FACTORS AFFECTING THE GLYCATION OF PROTEINS

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SUMMARY

Forty-two non-diabetics whose glycated haemoglobin levels could not be explained by their levels of fasting glycaemia or glucose tolerance were identified as part of the Islington Diabetes Survey. Mean glycated haemoglobin levels that were high relative to their blood glucose levels (HIGH GLYCATORS) were found in 22 subjects whilst 20 subjects had low relative levels (LOW GLYCATORS).

A repeat glucose tolerance test in 13 of the 42 subjects (7 high and 6 low glycators) showed that they remained categorised in the two groups four years after the discrepancy was identified. Mean blood glucose levels calculated from two 5-point diurnal profiles also failed to explain the glycated haemoglobin levels in the two groups.

Further biochemical and haematological tests revealed that glycated albumin showed a better relationship with blood glucose levels than did glycated haemoglobin. Intra-erythrocyte glucose concentrations were significantly lower than the plasma glucose in the low glycators, whilst the levels of erythrocyte 2,3-diphosphoglycerate (a known catalyst of glycation) were significantly higher in the high glycators. These findings may partially explain the original discovery of low and high glycators.

The effects of high fibre/low glucose and high glucose/low fibre diets on glycated proteins were studied in normal subjects. Only glycated albumin levels changed significantly, probably due to small changes in plasma glucose occurring on each diet, which were not mirrored by similar changes in the erythrocyte.

When another group of normal subjects received 1 g vitamin C daily for three months, significant reductions in glycated albumin and glycated haemoglobin were observed, with the former being reduced to a greater extent. This suggests that vitamin C has the potential of being used therapeutically to reduce glycation.

In conclusion, for normal subjects, glycated albumin is a more reliable indicator of blood glucose control than glycated haemoglobin.
ABBREVIATIONS

GHb  glycated haemoglobin
GA   glycated albumin
IDDM insulin-dependent diabetes mellitus
NIDDM non-insulin-dependent diabetes mellitus
OGTT oral glucose tolerance test
IGT  impaired glucose tolerance
EDTA ethylene diamine tetra-acetic acid
AA   ascorbic acid
DHAA dehydroascorbic acid
DKG  diketogulonic acid
SD   standard deviation
LDL  low-density lipoprotein
2HBG two-hour blood glucose
FBG  fasting blood glucose
MBG  mean blood glucose
FFI  2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole
CML  carboxymethyl-lysine
LL   lysino-lactic acid
AGE-PRODUCTS advanced glycosylation end-products
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INTRODUCTION
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Glycation is a non-enzymic post-translational modification of proteins which may be relevant not only to the long-term complications occurring in diabetes mellitus (Vlassara et al., 1986), but also to the normal process of ageing (Cerami et al., 1987).

1.2. DISCOVERY OF GLYCATED PROTEINS

Haemoglobin was the first protein to be discovered to undergo the process of glycation. In 1955 Kunkel and Wallenius reported the presence of both slow-moving (HbA₂) and fast moving minor fractions when haemoglobin samples were subjected to starch-gel electrophoresis. Allen, Schroeder and Balog in 1958 employed a cation-exchange column to separate from HbA a heterogeneous fast moving fraction (HbA₁). By modifying the conditions, the fast fraction was resolved into three minor components, HbA₁a, HbA₁b and HbA₁c. HbA₁c was found to be present in the greatest relative amount, comprising 5 - 7% of the total haemoglobin.

The independent discovery that HbA₁ (in particular HbA₁c) was increased two to three fold in diabetic subjects (Huisman and Dozy, 1962; Rahbar et al., 1969) led to extensive investigations into establishing the structure, biosynthesis and kinetics of formation of HbA₁c and the other minor components.

1.3. STRUCTURAL ANALYSIS OF HbA₁c AND THE OTHER MINOR COMPONENTS

In 1966 Holmquist and Schroeder demonstrated that the α-amino groups of the valine residues of the β-chains of HbA₁c were blocked by a small molecule held by a Schiff base or similar linkage. Subsequently, Bookchin and Gallop (1968) identified these as being hexoses and showed that two were bound per tetramer. Bunn et al., (1975) showed that glucose reacted with the amino terminal valines of the β-chains in HbA₁c. Periodate studies demonstrated a ketoamine linkage rather than an aldimine linkage between the glucose and the amino group. They proposed a two-step reaction scheme for the formation of HbA₁c (see Fig. 1.1). Initially a Schiff base is formed (aldimine) between glucose and the β-terminal valines followed by an Amadori rearrangement (Hodge, 1955) to form
FIGURE 1.1. REACTION SCHEME FOR THE FORMATION OF GLYCATED HAEMOGLOBIN

\[
\begin{align*}
HCOH + H_2N-R & \underset{K_1}{\overset{K_2}{\rightleftharpoons}} HC=N-R \\
& \underset{K_{-1}}{\overset{K_{-2}}{\rightleftharpoons}} HC-OH \\
& \underset{K}{\overset{K}{\rightleftharpoons}} C=O \\
\text{GLUCOSE} & \text{PROTEIN (HAEMOGLOBIN)} & \text{ALDIMINE (SCHIFF BASE)} & \text{KETOAMINE (AMADORI PRODUCT)} \\
\end{align*}
\]

\[
\begin{align*}
K_1 &= 0.096 \times 10^{-3} \text{ M}^{-1} \text{ SEC}^{-1} \\
K_{-1} &= 0.100 \times 10^{-3} \text{ SEC}^{-1} \\
K_2 &= 14.20 \times 10^{-6} \text{ SEC}^{-1} \\
K &= 1.700 \times 10^{-6} \text{ SEC}^{-1} \\
\text{(MORTENSEN AND CHRISTOPHERSON, 1983)}
\end{align*}
\]
a ketoamine. Further studies have confirmed an Amadori rearrangement occurs subsequent to Schiff base formation (Fluckiger and Winterhalter, 1976; Koenig et al., 1977; Bunn et al., 1979).

As well as the α-amino groups of the β-chains, other amino groups of the haemoglobin molecule have been shown to be glycated in vitro and in vivo. These include the amino termini of the α-chains and various ε-amino groups of both the α- and β-chains (Bunn et al., 1979). It is estimated that approximately 8 - 9% of haemoglobin in non-diabetic subjects is glycated through the ε-amino groups of lysyl residues of both chains (Bunn et al., 1979). As with HbA1c, glycation of these other sites has also been shown to be increased in diabetic subjects (Gabbay et al., 1979).

The structure of the other minor haemoglobins have also been studied. HbA1a has been shown to be further resolved into HbA1a1 and HbA1a2 (McDonald et al., 1978). HbA1a1 proved to have the N-terminal β-valine linked to fructose 1,6-diphosphate and possibly other red blood cell phosphates (Haney and Bunn, 1976) and HbA1a2 with glucose-6-phosphate (Haney and Bunn, 1976; McDonald et al., 1978). The structure of HbA1b has still not been elucidated, although it has been shown to be a covalent modification of the β-chains (Krishnamoorthy et al., 1977) and does not contain phosphate (McDonald et al., 1978). The HbA1a1, HbA1a2 and HbA1b fractions comprise 0.2, 0.2 and 0.4 percent of the total haemoglobin respectively in normal subjects. Levels of HbA1a1, HbA1a2 and HbA1b are not increased in diabetes (McDonald et al., 1978).

1.4. BIOSYNTHESIS AND KINETICS OF FORMATION OF GLYCATED HAEMOGLOBIN

The biosynthesis of HbA1 (and HbA1c) has been extensively studied both in vitro and in vivo.

1.4.1. IN VITRO STUDIES

Haney and Bunn (1976) and others subsequently (Bunn et al., 1976; Shapiro et al., 1979) incubated purified human haemoglobin (HbA0) with glucose and other sugars (mannose, lactose and galactose) for varying times and sugar concentrations. They showed that the sugars reacted with HbA0 to form HbA1c, which was identical with naturally occurring HbA1c isolated from erythrocytes of normal and
diabetic subjects (Koenig et al., 1977; Stevens et al., 1977 and MacDonald et al., 1978). Spicer et al., (1979) showed that HbA_1c increased linearly with time of incubation, glucose concentration and temperature of intact red cells in vitro.

The non-enzymic nature of the glycation process was indicated by the fact that red cell haemolysates and pure HbA_0 incubated with glucose or labelled glucose formed HbA_1c at identical rates (Fluckiger and Winterhalter, 1976). Also, L-[1^4C]-glucose and D-[1^4C]-glucose reacted similarly (Bunn et al., 1979).

The biokinetics of formation of the ketoamine HbA_1c, through a labile and reversible aldimine form has received intensive study. The initial rate for the aldimine condensation is very similar in several reports (Higgins and Bunn, 1981; Svendsen et al., 1981; Weykamp and Penders, 1982; Mortensen and Christophersen, 1983). It is formed very rapidly within a few hours of incubation of HbA_0 with glucose (for rate constants, see Fig. 1.1). In contrast, the formation of the more stable ketoamine is negligible during this time. The rate of the Amadori rearrangement has been calculated to be 1/60th the of the rate of dissociation of the aldimine back to glucose and haemoglobin. Bunn et al., (1979) incubated [1^4C]-[3H]-glucose with HbA_0 and used the 3H to 14C ratio in HbA_1c to measure accurately the Amadori rearrangement. After six days some 60% of the newly synthesized HbA_1c had undergone the Amadori rearrangement and an estimated 91% after 22 days.

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The formation of the ketoamine had been considered to be irreversible (Bunn et al., 1975). Graf et al. (1978) found a 50% conversion of haemoglobin to HbA_1c in vitro and predicted a saturable system of 23% glycation in vivo for severe states of glycaemia. However, prolonged saline incubation of pure HbA_1c (ketoamine) revealed a progressive decrease in HbA_1c concentration with the concomitant formation and increase in HbA_0 (Mortensen and Christophersen, 1983; Mortensen et al., 1984). The degradation of HbA_1c to the Schiff base was 12% of the rate of its formation.

1.4.2. IN VIVO STUDIES

The biosynthesis of HbA_1c was followed in humans after an injection of [59Fe] transferrin into a human volunteer (Bunn et al., 1976). The specific activity of HbA_1c increased slowly and at day 60 started to exceed that in HbA_0. It was concluded that HbA_1c is formed continuously at a slow rate over the 120 day lifespan of normal human red blood cells. In agreement with these findings, young red
cells separated by density gradient, have lower concentrations of HbA1c than do old cells (Fitzgibbons et al., 1976).

By injecting labelled reticulocytes into diabetic and normal mice, Koenig and Cerami (1975) showed that the rate of synthesis of HbA1c was much faster in diabetic mice. This is in agreement with Rahbar (1969) who found increased levels of HbA1c in diabetic subjects.

So, in summary, the formation of HbA1c is a two-stage process. The initial reaction to form the aldimine is rapid. Once formed it can either proceed by an Amadori rearrangement to form the ketoamine, or proceed to reverse liberating free glucose should the concentration of blood glucose decrease.

1.5. GLYCATION OF OTHER HAEMOGLOBINS

Normal haemoglobins other than HbA0, HbA2 and HbF also undergo glycation in humans (Bernstein, 1987). Glycation of fetal haemoglobin, HbF becomes particularly significant in diabetic pregnancy. Similarly glycation of variant haemoglobins C, D, E, G and S have also been reported (Bernstein 1987).

1.6. GLYCATION OF OTHER PROTEINS

Following the discovery and extensive study of glycated haemoglobin, it became evident that due to the non-specific nature of the glycation reaction, a wide variety of proteins with reactive α-amino or ε-amino groups in their composition could undergo variable degrees of glycation.

Glycation has since been demonstrated for many proteins ranging from those with short to very long half-lives. They have been found to be similarly raised in diabetic subjects compared to normal individuals.

1.6.1. SHORT-LIVED PROTEINS

A number of plasma proteins have been shown to be glycated in vitro and in vivo, however, glycated albumin has received most attention mainly because it is the most abundant of the plasma proteins (70-80% of the total) (Peters, 1985).

Glycation of horse serum albumin was first reported in 1956 (Bernstein, 1987). However, it was not until the phenomenon of human haemoglobin glycation was well accepted that glycated
albumin was found to be a normal constituent of human blood and also to be elevated in diabetic subjects (Day et al., 1979(a); Dolhofer and Wieland, 1979(b); Guthrow et al., 1979; Dolhofer and Wieland, 1980; Ney et al., 1981).

Experiments in vitro demonstrated that glycation occurred by the same mechanism as for glycated haemoglobin, with the initial formation of a Schiff base followed by a rearrangement to form a ketoamine (Day et al., 1979(a); Dolhofer and Wieland, 1979(b)). The biokinetics of albumin glycation studied with radioactive glucose (Baynes et al., 1984) indicated that the incorporation of glucose into the labile Schiff base was complete in 1 - 2 hours. Some 2% of the albumin contained the glucose bound in this form under physiological conditions (5.5mM glucose, pH 7.4, 37°C). From the kinetic constants involved in the formation and dissociation of the aldimine and its Amadori rearrangement product, an estimate of 29% glycation by the radiochemical procedure was found (Baynes et al., 1984). This is in close agreement with the value of 28% based on the furosine assay which measures lysine-bound glucose (Schleicher and Wieland, 1981). The in vivo glycation of 8% reflects the considerable binding capacity of albumin for endogenous metabolites, such as bilirubin and exogenous substances, such as drugs (Harding, 1985).

The ε-amino groups of lysine have been shown to be the main sites of glucose attachment in albumin (Dolhofer and Wieland, 1979; Garlick and Mazel, 1983). The principal site of glycation of human plasma albumin in vivo is lysine-525 (Garlick and Mazer, 1983; Iberg and Fluckiger, 1986). This differs from the main site found in vitro which is lysine-199 (Day et al., 1979(a)).

Other plasma proteins have been shown to be glycated in vivo and in vitro. These include transferrin (Kemp et al., 1984), fibrinogen and fibrin (Brownlee et al., 1983) and lipoproteins in particular low-density lipoproteins (Gonen et al., 1981; Schleicher et al., 1981; Harding, 1985). Glycation of erythrocyte membrane proteins have also been studied (Bailey et al., 1976; Harding, 1985). Glycation of insulin has been achieved in vitro (Dolhofer and Wieland, 1979(a)) and numerous enzymes have also been shown to be glycated in vitro (Coradello et al., 1981; Dolhofer et al., 1982).
1.6.2. LONG LIVED PROTEINS

The glycation of structural and tissue proteins have also been shown in vitro and in vivo, including lens crystallins (Monnier et al., 1979; Chiou et al., 1981; Mandel et al., 1983; Harding, 1985), collagen (Hamlin et al., 1975; Schneider and Kohn, 1981; Kohn and Schneider, 1982) and nerve proteins (Vlassara et al., 1981).

1.7. CHEMISTRY OF GLYCATION AND FACTORS AFFECTING THE REACTION

Non-enzymic glycation has been shown to be a reaction occurring in two phases (Bunn et al., 1975). The initial step involves a nucleophilic attack by an unprotonated amino group contributed by a free amino acid or protein, on the open chain, carbonyl form of a reducing sugar. This is a rapid, reversible reaction resulting in the formation of a labile Schiff base or aldimine. The Schiff base will be in equilibrium with the glycosylamine (Dixon, 1972). This intermediate product subsequently undergoes an Amadori rearrangement (Hodge, 1955) to form a 1-amino-1-deoxyfructose derivative (ketoamine or fructosamine) which in turn can cyclize to a ring structure (Fischer and Winterhalter, 1981).

The rate and extent of the Schiff base formation depends predominantly on the concentration of the two reactants. Since the concentration of the protein in vivo remains fairly stable, the glucose concentration together with the time of exposure of the protein to an increase in glucose concentration (i.e. degree and duration of hyperglycaemia) are the major determinants of the level of glycation in vivo.

The half-life of the protein will be an important factor in determining the extent of glycation in vivo. A reduction in the half-life of a protein can decrease the extent of glycation even without a change in the corresponding blood glucose levels. For example, there is a decreased content of glycated haemoglobin in the erythrocytes of patients with haemolytic anaemia and shortened red cell lifespan and also in normal subjects who have sustained an acute blood loss (Bunn et al., 1976). Conversely, polycythaemia as seen in smokers (Smith and Landaw, 19798) leads to an increase in glycated haemoglobin (Graham et al., 1980).
The permeability and availability of glucose in different tissues will clearly influence the extent of glycation of proteins. Higgins et al., (1982) showed that the permeability of erythrocytes to glucose was an important factor in affecting the glycation of haemoglobin.

Bunn and Higgins (1981) studied the reaction of various sugars with haemoglobin in vitro and showed that the rate of glycation is directly dependent on the extent to which the sugar exists in the open-chain form. The latter has been shown to be influenced by pH, with a decrease in the open-chain, reactive form occurring with decreasing pH (Cantor and Peniston, 1940). Also, aldose sugars such as glucose and galactose react more rapidly than ketose sugars, such as fructose. This is probably because the aldehyde carbonyl groups are relatively more electrophilic than ketone groups, making them more susceptible to nucleophilic attack by an unprotonated amino group (Bunn and Higgins, 1981). Stevens et al., (1977) showed that phosphorylated sugars react with haemoglobin more rapidly than glucose, including glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate and ribulose-5-phosphate. The rate of glycation of haemoglobin by glucose-6-phosphate was twenty times the rate with glucose. This is partly due to the fact that glucose-6-phosphate exists to a greater extent than glucose in the straight-chain form and partly due to the catalytic effect of the phosphate group (Watkins et al., 1987).

Although non-enzymic glycation can involve these different sugars and despite glucose being present in the lowest percentage of the reactive straight-chain form (0.002% compared to 0.02% for galactose and 0.7% for fructose) (Higgins and Bunn, 1981), it remains the major sugar involved in glycation in normal humans because of its relatively greater concentration.

There are circumstances where sugars other than glucose are pathologically raised and contribute to a greater extent to glycation. For example, in galactosaemia, glycation by galactose occurs to a considerable degree due to the increased rate of reaction of galactose as a result of the greater proportion of galactose that exists in the open-chain form (Urbanowski et al., 1982). Recently, glycation by fructose has received attention since fructose accumulates in organs where the sorbitol pathway is active (Suarez et al., 1989). This metabolic shunt mediates the conversion of glucose to fructose with the formation of sorbitol as an intermediate (Gabbay, 1975). In conditions of increasing glucose supply such as diabetes, the level of fructose in the lens may rise 23-fold becoming twice as concentrated as glucose (Suarez et al., 1989). Also, the rate of Schiff base formation between amino
groups of haemoglobin is 7.5 times higher for fructose than for glucose (Higgins and Bunn, 1981).

As well as the concentration of the reducing sugar present in the reactive form, the initial Schiff base formation depends on the pKa of the amino group and steric factors affecting its accessibility. The terminal amino acid residues (α-amino groups) and intra-chain lysine residues (ε-amino groups) which titrate with low pKa values are likely sites for Schiff base formation. The pH will clearly influence the formation of the Schiff base. However, its effect on the glycation reaction is extremely complex (Higgins and Bunn, 1981; Lowrey et al., 1985) and several mechanisms are probably involved. Although a decrease in pH will result in an increase in protonation of free amino groups and therefore a decrease in their reactivity, an increase in hydrogen ion concentration also catalyses the initial dehydration step. A decrease in pH also decreases the amount of glucose in the reactive open-chain form by affecting its tautomeric equilibrium (Cantor and Peniston, 1940). The overall effect is an increase in Schiff base formation with increasing pH.

The initial reaction between glucose and a protein may also be affected by other ketones and aldehydes which also react with α- and ε-amino groups. These may compete with glucose for binding and therefore may inhibit glycation.

Acetaldehyde is produced by the metabolism of alcohol in the liver and has been shown to react with amino groups on proteins in vitro and in vivo in a manner analogous to that of glucose. The reaction of acetaldehyde with haemoglobin in vitro yields five adducts which can also be identified in vivo and are raised in alcoholics compared to controls (Stevens et al., 1981; Hoberman, 1983).

The major cofactor of vitamin B₆, pyridoxal-5-phosphate also forms a Schiff base with protein amino groups through its reactive aldehyde functional group (Dempsey and Christiansen, 1962; Srivastava et al., 1972). Therefore, it should compete with glucose for binding to protein amino groups. Shepherd et al., (1985) showed that pyridoxal-5-phosphate inhibited the glycation of human serum albumin, ribonuclease A, lysozyme, human haemoglobin, human IgG and bovine myelin based protein in vitro. Pyridoxylated haemoglobin has also been demonstrated in vivo (Srivastava et al., 1972). Other metabolites of vitamin B₆, such as pyridoxal and pyridoxic acid have also been shown to react with haemoglobin (Benesch and Benesch,
Carbamylation of proteins by cyanate has long been recognised by food chemists and has been shown to occur in vitro and in vivo (see Fig. 1.2). Cyanate can react with both α- and ε-amino groups of all proteins. In haemoglobin the α-amino groups are preferentially carbamylated, partly due to their low pKa (Kilmartin and Rossi-Bernardi, 1971). Cyanate is formed from urea in vivo as follows:

\[
\begin{align*}
\text{NH}_2 & \quad \text{C} \quad \text{O} \\
\text{NH}_2 & \quad \text{UREA} \\
\text{NH}_2 & \quad \text{CYANATE} \\
\text{NH}_3 & \quad \text{AMMONIA}
\end{align*}
\]

Cyanate levels are increased in uraemia and an increase in the level of carbamylated haemoglobin has been shown in patients with renal failure (Fluckiger et al., 1981).

Aspirin has also been shown to modify the structure of a number of plasma proteins by a transacetylation reaction (see Fig. 1.3). Radioactivity from aspirin labelled in the acetyl group is incorporated into plasma proteins in vivo and in vitro (Defuria et al., 1973; Bridges et al., 1975). In haemoglobin the principal sites of acetylation are β-lys-59, β-lys-144 and α-lys-90 (Shamsuddin et al., 1974). Patients on high doses of aspirin may have 200 µM of acetylsalicylic acid in their plasma and approximately 5% of their haemoglobin may be acetylated (Bridges et al., 1975). The principal acetylation site on human serum albumin is lys-199 (Walker, 1976) and acetylation alters its drug-binding properties. Acetylsalicylic acid has been shown to inhibit the glycation of haemoglobin and albumin both in vivo and in vitro (Day et al., 1979; Rendell et al., 1986). The glycation of haemoglobin is far less susceptible to inhibition than is albumin glycation. This correlates well with the fact that the major glycation site on haemoglobin is the amino terminus of the β-chains whereas aspirin reacts preferentially at the ε-amino groups. In constrast to haemoglobin the reaction of glucose and albumin occurs mostly at the ε-amino residues. Also
FIGURE 1.2. THE REACTION OF CYANATE WITH PROTEIN AMINO GROUPS

\[
\text{Pr} - \text{NH} + \text{C} \rightarrow \text{PrNH} - \text{C} - \text{NH}_2
\]

PROTEIN AMINO GROUP  CYANATE  CARBAMYLATED PROTEIN
FIGURE 1.3. ACETYLATION OF PROTEIN AMINO GROUPS BY ASPIRIN

\[
\text{COO-} \quad \text{Pr NH}_2 \quad \rightarrow \quad \text{Pr NH - COCH}_3
\]

ASPIRIN (ACETYLSALICYLIC ACID)  \hspace{1cm} \text{ACETYLATED PROTEIN}
hydrolysis of acetylsalicylic acid occurs rapidly in the bloodstream with further hydrolysis occurring during membrane transport. The hydrolysis product, salicylic acid does not react with proteins. Therefore, haemoglobin in the erythrocyte is likely to be exposed to lower concentrations of acetylsalicylic acid than albumin in the plasma.

Dehydroascorbic acid, one of the oxidation products of ascorbic acid is another compound known to react with amino acids and proteins. This was first described by Dulkin and Friedeman in 1956 and has since been extensively studied by food chemists (Ranganna and Setty, 1974; Kurata and Fujimaki, 1976). The carbonyl group at the 2-position (see Chapter 5) is thought to undergo a nucleophilic reaction with both α- and ε-amino groups to form a Schiff base, which is probably reversible under physiological conditions. Stolba et al. (1987, 1988) have shown that both dehydroascorbic acid and ascorbic acid can affect the glycation of bovine serum albumin and collagen in vitro. They found that ascorbic acid inhibited glycation whilst dehydroascorbic acid appeared to enhance the incorporation of glucose into proteins. Khatami et al., (1988) on the other hand showed that ascorbic and dehydroascorbic acid both inhibited the glycation of bovine serum albumin in vitro. When insulin-dependent diabetic subjects were treated with 1.5g of ascorbic acid daily for three weeks, a significant decrease in the plasma fructosamine concentration occurred (Stolba et al., 1988).

Instead of compounds competing with glucose for binding to amino groups on proteins, free amino acids can react with glucose and may themselves compete with reactive amino groups on proteins for glucose attachment. Sensi et al., (1989) recently reported the inhibition of glycation of proteins by D-lysine in vitro. They investigated the effect of varying concentrations of D-lysine on glucose incorporation and ketoamine formation on short-lived proteins, such as albumin and immunoglobulin G and long-lived proteins, such as collagen and glomerular basement membrane. They found that under physiological conditions, inhibition of glycation of all the proteins increased linearly with increasing concentrations of D-lysine and that the degree of inhibition was greater with high concentrations of glucose.

The most important factor affecting the rate of conversion of the Schiff base to the ketoamine is its microenvironment within the protein (Watkins et al., 1987). The main determinants being the structure of the protein and ionic influences in the direct vicinity of
the Schiff base. A number of physiological factors have been shown to influence the extent of Amadori rearrangement both in vitro and in vivo. The effect of pH on the kinetics of formation of glycated haemoglobin has been studied in vitro (Smith et al., 1982; Lowry et al., 1985). The rate of formation of the stable ketoamine was found to increase with decreasing pH, which is in agreement with Hodge (1955) who showed that the Amadori rearrangement was acid catalysed. Also, Shapiro et al., (1989) reported that the glycation of lysine residues in haemoglobin occurs on lysines in the vicinity of carboxyl groups on the protein surface. They suggested that the latter may act as a local catalyst of glycation. They did not find a direct correspondence between the pKa of the amino group and the extent of glycation, suggesting that the kinetics of the Amadori rearrangement rather than the pKa of the amino group may be rate-limiting in determining the sites of glycation on the protein. Iberg and Fluckiger (1986) also noted that the lysine residues glycated in albumin were frequently located in sequences of basic amino acids. They suggested that ‘clusters’ of these basic amino acids in proteins may act as neighbouring group catalysts of the Amadori rearrangement on adjacent lysine residues.

Watkins et al. (1987) investigated the effects of phosphate on the kinetics and specificity of glycation of various proteins in vitro. They had previously found that reactive lysine residues involved in the glycation of RNAase were located in or near the phosphate region of the active site, a fairly basic region of the protein, rich in lysine, histidine and arginine residues (Watkins et al., 1985). They found that both inorganic and organic phosphates not only increased the glycation of all proteins studied by catalysing the Amadori rearrangement, but also apparently directed the specificity of the reaction. Incubation of the proteins in different buffers with varying concentrations of phosphate lead to a significant increase in glycation, which occurred in a concentration-dependent fashion. The pattern of glycation of amino-acid residues was different in the presence of phosphate. Haemoglobin was studied in greater detail, in particular to examine the effect of 2,3-diphosphoglycerate on glycation. The glycation of haemoglobin has been shown previously to be enhanced by 2,3-diphosphoglycerate in vitro (Smith et al., 1982; Lowry et al., 1985). Both inorganic phosphate and 2,3-diphosphoglycerate increased the overall extent of glycation of both valine and lysine residues, but the relative extent of glycation of valine residues was greater, particularly in the presence of 2,3-diphosphoglycerate. Phosphate is thought to increase the glycation of proteins by affecting both phases of the reaction. Phosphate is known to increase the proportion of the open-chain, reactive form of
reducing sugars in solution (Burton and McWeeny, 1963) and therefore will promote the initial Schiff base formation. Also, since its pKa is near neutral pH, phosphate can also serve as an acid-base catalyst of the Amadori rearrangement under physiological conditions. The effect of phosphate on the specificity of glycation is thought to be due to its binding to unique basic microenvironments in the protein (see Fig. 1.4), where it catalyses the rearrangement reaction. Thus, the preferential glycation of lysines in or near the active site of RNAase and the β-chain terminal valines in haemoglobin can be explained by the binding of phosphate in the active site and allosteric sites of these proteins respectively.

The pronounced effect of 2,3-diphosphoglycerate on both the overall kinetics and the specificity of glycation of haemoglobin in vitro suggests that the binding of organic phosphate is probably also significant in determining the rate and pattern of glycation of the protein in vivo. Several investigators have described a strong positive correlation between 2,3-diphosphoglycerate concentration and the kinetics of formation of glycated haemoglobin in vitro and in vivo (Smith et al., 1982; Roberts et al., 1984; Lowry et al., 1985). The enhanced rate of glycation of haemoglobin found under anaerobic conditions (Smith et al., 1982) is probably explained by the increased affinity of haemoglobin for 2,3-diphosphoglycerate under these conditions, which in turn will catalyse glycation.

The catalysis of the Amadori rearrangement by local amino acid residues and/or the binding of phosphate or other buffering ions probably explains the apparent preferential glycation of various residues in a given protein. It may also explain the fact that some proteins become more glycated than others and that there are differences between the sites and order of prevalence of glycation occurring in vivo versus in vitro. For example, although the amino group of the terminal valine of the β-chain of haemoglobin is the most reactive group in vivo and in vitro, several other sites on the β-chain as well as on the α-chain can be modified (Bunn et al., 1979). Incubation of purified HbA₀ with [¹⁴C]-glucose followed by ion-exchange chromatography and two-dimensional peptide mapping allowed identification of the major sites of glycation occurring in vitro. These were β-val-1, α-lys-16, β-lys-66, β-lys-17, α-val-1, α-lys-7 and β-lys-120. In contrast, the major sites of glycation occurring in vivo were identified as β-val-1, β-lys-66, α-lys-61, β-lys-17 and α-val-1 (Shapiro et al., 1980).
FIGURE 1.4. REACTION PATHWAY FOR GLYCATION OF PROTEIN, SHOWING THE CATALYTIC EFFECT OF PHOSPHATE

ALDIMINE

ENEAMINOL

KETOAMINE
The sites of glycation of albumin have also been studied in vitro and in vivo. Preliminary experiments in vitro and in vivo suggested that just the ε-amino groups of lysine residues were glycated (Dolhofer and Wieland, 1979; Day et al., 1979; Garlic and Mazer, 1983). However, recently Robb et al., (1989) showed that the N-terminal amino acids of albumin may also be glycated in vivo and in vitro. In vitro incubations of human (Day et al., 1979) and rat (Day et al., 1979) serum albumin with glucose suggested that the principal site of glycation was lysine-199. However, in vivo studies showed that approximately 40% of glycation of serum albumin occurred at lysine-525 (Garlic and Mazer, 1983; Iberg and Fluckiger, 1986). It was suggested that this difference may be due to the accessibility of a particular lysine ε-amino group. In vitro, the glycation of human serum albumin is enhanced when the fatty acids are removed from the albumin prior to incubation with glucose (Mereish et al., 1982). It is possible that in vivo the ε-amino group of lysine-199 is covered by a ligand, such as a fatty acid so that is is not accessible to glucose in vivo. For in vitro experiments a portion or all of the masking ligand may be lost in the purification process, uncovering the reactive ε-amino group, namely lysine-199.

The rate of non-enzymic glycation of human serum albumin in vivo appears to be markedly greater than that of human haemoglobin. In non-diabetics, serum albumin has a half-life of approximately 19 days and is 10 - 12% glycated whereas haemoglobin has a half-life of 120 days and is 5 - 7% glycated. Olufemi et al., (1987) investigated the relative extent of glycation of haemoglobin and albumin in vivo. They found that the ε-amino groups on albumin were more extensively modified than those on haemoglobin. They concluded that the average rate of reaction of lysyl residues in albumin is markedly faster than in haemoglobin, possibly due to acid catalysis by neighbouring aspartic acid and glutamic acid side-chains, which are abundant in albumin.

So, in summary, although the main determinants of the extent and rate of glycation of a given protein in vivo are the availability of reactive amino groups and the concentration of glucose in its reactive form, there are other important factors which may directly or indirectly influence one or both phases of the glycation reaction.
1.8. CLINICAL IMPLICATIONS OF PROTEIN GLYCATION

The process of glycation occurs continuously at a slow rate in normal subjects, but in diabetes mellitus due to the continual exposure to long periods of hyperglycaemia, glycation of proteins occurs at a faster rate and to a greater extent (Koenig and Cerami, 1975).

The discovery that proteins were glycated in a time-related fashion, dependent mainly on the circulating glucose concentration (Bunn et al., 1976), resulted in extensive evaluation of glycated proteins, in particular glycated haemoglobin (GHb) and glycated albumin (GA) as monitors of integrated glycaemia in diabetes.

The most intriguing aspect of glycation is the possibility of linking hyperglycaemia to the chronic complications occurring in diabetes mellitus (Brownlee et al., 1984).

Glycation may also play a role in the tissue changes associated with normal ageing (Cerami et al., 1987). The effect of diabetes on many organs and tissues is often described as accelerated ageing. Several of the complications that occur in people with diabetes, including cataracts, joint stiffness and atherosclerosis are identical with disorders that develop in the elderly. They merely develop earlier. If excess glucose does hasten the onset of these complications in people with diabetes, normal amounts of glucose could conceivably play a role in the slower onset seen in non-diabetics as they age.

Before discussing the pathophysiological effects of glycation, a brief outline of the history, classification and diagnosis of diabetes will be given together with a more detailed discussion of insulin and the use of glycated proteins in both the diagnosis and monitoring of blood glucose control.

1.9. DIABETES MELLITUS

1.9.1. DEFINITION (OLEFSKY, 1985)

Diabetes mellitus is a heterogeneous primary disorder of carbohydrate metabolism with multiple aetiologic factors that generally involve absolute or relative insulin deficiency or insulin resistance or both. It is characterised by chronic hyperglycaemia and is often associated with the development of specific microvascular complications, especially in the eye and kidney, and
an increased frequency of macrovascular disease, such as peripheral coronary artery disease.

1.9.2. HISTORY

Many books outline the history of diabetes and report on the investigations showing the association between insulin and diabetes (Oakley et al., 1975; Faris, 1982; Bennett, 1983).

The term of diabetes mellitus was originally used to describe a disorder characterised by the passage of sweet urine, excessive urination, thirst, excessive weight loss and hunger which over the course of several months resulted in death.

Over 100 years ago, it was recognised that the disease appeared in at least two distinct forms. One affected mainly obese adults and the other being more frequent in younger people, often children who were usually thin at the time of diagnosis.

Following the discovery of the role of the pancreas in regulating blood sugar and the demonstration that pancreatectomy resulted in a syndrome of hyperglycaemia, ketosis and death, it was assumed that the diabetes was the result of pancreatic disease. It was found that persons who died from diabetes had atrophy of the Islets of Langerhans and pancreatic fibrosis. Many attempts were made between 1890 and 1922 to demonstrate that extracts of the pancreas were effective in relieving the symptoms of diabetes. In 1916, Sharpey-Shafer concluded that the Islets of Langerhans must secrete a substance which regulates carbohydrate metabolism and proposed the name of insulin for this substance.

It was not until 1921 when Banting and Best isolated and purified insulin from pancreatic extracts that the first really effective preparation of insulin was produced for the treatment of diabetes.

Although insulin has led to increased life expectancy of diabetics, after years of exposure to high glucose levels and high insulin levels, standard treatment of insulin, diet or oral hypoglycaemic drugs does not prevent the development of chronic complications affecting the eyes, kidneys, nerves and arteries (Brownlee and Cerami, 1981). In the eye, retinal capillary damage and cataract formation eventually leads to blindness. Capillary damage in the glomerulus, associated with basement membrane thickening leads to chronic renal failure with proteinuria. In the diabetic peripheral nerve, axonal dwindling and segmental demyelination are
associated with a very high prevalence of motor, sensory and autonomic dysfunction. Increased atheromata in the medium and large arteries leads to coronary heart disease and peripheral arterial disease.

1.10. INSULIN

Since the discovery and isolation of insulin, extensive research has been carried out to elucidate its structure, biosynthesis and secretion from the pancreas and its mechanism of action.

1.10.1. STRUCTURE

The chemical sequence of insulin was determined in 1955 (Brown et al., 1955; Ryle et al., 1955). The human insulin molecule consists of two polypeptide chains. The A-chain contains 21 residues, whilst the B-chain is longer with 30 residues. The chains are held together by two inter-chain disulphide bonds. There is also a disulphide bridge within the A-chain.

In 1967 it was discovered that the hormone was synthesized in the pancreatic β-cells as pro-insulin, which is subsequently converted enzymatically to insulin in the storage vesicle (Steiner and Oyer, 1967). This cleavage reaction leads to the production of insulin and also a connecting peptide, C-peptide. The insulin and C-peptide are stored together in the β-granule until released into the intercellular space in response to an insulinotropic stimulus by the process of exocytosis (Lacy, 1961).

1.10.2. SYNTHESIS AND SECRETION

The regulation of the biosynthesis and secretion of insulin has recently been reviewed by Howell and Bird, (1989). The major physiological determinant of secretion in mammals is glucose, although a large number of physiological and pharmacological agents can also act as secretogogues. These can be divided into two groups. The first group are initiators, which are agents that are capable of provoking insulin release alone such as glucose and certain amino acids. The second group are potentiators which are agents that are effective alone, but will increase the insulin released in response to glucose or amino acids. The latter include glucagon and gastric-inhibitory polypeptide (GIP). The autonomic nervous system is also thought to affect insulin secretion.
The events leading to the secretion of insulin are not fully understood, but calcium is thought to play an essential role in the stimulus-secretion coupling in the pancreatic β-cells (reviewed by Wollheim and Sharp, 1981). The action of calcium to affect insulin secretion is thought to be via the calcium regulating protein, calmodulin (Sugden et al., 1979). Calmodulin has been implicated in a number of activities in the β-cells, but its major effects are probably produced via activation of plasma calmodulin-responsive protein kinase activity (McDonald and Kowlune, 1982), following which several proteins are specifically phosphorylated. Two possible target proteins are the microtubule subunit, tubulin and myosin light chains, with the implication that calmodulin-dependent phosphorylation may modulate granule movement leading to exocytosis (Howell, 1984).

1.10.3. RECEPTORS AND MECHANISM OF ACTION

Insulin exerts its metabolic actions by binding to specific receptors, which are found mainly on insulin-sensitive cells. Houslay and Siddle (1989) have recently reviewed the current knowledge on the structure and activity of the receptor.

The insulin receptor consists of two alpha subunits of approximately 135,000 Daltons and two beta subunits of approximately 90,000 Daltons held together covalently by both inter- and intra-subunit disulphide bridges (Czech, 1985). The alpha subunit provides the binding site for insulin and is wholly extracellular. The beta subunit is a transmembrane entity with globular domains at both the extracellular and cytosolic surfaces. It is assumed that the intracellular signal(s) elicited by the insulin receptor arise from the activity of the cytosolic domain of the beta-subunit after activation via conformational changes transmitted through the alpha subunit and the external domain of the beta-subunit. Evidence suggests that insulin action is mediated by reactions initiated by activation of an insulin receptor tyrosyl kinase. Many putative substrates have been suggested for the receptor kinase, however the most readily demonstrated substrate is the receptor itself. This autophosphorylation may be to enhance and prolong the activity of the kinase towards other cellular substrates involved in signalling pathways. Alternatively, the phosphorylation of the receptor may regulate its interaction with other proteins which are not themselves phosphorylated, but might nevertheless be important in signal transduction. However, experiments have shown that autophosphorylation and kinase activity are not the only mechanisms involved in signalling the metabolic response of insulin (Houslay and
Siddle, 1989). Insulin receptors have also been shown to interact with so called, G-proteins (Guanine-nucleotide binding regulatory proteins) which modulate cyclic-AMP concentrations and may control the production of inositol glycans. Cyclic-AMP and inositol glycans have been implicated as secondary messengers involved in exerting the metabolic effects of insulin along with calcium, cyclic-GMP and diacylglycerol. However, none of the mechanisms studied so far appear to exclusively mediate insulin action, but may be involved in controlling different aspects of its action.

Insulin has many actions. As well as lowering blood glucose concentrations, it lowers plasma amino acids, non-esterified free fatty acids and potassium levels by stimulating glycogen formation, triglyceride synthesis and protein synthesis. It also exerts an inhibitory effect upon gluconeogenesis and glycogenolysis.

Any disturbance in the secretion or action of insulin as occurs in diabetes mellitus, has profound effects on fat, protein and carbohydrate metabolism.

1.11. CLASSIFICATION OF DIABETES MELLITUS

The classification adopted by the WHO expert committee on diabetes mellitus in 1980 has since been revised by the committee in 1985 (WHO Report, Geneva, 1985) and is shown in Table 1.1. The most important change from the previous classification is the appearance of malnutrition-related diabetes as a major clinical subclass. So there are four major subclasses; insulin-dependent type, non-insulin-dependent type, malnutrition-related type and other types.

1.11.1. INSULIN-DEPENDENT DIABETES MELLITUS (IDDM)

IDDM is usually characterised by the abrupt onset of symptoms, insulinopenia, proneness to ketoacidosis and dependency on injected insulin to prevent ketosis and to sustain life. While classically the disease occurs in juveniles, it may appear at any age. Genetic determinants are believed to be important as evidenced by the association of this entity with increased and decreased frequencies of certain histocompatibility antigens (HLA). Islet cell antibodies are frequently present at the time of diagnosis. Autoimmune disorders are probably responsible for the disease in some instances, while evidence of a likely viral aetiology for some cases has been demonstrated.
TABLE 1.1 CLASSIFICATION OF DIABETES MELLITUS AND ALLIED CATEGORIES OF GLUCOSE TOLERANCE

A. CLINICAL CLASSES

(1) Diabetes mellitus (DM)
(a) Insulin-dependent diabetes mellitus (IDDM)
(b) Non-insulin-dependent diabetes mellitus (NIDDM)
   (i) Non-obese
   (ii) Obese
(c) Malnutrition-related diabetes mellitus (MRDM)
(d) Other types of diabetes associated with certain conditions and syndromes: (i) pancreatic disease; (ii) disease of hormonal aetiology; (iii) drug-induced or chemical-induced conditions; (iv) abnormalities of insulin or its receptors; (v) certain genetic syndromes; (vi) miscellaneous

(2) Impaired glucose tolerance (IGT)
(a) Non-obese
(b) Obese
(c) Associated with certain conditions and syndromes

(3) Gestational diabetes mellitus (GDM)

B. STATISTICAL RISK CLASSES
(SUBJECTS WITH NORMAL GLUCOSE TOLERANCE BUT SUBSTANTIALLY INCREASED RISK OF DEVELOPING DIABETES)

(1) Previous abnormality of glucose tolerance

(2) Potential abnormality of glucose tolerance
1.11.2. NON-INSULIN DEPENDENT DIABETES MELLITUS (NIDDM)

NIDDM is the term employed for subjects who are not insulin-dependent or ketosis-prone, although insulin may be used or required for the correction of symptomatic hyperglycaemia. Although not ketosis-prone under normal circumstances, they can develop ketosis under certain circumstances such as infection or other stress. The majority of the subjects with NIDDM are adults at the time of onset and are obese, but the disease can occur in children and non-obese adults. Evidence suggests there is a strong genetic basis for this type of diabetes. NIDDM is further characterised by the absence of islet-cell antibodies and is not associated with the HLA-types seen in IDDM. Nevertheless, the typical vascular complications seen in IDDM also frequently develop in persons with NIDDM.

1.11.3. MALNUTRITION-RELATED DIABETES MELLITUS

In tropical developing countries, young diabetics often present with a history of nutritional deficiency and a constellation of symptoms, signs and metabolic characteristics which fail to meet the criteria used to classify IDDM and NIDDM. Specific environmental factors interacting with genetic make-up are suspected to be involved in causing this type of diabetes. It is associated with damage of the pancreas caused by toxic substances and probably of inadequate protein intake.

1.11.4. OTHER TYPES

This group contains those forms of diabetes previously known as secondary diabetes, where the disease has an established aetiology or where it is part of a well-defined condition or syndrome (see Table 1.1). These cases of diabetes are in the minority.

1.11.5. IMPAIRED GLUCOSE TOLERANCE (IGT)

IGT is the term used to describe those persons whose glucose tolerance is neither unequivocally normal nor sufficiently abnormal to be diagnostic of diabetes. IGT is still the subject of research to determine its mechanisms and prognostic implications. A recent study by Kadowaki et al. (1984) suggested that Japanese patients with IGT have a higher risk of developing diabetes if they have low plasma insulin levels 30 minutes after the administration of a glucose load. This is independent of the two other positive
predictive factors which are high fasting and/or high post-load blood glucose concentration and the degree of adiposity.

1.11.6. GESTATIONAL DIABETES (GDM)

GDM denotes glucose intolerance that has its onset or is first recognised during pregnancy. As relatively mild degrees of glucose intolerance during pregnancy are associated with increased risk of perinatal mortality and morbidity, it is important to recognise its presence and provide appropriate management. Following parturition, the glucose tolerance may revert to normal, remain as IGT or persist as diabetes mellitus.

1.11.7. STATISTICAL RISK CLASSES

These are subjects who have had an abnormality of glucose tolerance in the past or who, by virtue of their genetic relationship with a diabetic or other characteristic, have an unusually high risk of developing diabetes. These are classified into two categories: previous and potential abnormality of glucose tolerance.

1.11.7.1. Previous Abnormality of Glucose Tolerance

These persons have had in the past diabetes, IGT or GDM. They are at risk of developing glucose intolerance if subjected to appropriate stresses.

1.11.7.2. Potential Abnormality of Glucose Tolerance

This is a group of people with normal oral glucose tolerance, but who have a statistically increased risk of developing diabetes.

1.12. DIAGNOSIS OF DIABETES MELLITUS

1.12.1. CLINICAL AND BIOCHEMICAL DIAGNOSTIC CRITERIA

The finding of the classical symptoms of diabetes such as thirst, polydipsia, polyuria, loss of weight and tiredness together with marked glycosuria (with or without ketonuria) and a random blood sugar of more than 10 mmol/L is diagnostic of diabetes mellitus (WHO Report, 1985). However, in many cases, particularly those with NIDDM, who have few symptoms and where glycosuria is frequently discovered by chance, the diagnosis is less obvious and an oral glucose tolerance test will be required.
1.12.2. ORAL GLUCOSE TOLERANCE TEST (OGTT)

The OGTT is the most important tool in the diagnosis of diabetes and is carried out according to the procedure recommended by the WHO Report (1985).

The test should be carried out in the morning after an overnight fast of 10 - 16 hours, during which water may be drunk. The test should be proceeded by at least three days of unrestricted carbohydrate intake (at least 150g) and usual physical activity. Outpatients should rest for at least half an hour before starting the test. They should also remain seated and refrain from smoking during the test. After collection of a fasting blood sample, the subject drinks 75g of glucose in 250 - 300mls of water over the course of five minutes. Another blood sample is collected after the test load. If appropriate, samples may also be taken every half-hour during this period. The glucose concentration is measured in either the whole blood or plasma of the collected samples in either venous or capillary blood. For the interpretation of the results see Table 1.2.

A number of factors should be taken into account when interpreting the results from the OGTT (Rifkin, 1983). The age of the patient will affect the results as there is a gradual decrease in glucose tolerance with increasing age (although not usually into the diabetic range). This is due to a decreased capacity of tissues to metabolize glucose. Also, a number of drugs can affect glucose tolerance. Chronic glucocorticoid administration, oral diuretics, the contraceptive pill (oestrogen) and nicotinic acid can all raise blood glucose and may give rise to false positives. Conversely, some drugs can lower blood glucose including salicylates, alcohol, monoamine oxidase inhibitors and propranolol. Ideally the patient should be off all medication when tested, to eliminate any possible influence upon the results. There are also a number of non-diabetic causes of an abnormal OGTT (see Table 1.3), which should be taken into account if diabetes is not conclusive.

1.12.3. GLYCATED PROTEINS

Glycated proteins have so far all proved to be insufficiently sensitive in the diagnosis of diabetes. A large number of studies of GHb in patients referred for OGTT have been carried out (Dods and Bolney, 1979; Dunn et al., 1979; Frazer and Shepherd et al., 1985; Forrest et al., 1987). The sensitivity of GHb in diagnosing patients with abnormal OGTT have varied from 20 - 80%. The vast range of
## TABLE 1.2 DIAGNOSTIC VALUES FOR THE GLUCOSE TOLERANCE TEST (WHO, 1985)

<table>
<thead>
<tr>
<th></th>
<th>WHOLE BLOOD (VENOUS) GLUCOSE (mmol/l)</th>
<th>WHOLE BLOOD (CAPILLARY) GLUCOSE (mmol/l)</th>
<th>PLASMA (VENOUS) GLUCOSE (mmol/l)</th>
<th>PLASMA (CAPILLARY) GLUCOSE (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DIABETES MELLITUS-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>≥6.7</td>
<td>≥6.7</td>
<td>≥7.8</td>
<td>≥7.8</td>
</tr>
<tr>
<td><strong>DIABETES MELLITUS-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2HR POST GLUCOSE</td>
<td>≥10.0</td>
<td>≥11.1</td>
<td>≥11.1</td>
<td>≥12.2</td>
</tr>
<tr>
<td><strong>IMPAIRED GLUCOSE TOLERANCE-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>&lt;6.7</td>
<td>&lt;6.7</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td><strong>IMPAIRED GLUCOSE TOLERANCE-</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2HRS POST GLUCOSE</td>
<td>6.7–10.0</td>
<td>7.8–11.1</td>
<td>&lt;7.8–11.1</td>
<td>6.9–12.2</td>
</tr>
<tr>
<td>Clinical State</td>
<td>Fasting Hyperglycaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver cell disease</td>
<td>Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic illness; prolonged physical inactivity; bed confinement</td>
<td>Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Acute stress&quot;: fever, trauma, major surgery, myocardial infarction, stroke</td>
<td>Often present for a few days after onset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starvation; under nutrition</td>
<td>Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium depletion (oral diuretics; alcoholism, ureamia, malnutrition, primary aldosteronism)</td>
<td>May be present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic renal disease</td>
<td>Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other disease of the endocrine glands:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active acromegaly</td>
<td>Present in about 25%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cushing's syndrome or glucocorticoid therapy (in potential diabetics only)</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary aldosteronism (secondary to potassium depletion)</td>
<td>May be present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulinoma</td>
<td>Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucagonoma</td>
<td>May be present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaeochromocytoma</td>
<td>May be present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyrotoxicosis</td>
<td>Only in prediabetic or diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
results corresponds to differences both in the criteria used for defining abnormal OGTT and in definition of an abnormal GHb concentration.

The first clinical assessment of glycated plasma proteins in the form of fructosamine (see Section 1.13.2.) suggested that the sensitivity was better than for GHb (Baker et al., 1983). A more recent comparison of GHb and fructosamine found however, that although fructosamine was slightly more sensitive than GHb in borderline diabetes, there was no difference in the sensitivity of patients with clearly abnormal OGTT, being 67% for both tests (Salemans et al., 1987).

Therefore, blood glucose measurement either with or without a preceding glucose load remain the basis for the diagnosis of diabetes mellitus or impaired glucose tolerance.

1.13. MONITORING GLYCAEMIC CONTROL USING GLYCATED PROTEINS

The regular monitoring of blood glucose concentration is used not only to assess the response of diabetic patients to new treatments, but also most studies have suggested that better glycaemic control is associated with the amelioration or less frequent development of long-term complications (Tschobroutsky, 1978; Eschwege et al., 1979; White et al., 1982).

Assessment of glycaemic control has depended upon sporadic blood glucose determinations, spot urine testing for glucose and 24 hour measurement of urine glucose. All these measurements are cumbersome, laborious and not always accurate or very reliable.

On the basis that the glycation of proteins occurs continuously during their lifespan and depends mainly on the prevailing glucose concentration, it was proposed that the measurement of glycated proteins should provide an objective means of quantitating the average blood glucose concentration in a time-related fashion, depending on the half-life of the protein involved (Bünn et al., 1976).

The circulating proteins in the blood were the obvious choice due to their easy accessibility and relative ease of measurement.
1.13.1. GLYCATED HAEMOGLOBIN

Haemoglobin was the first protein shown to be glycated in vivo and to be raised in diabetes mellitus. Bunn et al. (1976) showed that haemoglobin was glycated continuously throughout the lifespan of the erythrocyte (120 days) and that the extent of glycation depended on the glucose concentration within the erythrocyte. Due to the slight reversibility of the glycation reaction (Mortensen and Christophersen, 1983; Mortensen et al., 1984), the measurement of GHb therefore reflects the oscillations of glucose concentration in the erythrocyte over the preceding 4 - 6 weeks (Berstein, 1987).

Most investigators have reported a correlation of HbA₁ or HbA₁c levels with various parameters of glycaemic control in population studies employing both IDD or NIDD subjects. Random HbA₁ or HbA₁c levels have been found to correlate to variable degrees (r = 0.6 - 0.9) with fasting blood glucose, mean daily blood glucose during the preceding three months, maximum glucose concentration in the area under an OGTT curve, the highest mean blood glucose occurring during twelve hours of continuous glucose monitoring and 24 hour urine glucose obtained 4 - 12 weeks previously (Koenig et al., 1976; Gabbay et al., 1977; Gonen et al., 1977; Paisey et al., 1980; Boucher et al., 1981; Svendsen et al., 1982; Verillo et al., 1983).

The strongest correlations between levels of glycaemia and levels of GHb are found in NIDD subjects (Nathan et al., 1984). This is probably due to the relative stability of the blood glucose levels in NIDD subjects. In NIDDM the blood glucose concentrations remain fairly stable. There is little variability in fasting levels and close correlations are found between fasting and 24 hour integrated values (Holman and Turner, 1980; Paisey et al., 1980). In IDDM on the other hand considerable alterations of blood glucose may occur in minutes to hours, so the level at any time point may be misleading as to the integrated level of glycaemia. GHb levels and levels of glycaemia are often discrepant in IDDM. Kennedy et al. (1984) found that GHb and post-prandial plasma glucose levels in 302 IDD subjects, measured over a period of 9 - 12 months were only concordant in 40% of those studied. Schleicher et al., (1984) similarly found discrepancies between GHb and blood glucose levels in ten IDD subjects observed over a year.

Kennedy and Lyons (1989) suggest that regular monitoring of GHb is very useful in IDDM, but debatable in NIDDM, particularly in those not receiving insulin and should perhaps only be measured infrequently.
The time-relation between changes in glycaemic control and GHb levels have been studied in several longitudinal investigations. Studies assessing improved control have found GHb levels to lag 3-10 weeks behind achievement of improved diabetic control as judged by urine glucose excretion or mean daily blood glucose levels (Koenig et al., 1976; Gabbay et al., 1977). This variability probably reflects differences in individual rates of GHb formation, red blood cell destruction and also in pre-GHb (Schiff base) formation that follows acute changes in blood glucose concentrations (Widness et al., 1980). Many methods for GHb measurement quantitate both the labile Schiff base and the more stable ketoamine, which may give a falsely elevated GHb reflecting the blood glucose concentration at the time of sampling.

Boden et al., (1980) carried out in vivo studies that suggested that the rate of formation of GHb was considerably faster than the rate of its disappearance. They concluded that GHb is not suited to detect rapid metabolic improvements, but is useful to monitor rapidly deteriorating control.

There are certain clinical situations where the measurement of GHb will not be an accurate reflection of blood glucose concentration. This may occur in haemolytic anaemias (decreased erythrocyte survival), haemoglobinopathies, chronic renal disease (carbamylation of haemoglobin), alcoholism (acetaldehyde reacts with haemoglobin) and during the ingestion of certain drugs (e.g. aspirin).

So, although the regular determination of GHb levels has proved to be a useful index of metabolic control in diabetes (in particular IDDM), in certain instances it may give an unrepresentative measure of average glycaemia. Also, it cannot be used to monitor changes in glycaemia occurring in the short-term (i.e. week to week), which may be particularly important to assess the immediate response to new treatments and for the monitoring of diabetic pregnancies.

1.13.2. GLYCATED PLASMA PROTEINS

The measurement of glycated plasma proteins, especially glycated albumin have received much attention as monitors of short-term glycaemia due to their rapid turnover as compared to haemoglobin within the erythrocyte.
Although total glycated plasma proteins have been shown to be a sensitive indicator of hyperglycaemia (McFarland et al., 1979; Dolhofer and Wieland, 1980), the measurement of individual glycated plasma proteins is preferred due to problems in interpreting total glycated plasma proteins. The latter is a heterogeneous mixture of proteins with different half-lives, different numbers of reactive amino groups and competitive binding of particular proteins to other molecules.

Most attention has been devoted to glycated albumin (GA), which is the most abundant plasma protein (approximately 70-80% of the total) and has a half-life of about 20 days (Peters, 1985).

The correlations of GA with various indices of glycaemia are varied and depend particularly on the group of patients studied. This is due mainly to the differences in stability of blood glucose levels between NIDD and IDD subjects (as described in previous section on glycated haemoglobin). In all groups of subjects (including normal subjects) it has been found that GA correlates the strongest with the mean blood glucose level calculated from frequent daily blood glucose measurements (Dolhofer and Wieland, 1980; Ziel and Davidson, 1987).

A significant correlation is generally found between GHb and GA/total plasma glycated proteins in diabetic subjects (McFarland et al., 1979; Dolhofer and Wieland, 1980; Jones et al., 1983; Murtiashaw et al., 1983; Ziel and Davidson, 1987). Although, Guthrow et al., (1979) found a poor correlation between GHb and GA in both normals and diabetics. This finding may be partly due to the wide group of subjects studied, which included both NIDD and IDD subjects, with duration of diabetes ranging from 3 - 20 years and also normal subjects. All the diabetic subjects were defined as being poorly controlled, therefore the short-term changes in glycaemia may not mirror the long term changes in these patients. So, the lack of correlation between GA and GHb was probably due to the different half-lives of the proteins, thus detecting different periods of glycaemia.

The main advantage of measuring GA is that it appears to be a much more sensitive indicator of short-term changes in glycaemia. Several studies have been performed following the rate of change of GA and GHb with the start of therapy in newly diagnosed or poorly stabilized diabetics (Dolhofer and Wieland, 1980; Kennedy et al., 1981; Jones et al., 1983). The percentage decrease in GA was much greater than in GHb and occurred more rapidly (within the first week.
of treatment). So, changes in glycaemia in response to treatment can be monitored more closely with GA than with GHb.

Another important difference found between GA and GHb is the lack of overlap found between diabetic and non-diabetic subject groups when average glycaemia is assessed by GA (McFarland et al., 1979; Guthrow et al., 1979; Schleicher et al., 1984; Ziel and Davidson, 1987). This probably reflects both the shorter half-life of albumin and its greater tendency to become glycated (Olufemi et al., 1987), so that it detects smaller changes in blood glucose than GHb.

Despite GA having several advantages over GHb in the monitoring of blood glucose concentrations, GHb (either HbA1 or HbA1c) is generally used, probably reflecting methodological differences between the two proteins. Assays for GA are fairly laborious and lack automation, whereas GHb can be measured by one of several simple and relatively easily automated procedures. However, a different type of assay for the measurement of total glycated plasma proteins was developed by Johnson et al., (1982), the so-called fructosamine assay. It has since been automated (Lloyd and Marples, 1984) and has been shown to be cheap, precise and simple to perform. However, as well as having many methodological difficulties, it has been shown that only 50% of the fructosamine activity measured is due to glycated proteins (Howey et al., 1987; Schleicher et al., 1988). The nature of the other 50% is unclear but does not appear to be albumin associated and differs from patient to patient (Howey et al., 1987). Fluckiger et al., (1987) showed that there was vast intra-individual variation in fructosamine concentration that was not accounted for by correction for protein concentration, suggesting that other factors must be responsible for this variability.

Despite these findings, numerous clinical studies have shown that the fructosamine concentration correlates significantly with various indices of glycaemia and with other glycated proteins (Johnson et al., 1982; Lloyd and Marples, 1984; Baker et al., 1985; Mosca et al., 1987). Dominczak et al., (1988) followed fructosamine and GHb for 8 - 18 weeks in both IDD and NIDD subjects and found both tests of glycation to be useful monitors of glycaemic control. They concluded that the fructosamine and GHb assays should be used as complementary rather than alternative tests. Jerntorp et al., (1988) on the other hand found that in a group of diabetics, whilst only 4% had normal GHb levels, 39% had normal fructosamine concentrations. Also, after two weeks of treatment, fructosamine
and GHb both decreased, but after six weeks, although GHb had decreased further fructosamine remained unchanged.

So, although glycated plasma proteins when measured by specific assays (i.e. GA) have significant advantages over GHb for the monitoring of blood glucose levels, plasma glycated proteins as measured by the fructosamine assay does not appear to accurately reflect changes in blood glucose, probably due to a variable contribution made by a non-specific unglycated component.

Other plasma proteins studied as monitors of short-term blood glucose control, include transferrin which has a half-life of 8 days. Kemp et al., (1984) followed GA and glycated transferrin in 73 diabetic children. After 10 days of careful control mean GA fell from 16.4 to 14.6% and mean glycated transferrin fell from 8.2 to 6.7%.

1.14. PATHOPHYSIOLOGICAL EFFECTS OF GLYCATION

There are two mechanisms as to how non-enzymic glycation might lead to or contribute to the chronic complications of diabetes and perhaps also to the normal process of ageing. The first concept is that glycation might involve a site which is crucial to the specific functioning or normal metabolism of a protein. This might be particularly relevant for relatively short-lived proteins with highly specialised functions. The second concept is that glycation leads to an alteration in structure or physical properties of a protein. This would be especially relevant to long-lived tissue proteins.

1.14.1. SHORT-LIVED PROTEINS

1.14.1.1. Haemoglobin

The amino-terminal of the β-chain of haemoglobin to which glucose attaches is also the site where 2,3-diphosphoglycerate binds. 2,3-diphosphoglycerate influences the affinity of haemoglobin for oxygen through its ability to bind to deoxyhaemoglobin. When the amino-terminals of the β-chains are covalently linked to glucose, the availability of these sites for interaction with 2,3-diphosphoglycerate are compromised (Bunn and Briehl, 1970). Hence, GHb exhibits greater oxygen affinity than unglycated haemoglobin in the presence of 2,3-diphosphoglycerate (Ditzel et al., 1975; McDonald et al., 1979). When GHb is increased in diabetic red blood cells, the oxygen affinity of these cells in the presence of 2,3-
diphosphoglycerate is slightly greater than that of red cells from non-diabetic subjects (Arturson et al., 1974). Whether the minor shift in the haemoglobin-oxygen dissociation curve arising from increased GHb in diabetics has a major impact is unclear. Ditzel suggested that it promotes hypoxia, which in turn could contribute to diabetic complications (Ditzel & Standl, 1979; Ditzel et al., 1979; Ditzel, 1980). However, this interpretation rests on the extrapolation of in vitro data to in vivo situations. Ditzel et al., (1975) did find an increased oxygen affinity in the blood from diabetic children and it is possible that an increased GHb coupled with low red cell 2,3-diphosphoglycerate in diabetes (Standl and Kolb, 1973) might result in tissue hypoxia.

However, haemoglobinopathies produce greater shifts in the oxygen-dissociation curve without deleterious effects on tissue oxygenation, which makes it unlikely that the minor change in oxygen affinity associated with an increase in glycation of haemoglobin in diabetes significantly alters oxygen transport (Bunn et al., 1979).

1.14.1.2. Albumin

Although glycation of albumin does not affect its circulating half-life or metabolism (Day et al., 1979(b)), it can induce a conformational change in the protein altering its ligand binding properties (Shaklai et al., 1984). Williams (1981) showed that glycated albumin was taken up more avidly than native albumin by endothelial cells in vitro. Other experiments have shown that glycated albumin and other circulating glycated proteins in uncontrolled diabetes could increase transendothelial transport and contribute to increased capillary permeability associated with diabetes (Williams & Solenski, 1984; Williams & Segal, 1985). In support of this concept is the finding that the passage of glycated albumin through the glomerular filtration barrier is enhanced relative to that of normal albumin in diabetic patients with and without microalbuminuria as well as in normal people. McVerry et al., (1980) reported that the intravenous injection of glycated plasma proteins produces glomerular basement membrane thickening in non-diabetic mice. However, Nathka et al.(1984) performed a similar experiment and found no thickening of the glomerular basement membrane.
1.14.1.3. Lipoproteins

Glycation of low-density lipoproteins (LDL) has been implicated in the increased incidence of macrovascular disease associated with diabetes.

Initial in vitro studies showed that the binding and degradation of glycated LDL by cultured human fibroblasts and by umbilical vein endothelial cells was diminished compared to unglycated LDL and that the degree of reduction in degradation was greater with increased glycation (Kim & Karup, 1982; Saski & Cottam, 1982; Gonen et al., 1981 and Lorenzi et al. 1984).

However, it has since been argued that those in vitro studies used glycated LDL that had been reduced with sodium borohydride and hence represented unphysiological conditions. Schleicher et al. (1985) examined the effect of non-reductive glycation on the uptake and degradation of LDL in fibroblasts, hepatocyte macrophages and endothelial cells and found that glycation (1.8 - 4.6 glycated lysine residues per molecule of apolipoproteins) did not alter the interaction of LDL with its high affinity receptor. Only more heavily glycated LDL (more than 12 glycated lysine residues per molecule of apolipoproteins) or reduced glycated LDL exhibited reduced receptor-mediated internalisation and degradation by human fibroblasts.

The internalization and degradation of LDL from patients with IDDM and poor metabolic control is decreased compared to that of LDL isolated from normal subjects or from IDDM with good control (Lopez-Virella et al. 1982). There is little doubt that excess glycation of LDL occurs in vivo in diabetic patients with poor metabolic control, but whether glycated LDL plays an important role in the development of macrovascular disease is still equivocal.

1.14.1.4. Coagulation Proteins

Several pathophysiological consequences of excess glycation of factors involved in blood clotting have been suggested.

Increased glycation of lysine residues in fibrinogen may interfere with its function. However, in vitro studies showed that glycation of fibrinogen to a level of 3.8mol glucose/mole protein had no effect on clotting time nor factor VIII crosslinking of fibrinogen (Ney et al., 1985). Further in vitro studies showed that the glycation of
fibrin reduced its susceptibility to degradation by the enzyme plasmin (Brownlee, Vlassara & Cerami, 1984).

Antithrombin III is a coagulation-regulatory factor that binds to heparin and inhibits thrombin mediated cleavage of fibrinogen. Heparin binds at the ε-amino groups of lysine and is crucial to its function. In vitro glycation results in significant diminution of its thrombin-inhibitory activity (Brownlee, Vlassara & Cerami, 1984). Antithrombin III activity has also been shown to be reduced in patients with insulin-dependent and non-insulin dependent diabetes, the extent of the reduction correlating with fasting blood glucose concentrations and the GHb levels (Banerjee et al. 1974; Sowers et al. 1980). Glycation induced interference with antithrombin III activity could explain the accelerated disappearance of fibrinogen that is normalized by improving glycaemic control in diabetic patients (Jones & Peterson, 1979).

This inhibition of antithrombin III activity in conjunction with glycation induced changes in susceptibility of fibrin to degradation could play a role in the accumulation of fibrin reported to occur in several diabetic tissues affected by long-term complications (Brownlee et al., 1984).

Glycation may also alter platelet function. Although there is little information as to whether any of the changes described in diabetes, such as increased platelet adhesiveness and aggregation, derive from glycation of platelet proteins, there is evidence that glycation of collagen increases its aggregating potency (Le Pape et al., 1983).

1.4.1.5. Enzyme and Hormones

Even one first step in the glycation reaction, the reversible formation of the Schiff base could significantly alter the catalytic properties of certain types of enzymes. The most likely inactivation mechanism would involve the glucose attachment to a lysine ε-amino group essential for normal function of the active site.

In Ribonuclease A, for example, the loss of a single lysine at position 41 is known to cause total loss of enzyme activity. Incubation of the enzyme with glucose for 24 hours results in 50% loss of initial enzyme activity associated with glycation of two lysine residues per molecule (Brownlee et al., 1984).

Another study found that glycation of cathepsin B, an enzyme involved in proinsulin cleavage, partially inhibits the ability of the
enzyme to convert proinsulin to insulin in vitro (Coradello et al. 1981).

In vitro glycation of N-acetyl-D-glucosaminidase resulted in a reduction in its activity (Dolhofer et al., 1982). This enzyme participates in the catabolism of glycosaminoglycans which are integral constituents of connective tissues and extracellular matrices. Its activity is decreased in kidney and other tissues in diabetes. However, since the glycosaminoglycan content of basement membranes is reportedly decreased rather than increased in diabetes (Parathasarathy and Spiro, 1982; Cohen & Surma, 1984), it is unlikely that non-enzymic glycation of the enzyme in vivo is involved in the pathogenesis of diabetic basement membrane lesions.

In vitro evidence of insulin glycation has been reported (Dolhofer & Wieland, 1979). Glycation diminished the hormone's effect on the oxidation of $[^{14}C]$-glucose by adipose tissue, reduced its ability to stimulate lipogenesis in isolated adipocytes and decreased its antilipolytic activity. However, since insulin has a relatively short half-life, it is not clear whether in vivo glycation of the hormone would be to a sufficient extent to have impact on biologic function.

### 1.14.2. LONG-LIVED PROTEINS

The effect of glycation on the physical properties and structure of long-lived proteins is more complex than for the shorter-lived proteins and probably has more profound effects on their function.

Glycation of proteins resulting in the formation of the Amadori product is only the first step in a series of non-enzymic reactions long known to food chemists as the Maillard or Browning reaction (Hodge, 1953; Reynolds, 1963; Reynolds, 1965). The products of such reactions are highly cross-linked, insoluble, pigmented and fluorescent carbohydrate-protein polymers called melanoidins.

Maillard browning involves three phases, which were clarified over 25 years ago by Hodge (1953). Formation of the colourless deoxyketose derivative (Amadori compound) via the Schiff base intermediate is the first step and is sometimes referred to as the 'early Maillard reaction'. The next phase involves the removal of amino groups via 1,2- or 2,3-enolization reactions followed by dehydration, cyclization and fission reactions that generate secondary products such as hydroxymethylfurfural, reductones, aldehydes and dicarbonyls. In the third phase, polymerization reactions occur, yielding nitrogen-free brown pigments if the
complexing secondary phase products derive from the sugar moiety of the Amadori compound and nitrogen-containing polymers when secondary products react with amino groups (Finot, 1982) to form aldimes and ketimines. The first phase is relatively fast, whilst the second and third phases occur very slowly.

The formation of the Amadori product is well recognised and the evidence is overwhelming that it occurs in vivo. It is only recently that the possibility of advanced Maillard reactions with the formation of post-Amadori products might also occur in vivo and that they may be linked with both the pathogenesis of chronic complications occurring in diabetes and also with the normal process of ageing (Brownlee et al., 1984; Pongor et al. 1984).

The term now being adopted for substances which result from the further modification of the Amadori product is 'Advanced Glycosylation End (AGE) Products'. These AGE products form very slowly and irreversibly and may accumulate over many years, thus the biological half-life of the protein will be especially important in determining the extent to which these products form.

AGE products have been shown to be present in lens crystallin where they may result in cataract formation (Stevens et al. 1978; Monnier & Cerami, 1981) collagens where they may contribute to both micro and macro vascular disease (Brownlee et al., 1986), nerve tubulin and myelin affecting axoplasmic transport and therefore contributing to diabetic neuropathy (Williams et al., 1982) and also DNA (Bucala et al., 1984). The accumulation of AGE products on DNA may be responsible for age-dependent changes in the genetic material that include chromosomal aberrations, DNA stained breaks and a decline in DNA repair, replication and transcription. The amount of AGE products increases with age in normal subjects (Monnier et al., 1981). Diabetic individuals have a greater amount of AGE products relative to non-diabetics of similar age. This suggests that ageing of long-lived proteins is accelerated in subjects with diabetes.

The structure of one of the AGE products has been characterised, 2-furoyl-4(5)-(2-furany1) 1H-imidazole (FFI) (Pongor et al., 1984). However Njoroge et al. (1988) have recently shown that FFI can also form from the acid hydrolysis of Amadori products in vitro and may therefore be an artefact.

Preliminary evidence for the formation of a different type of compound, protein-bound pyrolles during the glycation of proteins in
vitro has been reported by Ghiggeri et al., (1985). Hayase et al. (1989) developed two different types of immunoassays to one of these glucose-derived pyroles, called pyrraline and found it to be present on albumin from both normals and diabetics and to a greater extent on diabetic albumin. They speculate that these pyroles might also be involved in ageing and diabetic complications.

Evidence has also been presented for the existence of two alternative pathways for the Amadori product that do not involve Maillard browning (Ahmed, 1986 and Ahmed, 1988) (see Fig. 1.5). Incubation of N-formyl-fructose lysine, an analogue of glycated lysine residues in proteins, under physiological conditions in vitro resulted in the formation of either carboxymethyl-lysine (CML) and erythronic acid or lysino-lactic acid (LL) and D-glyceraldehyde as a result of oxidation. Both CML and LL have been detected in human lens protein and in human urine suggesting that they are also formed in vivo by degradation of glycated proteins. They are described as non-browning pathways of the Maillard reaction and appear to compete with the browning reactions. These products are relatively inert compared to cross-linked products, such as FFI and may limit the consequences of protein glycation in the body. Differences in rates of browning versus non-browning pathways among individuals may be important in determining their relative susceptibility to tissue damage from these reactions. This may explain why some individuals develop complications more rapidly compared to others, despite having the same levels of glycaemia.

So, there is much evidence to suggest that hyperglycaemia-induced glycation of proteins is involved in the development of chronic complications in diabetes both through early Maillard reactions mainly affecting short-lived proteins and with the formation of AGE products on long-lived proteins.

The inhibition of the glycation reaction at any stage in the sequence of reactions may help to alleviate or prevent the development of these complications. Brownlee et al.(1986) have shown that a nucleophilic hydrazine compound, aminoguanidine reduces the formation of fluorescent AGE's and the formation of glucose-derived collagen cross-links in vivo. The administration of aminoguanidine to diabetic rats prevented the accumulation of fluorescent AGE in aortic connective tissue; and also inhibited the increase in collagen cross-linking seen in aortic tissue from untreated animals suggesting a potential role for aminoguanidine in the prevention and treatment of chronic diabetic complications.
FIGURE 1.5. PROPOSED PATHWAYS FOR FURTHER METABOLISM AND DEGRADATION OF NON-ENZYMATIC GLYCATION PRODUCTS AND HYPOTHETICAL ROLES IN THE FORMATION OR PREVENTION OF CHRONIC DIABETIC COMPLICATIONS

GLUCOSE → PROTEIN

AMADORI PRODUCT (KETOAMINE)

→ OXIDATIVE CLEAVAGE

ADVANCED GLYCATION END(AGE) PRODUCTS (eg. FFI)

→ DEVELOPMENT OF COMPLICATIONS?

CARBOXYMETHYLLYSINE + ERYTHRONIC ACID

LYSINOLACTIC ACID + GLYCERIC ACID

→ PREVENTION OF COMPLICATIONS?
1.15. BACKGROUND AND AIDS OF THESIS

Between 1983 and 1985 a two-phase screening survey for diabetes was conducted in North London, the so-called Islington Diabetes Survey (Forrest et al., 1986; Forrest et al., 1987).

A random sample of non-diabetic patients over the age of 40 registered with a single group practice were selected for study and 1084 of the 1644 eligible subjects (65.9%) were examined in phase I. They underwent a simplified glucose tolerance test consisting of a 75g glucose load taken in the form of lucozade in the morning after an overnight fast and a capillary blood sample taken 2 hours later for the estimation of glucose (2HBG) and GHB measured by electrophoresis without removal of the Schiff base. In phase II of the study a stratified sample of those originally examined were selected biased towards subjects with more marked degrees of glucose-intolerance. There were 347 subjects recalled and 223 (64.3%) were examined. The recall examination comprised a full OGTT with venous blood samples being taken at fasting, 1 hour and 2 hours after the glucose load for the measurement of glucose and for the assay of GHB by four different methods on the fasting and 2 hour samples. GHB was measured by agar gel electrophoresis, with and without the removal of the Schiff base, isoelectric focusing and affinity chromatography.

To overcome the problem of the use of capillary blood at screening and venous blood at recall, and by the different ranges for the various assays of GHB, all 223 subjects examined at recall were classed into centile rankings of 2HBG at screening and recall, and of GHB at screening and by each assay method both fasting and 2 hour-post glucose at recall. The mean centile ranking for 2HBG and GHB were then calculated.

An interesting observation in this study was the unusually poor correlation found between the mean GHB and the mean 2HBG (r = 0.51, see Fig. 1.6(a) and (b)). The relationship was the same between 2HBG at recall or screening and any of the individual GHB assays. There were 42 subjects (non-diabetics) in whom the mean 2HBG ranking differed by at least 30 centiles from their mean ranking for GHB (see Fig. 1.7). Twenty had a mean ranking for GHB exceeding 30 centiles higher than for 2HBG (HIGH GLYCATORS) and twenty-two had a mean GHB ranking more than 30 centiles lower than for 2HBG (LOW GLYCATORS). There was no difference in age, gender, body mass
FIGURE 1.6 (a) MEAN CENTILE RANKINGS OF TWO-HOUR BLOOD GLUCOSE AND OF ALL THE ASSAYS FOR GLYCATED HAEMOGLOBIN AT SCREENING AND RECALL

The line of agreement is indicated.

The corresponding levels of blood glucose, and of two-hour post-load assays of glycated haemoglobin are indicated.

Total GHb - agar-gel electrophoresis without the removal of the Schiff base

Stable GHb - agar-gel electrophoresis with prior removal of the Schiff base

Aff - affinity chromatography

IEF - isoelectric focusing
FIGURE 1.6.(b) ARITHMETIC MEAN LEVELS OF TWO-HOUR BLOOD GLUCOSE AND ALL OF THE ASSAYS FOR GLYCATED HAEMOGLOBIN

High glycators represented as (•) and low glycators as (▲).

Mean glycated haemoglobin %

r = 0.51

Mean 2h blood glucose mmol/l
FIGURE 1.7 MEAN CENTILE RANKINGS OF TWO-HOUR BLOOD GLUCOSE AND OF ALL OF THE ASSAYS FOR GLYCATED HAEMOGLOBIN AT SCREENING AND RECALL IN THE HIGH AND LOW GLYCATORS

The line of agreement is indicated.

The corresponding levels of blood glucose, and of two-hour post-load assays of glycated haemoglobin are indicated.

High glycators represented as (●) and low glycators as (▲).

Abbreviations as for Figure 1.6.
index or haemoglobin level between the high and low glycators (p > 0.1). Nine of the high (45%) and four of the low (18%) were cigarette smokers. Seven of the high glycators and five of the low glycators were taking medication; two high and one low glycator were taking non-steroidal anti-inflammatory drugs; two high glycators were on thiazides; one high and one low glycator were on nitrates and one high and one low glycator on digoxin; and one each was taking benzodiazepines (high), antacids (high), β-blockers (high), thyroxine (low) and aminophylline (low).

Due to the unphysiological nature of the OGTT the high and low glycators were also classed into rankings of fasting blood glucose (FBG) (see Fig. 1.8). There was a less marked difference between median rankings of GHb and of glycaemia than when classified by 2HBG, but the difference was still apparent. 18 out of 20 high glycators and 16 out of 22 low glycators remained in the same categories when classified by FBG.

The contribution of OGTT variability and of both assay and biological variability to the weakness of the relationship between GHb and glycaemia has also been assessed (Yudkin et al., in press), but were not found to be major determinants of the poor relationship.

So, the marked differences in individual levels of GHb found in this group of non-diabetics could not be explained by either fasting glycaemia or tolerance to a glucose load. Certain diets have been shown to have marked effects on both fasting glycaemia and tolerance to an unphysiological glucose load. Thus, starvation or a carbohydrate-restricted diet causes intolerance to a glucose load (Wilkerson et al., 1960), whilst causing a reduction in fasting blood glucose and GHb (Ktorza et al., 1985). A high carbohydrate intake has been reported to improve glucose tolerance (Brunzell et al., 1971). However, a high carbohydrate, high fibre diet may lower fasting blood glucose levels despite an increase in post-prandial levels of blood glucose (Simpson et al., 1979).

So, a dietary survey was carried out by 25 of the subjects (60%) (12 high glycators and 13 low glycators), a mean of 2.3 years after the original screening survey, to investigate whether differences in diet might explain the inappropriate levels of GHb relative to the degree of glycaemia (Yudkin et al., in press). Each subject was asked to record a 7-day food diary and then the daily intake of all nutrients were calculated from standard food tables. Table 1.4 shows the mean levels of nutrient intakes in the low and high glycators. There
FIGURE 1.8  CENTILE RANKINGS OF FASTING BLOOD GLUCOSE AND MEAN CENTILE RANKINGS OF ALL THE ASSAYS FOR GLYCATED HAEMOGLOBIN AT SCREENING AND RECALL IN THE LOW AND HIGH GLYCATORS

The line of agreement is indicated.

High glycators represented as (●) and low glycators as (▲).

The corresponding levels of blood glucose, and of two-hour post-load assays of glycated haemoglobin are indicated.

Abbreviations as for Figure 1.6.
TABLE 1.4. DAILY NUTRIENT INTAKE IN THE LOW AND HIGH GLYCATORS

<table>
<thead>
<tr>
<th></th>
<th>Low glycators (n=13)</th>
<th>High glycators (n=12)</th>
<th>Significance of difference (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>65.8 (13.0)</td>
<td>60.1 (0.3)</td>
<td>0.24</td>
</tr>
<tr>
<td>BMI</td>
<td>25.0 (3.4)</td>
<td>26.4 (4.3)</td>
<td>0.37</td>
</tr>
<tr>
<td>Total Carbohydrate (g)</td>
<td>195.9 (40.5)</td>
<td>194.6 (58.0)</td>
<td>0.95</td>
</tr>
<tr>
<td>Starches &amp; Dextrins (g)</td>
<td>111.6 (34.6)</td>
<td>108.5 (33.1)</td>
<td>0.87</td>
</tr>
<tr>
<td>Sugars (g)</td>
<td>75.1 (27.9)</td>
<td>76.0 (29.2)</td>
<td>0.94</td>
</tr>
<tr>
<td>Glucose Equivalents (g)</td>
<td>157.5 (35.2)</td>
<td>157.9 (48.4)</td>
<td>0.98</td>
</tr>
<tr>
<td>% Energy from Carbohydrates</td>
<td>41.7 (8.4)</td>
<td>38.8 (8.5)</td>
<td>0.39</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>67.1 (18.7)</td>
<td>67.9 (18.3)</td>
<td>0.91</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>1.15 (0.30)</td>
<td>1.35 (0.41)</td>
<td>0.19</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>53.5 (36.6)</td>
<td>67.9 (32.4)</td>
<td>0.31</td>
</tr>
<tr>
<td>Total Fibre (g)</td>
<td>16.6 (5.7)</td>
<td>19.2 (9.4)</td>
<td>0.42</td>
</tr>
<tr>
<td>Soluble Fibre (g)</td>
<td>7.3 (2.3)</td>
<td>8.2 (3.6)</td>
<td>0.49</td>
</tr>
<tr>
<td>Energy (Cals)</td>
<td>1806 (465)</td>
<td>1864 (429)</td>
<td>0.75</td>
</tr>
<tr>
<td>Alcohol (g) (median,range)</td>
<td>2.7 (0-36)</td>
<td>1.0 (0-57)</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Values are shown as mean (SD) for normally distributed data and median (range) for skewed data which are so indicated. The former are compared using Student's unpaired t-test and the latter by Mann-Whitney U-test.
was no significant difference in the intake of any of the major nutrients or of fibre between the two groups. In particular, the consumption of total carbohydrates, sugars, glucose equivalents, total fibre and soluble fibre were no different between high and low glycators. Also, no significant differences between the two groups could be identified when nutrient intake was expressed as a proportion of the total energy intake or as a percentage of body weight. Vitamin C and vitamin B₆ were found to be marginally higher in the high glycators. However, again these differences were no longer significant when corrected for body weight or total energy intake. So, dietary patterns also failed to explain the difference in levels of GHb relative to glycaemia in the high and low glycators.

Therefore the aims of this thesis were divided into three main parts, as follows:

1. INVESTIGATION OF THE HIGH AND LOW GLYCATORS

(a) Stability of the high and low glycators

This involved recalling the original 42 subjects and performing a repeat OGTT to determine whether they still remained classified in the same two groups, four years after the original screening survey.

(b) Measurement of ambient glycaemia in the low and high glycators

Blood glucose profiles were performed on the two groups of subjects and the mean blood glucose calculated to determine whether ambient glycaemia was responsible for the differences in GHb.

(c) Determination of the cause(s) of the high and low glycation

A detailed biochemical and haematological analysis was carried out on the two groups of subjects to investigate whether there may be determinants other than glucose affecting their glycation of haemoglobin.

2. INVESTIGATION OF THE EFFECT OF DIET ON GLYCATION

Although the dietary survey failed to show any significant differences between the two groups of subjects, the imprecision and poor reproducibility of the dietary history as a means of assessing...
intake is well known (Burke, 1947; James et al., 1980). Therefore a study was undertaken to investigate the effect of specific dietary components, carbohydrate and fibre on glucose tolerance and glycation of proteins in a small group of healthy volunteers.

3. INVESTIGATION OF THE EFFECT OF VITAMIN C ON GLYcation

Although the intake of another dietary component, vitamin C was not significantly different between the 2 groups it has been shown to specifically affect the glycation of proteins in vitro, therefore a third study was carried out investigating the effect of oral vitamin C on the glycation of proteins in vivo in healthy, human volunteers.
CHAPTER 2

EXPERIMENTAL METHODS
2.0 INTRODUCTION

The methods described in this chapter were used for all the analyses carried out in Chapters 3, 4 and 5.

All the reagents used were of Analar grade and obtained from BDH (BDH Limited, Poole, Dorset), unless otherwise stated.

2.1 GLYCATED HAEMOGLOBIN

Glycated haemoglobin was measured by three different methods, affinity chromatography (Gould et al., 1982), isoelectric focussing (Mortensen, 1980) and agar-gel electrophoresis (Menard et al., 1980).

2.1.1 AFFINITY CHROMATOGRAPHY

2.1.1.1 REAGENTS

GLYCOGEL B - m-aminophenylboronate agarose (Pierce and Warriner, Chester, U.K.).

WASH BUFFER-200 mmol/l ammonium acetate
   50 mmol/l magnesium chloride
   3 mmol/l sodium azide

The pH was adjusted to 8.5 using 1M sodium hydroxide or 1M hydrochloric acid.

ELUTION BUFFER-200 mmol/l sorbitol
   50 mmol/l disodium EDTA
   3 mmol/l sodium azide
   100 mmol/l tris (hydroxymethyl) methylamine

The pH was adjusted to 8.5 using 1M sodium hydroxide or 1M hydrochloric acid.

REGENERATION SOLUTION- 0.01M acetic acid.
2.1.1.2. APPARATUS

Minicolumns were obtained from Biorad Laboratories Limited (Watford, Herts, U.K.) for the affinity chromatography.

The absorbances of the fractions were measured on a Uvikon 860 Spectrophotometer (Kontron Instruments, Zurich).

2.1.1.3. PROCEDURE

100 μl of potassium EDTA packed erythrocytes was added to 2 mls of deionised water and vortex mixed to haemolyse the cells. 5 mls of wash buffer at 20 ±1°C was added to the affinity columns each of which contained 1 ml of the gel, so that the latter equilibrated at the correct pH and temperature. When drained, the columns were placed onto 25 ml measuring cylinders, 100 μl of haemolysate added to the top of the gel and allowed to soak in. This was followed by 1 ml and then a further 7 mls of wash buffer. Once this had drained through, the wash fraction collected in the measuring cylinder was made up to 15 mls with wash buffer. The columns were then placed onto 10 ml plastic tubes and the glycated haemoglobin eluted with 3 mls of elution buffer at 20 ±1°C. The wash fraction was mixed by inversion and the elution fraction by vortexing. The absorbances of the two fractions was measured at 414 nm, using the wash buffer as a blank. The percentage of glycated haemoglobin was determined by the following equation:

\[
GHb(\%) = \frac{A_e}{(A_w \times 5) + A_e} \times 100
\]

where \( A_e = \) Absorbance of elution fraction
\( A_w = \) Absorbance of wash fraction
2.1.2. ISO-ELECTRIC FOCUSING

2.1.2.1. REAGENTS

AMPHOLINE POLYACRYLAMIDE GELS for the determination of HbA₁c were obtained from LKB (LKB Products, Bromma, Sweden).

- 0.9% sodium chloride
- 1M potassium cyanide
- Carbon tetrachloride

ELUTION BUFFER - 0.05M Tris-HCl buffer, pH 8.3 containing 0.01M potassium cyanide and 0.1 mM EDTA.

ELECTRODE SOLUTIONS - Cathode - 0.1M sodium hydroxide
- Anode - 0.04M glutamic acid

2.1.2.2. APPARATUS

LKB (Bromma, Sweden) Multiphor II electrophoresis unit (2117).
- LKB Power supply
- Uvikon 860 Spectrophotometer (Kontron Instruments, Zurich, Germany).

2.1.2.3. PROCEDURE

The instructions provided by LKB with their gels were used to carry out the measurement of HbA₁c as follows:

Preparation of haemolysate

100 μl of potassium EDTA whole blood was added to 1 ml of 0.9% sodium chloride and vortex mixed. After centrifugation at 3000 g (room temperature) for 5 minutes, the supernatant was removed taking care not to disturb the erythrocyte pellet. This was followed by the addition of 600 μl of 0.01M potassium cyanide and 1 drop of carbon tetrachloride. After vortex-mixing, the sample was further centrifuged at 3000 g (room temperature) for 10 minutes. Then 0.5 mls of the clear supernatant was pipetted into a new tube.
Isoelectric focusing

15 - 30 minutes prior to electro-focusing, cold water was circulated in the cooling plate. A small volume of liquid paraffin was spread over the cooling plate to make a thin film. Then the gel was removed from the package and carefully lowered onto the cooling plate, avoiding getting air bubbles between the gel and the plate.

Each electrode strip (wick) was saturated with the appropriate electrode solution and the excess removed. These were then carefully laid onto the gel 1-2 mm from the edge on the correct sides for focusing (the strips should be cut to fit the gel length). The electrofocusing lid was then put on, positioning the electrodes on the wicks. The electrofocusing unit was then connected to the power supply and the voltage set to 2000V, the power to 20W and the current to 50 mA.

The gel was first run without samples for 30 minutes. Then the power supply was switched off and the sample application pieces placed on the gel surface, about 1cm from the cathode electrode strip and 3 mm apart. Up to 30 samples could be applied per gel. Then 15μl of each haemolysate was pipetted onto each sample application piece. Then the electrofocusing lid was replaced and the power switched on again. After 40 minutes the sample application pieces were removed and electrofocusing carried out for a further 70 minutes.

When focusing was complete the power supply was switched off and each HbA1c band cut out of the gel and put into 1 ml of eluting buffer. The HbA1c was eluted for 12 - 24 hours at 4°C with constant mixing. Total haemoglobin (HbA0) solutions were prepared by adding 15 μl of each haemolysate to 5 mls of the elution buffer and mixing well. The absorbance of the HbA1c and HbA0 fractions were measured at 415 nm and the percentage of HbA1c calculated as follows:

\[
\% \text{HbA}_{1c} = \frac{\text{Absorbance of HbA}_{1c}}{5 \times \text{Absorbance of HbA}_{0}}
\]
2.1.3. AGAR-GEL ELECTROPHORESIS

Glycated haemoglobin (HbA₁) was assayed with and without the removal of the labile Schiff base using the Glytrak TM Kit obtained from Corning Medical Limited, Halstead, Essex, U.K.

2.1.3.1. REAGENTS (all supplied by Corning)

AGAR GEL PLATES - 2% (w/v) agar gel containing 4% sorbitol, 0.036M sodium citrate, 0.002M citric acid and 0.003M disodium EDTA pH 6.3.

GLYOSYLATED HAEMOGLOBIN BUFFER - Supplied in vials each containing 27.9 g of citric acid trisodium salt dihydrate and 0.98 g citric acid. Each vial was made up to 11 with distilled water (0.1M, pH 6.3).

HAEMOLYSING REAGENT - Supplied in 15 ml vials, each containing 0.1% saponin and 0.05% EDTA in distilled water.

LABILE-REMOVING HAEMOLYSING REAGENT - Supplied in 15 ml vials, each containing 0.1% Saponin and 0.05% EDTA in potassium dithiolate buffer.

2.1.3.2. APPARATUS (supplied by Corning)

Cassette electrophoresis cell (No. 470130)
Incubator/oven (No. 470041/40)
Variable Voltage Power Supply (No. 470024)
Densitometer (No. 475710/360)

2.1.3.3. PROCEDURE

The procedure employed was as described in the instructions provided by the manufacturer, which are as follows:

Sample Preparation

100 µl of potassium EDTA whole blood was added to 300 µl of labile-removing haemolyzing reagent and also to 300 µl of the other haemolyzing reagent (measuring total HbA₁ inclusive of the Schiff base). The latter were vortexed mixed and then ready to use. The former samples were incubated at 37°C for 15 minutes prior to electrophoresis.
Electrophoresis

95 mls of buffer was placed in each chamber of the electrophoretic cell. Then 10 μl of each haemolysate was added to each of the wells in the gel plate. The gel plate was then loaded securely into the cassette holder and placed in the electrophoretic cell. Electrophoresis was carried out at 60V for 40 minutes. Then the gel plate was removed from the cassette holder and dried completely in the oven at 55 ±10°C (10 - 20 minutes). The gel was then scanned at 420 nm in the densitometer and the glycated haemoglobin (both total and with the labile removed) expressed as a percentage of the total.

2.2. GLYCATED ALBUMIN

Plasma glycated albumin was measured by first separating unglycated and glycated albumin by affinity chromatography, followed by the analysis of the albumin concentration in the two fractions using rocket immunoelectrophoresis (John and Jones, 1985).

2.2.1. AFFINITY CHROMATOGRAPHY

2.2.1.1. REAGENTS

GLYCOGEL B (Pierce and Warriner, Cheshire, U.K.)

WASH BUFFER - 250 mmol/l ammonium acetate
50 mmol/l magnesium chloride
3 mmol/l sodium azide

The pH was adjusted to 8.5 using 1M sodium hydroxide or 1M hydrochloric acid.

ELUTION BUFFER - 200 mmol/l sorbitol
50 mmol/l disodium EDTA
100 mmol/l tris(hydroxymethyl)methylamine
3 mmol/l sodium azide

The pH was adjusted to 8.5 using 1M sodium hydroxide or 1M hydrochloric acid.

REGENERATION SOLUTION - 0.01M acetic acid.
2.2.1.2. APPARATUS

Minicolumns were obtained from Biorad Laboratories Limited (Watford, Hertfordshire, U.K.) for the affinity chromatography.

2.2.1.3. PROCEDURE

100 µl of lithium heparin plasma was diluted in 1.5 mls of distilled water. 150 µl of the diluted plasma was then applied to a 1 ml affinity column already equilibrated with 5 mls of wash buffer (temperature 20 ±1°C). 1 ml of wash buffer was then added and allowed to soak in, followed by a further 8 mls of wash buffer. The entire eluate containing the unglycated albumin was collected and freeze-dried. The glycated albumin was eluted from the column with 3 ml of elution buffer (temperature, 20 ±1°C), which was also freeze-dried. The unglycated albumin fraction was reconstituted in 20 ml of distilled water and the glycated fraction in 0.5 ml of water.

2.2.2. ROCKET IMMUNOELECTROPHORESIS

This was carried out according to Laurell (1966).

2.2.2.1. REAGENTS

ELECTROPHORESIS BUFFER - 0.06M Barbitone buffer, pH 8.6
0.9% sodium chloride
1% agarose
50% polyethylene glycol

COOMASSIE BLUE STAIN - 1 g of Coomassie Brilliant Blue C (Sigma Chemical Co., Poole, Dorset, U.K.) was dissolved in 50 ml ethanol. 20 ml of glacial acetic acid and 130 ml of deionised water was added. Then it was left overnight and filtered before use.

DESTAIN SOLUTION - 50 ml methanol
100 ml glacial acetic acid
850 ml deionised water

ANTISERUM - Guildhay (Antisera Limited, Guildford, Surrey, U.K.)
Sheep anti-human albumin
2.2.2.2. STANDARD

Human albumin SPS01 (Sheffield Protein Reference Unit, Yorkshire, U.K.). 40 g/l diluted in 0.9% saline to produce a range of standards 2.5 - 40 mg/l.

2.2.2.3. APPARATUS

Glass plates 21 x 10 cm
Electrophoresis tank (Shandon Southern Products Limited, Runcorn, Cheshire, U.K.)
Power-pack SAE 2761 (Shandon Southern Products Limited)

2.2.2.4. Procedure

Preparation of gel

22.4 ml of molten agarose was added to 1.6ml of 50% polyethylene glycol (PEG) 6000, mixed thoroughly and warmed to 56°C. Then 40µl of anti-albumin antisera was added to the agarose/PEG solution. After mixing well, the mixture was drawn quickly into a warmed 30 ml syringe and smoothly injected into a mould (2 glass plates 21 x 10 cm clamped together). The gel was allowed to solidify and stored at 4°C in a moisture chamber at least overnight.

Electrophoresis

When required the glass plates were carefully separated and any air bubbles removed from under the gel. A row of 30 wells were cut into the gel using a 4 mm punch (0.6 cm apart and 1.5 cm from the lower edge).

The gel on the glass plate was then placed in the electrophoresis tank (which was kept cooled on a cold tray) in contact with filter paper (3 mm) wicks. A small voltage (5 - 10V) was applied across the gel whilst 10 µl of the standards and samples were quickly applied. The voltage was then set to 100V and left to run overnight (approximately 16 hours).

At the end of electrophoresis the gel was removed from the tank and compressed with a heavy weight. After 20 - 30 minutes, the gel was dried with a hair-dryer. The gel was then stained, destained and dried again. The peak heights of the rockets were measured and
the concentration of albumin in each fraction calculated by comparison with the standards.

The percentage of glycated albumin of the total albumin was calculated as follows:

\[
\% G.A. = \left( \frac{\text{GLYCATED ALBUMIN}}{\text{GLYCATED ALBUMIN} + 40 \times \text{UNGLYCATED ALBUMIN}} \right) \times 100
\]

where G.A. = GLYCATED ALBUMIN

2.3. FRUCTOSAMINE

Plasma fructosamine was measured using the method of Johnson and Baker (1982) adapted onto the Cobas Bio Centrifugal analyser (Lloyd and Marples, 1984).

2.3.1. REAGENTS

0.25 mmol/l nitroblue-tetrazolium (Sigma Chemical Company, Poole, Dorset, U.K.) prepared in 0.1 M carbonate buffer, pH 10.35.

150 mmol/l sodium chloride

40 g/l human albumin solution (Central blood products laboratory authority, Hertfordshire, U.K.) prepared in 150 mmol/l sodium chloride.

2.3.2. STANDARD

A 40 mmol/l stock solution of 1-deoxy-1-morpholinofructose (Sigma Chemical Company, Poole, Dorset, U.K.) was prepared in the human albumin solution. This was diluted in the human albumin solution to produce a series of standards 0 - 4 mmol/l.
2.3.3. APPARATUS

Cobas Bio sample cups, rotors and reagent trays (Roche Limited).

2.3.4. PROCEDURE

500 μl of plasma was pipetted into the sample cups in the sample rotor, which was then loaded onto the Cobas Bio, the reagent and standards were pipetted into the reagent rack which was also loaded onto the analyser.

The parameter listing for the fructosamine assay was as shown in Table 2.1.
TABLE 2.1  PARAMETER LISTING
FOR THE FRUCTOSAMINE ASSAY

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>UNITS</td>
<td>mmol/l</td>
</tr>
<tr>
<td>2</td>
<td>CALCULATION FACTOR</td>
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<tr>
<td>3</td>
<td>STANDARD 1 CONC.</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>STANDARD 2 CONC.</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>STANDARD 3 CONC.</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>LIMIT</td>
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</tr>
<tr>
<td>7</td>
<td>TEMPERATURE(°C)</td>
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</tr>
<tr>
<td>8</td>
<td>TYPE OF ANALYSIS</td>
<td>5</td>
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2.4. GLUCOSE

Plasma and whole blood glucose were measured on a Cobas Bio using a hexokinase kit (Roche Diagnostics, Limited). Intra-erythrocyte glucose was calculated from the whole blood and plasma measurements using the following formula:

\[
[\text{GLUCOSE}]_E = [\text{GLUCOSE}]_P - \frac{[\text{GLUCOSE}]_P - [\text{GLUCOSE}]_{WB}}{HcT}
\]

where E, P and WB are the erythrocyte, plasma and whole blood glucose concentrations respectively and Hct is the haematocrit (Higgins et al., 1982).

Glucose was also measured on capillary blood spots collected onto 1% boric acid impregnated filter paper using a glucose oxidase kit, (Boehringer Mannheim, Lewes, Sussex, U.K.) on the Cobas Bio (Burrin and Price, 1984).

2.4.1. WHOLE BLOOD AND PLASMA HEXOKINASE METHOD

2.4.1.1. REAGENTS

0.6M Perchloric acid (ice-cold)

0.9% sodium chloride

ASSAY REAGENT - supplied with kit

2.4.1.2. STANDARD

A 200 mmol/l stock solution of glucose was prepared in deionised water. This was diluted to produce a range of standards, 5, 10 and 20 mmol/l. These three standards were then diluted 1 in 4 with 0.6M perchloric acid, so that there were at the same pH and dilution as the samples.
2.4.1.3. APPARATUS

See section 2.3.2.

2.4.1.4. PROCEDURE

Sample preparation

200 μl of whole blood or plasma were added to 800 μl of ice-cold 0.6M perchloric acid and vortexed thoroughly. The samples were then centrifuged at 3000 g (at room temperature) for 10 minutes. The supernatants were then used for the assay.

The plasma and whole blood extracts were pipetted into the sample cups on the sample rotor which was then loaded onto the Cobas Bio. The reagent and standards were pipetted into the reagent tray which was also loaded onto the analyser. The parameter listing for the hexokinase assay is shown in Table 2.2.

To take into account the protein content of the plasma and whole blood the final results were multiplied by 0.94 for plasma (Cannon et al., 1974) and 0.85 for whole blood (Bergmeyer, 1984). These figures were then entered into the equation to calculate the erythrocyte glucose concentration. The water content of the erythrocyte is 71% (Hald and Eisenman, 1937) however approximately 15% of this is unavailable and is adsorbed onto haemoglobin (Drabkin, 1950). Therefore, the calculated intracellular glucose concentration was divided by 0.60 which is the fraction of water available in the erythrocyte.

2.4.2. BLOOD SPOT GLUCOSE OXIDASE METHOD

2.4.2.1. REAGENTS

1% boric acid

2.5% trichloroacetic acid

0.9% sodium chloride

31 ET chromatography paper (Whatman Limited, Maidstone, Kent, U.K.).

ASSAY REAGENT (supplied in kit).
2.4.2.2. STANDARDS

A 200 mmol/l glucose stock solution was prepared in 0.9% saline. This was used to prepare a range of standards 5, 10 and 20 mmol/l. These were diluted 1 in 50 with 2.5% trichloroacetic acid, so that they were at the same pH and dilution as the glucose in the eluant from the blood spots.

2.4.2.3. APPARATUS

See Section 2.3.3.

2.4.2.4. PROCEDURE

Preparation of Chromatography Paper

The chromatography paper was soaked in 1% boric acid for 5 - 10 minutes and then dried at 37°C.

Collection of Blood Spot

Each sample of capillary blood was collected in one large spot on the boric acid impregnated filler paper and then thoroughly dried at room temperature. These were stored at -20°C for 1 - 3 months.

Elution & Assay of Glucose in Blood Spot

A 6 mm (10 μl) disc of each blood spot was punched out and eluted in 500 μl of 2.5% trichloroacetic acid for 30 minutes. The eluants, standards and reagent were loaded onto the Cobas Bio and assayed for glucose using the parameter listing shown in Table 2.3.
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### TABLE 2.3. PARAMETER LISTING FOR GLUCOSE OXIDASE ASSAY

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2.5. INSULIN

Plasma insulin was measured by radioimmunoassay using an 'in house' reference method of St. Luke's Hospital Biochemistry Department, Guildford, Surrey.

2.5.1. REAGENTS

0.04M phosphate buffer, pH 7.4.

ASSAY BUFFER- 0.5% bovine serum albumin (Sigma Chemical Company, Poole, Dorset, U.K.) prepared in the 0.04M phosphate buffer.

Normal Guinea-pig serum (Guildhay Antisera Limited)

CHARCOAL-STRIPPED SERUM - obtained from colleagues in the Biochemistry Department at Guildford.

LABEL - A stock solution of iodinated insulin (I^{125}) obtained from Amersham, Bucks., U.K. was diluted 1 in 80 with assay buffer to produce the working label.

15% Polyethylene glycol 6000(dissolved in deionised water)

FIRST ANTISERUM - Guinea-pig anti-insulin antibody was supplied by Guildhay Antisera Limited in lyophilised form. Each vial contained 0.5 ml of diluted antiserum which was sufficient for either 100 or 1000 tubes with diluting the vials in 20.5 ml or 201 ml of assay buffer respectively. Normal guinea-pig serum was added to the first antiserum at a dilution of 1 in 500. Normal guinea-pig serum was also added to a small volume of assay buffer at a dilution of 1 in 500 for inclusion in the non-specific binding tubes.

SECOND ANTISERUM - Neat donkey anti-guinea pig antiserum supplied by Guildhay Antisera Limited was diluted 1 in 32 with the 15% polyethylene glycol solution.

2.5.2. STANDARD

A vial containing 1000 mU/l of insulin was obtained from Wellcome Diagnostics Limited, Dartford, Kent, U.K. This was diluted with charcoal-stripped serum to give a top standard of 200 mU/l. This
was used to prepare a working range of standards by double dilution in the stripped serum - 3.13, 6.25, 12.5, 25, 50 and 100 mU/l.

2.5.3. APPARATUS

J6B Centrifuge (MSE Scientific Instruments, Sussex, U.K.)
1260 Multigamma II gamma counter (LKB Wallac., Finland).

2.5.4 PROCEDURE

50 µl aliquots of stripped serum for non-specific binding (NSB) and zero standard tubes, standard sera, control sera or sample sera were pipetted into LP3 tubes (Luckham Limited, Burgess Hill, Sussex, U.K.). 200 µl of assay buffer containing the normal guinea-pig serum was added to the NSB tubes. To all the tubes, except the NSB and total tubes, 200 µl of working first antiserum was added, followed by 200µl of the working label to all of the tubes.

The tubes were vortex-mixed and incubated overnight (16 - 24 hours) at room temperature. Then 200 µl of the second antiserum working solution was added to all of the tubes except the totals. The tubes were vortex-mixed and incubated for a further two hours at room temperature.

Then all the tubes were centrifuged (except the totals) at 2500 rpm for 30 minutes at 4°C in the refrigerated J6B centrifuge (see apparatus). The supernatants were then aspirated off and the pellets counted in a gamma counter for 100 seconds.

2.5.5. CALCULATION OF RESULTS

The counts of the duplicates were meaned and the NSB counts subtracted from all the standard curve means, except the total. The mean NSB counts from each sample was subtracted from the sample mean counts. Then the percentage of the total counts bound was calculated from each mean i.e.
A standard curve was constructed of the % bound versus the concentration of unlabelled insulin. The test samples were then read off the standard curve.

2.6. INORGANIC PHOSPHATE

Plasma and whole blood inorganic phosphate were measured on the Cobas Bio using a kit obtained from Roche Diagnostics Limited, the method of which is based on that of Daly and Ertingshausen, 1972.

Intra-erythrocyte inorganic phosphate was calculated from the whole blood and plasma inorganic phosphate measurements using the same formula as for intra-erythrocyte glucose (see section 2.4).

2.6.1. REAGENTS

10% trichloroacetic acid

ASSAY REAGENT (supplied with kit)

2.6.2. STANDARD

A 1.5 mmol/l standard was supplied with the kit. This was diluted 1 in 4 with 10% trichloroacetic acid so that it was at the same dilution and pH as the samples.

2.6.3. APPARATUS

See section 2.3.3.

2.6.4. PROCEDURE

200 μl of lithium heparin whole blood or plasma was added to 800 μl of 10% trichloroacetic acid and vortexed thoroughly. The samples
were then centrifuged at 3000 g (at room temperature) for 10 minutes.

The whole blood and plasma extracts together with the standard and reagent were loaded onto the Cobas Bio. The parameter listing used to measure the inorganic phosphate in the samples is shown in Table 2.4.

The plasma and whole blood inorganic phosphate results were corrected for the protein content as described for glucose in Section 2.4.1.4. and similarly for the erythrocyte protein/water content in the calculation of the intra-erythrocyte inorganic phosphate concentration.

2.7. 2,3-DIPHOSPHOGLYCERATE

A kit for 2,3-diphosphoglycerate was obtained from Boehringer Mannheim (U.K.) the principle of which is based on the method of Ericson and De Verdier (1972). This manual method was adapted onto the Cobas Bio.

2.7.1. REAGENTS

0.6M perchloric acid (ice-cooled)

2.5M potassium carbonate

ASSAY REAGENTS A,B,C,D,E (supplied in kit) - Solutions A, B and C were mixed together in the proportions of 40:1:1 respectively (Reagent 1). Solutions D and E were mixed together in the proportions of 1:1 (Reagent 2).

2.7.2. STANDARD

A 8 mmol/l stock solution of 2,3-diphosphoglycerate (Sigma Chemical Company, Poole, Dorset, U.K.) was prepared in deionised water. This was double-diluted down to give a range of standards, 1,2 and 4 mmol/l. These were then subjected to the same preparations as the samples (see below, Section 2.7.4.) so that they were at the same dilution and pH.

2.7.3. APPARATUS

See Section 2.3.3.
2.7.4. PROCEDURE

500 µl of lithium heparin whole blood was added to 2.5 mls of ice-cold 0.6M perchloric acid (within 5 minutes of collection). This was vortex-mixed and centrifuged at 2500 rpm at 4°C in the J6B (MSE Ltd.) centrifuge for 10 - 15 minutes.

To 2 mls of the supernatant, 250 µl of 2.5M potassium carbonate was added. This was vortex-mixed and then left on ice for at least 30 minutes. Then it was centrifuged again at 3000 g (room temperature) for 10 minutes to remove the perchlorate precipitate. The supernatant was then used for the assay.

The sample and standard extracts together with reagent 1 (main reagent well) and reagent 2 (start reagent well) were loaded onto the Cobas Bio. The assay was carried out using the parameter listing shown in Table 2.5.

The final concentration obtained by the Cobas Bio was multiplied by 100/haematocrit to give the 2,3-diphosphoglycerate concentration in mmol/l erythrocytes.
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2.8. INTRA-ERYTHROCYTE PH

The pH inside the erythrocyte was measured using a method based on that described by Warth and Desforges (1978).

2.8.1. REAGENTS

5,5-dimethyl-[2-14C]oxazolidine-2,4-dione (Amersham International p.l.c., Bucks, U.K.)

10% trichloroacetic acid

Optiphase Scintillation fluid (LKB Ltd.,) Bromma, Sweden).

2.8.2. APPARATUS

1217 Rackbeta Liquid Scintillation Counter (LKB Wallac., Finland).

2.8.3. PROCEDURE

5 mls of venous blood was collected into a lithium heparin container. 50 µl of 5,5-dimethyl[2-14C]oxazolidine-2,4-dione was immediately added to the blood, mixed well and incubated for 20 minutes at 37°C. The pH (pHe) of the incubated whole blood was then measured and noted. The haematocrit was also measured (see Section 2.11).

The blood was then centrifuged at 3000 g (room temperature) for 10 minutes. From the resultant separated blood, 1 ml of plasma and 1 ml of packed erythrocytes were each added to 1 ml of deionised water separately and mixed thoroughly. 0.5 mls of the diluted plasma and the haemolyzed erythrocytes were then deproteinised each with 1 ml of 10% trichloroacetic acid in duplicate. The samples were then centrifuged at 3000 g (room temperature) for 10 - 15 minutes.

The two erythrocyte extracts and the two plasma extracts were then counted each in 10 mls of the Optiphase scintillation fluid using a β-counter. Counts per minute were converted to disintegrations per minute (dpm) using a quench curve.
2.8.4. CALCULATION

The plasma (external) dpm was converted to dpm/g of plasma water using 0.94 as the fraction of solvent water in plasma (Cannon et al., 1974). The erythrocyte (internal) dpm was also converted to dpm/g of erythrocyte water, utilizing 0.60 as the fraction of solvent water in the erythrocyte (Drabkin, 1950).

The dpm/g of plasma water and erythrocyte water were then entered into the following equation to calculate the intra-erythrocyte pH (Waddell and Beutler, 1959; Warth and Desforge, 1982):

$$pHi = pKa' + \log \left[ \frac{(DMO)_i (10^{pHe - pKa'} + 1) - (DMO)_e}{(DMO)_e} \right]$$

where

- $DMO = 5,5$-dimethyl[2-14C]oxazolidine-2,4-dione
- $pHi$ = intra-erythrocyte pH
- $pHe$ = whole blood pH
- $(DMO)_i$ = erythrocyte dpm/g water
- $(DMO)_e$ = plasma dpm/g water
- $pKa' = 6.13$ for DMO (Waddell and Beutler, 1959)

2.9. VITAMIN C

Plasma and intra-erythrocyte vitamin C were measured by a manual fluorometric method first introduced by Deutsh and Weeks (1959). This method was adapted for the measurement of vitamin C in physiological fluids by Brubacher and Vuilleumier (1974).

2.9.1. REAGENTS

- 0.9% saline
- 5% metaphosphoric acid
- 0.05M iodine solution
- 25% sodium acetate (trihydrate)(filtered)
SODIUM ACETATE-BORATE SOLUTION - 50 g of sodium acetate trihydrate and 20 g of boric acid dissolved in 200 mls deionised water and filtered.

0.1% o-phthalaldehyde (prepared freshly before use and protect from light).

0.5M sodium thiosulphate

2.9.2. STANDARDS

A 1 mmol/l stock solution of ascorbic acid was prepared in 5% metaphosphoric acid. This was double diluted down to produce a range of standards, 0.125, 0.25, 0.50 and 1.0 mmol/l. These were then used to produce both plasma and erythrocyte standards as follows:

2.9.2.1. PLASMA STANDARDS

2 mls of plasma was deproteinised with 18 mls of 5% metaphosphoric acid. This was then centrifuged at 3000 g (at room temperature) for 15 minutes. Then 20 μl of each standard solution was added to each of 2 ml samples of the plasma supernatant. (20 μl of 5% metaphosphoric acid was added to two further 2 ml samples for blanks).

2.9.2.2. ERYTHROCYTE STANDARDS

2 mls of packed erythrocytes were deproteinised with 18 mls of 5% metaphosphoric acid and then centrifuged at 3000 g (room temperature) for 10 minutes. 20 μl of each standard solution was added to each of 2 ml samples of the erythrocyte supernatant. Two blanks were also prepared by adding 20μl of 5% metaphosphoric acid to each of two further 2 ml samples.

2.9.3. APPARATUS

2.9.4. PROCEDURE

2.9.4.1. Sample Preparation

0.5 ml of plasma was deproteinised with 4.5 ml of 5% metaphosphoric acid and then centrifuged at 3000 g (at room temperature) for 10 - 15 minutes.

0.5 ml of packed erythrocytes were washed with 4.5 ml of 0.9% saline. After centrifuging (3000 g, 10 - 15 minutes) the supernatant was discarded and 4.5 ml of 5% metaphosphoric acid added. This was followed by mixing and centrifuging again at 3000 g for 10 minutes.

2.9.4.2. Analysis

To 2 ml of each deproteinised sample and each standard (both plasma and erythrocyte), 10 μl of 0.5M iodine solution was added followed by rapid mixing. After 10 seconds the excess iodine was destroyed by adding 5 μl of 0.5M sodium thiosulphate.

750 μl of each oxidized sample and standard was pipetted into two different tubes (one for the test and one as a blank). To all the test samples 750 μl of the sodium acetate solution was added and to all the blanks 750 μl of the sodium acetate-borate solution was added. These were all mixed well and then incubated in the dark at room temperature for 15 minutes.

Then 300 μl of the 0.1% o-phthalaldehyde solution was added to all of the tubes which were then incubated for a further 15 minutes in the dark at room temperature.

The fluorescence of the test and blank of each sample and standard was then measured on the fluorimeter, using an excitation wavelength of 348 nm and an emission wavelength of 423 nm.

Having subtracted the blank fluorescence from the test fluorescence for both the standards and the samples, a standard curve was constructed for the plasma standards and the erythrocyte standards which was used to obtain the concentration of vitamin C in the plasma and erythrocyte samples respectively.
Both the plasma and erythrocyte vitamin C concentrations were expressed as \(\mu\text{mol/l}\) of water using the correction factors described in Section 2.4.14.

2.10. AMINO ACIDS

Plasma, urine and erythrocyte amino acids were analysed using a Waters Picotag System (Waters Associates, Milford, Massachusetts, U.S.A.). This utilized pre-column derivatisation with phenylisothiocyanate (PITC), reverse-phase high-performance liquid chromatography (HPLC) and ultra-violet detection at 254 nm (Heinrikson and Meredith, 1984).

2.10.1. REAGENTS

All reagents were of HPLC grade.

2.10.1.1. Mobile Phase

ELUENT 1- 0.07M sodium acetate trihydrate prepared in milliQ water pH 6.45 + 2.5% acetonitrile. The solution was filtered using a 0.45 M nylon membrane filter (Gelman Sciences Limited, Northampton, U.K.) and degassed before use

ELUENT 2 - 45% acetonitrile, 40% MilliQ water and 15% methanol. The solution was degassed before use.

2.10.1.2. Drying Solution (Made up fresh)

2:2:1 (by volume) of methanol, 1M sodium acetate (filtered) and triethylamine

2.10.1.3. Derivatising Solution (made up fresh)


2.10.2. STANDARD

Basic amino acids (2.5 \(\mu\text{mol/l}\) ml) and acidic/neutral amino acids (2.5 \(\mu\text{mol/ml}\)) both from Sigma Chemical Co., Poole, Dorset, U.K. were diluted in milliQ water 1:1:4:25 (acidic/neutral:basic:water).

80
This standard solution was then mixed 1:1 with the internal standard (methionine sulphone, 0.4 mM in 0.1M hydrochloric acid). This resulted in a concentration of amino acids of 200 pmol/l.

2.10.3. APPARATUS

All obtained from Waters Associates as part of the Picotag System:
Automatic Sampler Wisp TM Model 712
Two (model 510) HPLC pumps
Model 680 Automatic Gradient Controller
Model 440 Absorbance Detector
Column Heater, Model 710B
Picotag reverse-phase column - 30 cm x 3.9 mm
Picotag Work Station for derivatization.

2.10.4. PROCEDURE

2.10.4.1. Sample Preparation

PLASMA - 50 µl of plasma was mixed with 50 µl of the internal standard solution (methionine sulphate, 0.4 mM in 0.1M hydrochloric acid).
Deproteinisation was carried out using a filtration device supplied by Waters Associates with an ultrafiltration membrane (retention rating, 10,000 NMWL) (Millipore (U.K.) Limited, Harrow, Middlesex). The resultant filtrate was used for the analysis.

ERYTHROCYTES - 1 ml of packed erythrocytes were washed twice in 0.9% saline and then deproteinised with 5 mls of ice-cold 0.6M perchloric acid. 50 µl of the extract was then mixed with 50 µl of the internal standard, which was then ready for analysis.

URINE - Urine was prepared by exactly the same procedure as for plasma.

2.10.4.2. Derivatisation

25 µl of the standard or prepared sample were pipetted into small glass tubes (6 x 50 mm). These were placed in a reaction vial (Waters Associates) and then dried down under vacuum using the Picotag Work Station (30 - 40 minutes). Then 10 µl of the drying solution was added to each tube (samples and standard). These were then re-dried under vacuum (15 - 20 minutes). When thoroughly dry, 20 µl of the derivatisation reagent was added to each sample and
standard. After vortex-mixing, the tubes were replaced in the reaction vial and incubated at room temperature (20 - 25°C) for 20 minutes.

The reaction vial was then returned to the work station and all the tubes dried down under vacuum (30 - 45 minutes). To ensure the samples (and standards) were thoroughly dry, 10 µl of methanol was added to each tube and re-dried under vacuum (20 - 30 minutes).

2.10.4.3. HPLC (analysis)

Each sample (and standard) was reconstituted with 100 µl of Picotag diluent and placed in the WispTM autosampler ready for analysis.

HPLC was carried out according to the manufacturer's instructions using the Picotag fully-automated HPLC system.

The concentration of the individual amino acids in each sample was calculated from the peak areas, using the standard amino acid peak areas as a reference.

The plasma and erythrocyte amino acid concentrations were corrected for the protein content (see Section 2.4.1.4.) in plasma and erythrocytes respectively. The urine amino acid concentrations were expressed as µmol/24hr/mmol creatinine.

2.11. HAEMATOCRIT

A very small volume of potassium EDTA whole blood was taken up into a capillary tube (Boehringer Mannheim, U.K.) and centrifuged in a microhaematocrit centrifuge (MSE Scientific Instruments, Sussex, U.K.) for 5 minutes. The haematocrit(HCT) was calculated as follows:

\[
HCT(\%) = \frac{\text{LENGTH OF PACKED ERYTHROCYTES (mm)}}{\text{LENGTH OF PACKED ERYTHROCYTES+PLASMA (mm)}} \times 100
\]
2.12. UREA

Plasma urea was measured on a Cobas Bio using a kit supplied by Roche Diagnostics Limited based on the method of Tiffany et al., (1972).

2.12.1. REAGENT

Assay reagent supplied with kit.

2.12.2. STANDARD

A standard urea solution of 7 mmol/l was supplied with the kit.

2.12.3. APPARATUS

See Section 2.3.3.

2.12.4. PROCEDURE

The plasma samples, standard and reagent were loaded onto the Cobas Bio and the assay carried out according to the parameter listing shown in Table 2.6.
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</tr>
<tr>
<td>19</td>
<td>PRINTOUT MODE</td>
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2.13. CREATININE

Urine creatinine was kindly measured by the Clinical Biochemistry Department at St. Luke's Hospital, Guildford, Surrey.

2.14. FREE FATTY ACIDS

Plasma free fatty acids were measured on a Cobas Bio using a kit obtained from Alpha Laboratories Limited, Hampshire, U.K., based on the method of Mulder et al., 1983.

2.14.1. REAGENT

Assay reagents supplied with the kit.

2.14.2. STANDARD

A standard free fatty acid solution of 1.0 mmol/l was supplied with the kit.

2.14.3. APPARATUS

See Section 2.3.3.

2.14.4. PROCEDURE

The plasma samples, standard and reagents were loaded onto the Cobas Bio and the assay carried out using the parameter listing shown in Table 2.7.
<p>| | | |</p>
<table>
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<td>STANDARD 1 CONC.</td>
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<td>STANDARD 2 CONC.</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>STANDARD 3 CONC.</td>
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CHAPTER 3

INVESTIGATION OF HIGH AND LOW GLYCATORS
3.1. INTRODUCTION

The Islington Diabetes survey identified a group of non-diabetic subjects, whose glycated haemoglobin (GHb) levels were inappropriate relative to their tolerance to a glucose load. Twenty subjects had high GHb levels relative to their two-hour blood glucose (2HBG) levels (HIGH GLYCATORS) and twenty-two subjects had low relative levels of GHb (LOW GLYCATORS) (see Figure 1.7). Biological or assay variability failed to account for the weak relationship found between GHb and 2HBG levels. Fasting glycaemia is the major determinant of GHb levels in non-insulin dependent diabetics (Paisey et al., 1980). It is also more representative of ambient blood glucose levels than the response to an unphysiological glucose load. However, fasting blood glucose levels also did not explain the differences in GHb levels found in these subjects (see Figure 1.8.)

It is well proven that during a glucose tolerance test, the response to the glucose load is very dependent upon the basic nutritional status of the subject (i.e. previous diet) (Conn, 1940). However, analysis of the dietary patterns of the high and low glycators found no significant differences between the two groups. This suggests that diet was not the causal factor determining the weak relationship between GHb and 2HBG. In general, the correlations found between GHb levels and glycaemia in non-diabetics are much weaker than those found in diabetic subjects (Boucher et al., 1981; Simon et al., 1985; John and Richardson, 1986). This may be partly because both assay and biological variability make a larger contribution to the variation at low levels of GHb and over the narrow range seen in non-diabetics.

Although correlation coefficients (r) of up to 0.98 have been found between GHb and blood glucose levels in diabetic subjects (Svendsen et al., 1982), there is considerable overlap of GHb levels between normal, impaired-glucose tolerant and diabetic individuals (Dunn et al., 1979; Verillo et al., 1983; Modan et al., 1988). This suggests the occurrence of similar GHb levels with quite different glucose tolerances. Also, two studies with diabetic subjects have shown unexplained discrepancies between GHb levels and blood glucose levels (Gonen et al., 1979; Verillo et al., 1983). This suggests that factors unrelated to blood glucose levels may result in differences in individual rates of glycation of haemoglobin. A number of
different factors have been shown to affect the rate and extent of glycation of proteins both in vivo and in vitro (see Chapter 1).

If individual differences in the degree of glycation of proteins were physiologically significant, the identification of factors other than glucose determining the rate of glycation of proteins in vivo may have important implications. In diabetic subjects, these factors may have bearing not only on the use of glycated proteins to monitor blood glucose control, but may also partly determine the rate of development of complications. Factors which can decrease glycation may help to alleviate or prevent the occurrence of these complications and could possibly delay the normal ageing process.

Therefore, a detailed biochemical investigation of the high and low glycators identified in the Islington Diabetes survey was carried out to determine the cause(s) of the weak relationship found between GHb and blood glucose levels. The chapter is divided into two parts (A and B). The first part of the study (A) was to recall the 42 subjects and to establish whether they remained classified in the two groups four years after the original screening survey. Then depending on the finding, the second part of the study (B) was to perform a thorough biochemical and haematological examination to determine why the glycation of haemoglobin in these subjects was occurring at a level inappropriate to their blood glucose levels.

**PART A - STABILITY OF THE HIGH AND LOW GLYCATORS**

**3.2. INTRODUCTION**

The high and low glycators have been classified because of their inappropriate level of GHb relative to their 2HBG level. Despite the fasting blood glucose (FBG) level being more physiological than the response to a glucose load, this measure of glycaemia also did not explain the differences in levels of GHb between the high and low glycators. However, the most accurate and representative measure of ambient glycaemia in all subjects is probably the mean blood glucose (MBG) level calculated from multiple daily blood glucose measurements. Very high correlations have been found between GHb levels and MBG measurements (Paisey et al., 1980; Svendsen et al., 1982).

Therefore, to investigate whether the high and low glycators had inappropriate levels of GHb relative to all measures of glycaemia,
daily blood glucose profiles were performed as well as repeat glucose tolerance tests.

3.3. METHODS

3.3.1. SUBJECTS

The high and low glycators were all patients of the St. John's Way Practice in Islington, North London. Initially the general practitioners in the practice were approached to allow permission to recontact the subjects for further study.

The original 42 subjects had been reduced to 30; 4 had moved away, 2 had died, 2 were unlocatable and 4 were too ill to take part in a further study. Therefore, 30 letters were sent out asking the subjects whether they would be interested in participating in a further study. Enclosed with each letter was a reply slip on which they could indicate whether they would agree to an informal visit, for a more detailed explanation of what the study involved. A reply-paid envelope was included in the letter.

Nineteen replies were received, of which 15 agreed to a home-visit to discuss the study. An appointment with each of the subjects was arranged by telephone. After the home-visits, 13 of the subjects agreed to take part in the study and letters were sent out later with appointment dates for both the daily blood glucose profiles and the glucose tolerance test.

The original letter was also re-sent to the 11 subjects who never replied in the first instance. Four more replies were received, of which 2 more subjects agreed to take part in the study. One further subject was contacted by telephone only and agreed to take part. So, a total of 16 subjects (38% of the original 42, or 53% of those traced and eligible) agreed to take part in the study. There were 12 female and 4 male subjects, aged 45 - 81 years. Nine were high glycators and 7 low glycators.

Before the start of the study the subjects were asked to fill in a brief questionnaire to check that physical activity, dietary habits and medication had not changed since the original screening survey.

3.3.2. GLUCOSE TOLERANCE TESTS

13 subjects participated in a full glucose tolerance test (7 high and 5 low glycators. The other 3 subjects who originally agreed to take
part, were for various reasons not able to get to the clinical investigation unit for the test.

Each subject visited the unit at the Whittington Hospital in North London separately to undergo the test. They were asked to fast for 10 - 12 hours prior to the glucose tolerance test and to arrive at the unit between 9.30 - 10.00a.m.

Initially a 30ml fasting venous blood sample was obtained. This was divided up into a number of different vessels containing either lithium heparin or potassium EDTA as the the anticoagulant. Most of this blood was collected for the measurement of a number of different analytes (see Part B). After consumption of a 75g glucose load dissolved in 200mls of water, further venous blood samples were collected at 30 minutes, 1 hour and 2 hours. At 30 minutes and 1 hour, 5mls of blood was collected into lithium heparin. At 2 hours, 10mls of blood was collected, 5mls into lithium heparin and 5mls into potassium EDTA. Lithium heparin whole blood was deproteinised immediately at all time points for the measurement of glucose. The extracts were stored at 4°C for 24 hours prior to analysis of glucose. Glycated haemoglobin was measured on the potassium EDTA whole blood collected at fasting and 2 hours after the glucose load, by affinity chromatography, iso-electric focussing and agar-gel electrophoresis with and without the removal of the Schiff base. The blood was stored at 4°C for 1 week prior to analysis.

3.3.3. BLOOD GLUCOSE PROFILES

16 subjects (9 high and 7 low glycators) performed daily blood glucose profiles. None of the volunteers were prepared to do the profiles themselves at home, which would have provided the best indication of their ambient blood glucose control. For this reason, they were invited to attend the clinical investigation unit at the Whittington Hospital from 8.30a.m. - 3.00p.m. in groups of 3 - 4 at a time. A 5-point profile was obtained employing finger-prick blood samples collected at 9.00a.m., 10.30a.m., 12.00p.m., 1.30p.m. and 2.30p.m. representing the fasting, 1 hour post-breakfast, pre-lunch, 1 hour and 2 hour post-lunch blood glucose concentrations respectively. The capillary blood was collected onto filter papers impregnated with boric acid, which were thoroughly dried before being stored at -20°C. The blood spots were analysed for glucose 1 month later.
Each subject was offered a range of food for breakfast and lunch, and selected items which most closely represented their usual meals. They were also encouraged to follow their usual level of activity (as far as was possible).

The blood glucose profile was repeated on a second day during the subsequent 4 weeks in 14 of the subjects. The mean blood glucose from the two profiles was calculated.

3.3.4. ASSAY METHODS

Full details are given in Chapter 2.

3.3.5. STATISTICAL METHODS

Pearson's correlation coefficient ($r$) was used to express the relationship between GHb levels and levels of glycaemia, between the different assays for GHb and also between the different measures of glycaemia in the low and high glycators. Groups were compared using Student's t-tests. Unless otherwise stated, values are presented as mean (±SD).

3.3.6. CALCULATION OF THE RESULTS

In the Islington Diabetes survey, the 223 subjects examined at recall were classed into centile rankings for 2HBG at screening and recall, and of GHb at screening and by each assay method both fasting and 2 hours post-load glucose at recall. The mean of the two centile rankings for 2HBG and for all nine GHb measurements were calculated. The use of centiles was to overcome the problem posed by the use of capillary blood at screening and venous blood at recall, and by the different ranges for the GHb assays.

The 42 high and low glycators were so classified because their mean 2HBG ranking differed by at least 30 centiles from their mean ranking for GHb. In order to reclassify the 13 subjects taking part in the present study, it was necessary to similarly class them into centile rankings for the various measurements of glycaemia (2HBG and FBG on the glucose tolerance test and MBG calculated from the profiles) and also for the four GHb measurements, for which the mean centile rank was calculated. The results for glycated haemoglobin and the different measures of glycaemia are also shown without the use of centiles.
3.4. RESULTS

Although 16 subjects took part in the glucose profiles, only 13 underwent the full glucose tolerance test with the measurements of GHb. Therefore, all the results presented are for these 13 subjects only.

3.4.1. CORRELATION OF GLYCATED HAEMOGLOBIN WITH BLOOD GLUCOSE LEVELS

3.4.1.1. Two-hour Blood Glucose

There was a stronger relationship between GHb and 2HBG levels in the subjects than found in the original study (see Fig. 3.1(a) compared to Fig. 1.6.). A change in grouping of the subjects has resulted in only one high and one low glycator still having a centile ranking for 2HBG differing by at least 30 centiles from the mean centile ranking for GHb. However, 85% of the subjects remain on the correct sides of the unity line. Only two subjects (1 low and 1 high glycator) have changed groups. One of the low glycators (No. 6) has become diabetic (D) and two other subjects have impaired glucose tolerance (1 low and 1 high glycator, No. 9 and No. 2 respectively).

The subjects were similarly distributed when the mean GHb and 2HBG levels were plotted without the use of centiles (see Fig. 3.1(b)), although the discrepancy between the two parameters in general, was less obvious.

3.4.1.2. Fasting Blood Glucose

All the low glycators remained classified with the exception of the new diabetic, who had an unusually low FBG (see Fig. 3.2(a)). The centile ranking for GHb and FBG were all at least 30 centiles apart for the low glycators. However, three of the high glycators had crossed the line of unity and none of the high glycators had a centile ranking for GHb and FBG 30 centiles apart.

When plotted without the use of centiles (see Fig. 3.2(b)), a similar relationship was found between GHb and FBG for the low and high glycators.
FIGURE 3.1(a) MEAN CENTILE RANKINGS OF ALL ASSAYS FOR GLYCA\nTED HAEMOGLOBIN AND CENTILE RANKING OF TWO-HOUR BLOOD GLUCOSE

The line of agreement and the +30 and -30 centile lines are indicated.

FIGURE 3.1(b) ARITHMETIC MEAN OF ALL THE ASSAYS FOR GLYCA\nTED HAEMOGLOBIN AND TWO-HOUR BLOOD GLUCOSE LEVEL

(D = new diabetic)
High glycators are represented as (❖) and the low glycator as (□).
FIGURE.3.2.(a) MEAN CENTILE RANKINGS OF ALL THE ASSAYS FOR GLYCA\NED HAEMOGLOBIN AND CENTILE RANKING OF FASTING BLOOD GLUCOSE

The line of agreement and the +30 and -30 centile lines are indicated.

FIGURE.3.2.(b) ARITHMETIC MEAN OF ALL THE ASSAYS FOR GLYCA\NED HAEMOGLOBIN AND FASTING BLOOD GLUCOSE LEVEL

(D=new diabetic)
High glycators are represented as (♦) and the low glycators as (□).
3.4.1.3. Mean Blood Glucose (Profiles)

A very poor relationship was found between the MBG and GHb level (see Fig. 3.3(a) and (b)). The grouping of the high and low glycators on either side of the unity line (see Fig. 3.3(a)) more closely resembled the original classification pattern (see Fig. 1.6). Only the new diabetic had changed groups. All the low glycators with the exception of one (No. 4), had a centile ranking for GHb and MBG 30 or more centiles apart. Two of the high glycators were actually on the unity line, whilst the majority of the others had 20-30 centiles or more between their ranking for GHb and MBG. The discrepancy between GHb and MBG was greater for the low than the high glycators. This poor relationship between GHb and MBG was also shown when the results were plotted without the use of centiles (see Fig. 3.3(b)).

3.4.2. CORRELATION OF DIFFERENT ASSAYS FOR THE MEASUREMENT OF GLYCATED HAEMOGLOBIN

The correlation between levels of GHb when assayed by unrelated physiochemical methods was much stronger than between any of the glycaemic parameters and GHb level (see Table 3.1). The mean correlation coefficient (r) was 0.83.

3.4.3. CORRELATION BETWEEN DIFFERENT MEASURES OF GLYCAEMIA IN THE LOW AND HIGH GLYCATORs

The correlation between FBG and 2HBG was much improved when the new diabetic was omitted (r = 0.84 vs 0.64) (see Fig. 3.4(a)). The diabetic had a very low FBG level in comparison to a very high 2HBG level. Significant correlations were found between FBG and MBG (r = 0.65) and between 2HBG and MBG (r = 0.63) (see Fig. 3.4(b) and (c)). The removal of the diabetic in the latter two situations made little difference to the correlations found.

The mean values for FBG, 2HBG and MBG were all higher for the low than the high glycators if the diabetic was omitted (see Table 3.2). Only the MBG calculated from the profiles was significantly greater for the low compared to the high glycators (p<0.01) and remained so whether the diabetic was included or omitted.
FIGURE 3.3(a) MEAN CENTILE RANKINGS OF ALL ASSAYS OF GLYCA TED HAEMOGLOBIN AND CENTILE RANKING OF MEAN BLOOD GLUCOSE (PROFILES)

The line of agreement and the +30 and -30 centile lines are indicated.

FIGURE 3.3(b) ARITHMETIC MEAN OF ALL ASSAYS FOR GLYCA TED HAEMOGLOBIN AND MEAN BLOOD GLUCOSE LEVEL (PROFILES)

High glycators are represented as (♦) and the low glycators as (□). (D=new diabetic)
**TABLE 3.1** CORRELATION (r) OF DIFFERENT ASSAYS FOR THE MEASUREMENT OF GLYCATED HAEMOGLOBIN

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<th>ISOELECTRIC FOCUSING(r)</th>
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<td>AFFINITY CHROMATOGRAPHY(r)</td>
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<tr>
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FIGURE 3.4 (a) CORRELATION BETWEEN FASTING BLOOD GLUCOSE AND TWO-HOUR BLOOD GLUCOSE IN THE LOW AND HIGH GLYCATORS

\[ r = 0.84 \text{(with diabetic)} \]
\[ r = 0.64 \text{(without diabetic)} \]

The regression line is indicated with \( r \) representing the correlation coefficient.

High glycators are represented as (❖) and the low glycators as (□).

(D = new diabetic)
FIGURE 3.4(b)  CORRELATION BETWEEN FASTING BLOOD GLUCOSE AND MEAN BLOOD GLUCOSE (PROFILES) IN THE HIGH AND LOW GLYCATORS

The regression line is indicated with \( r \) representing the correlation coefficient.

High glycators are represented as \((\Diamond)\) and the low glycators as \((\Box)\).

\(D=\) new diabetic
FIGURE 3.4(c)  CORRELATION BETWEEN TWO-HOUR BLOOD GLUCOSE AND MEAN BLOOD GLUCOSE (PROFILES) IN THE HIGH AND LOW GLYCATORS

The regression line is indicated with $r$ representing the correlation coefficient.

High glycators are represented as (❖) and the low glycators as (□).

(D=new diabetic)
**TABLE 3.2  FASTING, TWO-HOUR AND MEAN BLOOD GLUCOSE LEVELS IN THE HIGH AND LOW GLYCATORS**

<table>
<thead>
<tr>
<th>GLYCAEMIC MEASUREMENT</th>
<th>HIGH GLYCATORS</th>
<th>LOW GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FASTING BLOOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUCOSE (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DIABETIC</td>
<td>4.82 (0.67)</td>
<td>4.80 (0.74)</td>
<td>NS</td>
</tr>
<tr>
<td>-DIABETIC</td>
<td>4.82 (0.67)</td>
<td>5.06 (0.45)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>TWO-HOUR BLOOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUCOSE (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DIABETIC</td>
<td>5.12 (1.60)</td>
<td>6.45 (2.75)</td>
<td>NS</td>
</tr>
<tr>
<td>-DIABETIC</td>
<td>5.12 (1.60)</td>
<td>5.44 (1.32)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>MEAN BLOOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUCOSE (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+DIABETIC</td>
<td>5.45 (0.61)</td>
<td>6.75 (0.50)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>-DIABETIC</td>
<td>5.45 (0.61)</td>
<td>6.78 (0.59)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
3.4.4. DAILY BLOOD GLUCOSE PROFILES

The mean blood glucose calculated from the daily blood glucose profiles was clearly separated in the high and low glycators (see Fig. 3.3(a) and (b)). Not only was the mean blood glucose significantly greater in the low glycators, but also the mean blood glucose at each time-point (except fasting) was significantly greater in the low compared to the high glycators (see Fig. 3.5 and Table 3.3).

3.4.5. COMPARISON OF GLYCATED HAEMOGLOBIN AND FASTING/TWO-HOUR BLOOD GLUCOSE LEVELS WITH THOSE VALUES OBTAINED AT RECALL ON THE ORIGINAL SCREENING SURVEY

There was no significant differences found in the FBG or 2HBG levels between screening and the present study. With the exception of iso-electric focusing, all the methods for GHb were not significantly different four years after the original screening survey (see Table 3.4).

3.4.6. QUESTIONNAIRES

The physical activity and the medication of the subjects had not changed during the four years between screening and the present study (except for the diabetic). Three of the subjects reported a change in diet. One of these was the diabetic and the other two had both changed to reducing diets (one low and one high glycator).
FIGURE 3.5. MEAN BLOOD GLUCOSE LEVELS AT EACH TIME POINT DURING THE PROFILES IN THE HIGH AND LOW GLYCATORS (INCLUDING THE DIABETIC)

1 = 9.00 a.m.
2 = 10.30 a.m.
3 = 12.00 p.m.
4 = 1.30 p.m.
5 = 2.30 p.m.

The significance of the difference in mean blood glucose between the two groups at each time point is indicated.
<table>
<thead>
<tr>
<th>TIME OF SAMPLE</th>
<th>HIGH GLYCATORS (mmol/l)</th>
<th>LOW GLYCATORS (mmol/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASTING</td>
<td>4.58 (0.69)</td>
<td>5.03 (0.97)</td>
<td>NS</td>
</tr>
<tr>
<td>ONE-HOUR POST BREAKFAST</td>
<td>6.45 (1.48)</td>
<td>8.74 (1.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PRE-LUNCH</td>
<td>5.11 (0.88)</td>
<td>6.24 (1.04)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ONE-HOUR POST LUNCH</td>
<td>5.81 (0.84)</td>
<td>7.60 (0.99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TWO-HOURS POST LUNCH</td>
<td>4.62 (0.72)</td>
<td>6.30 (1.12)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>MEASUREMENT</td>
<td>SCREENING</td>
<td>AFTER 4 YEARS</td>
<td>SIGNIFICANCE OF DIFFERENCE (p)</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------</td>
<td>---------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>FASTING BLOOD GLUCOSE (mmol/l)</td>
<td>4.43 (0.34)</td>
<td>4.59 (0.45)</td>
<td>NS</td>
</tr>
<tr>
<td>TWO-HOUR BLOOD GLUCOSE (mmol/l)</td>
<td>5.05 (1.33)</td>
<td>5.74 (2.12)</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%)</td>
<td>6.89 (0.74)</td>
<td>7.08 (0.99)</td>
<td>NS</td>
</tr>
<tr>
<td>affinity chromatography</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%)</td>
<td>5.84 (0.48)</td>
<td>5.06 (0.72)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>isoelectric focusing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%)</td>
<td>7.63 (0.86)</td>
<td>7.67 (0.99)</td>
<td>NS</td>
</tr>
<tr>
<td>electrophoresis -total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%)</td>
<td>7.06 (0.45)</td>
<td>6.99 (0.85)</td>
<td>NS</td>
</tr>
<tr>
<td>electrophoresis -stable</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5. DISCUSSION

The tolerance to a glucose load (2HBG) did not produce the clear separation of the low and high glycators as it did four years previously. Although the majority of the subjects still had some discrepancy between the GHb and 2HBG levels, most do not fall into the original classification of high and low glycators, with the ranking for 2HBG and GHb differing by at least 30 centiles. This is probably not surprising as the blood glucose response to an oral glucose load is known to be highly variable in an individual. This is thought to be due to factors such as the absorption and distribution of glucose and the prevailing hepatic activity in glycolysis and gluconeogenesis. The insulin response is thought to exert a secondary modifying influence (Harding, Oakely and Wynn, 1973). Several studies have shown the poor reproducibility of the glucose tolerance test with increasing variability in fasting blood glucose, 1-hour blood glucose and 2-hour blood glucose respectively (Harding, Oakley and Wynn, 1973; Forrest, 1987). The within-subject coefficient of variation for 2 hour blood glucose has been shown to be 25% to 32% (Forrest, 1987). The glucose tolerance test is known to be affected by a number of factors including diet and certain drugs. No change in medication was reported between screening and the present study, but three subjects had recently gone onto reducing diets. This would be expected to cause an exaggerated blood glucose response during the glucose tolerance test. One of the three subjects on the reducing diet was the new diabetic who despite a low fasting blood glucose did show a markedly raised two-hour blood glucose. The other two did not show any abnormal response to the glucose load.

Another factor that might result in a redistribution of the subjects in relation to their 2HBG and GHb level is the phenomenon known as 'the regression to the mean' (Davis, 1976). This is a phrase used to describe the fact that a variable that is extreme on its first measurement will tend to be closer to the centre of distribution for a later measurement. This variability can be attributed to both the inherent variation in the phenomenon being measured and the variability of the measurement itself.

The fasting blood glucose also did not produce the marked separation of the low and high glycators as the two-hour blood glucose did in the original study. However, this was also the finding in the original study. Although fasting glycaemia is less variable than the two-hour blood glucose (Harding, Oakley and Wynn, 1973), it is not always an
accurate measurement as it is difficult to check whether the subjects are truly fasting. The majority of the subjects had fasting blood glucose levels which were increased from the original study which might explain why their GHb levels were not as discrepant as they had been previously and is possibly due to a non-fasting measurement. It is unlikely that fasting glycaemia is the whole explanation for the discrepant GHb levels in these subjects as the fasting blood glucose levels were higher in the low glycators compared to the high glycators.

When the mean blood glucose levels calculated from the two profiles were plotted against GHb levels, all the subjects remained classified with the exception of the new diabetic. Although the two 5-point blood glucose profiles may not accurately reflect 24 hour changes in blood glucose levels, the mean blood glucose calculated from the profiles is more representative of ambient blood glucose levels than the response to an unphysiological glucose load. The mean blood glucose may even be a more accurate measure of average glycaemia than fasting blood glucose if a number of the subjects were not truly fasting. Moreover, the mean blood glucose was significantly higher in the low compared to the high glycators. Also, the correlations between all the measures of glycaemia were much higher than the correlations between GHb and blood glucose levels. This would suggest that blood glucose levels are not the major determinant of GHb levels in these individuals. The high and low glycators demonstrate differences in absolute levels of GHb of 1 - 2% at the same blood glucose levels (see Fig. 3.3(b)).

The high correlation found between the different assays for GHb in both groups suggests that the weak relationship found between GHb and levels of glycaemia is not random. Also, because the assays rely on different physiochemical properties, it is unlikely that method interferences are responsible for the low and high levels of GHb. The affinity chromatography method separates only haemoglobin molecules with cis-diol sugar residues attached to them (Gould et al., 1982), therefore it is unlikely that other modifications of the haemoglobin molecule, such as occur in uraemia (Fluckiger et al., 1981) during treatment with salicylates (Bridges et al., 1975), or with abnormal haemoglobins (Abraham et al., 1983) are responsible for the existence of low or high glycation levels. No subject reported regular salicylate use or excess alcohol consumption both of which can lead to falsely high GHb levels when measured by certain methods (Stevens et al., 1981). There was no significant differences in the methods for GHb measurement between screening and the present study except for isoelectric focusing. The poor
reproducibility of isoelectric focusing is probably due to technical difficulties with the method itself. The lack of individual variation in GHb as measured by the other methods suggests that the small differences found between screening and the present study in the relationship between GHb and blood glucose levels are due to changes in glycaemia and not GHb for reasons already described.

One other study has investigated the relationship between levels of GHb and measures of glycaemia on glucose tolerance testing in subjects with normal glucose tolerance (Modan et al., 1988). They reported wide differences in levels of GHb which were poorly explained by fasting or 2 hour blood glucose levels. They also demonstrated the stability of high and low levels of GHb over a period of more than three years. This would suggest that biological and assay variability were not responsible for the discrepancy. However, this study employed only a single ion-exchange chromatography method for the assay of GHb, so it is possible that other modifications of the haemoglobin molecule may be in part responsible for their findings.

A recent study examined levels of post-load blood glucose and of GHb in 129 men (Starkman et al., 1987). They showed that 22% of the subjects had discordant elevation of one or other value to a level above the 97% centile of a normal population. The group of subjects with abnormalities in glucose tolerance but normal GHb levels demonstrated hyperinsulinaemia with insulin resistance; most would probably be defined as having impaired glucose tolerance by WHO criteria (1985) and most previous studies have reported normal levels of GHb in subjects with impaired glucose tolerance (Albutt et al., 1985; Simon et al., 1985). This study did not look further at the determinants of discordant elevations. Both the glucose tolerance test and the GHb assay were performed on a single occasion, therefore the problems of assay and biological variability remain.

It is likely that high and low glycation rates will be less obvious in diabetic subjects, where the high glucose levels may mask the effects of other factors on glycation. However, Gonen et al. (1979) found that in a mixed group of 230 diabetic subjects, 19% (24 subjects) had discrepancies between GHb and random blood glucose levels and 15% (18 subjects) had discrepancies between GHb and fasting blood glucose levels. The majority of these discrepant results (85%) could not be explained by 24 hour blood glucose monitoring or by method interferences. Therefore, high and low rates of glycation of haemoglobin appear to occur in the diabetic as well as the normal population. No studies have as yet been
undertaken to investigate possible determinants of high and low glycation.

Thus, in conclusion, although the repeat glucose tolerance test failed to classify the high and low glycators as they were originally categorized in the Islington Diabetes Survey, the mean blood glucose calculated from the profiles clearly separates the high and low glycators into their original groups. The difference in mean blood glucose level between the two groups strongly suggests that other mechanisms, possibly unrelated to blood glucose levels must be at least partially responsible for determining the GHb level in these subjects and that these factors remain constant over a period of 4 years of observation.
3.6. INTRODUCTION

Despite the fact that four years had elapsed between the original screening survey and the follow-up study described in Part 1, marked differences in the level of GHb relative to the mean blood glucose level were still apparent in the majority of the subjects. 92% were still classified as high and low glycators. One subject (a low glycator) had become diabetic in the interim and was omitted from further study. This consistent classification suggests that the phenomenon was not a transient occurrence and factors other than glucose were responsible for the differences in glycation levels of haemoglobin.

A number of physiological factors have been shown to directly affect the glycation reaction, both in vitro and in vivo. The glycation of haemoglobin might be affected by intra-erythrocyte pH (Lowry et al., 1985), 2,3-diphosphoglycerate (Smith et al., 1982; Lowry et al., 1985) and inorganic phosphate (Watkins, et al., 1985). There are also a number of compounds present in vivo that could compete with glucose for binding to haemoglobin, such as vitamin C (Stolba et al., 1987) and pyridoxal-5'-phosphate (Srivastova et al., 1972). High doses of aspirin and excess alcohol consumption can produce metabolites in vivo that have been shown to react with protein amino groups (Bridges et al., 1975; Stevens et al., 1981). Pathologically raised levels of urea, as occurs in renal disease, leads to the production of cyanate which can also react with amino groups (Kilmartin and Ross-Bernardi, 1971). Amino acids themselves can potentially react with glucose and therefore could compete with haemoglobin amino groups for binding of glucose.

The half-life of the erythrocyte also plays an important role in the extent of glycation of haemoglobin (Bunn et al., 1976). Therefore haemolytic diseases leading to decreased erythrocyte survival could lead to reduced levels of GHb (Starkman et al., 1983). Conversely, increased erythrocyte production leading to polycythaemia can produce increased GHb levels (Graham et al., 1980).

It is apparent that many other factors apart from blood glucose could potentially influence the glycation of haemoglobin. Therefore a detailed biochemical and haematological investigation of the high
and low glycators may determine the cause(s) of their inappropriate level of glycation of haemoglobin.

Although the blood glucose level does not appear to be related to the level of glycation of haemoglobin in these subjects, the intra-erythrocyte glucose levels to which haemoglobin is exposed may be different to plasma glucose levels and therefore will be measured.

GHb is the glycated protein most frequently used as a monitor of blood glucose control, but glycated plasma proteins, in particular glycated albumin, have also been used as short-term indices of glycaemia (see Chapter 1). It should be relevant to investigate whether the high and low levels of glycation of haemoglobin seen in our subjects are also true for other proteins and for this reason fructosamine and specifically glycated albumin will also be measured. Free fatty acids have been shown to bind to the same site on albumin as the major site of glucose attachment (Merish et al., 1982) in vitro. Therefore, the plasma level of free fatty acids will also be determined.

3.7. METHODS

3.7.1. SUBJECTS AND STUDY DESIGN

The 13 subjects (7 high and 5 low glycators) who took part in the glucose tolerance test agreed to donate a large fasting venous blood sample (30 ml) and a 24 hour urine specimen to carry out the biochemical and haematological analyses.

3.7.2. SAMPLES AND METHODS

(Details of all methodology are given in Chapter 2).

The blood collection and analyses for each subject is shown in Scheme 1.
SCHEME 1. COLLECTION OF BLOOD AND ANALYSES ON EACH SUBJECT

30 mls WHOLE BLOOD

- 5mls LITHIUM HEPARIN
- 5mls POTASSIUM EDTA
- 15mls LITHIUM HEPARIN

INTRAERYTHROCYTE pH

- 5mls POTASSIUM EDTA

HAEMATOLOGY
FULL BLOOD COUNT
RETICULOCYTES
COOMB'S TEST
ABNORMAL HAEMOGLOBINS

GLYCATED HAEMOGLOBIN (see part A)

BIOCHEMISTRY

WHOLE BLOOD

- HAEMATOCRIT
- GLUCOSE
- INORGANIC PHOSPHATE
- 2,3-DIPHOSPHOGLYCERATE

GLUCOSE
INORGANIC PHOSPHATE
FREE FATTY ACIDS
UREA
INSULIN
VITAMIN C
AMINO ACIDS
FRUCTOSAMINE
GLYCATED ALBUMIN

PLASMA

ERYTHROCYTE

- VITAMIN C
- AMINO ACIDS
- GLUCOSE
- INORGANIC PHOSPHATE
The measurement of intra-erythrocyte pH was carried out immediately after the collection of the blood. Lithium heparin whole blood was deproteinised immediately for the measurement of glucose, inorganic phosphate and 2,3-diphosphoglycerate. The extracts for glucose and inorganic phosphate were stored at 4°C prior to analysis within 24 hours. The extract for 2,3-diphosphoglycerate was stored at -80°C and analysed within 3 - 4 months. The remaining lithium heparin whole blood was centrifuged for 10 minutes at 3000 g (room temperature). Plasma was deproteinised for the measurement of glucose, inorganic phosphate and vitamin C. The extracts for vitamin C was stored at -80°C and analysed within 3 months. The rest of the plasma was divided up into small aliquots (200 - 500 µl) and stored at -80°C for the analysis of the other plasma constituents (see scheme1) 1 - 4 months later. After centrifugation of the whole blood and removal of the plasma, the packed red blood cells were deproteinised for the measurement of erythrocyte vitamin C and amino acids. The extracts were stored at -80°C until analysis 3 months later.

The whole blood samples collected at 30 minutes, 1 hour and 2 hours during the glucose tolerance test were also deproteinised for the measurement of glucose. The remaining whole blood at each time point was centrifuged for 10 minutes at 3000 g (room temperature). Some of the plasma was deproteinised for glucose analysis and the rest stored at -80°C for the measurement of insulin. The extracts for glucose were stored at 4°C and analysed within 24 hours as for the fasting blood.

The 24 hour urine collection was preserved with 20 mg of thiomersal (BDH Chemicals Limited, Poole, Dorset). After measurement of the volume, the urine was divided up into 5 x 20 ml aliquots which were stored at -80°C. These were analysed for creatinine and amino acids 1 - 3 months later.

The haematological analyses were kindly performed by the Haematology Department at the Whittington Hospital in North London.

3.7.3. CALCULATED ANALYSES

Intra-erythrocyte glucose and inorganic phosphate were calculated from the whole blood and plasma measurements together with the haematocrit (see Chapter 2). The intra-erythrocyte inorganic phosphate was calculated for the fasting blood sample only. Intra-erythrocyte glucose was calculated for all the blood samples.
collected during the glucose tolerance test including the fasting sample.

3.7.4. STATISTICAL METHODS

The glycated albumin and fructosamine levels were expressed in centiles in order to compare them to glycated haemoglobin and to examine their relationship with blood glucose levels.

Pearson's correlation coefficient ($r$) were used to examine the relationship between different glycated proteins and also between glycated proteins and the biochemical parameters measured. Student's t-tests were used to compare glycated proteins and all of the biochemical and haematological parameters measured between the high and low glycators. Paired Student's t-tests were used to compare plasma and intra-erythrocyte glucose concentrations. Unless otherwise stated values are shown as mean ($\pm$ SD).

3.8. RESULTS

3.8.1. RELATIONSHIP OF GLYCATED ALBUMIN AND FRUCTOSAMINE WITH BLOOD GLUCOSE LEVELS COMPARED TO GHB AND BLOOD GLUCOSE LEVELS

3.8.1.1. Two-hour Blood Glucose

Both glycated albumin and fructosamine showed a poor correlation with 2HBG (see Figs. 3.6(a)(ii) and 3.7(a)(ii)). The relationship between glycated albumin and 2HBG was marginally better than that of fructosamine ($r = 0.38$ and $r = 0.14$ respectively). However, both glycated albumin and fructosamine showed a markedly different relationship with 2HBG than GHB (see Figs. 3.6(a)(i) and 3.7(a)(i) compared to Fig. 3.3(a) + (b)). The high and low glycators were re-distributed on both sides of the unity line. Although three high glycators still had fructosamine levels 30 centiles higher than the centile for 2HBG, the other four had crossed the line of unity, with one high glycator now having a fructosamine 30 centiles lower than the 2HBG centile. Glycated albumin showed a similar change as for fructosamine except that only one high glycator still had a glycated albumin 30 centiles higher than the 2HBG centile. One low glycator still had a fructosamine 30 centiles lower than the 2HBG centile, but another two low glycators had fructosamines 30 centiles higher than the 2HBG centile. Glycated albumin in the low glycators showed a similar finding as fructosamine in relation to the 2HBG.
FIGURE 3.6(a) RELATIONSHIP BETWEEN GLYCATED ALBUMIN AND TWO-HOUR BLOOD GLUCOSE IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

![Graph showing the relationship between glycated albumin and two-hour blood glucose using centiles.]

The line of agreement and the +30 and -30 centile lines are indicated. High glycators are represented as (♦) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILE

![Graph showing the relationship between glycated albumin and two-hour blood glucose without using centiles.]

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 3.7(a) RELATIONSHIP BETWEEN FRUCTOSAMINE AND TWO-HOUR BLOOD GLUCOSE IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

The line of agreement and the +30 and -30 centile lines are indicated.
High glycators are represented as (♦) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILES

The regression line and correlation coefficient (r) are indicated.
High glycators are represented as (■) and low glycators as (□).
3.8.1.2. Fasting Blood Glucose

A significant correlation ($p < 0.05$) was found between glycated albumin and FBG ($r = 0.65$) whereas no correlation was found between fructosamine and FBG ($r = 0.093$) (see Figures 3.6(b)(ii) and 3.7(b)(ii)). When expressed in centiles (see Figure 3.6(b)(i) and 3.7(b)(i)) for glycated albumin, all the highs had crossed over the line of unity, although for fructosamine two highs remained on the original side of the line. The low glycators were distributed on both sides of the unity line for both glycated albumin and fructosamine (similarly to 2HBG). However, two low glycators still had very low glycated albumin and fructosamine levels in relation to their FBG level.

3.8.1.3. Mean Blood Glucose (Profiles)

As with FBG a significant correlation ($p < 0.05$) was found between glycated albumin and MBG ($r = 0.53$) whilst no apparent correlation was found between fructosamine and MBG ($r = -0.07$) (see Figures 3.6(c)(ii) and 3.7(c)(ii)). However, when expressed in centiles (see Figures 3.6(c)(i) and 3.7(c)(i)) the distribution of the subjects was similar for both measures of plasma glycation in relation to the MBG. The high glycators were again distributed on both sides of the unity line, only with fructosamine two high glycators still had relatively high fructosamine levels. These two subjects also had high fructosamine levels compared to their glycated albumin levels (see Figure 3.8(c) numbers 13 and 8). The low glycators were also distributed on both sides of the unity line. However, three of these subjects still had glycated albumin and fructosamine levels 30 centiles lower than the MBG centile.
FIGURE 3.6(b) RELATIONSHIP BETWEEN GLYCATED ALBUMIN AND FASTING BLOOD GLUCOSE IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

The line of agreement and the +30 and -30 centile lines are indicated. High glycators are represented as (♦) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILES

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 7.7. RELATIONSHIP BETWEEN FRUCTOSAMINE AND FASTING BLOOD GLUCOSE IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

The line of agreement and the +30 and -30 centile lines are indicated.
High glycators are represented as (◇) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILES

The regression line and correlation coefficient (r) are indicated.
High glycators are represented as (■) and low glycators as (□).
FIGURE 3.6(c) RELATIONSHIP BETWEEN GLYCATED ALBUMIN AND MEAN BLOOD GLUCOSE(PROFILES) IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

The line of agreement and the +30 and -30 centile lines are indicated. High glycators are represented as (♦) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILES

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 3.7. (c) RELATIONSHIP BETWEEN FRUCTOSAMINE AND MEAN BLOOD GLUCOSE (PROFILES) IN THE HIGH AND LOW GLYCATORS

(i) USING CENTILES

The line of agreement and the +30 and -30 centile lines are indicated. High glycators are represented as (❖) and the low glycators as (□).

(ii) WITHOUT THE USE OF CENTILES

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
3.8.2. CORRELATION OF GLYCATED ALBUMIN, FRUCTOSAMINE AND GLYCATED HAEMOGLOBIN.

A weak negative correlation \((r = -0.43)\) was found between glycated albumin and GHb (see Figure 3.8(a)), whilst no apparent correlation was found between fructosamine and GHb \((r = -0.18)\) (see Figure 3.8(b)). A weak positive correlation was found between glycated albumin and fructosamine \((r = 0.46)\) (see Figure 3.8(c)). There was one high glycator who had a relatively high fructosamine level compared to the glycated albumin level (No. 8).

3.8.3. COMPARISON OF GLYCATED ALBUMIN, FRUCTOSAMINE AND GLYCATED HAEMOGLOBIN LEVELS IN THE HIGH AND LOW GLYCATORS

Although the GHb levels were higher in the high glycators and the glycated albumin levels were higher in the low glycators, none of the differences were significant (see Table 3.5). The fructosamine levels were virtually identical in both groups of subjects.

3.8.4. PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS IN THE LOW AND HIGH GLYCATORS

Although there were no significant differences in either plasma or intra-erythrocyte glucose concentrations between the low and high glycators (see Table 3.6), there were significant differences between the two blood compartments at different time points within each group of subjects (see Figure 3.9(a) and (b)). There was no significant difference between the fasting plasma and intra-erythrocyte glucose concentrations in either the low or high glycators. However, following the glucose load, although no significant differences were found between plasma and intra-erythrocyte glucose concentrations in the high glycators, there were highly significant differences between the two compartments in the low glycators (see Table 3.7(a) and (b)). The total plasma concentration during the OGTT (i.e. swing) was significantly greater than the total intra-erythrocyte glucose concentration in both the high \((p<0.02)\) and low glycators \((p<0.005)\)
FIGURE 3.8(a)  CORRELATION BETWEEN GLYCATED ALBUMIN AND GLYCATED HAEMOGLOBIN IN THE HIGH AND LOW GLYCATORS

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 3.8.(b) CORRELATION BETWEEN GLYCATED HAEMOGLOBIN AND FRUCTOSAMINE

The regression line and correlation coefficient \( r \) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE.3.8.(c) CORRELATION BETWEEN GLYCATED ALBUMIN AND FRUCTOSAMINE

The regression line and correlation coefficient (r) are indicated. High glycators are represented as (■) and low glycators as (□).
<table>
<thead>
<tr>
<th>GLYCATED PROTEIN</th>
<th>LOW GLYCATORS</th>
<th>HIGH GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN GLYCATED HAEMOGLOBIN (%)</td>
<td>6.13 (0.97)</td>
<td>6.87 (0.97)</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCATED ALBUMIN (%)</td>
<td>2.28 (0.57)</td>
<td>1.99 (0.43)</td>
<td>NS</td>
</tr>
<tr>
<td>FRUCTOSAMINE (mmol/l)</td>
<td>2.55 (0.32)</td>
<td>2.53 (0.31)</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose Level</td>
<td>Low Glycators</td>
<td>High Glycators</td>
<td>Significance of Difference (p)</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mmol/l)</td>
<td>5.25 (0.58)</td>
<td>5.86 (0.66)</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting Intraerythrocyte Glucose (mmol/l)</td>
<td>4.60 (1.38)</td>
<td>5.01 (1.14)</td>
<td>NS</td>
</tr>
<tr>
<td>Two-Hour Plasma Glucose (mmol/l)</td>
<td>7.05 (1.48)</td>
<td>6.15 (1.99)</td>
<td>NS</td>
</tr>
<tr>
<td>Two-Hour Intraerythrocyte Glucose (mmol/l)</td>
<td>5.01 (1.73)</td>
<td>6.12 (1.94)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum Plasma Glucose - OGTT (mmol/l)</td>
<td>29.88 (4.35)</td>
<td>30.55 (5.57)</td>
<td>NS</td>
</tr>
<tr>
<td>Sum Intraerythrocyte Glucose - OGTT (mmol/l)</td>
<td>22.63 (6.52)</td>
<td>27.11 (5.97)</td>
<td>NS</td>
</tr>
</tbody>
</table>
FIGURE 3.9. CHANGES IN GLUCOSE CONCENTRATION IN THE PLASMA AND ERYTHROCYTES DURING THE GLUCOSE TOLERANCE TEST

(a) LOW GLYCATORS

(b) HIGH GLYCATORS

1 = BASAL (FASTING)
2 = 30 MINS POST-LOAD
3 = 1 HOUR POST-LOAD
4 = 2 HOURS POST-LOAD

The significance of difference between the plasma and erythrocyte glucose concentrations is indicated.
<table>
<thead>
<tr>
<th>TIME</th>
<th>PLASMA GLUCOSE (mmol/l)</th>
<th>INTRA-ERYTHROCYTE GLUCOSE (mmol/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASTING</td>
<td>5.25 (0.58)</td>
<td>4.60 (1.38)</td>
<td>NS</td>
</tr>
<tr>
<td>30 MINUTES</td>
<td>9.11 (2.08)</td>
<td>6.36 (1.49)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1-HOUR</td>
<td>8.49 (1.44)</td>
<td>6.68 (2.26)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2-HOURS</td>
<td>7.05 (1.48)</td>
<td>5.01 (1.73)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SUM</td>
<td>29.88 (4.35)</td>
<td>22.63 (6.52)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
**TABLE 3.7 (b) DIFFERENCES BETWEEN PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS IN THE HIGH GLYCATORS**

<table>
<thead>
<tr>
<th>TIME</th>
<th>PLASMA GLUCOSE (mmol/l)</th>
<th>INTRA-ERYTHROCYTE GLUCOSE (mmol/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASTING</td>
<td>5.86 (0.66)</td>
<td>5.01 (1.14)</td>
<td>NS</td>
</tr>
<tr>
<td>30 MINUTES</td>
<td>9.67 (1.45)</td>
<td>8.58 (2.96)</td>
<td>NS</td>
</tr>
<tr>
<td>1-HOUR</td>
<td>8.81 (2.35)</td>
<td>7.41 (1.91)</td>
<td>NS</td>
</tr>
<tr>
<td>2-HOURS</td>
<td>6.15 (1.99)</td>
<td>6.12 (1.94)</td>
<td>NS</td>
</tr>
<tr>
<td>SUM</td>
<td>30.55 (5.57)</td>
<td>27.11 (5.97)</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>
The ratio of intra-erythrocyte to plasma glucose, known as the permeability of the erythrocyte to glucose (Higgins et al., 1982) was significantly lower (p<0.05) in the low compared to the high glycators two hours after the glucose load. There were no significant differences between the two groups of subjects in permeability at fasting or at any other time (except at two hours) during the OGTT (see Table 3.8).

There was a significant (p<0.001) correlation between plasma and intra-erythrocyte glucose concentration at two hours, with the correlation being greater for the high (r = 0.87) than the low glycators (r = 0.67) (see Figure 3.10(b)). At fasting, the correlation between intra-erythrocyte and plasma glucose levels was weaker (r = 0.54), although there was a slightly better correlation for the low (r = 0.70) than the high glycators (r = 0.62) (see Figure 3.10(a)). A highly significant (p<0.001) correlation was found between intra-erythrocyte glucose concentration and the mean GHb level in the low glycators, particularly at fasting (r = 0.91) (see Figure 3.11(a) and (b)). The correlation between intra-erythrocyte glucose concentration and mean GHb level was much weaker in the high glycators, particularly at two hours (r = 0.22). At similar intra-erythrocyte glucose concentrations to the low glycators, the high glycators had similar or higher GHb levels. Similarly, the relationship between erythrocyte permeability to glucose and mean GHb levels was much stronger for the low than the high glycators (see Figure 3.12(a) and (b)).
<table>
<thead>
<tr>
<th>TIME</th>
<th>LOW GLYCATORS</th>
<th>HIGH GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>0.87 (0.24)</td>
<td>0.86 (0.17)</td>
<td>NS</td>
</tr>
<tr>
<td>30 MINUTES</td>
<td>0.71 (0.13)</td>
<td>0.88 (0.28)</td>
<td>NS</td>
</tr>
<tr>
<td>1 HOUR</td>
<td>0.77 (0.16)</td>
<td>0.87 (0.29)</td>
<td>NS</td>
</tr>
<tr>
<td>2 HOURS</td>
<td>0.70 (0.17)</td>
<td>1.01 (0.24)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
FIGURE 3.10. RELATIONSHIP BETWEEN PLASMA AND ERYTHROCYTE GLUCOSE CONCENTRATIONS IN THE HIGH AND LOW GLYCATORS

(a) FASTING

The regression lines and correlation coefficients ($r$) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 3.11. RELATIONSHIP BETWEEN MEAN GLYCAVED HAEMOGLOBIN AND ERYTHROCYTE GLUCOSE CONCENTRATIONS IN THE HIGH AND LOW GLYCATORS

(a) FASTING

(b) TWO-HOUR POST-LOAD

The regression lines and correlation coefficients (r) are indicated. High glycators are represented as (■) and low glycators as (□).
FIGURE 3.12. RELATIONSHIP BETWEEN ERYTHROCYTE PERMEABILITY TO GLUCOSE AND MEAN GLYCATED HAEMOGLOBIN IN THE HIGH AND LOW GLYCATORS

(a) FASTING

The regression lines and correlation coefficients (r) are indicated. High glycators are represented as [■] and low glycators as [□].
3.8.5. COMPARISON OF FACTORS DIRECTLY AFFECTING THE GLYCATION REACTION IN THE LOW AND HIGH GLYCATORS (Results are shown in Tables 3.9 and 3.10).

There was no significant difference in either plasma or intra-erythrocyte inorganic phosphate concentrations between the two groups, although there was a weak negative correlation between intra-erythrocyte inorganic phosphate and mean GHB (r = -0.38). The erythrocyte 2,3-diphosphoglycerate concentrations were significantly (p<0.001) greater in the high glycators compared to the low glycators. There was also a weak positive correlation between erythrocyte 2,3-diphosphoglycerate and mean GHB (r = 0.30).

There was no significant difference in intra-erythrocyte pH between the two groups. However, there was a significant (p<0.05) positive correlation (r = 0.55) between intra-erythrocyte pH and mean GHB.

3.8.6. COMPARISON OF COMPOUNDS COMPETING WITH GLUCOSE FOR BINDING TO PROTEINS IN THE LOW AND HIGH GLYCATORS (Results are shown in Tables 3.10 and 3.11).

There was no significant difference in either plasma or intra-erythrocyte vitamin C concentrations between the two groups, although there was a weak negative correlation (r = -0.40) between intra-erythrocyte vitamin C concentrations and mean GHB levels. Neither plasma urea nor free fatty acids were significantly different between the two groups.

3.8.7. COMPARISON OF AMINO ACID LEVELS IN THE HIGH AND LOW GLYCATORS

There were no significant differences in plasma, erythrocyte or urine total amino acids between the two groups (see Tables 3.12, 3.13 and 3.14). The plasma threonine and lysine concentrations were significantly greater (p<0.01 and <0.05 respectively) in the low glycators. Only the taurine concentration in the erythrocyte was significantly (p<0.05) greater in the low compared to the high glycators. A significant negative correlation was found between plasma total amino acids and mean GHB whereas only a very weak negative correlation was found between erythrocyte total amino acids and mean GHB (see Table 3.10).
TABLE 3.9 COMPARISON OF FACTORS DIRECTLY AFFECTING THE GLYCATION REACTION IN THE LOW AND HIGH GLYCATORS

<table>
<thead>
<tr>
<th>FACTOR AFFECTING GLYCATION</th>
<th>LOW GLYCATORS</th>
<th>HIGH GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA INORGANIC PHOSPHATE (mmol/l)</td>
<td>0.97 (0.19)</td>
<td>1.06 (0.07)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE INORGANIC PHOSPHATE (mmol/l)</td>
<td>0.46 (0.21)</td>
<td>0.36 (0.21)</td>
<td>NS</td>
</tr>
<tr>
<td>ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE (mmol/l)</td>
<td>4.81 (0.24)</td>
<td>5.61 (0.26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE pH</td>
<td>7.05 (0.19)</td>
<td>7.17 (0.11)</td>
<td>NS</td>
</tr>
</tbody>
</table>
### TABLE 3.10  CORRELATION (r) OF MEAN GLYCATED HAEMOGLOBIN WITH VARIOUS BIOCHEMICAL PARAMETERS MEASURED IN THE LOW AND HIGH GLYCATORs

<table>
<thead>
<tr>
<th>MEASUREMENT</th>
<th>CORRELATION COEFFICIENT (r)</th>
<th>SIGNIFICANCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRA-ERYTHROCYTE INORGANIC PHOSPHATE</td>
<td>-0.38</td>
<td>NS</td>
</tr>
<tr>
<td>ERYTHROCYTE 2,3-DIPHOSPHGLYCERATE</td>
<td>0.30</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE pH</td>
<td>0.55</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE VITAMIN C</td>
<td>-0.40</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE TOTAL AMINO ACIDS</td>
<td>-0.30</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA TOTAL AMINO ACIDS</td>
<td>-0.57</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
TABLE 3.11  COMPARISON OF COMPOUNDS COMPETING WITHIN GLUCOSE FOR BINDING TO PROTEINS IN THE LOW AND HIGH GLYCATORS

<table>
<thead>
<tr>
<th>COMPOUNDS COMPETING WITH GLUCOSE</th>
<th>LOW GLYCATORS</th>
<th>HIGH GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA VITAMIN C (μmol/l)</td>
<td>62.6 (36.8)</td>
<td>54.0 (22.4)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE VITAMIN C (μmol/l)</td>
<td>50.1 (20.1)</td>
<td>37.8 (29.9)</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA UREA (mmol/l)</td>
<td>4.64 (0.69)</td>
<td>4.64 (1.28)</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA FREE FATTY ACIDS (mmol/l)</td>
<td>0.49 (0.18)</td>
<td>0.61 (0.14)</td>
<td>NS</td>
</tr>
<tr>
<td>AMINO ACID</td>
<td>LOW GLYCATORS (μmol/l)</td>
<td>HIGH GLYCATORS (μmol/l)</td>
<td>SIGNIFICANCE OF DIFFERENCE (p)</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------</td>
<td>-------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>ASPARTIC ACID</td>
<td>N.D.</td>
<td>2.19 (3.97)</td>
<td>NS</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>34.3 (29.7)</td>
<td>40.0 (43.9)</td>
<td>NS</td>
</tr>
<tr>
<td>SERINE</td>
<td>49.9 (14.7)</td>
<td>75.3 (49.6)</td>
<td>NS</td>
</tr>
<tr>
<td>ASPARAGINE</td>
<td>47.8 (28.3)</td>
<td>29.0 (22.3)</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>118.5 (56.8)</td>
<td>98.2 (51.9)</td>
<td>NS</td>
</tr>
<tr>
<td>TAURINE</td>
<td>28.3 (47.4)</td>
<td>19.6 (13.8)</td>
<td>NS</td>
</tr>
<tr>
<td>HISTIDINE</td>
<td>43.3 (25.4)</td>
<td>25.4 (25.5)</td>
<td>NS</td>
</tr>
<tr>
<td>CITRULLINE</td>
<td>17.0 (11.0)</td>
<td>17.5 (10.2)</td>
<td>NS</td>
</tr>
<tr>
<td>THREONINE</td>
<td>65.8 (16.3)</td>
<td>27.3 (22.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALANINE</td>
<td>178.0 (32.6)</td>
<td>166.3 (92.0)</td>
<td>NS</td>
</tr>
<tr>
<td>ARGinine</td>
<td>48.7 (10.1)</td>
<td>49.9 (27.8)</td>
<td>NS</td>
</tr>
<tr>
<td>PROLINE</td>
<td>108.1 (65.1)</td>
<td>71.7 (59.1)</td>
<td>NS</td>
</tr>
<tr>
<td>TYROSINE</td>
<td>35.7 (21.8)</td>
<td>46.1 (15.1)</td>
<td>NS</td>
</tr>
<tr>
<td>VALINE</td>
<td>114.4 (24.4)</td>
<td>129.4 (39.9)</td>
<td>NS</td>
</tr>
<tr>
<td>METHIONINE</td>
<td>15.3 (2.6)</td>
<td>16.3 (13.1)</td>
<td>NS</td>
</tr>
<tr>
<td>ISOLEUCINE</td>
<td>28.5 (15.3)</td>
<td>42.2 (18.0)</td>
<td>NS</td>
</tr>
<tr>
<td>LEUCINE</td>
<td>88.5 (72.1)</td>
<td>102.8 (60.4)</td>
<td>NS</td>
</tr>
<tr>
<td>PHENYLALANINE</td>
<td>50.4 (21.0)</td>
<td>34.1 (19.7)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Value 1 (SD)</td>
<td>Value 2 (SD)</td>
<td>p-value</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>40.4 (20.4)</td>
<td>28.1 (10.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Ornithine</td>
<td>33.5 (26.8)</td>
<td>37.8 (26.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Lysine</td>
<td>105.6 (16.4)</td>
<td>84.0 (14.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Total Amino Acids</strong></td>
<td>1280 (160)</td>
<td>1090 (160)</td>
<td>NS</td>
</tr>
</tbody>
</table>

N.D. = NOT DETECTED
<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>LOW GLYCATORS (µmol/l)</th>
<th>HIGH GLYCATORS (µmol/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERINE</td>
<td>66.0 (15.7)</td>
<td>56.9 (11.5)</td>
<td>NS</td>
</tr>
<tr>
<td>ASPARAGINE</td>
<td>126.4 (19.5)</td>
<td>109.6 (19.5)</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>186.8 (66.2)</td>
<td>183.0 (58.1)</td>
<td>NS</td>
</tr>
<tr>
<td>TAURINE</td>
<td>32.4 (7.8)</td>
<td>9.6 (16.5)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>CITRULLINE</td>
<td>7.5 (10.4)</td>
<td>7.2 (13.0)</td>
<td>NS</td>
</tr>
<tr>
<td>THREONINE</td>
<td>N.D.</td>
<td>8.7 (22.9)</td>
<td>NS</td>
</tr>
<tr>
<td>ALANINE</td>
<td>179.9 (29.6)</td>
<td>191.0 (26.4)</td>
<td>NS</td>
</tr>
<tr>
<td>PROLINE</td>
<td>50.8 (32.6)</td>
<td>28.2 (42.3)</td>
<td>NS</td>
</tr>
<tr>
<td>VALINE</td>
<td>63.0 (18.5)</td>
<td>42.6 (11.8)</td>
<td>NS</td>
</tr>
<tr>
<td>METHIONINE</td>
<td>5.5 (12.3)</td>
<td>N.D.</td>
<td>NS</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>285.0 (108.0)</td>
<td>160.9 (65.7)</td>
<td>NS</td>
</tr>
<tr>
<td>LEUCINE</td>
<td>28.4 (24.1)</td>
<td>38.1 (49.9)</td>
<td>NS</td>
</tr>
<tr>
<td>ORNITHINE</td>
<td>45.0 (11.9)</td>
<td>35.9 (21.3)</td>
<td>NS</td>
</tr>
<tr>
<td>LYSINE</td>
<td>75.3 (42.5)</td>
<td>48.8 (9.7)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>TOTAL AMINO ACIDS</strong></td>
<td><strong>1120 (170)</strong></td>
<td><strong>980 (120)</strong></td>
<td><strong>NS</strong></td>
</tr>
</tbody>
</table>

N.D. = NOT DETECTED
TABLE 3.14  TOTAL URINE AMINO ACIDS IN THE HIGH AND LOW GLYCATORS

<table>
<thead>
<tr>
<th></th>
<th>LOW GLYCATORS</th>
<th>HIGH GLYCATORS</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mmol/24hours/mmol creatinine)</td>
<td>0.29 (0.14)</td>
<td>0.23 (0.11)</td>
<td>NS</td>
</tr>
</tbody>
</table>
3.8.8. COMPARISON OF PLASMA INSULIN LEVELS IN THE LOW AND HIGH GLYCATORS

Although the plasma levels of insulin were higher in the high glycators than the low glycators at all time points except two-hours, the differences failed to reach significance (see Table 3.15).

3.8.9. COMPARISON OF HAEMATOLOGICAL VARIABLES IN THE LOW AND HIGH GLYCATORS

No abnormality in haematology was detected in any of the subjects. None of the tests suggested any differences in erythrocyte survival between the low and high glycators and no abnormal haemoglobins were detected in the subjects (see Table 3.16). All the subjects had a negative Coomb's test indicating that antibodies to erythrocytes as a cause of haemolysis were not present.

3.8.10. COMPARISON OF TWO LOW GLYCATORS WITH RELATIVELY LOW GLYCATED ALBUMIN LEVELS WITH THE OTHER LOW GLYCATORS

None of the biochemical parameters measured were significantly different between the subgroup of low glycators who had relatively low glycated albumin levels as well as low GHb levels compared to the other low glycators who despite having relatively low GHb levels had appropriate levels of glycated albumin (see Table 3.17).
### TABLE 3.15 COMPARISON OF PLASMA INSULIN LEVELS DURING THE GLUCOSE TOLERANCE TEST IN THE LOW AND HIGH GLYCATORS

<table>
<thead>
<tr>
<th>TIME OF SAMPLE</th>
<th>LOW GLYCATORS (mU/l)</th>
<th>HIGH GLYCATORS (mU/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASTING</td>
<td>11.0 (3.8)</td>
<td>12.5 (3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>30 MINUTES</td>
<td>64.1 (29.6)</td>
<td>93.2 (43.5)</td>
<td>NS</td>
</tr>
<tr>
<td>1 HOUR</td>
<td>76.5 (55.7)</td>
<td>116.1 (71.8)</td>
<td>NS</td>
</tr>
<tr>
<td>2 HOURS</td>
<td>77.7 (42.2)</td>
<td>65.8 (60.9)</td>
<td>NS</td>
</tr>
<tr>
<td>SUM</td>
<td>235 (136)</td>
<td>289 (153)</td>
<td>NS</td>
</tr>
<tr>
<td>HAEMATOLOGICAL VARIABLE</td>
<td>LOW GLYCATORS</td>
<td>HIGH GLYCATORS</td>
<td>SIGNIFICANCE OF DIFFERENCE (p)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>----------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>ERYTHROCYTE COUNT (x 10¹²/l)</td>
<td>4.36 (0.37)</td>
<td>4.30 (0.42)</td>
<td>NS</td>
</tr>
<tr>
<td>HAEMOGLOBIN (g/100ml)</td>
<td>13.9 (0.9)</td>
<td>13.5 (0.9)</td>
<td>NS</td>
</tr>
<tr>
<td>PACKED CELL VOLUME (%)</td>
<td>41 (4)</td>
<td>40 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>MEAN CELL HAEMOGLOBIN (pg)</td>
<td>31.9 (1.2)</td>
<td>31.8 (2.1)</td>
<td>NS</td>
</tr>
<tr>
<td>MEAN CELL HAEMOGLOBIN CONCENTRATION (g/100ml)</td>
<td>33.5 (2.1)</td>
<td>33.1 (1.4)</td>
<td>NS</td>
</tr>
<tr>
<td>RETICULOCYTES (%)</td>
<td>1.6 (0.6)</td>
<td>1.8 (0.5)</td>
<td>NS</td>
</tr>
</tbody>
</table>
TABLE 3.17 COMPARISON OF THE TWO LOW GLYCATORS (LL) WITH RELATIVELY LOW GLYCATED ALBUMIN TO THE OTHER LOW GLYCATORS (L) WHO HAVE APPROPRIATE LEVELS OF GLYCATED ALBUMIN

<table>
<thead>
<tr>
<th>BIOCHEMICAL MEASUREMENT</th>
<th>LOW GLYCATORS (LL)</th>
<th>LOW GLYCATORS (L)</th>
<th>SIGNIFICANCE DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAST PLASMA GLUCOSE (mmol/l)</td>
<td>5.64 (0.48)</td>
<td>4.99 (0.54)</td>
<td>NS</td>
</tr>
<tr>
<td>2HOUR PLASMA GLUCOSE (mmol/l)</td>
<td>6.39 (0.74)</td>
<td>7.49 (1.84)</td>
<td>NS</td>
</tr>
<tr>
<td>SUM OF PLASMA GLUCOSE-OGTT (mmol/l)</td>
<td>31.20 (4.67)</td>
<td>29.00 (4.91)</td>
<td>NS</td>
</tr>
<tr>
<td>FAST INTRA-ERYTHROCYTE GLUCOSE (mmol/l)</td>
<td>3.84 (0.79)</td>
<td>2.44 (0.99)</td>
<td>NS</td>
</tr>
<tr>
<td>2HOUR INTRA-ERYTHROCYTE GLUCOSE (mmol/l)</td>
<td>3.44 (0.34)</td>
<td>2.92 (1.46)</td>
<td>NS</td>
</tr>
<tr>
<td>SUM OF INTRA-ERYTHROCYTE GLUCOSE - OGTT (mmol/l)</td>
<td>16.45 (0.50)</td>
<td>12.89 (5.17)</td>
<td>NS</td>
</tr>
<tr>
<td>FAST PLASMA INSULIN (mU/l)</td>
<td>14.9 (2.2)</td>
<td>8.4 (1.1)</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA INORGANIC PHOSPHATE (mmol/l)</td>
<td>1.06 (0.04)</td>
<td>0.91 (0.24)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE INORGANIC PHOSPHATE (mmol/l)</td>
<td>0.37 (0.09)</td>
<td>0.53 (0.26)</td>
<td>NS</td>
</tr>
<tr>
<td>ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE (mmol/l)</td>
<td>4.77 (0.28)</td>
<td>4.84 (0.27)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE pH</td>
<td>7.14 (0.21)</td>
<td>7.00 (0.19)</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA VITAMIN C (μmol/l)</td>
<td>34.5 (44.2)</td>
<td>38.2 (28.0)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE VITAMIN C (μmol/l)</td>
<td>39.7 (12.0)</td>
<td>77.6 (42.0)</td>
<td>NS</td>
</tr>
<tr>
<td>MEAN GLYCATED HAEMOGLOBIN (%)</td>
<td>6.58 (0.70)</td>
<td>5.83 (1.14)</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCATED ALBUMIN (%)</td>
<td>1.70 (0.10)</td>
<td>2.67 (0.17)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>FRUCTOSAMINE (mmol/l)</td>
<td>2.26 (0.03)</td>
<td>2.94 (0.23)</td>
<td>NS</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Value 1</th>
<th>Value 2</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN BLOOD GLUCOSE-PROFILES (mmol/l)</td>
<td>6.67 (0.46)</td>
<td>6.85 (0.75)</td>
<td>NS</td>
</tr>
<tr>
<td>PLASMA TOTAL AMINO ACIDS (mmol/l)</td>
<td>1.34 (0.11)</td>
<td>1.24 (0.20)</td>
<td>NS</td>
</tr>
<tr>
<td>INTRA-ERYTHROCYTE TOTAL AMINO ACIDS (mmol/l)</td>
<td>1.27 (0.06)</td>
<td>1.02 (0.14)</td>
<td>NS</td>
</tr>
<tr>
<td>URINE TOTAL AMINO ACIDS (mmol/24hrs/mmol creatinine)</td>
<td>0.29 (0.19)</td>
<td>0.31 (0.15)</td>
<td>NS</td>
</tr>
</tbody>
</table>
DISCUSSION

The majority of the subjects studied in Part 1 who had inappropriate levels of GHb relative to their blood glucose levels did not have glycated albumin levels that were inappropriate relative to their levels of glycaemia. All of the so-called high glycators and most of the so-called low glycators had appropriate levels of glycated albumin in relation to their blood glucose levels. The fructosamine levels followed a similar pattern to the glycated albumin levels, but in general there was a much weaker relationship between fructosamine and blood glucose levels compared to glycated albumin and glycaemia. This probably reflects the non-specific nature of the fructosamine reaction which has a number of known interferences (Schleicher et al., 1988). These will also be more obvious in normal subjects where they probably make a larger contribution to the overall fructosamine activity measured. Albumin makes up at least 60% of the total plasma proteins (Peters, 1985) and its specific measurement is a more accurate reflection of blood glucose levels than fructosamine, which is a measure of all plasma glycated proteins, a heterogeneous mixture with different half-lives (Seng and Staley, 1986). In general, the high and low glycators for haemoglobin did not also show high and low glycation of albumin. This indicates that the glycation of haemoglobin and albumin were occurring at different levels.

Although albumin and haemoglobin are both found in the blood, they are located in different compartments. Albumin is free to circulate in the plasma and extravascular spaces, whilst haemoglobin is confined to the erythrocyte. Albumin has a half-life of approximately 19 days (Peters, 1985). There is no specific site for its metabolism. It is thought to be taken up by cells throughout the body and degraded in lysosomes (Baynes and Thorpe, 1981; Henderson et al., 1982). Haemoglobin circulates within the mature erythrocytes for 120 days, after which they are taken up and destroyed mainly by the spleen (Harris and Kellermeyer, 1970).

Both albumin and haemoglobin are glycated in vivo and have been shown to reflect blood glucose levels over the previous 2 - 3 weeks and 4 - 6 weeks respectively (Kennedy et al., 1981; Bunn et al., 1978). Haemoglobin is glycated at both the N-termini of the α- and β-chains and also at various ε-amino groups of lysine residues throughout the molecule. The major sites of glycation of haemoglobin in vivo are in order of prevalence, β-val-1, β-lys-66,
α-lys-61, β-lys-17 and α-val-1 (Shapiro et al., 1980). Albumin on the other hand has been shown to be glycated mainly at the e-amino groups of lysine residues, although the end terminal amino acids have recently been shown to be glycated in vivo (Robb et al., 1989). Lys-525 has been shown to be the major site of glycation of albumin in vivo (Garlick and Mazer, 1983; Iberg and Fluckiger, 1985). At least nine other lysine residues in albumin have been shown to be glycated in vivo (Iberg and Fluckiger, 1986). These are lys 439, 199, 281, 233, 317, 351, 534 and 12. The specificity of glycation of both albumin and haemoglobin has been suggested to be partially due to the pKa of the amino group but appears to be predominantly due to the location of the Schiff base within the microstructure of the protein (Watkins et al., 1988). Acid-base catalysis of the Amadori rearrangement by acidic or basic amino acids in close proximity to the Schiff base is thought to be the main factor in directing the specificity of glycation of albumin (Olufemi et al., 1987). With haemoglobin, although neighbouring amino acids may also play a role catalysing the rearrangement, the specific glycation of the β-chain terminal valine residue is thought to be due to its location within the 2,3-diphosphoglycerate binding pocket. It is thought that the anionic phosphate groups catalyse the glycation reaction at this site (Watkins et al., 1987). Approximately 4 - 5% of haemoglobin is glycated at the β-chain termini in normal subjects whereas another 9 - 10% of haemoglobin is thought to be glycated at lysine residues (Bunn et al., 1979).

The percentage of glycation of albumin in normal subjects has been shown to be 2 - 12%. The inconsistency in its measurement appears to be due to differences in the methodology (Yatscoff et al., 1984; Ziel and Davidson, 1987). With the methodology used in the present study (affinity chromatography and immunoelectrophoresis) a normal range of 1 - 3% was found.

Several studies have been carried out to investigate the relative merits of using glycated haemoglobin and/or glycated albumin in the monitoring of blood glucose levels (Yue et al., 1979; Schleicher et al., 1984; Ross et al., 1986). Although in general both glycated proteins have been shown to correlate well with measures of glycaemia in diabetic subjects, evidence does appear to suggest that haemoglobin and albumin are glycated at different levels. For example, the correlation of the two glycated proteins with various glycaemic indices is often different in the same subject. Schleicher et al., (1984) found that in a group of diabetic subjects followed over several months, the correlation between glycated plasma
proteins with the mean blood glucose was significantly greater than for glycated haemoglobin. Quite poor correlations are also often found between glycated plasma proteins and glycated haemoglobin (Guthrow et al., 1979; Kennedy et al., 1981; Yatscoff et al., 1984; Ross et al., 1986). Guthrow et al., (1979) found that whilst glycated haemoglobin levels were in the normal range in a group of diabetic subjects, glycated albumin levels were much higher in the same individuals.

This difference between the level of glycation of haemoglobin and albumin has been mainly attributed to the different half-lives of the two proteins, therefore reflecting different periods of glycaemia. This may be true in diabetics, particularly insulin-dependent diabetics where large fluctuations in blood glucose occur, but it is unlikely to provide an explanation for the discrepancy found in the non-diabetic subjects between glycated albumin and glycated haemoglobin in this study. Significant correlations were found between the different measures of glycaemia with both the high and low glycators.

The glucose concentration within the erythrocyte has been shown to be 60 - 80% of the plasma concentration (Somogyi, 1933; Higgins et al., 1982). In the present study, the erythrocyte glucose concentration was found to be approximately 80% (mean) of the plasma glucose concentration. However, there was a large variability in the ratio of glucose in the erythrocyte to glucose in the plasma between individuals and at different time points during the glucose tolerance test. The low glycators had significantly lower intra-erythrocyte glucose concentrations compared to plasma at all time points during the glucose tolerance test (except at fasting). This may be a contributory factor in determining the low GHb levels relative to their plasma glucose levels in these subjects.

Although no significant difference was found between the fasting or two-hour intra-erythrocyte glucose concentrations between the low and high glycators, a much stronger correlation was found between the intra-erythrocyte glucose concentrations and the GHb levels in the low compared to the high glycators. This would suggest that differences between plasma and erythrocyte glucose concentrations might be responsible for the discrepancy found between glycated albumin and GHb in most of the low glycators. The high glycators had similar intra-erythrocyte glucose concentrations to the low glycators whilst having significantly higher GHb levels. This would therefore suggest that some other additional factor within the
erythrocyte was causing a higher rate of glycation of haemoglobin relative to their erythrocyte glucose concentrations.

Any difference between plasma and erythrocyte glucose levels must be determined by transport of glucose into or out of the erythrocyte and/or metabolism of glucose within the erythrocyte. The principal route for the consumption of glucose in the erythrocyte is the Embden-Meyerhof pathway and more than 95% of glucose utilized by the cells pass through it (Grimes, 1980). The remainder passes through the hexose monophosphate shunt. However, metabolism of glucose via either of these pathways is unlikely to be the main explanation of the relatively low erythrocyte glucose levels because the transport of glucose into the erythrocyte has been shown to be 250 times the rate of glucose utilization (Widdas, 1954). No relationship has been found between the transport of glucose into the erythrocyte and the rate of glycolysis (Somogyi, 1933; Laris, 1958).

Glucose is taken up by the erythrocyte by facilitated diffusion (Grimes, 1980). Brahm (1983) studied the kinetics of glucose transport into the human erythrocyte in vitro and found it to be both concentration and pH dependent. The maximal transport occurred at pH 7.2 and decreased progressively with an increase in acidic or alkaline conditions. Therefore at a physiological whole blood pH of 7.4, the transport system is not working at its maximal rate. This may contribute to the difference found between plasma and erythrocyte glucose levels. Glucose may be transported in either direction, with the influx depending on the extracellular pH and the efflux from the cell depending on the intra-erythrocyte pH. The weak relationship found between plasma and erythrocyte glucose levels in the low glycators compared to the high glycators could be explained by a failure of the transport system to work at a maximal rate, perhaps due to a pH effect. The intra-erythrocyte pH was found to be very close to the pH causing maximal transport in the high glycators but was lower in the low glycators. The extracellular pH (in the calculation of intra-erythrocyte pH) was higher in the high glycators and was nearer the maximal rate in the low glycators. Therefore, if there is a difference in the rate of transport into the erythrocyte between the low and high glycators it is not explained by pH differences.

There is also some evidence for the conversion of glucose to glycogen within the erythrocyte. Guarner & Alvarez-Buylla (1989) studied glucose homeostasis in rats in vitro and in vivo. They found that the erythrocytes incorporated glucose into their glycogen
stores when plasma glucose was high and released it when the plasma glucose was low. Therefore, once the enzymes for the Embden-Meyerhof and hexose monophosphate pathways are saturated, increased conversion of glucose to glycogen might provide part of the explanation of the relatively low erythrocyte glucose levels compared to plasma levels.

The permeability of the erythrocyte to glucose has been shown to play an important role in determining the extent of glycation of haemoglobin in different species (Higgins et al., 1982). They found strong positive association between permeability and the GHb level. A better correlation between permeability and GHb levels was found in the low compared to the high glycators who had higher GHb levels with similar permeabilities (and erythrocyte glucose levels) to the low glycators. This suggests that the permeability of the erythrocyte to glucose may play a major role in determining the extent of glycation of haemoglobin in the low glycators. The weak relationship found between plasma and erythrocyte glucose levels in the low glycators would suggest some transport defect. This would also explain the discrepancy between GHb and glycated albumin levels in the low glycators as the swings in plasma glucose were significantly greater than those occurring in the erythrocyte.

The weak relationship found between intra-erythrocyte glucose and GHb levels in the high glycators would suggest that erythrocyte glucose was not the major determinant of the GHb levels. One of the major metabolites of glucose within the erythrocyte, 2,3-diphosphoglycerate has been shown to be a potent catalyst of haemoglobin glycation (Smith et al., 1982; Lowrey et al., 1985). The 2,3-diphosphoglycerate levels were significantly higher in the high glycators and may be the additional factor stimulating glycation of haemoglobin resulting in high GHb levels, relative to both the plasma and erythrocyte glucose levels. 2,3-diphosphoglycerate is produced along with 3-phosphoglycerate from the common substrate 1,3-diphosphoglycerate (see Figure 3.13). It undergoes conversion to 3-phosphoglycerate and therefore acts as a bypass to the phosphoglycerate kinase step. The two pathways compete for their substrate and form either adenosine triphosphate or 2,3-diphosphoglycerate. This section of the Embden-Meyerhof pathway is called the Rapoport-Luebering Shuttle and the regulation between the two branches is complex (Grimes, 1980). The two steps in the 2,3-diphosphoglycerate branch are catalysed by two different enzymes, 2,3-diphosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase (Rose and Liebowitz, 1970). 2,3-diphosphoglycerate like adenosine triphosphate is an inhibitor of its
FIGURE 3.13. THE FORMATION OF 2,3-DIPHOSPHOGLYCERATE VIA THE RAPOPORT-LUEBERING SHUTTLE

GLUCOSE
↓
1,3-DIPHOSPHOGLYCERATE

ADP

ATP

1,3-DIPHOSPHOGLYCERATE

mutase

2,3-DIPHOSPHOGLYCERATE

phosphatase

3-PHOSPHOGLYCERATE

2-PHOSPHOGLYCERATE

LACTATE
own synthesis because of its action on the mutase. In addition to changes in 2,3-diphosphoglycerate levels brought about by modulations within the shuttle, 2,3-diphosphoglycerate levels may be affected secondarily to direct effects upon overall glycolysis. Glycolysis is very pH sensitive, with a fall in pH decreasing the rate of glycolysis, leading to a reduction in 2,3-diphosphoglycerate. The level of 2,3-diphosphoglycerate is further decreased by an increase in phosphatase activity mediated by a fall in pH. An increase in pH leads to an increase in glycolytic activity and therefore an increase in 2,3-diphosphoglycerate levels. Astrup (1969) showed that a change of pH of 0.01 units corresponded to a change in 2,3-diphosphoglycerate of 4% in vivo. Inorganic phosphate can also stimulate glycolysis independently of pH increasing levels of 2,3-diphosphoglycerate (Grimes, 1980).

The importance of 2,3-diphosphoglycerate in the erythrocyte lies in its ability to alter the extent to which haemoglobin binds oxygen. This was discovered by two independent groups of workers, Benesch and Benesch and Chanutin and Curnish in 1967 (Grimes, 1980). Arnone (1972) carried out x-ray studies which indicated that 2,3-phosphoglycerate binds in the central cavity of deoxyhaemoglobin between the two β-chains by salt bridges. At the entrance to the central cavity there are β-chain cationic groups, and 2,3-diphosphoglycerate which is anionic is believed to form seven salt bridges with val-1, his-2 and lys-43 of the two β-chains together with one of the lys-82 residues. Oxygenation leads to a conformational change leading to the expulsion of 2,3-diphosphoglycerate from the central cavity (Grimes, 1980). Changes in the 2,3-diphosphoglycerate level permit adjustment of the dissociation of oxygen from haemoglobin according to the physiological conditions.

Hypoxia which may arise due to pulmonary disease, circulatory disease, high altitude or severe exercise leads to an increase in 2,3-diphosphoglycerate levels by two mechanisms. Firstly, the increased binding of 2,3-diphosphoglycerate to deoxyhaemoglobin leads to a fall in free 2,3-diphosphoglycerate, removing the inhibition of the mutase in the shuttle, which results in increased formation of 2,3-diphosphoglycerate. This leads to an increase in total levels of both free and bound. Secondly, hypoxia may stimulate glycolysis by a pH effect because deoxyhaemoglobin is more alkaline than oxyhaemoglobin, leading to an increase in intra-erythrocyte pH. It is likely that the latter mechanism is the predominant one (Gerlach and Duhm, 1972). Anaemia also leads to a slight increase in
pH due to tendency to hyperventilate and also because the ratio of deoxy- to oxyhaemoglobin increases which results in an increase in 2,3-diphosphoglycerate levels. An increase in plasma inorganic phosphate, as occurs in renal disease, also leads to an increase in 2,3-diphosphoglycerate synthesis (Grimes, 1980). There are at least two inborn errors of metabolism that can affect the glycolytic process in the erythrocyte and therefore the 2,3-diphosphoglycerate level (Newsholme and Leech, 1985). Pyruvate kinase deficiency leads to an increase in 2,3-diphosphoglycerate levels whilst hexokinase deficiency leads to a dramatic fall in 2,3-diphosphoglycerate levels. There is also some evidence that genetic factors may influence 2,3-diphosphoglycerate levels in individuals (Brewer et al., 1970). Several studies have shown a positive correlation between erythrocyte 2,3-diphosphoglycerate and GHb levels in vivo (Roberts et al., 1984; Story et al., 1986) and in vitro (Smith et al., 1972; Lowrey et al., 1985). It has also been suggested that the glycation of haemoglobin leads to a compensatory increase in 2,3-diphosphoglycerate levels in subjects with diabetes. Bunn and Briehl (1970) showed that GHb had an increased oxygen affinity in vitro. They suggested that this was due to the fact that in GHb glucose attaches to the end terminal valines of the β-chains, therefore inhibiting the binding of 2,3-diphosphoglycerate. This led to the proposal that in diabetics, glycation of haemoglobin might lead to tissue hypoxia and that 2,3-diphosphoglycerate synthesis is increased to prevent this from occurring (Ditzel et al., 1975). The levels of 2,3-diphosphoglycerate have been shown to be raised in diabetics (Ditzel et al., 1975; Roberts et al., 1984), but there is no conclusive evidence that the increase in 2,3-diphosphoglycerate is due to the inability of 2,3-diphosphoglycerate to bind to GHb.

On the other hand, 2,3-diphosphoglycerate has been shown to catalyse the reaction of glucose with haemoglobin in vitro (Smith et al., 1972; Lowry et al., 1985; Watkins et al., 1987). It appears to enhance the glycation of both valine and lysine residues selectively by binding to basic micro-environments within the haemoglobin molecule and catalysing the conversion of the Schiff base to the ketoamine (see Figure 1.4). The significantly higher 2,3-diphosphoglycerate levels found in the high glycators may be the additional factor responsible for the inappropriately high GHb levels in these subjects.

The mechanism for the increased 2,3-diphosphoglycerate levels is unclear. The presence of an inborn error of metabolism is unlikely as the effect of an enzyme deficiency has a much more marked effect on the 2,3-diphosphoglycerate levels. They rise to 10 mmol/l
in subjects with pyruvate kinase deficiency (Newsholme and Leech, 1985). Also, glycation of haemoglobin is unlikely to be the cause of the increased 2,3-diphosphoglycerate levels as the high glycators only had 6 - 8% of their haemoglobin glycated. The plasma levels of inorganic phosphate and the intra-erythrocyte pH were not significantly higher in the high compared to the low glycators. However, the finding that a change in pH of only 0.01 units could result in a 4% increase in 2,3-diphosphoglycerate might be part of the explanation for the higher 2,3-diphosphoglycerate levels in the high glycators, who had a mean intra-erythrocytic pH 0.12 units higher than that of the low glycators. The cause of the slightly higher pH in the high glycators is not clear. None of the subjects had evidence of pulmonary or circulatory disease and no evidence for anaemia was suggested by the haematology results. It is possible that the higher 2,3-diphosphoglycerate levels in the high glycators is a genetic phenomenon.

Another explanation for a discrepancy between glycated haemoglobin and blood glucose levels is the competition of other substances with glucose for binding to haemoglobin. However, there was no significant differences in erythrocyte vitamin C concentrations between the two groups, which has been shown to inhibit the glycation of plasma proteins (Stolba et al., 1987, 1988). Also, no significant differences in plasma urea were found, which at high levels is converted to cyanate, which can subsequently react with protein amino groups (Fluckiger et al., 1981). The questionnaire revealed no differences in the intake of alcohol or salicylates, whose metabolites in vivo can also react with amino groups (Stevens et al., 1981; Bridges et al., 1975). Pyridoxal-5-phosphate is known to bind to protein amino groups covalently (Harding, 1985). However, due to technical difficulties this was not measured in the low and high glycators. Amino acids themselves can react with glucose (Sensi et al., 1989) and thereby reduce the amount available to react with protein amino groups. The total amino acid concentrations in the plasma, erythrocyte and urine were not significantly different between the low and high glycators. Although the erythrocyte taurine concentration was higher in the low glycators, it only represents approximately 3% of the total amino acid concentration in the erythrocyte and is therefore unlikely to contribute to the discrepancy found between the GHb and blood glucose levels in the low glycators.

There were two low glycators who as well as having inappropriately low GHb levels, also had glycated albumin levels that were low compared to their blood glucose levels. None of the biochemical
parameters measured were found to be significantly different between this small subgroup and the other low glycators who had appropriate levels of glycated albumin. It is possible that the factor(s) responsible for the relatively low glycated albumin levels were different to those causing the low GHb levels. One of the important roles of albumin is the transport of substances throughout the body (Peters, 1985), including endogenous and exogenous substances such as therapeutic drugs. The questionnaire revealed that one of the subgroup of low glycators was on naproxen, a non-steroidal anti-inflammatory drug, which has been shown to covalently bind to albumin (Peters, 1985) and the other subject with a low glycated albumin was on thyroxine and valium, both of which are known to bind to albumin (Peters, 1985). The binding of these drugs may influence the extent to which glucose can react with albumin. None of the other low glycators were on regular medication known to bind to albumin. Therefore, the relatively low glycated albumin levels in the subgroup of low glycators may be due to the inhibition of glycation by therapeutic drugs.

This study has shown that in normal, non-diabetic subjects, the levels of glycated albumin and in particularly GHb may not always be appropriate to the level of glycaemia. There are subjects with high levels of GHb relative to their blood glucose levels who have appropriate glycated albumin levels. At least part of this discrepancy appears to be due to increased levels of erythrocyte 2,3-diphosphoglycerate which may be due to relatively high intra-erythrocytic pH or perhaps genetically determined. There are also subjects with low levels of GHb relative to their blood glucose levels who have appropriate levels of glycated albumin. This may be at least partially due to the relatively low intra-erythrocyte glucose levels compared to plasma and also the apparent ability of their erythrocytes to 'damp out' the swings in glucose occurring in the plasma post-prandially. There are also some subjects who have relatively low GHb and glycated albumin levels. The low GHb levels are probably due to the relatively low intra-erythrocyte glucose levels, whilst the low glycated albumin levels may be due to the presence of therapeutic drugs which compete with glucose for binding to albumin, thereby inhibiting glycation.

Therefore, in conclusion, GHb may not always accurately reflect mean blood glucose levels due to intra-individual differences in erythrocyte permeability and/or metabolism. In general, glycated albumin appears to provide a more accurate reflection of mean blood glucose levels, except when certain drugs are being taken which interfere with the reaction of glucose and albumin. The possibility
that similar factors could affect the glycation of proteins in diabetic subjects needs to be investigated.
CHAPTER 4

THE EFFECT OF DIET ON THE GLYCATION OF PROTEINS
4.1. INTRODUCTION

By affecting blood glucose levels, diet may indirectly influence the glycation of proteins in vivo.

The blood glucose concentration depends on the balance between the glucose inflow and outflow (Marks and Rose, 1981). The main source of glucose comes from the diet, which may be in the form of complex carbohydrates such as starch, disaccharides such as lactose or free in its monosaccharide form.

The rate of absorption of glucose from the gut and hence the post-prandial rise in blood glucose concentration is dependent on the size and composition of the meal ingested (Newsholme and Leech, 1983). Two major dietary components known to have marked effects on blood glucose levels in normal and diabetic subjects are carbohydrates (Brunzell et al., 1971; Simpson et al., 1979) and soluble fibre (Jenkins et al., 1976, 1977; Krotkiewski, 1984). The type of carbohydrate will affect the rate of absorption. For example, complex carbohydrates, such as starch are relatively slowly digested. Although digestion begins in the mouth, it is not completed until it reaches the duodenum, where the free glucose is then transported across the intestinal mucosa. In contrast, the ingestion of free glucose will result in a more rapid absorption and therefore a more rapid rise in blood glucose concentration (McMahon et al., 1989).

The amount of carbohydrate in the diet will also affect the blood glucose concentration occurring post-prandially. A carbohydrate restricted diet causes an intolerance to a glucose load (Wilkerson et al., 1960) whilst causing a reduction in fasting blood glucose (Ktorza et al., 1985).

The effect of a high carbohydrate intake on blood glucose levels has also been investigated. Brunzell et al., (1971) found that a high carbohydrate diet led to a fall in fasting blood sugar and to an improvement in oral glucose tolerance in both normal and mildly diabetic subjects. However Bierman and Nalmin (1961) found that a high carbohydrate diet had no effect on blood glucose levels in diabetic subjects whilst Grey and Kipnis (1971) found a worsening of these glucose parameters on a high carbohydrate diet in obese subjects. These contradictory findings were probably partly due to
the studies being carried out with different groups of subjects (normals, obese or diabetic), with in some instances no adequate control of calorie intake and body weight. Also, the diets utilized varied greatly in composition and duration, as did the type and consistency of the meal used as a test dose.

These conclusions were supported by Silva et al., (1987) who evaluated the short-term (3 weeks) and long-term (6 weeks) effects of a high carbohydrate diet on carbohydrate metabolism in normal, obese and diabetic subjects. Fasting blood glucose and insulin were measured twice weekly. The tolerance to carbohydrate was assessed by the blood glucose and insulin responses to oral and intravenous glucose loads and to a standard liquid meal. It was found that the long-term diet improved the tolerance to oral glucose in the obese and diabetic subjects whilst having no effect on their fasting glucose or insulin levels. In the normal subjects, the long-term diet had no significant effects on tolerance to oral glucose or on fasting blood glucose, although it did increase both the fasting and post-load insulin levels significantly. However, in response to the liquid test meal, both the blood glucose and insulin levels were significantly increased in all the subjects. The response to the intravenous glucose load was also different. There were no significant differences in blood glucose or insulin levels following the intravenous glucose bolus after the high carbohydrate diet in any of the subjects. These contradictory results found between the different methods of glucose tolerance assessment were probably due to differences in the rate of entry of glucose into the blood and therefore of the insulin response.

The fibre content of a meal will also have a major influence on the rate of absorption of glucose from the gut and therefore on the post-prandial blood glucose levels (Newsholme and Leech, 1983). Fibre is a general term used to describe a range of different substances including cellulose, hemi-cellulose, lignin, pectin and gums and mucilages. The metabolic effects of different types of fibre may vary. Gel-forming fibres such as guar gum have been shown to reduce post-prandial glucose and insulin levels in healthy subjects (Jenkins et al., 1978) and in diabetic subjects (Jenkins et al., 1976). Likely reasons for this are a reduced rate of carbohydrate absorption due to a slower gastric-emptying rate (Wilmshurst and Crawley, 1980) as well as a reduced secretion of insulin secretagogues like gastric inhibitory polypeptide.

Krotkiewski (1984) investigated the acute and long-term effects of guar-gum on post-prandial glucose levels in obese non-diabetic
subjects. Both acute and long-term treatment (8 weeks) with guar resulted in a decrease in mean post-prandial blood glucose level (following a standard test meal containing guar). However, in the long term study this reduction in mean post-prandial blood glucose was due to two particular patients who had higher initial post-prandial blood glucose levels than the rest of the group. After the long-term treatment with guar the post-prandial blood glucose level decreased by 26% in these two subjects but was not significantly different in the rest of the group. The mean fasting blood glucose was not significantly different after the acute or long-term treatment with guar.

It is apparent that the effects of a high carbohydrate or high fibre diet on blood glucose levels depends on whether normal, obese or diabetic subjects are studied and particularly on the type of test used to assess glucose tolerance. The difference between the subjects is probably due to differences in insulin secretion and tissue sensitivity to insulin.

The effect of a long-term high carbohydrate or high fibre diet on blood glucose levels is probably better assessed by measuring glycated protein levels which are a reflection of ambient blood glucose levels (Bunn et al., 1976). If the carbohydrate and fibre content of the diet primarily affect the post-prandial changes in blood glucose levels, glycated albumin in the plasma is more likely to indicate these changes than GHb in the erythrocyte. Glycated haemoglobin appears to be less sensitive to small changes in blood glucose concentration partly due to its longer half-life and also because albumin appears to be glycated at a faster rate compared to haemoglobin (Olufemi et al., 1987).

In the high and low glycators described in Chapter 3, the carbohydrate and fibre content of their diets (calculated from 7-day food diary) did not explain the discrepancy found between their GHb and blood glucose levels. Also, the glucose profiles showed that the ambient blood glucose level was not the main determinant of the GHb levels. However, the dietary survey as a method of assessing nutrient intake has been criticised for its imprecision and poor reproducibility (Burke, 1947; James et al., 1980). Therefore, the effect of diet, specifically a high glucose/low soluble fibre and a high soluble fibre/low glucose diet on the glycation of both albumin and haemoglobin in a group of normal subjects was investigated. Glucose tolerance at the start and following each dietary period was assessed by a 75 g oral glucose load. Plasma glucose and insulin
levels were measured at fasting, one hour and two hours after the load.

4.2. METHODS

4.2.1. SUBJECTS AND STUDY DESIGN

11 non-diabetic subjects, 7 male and 4 female, aged 22 - 45 participated in the study. This comprised a six week period on a high glucose/low soluble fibre diet (A) and six weeks on a low glucose/high soluble fibre diet (B) in random sequence.

Each subject was instructed to follow a baseline diet which was low in both sugar and soluble fibre. During the period of diet A subjects had their diets supplemented with 100 g glucose/day taken in the form of a high glucose drink (Lucozade, Beecham Laboratories p.l.c., Slough, Buckinghamshire, U.K.). Diet B was supplemented with a guar preparation (Guarem, Rybar Laboratories Limited, Amersham, Buckinghamshire, U.K.) initially in a dose of 5 g/day and increasing to 5 g three times a day over two weeks. This was taken either mixed into or sprinkled over food or in a drink just before or during the meal.

Subjects had their usual dietary intake assessed by a dietitian and were advised on any caloric intake changes required for either diet to ensure that an isocaloric intake was maintained throughout the study. All participants were provided with low sugar/low fibre food and drinks to encourage compliance throughout the twelve week period.

Subjects were instructed to avoid any drugs such as aspirin, or vitamin supplements such as vitamin C or B₆ which are known to affect glycation. Alcohol intake, smoking and exercise were continued as habitual for each individual.

Height and weight were measured at the beginning of the study and at the end of each dietary period. Compliance with the glucose drink and guar preparation were estimated by counting the number of unfinished bottles and sachets respectively.

4.2.2. SAMPLE COLLECTION

At the beginning of the study and at the end of each six week period, each subject had a glucose tolerance test with venous blood being taken fasting, 1 hour and 2 hours after a 75 g glucose load. For the
fasting sample 10 ml of blood was collected into a lithium heparin container and 5 ml into a potassium EDTA container. At 1 hour and 2 hours after the glucose load 5 ml blood samples were collected into lithium heparin containers. Some of the lithium heparin whole blood at each time point was deproteinised and the extracts stored at 4°C for 24 hours prior to glucose analysis. Fasting lithium heparin whole blood was also deproteinised for the measurement of 2,3-diphosphoglycerate. The extracts were stored at -80°C until analysis up to 3 months later. The rest of the whole blood at each time point was centrifuged (at room temperature) for 10 minutes at 3000 g. Some of the plasma at each time point was deproteinised and the extracts stored at 4°C for 24 hours prior to glucose analysis. The rest of the plasma was stored at -80°C for up to 3 months prior to the measurement of insulin and glycated albumin (on the fasting plasma only). Haematocrits were measured on the potassium EDTA whole blood samples, the rest of which were stored at 4°C for 1 week prior to the analysis of GHb by agar-gel electrophoresis (with and without the removal of the Schiff base), affinity chromatography and iso-electric focusing.

The intra-erythrocyte glucose concentration was calculated from the whole blood and plasma measurements together with the haematocrit (see Chapter 2).

4.2.3. ASSAY METHODS

The full details of all the methods are given in Chapter 2.

4.2.4. STATISTICAL METHODS

Unless otherwise stated, all the plasma and erythrocyte measurements are expressed as the mean (± SD). The measurements after each dietary period were compared to the basal measurements using paired Student's t-tests.
4.3. RESULTS

4.3.2. PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS BEFORE AND FOLLOWING THE TWO DIETARY PERIODS

4.3.2.1. Post - High Soluble Fibre/Low Glucose Diet

There were no significant changes from the basal concentrations in fasting or two-hour plasma or intra-erythrocyte glucose levels following the high soluble fibre/low glucose diet (see Table 4.1). However, the one-hour and total plasma glucose concentrations were significantly increased from the basal concentrations (p < 0.025 and p < 0.01 respectively). The intra-erythrocyte one-hour and total glucose concentrations, on the other hand remained unchanged following the high soluble fibre/low glucose diet.

4.3.2.2. Post - High Glucose/Low Soluble Fibre Diet

There were no significant changes from the basal concentrations in plasma glucose at any time point during the glucose tolerance test following the high glucose/low soluble fibre diet (see Table 4.1). Although there were no significant changes from the basal concentrations in intra-erythrocyte glucose at fasting and two-hours post-glucose load, the one-hour intra-erythrocyte glucose concentration was significantly (p < 0.005) decreased following the high glucose/low soluble fibre diet.

4.3.2. COMPARISON OF PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS BASALLY AND AFTER EACH DIETARY PERIOD

There was no significant difference between the plasma and intra-erythrocyte glucose concentrations at the different time points of the glucose tolerance test performed at the start of the study. However, following both dietary periods, the differences in glucose concentration between the two compartments was markedly significant at all time points including fasting (see Table 4.2.). The mean intra-erythrocyte glucose concentration was 80 - 90% of the mean plasma glucose concentration.
### TABLE 4.1 PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS DURING THE GLUCOSE TOLERANCE TEST BASALLY AND AFTER EACH DIETARY PERIOD

<table>
<thead>
<tr>
<th>GLYCAEMIC PARAMETER</th>
<th>BASAL MEASUREMENT</th>
<th>HIGH GLUCOSE /LOW SOLUBLE FIBRE</th>
<th>HIGH SOLUBLE FIBRE/LOW GLUCOSE FIBRE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLASMA GLUCOSE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>5.70 (1.00)</td>
<td>6.12 (0.96)</td>
<td>6.00 (0.88)</td>
</tr>
<tr>
<td>1 Hour</td>
<td>5.73 (1.15)</td>
<td>5.51 (1.83)</td>
<td>7.23 (2.49)*</td>
</tr>
<tr>
<td>2 Hours</td>
<td>5.85 (1.39)</td>
<td>5.65 (1.53)</td>
<td>6.39 (1.51)</td>
</tr>
<tr>
<td>SUM</td>
<td>17.58 (2.88)</td>
<td>18.04 (4.06)</td>
<td>20.10 (3.95)**</td>
</tr>
</tbody>
</table>

| **INTRA-ERYTHROCYTE GLUCOSE** | | | |
| mmol/l | | | |
| Fasting | 5.37 (1.25) | 5.21 (1.16) | 5.65 (1.05) |
| 1 Hour  | 5.47 (1.10) | 4.40 (0.81)** | 6.28 (1.95) |
| 2 Hours | 5.78 (1.55) | 5.19 (1.00) | 5.67 (1.43) |
| SUM     | 16.6 (3.20) | 14.81 (2.69) | 17.69 (3.67) |

The glucose concentrations following each dietary period were compared to the basal concentrations.

* p < 0.025
** p < 0.01
*** p < 0.005
TABLE 4.2 COMPARISON OF PLASMA AND INTRA-ERYTHROCYTE GLUCOSE CONCENTRATIONS DURING THE GLUCOSE TOLERANCE TESTS BASALLY AND AFTER EACH DIETARY PERIOD

<table>
<thead>
<tr>
<th>TIME OF SAMPLE</th>
<th>PLASMA GLUCOSE (mmol/l)</th>
<th>INTRA-ERYTHROCYTE GLUCOSE (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BASAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASTING</td>
<td>5.70 (1.00)</td>
<td>5.37 (1.25)</td>
</tr>
<tr>
<td>1HOUR</td>
<td>5.73 (1.15)</td>
<td>5.47 (1.10)</td>
</tr>
<tr>
<td>2HOURS</td>
<td>5.85 (1.39)</td>
<td>5.78 (1.55)</td>
</tr>
<tr>
<td>SUM</td>
<td>17.58 (2.88)</td>
<td>16.60 (3.20)**</td>
</tr>
<tr>
<td><strong>HIGH GLUCOSE/LOW SOLUBLE FIBRE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASTING</td>
<td>6.12 (0.96)</td>
<td>5.21 (1.16)****</td>
</tr>
<tr>
<td>1HOUR</td>
<td>5.51 (1.83)</td>
<td>4.40 (0.81)*</td>
</tr>
<tr>
<td>2HOURS</td>
<td>5.65 (1.53)</td>
<td>5.19 (1.05)*</td>
</tr>
<tr>
<td>SUM</td>
<td>18.04 (4.06)</td>
<td>14.81 (2.69)****</td>
</tr>
<tr>
<td><strong>HIGH SOLUBLE FIBRE/LOW GLUCOSE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASTING</td>
<td>6.00 (0.88)</td>
<td>5.65 (1.05)***</td>
</tr>
<tr>
<td>1HOUR</td>
<td>7.23 (2.49)</td>
<td>6.28 (1.95)****</td>
</tr>
<tr>
<td>2HOURS</td>
<td>6.39 (1.51)</td>
<td>5.67 (1.43)**</td>
</tr>
<tr>
<td>SUM</td>
<td>20.10 (3.95)</td>
<td>17.69 (3.67)****</td>
</tr>
</tbody>
</table>

The plasma and intra-erythrocyte glucose concentrations are compared at each time point during the three glucose tolerance tests.

* p < 0.025
** p < 0.01
*** p < 0.005
**** p < 0.0025
***** p < 0.0005
The sum of the glucose concentrations measured at the different time points during each of the three glucose tolerance tests (total glucose) was significantly (p <0.005) lower in the erythrocyte compared to the plasma.

4.3.3. PLASMA INSULIN LEVELS

There were no significant changes from the basal levels in fasting or post-load plasma insulin levels following either dietary period (see Table 4.3).

4.3.4. ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE LEVELS

The erythrocyte 2,3-diphosphoglycerate levels significantly decreased from the basal level after both the high soluble fibre/low glucose (p <0.001) and high glucose/low soluble fibre (p <0.05) diets (see Table 4.4).

4.3.5. GLYCATED PROTEIN LEVELS

There were no significant changes in GHb by any of the methods of measurement following either dietary period (see Table 4.5).

Glycated albumin significantly decreased following the high soluble fibre/low glucose diet (p=0.01) and significantly increased following the high glucose/low soluble fibre diet (p = 0.02) (see Table 4.4). The individual changes in glycated albumin are shown in Figure 4.1, which shows that the level increased in all but one subject after the high glucose diet and fell in all but two subjects after the diet high in soluble fibre.

No significant changes were found in plasma fructosamine concentration following either dietary period (see Table 4.5).

4.3.6. COMPLIANCE AND BODY WEIGHT

All the subjects completed both dietary periods. Compliance with the high soluble fibre diet, as evidenced by counting unconsumed sachets of guar gum was 89.7 ± 7.5% and with the high glucose diet, using counts of unconsumed Lucozade, 97.1 ± 3.5%.

There were no significant changes in BMI during either dietary period, 21.63 ± 3.10 (Basal), 21.74 ± 3.38 (high glucose) and 21.61 ± 3.10 (high soluble fibre).
TABLE 4.3  PLASMA INSULIN LEVELS DURING THE GLUCOSE TOLERANCE TESTS BASALLY AND AFTER EACH DIETARY PERIOD

<table>
<thead>
<tr>
<th>TIME</th>
<th>BASAL</th>
<th>HIGH GLUCOSE/LOW SOLUBLE FIBRE</th>
<th>HIGH SOLUBLE FIBRE/LOW GLUCOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FASTING (mU/l)</td>
<td>6.4 (2.1)</td>
<td>7.7 (3.2)</td>
<td>6.7 (2.1)</td>
</tr>
<tr>
<td>1HOUR (mU/l)</td>
<td>29.4 (22.6)</td>
<td>32.7 (26.7)</td>
<td>37.6 (24.2)</td>
</tr>
<tr>
<td>2HOURS (mU/l)</td>
<td>20.2 (18.4)</td>
<td>21.7 (23.1)</td>
<td>22.2 (14.8)</td>
</tr>
<tr>
<td>SUM (mU/l)</td>
<td>56.0 (40.7)</td>
<td>59.8 (49.5)</td>
<td>66.5 (33.9)</td>
</tr>
</tbody>
</table>

** p < 0.05
**** p < 0.001

TABLE 4.4  FASTING ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE CONCENTRATIONS BASALLY AND FOLLOWING EACH DIETARY PERIOD

<table>
<thead>
<tr>
<th>BASAL MEASUREMENT (mmol/l)</th>
<th>HIGH GLUCOSE/LOW SOLUBLE FIBRE DIET (mmol/l)</th>
<th>HIGH SOLUBLE FIBRE/LOW GLUCOSE (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.78 (0.49)</td>
<td>4.41 (0.34)**</td>
<td>4.31 (0.34)****</td>
</tr>
<tr>
<td>GLYCATED PROTEIN</td>
<td>BASAL</td>
<td>HIGH GLUCOSE/LOW SOLUBLE FIBRE</td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%) (affinity chromatography)</td>
<td>5.88 (0.47)</td>
<td>6.02 (0.44)</td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%) (isoelectric focusing)</td>
<td>4.69 (0.24)</td>
<td>4.60 (0.48)</td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%) (electrophoresis-total)</td>
<td>7.19 (0.53)</td>
<td>6.94 (0.62)</td>
</tr>
<tr>
<td>GLYCATED HAEMOGLOBIN (%) (electrophoresis-stable)</td>
<td>6.42 (0.61)</td>
<td>6.16 (0.72)</td>
</tr>
<tr>
<td>FRUCTOSAMINE (mmol/l)</td>
<td>2.05 (0.12)</td>
<td>2.12 (0.06)</td>
</tr>
<tr>
<td>GLYCATED ALBUMIN (%)</td>
<td>1.71 (0.35)</td>
<td>1.95 (0.23)*</td>
</tr>
</tbody>
</table>

* p=0.02
** p=0.01
FIGURE 4.1. INDIVIDUAL CHANGES IN GLYCATED ALBUMIN FOLLOWING THE TWO DIETARY PERIODS

1 = BASAL
2 = POST-HIGH GLUCOSE/LOW SOLUBLE FIBRE DIET
3 = POST-HIGH SOLUBLE FIBRE/LOW GLUCOSE DIET
4.4. DISCUSSION

This study has shown that dietary supplementation with glucose or soluble fibre in normal subjects has a marked effect on albumin glycation, whilst having no apparent effect on the glycation of haemoglobin. The high glucose/low soluble fibre diet resulted in a 14% increase in glycated albumin and the high soluble fibre/low glucose diet led to a 22% decrease in glycated albumin. These results suggest that the diets led to changes in plasma glucose levels, which subsequently resulted in alterations in the extent of glycation of albumin. These results are apparently at variance with the results for plasma glucose levels obtained during the glucose tolerance tests (Table 4.1). For the high glucose diet, no significant changes from the basal glucose levels were observed while for the high soluble fibre diet the observed significant increases at 1 hour and for the total change in plasma glucose are contrary to the decrease in glycated albumin that was found.

Most of the previous studies with guar (Jenkins et al., 1976, 1977) have shown a decrease in post-prandial blood glucose levels after long- or short-term treatment. However, in these studies, guar was given with or immediately before the test meal or glucose load. This would have the effect of decreasing post-prandial blood glucose levels by reducing the rate of absorption of glucose from the gut. In the present study, the subjects were not only put on a high fibre diet but also on one containing reduced amounts of glucose. Also, no guar was consumed before or during the glucose tolerance test. Therefore, following the ingestion of a 75 g glucose load after six weeks on a diet low in sugar and high in fibre, an increased glucose response would likely result.

The high glucose/low soluble fibre diet had no apparent effects on plasma glucose tolerance. However, during the glucose tolerance test performed at the start of the study (basal), in the majority of the subjects, the one-hour plasma glucose level had decreased to a level below that of fasting, suggesting that the peak in plasma glucose was occurring much earlier. Therefore, the 'true' tolerance to the glucose load was not measured. A blood sample taken at 30 minutes after the glucose load may have given a better assessment of tolerance.

Following a long-term (6 weeks) high carbohydrate diet, Silva et al., (1987) similarly found no effects on fasting blood glucose or tolerance to an oral glucose load in normal subjects. However they
did find a significant increase in both fasting and post-load insulin levels following the high carbohydrate diet. No significant changes in fasting or post-load insulin levels occurred in the present study, although again the peak insulin response may have occurred earlier than the sample taken one-hour post dose. Most studies that have demonstrated an improvement in glucose tolerance have been with very high carbohydrate diets, such as 85% carbohydrate (Brunzell et al., 1971). In the present study, in order to maintain an iso-caloric diet, the main change was in the type of carbohydrate and not the total consumed. Anderson (1974) showed that in normal subjects, as dietary carbohydrate increased glucose tolerance improved, but the actual source of carbohydrate made no difference.

Glycated albumin has been shown to correlate the strongest with mean blood glucose levels calculated from frequent daily measurements (Dolhofer and Wieland, 1980; Ziel and Davidson, 1987) as compared to both fasting and post-load blood glucose levels. Therefore alterations in the tolerance to an unphysiological glucose load after each diet are unlikely to be helpful in explaining the changes in glycated albumin that occurred during the diet. Glycated albumin is a very sensitive indicator of short-term changes in blood glucose level (Dolhofer & Wieland, 1980; Kennedy et al., 1981; Jones et al., 1983) due to its relatively short half-life and its greater tendency to become glycated (compared to haemoglobin) (Olufemi et al., 1987). Therefore it is probably representative of the small changes in blood glucose level that occur throughout the day, including those occurring post-prandially. It has been shown that the proportion of time that a person is in a genuinely fasted state is fewer than nine hours (Marks and Rose, 1981). The proportion of time that a person is fasting depends on the frequency of feeding, the size and composition of the meals, the rate of gastric emptying and intestinal absorption. The last three factors are all affected by both the carbohydrate and fibre content of the meals. Therefore, by influencing the extent and duration of the post-prandial changes in blood glucose level, dietary carbohydrate and fibre are likely to affect the glycation of albumin. The effect of different types of carbohydrate and fibre on the blood glucose response can be defined in terms of a glycaemic index (GI) (Jenkins et al., 1984; Chantelau et al., 1986; Riccardi and Rivellese, 1987). This is calculated as follows:

\[ GI = \frac{\text{BLOOD GLUCOSE AREA OF TEST FOOD}}{\text{BLOOD GLUCOSE AREA OF REFERENCE FOOD}} \times 100 \]
where the available carbohydrate content of the test and reference foods is the same. The glycaemic index of different foods depends on the rate at which they are digested, the presence of other types of food (e.g. fats, proteins) and on the metabolic response to the absorbed glucose (Jenkins et al., 1984). Thus, free glucose has a high glycaemic index whilst guar has a low glycaemic index.

Unfortunately no daily glucose profiles were collected during the study which might have been useful in explaining the changes in glycated albumin. However, the increase in glycated albumin during the high glucose/low soluble fibre and the decrease in glycated albumin on the high soluble fibre/low glucose diets can probably be explained by the theoretical glycaemic indexes of the two diets (high soluble fibre - low glycaemic index, high glucose - high glycaemic index).

In contrast to glycated albumin, the plasma fructosamine concentration did not significantly change after either dietary period. This is probably due partly to the fact that fructosamine is measuring the glycation of a wide variety of proteins, which may be affected to different extents (Seng and Staley, 1986; Austin et al., 1987). Also, the variable contribution of non-specific components to the fructosamine reaction (Schleicher et al., 1988) thought to partly account for the variability of the measurement (Howey et al., 1987) may mask the effect of small changes in blood glucose level on glycation.

Glycated haemoglobin was also not significantly altered by either diet. Although, GHb has a longer half-life than glycated albumin, due to the slight reversibility of the glycation reaction (Mortensen and Christophersen, 1983; Mortensen et al., 1984) GHb reflects blood glucose levels over the previous 4 - 6 weeks (Berstein, 1987). Therefore any changes in blood glucose levels occurring within each dietary period of six weeks would be expected to be reflected in changes in the GHb level. GHb has previously been shown to increase on a carbohydrate-rich diet (Serog et al., 1982) and decrease during caloric restriction (Ktorza et al., 1985), in normal subjects. However, this increase was explained by an increase or decrease in fasting blood glucose respectively and was associated with a gain or loss in weight. No measurement of intra-erythrocyte glucose or glycated plasma proteins were obtained, but the results suggest that an increase or decrease in basal intra-erythrocyte glucose was responsible for the increase or decrease in GHb respectively.
There were no significant changes in fasting or two-hour intra-erythrocyte glucose levels similarly to plasma following either diet. However, daily post-prandial changes in intra-erythrocyte glucose levels were not measured and may have provided a possible explanation for the lack of alteration in GHb compared to glycated albumin. In Chapter 3, it was shown that plasma changes in glucose concentration are not always mirrored by similar changes in glucose concentration in the erythrocyte. In the present study, the total change in intra-erythrocyte glucose was found to be consistently, significantly lower than the total change in plasma glucose occurring during the glucose tolerance tests. The fasting intra-erythrocyte glucose levels were 80 - 100% of the fasting plasma glucose levels, but during the glucose tolerance tests, the intra-erythrocyte glucose levels varied from 60 - 140% of the plasma glucose levels. This would suggest that albumin and haemoglobin are not only exposed to different basal glucose concentrations but also different post-prandial swings in glucose concentration. The lack of alteration of GHb following each diet would suggest that any changes occurring in daily post-prandial plasma glucose were not mirrored by similar changes in intra-erythrocyte glucose concentration. This apparent difference in the glucose concentration between plasma and erythrocytes is probably due to the rate of glucose transport to the erythrocyte and/or metabolism within the erythrocyte as discussed in Chapter 3.

Apart from the glucose concentration within the erythrocyte, other factors may influence the glycation of haemoglobin. In Chapter 3, 2,3-diphosphoglycerate was found to be an important factor affecting haemoglobin glycation. In the present study, although erythrocyte 2,3-diphosphoglycerate levels decreased significantly after both diets there were no significant changes in GHb. The percent changes in 2,3-diphosphoglycerate were small for both diets but were significant because of the small individual variation. With most methods for measuring GHb there was a trend to a decreased value with both diets but the individual variation was much greater.

In conclusion, the changes in glycated albumin following dietary supplementation with glucose or guar were probably the result of small changes in daily post-prandial plasma glucose levels. The lack of alteration of GHb is probably due to its location within the erythrocyte, where the changes in glucose concentration do not mirror those occurring in the plasma at the same time. This may also explain why the dietary patterns in the high and low glycators failed to explain the discrepancy found between GHb and blood glucose levels.
This study substantiates current literature that glycated albumin is a more sensitive indicator of short-term changes in blood glucose than GHb. This is not only because of the difference in half-life of the two proteins and the apparent increased rate of glycation of albumin, but also because of their different locations within the blood, where they are exposed to different concentrations of glucose. This compartmental difference in glucose concentration may partly explain the large variation found in the relationship between different glycated proteins and between different glycated proteins and blood glucose levels.

Further work needs to be carried out to investigate whether differences in intra-erythrocyte and plasma glucose concentration occur post-prandially after 'normal' meals in both normal and diabetic subjects and how variable the differences are in individuals and between individuals.
CHAPTER 5

THE EFFECT OF VITAMIN C ON THE GLYCATION OF PROTEINS
5.1. INTRODUCTION

Vitamin C has recently been shown to affect the glycation of proteins (Stolba et al., 1987; Khatami et al., 1988). However, the effects found are confusing and conflicting. Stolba et al., (1987, 1988) investigated the effect of ascorbic acid (AA) and its metabolite dehydroascorbic acid (DHAA) on the glycation of bovine serum albumin and collagen in the presence of varying concentrations of glucose in vitro. It was found that whilst AA inhibited glycation, DHAA apparently enhanced glycation. Khatami et al., (1988) also studied the effect of AA and DHAA on the glycation of bovine serum albumin in vitro, but found that both compounds inhibited glycation. The only apparent difference between the two studies was the concentration of vitamin C used. Stolba et al., (1987) used concentrations up to 1 mM, whilst Khatami et al., (1988) used 5 mM AA and DHAA. Stolba et al., (1987) also performed an in vivo study in which ten insulin-dependent diabetic subjects were supplemented with 1.5 g of vitamin C daily for three weeks. A significant fall in fructosamine concentration was found to occur, thus suggesting an inhibition of glycation of plasma proteins by vitamin C as no change in glycaemia was observed.

In the study described in Chapter 3, vitamin C was not found to be a significant contributory factor in reducing haemoglobin glycation, although a weak negative correlation was found between erythrocyte vitamin C levels and GHb levels. Differences in erythrocyte vitamin C levels in the high and low glycators might have helped to explain the discrepancies found between GHb and blood glucose levels. However, the effects of vitamin C on glycation have so far been shown to occur only at unphysiologically high levels that are produced in vivo by supplementation with large doses of vitamin C. So it would have been unlikely that at the physiological levels of vitamin C found in low and high glycators effects on glycation would be apparent. Vitamin C, in particular DHAA has long been known to food chemists to react with amino groups of both amino acids and proteins (Kurata et al., 1973; Ranganna and Setty, 1974; Kurata and Fujimaki, 1976). Benesch (1981) using electron-spin-resonance studies showed that AA could bind to proteins via an ionic interaction. In physiological fluids it is likely that DHAA can form a reversible Schiff base with amino groups (Tolbert and Ward, 1982), but cannot undergo the Amadori rearrangement to form a ketoamine because it lacks the two hydrogens corresponding to the carbon-2 on glucose which are needed for the reaction (see Figure 5.1). It is also
possible that the carbonyl groups of ascorbic acid and the further oxidation product of DHAA, diketogulonic acid (DKG) could also react with amino groups to form Schiff bases. DKG may be able to undergo the Amadori rearrangement due to the location of two hydrogens on carbon-4 next to the carboxyl group, (see Fig. 5.1). Thus, all three compounds AA, DHAA and DKG could compete with glucose for binding to proteins and thereby inhibit glycation.

There are, however, three indirect mechanisms by which vitamin C could affect glycation. AA has been shown to influence glucose tolerance by affecting the production and/or secretion of insulin (Sylvest et al., 1942; Arendt and Patee, 1956; Scarlett et al., 1970). AA is also involved in the catalysis of many enzyme reactions, including those involved in carbohydrate metabolism (Mapson, 1967). Therefore, ascorbic acid may indirectly affect glycation by influencing the blood glucose levels. The second indirect mechanism is the effect of AA and DHAA on the glycation of intracellular proteins. By affecting the transport of glucose into cells, AA and DHAA could influence the intracellular glucose concentration to which proteins are exposed. Bigley et al., (1983) suggested that DHAA transport into human neutrophils and fibroblasts is mediated by glucose transport systems. Similarly to glucose, uptake may be dependent on insulin in certain tissues. This would suggest that AA/DHAA may compete with glucose for uptake into cells. Mooradian (1987) found that DHAA (but not AA) competed with glucose for uptake into the brain but not into muscle in vitro. The effect appears to be tissue specific.

The third mechanism by which AA and DHAA could affect glycation is a specific effect on haemoglobin. Both in vitro (Wood and Beutler, 1973) and in vivo (Moore et al., 1977) studies have shown that AA appears to increase the levels of 2,3-diphosphoglycerate in erythrocytes. This is thought to be due to some effect of vitamin C on the metabolism of glucose within the erythrocyte. As 2,3-diphosphoglycerate is known to catalyse glycation (Smith et al., 1982; Lowrey et al., 1985) vitamin C could in this way indirectly lead to an increase of glycation of haemoglobin.

The effect of AA and its metabolites on the glycation of proteins is therefore likely to be very complex in vivo. Different proteins may be affected in different ways depending on their location. However, if the overall effect of vitamin C is to inhibit the glycation of proteins, it could have the potential of being used therapeutically to ameliorate or prevent the chronic complications of diabetes and
FIGURE 5.1. THE STRUCTURE OF ASCORBIC ACID AND ITS METABOLITES, DEHYDROASCORBIC ACID AND DIKETOGLULONIC ACID
perhaps even the normal process of ageing. Although Stolba et al., (1987) showed a significant decrease in fructosamine concentration in vivo with vitamin C supplementation, the effect on glycation of intracellular proteins such as haemoglobin was not determined and no attempt was made to try and elucidate the mechanisms involved. The fructosamine assay is also known to be unreliable with many unidentified non-specific interferences (Howey et al., 1987; Schleicher et al., 1988). Therefore, to study the mechanisms in more detail, the effect of oral vitamin C in a group of healthy volunteers on the glycation of proteins was investigated.

Before describing this study, the absorption, distribution and metabolism of vitamin C in normal subjects will be discussed.

5.1.1. ABSORPTION, DISTRIBUTION AND METABOLISM OF VITAMIN C.

Vitamin C from the diet is rapidly absorbed through the buccal mucosa, from the stomach and the small intestine. The rate of absorption from the small intestine depends on the local pH and the vitamin C concentration. Quantitatively absorption from the small intestine is most important. AA dissociates in aqueous solution to the ascorbate radical which is not absorbed as the charge on the molecule interferes with its transport (Hornig et al., 1971). DHAA is electrically neutral and is therefore the more suitable ligand for transport (Bigley et al., 1983). Vitamin C transport has been shown to follow saturation kinetics and exhibits a high degree of specificity for the L-isomer suggesting a carrier mediated process. Evidence would suggest vitamin C transport occurs by facilitated diffusion (Hornig et al., 1971).

The metabolism of AA is illustrated in Fig. 5.2. The principal pathway involves the loss of two successive electrons. The intermediate free radical immediately and reversibly forms DHAA. Subsequent irreversible hydrolysis yields the biologically inactive DKG which undergoes degradation by alternate pathways; decarboxylation to carbon dioxide and five-carbon fragments such as L-xylose, and the formation of oxalic acid and four-carbon fragments, such as threonic acid (Jaffe, 1984). The formation of ascorbic acid 2-hydrogen sulphate has also been demonstrated as a metabolic product in humans, but has no known biological significance.
FIGURE 5.2. THE METABOLISM OF ASCORBIC ACID IN MAN
The plasma level of vitamin C of an individual will depend on the dietary intake, the rate of absorption through the gastrointestinal tract, transport between plasma and tissues and the rate of renal excretion. The relative proportions of AA and its metabolites will depend on rates of release and uptake by blood cells and tissues together with the rate of oxidation. It has been found that AA makes up a major proportion of total vitamin C in plasma (approximately 75%) of normal subjects, DHAA contributes only 5 - 10% of total vitamin C whilst DKG makes up at least 20% of the total. (Cox and Whichelow, 1975). Supplementation with vitamin C produced a different picture, with plasma DHAA rising 100-fold, whilst AA levels remained unchanged such that the concentration of DHAA exceeds that of AA by two-fold. The DKG levels were not quantitated in this case (Chatterjee et al., 1973).

The distribution of vitamin C between various cellular components of blood in normal individuals and its relation to the plasma concentration has been investigated by Barkhan and Howard (1958) and again by Evans et al., (1982). It is found that although the leucocytes and platelets have the highest concentration of AA in the blood (40 to 80-fold greater than plasma concentration) the erythrocytes and plasma make up approximately 70% of the total blood AA due to their relatively greater proportion. Erythrocytes and plasma have approximately the same concentration of AA, which suggests that erythrocytes do not concentrate AA to any appreciable extent. Daily supplementation of normal individuals with 2 g of vitamin C results in a more than two and a half fold increase in plasma AA concentration whilst smaller increases are seen in the cellular components of the blood. The AA content of platelets increases by 78%, erythrocytes by 50% and leucocytes by 33% (Evans et al., 1982).

All the metabolites (as well as AA) are excreted in the urine. The relative proportions vary from species to species and the amount ingested. In humans, with physiological doses of 60-100 mg/day urinary oxalate is the major metabolite, with 30-50 mg/day being formed. But when vitamin C is given in larger doses up to 10 g/day, the urinary oxalate is increased by only 10-30 mg/day and the vitamin is largely excreted unmetabolized in the faeces (Hornig and Moser, 1981). Substantial quantities of AA are excreted in the urine after the plasma concentration exceeds its renal threshold of 1.4 mg/100 ml (79 μmol/l). The half-life of ascorbic acid is inversely related to the daily intake and is 13-40 days in humans (Jaffe, 1984).
Very little is known of the factors which control the rate of turnover of AA. Studies by Bates et al., (1981) showed that the rate of turnover in healthy male subjects was related to the pool of AA and for a given pool size was remarkably constant between individuals. It seems to vary depending on daily intake but may also be influenced by external factors such as smoking and stress (Jaffe, 1984).

It is likely that the extent of the effect of vitamin C on the glycation of proteins in vivo will vary considerably between subjects due to intra-individual differences in absorption, distribution, metabolism and renal excretion of the vitamin. To investigate the effect of vitamin C on glycation, during supplementation plasma and erythrocyte levels of total vitamin C were monitored and related to both glycated albumin and GHb levels. Plasma and intra-erythrocyte glucose concentrations were also measured to monitor any changes in glycaemia occurring during supplementation with vitamin C. Plasma insulin levels were measured and also erythrocyte 2,3-diphosphoglycerate due to the evidence that the levels within the erythrocyte may be affected by vitamin C.

5.2. METHODS

5.2.1. SUBJECTS AND STUDY DESIGN

Twelve healthy volunteers agreed to participate in the study. These were seven males and five females aged 22 - 50. Ascorbic acid in the form of 500 mg tablets were supplied by Roche (Roche Products Limited, Welwyn Garden City, Hertfordshire). The subjects were asked to take 1g (2 x 500 mg) of vitamin C daily for three months. 15 ml fasting venous blood samples were collected at the start of the study and at the end of each month. Two further samples were obtained at one month (15 mls) and two months (5 mls) after cessation of the vitamin C supplement (6 samples in total). The subjects were asked to follow their usual diet, but to refrain from the consumption of excess alcohol and the ingestion of aspirin and dietary supplements (other than the vitamin C).

5.2.2. BLOOD SAMPLES

The 15 ml blood samples were each divided up into two aliquots. 10 mls was transferred into a lithium heparin tube and 5 mls into a potassium EDTA tube. Some of the lithium heparin whole blood was deproteinised immediately for the measurement of glucose and 2,3-
diphosphoglycerate. The remaining lithium heparin whole blood was centrifuged at 3000 g (room temperature) for 10 minutes. Some of the resulting plasma was deproteinised for measurement of glucose and vitamin C. The remaining plasma was stored at -80°C in aliquots for the analysis of insulin, fructosamine and glycated albumin three months later. The packed red cells were also deproteinised for the measurement of vitamin C. The whole blood and plasma extracts for glucose were stored at 4°C and analysed within six hours of collection. The plasma and red blood cell extracts for measurement of vitamin C were stored at -80°C and analysed within one week. A very small amount of the potassium EDTA whole blood was used immediately to measure the haematocrit. The rest was stored at 4°C for one week prior to the analysis of GHb by affinity chromatography and agar-gel electrophoresis (with removal of the Schiff base). The 5 ml blood sample collected two months after cessation of the vitamin C supplement was transferred into a potassium EDTA container. It was stored at 4°C and analysed for glycated haemoglobin by affinity chromatography and electrophoresis within one week of collection.

The intra-erythrocyte glucose was calculated from the whole blood and plasma measurements together with the haematocrit (see Chapter 2).

5.2.3. ASSAY METHODS

The full details of all the methods are given in Chapter 2.

5.2.4. STATISTICAL METHODS

All the plasma and erythrocyte measurements are expressed as the mean (± SD). The measurements at different time points were compared to the basal levels using paired Student’s t-tests.

5.2.5. IN VITRO EXPERIMENT TO INVESTIGATE THE EFFECT OF VITAMIN C ON GLYCATED HAEMOGLOBIN METHODOLOGIES

Haemoglobin obtained from freshly haemolyzed human red blood cells was incubated with AA or DHAA in the presence or absence of glucose. Following dialysis GHb was measured on each of the incubates by both affinity chromatography and agar-gel electrophoresis (with removal of Schiff base).
5.2.5.1. Reagents

All the reagents were of "Analar" grade and obtained from BDH (BDH Products Limited, Poole, Dorset).

A 0.1M phosphate buffer with 0.001% sodium azide was prepared and adjusted to pH 7.4. This buffer was then used to prepare five other solutions as follows:

(i) Glucose, 25 mmol/l.
(ii) Glucose, 25 mmol/l and AA, 1 mmol/l
(iii) Glucose, 25 mmol/l and DHAA, 1 mmol/l
(iv) AA, 1 mmol/l
(v) DHAA, 1 mmol/l

The pH of each solution was re-adjusted to pH 7.4 if necessary.

A 0.9% saline solution was prepared for dialysis.

The reagents for the affinity chromatography and electrophoresis methods for GHb are as described in Chapter 2.

5.2.5.2. Procedure

10 mls fasting whole blood was obtained from a healthy volunteer and transferred into a lithium heparin container. It was immediately centrifuged for 10 minutes at 3000 g (room temperature) and the plasma discarded. 500 µl of the packed red blood cells were diluted in 3.5 mls of each of the five incubation solutions containing glucose and/or vitamin C. A further 500 µl of packed red blood cells were diluted in the phosphate buffer alone.

After vortexing thoroughly, the six samples were incubated at 37°C for 3 days. This was followed by dialysis of all of the samples against 25 l of 0.9% saline over 48 hours.

The dialysates containing the modified haemoglobin were then subjected to affinity chromatography and electrophoresis for the remeasurement of GHb. The procedures for the latter two methods were followed as described in Chapter 2.
5.3. RESULTS

5.3.1. FASTING PLASMA AND ERYTHROCYTE VITAMIN C LEVELS BEFORE, DURING AND AFTER SUPPLEMENTATION

The total plasma vitamin C levels increased by 49% after one month and remained elevated during supplementation. The percentage by which the vitamin C levels were elevated above the basal level varied from month to month. After cessation of the vitamin C supplement the plasma levels of vitamin C were lower than the original basal level (see Fig. 5.3).

The erythrocyte levels of vitamin C followed a different pattern to the plasma levels with supplementation. After one month there was no significant change in levels, but after two months it rose by 21% and by 40% after three months. After one month cessation of vitamin C the levels of vitamin C within the erythrocyte were lower than the basal level (see Fig. 5.3).

Comparison of the plasma and the erythrocyte levels of vitamin C (see table 5.1) show that initially the plasma levels were 23% higher than the erythrocyte levels of vitamin C. However, after one month of supplementation because there was no change in erythrocyte levels, the plasma vitamin C level was 85% higher than the erythrocyte level (p <0.0005). After 2 months supplementation there was less difference between plasma and erythrocyte vitamin C levels because the erythrocyte vitamin C level had also increased, but the difference still remained significant (p <0.0005). After 3 months there was no longer a significant difference between the two compartments (p >0.05). One month following the cessation of supplementation, the difference between the plasma and erythrocyte vitamin C levels became significant again (p <0.01).

5.3.2. EFFECT OF VITAMIN C ON FASTING GLYCAEMIA (PLASMA AND ERYTHROCYTE)

There were no significant changes in either fasting plasma or erythrocyte glucose concentration throughout the study (see Table 5.2 and Table 5.3).
FIGURE 5.3. CHANGES IN VITAMIN C CONCENTRATION IN THE PLASMA AND ERYTHROCYTES DURING SUPPLEMENTATION

![Graph showing changes in vitamin C concentration in plasma and erythrocytes during supplementation.]

1 = BASAL
2 = 1 MONTH SUPPLEMENTATION
3 = 2 MONTHS SUPPLEMENTATION
4 = 3 MONTHS SUPPLEMENTATION
5 = 1 MONTH POST-CESSATION OF SUPPLEMENT

The significance of difference of the plasma and erythrocyte vitamin C levels during supplementation from the basal levels is indicated.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PLASMA VITAMIN C CONCENTRATION (µmol/l)</th>
<th>ERYTHROCYTE VITAMIN C CONCENTRATION (µmol/l)</th>
<th>SIGNIFICANCE OF DIFFERENCE (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BASAL</td>
<td>73.3 (13.9)</td>
<td>59.5 (17.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1 MONTH SUPPLEMENTATION</td>
<td>109.2 (18.6)</td>
<td>59.1 (9.5)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>2 MONTHS SUPPLEMENTATION</td>
<td>119.1 (19.9)</td>
<td>73.8 (17.1)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>3 MONTHS SUPPLEMENTATION</td>
<td>93.3 (16.8)</td>
<td>83.6 (10.8)</td>
<td>NS</td>
</tr>
<tr>
<td>1 MONTH AFTER CESSION OF SUPPLEMENTATION</td>
<td>59.2 (12.9)</td>
<td>43.3 (16.8)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
### TABLE 5.2  EFFECT OF VITAMIN C ON FASTING PLASMA GLUCOSE LEVELS

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>1 Month on Vitamin C</th>
<th>2 Months on Vitamin C</th>
<th>3 Months on Vitamin C</th>
<th>1 Month Post Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>5.05 (0.34)</td>
<td>4.94 (0.24)</td>
<td>5.01 (0.29)</td>
<td>4.86 (0.47)</td>
<td>4.82 (0.40)</td>
</tr>
</tbody>
</table>

Sig. of Difference (p): NS NS NS NS NS

### TABLE 5.3  EFFECT OF VITAMIN C ON FASTING INTRAERYTHROCYTE GLUCOSE LEVELS

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>1 Month on Vitamin C</th>
<th>2 Months on Vitamin C</th>
<th>3 Months on Vitamin C</th>
<th>1 Month Post Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-erythrocyte Glucose (mmol/l)</td>
<td>4.07 (0.60)</td>
<td>4.23 (0.61)</td>
<td>4.32 (0.60)</td>
<td>3.96 (0.97)</td>
<td>3.78 (0.55)</td>
</tr>
</tbody>
</table>

Sig. of Difference (p): NS NS NS NS NS
5.3.3. EFFECT OF VITAMIN C ON FASTING PLASMA INSULIN LEVELS

There was no significant difference in plasma insulin levels, although there was a slight increase initially after two and three months (not significant) (see Table 5.4).

5.3.4. EFFECT OF VITAMIN C ON ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE LEVELS

Although at one and two months following supplementation the 2,3-diphosphoglycerate levels increased, it was not statistically significant (see Table 5.5). However, after three months, the increase in 2,3-diphosphoglycerate was significant ($p < 0.05$). After cessation of the vitamin C, the 2,3-diphosphoglycerate returned to the basal level.

5.3.5. EFFECT OF VITAMIN C ON GLYCATED PROTEINS

5.3.5.1. Fructosamine

Supplementation with vitamin C produced a small percentage decrease in fructosamine concentration. At one and three months the decrease was significant ($p < 0.005$ and $p < 0.01$ respectively) but at two months the decrease was not statistically significant. A month after cessation of vitamin C, the fructosamine concentration returned to the basal level (see Fig. 5.4).

5.3.5.2. Glycated Albumin

The glycated albumin decreased by 33% after one month and remained at this level during supplementation with vitamin C. After one month cessation of vitamin C the glycated albumin level increased by 12% (see Fig. 5.5).
### TABLE 5.4  EFFECT OF VITAMIN C ON FASTING PLASMA INSULIN LEVELS

<table>
<thead>
<tr>
<th></th>
<th>BASAL</th>
<th>1 MONTH ON VITAMIN C</th>
<th>2 MONTHS ON VITAMIN C</th>
<th>3 MONTHS ON VITAMIN C</th>
<th>1 MONTH POST VITAMIN C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA INSULIN (mU/l)</td>
<td>6.98 (2.82)</td>
<td>7.68 (3.28)</td>
<td>8.18 (3.29)</td>
<td>6.49 (2.40)</td>
<td>7.93 (2.94)</td>
</tr>
<tr>
<td>SIG. OF DIFF. (p)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

### TABLE 5.5  EFFECT OF VITAMIN C ON ERYTHROCYTE 2,3-DIPHOSPHOGLYCERATE(DPG) LEVELS

<table>
<thead>
<tr>
<th></th>
<th>BASAL</th>
<th>1 MONTH ON VITAMIN C</th>
<th>2 MONTHS ON VITAMIN C</th>
<th>3 MONTHS ON VITAMIN C</th>
<th>1 MONTH POST VITAMIN C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERYTHROCYTE 2,3 DPG (mmol/l)</td>
<td>4.64 (0.69)</td>
<td>4.80 (0.64)</td>
<td>4.87 (0.61)</td>
<td>5.12 (0.83)</td>
<td>4.61 (0.64)</td>
</tr>
<tr>
<td>SIG. OF DIFFERENCE (p)</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
FIGURE 5.4. EFFECT OF VITAMIN C ON FRUCTOSAMINE

1 = BASAL
2 = 1 MONTH SUPPLEMENTATION
3 = 2 MONTHS SUPPLEMENTATION
4 = 3 MONTHS SUPPLEMENTATION
5 = 1 MONTH POST-CESSATION OF SUPPLEMENT

* P < 0.05
** P < 0.005
FIGURE 5.5. EFFECT OF VITAMIN C ON GLYCATED ALBUMIN

1 = BASAL
2 = 1 MONTH SUPPLEMENTATION
3 = 2 MONTHS SUPPLEMENTATION
4 = 3 MONTHS SUPPLEMENTATION
5 = 1 MONTH POST-CESSATION OF SUPPLEMENT

* P < 0.05
**** P < 0.0001
5.3.5.3. Glycated Haemoglobin (Affinity Chromatography)

The GHb level decreased by 18% at one month and remained at about this level during supplementation and even at one month after cessation of vitamin C. Two months after the end of vitamin C supplementation the GHb level had almost returned to the basal level (see Fig. 5.6(a)).

5.3.5.4. Glycated Haemoglobin (Electrophoresis)

The GHb levels measured by electrophoresis initially fell after one month (by 11%) but after two and three months the levels increased progressively and remained raised even 1 month after vitamin C supplementation had finished. The GHb level had almost returned to the basal level two months following cessation of the vitamin C supplement (see Fig. 5.6(b)).

5.3.7. EFFECT OF VITAMIN C ON THE METHODOLOGIES FOR GLYCATED HAEMOGLOBIN (IN VITRO)

1 mM AA or DHAA had no significant effect on the glycation of haemoglobin in the absence or presence of glucose when affinity chromatography was used (see Table 5.6). However, when electrophoresis was used to measure glycated haemoglobin, a significant increase was recorded in the presence of both AA and DHAA. In the presence of only AA or DHAA and no additional glucose a significant increase in glycated haemoglobin was still measured by electrophoresis. This increase was greater than that observed when haemoglobin was incubated with glucose alone.
FIGURE 5.6 EFFECT OF VITAMIN C ON GLYCATED HAEMOGLOBIN

(a) AFFINITY CHROMATOGRAPHY

(b) ELECTROPHORESIS

1 = BASAL
2 = 1 MONTH SUPPLEMENTATION
3 = 2 MONTHS SUPPLEMENTATION
4 = 3 MONTHS SUPPLEMENTATION
5 = 1 MONTH CESSATION SUPPLEMENTATION
6 = 2 MONTHS CESSATION SUPPLEMENTATION
### TABLE 5.6  EFFECT OF VITAMIN C ON THE METHODOLOGIES FOR GLYCATED HAEMOGLOBIN (IN VITRO)

<table>
<thead>
<tr>
<th></th>
<th>PHOSPHATE BUFFER (0.1M)</th>
<th>GLUCOSE (25mM)</th>
<th>GLUCOSE (25mM) +AA (1mM)</th>
<th>GLUCOSE (25mM) +DHAA (1mM)</th>
<th>AA (1mM)</th>
<th>DHAA (1mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHb(%) (E)</td>
<td>5.50</td>
<td>8.00</td>
<td>16.90</td>
<td>16.80</td>
<td>12.80</td>
<td>17.10</td>
</tr>
<tr>
<td>(%) CHANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>114</td>
<td>113</td>
<td>73</td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>GHb(%) (A.C.)</td>
<td>5.76</td>
<td>9.00</td>
<td>9.00</td>
<td>9.30</td>
<td>6.16</td>
<td>6.36</td>
</tr>
<tr>
<td>% CHANGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

**E** = ELECTROPHORESIS (WITH LABILE REMOVED)

**A.C.** = AFFINITY CHROMATOGRAPHY
5.4. DISCUSSION

This study has confirmed previous work that vitamin C affects the glycation of proteins. It has also provided a better insight into the possible mechanisms involved in vivo and shown that the methodology used for the measurement of glycated proteins has important consequences on the interpretation of the results.

The in vitro experiment showed that when haemolysates were incubated with vitamin C in the absence or presence of glucose, measurement of GHb by electrophoresis and affinity chromatography produced very different results. AA and DHAA had no apparent effect on the glycation of haemoglobin as measured by affinity chromatography, but apparently increased the glycation of haemoglobin by more than 100% when electrophoresis was used to measure it. This discrepancy between electrophoresis and affinity chromatography is probably due to the two methods relying on completely different properties of GHb for their measurement. The affinity chromatography method relies on the structure of the carbohydrate moiety attached to the protein amino groups, specifically the presence of cis-diols (Gould and Hall, 1987) to separate glycated from unglycated haemoglobin. The electrophoresis method on the other hand depends on the small charge difference between glycated and unglycated haemoglobin to separate them. Vitamin C (AA or DHAA) in the absence of glucose resulted in an increase in GHb when measured by electrophoresis suggesting that vitamin C in one form or another was reacting with haemoglobin to produce a charge change similar to the addition of glucose to haemoglobin. For the electrophoresis method, the Schiff base is removed prior to the assay of GHb. Therefore, the increase in GHb measured in the presence of vitamin C must be the result of vitamin C reacting with haemoglobin to form a stable linkage, analogous to the ketoamine formed with glucose. With the affinity chromatography method no change in GHb in the presence of vitamin C was found. This suggests that if vitamin C is reacting with haemoglobin it does not have the correct structure or configuration for binding to the boronate gel. Therefore, it is apparent that affinity chromatography is measuring 'true' glycated haemoglobin whilst electrophoresis is measuring vitamin C bound to haemoglobin in addition to 'true' glycated haemoglobin. Vitamin C did not appear to inhibit the addition of glucose to haemoglobin in this in vitro situation, unlike the inhibition of glycation of albumin by AA shown by Stolba et al.(1987) and Khatami et al.(1988). This may be partly
due to differences in the relative concentrations of vitamin C, glucose and the proteins used in the experiments.

Supplementation of the subjects diet with vitamin C during the in vivo study produced a significant decrease in GHb when it was measured by affinity chromatography, whereas electrophoresis measured a significant increase in GHb (except at one month after supplementation). The in vitro study showing the different effect of vitamin C on the two methods for GHb provides the likely explanation for this finding. Affinity chromatography is therefore measuring a true decrease in GHb. Plasma glycated proteins were also significantly decreased during vitamin C supplementation as measured by fructosamine and specifically glycated albumin (affinity chromatography). The decrease in fructosamine was much less marked than for glycated albumin. This is probably due partly to the fact that fructosamine is measuring the glycation of a wide variety of proteins, which may be affected to different extents (Seng and Staley, 1986; Mosca et al., 1987). Also, the variable contribution of non-specific components to the fructosamine reaction (Schleicher et al., 1988) thought to partly account for the variability of the measurement (Howey et al., 1987) may mask the effect of vitamin C on glycation. The decrease in glycated albumin and GHb during supplementation would suggest that by some mechanism vitamin C was inhibiting glycation. The decrease in glycated albumin was approximately twice that of GHb. This may be due in part to structural differences of the two proteins and availability of sites for glycation or vitamin C binding, but is probably mainly due to the higher concentration of vitamin C in the plasma compared to the erythrocyte during supplementation.

Following one month cessation of vitamin C glycated albumin (and fructosamine) had returned to their basal levels. However, GHb (measured by both techniques) did not return to the basal level after one month, but had done so two months after finishing the vitamin C supplement. This is probably due to the relatively slow turnover of haemoglobin in the erythrocytes compared to the more rapid turnover of albumin.

The mechanism for the effect of vitamin C on glycation is complex. The in vitro experiment suggested that vitamin C could react with haemoglobin to form a stable linkage. Therefore the most likely explanation for the decrease in glycated proteins measured during supplementation with vitamin C in vivo is the competition of vitamin C with glucose for reaction with the protein amino groups, thereby inhibiting glycation. Vitamin C has previously been shown
to react with amino groups on proteins, possibly via an ionic interaction (Benesch, 1987). The structure of DHAA (and AA) would suggest that they could only form a Schiff base, whereas the structure of DKG may allow it not only to form a Schiff base, but also to undergo the Amadori rearrangement to form a ketoamine. Cox and Whichelow (1974) found that in normal subjects DKG made up a surprising 20% of the total plasma vitamin C, whilst DHAA made up only 5% of the total. Furthermore, Chatterjee et al., (1973) found that supplementation with 2-4 g vitamin C produced a much greater increase in plasma levels of DHAA than AA. DKG was not measured in that study, but it is possible that DKG levels may increase similarly to DHAA. No measurement of erythrocyte levels of DKG or DHAA during supplementation have been carried out. The binding of DKG to haemoglobin and to other proteins would not only explain most of the in vivo findings, but also the in vitro increase when measured by electrophoresis in GHb. Although the incubation solutions only contained AA or DHAA at the start, it is likely that further oxidation occurred during the three-day incubation, resulting in a substantially greater proportion of DKG compared to AA and DHAA. The absence of the cis-diol group in DKG would explain why if it reacted with haemoglobin it would not interact with the boronate gel during affinity chromatography, thus explaining the fact that no change in GHb was measured in the presence of vitamin C in vitro.

However, the competitive reaction of vitamin C with glucose for reaction with protein amino groups does not explain all the results found in either the in vivo or in vitro study. This is reflected by the fact that at one month following the start of the vitamin C supplement, the fall in GHb measured by affinity chromatography was mirrored by a similar decrease in GHb measured by electrophoresis. After this GHb measured by electrophoresis progressively increased until at three months the percentage increase was equivalent to the percentage decrease measured by affinity chromatography. From the results of the in vitro experiment, reaction of vitamin C with haemoglobin produced a falsely elevated GHb when measured by electrophoresis. This is in conflict with the initial fall in GHb measured by electrophoresis at one month. This would suggest that the decrease in GHb (also measured by affinity chromatography) at one month was not due to the reaction of vitamin C with haemoglobin, but due to some other effect. In keeping with this is the fact that the erythrocyte vitamin C level did not increase after the first month, but did so progressively at two and three months. Although no change in fasting plasma or erythrocyte glucose or plasma insulin was observed during the study, no measurements were made closely
following the ingestion of the vitamin C. Therefore, an acute effect of vitamin C on glucose metabolism or on the transport of glucose into the erythrocyte cannot be ruled out.

Therefore, the decrease in GHb found in vivo may be due to a combined effect of vitamin C. It may directly inhibit glycation by competition with glucose, but an indirect effect possibly on glucose metabolism or glucose transport into the erythrocyte also appears to occur. The decrease in glycated albumin may also be due to an effect of vitamin C on glucose tolerance as well as a direct effect on glycation by competition with glucose.

An unexpected finding was the increase in erythrocyte 2,3-diphosphoglycerate levels found to occur with vitamin C supplementation. This would have the opposite effect on GHb levels as it is a known catalyst of glycation (Smith et al., 1982; Lowrey et al., 1985). It may be a contributory factor explaining why GHb levels did not decrease as much as the glycated albumin levels. However, 2,3-diphosphoglycerate may also catalyse the reaction of vitamin C as well as glucose with haemoglobin.

If vitamin C does react with proteins to produce a stable linkage, it may have deleterious effects similar to glucose. It has been shown in vitro that ascorbic acid appears to react with lens crystallin proteins eventually producing extensive cross-linking analogous to glucose in non-enzymic glycation (Benesch et al., 1985; Ortwerth and Olesen, 1988). It has therefore been suggested that ascorbic acid is involved in the development of senile cataract. However, these in vitro experiments used very high concentrations of ascorbic acid (20 - 100 mM) compared to normal plasma concentrations, 40 - 90 μM and an in vivo experiment in which mice were fed a diet high in vitamin C for a year was found to have no effect (Benesch et al., 1985). However, further work is required to investigate the long-term effects of high doses of vitamin C, in vivo on both short-lived and long-lived proteins.

If vitamin C can inhibit the glycation of proteins at levels unlikely to cause deleterious effects, it may be of use in the treatment of diabetics. Some of the chronic complications occurring in diabetes are thought to be due mainly to the further reactions of glycated proteins (Brownlee et al., 1984), therefore the administration of vitamin C may be a simple way of slowing their progression. If glycation is also involved in the normal process of ageing (Cerami et al., 1987), long term vitamin C supplementation may even slow down this natural process.
In conclusion, this study has shown that in normal subjects vitamin C can reduce the glycation of short-lived proteins such as albumin and haemoglobin in vivo. Competition of vitamin C with glucose for reaction with protein amino groups does not appear to be the sole explanation for the inhibition effect. Further work is required to investigate the acute changes in glycaemia occurring following ingestion of vitamin C. Monitoring plasma and intra-erythrocyte glucose levels every hour for 6 - 12 hours after vitamin C intake might provide a more accurate indication of glucose tolerance than just the fasting glycaemia. The relative levels of AA, DHAA and DKG in the plasma and erythrocyte in normal subjects following supplementation would also be useful to quantitate. The reaction of AA, DHAA and DKG with protein amino groups also needs to be studied to determine whether they bind irreversibly or reversibly. This may have important consequences on whether vitamin C could be used therapeutically to delay or prevent the chronic complications occurring in diabetes.
CHAPTER 6

GENERAL DISCUSSION
The main conclusion that can be drawn from the studies described in this thesis is that the location of a protein in vivo appears to have an important affect on the extent of its non-enzymatic modification by glucose (glycation).

Although the two proteins studied were both in the vascular compartment, albumin is free to circulate in the plasma whilst haemoglobin is confined to the erythrocyte. Chapter 3 showed that this difference in location resulted in albumin and haemoglobin being glycated to markedly different extents, such that in general only albumin was appropriately glycated in relation to the blood glucose level. In Chapter 4, changes in dietary fibre or carbohydrate in normal subjects resulted in changes in glycated albumin, with no apparent effect on GHb. This effect also appears to be a direct result of the different locations of albumin and haemoglobin in the blood. In Chapter 5, supplementation with oral vitamin C in normal subjects resulted in the inhibition of the glycation of both albumin and haemoglobin. However, albumin was inhibited to a much greater extent than haemoglobin, which appears to be at least in part due to the different concentrations of vitamin C reached in the plasma and erythrocytes during supplementation. In all of the studies the differences in the half-life of the two proteins did not explain their differences in glycation level.

As discussed in Chapter 1, the main factors affecting the glycation of proteins in vivo are the glucose concentrations to which it is exposed and its half-life (Bunn et al., 1976). As albumin and haemoglobin are both present in the blood, it is assumed that they are exposed to the same basal and post-prandial concentrations of glucose. However, the results presented in Chapters 3 and 4 would suggest that not only is there sometimes a difference between the basal concentrations of glucose between the plasma and the erythrocyte, but also the total change in glucose concentration that occurs in the plasma following a glucose load is not mirrored in most cases, by the same change in glucose concentration in the erythrocyte. In general, the total change in erythrocyte glucose concentration was substantially lower compared to that occurring in the plasma. Not only is albumin in the plasma exposed to higher concentrations and greater swings in glucose concentration than haemoglobin in the erythrocyte, but it is also more readily glycated than haemoglobin (Olufemi et al., 1987). Therefore, glycated albumin is likely to reflect changes in blood (plasma) glucose more
accurately than GHB in the erythrocyte. The daily post-prandial changes in plasma glucose concentration occurring in response to standard meals, in normal subjects are probably not as great as those occurring following an un-physiological glucose load. However, because albumin is more reactive, even small changes in the blood glucose concentration will be reflected by changes in glycated albumin. GHB on the other hand, may be unchanged because small changes in plasma glucose may not be mirrored by similar changes in erythrocyte glucose and because haemoglobin is less reactive. These differences would account for the results presented in Chapter 4, where changes in dietary carbohydrate or fibre resulted in marked changes in glycated albumin whilst having no significant effect on GHB.

This difference between plasma and erythrocyte glucose concentrations is discussed in Chapter 3. It may be partly due to the rate of transport of glucose into the erythrocyte, which is affected by pH-dependent carriers in the membrane and also by metabolism of glucose inside the erythrocyte.

Although albumin and haemoglobin have markedly different half-lives and therefore reflect different periods of glycaemia, all the subjects studied in this thesis were non-diabetics. Therefore, day to day and week to week fluctuations in blood glucose levels comparable to those that occur particularly in IDD subjects are unlikely. Therefore, in normal subjects albumin and haemoglobin should in theory be exposed to consistent, relatively small daily changes in blood glucose levels.

If these differences between erythrocyte and plasma glucose concentrations also occurred in diabetic subjects, it might explain why GHB tends to correlate most strongly with fasting blood glucose levels (Dunn et al., 1979; Dods et al., 1979) and glycated albumin with mean blood glucose levels (ambient) (Dolhofer and Wieland, 1980; Ziel and Davidson, 1987). In diabetics, particularly IDD subjects where the rapid fluctuations in blood glucose concentrations result in random or fasting blood glucose levels being of little use in their management and a poor reflection of mean blood glucose levels, glycated albumin should be a much better indicator of short-term changes in blood glucose levels than GHB. Further work needs to be carried out to investigate how plasma and erythrocyte glucose concentrations change in response to glucose loads in diabetic subjects.
In Chapter 4, despite the subjects being on the different diets for 6 weeks, no changes in GHb occurred. However, glycated albumin increased significantly on the high glucose/low soluble fibre diet and decreased significantly on the high soluble fibre/low glucose diet. As no changes in fasting or post-load blood glucose levels were apparent, it was concluded that the changes in glycated albumin were probably the result of small changes in post-prandial glucose levels during each dietary period. The lack of alteration of GHb would suggest that haemoglobin in the erythrocyte was not exposed to comparable changes in glucose concentration. These results again suggest that glycated albumin might be a more accurate monitor of daily changes in blood glucose.

It was apparent in Chapter 3 that factors other than the glucose concentration and the half-life of the protein may influence the glycation of proteins. The erythrocyte 2,3-diphosphoglycerate concentration was found to be significantly greater in the high compared to the low glycators and may account for the inappropriately high GHb levels in these subjects in relation to both their plasma and intra-erythrocyte glucose concentrations. 2,3-Diphosphoglycerate has been shown to be a potent catalyst of glycation in vitro (Smith et al., 1982; Lowrey et al., 1985), but the mechanism for the comparatively raised levels in the high glycators was unclear. There was no evidence for metabolic changes such as hypoxia to account for the raised levels of 2,3-diphosphoglycerate, but genetic factors have been suggested to play a role in determining the erythrocyte 2,3-diphosphoglycerate level (Brewer et al., 1970) in vivo and thus may be the cause of the relatively high levels in these subjects.

This independent effect of 2,3-diphosphoglycerate on the glycation of haemoglobin may have important implications in the use of GHb as a reliable and accurate monitor of blood glucose control in diabetic subjects. It may explain why GHb does not always correlate very well with glycated albumin (Guthrow et al., 1979; Kennedy et al., 1981; Yatscoff et al., 1984; Ross et al., 1986) in diabetic subjects.

In all three studies described in this thesis, the plasma fructosamine concentration showed a poor correlation with other glycated protein levels and with blood glucose levels, whether fasting, in response to glucose load or calculated from daily blood glucose profiles. In Chapter 3 fructosamine correlated weakly with glycated albumin, but whilst the latter showed a good relationship with blood glucose levels (especially ambient blood glucose levels), fructosamine showed no relationship with any of the glycaemic
parameters measured. In Chapter 4, despite significant changes in glycated albumin during each diet, no significant changes in fructosamine occurred. In Chapter 5, supplementation with vitamin C resulted in both significantly reduced glycated albumin and glycated haemoglobin, whilst having little or no effect on fructosamine.

These findings may be partly due to the fact that only non-diabetics were studied. The narrow reference range for both fructosamine and blood glucose levels in non-diabetic subjects may partly account for their poor correlation. However, the fructosamine assay has been shown not to be specific for glycated proteins. Schleicher et al. (1988) found that at least 50% of the fructosamine activity was ascribable to non-specific reducing components. The nature of the other 50% is unclear but does not appear to be albumin associated and differs from patient to patient (Howey et al., 1987). Fluckiger et al., (1987) showed that there was vast intra-individual variation in fructosamine concentration that was not accounted for by correction for protein concentration, suggesting that other factors must be responsible for this variability. It is likely that there is a greater contribution of these unidentified factors to the fructosamine concentration measured in non-diabetic subjects, such that they are able to mask the effect of small changes in blood glucose or other factors (e.g. vitamin C) on glycation. This may account for the lack of change in fructosamine during the diet study and the small change in fructosamine during vitamin C supplementation. A longitudinal study was recently carried out by Suhonen et al., (1989) in pregnant IDD diabetic subjects comparing the relative merits of using different glycated proteins to monitor blood glucose levels (measured every fortnight for two months). They showed that whilst GHb, glycated total proteins and glycated albumin correlated significantly with ambient blood glucose levels, no correlation was found between fructosamine and ambient blood glucose levels. Also, whilst the changes in GHb, glycated total proteins and glycated albumin paralleled each other, the changes in fructosamine were much smaller and were often opposite to the changes in the other assays. These findings support those found with fructosamine measurement in the non-diabetics studied in this thesis, which suggest that the measurement of a specific glycated protein is a more accurate indicator of blood glucose levels than is the measurement of fructosamine.

In Chapter 3 there were also two low glycators who not only had inappropriately low GHb levels, but who also had inappropriately low glycated albumin levels. This was explained by the presence of
therapeutic levels of thyroxine and valium in one of the subjects and naproxen in the other subject. These therapeutic drugs are all known to bind to albumin (Peters, 1985) and are therefore able to compete with glucose for binding, reducing glycation if comparable concentrations are reached. Many therapeutic drugs bind to albumin in vivo. Some of these are tightly but reversibly bound by hydrophobic or ionic interactions (such as thyroxine, diazepam), whilst others interact irreversibly by forming a covalent bond (such as aspirin, penicillin and possibly naproxen) (Peters, 1985). Further work is required to investigate the effect of drugs on the glycation of albumin in vitro and in vivo. The effect of drugs on the glycation of haemoglobin will depend on whether they are able to cross the erythrocyte membrane and also on whether the binding to albumin is specific. Also, some drugs are rapidly metabolised in the liver and blood to compounds which either are no longer able to cross the erythrocyte membrane or no longer react with protein amino groups. For example, aspirin (acetylsalicylic acid) is rapidly metabolised to salicylic acid which no longer reacts with proteins. The glycation of haemoglobin has been shown to be far less susceptible to inhibition by aspirin than albumin (Rendell et al., 1986). This is because the conversion to salicylic acid occurs during its transport across the erythrocyte membrane, such that haemoglobin is exposed to much lower concentrations of acetylsalicylic acid than albumin in the plasma. As the major site of glycation on haemoglobin is the amino terminus of the β-chains, it will probably be less affected by drugs which mainly bind to ε-amino groups. It is likely that naproxen, diazepam and thyroxine are able to cross the erythrocyte membrane and may have contributed to the inappropriately low glycated haemoglobin in the low glycaters. A better knowledge of drug interference is therefore needed for the correct interpretation of glycated protein results.

Although drugs may affect the glycation of proteins, it is less likely that the fructosamine measurement will be affected by drugs, as it appears to be less sensitive to small changes in glycation than the measurement of specific glycated proteins.

The fact that GHb and glycated albumin do not always reflect mean blood glucose levels accurately, due to other factors influencing the glycation process, suggests that the glycation of other proteins particularly those that are intracellular may not occur at the same level as those in the blood. The glycation of these proteins may be dependent not only on the extracellular or intracellular glucose concentration (which will generally differ to the glucose
concentration in the blood), but also on the presence of other factors influencing the glycation process.

The identification of this locational difference in the individual level of glycation of proteins may be important in helping to determine the rate of development of the complications occurring in diabetes in tissues such as the eye and the kidney.

The results in Chapter 5 showed that supplementation with oral vitamin C resulted in a plasma and erythrocyte vitamin C concentrations which reduced the glycation of both albumin and haemoglobin. Vitamin C concentrations in the plasma and erythrocyte are known to be one of the lowest compared to other cells and organs of the body (Jaffe, 1984). Therefore, the effect of vitamin C on glycation may be more profound with other proteins located extravascularly. There is also the possibility that vitamin C may react further with proteins to produce complex, cross-linked compounds similar to the AGE-products that occur with glucose (Brownlee et al., 1984; Pongor et al., 1984). However, at present the evidence for these vitamin C/protein cross-linked products has come from in vitro experiments using very high concentrations of vitamin C (Benesch et al., 1985; Ortwerth and Olesen, 1988). Further work needs to be carried out to investigate the reaction of vitamin C and proteins both in vitro and in vivo (in normal and diabetic subjects). Furth and Harding (1989) have recently reviewed glycation and post-amadori product formation, particularly substances that may be used to block either the initial glycation process or the formation of the AGE-products. They discuss the use of vitamin C in its anti-oxidant capacity to act as an anti-glycation agent. However, this is assuming that the damage to proteins occurs through free radicles which are produced from the reaction of glucose and oxygen and not by the glycation of proteins.

In conclusion, the measurement of a specific glycated protein provides a more accurate indication of blood glucose control than the measurement of fructosamine concentration, with glycated albumin probably being a more reliable indicator of blood glucose control than GHB. More work is required to assess the validity of using glycated albumin as a general indicator of the level of protein glycation throughout the body. Vitamin C was shown to reduce the glycation of albumin and haemoglobin in vivo, but again more work is needed to determine whether it should be used therapeutically in diabetic subjects to delay the onset of complications.
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