Blood and Urinary Glycosylated Proteins

In Diabetes Mellitus

By

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SUMMARY

In this thesis, the development of an immunoassay system for glycosylated albumin is described. The technique that was used consisted of affinity separation of glycosylated from non-glycosylated albumin, followed by an enzyme-linked immunosorbent assay (ELISA) for albumin. The assay sensitivity was 2 mg/l and was sufficiently sensitive to measure the glycosylated albumin concentration in the urine and plasma of both diabetic and non-diabetic subjects.

An overnight timed urine sample and blood sample were collected from 85 diabetic subjects (56 IDDM, 29 NIDDM) and 39 age and sex matched non-diabetic control subjects. Plasma and urinary glycosylated albumin, urinary α₁-microglobulin, glycosylated haemoglobin, plasma and urinary creatinine were measured. Duration of diabetes, body weight and presence of retinopathy were noted.

The diabetic subjects were grouped according to their albumin excretion rate (AER) and a relationship was found between urinary glycosylated albumin and albumin excretion rate. An association was found between increasing albumin excretion rate and elevated protein glycosylation and an enhanced excretion of glycosylated albumin was demonstrated.

Affinity chromatography was used to measure glycosylated haemoglobin as part of the Islington Diabetes Survey. Some of the screening samples and almost all of the follow-up samples were measured. Three glycosylated haemoglobin methods were compared as screening tests for diabetes mellitus, the other methods were carried out at the Whittington Hospital. Affinity chromatography was found to have the highest specificity and predictive value (positive) tests of the three methods, and was shown to be potentially useful as a screening test.
The recognition that protein glycosylation is increased in the diabetic state has resulted in investigation of its usefulness as a screening tool and of its involvement in the pathogenesis of diabetic complications. Glycosylated proteins may have a role in population screening for diabetes, and may be involved in, and indicative of, the development of the sequelae of diabetes mellitus.
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CHAPTER 1

INTRODUCTION
When this study was planned, there were no direct assays available for the measurement of glycosylated albumin (Gly-Alb), and so it was decided to develop a sensitive immunoassay for Gly-Alb, and to use this assay in an investigation of the relationship between glycosylated proteins and microalbuminuria in diabetes mellitus. We also carried out glycosylated haemoglobin (GHb) measurements as part of the Islington Diabetes Survey.

This introductory Chapter, therefore, begins with a brief description of the classification and diagnosis of diabetes mellitus and then reviews the literature on GHb, its discovery, chemical nature, and methods for its measurement. The applications of GHb measurement are then reviewed, followed by glycosylation of other proteins and their roles in the development of diabetic complications, with emphasis on the renal changes that are common in diabetes mellitus.
Diabetes mellitus is a universal health problem, which affects societies all over the world. At least 30 million people are thought to be involved throughout the world (WHO 1985) and the numbers of reported cases are increasing rapidly. This increase may be due to a number of factors, including ageing of populations, changes in lifestyles and improvement in diagnosis. Although it may appear to be a more obvious problem in affluent societies, it is in fact found in all societies. In developing countries, diagnosis and mortality data are more difficult to obtain.

Obesity is strongly associated with diabetes in some societies, whereas in others, malnutrition may be an important determinant. Infection and food toxins are also thought to play a part; environmental factors interact with varying degrees and types of inherited susceptibility. Research is ongoing into the causes of diabetes, its aetiology and the prevention of the complications which can develop, improvements in treatment and assessment of the efficacy of treatment.
Definition, Classification and Diagnosis of Diabetes

1:3:1 Definition

Diabetes mellitus is a state of chronic hyperglycaemia, resulting from genetic and environmental factors. This state is brought about by the inability of the beta cells of the Islets of Langerhans to produce sufficient insulin, or by an excess of factors opposing its action, resulting in poor control of blood glucose concentration (Newsholme & Leech 1983). This imbalance leads to abnormalities of carbohydrate, lipid and protein metabolism. Characteristic symptoms include ketoacidosis, thirst, increased urine volume and raised blood glucose concentration. As the condition progresses complications frequently develop. These include damage to peripheral nerves, capillaries of the kidneys and retina and excessive arteriosclerosis.

1:3:2 Classification

A classification of diabetes and other categories of glucose intolerance has been developed and recommended by the National Diabetes Data Group (National Diabetes Data Group 1979), and by the Expert Committee on Diabetes of the World Health Organisation (WHO 1980). There has been general acceptance of this classification, but more recently, malnutrition-related diabetes mellitus has been included as a major clinical sub-class, ranking with insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) (WHO 1985). Table 1:1 shows the revised classification system described by the WHO Study Group in 1985.
### Table 1:1

#### Classification of Diabetes Mellitus and Other Categories of Glucose Tolerance

<table>
<thead>
<tr>
<th>Class Name</th>
<th>Characteristic</th>
</tr>
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<tbody>
<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td></td>
</tr>
<tr>
<td>Insulin-dependent diabetes mellitus (IDDM)</td>
<td>Insulin required to prevent ketosis and preserve life</td>
</tr>
<tr>
<td>Non-insulin-dependent diabetes mellitus (NIDDM) (i) Non-obese (ii) Obese</td>
<td>Non-insulin requiring or ketosis prone, but may use insulin at times. Serum insulin may be normal, elevated or depressed. In obese subjects, weight loss may improve glucose tolerance</td>
</tr>
<tr>
<td>Malnutrition-related diabetes mellitus (MRDM)</td>
<td>Severe hyperglycaemia present, requiring insulin for control, not usually ketosis prone. Some residual insulin production</td>
</tr>
<tr>
<td>Other types of diabetes associated with certain conditions and syndromes</td>
<td>DM present in addition to (i) pancreatic disease; (ii) disease of hormonal etiology; (iii) drug or chemical-induced conditions; (iv) abnormalities of insulin or receptors; (v) genetic syndromes; (vi) other.</td>
</tr>
<tr>
<td>Impaired glucose tolerance (i) Non-obese, (ii) Obese, (iii) associated</td>
<td>Non-diagnostic fasting BG and glucose tolerance between normal and DM</td>
</tr>
<tr>
<td>with other syndromes</td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>Glucose intolerance, onset or recognition during pregnancy. Reclassification needed post partum</td>
</tr>
<tr>
<td><strong>Statistical Risk Classes</strong></td>
<td></td>
</tr>
<tr>
<td>Previous abnormality of glucose tolerance</td>
<td>Normal glucose tolerance but substantially increased risk of developing DM</td>
</tr>
<tr>
<td>Potential abnormality of glucose tolerance</td>
<td>Normal glucose tolerance but previous abnormal glucose tolerance found</td>
</tr>
</tbody>
</table>
Diagnosis

The diagnosis of diabetes is usually based on characteristic signs and symptoms, such as increased thirst and urine volume, glycosuria, unexplained weight loss and, in severe cases, drowsiness and coma (WHO 1985). Unequivocally raised blood glucose levels may establish diagnosis, and may also do so when symptoms are less severe. In cases where the signs are less clear and blood glucose levels less clearly raised, measurements made under standard conditions may be needed to assist the confirmation of diagnosis. The oral glucose tolerance test (OGTT) has become widely accepted as a diagnostic tool, but differences in the execution of the test and interpretation of the results still remain. The situation is further complicated by day-to-day variations in individuals and the knowledge that results are affected by many factors including previous diet, emotion, trauma, other diseases, drugs and time of day (Modan et al. 1984).

Three expert committees have published standard criteria for diagnosis of diabetes mellitus (Keen et al. 1979; National Diabetes Data Group 1979; WHO 1980), which are in overall agreement. The National Diabetes Data Group (NDDG) and WHO criteria for diabetes, as shown in Table 1:2 are virtually identical. Both permit a diagnosis based on the presence of the classic diabetic symptoms and random plasma glucose values of 11.1mmol/l or greater; both also permit a diagnosis of diabetes based on a fasting plasma glucose concentration of 7.8 mmol/l or greater. In persons without unequivocal symptoms and in those with lower random or fasting plasma glucose levels, both require a glucose tolerance test in which blood glucose concentration is measured 2 hours after a 75g oral glucose challenge. The NDDG and WHO both require that, for diagnosis of diabetes, this 2-hour value be equal to, or greater than, 11.1mmol/l; the NDDG also requires that a mid-test blood sample be taken and that the glucose concentration of this sample
<table>
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<th>Glucose Concentration, mmol/l</th>
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<td></td>
<td>Whole Blood</td>
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<tr>
<td></td>
<td>Venous</td>
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<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td></td>
</tr>
<tr>
<td>Fasting value</td>
<td>&gt;6.7</td>
</tr>
<tr>
<td>2-hours after 75g glucose load</td>
<td>&gt;10.0</td>
</tr>
<tr>
<td><strong>Impaired glucose tolerance</strong></td>
<td></td>
</tr>
<tr>
<td>Fasting value</td>
<td>&lt;6.7</td>
</tr>
<tr>
<td>2-hours after 75g glucose load</td>
<td>6.7-10.0</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td><strong>Plasma</strong></td>
<td></td>
</tr>
<tr>
<td>Venous</td>
<td>&gt;7.8</td>
</tr>
<tr>
<td>Capillary</td>
<td>&gt;11.1</td>
</tr>
<tr>
<td>Venous</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>Capillary</td>
<td>7.8-11.1</td>
</tr>
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</table>
also be equal to or greater than 11.1 mmol/l. This requirement for a mid-test value by NDDG makes only a slight difference (Harris 1985); 91% of individuals in a sample survey of the United States population whose 2-hour value exceeded 11.1 mmol/l also had 1-hour values exceeding 11.1 mmol/l.
Impaired glucose tolerance (IGT) describes individuals whose fasting plasma glucose concentration is lower than that required for a diagnosis of diabetes and whose plasma glucose level 2 hours after a 75g oral glucose challenge is between 7.8 and 11.1mmol/l.

Long-term results of follow-up studies of diabetes such as the Bedford Survey (Sharp et al. 1964) and the Whitehall Study (Reid et al. 1974) have shown the predictive ability of a blood glucose measurement, made 2 hours after an oral glucose load, for increased incidence of atherosclerotic disease and risk of cardiovascular death over a ten-year period. The groups were defined by a 2-hour post load blood glucose criteria described in Table 1:2. Both diabetic and IGT subjects showed an increase in development of vascular disease but only the new diabetics carried an increased risk of developing retinopathy. These findings contributed to the setting-up of the WHO diagnostic criteria and are evidence of the relevance of the OGTT as a diagnostic test for diabetes.

As these studies used only the 2-hour blood glucose measurement, other criteria cannot be assessed from these results. The new criteria include fasting blood glucose levels, but this value is more difficult to standardise experimentally.

An effective method of population screening should be sensitive enough to identify most, if not all, of the diseased population, and be relatively cheap, quick and easy to perform. It must also have a reasonable positive predictive value so that subsequent, more specific, and costlier final diagnostic tests will have a high yield. At present this diagnosis rests on 'abnormally' increased blood glucose levels in both the fasting state and following an oral glucose load.
The classification system based on the OGTT has gained wide acceptance in studies of diabetes. However, the test is time-consuming, unpleasant for the patients and problematic in large-scale population screening. Two-stage screening, i.e., the measurement of fasting blood glucose levels in the population followed by OGTTs for individuals with abnormally high values, has been found to be unsatisfactory. This is due to the failure to detect some individuals with glucose intolerance (National Diabetes Data Group 1979). In order to ease the task of screening a large population, some workers have simplified the OGTT by taking only one blood sample, usually 2 hours following the oral glucose load. The relative usefulness of these much reduced tests have yet to be established (Modan et al. 1984).
Discovery

Until the mid-1950's, it was assumed that haemoglobin was homogeneous, as careful crystallization techniques had confirmed. However, in 1958 Allen et al. carried out ion exchange chromatography on crystallized haemoglobin and found a minor component which was not an artifact of preparation or chromatography. Nor could it be removed by crystallization under the most exacting conditions. Over 90% of the soluble protein within normal adult human red cells is haemoglobin A, consisting of two alpha chains and two beta chains ($\alpha_2\beta_2$). In addition, they found several minor haemoglobin components which they designated HbA$\alpha$, HbA$\beta$, and HbA$\gamma$ in the order of their elution from the chromatography column, the last of which is the most abundant.

Holmquist & Schroeder (1966) showed that HbA$\gamma$ is structurally identical to HbA except that an N-terminal amino group of a $\beta$ chain is attached to a carbonyl group of an aldehyde or ketone by a Schiff base. Bookchin & Gallop (1968) confirmed that there was a Schiff base linkage on both beta chains, and showed that the N-terminal valine residues of both beta chains were linked to a hexose. These techniques could not, however, distinguish between various hexose isomers.

Interest in HbA$\gamma$ was enhanced by the demonstration by Rahbar (1968) that this component was increased about 2-fold in patients with diabetes mellitus. Starch gel electrophoresis was used to separate the HbA$\gamma$ component and illustrate the difference between diabetic and non-diabetic haemolysates.

The Chemical Nature of Glycosylated Haemoglobin

Trivelli et al. (1971) clarified the increase of the glycosylated haemoglobins in diabetic subjects using ion exchange chromatography. They found that one or
more hexoses were bound to the beta chains, and that 75-80\% of the 'fast' fraction of the haemoglobin was accounted for by HbA\textsubscript{1c}. No relationship was found in this early study between the level of glycosylation and age, duration, therapy or complications in the diabetic subjects.

Bunn et al. (1975) investigated the nature and mode of linkage of the carbohydrate moiety in HbA\textsubscript{1c} from normal and diabetic individuals. Glucose and mannose were both found following hydrolysis of the molecule. They found that the hexose was bound to the alpha amino terminal position of the beta chain (valine) of the haemoglobin in a Schiff base linkage. This aldimine linkage could rearrange in a reversible manner to form a ketoamine linkage (Figure 1:1) which was stable to acid hydrolysis. Several minor haemoglobin components were found, of which HbA\textsubscript{1c} was the major constituent.

Fluckiger & Winterhalter (1976) carried out in vitro synthesis of HbA\textsubscript{1c} to determine its structure and synthetic pathway. They incubated whole blood with radioactive-labelled glucose and analysed the products compared with native HbA\textsubscript{1c}. Tryptic peptide mapping techniques and the thiobarbituric acid (TBA) assay were used. In this test, acid hydrolysis releases the ketoamine-linked sugar as 5-hydroxymethyl furfural which then forms a colour complex with TBA. They showed that the product formed was indistinguishable from native HbA\textsubscript{1c}. Between 2-2.4 moles of labelled glucose bound to each haemoglobin tetramer, with ten times more bound to the beta chains than to the alpha chains. Over 90\% of the labelled glucose was found to be attached to the N-terminal peptides. The overall reaction was summed up as a nucleophilic attack on the carbohydrate by the unprotonated amino group of the haemoglobin molecule. Their work was one of the first major contributions to our understanding of the structure of GHb and led the way for many groups of workers to investigate this material.
Figure 1:1 Reaction scheme for the non-enzymatic glycosylation of proteins

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{H}_2\text{C} = \text{N} - \text{P} & \quad \text{H}_2\text{C} - \text{NH} - \text{P} \\
\text{HCOH} & \quad \text{OHCH} & \quad \text{C} = \text{O} \\
\text{OHCH} & \quad \text{OHCH} & \\
\text{HCOH} + \text{H}_2\text{N} - \text{P} & \quad \text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} & \quad \text{HCOH} \\
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{Glucose} & \quad \text{Schiff Base} & \quad \text{Ketoamine} & \quad \text{Glycosylated Protein}
\end{align*}
\]

\( p = \text{protein molecule} \)
Bunn and co-workers also carried out extensive work in this area. Their investigation of the formation of HbA\textsubscript{1c} showed that it was formed slowly throughout the life-span of the red cell (Bunn et al. 1976). Red cells with reduced life-spans due to haemolysis, had lower levels of HbA\textsubscript{1c}. They described three negatively charged minor components of haemoglobin, HbA\textsubscript{1a}, HbA\textsubscript{1b}, and HbA\textsubscript{1c}, comprising 1.6, 0.8 and 4% of haemoglobin respectively in normal individuals.

Koenig et al. (1977) investigated the nature and linkage of the carbohydrate moiety, by studying the enzymatic digests of borohydride reduced beta HbA\textsubscript{1c} chains separated by ion exchange chromatography. They suggested that the carbohydrate was glucose-6-phosphate, but this was contradicted by McDonald et al. (1978) who showed that phosphate was present in HbA\textsubscript{1a} and HbA\textsubscript{1a2} but not in HbA\textsubscript{1b} or HbA\textsubscript{1c}.

Dolhofer & Wieland (1978) showed that HbA\textsubscript{1c} was formed post-ribosomally by the attachment of glucose to the N-terminal valine via covalent bonds. They confirmed that glucose-6-phosphate was the carbohydrate moiety in HbA\textsubscript{1a} and HbA\textsubscript{1b} but not in HbA\textsubscript{1c}. Further studies of the minor haemoglobin components (Bunn et al. 1979) showed that there were four components which were separable by ion exchange chromatography from the main haemoglobin constituent, designated HbA\textsubscript{o}. The glucosyl ketoamine linkage in HbA\textsubscript{1c} was detected colorimetrically using the TBA test. On the ion exchange column, HbA\textsubscript{o} eluted after HbA\textsubscript{1c} and the leading edge of HbA\textsubscript{o}, as well as HbA\textsubscript{1c}, contained carbohydrate. Both glycosylated components were increased in diabetic individuals. Synthetic HbA\textsubscript{1c} and glycosylated HbA\textsubscript{o} both underwent an Amadori rearrangement to the more stable ketoamine linkage. 8-10% of the HbA\textsubscript{o} in
normal individuals was found to be glycosylated. Several sites on the alpha and
beta chains of HbA\textsubscript{0} were glycosylated, where glucose had linked to the epsilon-
amino group of the lysine residues on the alpha and beta sub-units as well as at the
amino terminal valine of the alpha chain. Even though glucose binds covalently to
a number of different amino groups on both haemoglobin chains, only one
modification, that of the addition of glucose to the beta chain amino terminus,
resulted in the chromatographically different component of HbA\textsubscript{lc}.

The synthesis of HbA\textsubscript{lc} \textit{in vitro} has been extensively studied, and the effects
of time, glucose concentration and incubation temperature investigated. Spicer \textit{et al.} (1979) reported that the level of HbA\textsubscript{lc} in intact erythrocytes increased with
time, glucose concentrations (from 5 to 500mM) and incubation temperature (4-
37\textdegree C). Higgins \textit{et al.} (1982) showed that glycosylation was determined not only by
the mean blood glucose level, but also by the red cell life span and red cell
permeability. They indicated that it was important to consider the clinical status
of the patient, as many diseases decrease the red blood cell life-span. The rate of
synthesis of HbA\textsubscript{lc} is affected by the presence of 2,3 diphosphoglycerate (2,3 DPG)
and carbon dioxide, both of which normally interact with the amino terminus of the
beta chain, and by O\textsubscript{2} saturation and intracellular pH. Kinetic analysis of the
formation of HbA\textsubscript{lc} (Higgins & Bunn 1981) yielded rate constants for the three
processes involved; (a) condensation of glucose and haemoglobin to form the Schiff
base intermediate (pre-A\textsubscript{lc}) (b) dissociation of glucose from haemoglobin and (c)
rearrangement of the Schiff base to the stable ketoamine form. Their studies
showed that during the incubation of haemoglobin with glucose, the labile aldimine
increased to reach an equilibrium plateau within a few hours. During this time only
a minute amount of ketoamine was formed since the rate of rearrangement was
only 1/60 that of the rate of dissociation back to Hb
and glucose. More than one active site was shown in the Hb molecule; although the beta chain amino terminus is favoured, other sites are also glycosylated, but more slowly. They calculated that at a blood glucose level of 5mmol/l, approximately 0.5% of the total Hb would be pre-\( \text{A}_{\text{lc}} \) or approximately 10% of the total HbA\(_{\text{lc}}\) in normal red cells.

These studies, and those of other workers (Acharya & Manning 1980; Garel et al. 1979; Shapiro et al. 1980) confirmed that the non-enzymatic glycosylation of Hb involved the initial formation of a reversible aldimine precursor.

By simultaneous determination of HbA\(_{\text{lc}}\) and the labile fraction (or HbA\(_{\text{ld}}\)) and the corresponding blood glucose level it has been ascertained that the HbA\(_{\text{lc}}\) level remains constant, whereas the HbA\(_{\text{ld}}\) fraction varies in parallel with the blood glucose level (Stickland et al. 1982). Further detailed studies of the kinetics of HbA\(_{\text{lc}}\) formation from pre-HbA\(_{\text{lc}}\) or HbA\(_{\text{ld}}\) have shown that the process is more readily reversible than was previously thought (Mortensen 1985). The first step, formation of the Schiff base is rapid and rearrangement to the stable form is much slower. This second reaction is also reversible, however, and this must be taken into account when HbA\(_{\text{ld}}\) is eliminated by saline incubation. Not only is the labile component removed by this incubation, but also the concentration of HbA\(_{\text{lc}}\) is significantly lowered. This means that when pre-treatment of samples has been carried out using an incubation step to remove the Schiff base, the concentration of the stable adduct will also be artificially lowered.

Investigation of the carbohydrate moieties involved in the glycosylation of Hb showed that the minor Hb components contained different sugars (Bunn 1981a). Sugars that have been found to be precursors of GHb are readily transported into
the erythrocyte. The formation of the ketoamine adduct was influenced not only by the concentration of the sugar but also by the equilibrium between the open and ring formations of the sugar. The chain structure was found to be more reactive than the ring structure. Sugar molecules that combined with the protein molecule were in the open (chain) formation, and subsequent rearrangement of the glucose molecule takes place, to the ring structure.

Glucose-6-phosphate was found to react faster with Hb and to have a less stable ring structure than glucose. More glucose-6-phosphate molecules than glucose were in the active aldehyde form.

The carbohydrate components of the minor Hb's have been shown to be fructose-1,6-diphosphate, glucose-6-phosphate and glucose incorporated into HbA₁a', A₁a₂ and A₁c respectively (McDonald et al. 1978; Abraham et al. 1983). It has been suggested (Krishmanoorthy et al. 1977) that HbA₁b is non-glycosylated and is a deamidation product of HbA₀ (Monnier & Cerami 1982a). However, Garrick et al. (1980) showed that HbA₁a₁ and HbA₁a₂ contained sugar phosphates, but HbA₁b and HbA₁c contained non-phosphorylated sugars.

Further kinetic studies of the formation of GHb have shown that the stable HbA₁c formed in 24 hours was equal to, on average, 0.006% of the total Hb per mmol of glucose (Svendsen et al. 1981). They also showed that an increase of blood glucose level of 1 mmol/l increased the level of HbA₁c by 0.058% of the total Hb concentration in 24 hours.

Detailed investigation of the sites of incorporation of glucose in the Hb molecule (Foldi et al. 1982) showed that 65% of the incorporated glucose was bound
to five peptides in the alpha chain. The major sites of glycosylation in the beta chain were described by Mortensen & Christophersen (1982). Garlick et al. (1983) showed that 88% of the glucose in HbA$\text{ic}$ was attached to the beta chain amino terminal valine, and that the remaining 12% was probably linked to the same lysine and valine groups as in HbA$_0$. The relative stabilities of the sites of incorporation have been studied and the ketoamine linkages of aldotriose at the alpha amino groups are almost irreversible. The higher stability of ketoamine linkages of Val-1 (β) suggests that some of the structural features of the protein around the alpha amino groups of the beta chain confer a degree of stability to the ketoamine adducts there (Acharya and Sussman 1984).
The non-enzymatic glycosylation reaction is not limited to glucose, and a variety of sugars and sugar phosphates including ribose, galactose and glucose-6-phosphate can covalently attach to proteins (Dolhofer & Wieland 1978). Ribose is one of the most effective, whereas glucose is the least effective in glycosylation, and the rates of reaction of different monosaccharides with protein (using haemoglobin as the model) can vary over a 300-fold range. Aldohexoses as well as ketohexoses, such as fructose and xylulose can condense with protein, although in general the rate of interaction of the latter group is less than that of the former. This appears to be due to the fact that aldehyde carbonyl groups are relatively more electrophilic than ketone carbonyl groups, and the reaction proceeds by a nucleophilic attack of an unprotonated amino group on the carbonyl group of the sugar. The relative reactivity of individual monosaccharides is strongly influenced by the extent to which the compound exists in the open (acyclic) form, which in turn depends on the equilibrium between the open and ring configurations.

Although non-enzymatic glycosylation can involve different sugars, glucose is the major carbohydrate nutrient in humans. In view of the mounting evidence that the reaction exerts deleterious effects on structure-function relationships of involved proteins, it is of interest to consider factors that protect humans from non-enzymatic glycosylation and its potentially injurious consequences. According to Bunn & Higgins (1981) this relates to the stability of the ring structure of glucose, which under normal circumstances and at physiological concentrations of glucose, limits the forward reaction that attaches glucose to proteins. Bunn & Higgins proposed that the emergence of glucose rather than other monosaccharides as the most important metabolic fuel coincided with the stability of its ring structure, which limits undesirable side reactions attributable to covalent modification of proteins via non-enzymatic glycosylation.
Initially it was thought that glycosylation of Hb would affect its function. McDonald et al. (1979) studied the functional properties of the minor components of Hb, and showed that they each possessed unique properties which differed from HbA₀. They suggested that the glucose moieties attached to the amino terminus of the beta chain might interfere with the binding of 2,3 DPG, a regulator of Hb function and thereby affect oxygen binding capacity. The two most acidic components HbA₁₈ and HbA₁₉ have lower oxygen affinities than HbA (Imagawa et al. 1982). The slight increase in oxygen affinity (about 2 mm Hg) of diabetic blood has been attributed to increased levels of HbA₁c, which has a raised oxygen affinity.

Bunn (1981a) showed that no significant influence on oxygen transport had been found. Even in diabetic subjects with two or three times the normal level of HbA₁c only a very slight change in oxygen unloading was evident. It is unlikely that this change has any clinical significance since patients with mutant Hbs and higher oxygen affinities do not show signs of poor oxygenation of their peripheral tissues (Monnier & Cerami 1982a).
Assay methods for the analysis of GHb that are in routine use can be divided into three main categories based on the manner in which glycosylated and non-glycosylated Hb components are measured (Table 1:3). The first group of methodologies considered are the techniques which use charge differences as a means of separation, of which there are three types, followed by a method which is based on the reactivity of the carbohydrate group. Finally, a method based on the structural characteristics of the GHb molecule is described.

1:7:1 Cation Exchange Chromatography

This technique has been widely used and adapted and much of the early work investigating the chemical nature of GHb has involved ion exchange chromatography. Schnek & Schroeder (1961), Holmquist & Schroeder (1966) and Trivelli et al. (1971) developed and adapted a technique using a cation exchange resin, Bio-Rex 70 (Bio-Rad Laboratories, Richmond, California, USA). The principle of the method is that most GHb species, including HbA^c, are more negatively charged than HbA and bind less well to a resin that attracts haemoglobin components based on their charge. The more negatively charged haemoglobin components, HbA^la, HbA^lb and HbA^lc elute before the main haemoglobin fraction HbA and their proportion of the total haemoglobin can be determined using a spectrophotometer. McDonald et al. (1978) adapted the technique and used a sodium chloride gradient from 0.1M to 1.0M to elute the minor components followed by HbA. These studies investigated the structure and function of GHb and involved the large-scale separation of haemoglobin components, with the application of up to 6g of haemolysate onto chromatography columns up to 5 cm x 40 cm.
Table 1:3

Classification of Glycosylated Haemoglobin Assay

Methods Used in Routine Clinical Laboratories

1. Methods based on charge differences between glycosylated and non-glycosylated haemoglobins.
   (a) Cation exchange chromatography (including HPLC): glycosylated HbA\textsubscript{1}, (HbA\textsubscript{1a1}, HbA\textsubscript{1a2}, HbA\textsubscript{1b}, HbA\textsubscript{1c} and HbA\textsubscript{1d}) all separated from HbA.
   (b) Electroendosmosis: glycosylated HbA\textsubscript{1} (HbA\textsubscript{1a1}, HbA\textsubscript{1a2}, HbA\textsubscript{1b}, HbA\textsubscript{1c} and HbA\textsubscript{1d}) all separated from HbA.
   (c) Isoelectric focusing: HbA\textsubscript{1c} separated from remainder of HbA\textsubscript{1} and HbA.

2. Methods based on chemical reactivity of carbohydrate groups on haemoglobin.
   (a) Hydroxymethylfurfural - thiobarbituric acid (colorimetric) method: ketohexoses on the N-terminal valine as well as lysine groups on the \(\alpha\) and \(\beta\) chains are measured.

3. Methods based on the structural characteristics of the carbohydrate groups on haemoglobin.
   (a) Affinity chromatography: haemoglobin bound ketohexoses on N-terminal valine of \(\beta\)-chain as well as on non-\(\beta\) N-terminal loci are separated.

4. Other methods
   (a) Radioimmunoassay
   (b) Fructosamine
The method has been adapted for the measurement of GHb levels in large numbers of samples, eluting \( \text{HbA}_1 \) (\( \text{HbA}_{1a} \), \( \text{HbA}_{1b} \) and \( \text{HbA}_{1c} \)) separately from \( \text{HbA}_0 \). Kynoch & Lehman (1977) were the first to develop a rapid smaller scale assay using Bio-Rex 70, but this took 2½ hours to complete and required large quantities of potassium cyanide.
Welch & Boucher (1978) developed a rapid micro-scale method, which required only 0.05ml of blood and took forty minutes to complete. This method not only reduced the time needed for each GHb estimation but also reduced the quantity of potassium cyanide used to prevent the conversion of haemoglobin to met-haemoglobin, making the technique more suitable for routine hospital laboratories.

Other groups have investigated the effects of pH and tried to make the method faster to perform. Chou et al. (1978) adapted the method of Trivelli et al. (1971) using 10ml resin columns and the same buffer systems as Schnek & Schroeder (1961). Clarke & Canivet (1979) developed a methodology that permitted up to 30 columns to be run simultaneously, with the measurement of HbA\textsubscript{1c} instead of HbA\textsubscript{1} as in the other micro-column methods. In this technique, by varying the number of binding sites (i.e., the product of the height of the column and the diameter), the pH of the resin and the buffers and the osmolarity of the buffers, they were able to increase the differential separation to a point at which HbA\textsubscript{1a} and HbA\textsubscript{1b} were eluted as a separate peak from HbA\textsubscript{1c}. However, the pH of the buffers used was found to be critical, as was the temperature making the method less suitable for routine use, despite its specificity for HbA\textsubscript{1c}.

Worth et al. (1980) investigated the effects of temperature on the micro-column assay and found that variations in temperature had a major influence on the results obtained. When samples from pooled haemolysates were run at room temperature on consecutive days, inter-assay coefficients of variation were several times greater than those achieved when temperature was maintained at a constant level.
These findings were confirmed by Simon & Eissler (1980) who defined tolerance limits for osmolarity and pH of the buffers and temperature of the columns. Within strictly set limits for these parameters, they were able to obtain good reproducibility. However, they pointed out that HbF interfered with the assay, as it co-eluted with HbA₁.

Nikolov & Damianova (1983) compared a slightly adapted version of the micro-scale assay (Welch & Boucher 1978) with a method using Amberlite resin (Fluka, Buchs, Switzerland), they found that using the latter technique shortened the procedure, and that performing it at 22°C improved reproducibility.

The next step from rapid, micro-scale methods was to develop an automated technique using the Bio-Rex 70 resin. This was described by Blouquit et al. (1983) who used a three buffer system to separate HbA₁a and HbA₁b and then HbA₁c from HbA₀. They used an increase in sodium chloride content in the buffers to remove HbA₁c and HbA₀ once the other faster-moving fractions had been eluted. The chromatographic system consisted of a sample injector, peristaltic pump and two resin-filled columns, one being equilibrated while the other was running.

Allen (1983) described a thorough investigation of the effects of haemoglobin variants on HbA₁c measurement using Bio-Rad columns. HbA₁c levels were found to be low in patients heterozygous for HbS or HbC. However, results from patients homozygous for HbS or HbC, or with high levels of HbF could not be interpreted as values were considerably raised. This can lead to difficulties in using the results as prior knowledge of the existence of a haemoglobin variant is not usually available.
Electroendosmosis

This is a type of electrophoresis, in which, when a haemolysate is applied to an agar gel and an electrical potential is applied across the gel plate, the various components can be separated according to charge. The gel contains charged groups which interact with the haemoglobin components depending on their charge. When the electrical current is applied, migration of HbA_0 is retarded more than that of the minor components, HbA_a, HbA_1b, and HbA_1c. The method was developed by Menard et al. (1980) and is widely used in routine hospital laboratories, usually with equipment and reagents made by Corning Limited (Corning, Halstead, Essex, UK). The equipment required is expensive but running costs can be reduced by making the agar gel plates instead of purchasing commercially prepared ones.

The assay measures all of the more negatively charged minor components of haemoglobin HbA_1a, HbA_1b and HbA_1c (HbA_1) as well as the Schiff base and HbF. HbC and HbS however, do not interfere (Boucher et al. 1983). The technique allows many samples to be run simultaneously, and the whole procedure requires under 2 hours to be completed. Sample preparation is very straight-forward requiring the addition of a haemolysing reagent containing saponin.

The technique used by Menard et al. (1980) is now widely used in conjunction with the Corning equipment and subsequent workers using the method have made few significant alterations to this standard method (Boucher et al. 1983; Mortensen et al. 1983). There are now, however, commercially prepared haemolyzing reagents which are used to pre-treat the blood samples, and remove the labile fraction. The reagent contains potassium diphthalate, saponin and EDTA and is used to incubate the red cells (Nathan et al. 1984; Moore et al. 1986).
**Isoelectric Focusing**

In this special type of electrophoresis substances such as amino acids and peptides are separated in a column along which there is both a pH gradient and a voltage gradient. Each compound migrates towards the region in the column where the pH corresponds to that of its isoelectric point and is immobilized (focused) there (Williams & Wilson 1976).

Schoos et al. (1978) developed a method suitable for routine use, using thin-layer polyacrylamide gel. Migration and focusing of haemoglobin were carried out and, after focusing, the proteins were precipitated, and read in a densitometer.

Jeppsson et al. (1978) adapted the polyacrylamide gel, by the addition of beta alanine to the ampholytes and so improved the critical part of the pH gradient to increase specificity of the method. Poon & Whittle (1980) found this method to be independent of ambient temperature and to compare favourably with the ion-exchange method.

Mortensen and Marshall (1983) stated that pre-treatment of erythrocytes was unnecessary as the HbA\textsubscript{1c} fraction was well separated from the labile fraction. They did, however, study the effects of saline incubation on the red cell content of HbA\textsubscript{1c} as measured by isoelectric focusing, and found that incubation in isotonic saline for 6 hours at 37\degree C significantly decreased the concentration of HbA\textsubscript{1c}.

**Colorimetric Technique**

This method is based on the chemical reactivity of the GHb molecule. The technique is widely used, and was originally developed for use in the measurement of GHb by Flückiger & Winterhalter (1976). The thiobarbituric acid (TBA) reaction
involves the release of bound glucose from GHb by acid hydrolysis and conversion to 5-hydroxymethylfurfural (5HMF). After a lengthy incubation at 100°C the haemolysate and oxalic acid mixture is cooled rapidly to room temperature. This drives the Amadori rearrangement, which normally proceeds slowly, and liberates 5HMF from the protein (Winterhalter 1981). The addition of trichloroacetic acid precipitates the haemoglobin, which can then be removed by filtration or centrifugation. The supernatant is then incubated with thiobarbituric acid which reacts with 5HMF to form a compound with an absorption maximum at 443nm.

Different incubation times and acid conditions have been investigated by various groups. In the earliest methods (Flückiger & Winterhalter 1976) the hydrolysis was carried out for 1 hour with 0.3M oxalic acid, but the incubation time was later increased to 4.5 hours in the presence of 1M oxalic acid (McFarland et al. 1979) making it a very time consuming process. These modified hydrolysis conditions released the maximum amount of 5HMF from the protein. Gabbay et al. (1979) also modified the TBA technique to increase the colour yield and enhance reproducibility by increasing the oxalic acid concentration and the duration of heating of the mixture. Their method involved a 5-hour incubation at 100°C with 1M oxalic acid. Pecoraro et al. (1979) characterized various steps from the method of Gabbay and co-workers that were found to be crucial to precision and compared determinations made with the TBA method with corresponding results by the traditional chromatographic technique described in Section 1:7:1. The major advantages of the colorimetric method, they concluded, included its yield of stable results from samples stored frozen before assay and its ability to determine glycosylation of variant haemoglobins. The method is quantitated using standards containing known concentrations of 5HMF or fructose (Pecoraro et al. 1979).
The method has been used and adapted for the measurement of glycosylated plasma proteins by various groups who investigated ways of eliminating the interference caused by the presence of glucose. This was achieved by dialysis of the samples against saline prior to hydrolysis (Dolhofer & Wieland 1981). Another approach was to precipitate the protein with trichloroacetic acid before hydrolysis, thus removing free glucose (Ma et al. 1981).

Prior to the measurement of GHb on the samples, the haemoglobin concentration of each sample has to be measured and each sample diluted to the same haemoglobin concentration so that aliquots of haemolysate containing standard concentrations of haemoglobin can be tested in the assay (Pecoraro et al. 1979). This extra step, and the problem of high reagent blanks, makes this technique time consuming and cumbersome.

Automated methods have been developed, as the colorimetric technique in common use is laborious. Burrin et al. (1980) modified the method for use with standard autoanalyser apparatus consisting of a sampler unit, peristaltic pump, heating baths, dialysers and a recording spectrophotometer. The system enabled the analysis of 200 samples per day using a modified colorimetric system based on the method of Fluckiger & Winterhalter (1976) with a reduction in sample size, reduction in incubation time with oxalic acid and dialysis of the sample instead of TCA precipitation. The running time of the system was approximately 2 hours from sampling to peak production on the recorder. However, this system did not function well and has never been in widespread use. A similar system was developed by Ross & Gibson (1979) which required a 2-hour incubation with oxalic acid.
By carrying out the hydrolysis stage in an autoclave, Parker et al. (1981) were able to reduce the assay time. The method yielded good within and between assay coefficients of variation (2 and 3% respectively) and correlated well with an ion-exchange method.

The derivatives of phosphorylated sugar give small amounts of coloured reaction products; these, however, do not significantly influence the optical density reading at 443 nm (Winterhalter 1981). The method measures all the non-enzymically glycosylated proteins in the haemolysate or whole blood, but the labile fraction of GHb contributes very little to the total measured. Klenk et al. (1982) investigated the effect of incubating the erythrocytes in isotonic saline for 5 hours prior to the assay and found that the TBA technique showed only a 1.6% decrease.

1:7:5 Affinity Chromatography

Affinity chromatography is a technique for separating large molecules based on their chemical structure. Shapiro et al. (1980) first mentioned the ability of amino-1-deoxysorbitols to form a complex with borate and suggested its use as a potentially powerful tool for the purification of non-enzymatically glycosylated proteins. Mallia et al. (1981) developed an immobilized boronate ligand on agarose which would selectively bind with the cis-diol groups of GHb. Aminophenylboronic acid in the hemisulphate form, as prepared by the method of Seaman & Johnson (1931) was covalently attached to activated agarose gel. The gel was activated following the method of Bethell et al. (1979) and then aminophenylboronic acid allowed to react with it. Quantities of this affinity matrix were washed with water, loaded into microcolumns to a bed volume of 1ml and equilibrated with 'wash buffer'. This contained ammonium acetate and magnesium chloride at pH 8.5. Red blood cells were haemolysed and then applied to the gel. Wash buffer was
applied and the unbound (non-glycosylated) haemoglobin collected. Elution buffer containing 200 mM sorbitol to compete with the cis-diol groups of the GHb, was applied and the bound fraction collected separately. The non-bound fraction was diluted, the absorbances of both fractions measured in a spectrophotometer, and the percentage glycosylated haemoglobin calculated from the values (Klenk et al. 1982).

Bouriotis et al. (1981) developed a method which also used immobilised phenylboronic acid to selectively bind diols. Their wash buffer contained morpholine-HCl and the elution buffer the same with sorbitol added. They carried out the separation at 4°C instead of room temperature. A reasonable correlation was found between this method and an ion-exchange method, and the affinity technique showed an improved separation of diabetic from non-diabetic samples.

Gould et al. (1982) carried out a detailed study to investigate the effects of temperature and pH on the affinity chromatography method and compared the results with those from an electrophoretic method. They found that an increase in temperature from 7°C to 37°C caused a decrease in the percentage of haemoglobin that bound to the gel. An increase in pH from 8.1 to 8.9 also resulted in a decrease in the bound fraction. They also removed the labile GHB fraction under a range of conditions. A good correlation was found between the two techniques and a running temperature of 20°C ± 1°C and pH of 8.6 ± 0.1 were selected as yielding a very low coefficient of variation and being convenient to achieve.

Yu et al. (1982b) compared the affinity chromatography method with ion-exchange and TBA methods and found that the former showed the best separation between diabetic and non-diabetic samples, correlated best with plasma glucose levels and showed the least effect from changes in temperature.
Storage of blood samples at 4°C for up to a week was shown to have little effect on the results obtained from affinity chromatography, ion-exchange chromatography, or the TBA method (Little et al. 1983a). The affinity chromatography and TBA methods also showed sample stability after storage at 20°C for a week or more. However, the ion-exchange method showed a marked increase in GHb values after only a few days. They also showed that whole blood stored at room temperature in sealed capillary tubes for 21 days gave the same level of GHb as fresh samples when measured by affinity chromatography (Little et al. 1983b).

The affinity chromatography method has been developed for routine clinical use and a kit is available which allows a large number of samples to be measured, up to sixty in a working day (Hall et al. 1983b). The method can also be used to measure glycosylated plasma proteins (Hall et al. 1983a) and glycosylated haemoglobin (Talwar et al. 1983) in adult and foetal blood.

There is still no consensus method for the affinity chromatography technique, resulting in poor between-laboratory agreement in an external quality assessment scheme (John 1986). A widely used method is that of Gould et al. (1982), but there are many variations in the buffer volumes used, temperature and pH of the buffers. It is, however, an inexpensive and rapid technique, which can be carried out at room temperature, does not require the removal of labile GHb and is not subject to common interferences (Willey et al. 1984) and so is frequently the method of choice in routine use. It is also a flexible technique that can be adapted for the measurement of glycosylated plasma proteins (Gould et al. 1984) and albumin (John & Jones 1985).
Detailed comparison of ion-exchange and affinity chromatography (Fluckiger et al. 1984) has shown that non-β-N-terminal glycosylation accounts for a significant proportion of the total GHb as measured by affinity chromatography. As ion-exchange chromatography only measures the β-N-terminal glycosylation, it does not give as complete an estimate of total glycosylation as the affinity chromatography method.

**1:7:6 Immunoassay**

A radioimmunoassay technique has been described by Javid et al. (1978) in which a sheep antiserum was raised against HbA$_{1c}$ and the antibodies to the common determinants of HbA$_{1c}$ and HbA$_0$ removed by adsorption. The majority of the antibodies, however, recognised these common determinants, so that repeated adsorption was required to remove the cross-reacting antibodies. The method has not yet been developed for general use.

Monoclonal antibodies have been generated and characterized that bind glycosylated proteins, but do not react with normal plasma proteins (Curtiss & Witztum 1983). The antibodies recognised glucitollysine, the reduced hexose alcohol form of glucose conjugated to the epsilon amino group of lysine. An immunoassay for glycosylated proteins using monoclonal antibodies could offer a number of advantages, including sensitivity, specificity and the ability to handle a large number of samples. However, antibody production is time-consuming and costly initially, and so this method has not been widely adopted for routine or research purposes.
Two other groups of workers have recently produced monoclonal antibodies with specificity for HbA$_{1c}$ and cross-reactivity studies have shown that the antibodies bind only to the haemoglobin fractions with beta-subunits that were glycosylated at the N-terminus (Knowles et al. 1986; Kruse et al. 1986).

1:7:7 The Fructosamine Assay

The 'fructosamine' method is a recently developed colorimetric test which measures all glycosylated proteins and lipoproteins and relies on the ability of ketoamines (fructosamines) to act as reducing agents in alkaline solution. A manual method was developed by Johnson et al. (1982) that would allow the measurement of up to 40 samples per hour and required only simple equipment.

The procedure adopted was as follows: serum was added to carbonate buffer containing nitroblue tetrazolium, the absorbance was measured at 10 and 15 minutes after mixing and compared with that of standards of 1-deoxy, 1-morpholinofructose plus albumin at 40 g/l treated identically.

The assay was ideal to be adapted for use in a centrifugal analyser as only one reagent addition and measurement of the absorbance change over 5 minutes, are required (Lloyd & Marples 1984). The automated method was developed on a Cobas Bio analyser (Roche Products Limited, Welwyn Garden City, UK) and enabled the measurement of 75 samples in an hour.

The technique has been shown to be a viable alternative to the measurement of GHb. Baker et al. (1984) showed that the fructosamine concentration was more sensitive to a deterioration in diabetic control than HbA$_{1c}$ measurements. Hindle et al. (1985) also used the automated method and showed a good correlation between fructosamine concentrations and HbA$_{1}$ levels.
The method estimates the quantity of ketoamines in the blood sample and so includes all glycosylated proteins and lipoproteins. This is a less specific test than the one for GHb, as all glycosylated serum constituents are included, and indeed, exactly what is being measured is still not known. It is this uncertainty, with the enormous cost of the equipment needed for the automated technique, that has prevented widespread adoption of this method.

The albumin concentration in the sample may give rise to misleading fructosamine results. Plasma albumin levels below 35g/l or above 53g/l were shown to cause significant changes in the fructosamine assay that did not reflect the true degree of glycaemia, and some form of albumin correction may therefore be necessary in such cases (Lloyd & Marples 1984).
Fast Glycosylation of Haemoglobin

Glycosylation of haemoglobin was thought to be a slow, non-enzymatic process (Bunn et al. 1976) occurring within the erythrocyte and influenced by blood glucose level. Because the glucose molecule proved so difficult to remove from the haemoglobin it was thought that the formation of GHb was irreversible. Svendsen et al. (1979) suggested that glycosylation might be both rapid and reversible. They incubated whole blood from diabetic and non-diabetic subjects in saline with or without added glucose and measured HbA\textsubscript{1a} and HbA\textsubscript{1b} and HbA\textsubscript{1c} by ion exchange chromatography. An increase in HbA\textsubscript{1c} level was found in all samples incubated with glucose, the increase being greater in the samples with lower initial HbA\textsubscript{1c} values (i.e., non-diabetic). A return to pre-incubation levels of HbA\textsubscript{1c} was noted after 17 hours of incubation in a glucose-free medium.

This evidence for fast glycosylation and dissociation was investigated by other groups of workers who suggested that the rapid increase was due to the formation of the Schiff base, or labile form (Welch 1979; Leslie et al. 1979). Welch made the observation that Svendsen and co-workers presented no evidence that the molecule formed during the incubation with glucose was stable HbA\textsubscript{1c} and suggested that it was more likely to have been the labile form.

This major criticism of the validity of HbA\textsubscript{1c} measurement has since been studied and discussed at length. Ploybutr et al. (1982) confirmed that the addition of glucose to normal or diabetic blood increased the level of HbA\textsubscript{1} in proportion to the glucose concentration. However, this rapid glycosylation was detected only by the ion-exchange chromatographic method and not by the colorimetric method.
This had previously been demonstrated in vivo (Svendsen et al. 1980) and Goldstein et al. (1980) had suggested that the labile form might be the result of short-term fluctuations in blood glucose concentrations. Similar observations regarding the rapid fluctuations of HbA1c following oral glucose tolerance tests were made by Widness et al. (1980), Shenouda et al. (1982), Scobie et al. (1981), Goebel and Fuessl (1982) and Botterman (1981). There were differences of opinion however, on the time scale of the increase; Scobie et al. reported the increase 10 days after the oral glucose tolerance test, whereas Goebel and Fuessl reported the increase after 1 hour. Maquart et al. (1978) showed a slight increase in HbA1c 5 days after the glucose load, but reaching a maximum level after 30 days. The studies of Gachon et al. (1982) demonstrated that fast deglycosylation was slower than glycosylation, and that an oral glucose tolerance test caused an increase of 10% in HbA1c values (Kitzis et al. 1982). These findings of raised HbA1c values following an OGTT must be due to the presence of the labile adduct, as one period of hyperglycaemia resulting from the oral glucose load would be unlikely to cause a rise in stable HbA1c 30 days later. The labile HbA1c, or pre-HbA1c, created as a result of an OGTT interferes with the electroendosmosis and ion-exchange chromatography results unless removed before the assay (Forrest et al. in press b).

The outcome of these studies and conflicting results has been, in general, an agreement that the blood samples should be treated in some way to remove the labile HbA1c before using the ion-exchange chromatography and electroendosmosis methods. As these rapid variations depend on the synthesis (at high glucose level) or dissociation (at low glucose level) of the labile GHb adduct, the interference caused to the final HbA1 result is unpredictable. Compagnucci et al. (1981) proposed the separate estimation of the total, stable and labile fractions of Hb. The labile fraction was removed by sample incubation in saline at 37°C for 6 hours.
Nathan et al. (1981) described a rapid chemical means for removing the labile portion, involving a 30 minute incubation of the erythrocytes in 30mM semi-carbazide and 12 mM aniline at pH 5.0, 37°C. They found this method to be as effective as a 14-hour incubation in isotonic saline.

The method of Bisse et al. (1982) made use of the instability of the Schiff base adducts in acid conditions. They carried out the haemolysis of the samples with a 15-minute incubation in potassium biphthalate buffer, pH 5, at 37°C.

Commercial methods are now available for the removal of labile GHb (Nathan et al. 1984) which have been developed by the manufacturers of a mini-column chromatographic kit and of an electrophoresis method. The first involved the use of a borate buffer, 0.6M, pH 6.7 to elute the haemoglobin, and the second involved haemolysis of the samples for 15 minutes at 38°C in a buffer containing potassium diphthalate and saponin at pH 5.0. These methods were compared with incubation of the erythrocytes in isotonic saline at room temperature for 14 hours followed by HPLC, using the ion-exchange resin. The chemical methods were as effective as the long incubation, and so are useful alternatives when ion-exchange chromatography or electrophoresis methods are used for routine assays.

1:8:2 Comparison of Techniques

Many studies have been carried out to compare the different methods for measuring GHb. Early comparisons were made between the colorimetric method and ion-exchange chromatography (Pecoraro et al. 1979). The advantages of the former method were its reproducibility when samples had been stored as haemolysates or packed erythrocytes for several months, and its capability to estimate GHb levels when variant haemoglobins, including sickle and foetal haemoglobins were present.
Since the development of the affinity chromatography technique, comparisons have been made between these three methods (Klenk et al. 1982) to investigate the effects of common interferences. Results from the affinity chromatography technique were shown to be least affected by changes in temperature, length of time over which the samples had been stored and least affected by the removal of the labile GHb fraction of the three techniques. Re-chromatography by ion-exchange chromatography and isoelectric focusing analysis of the fractions following an affinity separation revealed a substantial population of glycosylated haemoglobins not measured by the ion-exchange method. Klenk and co-workers concluded that the affinity method offered a rapid, simple and precise alternative to the ion-exchange and colorimetric method already in use that was free of many of the common interferences.

Six methods were compared by Mortensen et al. (1983): three ion-exchange chromatographic systems, the TBA method, iso-electric focusing and electroendosmosis. All assays showed a correlation to HbA_1c determined by chromatography better than 0.96 and intra-assay variations were about 2%. They concluded that the inclusion of HbA_{1a} and HbA_{1b} in the electroendosmosis measurement confused the result, and the HbA_{1c} result from the ion-exchange method was related more closely to mean blood glucose. The TBA method was shown to be too laborious to be of routine use.

A collaborative study by Boucher et al. (1983) compared four methods in seven laboratories. The measurements were made using small column separation, colorimetric analysis, isoelectric focusing and electroendosmosis. As expected, the column separation methods gave higher results than the colorimetric method, due, in part, to the presence of the labile component. None of the methods stood out as
being the most cost-effective and as no inter-laboratory standards could be identified, it was recommended that each laboratory develop its own 'in-house' quality controls and normal range.

The feasibility of references and standards were also considered by Peterson et al. (1984), who compared ion-exchange, colorimetric and affinity techniques. The micro-column ion-exchange method proved to be the most discriminating between diabetic and non-diabetic samples, but all methods gave reproducible values and correlated well with HbA\textsubscript{1c} by HPLC. Sample storage was also studied and the findings of other groups confirmed, that the colorimetric method was the least affected (Little et al. 1983a). It was therefore concluded that the colorimetric method would provide a method which could be utilized for the establishment of stored standards. Peacock (1984) reviewed the widely used methods, and agreed that generally acceptable standards for all methods were not yet available but each laboratory much establish its own.

Electroendosmosis, ion-exchange and affinity chromatography were compared by Kortlandt et al. (1985) to determine the influence of pre-HbA\textsubscript{1c}, temperature and haemoglobin concentration. All three methods showed acceptable precision and correlated well with each other, but only the affinity chromatography method was independent of the amount of pre-A\textsubscript{1c} present. The electroendosmosis method was the only technique that they found to be independent of temperature. All three methods were dependent on the haemoglobin concentration and protein content of the haemolysate. They concluded that, with careful handling, all three methods were suitable for clinical use, with the affinity method the easiest and fastest to perform.
A thorough study by Moore et al. (1986) compared isoelectric focusing, electroendosmosis, ion-exchange, affinity and colorimetric methods. Isoelectric focusing was used as the reference method, as suggested by Boucher et al. (1983) but it was too labour-intensive, lengthy and expensive for routine use. The other four methods correlated well, but as the different methods do not discern identical molecular species, it is understandable that the correlation values fell between 0.74 and 0.88. Each of the methods has its advantages, and the choice of a method depends on many factors, including number of samples anticipated and the cost of establishing a particular method (Goldstein 1986).
Clinical Application of Glycosylated Haemoglobin Measurement

Glycosylated Haemoglobin in the Diagnosis of Diabetes

GHb has been suggested as a diagnostic test for glucose intolerance in population surveys and in clinical situations (Dods et al. 1979; Boucher et al. 1981; Hall et al. 1984). Koenig et al. (1976) reported a good correlation between HbA\textsubscript{1c} levels and the area under the OGTT curve. Santiago et al. (1978) reported a correlation between HbA\textsubscript{1c} and the response to glucose loading at 1-hour and 2-hour of the OGTT. Dods and co-workers, however, showed that between 37% and 64% of the individuals in their study judged to be diabetic by the OGTT were classified as non-diabetic by the HbA\textsubscript{1} assay.

HbA\textsubscript{1} assay as a diabetes screening technique has the advantage of being insensitive to various individual factors that adversely affect the results of the OGTT. Dods et al. (1979) concluded that HbA\textsubscript{1}, used as a screening test for diabetes, necessitates the use of conservative criteria in the diagnosis of diabetes mellitus. Ferrell et al. (1984) compared GHb measurements with blood glucose levels taken at 0 hours, 1-hour and 2-hour time points of an OGTT. They found considerable overlap in the distributions of GHb in individuals with fasting blood glucose levels above and below 7.8mmol/l. Although this suggests a limited usefulness of the GHb measurement for diagnosis, the group tested had elevated random blood glucose levels, by which they had been selected following a previous test. Their results indicate that the measurement of GHb in an epidemiologic survey was useful and that, as a single criterion, it was superior to a random blood glucose measurement. They found that fasting blood glucose measurement was only slightly more reliable than GHb as a diagnostic tool.
Fasting samples are, however, much more difficult to obtain, whereas random samples, though easier to obtain, yield a high number of false positives, necessitating a second test for those individuals.

Modan et al. (1984) compared five short-cut methods of population screening for glucose intolerance. These were total $\text{HbA}_{1c}$ measured by ion-exchange chromatography, (i.e., labile $\text{HbA}_{1c}$ was not removed), fasting plasma glucose, combinations of fasting plasma glucose and $\text{HbA}_{1c}$, plasma glucose 1 hour post oral glucose load and 2 hour post oral glucose load. Their results suggest that fasting plasma glucose was more effective than $\text{HbA}_{1c}$ in screening for IGT and DM. Combinations of the two measurements did not improve prediction over fasting glucose alone. The most effective single test was a 2-hour post glucose load blood glucose level. They concluded, however, that a full OGTT, with blood glucose measurement made at the 0-hour, 1-hour and 2-hour time-points was the best method for screening for both IGT and DM.

Hall et al. (1984) measured total and stable GHb and glycosylated plasma proteins using the affinity chromatography method on patients referred for an OGTT. Significant correlations were found with fasting blood glucose or 2-hour glucose and the area under the glucose tolerance curve, but no significant correlations were found with labile (i.e., total minus stable) GHb levels. No overlap was found in the GHb levels between non-diabetic, IGT and DM subjects (as diagnosed by the WHO criteria).

More recently, Lester et al. (1985) compared the standard WHO 75 g OGTT and total GHb in 168 subjects who had been referred for the diagnosis of DM. When divided into normal, impaired and diabetic responses to the OGTT,
considerable overlap was found in the GHb values. A significant correlation was found between the total areas under the glucose tolerance test curve and GHb. A significant difference was found between the fasting and 2-hour GHb values in subjects with diabetic response, but as the electrophoretic method had been used, this would be expected, as labile material is included in this measurement unless removed prior to measurement.

GHb has also been assessed as an alternative marker for diabetes by Albett et al. (1985). 535 patients who had been referred for diagnosis of diabetes, were given a standard OGTT. They found a significant correlation between HbA1c and fasting blood glucose, but also considerable overlap between the HbA1c values in the three groups (normal, IGT and DM). The results showed that many patients with IGT or newly developed DM and HbA1c values within the normal range of their study. They concluded that a full OGTT was still the method of choice for definition of glucose tolerance, except where the patient has marked chronic hyperglycaemia.

Jovanovic et al. (1981) found that raised HbA1c levels were highly specific for the detection of diabetes, but less sensitive than the OGTT. These conclusions were confirmed by Mayer & Freedman (1983). Verrillo et al. (1983) suggested that GHb might be a useful test to improve the specificity of the OGTT to select and follow-up subjects with impaired tolerance.

1:9:2 Glycosylated Haemoglobin and the Monitoring of Metabolic Control

As early as 1962 Huisman & Dozy reported the presence of elevated levels of HbA1c in patients with diabetes but did not recognize this as a significant finding. After the finding of a 'diabetic haemoglobin component' by Rahbar (1968), Trivelli et al. (1971) published the first set of figures in diabetic patients. With their
macrocolumn technique they found a reference range for HbA$_{1c}$ of 5.0-8.0% and values of up to 15% in adult diabetics. High levels of HbA$_{1c}$ in poorly controlled diabetic patients decreased after effective therapy: 6.8-12.1% before therapy; 4.2-7.6% after three months' intensive treatment (Koenig et al. 1976). These results initiated many studies regarding the clinical application of the measurement of GHb in the management of diabetes mellitus (Fraser et al. 1979; Turner 1985).

Gabbay et al. (1977) found a good correlation between the actual HbA$_{1c}$ level and the urinary glucose measured two months earlier. Subsequently, Casparie & Miedema (1977) found very good correlations between HbA$_{1c}$ and clinical impression and mean blood glucose level over the previous weeks. However, good correlation with urinary glucose level could not be confirmed.

These findings have been confirmed in several studies during the last few years, where a good correlation between HbA$_{1c}$ and mean blood glucose was uniformly confirmed (Graf et al. 1978; Distiller & Zail 1979). GHb has been shown to remain nearly constant despite transient periods of hyperglycaemia (Compagnucci et al. 1981) and is a more valuable method of assessing overall control than random blood glucose measurements (Bunn 1981a).

It was initially thought that GHb reflected overall metabolic control over a period of 3-4 months (Bunn et al. 1976) and that measurements made every 3 months would be adequate to assess control. A study of juvenile diabetic subjects attending a summer camp, however, appeared to show that HbA$_{1c}$ levels fluctuated more acutely (Dunn et al. 1979). Tornqvist et al. (1983) showed that these short-term fluctuations in HbA$_{1c}$ levels in children were due to the labile pre-HbA$_{1c}$ and found a clear correlation between pre-HbA$_{1c}$ and urinary glucose concentration.
during the 12 hours preceding blood sampling. A study of repetitive measurements in out-patients, however, showed that HbA\textsubscript{1c} values were as stable for this population as for non-diabetic subjects (Dunn \textit{et al.} 1981). Svendsen \textit{et al.} (1982) demonstrated a close relationship between HbA\textsubscript{1c} and blood glucose profiles over a 5-week period, but the curvilinear relationship suggested that the HbA\textsubscript{1c} was not a very sensitive index of glycaemic control. Most investigators have confirmed that HbA\textsubscript{1} is a useful measure of diabetic control, and that it has a role in patient education and as a care reinforcement tool (Goldstein \textit{et al.} 1982).

Paisey \textit{et al.} (1980) compared serial capillary blood glucose measurements over 24-hour periods at fortnightly intervals and compared the values with HbA\textsubscript{1} levels over three months. Strong correlations found between mean blood glucose levels over the three months and GHb measured by both ion-exchange chromatography and by the colorimetric method. However, Mehl \textit{et al.} (1983) found a poor correlation between fasting blood glucose and GHb in IDDM and NIDDM subjects, which suggest that mean blood glucose levels are more suitable for comparison with GHb.

Further studies have compared GHb with other metabolic parameters in diabetic subjects. Positive correlations were found between HbA\textsubscript{1} and triglyceride levels and mean diurnal blood glucose. A negative correlation was found between HbA\textsubscript{1} and high density lipoprotein. Several factors are responsible for significant variations in lipid metabolism: caloric intake, alcohol in the diet, physical activity and particularly insulin regime, which are also correlated to the quantity of carbohydrate ingested. Optimisation of control in diabetic subjects requires not only the normalisation of blood glucose and lipid metabolism, but also the maintenance of HDL-cholesterol as a protective factor (Odetti \textit{et al.} 1983).
The level of HbA$_1$ in ambulatory diabetic patients was compared with their physicians' ratings of the degree of control and their fasting plasma glucose levels (Gonen et al. 1977). A close correlation was found between these parameters, and in a group of patients in hospital for closer study, HbA$_1$ correlated significantly with mean fasting and mean daily glucose values and with maximum daily glucose levels.

GHb values were compared with practitioners' estimates of glucose control over the preceding 10 weeks (Nathan et al. 1984). 24% of the estimates, which were based on historical and laboratory data collected during a routine clinic visit, differed from the actual mean blood glucose levels calculated from the GHb assay. When examined individually or in aggregate, historical information, such as polyuria, nocturia, or home urine testing for glucose and laboratory information, such as fasting or random blood glucose levels, were weak predictors of actual mean concentration of blood glucose. These workers concluded that the GHb assay provided useful information about the long-term glycaemic control of the patients that was otherwise unobtainable from the other information gathered in the clinic setting.

There is now little doubt that the periodic monitoring of GHb is useful for documenting the degree of glucose control over a period of several weeks before the sample is taken. Evidence supporting the correlation between GHb levels and traditional methods of assessing glucose control is abundant (Gabbay et al. 1977; Koenig et al. 1976). GHb levels correlate with long-term metabolic control, regardless of whether such control is achieved by multiple or single insulin injections or insulin pump therapy (Agardh et al. 1985; Dahlquist et al. 1982). Thus, periodic measurement of GHb levels provides an objective assessment of glycaemic status that complements and extends information gained from other methods of evaluating glucose control and treatment regimens.
There are some circumstances in which the measurement of GHb levels are particularly useful, for instance, in the management of insulin-dependent diabetic children with poor metabolic control (Miedema & Casparie 1984), or to compare the efficacy of different modes of treatment (Mecklenburg et al. 1982).

Ditzel & Kjaergaard (1978) measured GHb levels in newly diagnosed diabetics before and after diet and insulin treatment. The average concentration of HbA$_{1c}$ was 11.4% in untreated diabetics as compared with 4.3% in healthy controls. With prolonged optimal regulation of blood glucose HbA$_{1c}$ slowly decreased to a mean concentration of 5.5%. The concentration of HbA$_{1c}$ correlated significantly with the fasting blood glucose value.

Koenig et al. (1976) reported on a group of hospitalised diabetic patients whose mean HbA$_{1c}$ levels fell from 9.8% to 5.8% following a period of improvement in their metabolic control over one to two months.

Tchobroutsky et al. (1980) reported a significant inverse correlation between HbA$_{1c}$ levels and the number of daily insulin injections, but this has since been contradicted (Boucher et al. 1980; Agardh & Tallroth 1985). A study by Dahlquist et al. (1982) of 131 IDDM subjects also showed no correlation between insulin dose or the number of insulin injections and HbA$_{1c}$ concentration. However, a low but significant positive correlation was found between duration of diabetes and HbA$_{1c}$ levels.

A study to investigate the effects of sulphonylurea therapy in well controlled NIDDM subjects, who were given placebo treatment until the fasting blood glucose values had risen significantly and were then returned to drug treatment with diet,
showed changes in $\text{HbA}_{1c}$ levels with a lag of four to six weeks (Schultz et al. 1981). Newly diagnosed NIDDM subjects were treated with diet alone, additional sulphonylurea or with basal insulin supplement, and the aim was to lower the fasting blood glucose level to below 6mmol/l (Turner 1985). The degree to which these treatments reduced $\text{HbA}_{1c}$ concentration was studied over one year. Sulphonylurea and insulin treatment lowered blood glucose and $\text{HbA}_{1c}$ levels significantly, both groups having $\text{HbA}_{1c}$ levels significantly lower than the diet treated group at one year. This group had little change in blood glucose or $\text{HbA}_{1c}$ concentrations.

Estimation of $\text{GHb}$ levels provide an important tool for clinical research in diabetes, particularly in patients whose blood glucose levels may be subject to wide fluctuations. $\text{GHb}$ measurements can be performed in the non-fasting state, and hence, may be more convenient in the management of diabetes. Comparison of different treatment regimens or patient groups are possible. As a diagnostic tool, estimation of $\text{GHb}$ may not be as precise as the OGTT (Flock et al. 1979; Miedema & Casparie 1984) but its convenience and lack of acute dependence on variables such as patient cooperation, time of day, stress and food intake, make it an invaluable clinical tool (Gabbay 1982).

1:9:3 Glycosylated Haemoglobin in Pregnancy

The measurement of $\text{GHb}$ is well established as a retrospective index of glucose control in diabetes (Goldstein et al. 1982), but it is perhaps most useful in periods of labile control, such as during pregnancy (Jovanovic & Peterson 1981). In non-diabetic women, $\text{HbA}_{1c}$ levels progressively decrease from the first to the third trimester of pregnancy (Kjaergaard & Ditzel 1979). Levels also decrease during the third trimester in diabetic women (Schwartz et al. 1976), but are higher
than those in non-diabetic pregnant women (Hall et al. 1983a). The fall in GHb levels in non-diabetic women may be due to the increase in red cell volume and decrease in fasting blood glucose concentration that occurs during pregnancy (Lind & Cheyne 1979).

Miller and co-workers (1981) compared the total HbA\textsubscript{1c} levels in early pregnancy of diabetic women who delivered infants with major congenital anomalies with levels in women who delivered infants free of anomalies. Their results revealed a significantly higher incidence of anomalies in the infants of subjects with raised HbA\textsubscript{1c} levels. They concluded that poorly controlled diabetes in early pregnancy was associated with an increased risk of major structural malformations in the offspring, but as these malformations have probably been caused before the subject has come to medical attention, good metabolic control should be attained prior to conception, where possible.

Glycosylated plasma proteins in addition to GHb have been studied in maternal and cord blood samples in non-diabetic and diabetic women at delivery (Hall et al. 1983b). The maternal blood samples showed significantly higher levels of glycosylated plasma proteins, GHb and glucose concentration than cord blood in non-diabetic and diabetic women. The affinity chromatography method was used so that fetal haemoglobin would not interfere with the GHb results, which was demonstrated by their results. The lower levels of glucose and glycosylated proteins suggest that the fetus utilises glucose more rapidly than the mother.

It has been suggested that GHb levels during pregnancy can be used to predict infant birth weight, as maternal hyperglycaemia results in excess glucose crossing the placental barrier, which promotes fetal insulin production and may result in
increased birth weight (Cohen 1986a). However, there are conflicting results on this hypothesis, possibly due to the fact that macrosomia develops by the 32nd week of gestation in women who have been hyperglycaemic for several weeks. If the glycaemic control normalised after this time, GHB levels would be lowered by delivery, whereas the infant would still be large for its gestational age, as a result of the previous hyperglycaemia (Jovanovic & Peterson 1981).

Attempts to use HbA\textsubscript{1c} levels during pregnancy for diagnostic or screening purposes to detect gestational diabetes have not been successful, due to the overlap of the ranges of results (Miller et al. 1979). GHB values are, however, useful in the monitoring of glycaemic control in pregnant diabetic women. Glycosylated plasma proteins may also be a useful index of glycaemia over a shorter time period than GHB, and so measurement of both these parameters would yield useful information during pregnancy.
The general consensus now subscribes to the view that sustained hyperglycaemia contributes to the pathogenesis of several complications of diabetes. During hyperglycaemia, glucose may be over-utilised by the following pathways: (i) the enzymatic conversion to sorbitol and fructose, leading to osmotic damage; and (ii) non-enzymatic attachment of glucose to free amino groups of proteins, which may alter their structural and functional properties (Wieland 1983). The sorbitol pathway will be briefly described in the following section, followed by non-enzymatic glycosylation of haemoglobin and other proteins and their role in the development of diabetic complications.

1:10:1 The Sorbitol Pathway and Diabetic Complications

In those tissues of the body which are relatively impermeable to glucose in the absence of insulin, there are substantial periods when intracellular glucose concentrations are depressed in diabetes. Following insulin therapy, there is a sudden flux of glucose into the cell. These tissues include adipose tissue, muscle and erythrocytes. In tissues which are freely permeable to glucose, there are long periods of raised intracellular glucose concentration, with short periods of reduced concentration following insulin therapy. These tissues include liver, kidney, blood vessels, retina, lens, brain and peripheral nerves, and it is amongst this group of tissues that the major complications of diabetes occur (Alberti & Press 1982).

The sorbitol pathway consists of two enzymes that catabolize the conversion of free glucose to its sugar alcohol, sorbitol, and the further conversion of sorbitol to fructose. The enzymes are, respectively, aldose reductase and sorbitol dehydrogenase. There is evidence to suggest that aldose reductase is
highly localised to certain cell types within tissues and this localisation is important for correlating the site of sugar alcohol accumulation with the site of abnormality (Gabbay 1973).

Aldose reductase has a low affinity for glucose, but in diabetes the raised intracellular concentration of glucose causes increased formation of sorbitol. Once formed, sorbitol cannot easily penetrate the cell membrane, and so the molecules are trapped, contributing to the accumulation (Gabbay 1973). The sorbitol pathway has been demonstrated in many tissues, but it is only in the lens, sciatic nerve and renal papilla that sufficiently high concentrations of sorbitol have been demonstrated which may be of osmotic importance.

In the lens, the effect of sorbitol may be an osmotic one, with increased uptake of water and concomitant influx of sodium ions and loss of potassium ions. The amino acid concentrating mechanism loses its function and the process of swelling and electrolyte imbalance continues. These changes lead to cataract formation in diabetes, and initial animal studies have shown that inhibition of the aldose reductase enzyme, which prevents accumulation of sorbitol, can prevent the osmotic damage (Gonzalez et al. 1983).

Increased peripheral nerve polyol pathway activity, resulting from hyperglycaemia and leading to abnormal nerve sorbitol and fructose accumulation, has been proposed as a pathogenic factor in diabetic neuropathy. Osmotic swelling is thought to produce functional and morphological changes in diabetic nerve and this theory has primarily been derived from animal studies. However, the concentration of sorbitol reached in nerve cells is much lower than that reached in lens tissue (Finegold et al. 1983). Winegrad et al. (1976) showed that depletion of
myo-inositol was involved in the development of neuropathy, and it has since been demonstrated that aldose reductase inhibitors prevent the accumulation of polyols and the depletion of myo-inositol, but the interrelation of these processes has not yet been explained (Finegold et al. 1983).

Recently, much interest has been shown in the prevention or reversal of neuropathy by aldose reductase inhibitors, but the evidence is still inconclusive (Yue et al. 1982a; Fagius et al. 1983). The mechanisms by which increased sorbitol levels in nerve cells contribute to the development of neuropathy is not yet fully understood, but similar changes, also prevented by aldose reductase inhibition, have been shown in the glomeruli of rats with experimental diabetes (Cohen et al. 1985). Sorbitol accumulation and myo-inositol depletion seem to have a role in the development of neuropathy, but prevention of these changes has only a limited effect on vascular changes, where increased permeability can be lessened, but not the increased cross-linking of collagen in vascular tissue (Williamson et al. 1985).

1:10:2 Glycosylated Haemoglobin and Diabetic Complications

Investigation of the effects of glycosylation of haemoglobin on its affinity for oxygen have been shown to be insignificant (Samaja et al. 1982) as described in Section 1:6.

The oxygen affinity of diabetic red cells, due to the presence of increased level of GHb, is slightly greater than that of normal red cells, but this does not have any serious effect on oxygen or carbon dioxide transport (Bunn et al. 1978). Certain haemoglobinopathies produce greater shifts in the oxygen dissociation curve without deleterious effects on tissue oxygenation, which confirms the findings that the minor changes in oxygen affinity associated with increased
glycosylation have no appreciable effect on oxygen transport (Bunn 1981c). Thus, although investigations of GHbs have provided critical insights into structure-function relationships, the principal clinical contribution derived from studies of the minor haemoglobin components in diabetes remains the conclusive demonstration that their measurement can reliably assess integrated blood glucose levels over the preceding 6-8 weeks (Cohen 1986a).

In animal studies designed to address the question of the relationship between glucose control and the sequelae of diabetes, certain evidence has been conclusive. For example, Bloodworth & Engerman (1972) clearly showed that insulin therapy that normalised serum glucose in dogs eliminated retinopathy, whereas insulin therapy that did not succeed in normalizing glucose levels was associated with retinopathy and cataract formation.

In man, studies have been more controversial. Determination of GHb has allowed re-evaluation of the relationship between hyperglycaemia and complications. The red cell may serve as a model for several types of cells in the body that do not require insulin for glucose uptake; the sequelae of diabetes are associated with those tissues that are insulin-dependent (Cerami et al. 1979).

Long-term prospective clinical studies, in which GHb is monitored for years and the incidence of complications in patients with normal or elevated levels are compared, would be needed before the contribution of hyperglycaemia to the development of complications could be assessed. Short-term studies have, however, shown promising results with respect to uncovering the links between the severity and duration of hyperglycaemia and development or progression of sequelae. For example, the significant correlation between GHb and cholesterol
levels that has been found in some studies (Peterson et al. 1977) may suggest a role for hyperglycaemia in the development of heart disease. In some subsequent studies this relationship between triglyceride concentrations and GHb levels was not found (Klujber et al. 1979), but most studies have confirmed the relationship (Moore et al. 1979).

With respect to microangiopathic complications the results of one study failed to show a correlation between muscle capillary basement membrane thickness and GHb (Koenig et al. 1976) whereas another reported a decrease in quadriceps capillary basement membrane width after 8 to 10 months of optimum diabetic control and GHb concentrations (Peterson et al. 1980). After two years of treatment with continuous subcutaneous insulin infusion, GHb levels and skeletal muscle capillary basement membrane width in 13 IDDM patients were both significantly reduced compared to values obtained before initiation of the intensified insulin regimen (Raskin et al. 1983). Ten control patients in this study received conventional insulin therapy and showed no change in GHb levels or capillary basement membrane width. Capillary basement membrane thickness correlated with GHb levels in a study of 39 IDDM patients over 2½ years (Sosenko et al. 1984). An association between raised GHb and presence of retinopathy was found in a study of 149 NIDDM patients (Howard-Williams et al. 1984). The patients with retinopathy had significantly greater glycaemia, shown by higher fasting plasma glucose levels at diagnosis, larger mean values for fasting plasma glucose 1, 3 and 5 years later in addition to higher random plasma glucose and GHb 7 years later. A significant relationship was found between HbA1 levels and prevalence of diabetic retinopathy in a randomly selected group of IDDM and NIDDM subjects (Monson et al. 1986). The Kroc Collaborative Study Group, however, found an initial worsening of retinopathy in patients with IDDM treated
with continuous subcutaneous insulin infusion for eight months (Kroc 1984). These results suggested that a longer-term trial may be needed to study retinopathy changes, as pre-trial elevated AERs fell in the group treated with continuous infusion. A group of NIDDM subjects demonstrated a close relationship between GHb levels and the presence of retinopathy (Nathan et al. 1986) and although the study was cross-sectional in design, they were also able to show that duration was another important, independent factor.

A large-scale detailed screening study involving over 500 diabetic subjects (IDDM and NIDDM) revealed a strong association between GHb and presence of retinopathy (Nielsen & Dietzel 1985). No relationship was found between GHb and macrovascular disease, and the results emphasized the suggestion that the development of microvascular disease depends on the quality of blood glucose control whereas that of macrovascular disease appears unrelated to chronic hyperglycaemia.

Since Tchobroutsky wrote his review on the association of glycaemic control and the development of microvascular complications in 1978, techniques such as home blood glucose monitoring, GHb measurements and insulin pump treatment have become widely available. In a recent review of the findings of many studies over the intervening years, Hanssen et al. (1986) concluded that glycaemic control was an important factor for the development and progression of microangiopathy. Monitoring the GHb levels at regular intervals may therefore be a crucial factor in the attempt to retard the progression of complications.
It is clear that more analyses are needed, which focus on specific clinical parameters and levels of glycosylation (or advanced glycosylation end products; see Section 1:11) over time in individual tissues. Unfortunately, the limited amounts of types of tissues that can be obtained by biopsy from living subjects present obstacles to these kind of studies. Skin, for example, is one tissue that can be sampled relatively easily, and Lyons & Kennedy (1985) found a correlation between GHb and glycosylation of skin tissue.

Measurement of glycosylated proteins in the tissues of the body, particularly those involved in the complications of diabetes, should help to elucidate the processes involved in their development. The same chemical reactions that are involved in glycosylation of haemoglobin cause similar modifications in other proteins.
Non-enzymatic glycosylation begins in all cases with the attachment of glucose to the groups of protein via nucleophilic addition, resulting in the formation of a Schiff base. The labile adduct rapidly reaches equilibrium in vivo reflecting ambient glucose levels.

Over a period of weeks a slow chemical rearrangement takes place, resulting in the formation of a stable but chemically reversible product (Higgins & Bunn 1981). The Amadori products do not accumulate indefinitely even on long-lived proteins, because equilibrium is reached after a few weeks. Once equilibrium has been attained, levels of stable glycosylated protein reach a steady-state value. The quantity of Amadori product found on a protein reflects not only blood glucose concentration, but also the half-life of the protein (Cerami 1985).

The equilibrium is reached after 21-28 days, which accounts for the shorter than expected integral of ambient glucose found on haemoglobin, since the red cell has a half-life of 60 days. The amount of Amadori product remains constant (after equilibrium has been reached) whether the protein has a half-life of weeks or years.

Proteins that have a much slower turn-over rate than, say, haemoglobin, accumulate advanced glycosylation end-products (Brownlee et al. 1984). Crystallins, collagen, myelin and elastin, for example, form these products very slowly from the Amadori adducts, through a series of further reactions, rearrangements and dehydrations. The end-products have a characteristic yellow-brown pigmentation, fluoresce and cause cross-linking between proteins (Finot 1982). The formation of these end-products is extremely slow, but as they are chemically irreversible, there is a steady build-up throughout the life of the protein.
Recent studies by Chang and co-workers demonstrated the presence of advanced glycosylation products on albumin and globulin molecules in normal subjects (Chang et al. 1985). The substance measured was 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) which had been isolated from acid hydrolysates of bovine serum albumin and poly-L-lysine which had been incubated with glucose. Antibodies were raised to this molecule, and a radioimmunoassay developed for its measurement. The research group is continuing with this investigation, to discover if FFI is present in greater than normal quantities in proteins from ageing and diabetic subjects.

Brownlee et al. (1985) have discovered that FFI has cytotoxic potential. Furan rings have been associated with cytotoxicity, so it was thought that the advanced glycosylation end-products, containing furan, might also be cytotoxic. FFI was shown to be capable of causing cell death at concentrations as low as 0.5 mmol/l, and the percentage of lymphocytes killed increased with FFI concentrations in a dose-dependent manner. The ability of the cytochrome P-450 mono-oxygenase system to mediate the covalent binding of FFI to macromolecules was studied. In the absence of the mono-oxygenase system binding was inhibited. The results suggest that the development of diabetic complications have not only a hyperglycaemia-induced metabolic component (i.e., increased FFI) but also a genetic component, involving inherited variation in levels of P-450 enzymes.
In recent years it has become increasingly clear that non-enzymatic glycosylation, long known to food chemists as the 'browning' reaction (or Maillard reaction) that occurs in baked or stored foodstuffs (Saltmarch & Labuza 1982) has considerable relevance in diabetes. The Maillard reaction, which occurs between reducing sugars and free amino acids and proteins, occurs readily during the preparation and storage of foods. In food proteins, the reaction involving lysine is the most important nutritionally. The deoxyketosyl-lysine molecule formed in the early stage of the reaction is not available biologically. In the later stage of the reaction, which produces browning, the digestibility of the protein is decreased and other amino acids are destroyed (Finot 1982).
Lens Crystallins

The effect of non-enzymatic glycosylation on structural properties of proteins has been best studied in the lens, which was one of the first tissues examined in detail (Stevens et al. 1978). Lens crystallins were shown to aggregate and precipitate more rapidly in solutions containing glucose than in sugar-free medium. Inhibition of this aggregation by reducing agents, coupled with the increased disulphide content of cataractous lens, led to the hypothesis that glycosylation increased the susceptibility of sulphydryl groups to oxidation, inducing formation of disulphide-linked protein aggregation (Stevens et al. 1978). Subsequent studies have shown an increase in protein glycosylation in cataractous lenses in diabetic subjects (Kasai et al. 1983).

Incubation of lens protein with glucose in vitro leads to non-enzymatic glycosylation, intermolecular disulphide bond formation, and protein aggregates that diffract light. These findings mimic the state of proteins found in the diabetic lens. With longer incubation times, these non-enzymatically glycosylated proteins can undergo further re-arrangements to form yellow-brown pigments that act as molecular cross-links (Monnier & Cerami 1982). In initial studies Cerami and co-workers demonstrated that the ε-amino groups of lysine residues in bovine and rat lens crystallins became glycosylated when incubated in vitro with glucose (50mM) or glucose-6-phosphate (5mM) and that under these conditions the lens also developed an opalescence reminiscent of the cloudy cataracts of diabetes (Cerami et al. 1979). The greatest extent of glycosylation, measured as 14C hexose incorporation was found to involve α-crystallins, although carbohydrate was found associated with all the crystallins. This increase in glycosylation has been demonstrated in vivo in alloxan-induced diabetic rats (Cerami et al. 1979).
Since neutralization of a charged lysine residue can create conformational changes in α-crystallins that expose inaccessible sulphhydril groups, it is believed that non-enzymatic glycosylation exerts a similar effect, altering the tertiary structure of the protein and unmasking hidden sulphhydril groups. These exposed groups then become susceptible to oxidation and can form disulphide cross-links (Cohen 1986).

Recent detailed studies by Liang et al. (1986) have shown that α-crystallins are glycosylated to a greater degree than other crystallins (two to four times as much). The extent of glycosylation depends not only on the lysine content, which does not vary much among the crystallins, but also on the accessibility of the surface areas where lysine residues are located.

Non-enzymatic glycosylation is a normal ageing process which is enhanced in diabetic patients (Kennedy & Baynes 1984). Garlick and co-workers (Garlick et al. 1984) found that glycosylation increased with ageing in non-diabetic lenses and was two-fold higher than normal in diabetic lenses.

The accumulation of sorbitol in the lens, as a consequence of poor diabetic control, also has a role in the development of cataracts (Wieland 1983) and it is not yet clear which of these metabolic changes is the more significant (Kennedy & Baynes 1984).

1:13:2 Nerve Proteins

Increased glycosylation of myelin and myelin-associated proteins has been noted in both the peripheral and central nervous system in diabetes (Vlassara et al. 1983). The two- to five-fold increase in glycosylation of these proteins is
comparable to increases measured for plasma proteins. Proteins in sciatic nerves from alloxan-diabetic rats and in femoral nerves from alloxan-diabetic dogs were shown to have increased glycosylation compared to nerve proteins in non-diabetic animals (Vlassara et al. 1981). The measurements were made using an adaptation of the affinity chromatography technique, and a three-fold difference was found between diabetic and non-diabetic samples. The exact nature of the proteins undergoing the glycosylation in vivo was not identified, but they were believed to consist of axonal and myelin proteins. Subsequent studies by Vlassara and co-workers (Vlassara et al. 1983) have demonstrated myelin to be the principal nerve constituent undergoing excessive glycosylation. Since the involved peripheral nerve myelin is the main structural protein of the myelin sheath, the suggestion that non-enzymatic glycosylation might alter its integrity or the functional properties of the nerve, seems likely. The excessively glycosylated high molecular weight species of central nervous system myelin may represent cross-linked proteins derived from interactions as a result of advanced glycosylation end-products.

An important insight into a possible mechanism by which excess glycosylation contributes to the development of diabetic neuropathy was provided by the demonstration that macrophages specifically recognise glycosylated myelin. Vlassara and co-workers showed that this recognition led to endocytosis and intracellular accumulation of myelin, and demonstrated that this accumulation was two to three times higher in diabetic than in non-diabetic subjects (Vlassara et al. 1985).
Collagen and Basement Membrane Proteins

Collagen is the major fibrous protein of connective tissues and is a principal constituent of basement membranes. Collagenous proteins are rich in lysine and hydroxylysine, generally have a long half-life and are continuously exposed to ambient levels of glucose in the vascular compartment and extracellular fluids. Since variables such as the number of free amino groups in and the resident time of a protein determine the extent of glycosylation in vivo, collagen would be expected to be excessively glycosylated in diabetic subjects. Examination of several collagens has confirmed that this is the case, and a two- to three-fold increase has been found when non-enzymatic glycosylation of collagens from tissues of diabetic subjects were compared to control samples (Vogt et al. 1982). Type I and type II collagens and glomerular basement membrane, as well as aortic, skin and tendon collagen all undergo glycosylation (Cohen et al. 1981; Kohn & Schnider 1982).

Kohn and co-workers studied human collagen from a number of sites and found an age-related decrease in solubility and an increase in glycosylation of skin collagen, both of which were also marked in diabetes. Many of the debilities that characterise the ageing syndrome can be explained by the cross-linking of macromolecules such as collagen, and these changes have also been demonstrated in diabetes. Glycosylation of skin collagen was found to be less than that of tendon, possibly due to the greater turnover of skin collagen. Collagen alterations in diabetes were most apparent in samples from younger adult subjects, suggesting than an equilibrium had been reached in the older samples.

Tissue proteins from heart, liver and kidney samples from diabetic rats demonstrated higher non-enzymatic glycosylation (Yue et al. 1983a). This group
went on to show enhanced glycosylation in tendon collagen in diabetic rats, and showed that levels of glycosylation correlated with increased thermal stability of collagen (Yue et al. 1983b). Thermal stability of collagen normally increases with age, but this change had been brought about after only 4-8 weeks duration of diabetes in the rat.

Lyons & Kennedy (1985) clearly demonstrated enhanced skin collagen glycosylation in diabetic patients, and found a positive correlation with GHb values. However, when samples from patients with limited joint mobility were compared with those without limited mobility, no difference in levels of glycosylation of collagen were found.

Interstitial and basement membrane collagens form intermolecular cross-links that play an important role in the stability and physicochemical properties of connective tissue. The first step in the generation of these cross-links is an oxidative deamination of specific lysine and hydroxylysine residues, which enables the formation of cross-links by condensation between these reactive aldehydes of lysine and hydroxylysine and free ε-amino groups on these amino acids. Since many of these ε-amino groups have been glycosylated, it is possible that the normal cross-linking is not initiated (Cohen 1986a). Such a result would affect the packing and permeability of basement membranes and may help to explain the increased permeability of membranes to serum proteins.

However, this seems to contradict the findings of Yue et al. (1983b) of increased thermal stability in excessively glycosylated collagen. The explanation may relate to the formation of abnormal cross-links or covalent interactions, particularly in the insoluble fraction rather than in newly synthesised collagen.
Glycosylation leads to the formation of advanced glycosylation end-products, which may lead to formation of abnormal cross-links, and the reactive groups formed can trap, condense or form covalent bonds with non-glycosylated proteins such as serum albumin, IgG or lipoprotein (Brownlee et al. 1984; Brownlee et al. 1983). This may explain the increased concentration of albumin in basement membranes of patients with diabetic nephropathy (Michael & Brown 1981).

The trapped or bound proteins retain their ability to form immune complexes with appropriate antibody or antigen, suggesting a mechanism by which non-enzymatic glycosylation could initiate or perpetuate immune injury. Bassiouny and co-workers (1983) showed that glycosylated collagen induced antibody production specific to the modified sites in the collagen. However, as they glycosylated collagen in vitro and produced collagen with a glucitollysine-collagen adduct in which the ketoamine structure had been chemically reduced, rather than glycosylated collagen which would be produced in vivo, the results do not correspond to the in vivo situation. More recently, auto-antibodies were shown to be present for native albumin, but none were detected that were specific for glycosylated albumin (Gregor et al. 1986). Their results showed that there was a five-fold increase in incidence of albumin antibodies in a diabetic population. It is still not known why an endogenous substance, such as albumin, becomes antigenic, but one theory is that association with an antigenic region provided, for example, by a viral particle, might render albumin antigenic.

There is however, a characteristic deposition of albumin and IgG that has been detected with immunofluorescent techniques in the microvascular matrix of tissues from diabetic patients (Chavers et al. 1981), that has not yet been fully explained.
The significance of capillary basement membrane changes with respect to nephropathy in diabetes will be discussed in a later section.
Glycosylation of Blood Components

1:14:1 Coagulation Proteins

The amount of glucose bound to lysine residues in fibrinogen purified from the plasma of poorly controlled insulin-dependent diabetics is approximately 1.5 times that bound to fibrinogen purified from non-diabetic subjects (Lutjens et al. 1985). Since lysine is the amino donor for fibrin cross-linking and since fibrin and fibrinogen (from which the fibrinopeptides A and B are split by the action of thrombin to form fibrin) are glycosylated to a similar extent, it is possible that glycosylation of lysine residues interferes with fibrinogen and/or fibrin function or processing. One such suggested effect of the attachment of glucose would be to compromise the availability of lysine residues in fibrin to the fibrin-stabilising factor (Factor XIII) and, hence, the formation of intramolecular cross-links. However, a recent study examining the effects of in vitro glycosylation of fibrinogen did not confirm this suggestion, in that glycosylation at a level of 3.8 mol of glucose per mole of protein altered neither clotting time nor Factor XIII cross-linking of fibrinogen (Ney et al. 1985). Glycosylation of fibrin achieved by in vitro incubation with glucose results in a reduced susceptibility to degradation by the fibrinolytic enzyme, plasmin (Brownlee et al. 1983). Since lysyl residues of fibrin are specific sites of plasmin hydrolysis, excess non-enzymatic glycosylation could interfere with the degradation of deposited fibrin and lead to its accumulation in the tissues. A high level of glycosylation is apparently required to produce this effect on plasmin-mediated fibrinolysis, since the study by Ney and co-workers demonstrated that the susceptibility to plasmin degradation of fibrin formed from fibrinogen glycosylated at a level of 3.8 mol/mol was not reduced compared to that formed from native fibrinogen. However, even this level of glycosylation was sufficient to slow the rate of cleavage of fibrinogen by plasmin.
1:14:2  **Erythrocyte Membrane Proteins**

Another advance of potential pathochemical interest, was the observation that glucose was also bound by the ketoamine linkage to erythrocyte membrane proteins, and again there is a higher degree of glycosylation in red cells from diabetic patients (Miller *et al.* 1980; McMillan & Brooks 1982). There was a close correlation between lysine-bound glucose of erythrocyte membranes and that of haemoglobin, suggesting that the ketoamine linkages persist throughout the life span of the erythrocytes (Wieland 1983). The increased glycosylation may alter the permeability of the red cell. Diabetics have been shown to have slightly decreased red cell survival, perhaps owing to decreased deformability, but whether increased glycosylation contributes to these changes is unclear (Bunn 1981d).

1:4:3  **Lipoproteins**

Non-enzymatic glycosylation of low-density lipoproteins (LDL) has been implicated in the pathogenesis of macrovascular disease, which has an increased incidence in diabetes (Wieland 1983). Incubation of human serum lipoproteins with glucose *in vitro* results in the covalent binding of glucose to $\varepsilon$-amino groups of lysine residues in the apolipoproteins of LDL, VLDL (very-low-density lipoproteins) and HDL (high-density lipoproteins). Glycosylation of apolipoprotein-B of LDL purified from serum of diabetic subjects showed a two- to three-fold increase compared with non-diabetic samples (Witztum *et al.* 1982). This modification is of special interest as it is known that chemical modification of lysine amino groups of apolipoprotein-B, e.g., by acetylation, interferes with the specific receptor-mediated cellular LDL uptake and favours cholesterol accumulation (Wieland 1983). A decreased uptake of glycosylated LDL was demonstrated by Witztum and co-workers which may lead to prolonged residence of LDL in plasma, contributing to elevated plasma levels. If the life-time of glycosylated LDL in plasma is
prolonged, it may undergo denaturation or enzyme-catalyzed modifications that then target it to tissues other than those normally involved in LDL degradation. Glycosylated LDL may also be metabolised differently at crucial sites, such as the arterial wall (Witztum et al. 1982).

More recent studies using guinea pig samples (Steinbrecher & Witztum 1984) showed clear indications that glycosylation of as little as 2-5% of the lysine residues of LDL decreased LDL catabolism by 5-25% and the degree of inhibition of catabolism was linearly related to the extent of LDL glycosylation. Curtiss and Witztum (1983) showed that excess non-enzymatic glycosylation of LDL also occurs in human diabetic subjects, the overall level of glycosylation in poorly controlled patients was up to four times greater than in non-diabetic subjects.

1:14:4  Plasma Proteins

The discovery of glycosylated albumin and other plasma proteins and their elevation in diabetes (McFarland et al. 1979; Dolhofer & Wieland 1979; Guthrow et al. 1979; Yue et al. 1980; Ma et al. 1981) offered another tool for monitoring metabolic control, differing from the glycosylated haemoglobin measurements, in that they reflect fluctuations and glycaemia over a shorter period of time. McFarland and co-workers demonstrated close correlations between glycosylated serum proteins and GHb, glycosylated albumin and fasting blood glucose, and suggested that glycosylated serum protein measurements were a sensitive indicator of glycaemic control. Kennedy et al. (1979) obtained a much poorer correlation when they compared GHb with protein-bound hexose, but a clear difference was seen between diabetic and non-diabetic levels. Glycosylated serum proteins were assessed for usefulness as a measure of short-term glycaemia. Treatment aimed at improving control in poorly controlled diabetic patients resulted in a fall of 37% in
glycosylated serum protein in one week, whereas GHb decreased only 8% (Kennedy et al. 1981). These results were in agreement with a study (Day et al. 1980) comparing glycosylated serum proteins and GHb levels after changes in blood glucose levels in rats. Glycosylated albumin and serum protein levels were more sensitive to changes in glycaemic control than GHb levels, as a result of the shorter half-life of serum proteins (Dolhofer & Wieland 1980).

The importance of increased glycosylation of serum proteins was suggested to be due to the reduced solubility of the affected protein (Kennedy et al. 1982). Kennedy and co-workers demonstrated reduced solubility of glycosylated albumin, but were unable to show any changes in plasma viscosity as a result of increased glycosylation of plasma proteins.

Human serum albumin is a single-chain polypeptide of 585 residues which comprises about 60% of the plasma protein and is the major contributor to the oncotic pressure of blood.

There is now evidence implicating the non-enzymatic glycosylation of albumin in the pathophysiology of diabetic microangiopathy by methods quite distinct from those invoked for GHb. Although glycosylation of albumin does not alter its circulating half-life or catabolism, it can induce a conformational change in the protein and alter its ligand-binding properties (Shaklai et al. 1984). Hemin affinity was unaltered by glycosylation in vivo whereas the affinity of bilirubin for glycosylated albumin was about 50% of its value for the non-glycosylated form. The glycosylated form appears to be taken up more avidly than native albumin by endothelial cells (Williams et al. 1981). This process involves micropinocytic vesicles of endothelial cells, which participate in the bidirectional transport of
proteins across the capillary wall. Glycosylation of albumin and other circulating proteins in poorly controlled diabetes may increase trans-endothelial transport and contribute to the increased capillary permeability associated with diabetes. In support of this concept is the finding that the passage of glycosylated albumin through the glomerular filtration barrier is enhanced relative to that of native albumin in diabetic patients with and without microalbuminuria as well as in non-diabetic subjects (Ghiggeri et al. 1984). In diabetic subjects, the clearance of albumin correlated with the serum concentration of glycosylated albumin, and the urinary-to-serum glycosylated albumin ratio correlated inversely with albumin clearance. The reason for the preferential excretion of glycosylated albumin was suggested to be a result of the increased negative charge on the molecule (Ghiggeri et al. 1985). A recent report by Kverneland et al. (1986) demonstrated an increased anionic charge as a result of glycosylation, and suggested that this might affect its filtration by the glomerular barrier.

Glycosylated albumin, with respect to the sites of glycosylation, has been studied in detail. Candiano et al. (1984) showed that glycosylated albumin was a heterogeneous molecule, and that the addition of carbohydrates could occur at many sites. The rate of non-enzymatic glycosylation of albumin in vivo is approximately 9 times that of haemoglobin (Garlick & Mazer 1983).

A report that demonstrated that repeated intravenous injection of glycosylated plasma proteins produced glomerular basement membrane thickening in non-diabetic mice is also of interest (McVerry et al. 1980). This does not appear to be due to binding of exogenously administered glycosylated albumin to the basement membrane (Jeraj et al. 1983) although binding of endogenous albumin is known to occur (Miller & Michael 1976).
Albumin is not only the major contributor to plasma oncotic pressure, but also serves as a plasma carrier for many drugs and metabolites. In plasma less than 0.1% of non-esterified fatty acids are free in solution, the remainder circulating as a fatty acid/albumin complex. Serum albumin has a great capacity to bind fatty acids, and it has been suggested that glycosylation might interfere with this function. A study reported recently, however, showed that albumin glycosylated to a level of 1-3 mol glucose per mol albumin, had a similar affinity for binding palmitic acid (Murtiashaw & Winterhalter 1986).

Thus, the usefulness of glycosylated albumin as an assessment of medium-term blood glucose control has been established (Kemp et al. 1984), but its role in the pathogenesis of diabetic complications remains unclear. The changes that occur during the development of nephropathy, and the possible roles of glycosylated proteins, will be described.
It has been recognised for some time that renal disease is an almost invariable, though initially insidious, concomitant of diabetes. Interest in the renal aspects of diabetes was kindled by the report of Kimmelstiel & Wilson (1936) on nodular glomerulosclerosis. Subsequent studies have demonstrated that prolongation of life by means of insulin and antibiotics was often at the cost of renal disease (Churg & Dolger 1979).

The natural history of renal involvement in diabetes follows a characteristic pattern. After a long period, 10-20 years or more in IDDM, and usually less in NIDDM, structural components in the kidney are enlarged with concomitant hyperfunction. Thereafter, in a large proportion of the patients (around 30-50%) renal function starts to deteriorate, first indicated by proteinuria. The initial phase of abnormal increase in protein excretion is not detectable by the commonly used method, i.e., Albustix (Ames Limited, Stoke Poges, UK) more sensitive methods such as radioimmunoassay are required (Keen & Chlouverakis 1963).

A series of stages in the development of renal changes in diabetes has been described (Mogensen et al. 1983) and are as follows:

Stage 1 is characterized by early hyperfunction and hypertrophy, the changes may be detectable at diagnosis, before treatment begins. Urinary albumin excretion is increased, to a greater extent during exercise. Onset of treatment may partially reverse these changes.
Stage 2 develops over many years, and is characterized by morphologic lesions with no signs of clinical kidney disease. GFR is raised, albumin excretion is normal, except during exercise. When metabolic control is inadequate, albumin excretion rises. Some patients do not progress any further than this stage.

Stage 3, called incipient diabetic nephropathy, is characterised by continually raised albumin excretion, or microalbuminuria. This is greater than normal excretion, but below that measurable by Albustix. This will be defined in more detail in a later section. Albumin excretion and blood pressure may rise slowly over a number of years, and GFR is also elevated.

Stage 4 can be defined as overt diabetic nephropathy, characterised by persistent proteinuria (> 0.5g/24h). Blood pressure is raised and GFR declines.

Stage 5 is end-stage renal failure, with development of uraemia as a result of the nephropathy.

The first three stages will be considered in more detail, with particular emphasis on microalbuminuria and the possible involvement of increased protein glycosylation on its development.

1:15:1 Evolution of Diabetic Nephropathy

It is now well established that GFR is raised in early IDDM (Mogensen 1971a; Mogensen 1971b). The metabolic derangement associated with diabetes has been shown to be involved in the changes in GFR. There are, however, conflicting reports of the effects of raising the blood glucose level on GFR. Mogensen (1971c) and co-workers demonstrated that elevated blood glucose was not alone sufficient
to cause a significant increase in GFR, but a more recent study by Christiansen et al. (1986) showed a significant increase in GFR following an oral glucose load, in diabetic patients with near-normal blood glucose levels preceding the test. By contrast, a similar challenge to patients with hyperglycaemia was followed by a decline in GFR. Thus, the blood glucose values before the glucose load are of great importance to the outcome of this challenge on renal haemodynamics; an observation which explains the different results in the studies. Christiansen and co-workers concluded that the glucose-mediated elevation of GFR was, at least in part, explained by vasodilation of the renal arterioles.

Changes in the diabetic kidney occur very early in the course of the disease, and abnormalities may even be present at diagnosis. Following the onset of treatment for diabetes, these changes may be normalised (Viberti et al. 1981). There are, however, conflicting views on whether or not these early changes are a result of kidney enlargement and concomitant glomerular changes. Mogensen et al. (1983) state that the kidney may be enlarged at an early stage of diabetes, but Viberti et al. (1981) state that no glomerular changes can be seen for the first one or two years after diagnosis. Early reversible kidney enlargement at diagnosis is, however, frequently demonstrated (Gatling 1985).

The second stage of the development of renal changes is characterised by the development of glomerular lesions, with raised GFR (Mogensen 1985). This stage may last many years, with some patients remaining at this stage for the rest of their lives. However, in a large number of diabetic patients, the renal changes progress slowly until microalbuminuria is a permanent finding (Keen & Viberti 1981).
Microalbuminuria can be defined as an albumin excretion level which is greater than normal but lower than Albustix-positive or clinical proteinuria, which is detectable with the Albustix at 30mg/100ml or 0.5g per 24 hours (Christiansen et al. 1985).

Studies have shown that in IDDM, a raised AER is a good predictor of the development of Albustix-positive proteinuria and renal failure (Viberti et al. 1982; Parving et al. 1982; Mathiesen et al. 1984; Mogensen & Christensen 1984). Two other studies have demonstrated that a raised AER predicts morbidity and mortality in NIDDM patients (Jarrett et al. 1984; Mogensen 1984a). However, each of the groups carrying out these studies has used different conditions for measuring AER and different terminology.

A summary of the urine collections used and definitions of microalbuminuria from the various groups of workers are shown in Table 1:4. The level of AER that was found to predict clinical proteinuria differed between the groups. The Steno Memorial Hospital Group (Parving, Mathiesen and co-workers) reported a value of 28μg/min as the upper limit of their normal range, above which subjects were more likely to develop proteinuria (Parving et al. 1982). A later study showed that a value of 70μg/min predicted proteinuria (Mathiesen et al. 1984) both studies used a 24-hour urine sample. The Aarhus group (Mogensen and co-workers) defined 15μg/min on a short-term urine sample at rest, as the predictive value in IDDM subjects (Mogensen & Christensen 1984), but defined 30μg/min on an early morning sample, as the predictive value in NIDDM subjects (Mogensen 1984). The Guy's Hospital group (Viberti, Jarrett and co-workers) showed that a level of 30μg/min on a timed overnight sample was predictive of
## Definitions of Microalbuminuria by Different Groups

<table>
<thead>
<tr>
<th>Study</th>
<th>Urine Sample Collected</th>
<th>At Risk Level of AER</th>
<th>IDDM or NIDDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parving et al. 1982</td>
<td>24-hour 28µg/min</td>
<td>IDDM</td>
<td></td>
</tr>
<tr>
<td>Viberti et al. 1982</td>
<td>Timed overnight 30µg/min</td>
<td>IDDM</td>
<td></td>
</tr>
<tr>
<td>Mathiesen et al. 1984</td>
<td>24-hour 70µg/min</td>
<td>IDDM</td>
<td></td>
</tr>
<tr>
<td>Mogensen and Christiansen 1984</td>
<td>Short-term at rest 15µg/min</td>
<td>IDDM</td>
<td></td>
</tr>
<tr>
<td>Mogensen 1984</td>
<td>Morning sample 30µg/min</td>
<td>NIDDM</td>
<td></td>
</tr>
<tr>
<td>Jarrett et al. 1984</td>
<td>Timed overnight 30µg/min</td>
<td>NIDDM</td>
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later proteinuria in both IDDM and NIDDM subjects (Viberti et al. 1982; Jarrett et al. 1984). These differences may be due to different lengths of follow-up (6 years for the Steno group, 10 years for the Aarhus group and 14 years for the Guy’s group) and differences in sample collection and timing.

Epidemiological studies measuring the prevalence of microalbuminuria have shown that it is common among a diabetic clinic population. Mogensen (1983) found that almost 12% of the clinic population could be classed as having microalbuminuria, with a further 12% showing intermittent or persistent Albustix-positive proteinuria. Many groups have followed the development of nephropathy in patients with microalbuminuria over a number of years (Viberti & Wiseman 1983; Parving et al. 1982; Andersen et al. 1983).
The studies find that approximately 40% of IDDM patients will, in time, develop clinical proteinuria. When only those patients with microalbuminuria at the start of the study were considered, almost all had developed proteinuria at the follow-up (Mogensen & Christensen 1984; Viberti et al. 1982). A large survey of IDDM subjects by Steno group (Borch-Johnsen et al. 1985) showed that the development of proteinuria was a major life-threatening complication, as more than 80% of these subjects died, compared with less than 25% without proteinuria.

1:15:3 Development of Kidney Changes

The healthy kidney acts as an extremely good filter system. The nephron is the functional unit in the kidney, filtration taking place in the glomerulus. This is made up of a small tuft of capillaries surrounded by the epithelial cells of the Bowman's capsule. The mesangial cells form a supporting network for the capillaries and are involved in basement membrane turnover. This membrane is made up largely of glycoproteins, one of the major ones being negatively charged sialoglycoproteins.

The passage of molecules through the filtration barrier of the glomerulus depends on shape, size and electrical charge (Aronoff et al. 1981). Studies using neutral dextran molecules have shown that up to a radius of 2nm, there is no restriction to filtration, but above that, measurable restriction occurs up to a radius of 4.2nm, beyond which there is complete restriction (Brenner et al. 1978). Albumin has a radius of 3.6nm and so could, in theory, pass freely through the glomerular filtration barrier if filtration rate was based on size alone. Studies using uncharged dextran molecules, and negatively charged dextran sulphate molecules have demonstrated that the filtration of negatively charged molecules is retarded in comparison with uncharged molecules. This is thought to be due to the
negatively charged glycoproteins in the glomerular basement membrane (Brenner et al. 1978). Changes in both structure and composition of the glomerular capillary wall have been detected within a few years of the onset of IDDM, including progressive thickening of the glomerular basement membrane and reduction of the negatively charged sialoglycoprotein content (Friedman et al. 1983). Despite these early changes, the filtration properties of the glomerular capillary wall in a diabetic subject may remain quite normal for several years. A normal or slightly elevated rate of glomerular filtration occurs. Selectivity for neutral dextran macromolecules remains unchanged and leakage of large plasma proteins into the urine is negligible (Mogensen 1971a). However after a number of years there are important changes in the membrane properties of the glomerular capillary wall, with concomitant lowering of GFR and the development within the glomerular membrane of a small number of large pores which allow large plasma proteins to pass through.

Osterby et al. (1983) have studied glomerular basement membrane changes throughout the development of clinical nephropathy. During the early stages, up to five years duration of diabetes, there is considerable thickening of the glomerular basement membrane, developing to a 100% increase in membrane thickness when clinical nephropathy has been diagnosed. The causes of the early increase in GFR have still not been determined. The role of the increase in kidney volume and by implication glomerular filtration surface area, in the elevation of GFR has been investigated by Wiseman & Viberti (1983). They found a positive correlation between GFR and kidney volume in those diabetic patients with raised GFR, but no correlation in non-diabetic subjects or those diabetic subjects with normal GFR. They concluded that an increased kidney volume was necessary for the development of elevated GFR, but would not cause the elevation of GFR.
One model of the development of albuminuria suggests that the observed increases in mesangial volume and capillary basement membrane thickness leads to a decrease in the available glomerular filtration surface, which occurs unevenly, so that a compensatory increase occurs in those nephrons which have been spared. This results in an increase in the single nephron glomerular flow and trans-capillary hydraulic pressure, causing a loss of the selectivity function of the glomerulus leading to increased protein excretion (Mauer et al. 1983). This hypothesis is in agreement with clinical observations (Mogensen 1982; Parving et al. 1983), but it implies that clinical nephropathy only develops in patients who have advanced glomerulosclerosis. More recent studies have, however, shown that thickening of the glomerular basement membrane and mesangial expansion have been found in patients with long-standing diabetes who had no proteinuria (Thomsen et al. 1984).

Deckert and co-workers (1984) compared kidney biopsies from diabetics with no sign of clinical nephropathy with diabetics with early clinical nephropathy, and found no difference in the amount of mesangial tissue or the area of open capillaries between the two groups. They concluded that the development of albuminuria was not due to these morphological changes alone, and suggested that a change occurs in the glomerular basement membrane that is not linked with the basement membrane thickening or glomerular haemodynamics. This initiating change might be a reduction of the negative charge of the glomerular filtration barrier, causing a lowering of the charge selective properties of the glomerulus.

Haemodynamic factors such as renal plasma flow (Mogensen 1971b) and increased hydraulic pressure are most likely to contribute to the elevation of GFR (Mogensen et al. 1981), but whether or not these are more important than the morphologic alterations is still unknown.
The increased levels of albuminuria are known to be glomerular in origin in the early stages, as normal tubular function has been demonstrated by the presence of normal levels of urinary β2-microglobin (Viberti et al. 1982a). Tubular proteinuria is characterised by large increases in β2-microglobin (molecular weight 11,800 daltons, radius 1.6nm) and indicates loss of the reabsorptive capacity of the tubules, found in nephropathy.

The relative urinary concentrations of albumin and immunoglobulin (IgG) a larger and essentially neutral molecule (molecular weight 160,000 daltons, radius 5.5nm) change as the level of microalbuminuria increase. At low excretion rates the ratio is the same as in a non-diabetic subject, indicating that the selectivity of the pores for molecular size and charge is unaltered. However, as the levels of microalbuminuria rises, the level of urinary IgG does not rise at the same rate, indicating that pore size is unchanged, but that the membrane has lost some of its charge selectivity. As albumin is a polyanionic molecule, its increased filtration may be due to a loss of the fixed negative charge on the glomerular membrane (Viberti & Keen 1984). In advanced nephropathy size selectivity is also lost (Viberti et al. 1983).

Recent cytochemical studies (Vernier et al. 1986) demonstrated the presence of anionic sites in the normal human kidney, and a decrease in the number of these sites with increasing albumin excretion.

The effects of glucagon and growth hormone on glomerular hyperfiltration have been investigated by Wiseman et al. (1985). They found that diabetic subjects with raised GFR had similar plasma concentration of glucagon and growth hormone when compared with diabetic subjects with normal GFR.
Detailed studies in the streptozotocin-diabetic rat have helped to identify a number of biochemical abnormalities implicated in the accumulation of glomerular basement membrane and its increased permeability (Cohen 1986b). Three major mechanisms have been described which link hyperglycaemia with tissue damage. The first involves changes in glomerular basement membrane collagen synthesis and turnover. In vivo studies have demonstrated increased collagen synthesis associated with diabetes and decreased turnover, which may contribute to the accumulation of the glomerular basement membrane. The second mechanism involves increased non-enzymatic glycosylation of collagen, as described earlier. Excess glycosylation of basement membrane collagen may interfere with normal cross-link formation and may promote the formation of abnormal cross-links, possibly via the condensation reaction of two \( \varepsilon \)-amino bound glucose molecules (Brownlee et al. 1984). Increased levels of glycosylated proteins have been found in renal biopsies taken from diabetic patients with nephropathy (Miura et al. 1985) supports this hypothesis. Finally, changes in the polyol pathway, resulting in the intracellular accumulation of sorbitol, have also been implicated (Cohen 1986a).

Structural data have recently been obtained from kidney biopsy material from young, long-term diabetics with signs of kidney dysfunction. Osterby and co-workers (1986) studied biopsy material from patients showing a range of severity of microalbuminuria through to clinical nephropathy. The long-term diabetic samples demonstrated thickening of the peripheral basement membrane, increased mesangial volume and mesangial basement membrane-like material as a fraction of the tuft volume, when compared with short-term diabetic samples. Duration of diabetes was found to correlate positively with these structural changes.

A marked glomerular hypertrophy was found in the long-term diabetics, greater than that found in the early hyperfiltration stage. This hypertrophy in
long-term diabetics may be due to compensatory mechanisms in the open glomeruli when a certain number of glomerular tufts have become occluded and ceased functioning. With increasing duration of diabetes, a decrease of total peripheral capillary wall surface was found which correlates with GFR. Glomerular closure may be the most important event in the later stages of diabetic glomerulopathy.

Horlyck et al. (1986) showed that the phenomenon of glomerular occlusion occurs in clusters and is not randomly distributed throughout the renal cortex. When advanced glomerulopathy has developed, compensatory mechanisms take place in an attempt to retain glomerular function. Studies have shown clear-cut relationships between structure and function in relation to the changes in GFR; the early elevation and later decline closely follow the total filtration surface area (Steffes et al. 1986).

Despite these recent advances, more information is still needed on the complex interactions between the morphologic lesions leading to glomerular sclerosis and the clinical manifestations of advanced nephropathy, hypertension, falling GFR and proteinuria.
Factors Which May Affect Microalbuminuria

Blood Pressure and Microalbuminuria

It has been known for several years that raised blood pressure, or hypertension, is associated with elevated albumin excretion (Parving et al. 1974). The albumin excretion rate in patients treated effectively for hypertension was found to be significantly lower (within the normal range) than that in patients with untreated hypertension. Mogensen (1976) demonstrated that antihypertensive treatment reduced albumin excretion in long-term diabetic patients with proteinuria. The trans-capillary escape rate was found to be higher in diabetic subjects with hypertension than normotensive diabetics (O'Hare et al. 1983). These workers showed that hypertension elevated the trans-capillary escape rate independently of other factors, such as poor metabolic control and microangiopathy. Blood pressure correlated strongly with AER, and hypertension was shown to characterise those patients at risk of developing proteinuria (Wiseman et al. 1984). Hasslacher et al. (1985) confirmed these findings but concluded that metabolic control determines the early course of nephropathy, while blood pressure is more important in the later stages.

The effect of anti-hypertensive treatment on albumin excretion rate was investigated in IDDM patients with microalbuminuria, or incipient nephropathy, (Hommel et al. 1986) and clinical nephropathy (Parving et al. 1985). The patients with microalbuminuria had lowered AER during acute blood pressure reduction, which strongly suggests that haemodynamic factors are important in the development of microalbuminuria. The enhanced albumin excretion in long-term IDDMs was also shown to be pressure dependent. A simple explanation may be that reducing arterial blood pressure in turn reduces glomerular capillary pressure.
It is well documented that hyperfiltration and hyperfusion are frequently present in IDDM patients without clinical microangiopathy (Christiansen et al. 1981). GFR was within the normal range in the majority of patients with microalbuminuria (Wiseman et al. 1984), however single nephron GFR may be elevated as a result of a reduction in the number of functioning glomeruli.

Long-term antihypertensive treatment can reduce albuminuria and prevent the decline in GFR in patients with microalbuminuria (Christensen & Mogensen 1986), which confirms the findings of the other acute studies of antihypertensive therapy. All of these studies support the suggestion that elevated trans-glomerular hydrostatic pressure and increased transglomerular protein passage are important factors in the development and progression of diabetic glomerulopathy (Viberti 1983). The results support the conclusions of Hommel and co-workers (1986) that early treatment with antihypertensives may be able to retard the development of microangiopathy. This would prevent the enhanced loss of proteins through, and their deposition in, the walls of the microvasculature, that are so frequently found in diabetic individuals.

1:16:2 Metabolic Control and Microalbuminuria

Prospective studies (Viberti et al. 1982a; Parving et al. 1982) have shown that certain rates of AER above the normal range, but falling short of clinical proteinuria, in IDDM patients are highly predictive of later proteinuria. However, these studies were not designed to discover the factors responsible for the microalbuminuria. Several reports have suggested a relationship between glycaemia and urinary albumin excretion in man (Viberti et al. 1979; Viberti et al. 1981; Parving et al. 1976). A detailed study by Wiseman et al. (1984) in which blood pressure and glycaemic control were compared between IDDM subjects with
varying degrees of microalbuminuria. The groups with higher levels of microalbuminuria had significantly higher GHb values and blood pressure. Similar relationships between glycaemic control and AER have been confirmed (Rowe et al. 1984; Taylor et al. 1986) and Young and co-workers (Young et al. 1986) also demonstrated an association between glycaemic control and a number of neural and microvascular complications.

None of these studies, however, indicate whether or not the glycaemic control has a causal role in the development and progression of microalbuminuria. Viberti et al. (1983) investigated the effect of rigorous blood glucose control on AER and showed that microalbuminuria could be reversed, but clinical proteinuria remained unchanged. Short-term improvements in glycaemic control, using continuous subcutaneous insulin infusion (CSII) can substantially reduce microalbuminuria in as little as three days (Viberti et al. 1979), three weeks (Viberti et al. 1981) or four weeks (Milenyi et al. 1983). Christiansen & Parving (1984) were unable to reduce albuminuria in a group of IDDM subjects with clinical nephropathy and normal GFR after five days of strict metabolic control. Improved metabolic control for one week in short-term IDDM subjects lowered cardiac output, forearm blood flow and blood pressure significantly (Mathiesen et al. 1983). These results indicate that poor metabolic control of short duration is associated with elevated cardiac output and peripheral blood flow, which can be corrected by short-term improved glycaemic control. The early stages of microalbuminuria can be reversed, but clinical nephropathy cannot be improved by strict glycaemic control (Christiansen & Parving 1984).

Longer-term correction of metabolic control has also been investigated using CSII. Raskin et al. (1983) demonstrated that two years of meticulous control
significantly reduced the width of skeletal muscle capillary basement membrane. One year of CSII reduced GFR to normal but did not reduce kidney volume in IDDM patients with elevated GFR and enlarged kidneys (Wiseman et al. 1985). A close correlation has previously been demonstrated between these two variables (Christiansen et al. 1981) but Wiseman and co-workers were unable to explain fully why GFR should respond to improvement of glycaemic control but kidney volume did not.

There are conflicting results on the effects of long-term strict glycaemic control. Viberti et al. (1983) showed no improvement in AER in a group of patients with clinical nephropathy, whereas the Kroc Collaborative Study Group (1984) demonstrated a reduction in mean AER in a group of patients with a range of levels of initial AER. Most recently studies investigating improved glycaemic control for one year or two years, using CSII in a group of patients with microalbuminuria resulted in reduced kidney size (Feldt-Rasmussen et al. 1986) but no change in AER or GFR in either study (Hanssen et al. 1986).

The effect of improved glycaemic control is therefore equivocal and may depend on the level of albuminuria and disease duration when the improvement is initiated (Feldt-Rasmussen et al. 1986).

1:16:3 Protein Intake and Microalbuminuria

The effect of lowering the dietary protein intake on GFR and AER has recently been investigated (Viberti et al. 1986). This study suggests that diabetics with raised GFR respond to changes in glycaemia and protein intake in a different way from diabetics with normal GFR or non-diabetic subjects. Dietary protein appears to be a strong and independent influence on GFR, as reduction in protein
intake led to a prompt fall in GFR in all subjects with microalbuminuria. Lowering the protein intake (77 ± 22 g/day reduced to 47 ± 7 g/day) in a group of patients with clinical nephropathy, however, reduced AER but left GFR unchanged (Bending et al. 1986a). Blood glucose and blood pressure were the same for the groups on a normal or reduced protein intake.

The reasons for these heterogeneous responses to reduced protein intake are still unclear but improvements to the deterioration of glomerular charge and size selectivity that occur in diabetes has been suggested (Bending et al. 1986b).
Research Objectives

The aims of the research work were:

(i) To develop a sensitive immunoassay system suitable for measuring Gly-Alb in plasma and urine

(ii) To apply the technique in a study of the effect of glycosylation on the elimination of albumin

and

(iii) To investigate the application of GHb measurement as a screening tool for diabetes mellitus.
CHAPTER 2

DEVELOPMENT OF AN ASSAY SYSTEM FOR GLYCOSYLATED ALBUMIN
Introduction to Enzyme Immunoassay

Since the development of the competitive binding assay based on the use of radiolabelled ligands, as published by Yalow & Berson (1959), this technique has found widespread and diversified application. The specificity of radioimmunoassay (RIA) led to increasing acceptance and use of this methodological principle, the sensitivity of the method allows accurate quantitative measurements of many biologically active compounds. However, $^{125}$I-labelled reagents have a relatively short shelf-life, costly equipment is required for obtaining the results, and special safety measures have to be observed in the handling and disposal of the reagents.

As a result of these disadvantages, alternatives to the radio-label have been sought. Several possibilities have been investigated, including chemiluminescent and bioluminescent groups (Velan & Halmann 1978; Pazzagli et al. 1981), fluorescent groups (Soini & Hemmila 1979), metal atoms and stable free radicals (Schall & Tenoso 1981). These alternatives have all been found to have their own advantages and disadvantages, as discussed by Schall & Tenoso (1981) who concluded that no one label or method could be singled out as the best replacement for RIA.

Enzymes have also been used as labels in immunoassay systems, and their applications and limitations discussed in detail (Scharpe et al. 1976; Wisdom 1976; and Blake & Gould 1984). Enzyme immunoassay (EIA) has provided the most convenient means by which the sensitivity and specificity of RIA can be more generally applied. The reagents used are already employed in many laboratories
and are thus not associated with special hazards. The enzymes used as labels are cheap and may be stored for several months at 4°C. Once conjugated to an antibody or antigen, the labelled substance also has a shelf-life of up to a year.

There are several different types of EIA, among the most frequently used being enzyme linked immunosorbent assays (ELISA), in which the separation step of the assay is facilitated by the attachment of either a soluble antigen or antibody to an insoluble solid phase. These techniques were pioneered by Van Weemen & Schuurs (1971) and Engvall & Pearlman (1972) and can be sub-divided into three main types: competitive ELISA, sandwich ELISA for antibody or antigen, and double antibody ELISA. The methods used in the development of the assay in this study were all adaptations of a sandwich ELISA technique.

2:1:1 Sandwich ELISA for Antibody or Antigen

In the assay for antibody, antigen is adsorbed onto the solid phase. The sample containing the antibody to be measured is added, followed by the addition of a second antibody specific to the antibody to be measured. The amount of enzyme activity bound to the solid phase is therefore proportional to the amount of antibody present in the sample.

In the assay for antigen, antibody is attached to the solid phase in excess followed by incubation with the sample containing antigen to be measured. After washing, enzyme-labelled antibody is added to the solid phase. The enzyme activity bound to the solid phase is proportional to the quantity of antigen present in the sample (Clark & Adams 1977). This technique is only suitable for antigens with more than one antigenic determinant.
There have been other adaptions to the basic ELISA system and new assays are being developed all the time, but the principles and practical aspects involved remain the same. Either antibody or antigen is adsorbed on to a solid phase. This is a passive process, the speed of which can be varied by altering the temperature of this first incubation. After adsorption has taken place, the excess, unbound antibody or antigen, must be removed by thorough washing.

The solid phase can come in a variety of forms; particles of cellulose, polyacrylamide, silicone rubber, cross-linked dextrans, microcrystalline glass and plastic have all been used. These, however, all need a centrifugation step which complicates the assay.

Tubes, beads, discs and microplates have been tested, removing the need for centrifugation and of these, microplates have been found to be the most convenient. They can be made of polystyrene, polyvinyl or polypropylene. Microplates made of polyvinyl have been found to adsorb immunoglobulin more thoroughly than the polystyrene plates (Voller et al. 1979).

The coating incubation is often carried out at an alkaline pH and the concentration of protein which gives satisfactory coating is normally between 1 and 10µg/ml (Voller et al. 1979). This process can be carried out overnight at 4°C or more quickly, in one to three hours, at 20-25°C.
Once the solid phase has been coated, excess coating reagent must be removed and the microplate washed. Phosphate buffered saline (PBS) containing a detergent such as Tween 20, and a protein, such as gelatin is used. These additives block the remaining free binding sites and so reduce the background which is caused by the non-specific uptake of protein.

2:1:3 Conjugates and Conjugation Techniques

A wide variety of enzymes have been used for labelling antibodies and antigens, including alkaline phosphatase, horseradish peroxidase, β-D-galactosidase, glucose oxidase, urease, acetyl cholinesterase and glucoamylase. The first two have been most widely used because of their low cost and ease of conjugation. The reactive groups necessary for the linkage are shown in Table 2:1.

2:1:3:i Glutaraldehyde Method

The simplest way to link antibody or antigen to an enzyme is by using a cross-linking agent such as glutaraldehyde (Avrameas 1969). The cross-linking can be achieved in a one-step procedure where enzyme, antibody and glutaraldehyde are mixed together. A free NH$_2$ group is needed on each molecule to be conjugated, and the glutaraldehyde group forms a bridge between the two. As the method is so non-specific, it yields a heterogeneous conjugate mixture, resulting in a loss of antibody activity.

In order to prevent this polymerisation, a two-step method was developed for use with horseradish peroxidase. This method is based on the principle that glutaraldehyde in excess reacts with one or two free amino groups present in
<table>
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<th>Summary of the Reactive Groups Required for Conjugation Reactions on Enzyme and Antibody</th>
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<tr>
<td>i</td>
<td>GLUTARALDEHYDE -NH$_2$ + NH$_2$ -</td>
</tr>
<tr>
<td>ii</td>
<td>PERIODATE 2 vicinal -OH groups on sugar + NH$_2$ -</td>
</tr>
<tr>
<td>iii</td>
<td>DIMALEIMIDE -RSH + -RSH</td>
</tr>
<tr>
<td>iv</td>
<td>MALEIMIDE ESTER -NH$_2$ + -RSH</td>
</tr>
<tr>
<td>v</td>
<td>MIXED ANHYDRIDE -COOH + -NH$_2$</td>
</tr>
<tr>
<td>vi</td>
<td>CARBODIIMIDE -COOH + -NH$_2$</td>
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</table>
peroxidase, via only one of its two active aldehyde radicals. After removal of the excess glutaraldehyde, the enzyme, now with active aldehyde groups, is reacted with added antigen or antibody in a second reaction (Avrameas & Ternyck 1971).

Glutaraldehyde reacts mainly through the ε-amino groups of lysine residues under mild conditions and a Schiff base is formed with a five-carbon spacer group.

2:1:3:ii Periodate Method

This method is most suitable for peroxidase as it requires carbohydrate groups on the enzyme, of which peroxidase has eight per molecule (Butler & Chen 1967). The carbohydrate chains are oxidised by sodium periodate to form aldehyde groups which react with ε-amino groups on the antibody to form a Schiff base. The cross linkages are then stabilised by reduction with sodium tetraborate.

2:1:3:iii Dimaleimide Method

Dimaleimide derivatives react with thiol groups of proteins. The most commonly used of these reagents is N, N'-0-phenylenedimaleimide, which has been used with β-galactosidase (Kato et al. 1975). The protein to be conjugated, for example, immunoglobulin G (IgG), is first treated with 2-mercaptoethylamine to reduce the sulphide bond in the hinge of IgG to thiol groups, without affecting the other disulphide bonds in the molecule. The treated IgG is then combined with dimaleimide and conjugated with β-galactosidase (Ishikawa et al. 1980).

2:1:3:iv Maleimide Ester Method

This was first carried out by Kitigawa in 1976 who coupled β-galactosidase to insulin. Maleimide groups are introduced into the protein to be conjugated by
reacting with the N-hydroxysuccinimide ester of maleimidobenzoic acid, with the amino groups of the insulin. After removal of the excess reagent, β-galactosidase is added and thioester bonds form between the thiol groups of the enzyme and the double bond of the maleimide moiety.

2:1:3:vi  **Mixed Anhydride Method**

This is a simple, direct procedure that does not require the preparation and isolation of an active derivative (Erlanger et al. 1957). The protein to be conjugated is converted to an acid anhydride at a low temperature in organic solvents, which is then mixed with the pre-cooled enzymes and the anhydride groups react with the amino groups of the enzyme.

2:1:3:vi  **Carbodiimide Method**

This method has been widely used, most commonly to couple peroxidase or alkaline phosphatase to antigens or antibodies in water-soluble conditions. Carbodiimides activate carboxyl groups present in proteins, with subsequent coupling of these groups to free amino groups and the formation of a peptide bond (Goodfriend et al. 1964).
2:2 Introduction to Methods

The conjugation methods used, and the ELISA systems investigated, will be described first, followed by the results of the various methods. Discussion of the results will follow, with reasons for the various systems that were tested. The aim was to develop a specific assay for Gly-Alb using the affinity ligand amino phenyl boronic acid in place of a specific antibody for Gly-Alb, as glycosylated proteins have been shown to be poor immunogens (Brownlee et al. 1984). As APBA is specific for glycosylated proteins (Mallia et al. 1981) it was thought that a specific sensitive assay system could be developed for Gly-Alb without raising antisera to the molecule.

2:2:1 Materials

Poly-L-lysine, glyceraldehyde, horseradish peroxidase, alkaline phosphatase (from calf intestine), amino phenyl boronic acid, O-phenylenediamine, Tween 20, gelatin, p-nitrophenylphosphate, and human albumin were all obtained from Sigma Chemical Company, Poole, Dorset. Sephacryl was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sheep anti-human albumin (Batch HP/S/641 IIIA) was obtained from Guildhay Antisera, University of Surrey, Guildford. Glyco-Gel B was obtained from Pierce and Warriner (UK) Limited, Chester, UK.

All other chemicals were obtained from British Drug Houses Chemicals Limited, Poole, Dorset.

The microplate washer was obtained from Titertek, Flow Laboratories Limited, Scotland, UK. The MR600 microplate reader and PVC microplates were obtained from Dynatech Laboratories Limited, Billingshurst, West Sussex, UK.
Methods
Preparation of Reagents

The following buffers were prepared for use in the methods.

Buffer A: 0.05M sodium carbonate bicarbonate buffer, pH 9.6, containing 1.59g \( \text{Na}_2\text{CO}_3 \) and 2.93g \( \text{NaHCO}_3 \) per litre.

Buffer B: 0.01M phosphate buffered saline, pH 7.4, containing 8g NaCl, 0.2g \( \text{KH}_2\text{PO}_4 \), 2.9g NaH\(_2\)PO\(_4\), 0.2g KCl and 0.5ml Tween 20 per litre.

Buffer C: Buffer B containing 0.5g gelatin per litre.

Buffer D: Substrate buffer for alkaline phosphatase, containing 97ml diethanolamine, 100mg MgCl\(_2\cdot6\)H\(_2\)O, 0.2g NaN\(_3\) per litre. Adjusted to pH 9.8 with 1M HCl. 1mg p-nitrophenyl phosphate added.

Buffer E: Substrate buffer for horseradish peroxidase, 0.2M phosphate, pH 6.0, containing 24.3ml 0.1M citric acid (1.92g/100ml) and 25.7ml 0.2M phosphate (2.84g \( \text{Na}_2\text{HPO}_4 \)/100ml) 40mg O-phenylenediamine and 40\( \mu \)l 30% \( \text{H}_2\text{O}_2 \) added immediately prior to use.

Buffer F: 0.2M sodium carbonate, pH 9.5, containing 21.2g \( \text{Na}_2\text{CO}_3 \) and 16.8g NaH\(_2\)O\(_3\) per litre.

Buffer G: 1mM sodium acetate buffer, pH 4.4, containing 25mls sodium acetate solution (8.2g/l) and 75mls acetic acid (6.0g/l).

Buffer H: 0.01M sodium carbonate, pH 9.5, containing 1.06g \( \text{Na}_2\text{CO}_3 \) and 0.84g NaH\(_2\)O\(_3\) per litre.

Buffer I: 250mM ammonium acetate buffer, pH 8.5, containing 19.27g ammonium acetate, 10.16g magnesium carbonate per litre. Adjusted to pH 8.5 with 1N NaOH.

Buffer J: 200mM sorbitol buffer, containing 36.4g sorbitol, 14.6g sodium ethylenediaminetetraacetic acid (EDTA) and 12.11g Tris hydroxymethyl aminomethane (TRIS) per litre.
2:3 Preparation of Conjugates

2:3:1 Conjugation of Poly-L-Lysine to Aminophenylboronic Acid

10mg poly-L-lysine was dissolved in 5ml of degassed H₂O and 5mg of aminophenylboronic acid (APBA) were added. Following the method of Avrameas (1969), 100μl of glutaraldehyde were added and the mixture stirred at room temperature for three hours. The mixture was dialysed against Buffer A overnight.

A 20ml column of Sephacryl (2cm diameter) was washed with Buffer A, and the dialysed conjugate mixture allowed to flow through the gel. 1ml fractions were collected in glass tubes and each fraction scanned using a Cary Scanning Spectrophotometer (Varian Associates Limited, Walton-On Thames, UK), in quartz cuvettes between 220nm and 330nm.

The fractions containing conjugate, detected by a change in peak wavelength from the characteristic peak wavelengths of PLL and APBA were pooled (Figure 2:1) dialyzed against distilled water, freeze-dried and stored at 4°C.

2:3:2 Partial Purification of Sheep Anti-Human Albumin IgG and its Conjugation to Horseradish Peroxidase

The immunoglobulin was first partially purified by salt precipitation (Campbell et al. 1970). Saturated ammonium sulphate was prepared by dissolving solid ammonium sulphate in water heated to 50°C and then cooled. 50ml sheep anti-human albumin antisera were placed in a beaker and 25ml saturated ammonium sulphate added dropwise with continual stirring. This avoided localised high concentrations of ammonium sulphate leading to non-specific precipitation of protein.
Figure 2:1 Graph to demonstrate the change in peak wavelength following conjugation of poly-L-lysine (PLL) and aminophenylboronic acid (APBA)
After stirring for 30 minutes at room temperature, the mixture was centrifuged at 1500g (in a Beckman J6 centrifuge) for 20 minutes. The supernatant was discarded and the pellet resuspended in saturated ammonium sulphate and then centrifuged.

The pellet was resuspended in saturated ammonium sulphate and centrifuged. The IgG pellet was resuspended in distilled H₂O and then dialyzed extensively against distilled H₂O. The sheep anti-human albumin IgG was then freeze-dried and stored at 4°C.

The Conjugation Reaction

4mg horseradish peroxidase were dissolved in 1ml distilled water (Wilson & Nakane 1978) and 200μl freshly made 0.1M sodium periodate added and the mixture stirred for 20 minutes at room temperature. The mixture was dialyzed against Buffer G at 4°C.

20μl Buffer F were added and immediately 8mg of the partially purified, freeze-dried anti-human albumin IgG dissolved in 1.0ml Buffer H added. The mixture was stirred for two hours at room temperature. 100μl fresh sodium borohydride solution (4mg/ml in H₂O) were added, and allowed to stand for two hours at 4°C. An equal volume (to the conjugate mixture) of saturated ammonium sulphate was added and the mixture centrifuged at 1500g for 20 minutes. The precipitate was washed twice using 50% saturated ammonium sulphate.

Finally, the pellet was dissolved in Buffer B and dialyzed extensively against Buffer B. An equal volume (to the dialysate) of glycerol was added for storage at 4°C.
Conjugation of Alkaline Phosphatase to APBA

0.1ml alkaline phosphatase solution (containing 4nmol of enzyme) was added to 0.5ml of 1,4 dioxan in a stoppered glass tube (Erlanger 1957). 28.5μl (119μmol) of tri-n-butylamine were added, mixed, and the tube cooled in a water and ice mixture to 10°C. 7.8μl (59.7μmol) of isobutylchlorocarbonate were added quickly and mixed, the reaction mixture was then left to stand for 30 minutes at 10°C. 1.48μg (8nmol) of APBA were dissolved in 2ml deionised water and cooled to 4°C, 3 minutes before the end of the 30 minutes incubation of the enzyme mixture, 0.1ml NaOH was added to the stirred, cooled APBA solution. With fast stirring 2ml 1,4 dioxan were added dropwise to the APBA solution, followed by the enzyme mixture. The final solution was stirred at 4°C for four hours. The conjugate of alkaline phosphatase linked to APBA was dialyzed extensively against distilled water and stored at 4°C.

Conjugation of APBA to Horseradish Peroxidase (a)

Bethell et al. (1979) described a method by which APBA could be attached to an activated Sepharose (Pharmacia) matrix. This technique was investigated as a possible mode of attachment of APBA to an enzyme.

2mg horseradish peroxidase were dissolved in 0.2ml with stirring. 100μg 1,1-carbonyl diimidazole were added and stirred for 30 minutes at room temperature. 60μg of APBA in 0.1ml 1M sodium carbonate solution was added and stirred overnight at 4°C. The conjugate was dialyzed extensively against Buffer I and stored at 4°C.
Conjugation of APBA to Horseradish Peroxidase

An adaption of the periodate method of Wilson & Nakane (1978) was attempted, substituting APBA for the antibody.

4mg of horseradish peroxidase was dissolved in 1ml of distilled water, 0.2ml freshly made 0.1M sodium periodate was added and the mixture stirred for 20 minutes at room temperature. The mixture was dialyzed against Buffer G overnight. 20μl of Buffer F was added, followed immediately by 1mg of APBA in Buffer H.

The reaction mixture was stirred for two hours at room temperature, 0.1ml of freshly prepared sodium borohydride solution (4mg/ml) was added and the conjugate mixture allowed to stand for two hours at 4°C. After extensive dialysis against Buffer B the conjugate was stored at 4°C.
Measurement of Glycosylation of Anti-Human Albumin

IgG, Horseradish Peroxidase and Alkaline Phosphatase

5μg of IgG was dissolved in distilled water, and the affinity separation method described in detail in the next Chapter in Section 3:3 carried out. The absorbance at 595nm of both fractions was found and percentage glycosylation calculated. 1mg of horseradish peroxidase (or alkaline phosphatase) was taken and dissolved in 10ml of distilled water. A 1:5 dilution of this stock enzyme solution was made. 0.2ml of the diluted enzyme solution was applied to a 1ml affinity gel column with 150μl of a 1:20 dilution of haemolysate, to reduce non-specific binding of the enzyme to the gel. 7ml of Buffer I was added, followed by 3ml of Buffer J.

The wash and elution fractions were assessed for enzyme activity in the following manner:

2ml of Buffer I, 0.2mls of the wash or elution fraction, and 0.5ml substrate solution (Buffer D or E) were mixed together in a quartz cuvette. The reaction at 37°C estimated by change in absorbance, (i.e. rate of colour production) was found for wash and elution fractions using a reaction rate spectrophotometer calculator attached to the Cecil 292. The following calculation was carried out to estimate the percentage glycosylation of both enzymes.

\[
\text{Percentage glycosylation} = \frac{\Delta A \text{ elution fraction } \times 3}{\Delta A \text{ wash fraction } \times 8 + \Delta A \text{ elution fraction } \times 3} \times 100
\]

\(\Delta A = \text{change in absorbance in 1 minute}\)
### 2:5 ELISA Methods

#### 2:5:1 ELISA using Poly-L-Lysine and APBA Conjugate

Freeze-dried PLL-APBA conjugate was diluted in Buffer A at concentrations of 1-20μg/ml. Microplate wells were filled in triplicate with 200μl of these conjugate dilutions. The plate was washed with Buffer A to remove excess conjugate. Glycosylated albumin previously separated from human albumin, as described in Section 3:3, was diluted in Buffer I to give a concentration of 500μg/ml. 150μl were added to each well. A row of wells were incubated with Buffer I containing no albumin. Horseradish peroxidase anti-albumin IgG conjugate, as prepared in Section 2:3:2 was diluted in Buffer B. Doubling dilutions from 1:200 to 1:6400 were made and 150μl of the dilutions added to the wells in duplicate for each coating concentration and incubated at room temperature for three hours. The plates were washed three times with Buffer B and the wells aspirated. 150μl of substrate Buffer E were added to each well and incubated at 37°C for 30 minutes. 50μl of 2.5M H₂SO₄ were added to stop the reaction. The absorbances of the wells were read at 490nm.

The method was repeated with the following modifications: (a) further dilution of the coating conjugate; (b) addition of either gelatin or bovine gammaglobulin to Buffer I at 0.1%; (c) addition of gelatin or bovine gammaglobulin to Buffer B at 0.1%.

#### 2:5:2 ELISA using APBA-Alkaline Phosphatase Conjugate

Anti-human albumin IgG was dissolved in Buffer A (25μg/ml) and incubated for 3 hours at room temperature in polyvinyl chloride (PVC) microplates.
The wells were washed with Buffer C, human albumin was dissolved in Buffer C (5-100μg/ml) and 200 μl aliquots of each concentration incubated in the wells for two hours at room temperature. Ten wells were incubated with Buffer C containing no human albumin. The wells were washed with Buffer C, and then 200μl aliquots of APBA-alkaline phosphatase conjugate in Buffer C at doubling dilutions (1:500 -1:4000) incubated in duplicate, overnight at 4°C. The wells were washed with Buffer C, and 200μl of Buffer D were incubated for 30 minutes at 37°C. The reaction was stopped with the addition of 50μl of 3M NaOH, and the absorbance measured at 405nm.

The method was repeated using the following modifications: (a) lower and higher coating concentrations and (b) lower and higher APBA-alkaline phosphatase conjugate concentrations.

2.5:3 ELISA using the APBA-horseradish Peroxidase Conjugate, Prepared in Section 2:3:4

Anti-human albumin IgG was dissolved in Buffer A (10, 50 and 100μg/ml), and 150μl aliquots incubated in microplate wells overnight at 4°C. The wells were washed with Buffer C. Glycosylated albumin, diluted in Buffer C (20-500μg/ml) was added to the wells and incubated at room temperature for two hours. The wells were washed in Buffer C, then the APBA-horseradish peroxidase (prepared in Section 2:3:4) was added to the wells, diluted in Buffer C (1:100, 1:200 and 1:500 dilutions). The enzyme conjugate was incubated at room temperature for three hours. After washing with Buffer C, substrate was added to the wells (Buffer E) and incubated at 37°C for 30 minutes. 50μl of 2.5M H₂SO₄ were added to the wells to stop the reaction and the absorbances read at 490nm.
The method was repeated to investigate several modifications: (a) further dilution of glycosylated albumin, (b) further dilution of enzyme conjugate, (c) the coating incubation was carried out for three hours at 4°C and (d) addition of an extra incubation step. This consisted of incubating normal sheep antiserum in Buffer C (500 and 100μg/ml) in the wells at 37°C for two hours after excess coating conjugate had been washed off (Ishikawa et al. 1980).

2:5:4 ELISA using APBA-horseradish Peroxidase Conjugate Prepared in Section 2:3:5

Microplate wells were coated with anti-albumin IgG diluted in Buffer A (10, 50 and 100μg/ml) and incubated at 4°C for three hours, or overnight. The wells were washed with Buffer C, and then incubated with glycosylated albumin diluted in Buffer C (50-500μg/ml), the microplates were then incubated at room temperature for two hours. After washing with Buffer C, peroxidase labelled APBA (as prepared in Section 2:3:5) was added to the wells. 1:200 and 1:500 dilutions were made in Buffer C, and incubated for three hours at room temperature or overnight at 4°C. The microplate wells were washed with Buffer C, and substrate (Buffer E) added. 50μl of 2.5M H₂SO₄ were added to each well and the absorbances read at 490nm. An extra step was also tested in this system. Sheep antiserum IgG was diluted in Buffer C (100 and 500μg/ml) and incubated for two hours at 27°C after the plates had been coated and washed.
Results


Anti-human albumin IgG was shown to be 6.8% glycosylated; horseradish peroxidase, approximately 5% glycosylated, and alkaline phosphatase, less than 0.1% glycosylated.

2:6:2 APBA Poly-L-Lysine Conjugate Coated Plates

The absorbance values for this system showed no response to altering the concentration of any of the components of the method. Even the lowest concentrations of coating conjugate, antigen and antibody-enzyme conjugate combined yielded absorbance values greater than 2 (off the scale of the microplate reader). The addition of gelatin or bovine γ-globulin did not cause any improvement in the results and absorbances of greater than 2 were still produced.

2:6:3 APBA Alkaline Phosphatase Conjugate Used as Enzyme Label

No standard curves could be constructed from the results of this method, as changes in conjugate or coating concentrations resulted in only minor changes in absorbance values. Thus, approximately horizontal lines would have resulted. A higher coating concentration of conjugate in the wells resulted in lower absorbance values.

2:6:4 APBA Horseradish Peroxidase Conjugate Used as Enzyme Label (Produced by the Method of Bethel et al., 1979)

The blank wells, i.e. wells to which no antigen (albumin) had been added, gave higher absorbance values than the test wells. Different timing schemes and
combinations of concentrations of the various constituents of the technique did not improve the results sufficiently.

As there was no significant response to different coating concentrations and dilutions of antigen no standard curves could be plotted. The non-specific binding or test:background ratio was between 60% and 90%.

2:6:5 APBA Horseradish Peroxidase Conjugate Used as Enzyme Label (Produced by the Method of Wilson & Nakane 1978)

This method also had background (NSB) absorbance values greater than 70% of the test values. The addition of the normal sheep IgG incubation step in the wells did not reduce the non-specific binding below 70%.
The APBA moiety was investigated as a substitute for specific antibodies to glycosylated albumin, as the Gly-Alb molecule is so little different from the albumin molecule (Shaklai et al. 1984), the production of specific antisera would be difficult. Javid and co-workers (1978) raised antisera to human GHb, but they were not sufficiently avid antibodies, and the method is not now in use. The Amadori adduct has been shown to be a poor immunogen, producing low titres of low affinity antibodies (Brownlee et al. 1984).

As APBA had been shown to be specific for glycosylated proteins (Mallia et al. 1981), it was decided to develop an ELISA system using this molecule instead of one of the antibody components normally used in such a system, and to immobilise it onto a solid phase. Poly-L-lysine was used as a linking agent as it is frequently used to attach other molecules to plastics and glass. It was also thought that this conjugation would involve the amine group of the APBA molecule, thus leaving the cis-diol groups free to bind to Gly-Alb. The results indicate that this did not take place. The Gly-Alb molecule may have initially bound to the APBA, but been subsequently removed during the washing steps. The NSB was high, suggesting that the Gly-Alb, and subsequently IgG/HRP conjugate, were binding to the walls of the wells also. The addition of gelatin and bovine γ-globulin did not lower this NSB of antigen and enzyme-labelled conjugate sufficiently.

APBA was then substituted for the antibody linked to the enzyme. This method initially showed more promise, as there was some response to alterations in conjugate and antigen concentration. However, the greater absorbance values obtained with lower coating concentrations indicate that the non-specific
attachment of the enzyme conjugate to the wells was greater than the specific attachment to albumin bound to the IgG coated on the wells. Alkaline phosphatase was used initially, but as this system yielded no acceptable standard curve, horseradish peroxidase was tested instead.

Two conjugation methods were used to attach APBA to horseradish peroxidase. The first was an adaption of the method used by Bethell et al. (1978), to attach APBA to an agarose gel, the second was similar to that of Wilson & Nakane (1978) using the carbohydrate shell of horseradish peroxidase, oxidised to form aldehyde groups, to react with the amino group of the APBA molecule forming a covalent coupling.

The ELISA systems utilizing these enzyme-labelled APBA conjugates were not successful, yielding very high background binding (60-90%), and no standard curve.

When all these variations had been tested it became apparent that the affinity of APBA for Gly-Alb was weaker than that of the other components in the system for (a) the plastic well, or (b) the un-bound IgG coating the well. The affinity of APBA for Gly-Alb may be lower than that of a specific antibody for an antigen, and as a result, the bond between APBA and Gly-Alb may be broken during the washing steps. These observations led to the conclusion that APBA was not a suitable substitute for a specific antisera to Gly-Alb.

However, specific antibodies to human albumin were freely available, and so an ELISA for albumin proceeded by a separation of Gly-Alb from non-Gly-Alb was considered. The original intention had been to develop a single step assay using APBA, but a two-step assay was finally attempted as the simpler method was unsuccessful.
A straightforward sandwich-type ELISA (Voller et al. 1979) was chosen as being the most suitable system (Mohamed et al. 1984).

PVC microplates were chosen for use as they are more suitable for binding antibody (Voller et al. 1979). The diagram (Figure 2:2) illustrates the stages involved in the ELISA system. Initially, a range of concentrations for each component was tested to investigate which combination yielded the most suitable standard curve and lowest NSB.

2:8:1 Method

Sheep anti-human albumin IgG (as prepared in Section 2:3:2) was dissolved in Buffer A to give concentrations from 0.1 to 100μg/ml. The coating incubation was carried out overnight at 4°C or for three hours at 4°C. The wells were washed three times with Buffer C, containing gelatin to prevent non-specific binding to the wells (Sauer et al. 1981), and aspirated. Then human albumin was dissolved in Buffer C at concentrations from 0.19 to 1000μg/ml added to the wells, and incubated in a moist environment at room temperature. After the wells were washed three times with Buffer C, horseradish peroxidase-labelled anti-human albumin IgG (as prepared in Section 2:3:2) at concentrations from 1:100 to 1:5000 in Buffer C was added and incubated for three hours at room temperature or overnight at 4°C. After three further washes with Buffer C, 150μl of substrate (Buffer E) were incubated in the wells for 30 minutes at 37°C in the dark. Then 50μl of 2.5M H₂SO₄ were added to stop the reaction, and the absorbances read at 490nm.
Figure 2:2 Scheme to show the stages in the albumin ELISA method

Albumin in the sample or standard was mixed with excess solid-phase albumin antibodies. After washing of the solid phase, peroxidase-labelled anti-albumin was added. After further washing, the label which remained bound was measured by the addition of substrate and measurement of the colour produced.
2:8:3 Coating Concentration

The graph (Figure 2:3) shows a comparison between three concentrations of anti-human albumin IgG used for coating.

1μg/ml does not give the maximum possible absorbance values when the enzyme-labelled IgG was used at 1:1000 dilution. 4μg/ml showed a plateau effect, and could only be used with concentrations of antigen up to 25μg/ml. However, when 2μg/ml coating was used, changes in antigen concentration could be detected up to 100μg/ml.

2:8:4 Timing of the Coating Step

2μg/ml of anti-human albumin in Buffer A was incubated in the wells overnight at 4°C or for different times at 4°C. Figure 2:4 shows the standard curves for these conditions. The enzyme IgG conjugate was used at a dilution of 1:1000 throughout.

2:8:5 Timing of the Sample Incubation

Figure 2:5 shows a comparison between four different sample incubation times, from 30 minutes to two hours, all at 4°C. All other parameters were identical, a coating concentration of 2μg/ml IgG, for three hours at 4°C. The enzyme-labelled conjugate was used at a dilution of 1:1000 throughout.

The incubation for two hours resulted in the most suitable standard range and sensitivity.
Figure 2:3 Graph to show the effect of different coating concentrations of IgG

- ■ = 4μg IgG/ml
- ● = 2μg IgG/ml
- ▲ = 1μg IgG/ml
Figure 2:4 Graph to show the effect of different incubation times of the plate coating stage

- = 3 hours at 4°C
△ = 2 hours at 4°C
■ = 1 hour at 4°C
Figure 2:5 Graph to illustrate the effect of sample incubation time

- = 2 hour incubation
△ = 1½ hour incubation
○ = 1 hour incubation
■ = ½ hour incubation
Three hours at 4°C was shown to be the most suitable incubation time for this system, as the standard curve shows most sensitivity over the widest range of antigen concentrations.

2:8:6 Timing of the Enzyme-Label Incubation

Figure 2:6 shows the effects of time and temperature on the enzyme-label incubation. The overnight incubation at 4°C gave the most suitable standard curve, with the best standard range and sensitivity.

2:8:7 Summary of the Optimised ELISA for Albumin

200 µg of anti-human albumin IgG (2 µg/ml) in Buffer A were incubated in each well for three hours at 4°C. Then the wells were washed three times in Buffer C. 200 µl diluted test sample, or standard (0.19-50 µg/ml) or sample added to wells and incubated for two hours at 4°C. Again, the wells were washed three times in Buffer C. Anti-human albumin IgG-horseradish peroxidase conjugate was diluted 1:1000 in Buffer C, and 200 µl were added to each well and incubated overnight at 4°C. The wells were washed three times in Buffer C followed by the addition of 150 µl of substrate (Buffer E) and the microplate incubated for 30 minutes at 37°C. The reaction was stopped by adding 50 µl of 2.5 M H₂SO₄ and the absorbances were read at 490 nm. Figure 2:7 shows a typical standard curve obtained from this assay method.
Figure 2.6  Graph to show the effect of enzyme conjugate incubation time on the albumin ELISA standard curve.

- = overnight at 4°C
Δ = 3 hours at room temperature
○ = 2 hours at room temperature
■ = 1 hour at room temperature
Figure 2:7 Graph to show a sample standard curve for the albumin ELISA method with S.D. 

\[ n = 10 \]
2:9 Validation of the Assay

2:9:1 Loading of the Column

Plasma samples were taken and diluted before, before and after, or only after separation by affinity chromatography. A diabetic plasma sample was diluted 1:20 in distilled water. 150μl of this dilution was applied to each of two Glyco-Gel B affinity columns. The diluted plasma was taken and diluted further, 1:50 and 1:2500, giving final dilutions of 1:1000 and 1:50000, respectively, in distilled water. 150μl of each of these dilutions was applied to two affinity columns. The affinity separation was carried out as in Section 3:3. and further dilutions made as summarised in Table 2:2. The ELISA method was then used to measure albumin concentration, each sample being measured in triplicate, and the percentage glycosylation calculated as before.

The results show that the dilution step taking place before or after separation does not affect the percentage glycosylation. It has been shown by Gould et al. (1984) that a protein load of up to 2mg per 1ml column of gel does not affect the percentage glycosylated. Therefore, the loading used in this experiment was below this value.

2:9:2 Precision

'Within-run' precision was estimated by measuring one sample thirty times, on one microtitre plate. The 'between-run' precision was estimated measuring two samples in triplicate each time the assay was run.

The results can be seen in Table 2:3. The quality control data can be seen in Table 2:4.
Table 2:2

Table Showing the Effect of Loading Different Quantities of Albumin onto the Affinity Column on the Subsequent ELISA Results

<table>
<thead>
<tr>
<th>Dilution Before Separation</th>
<th>Dilution After Separation</th>
<th>Glycosylated Albumin %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:20 x 1:500</td>
<td>1:50</td>
<td>6.0</td>
<td>0.14</td>
</tr>
<tr>
<td>1:20 x 1:2500</td>
<td>None</td>
<td>6.2</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Table 2:3

Table Showing 'Between-Run' and 'Within-Run' Precision Data

<table>
<thead>
<tr>
<th>Mean albumin concentration mg/l</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between run n = 72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.80</td>
<td>0.81</td>
<td>7.5</td>
</tr>
<tr>
<td>Within run n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.65</td>
<td>0.47</td>
<td>4.5</td>
</tr>
</tbody>
</table>
**Table 2:4**  
Table Showing the Quality Control Data

<table>
<thead>
<tr>
<th>Mean Value mg albumin/l</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0.28</td>
<td>5.1</td>
</tr>
<tr>
<td>25.8</td>
<td>1.62</td>
<td>6.3</td>
</tr>
<tr>
<td>40.5</td>
<td>1.98</td>
<td>6.9</td>
</tr>
</tbody>
</table>

**Table 2:5**  
Table Demonstrating The Parallelism of the ELISA Method

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Value from graph albumin/ml mg/l</th>
<th>Calculated albumin conc. mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:125</td>
<td>9.02</td>
<td>1.12</td>
</tr>
<tr>
<td>1:250</td>
<td>4.58</td>
<td>1.14</td>
</tr>
<tr>
<td>1:500</td>
<td>2.32</td>
<td>1.16</td>
</tr>
<tr>
<td>1:1000</td>
<td>1.19</td>
<td>1.19</td>
</tr>
</tbody>
</table>

| Mean     |                                  | 1.15                          |
| SD       |                                  | 0.025                         |
| CV       |                                  | 2.17%                         |
2:9:3 Parallelism

A urine sample was taken and diluted 1:125, 1:250, 1:500 and 1:1000. Each dilution was aliquoted and its albumin concentration measured in triplicate. The results are shown in Table 2:5.

2:9:4 Sensitivity

Uncoated wells were used in the assay to estimate non-specific binding and were incubated with Buffer C, containing no albumin. The absorbance value (mean of ten samples) was 0.06.

Coated wells were incubated with Buffer C containing no albumin, giving a zero standard value. The zero standard values for ten consecutive assays and the corresponding values for a concentration of 0.2 µg/ml were found to be significantly different (P<0.0001).

2:9:5 Recovery of Albumin

A known concentration of albumin was measured in the ELISA method (2.5, 10 and 20µg/ml) in triplicate. The results are shown in Table 2:6.

2:9:6 Comparison with an Electrophoretic Method

As described in Section 3:3 albumin was measured in all the urine samples by an electrophoretic method. The correlation between the two methods was 0.99 (see Figure 2:8) (P<0.0001).
<table>
<thead>
<tr>
<th>Sample conc. Albumin µg/ml</th>
<th>Measured albumin conc. mg/l</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>2.6</td>
<td>104</td>
</tr>
<tr>
<td>10</td>
<td>9.5</td>
<td>95</td>
</tr>
<tr>
<td>20</td>
<td>19.2</td>
<td>96</td>
</tr>
</tbody>
</table>
Figure 2:8 Graph to show the relationship between the ELISA method and an electrophoretic method.

\[ n = 124, \ r = 0.99 \]
Cross-Reactivity

A standard curve was measured and a simultaneous set of standards measured which had 1μg/ml human IgG added to each standard. Figure 2:9 shows that no cross-reactivity could be detected.

Discussion

As human albumin is a large molecule (molecular weight = 69,000), it is very likely that it has several sites at which it can bind to its specific antibody. This has been shown to be true, as a sensitive, specific assay has been developed where only one specific antiserum has been used. Other workers (Mohamed et al. 1984) also developed a comparable assay, which made use of this capacity of the albumin molecule, confirming the validity of the hypothesis. The sensitivity of the assay was 0.2 mg/l, and the standard range 0.39 - 100 mg/l, and inter- and intra-assay variation were both below 8%. The parallelism of the assay was good, and recovery of albumin from the samples was approximately 100%.

When the albumin ELISA had been developed and optimized, the addition of a preceding step was made, to make possible the measurement of glycosylated albumin. The development of the complete assay is described in the following section.
Figure 2:9 Graph to show cross-reactivity with IgG

- = albumin standard curve
○ = albumin standard curve with IgG (μg/ml)
2:10 Measurement of Glycosylated Albumin Using Affinity Chromatography Followed by ELISA for Albumin

2:10:1 Method for Urine Samples

0.5 ml of urine (which had been frozen, thawed and centrifuged) were applied to a 1 ml column of affinity gel, and the method followed as in Section 3:3. The wash and elution fractions were kept separately, 100 µl of each were made up to 5 ml with Buffer C (i.e., 1:50 dilution) so that the samples were in the correct buffer for the ELISA and at a suitable concentration. 200 µl aliquots, in triplicate, of both diluted wash and elution fractions were pipetted into coated wells and albumin concentration measured as in Section 2:7:7.

Total albumin was measured in each urine sample, diluted 1:250 and 1:500 before use in the assay. If this result was out of the range of the standard curve, the sample was diluted further and the assay repeated.

Urine contains high concentrations of urea, which can interfere with immunological reactions at high levels. Thus, in a urine assay, it was necessary to assess the effect of urea on the system. Figure 2:10 shows that concentrations of urea up to 1M added to the antigen incubation had no effect on the standard curve.

2:10:2 Method of Plasma Samples

20 µl of plasma were mixed with 400 µl of distilled water and mixed well. 100 µl was taken and made up to 5 mls with distilled water. 150 µl of this second dilution was applied to a 1 ml affinity gel column and the separation carried out as in Section 3:3. In this case, however, the wash fraction was made up to 24 ml with wash buffer after separation.
Figure 2:10 Graph to show the effect of the addition of urea on the albumin ELISA standard curve

- = albumin standard curve
△ = albumin standard curve + 0.2M urea
■ = albumin standard curve + 1.0M urea
100 µl of wash and elution fraction were taken and diluted 1:50 with Buffer C. 200 µl aliquots of these dilutions were pipetted, in triplicate, into coated microplate wells, and the albumin assay carried out as in Section 2:7:7.

Nine standards, a zero standard and three quality control samples were measured in triplicate on each microplate. The absorbances were read on a Dynatech plate reader, and the values transferred to a BBC computer. A spline function programme computed a standard curve and calculated the test values.

Using these results, percentage glycosylation was calculated using the following equation:

\[
\text{Percentage} = \frac{\text{Alb E}}{(\text{Alb W} \times 8) + \text{Alb E}} \times 100\% 
\]

where Alb E = concentration of albumin in the elution fraction and Alb W = concentration of albumin in the wash fraction
An ELISA system, preceded by the affinity chromatography separation, was developed to measure Gly-Alb in urine and plasma of diabetic and non-diabetic subjects. When this assay was developed, low concentrations of urinary albumin were usually measured by radioimmunoassay (Keen & Chlouverakis 1963). Other groups developed similar radioimmunoassay methods (Miles et al. 1970; Woo et al. 1978) with improved sensitivity. A double antibody ELISA was developed by Fielding and co-workers (1983) with greater sensitivity, but a more complex technique. Thus, a simple assay, sufficiently sensitive for the measurement of urinary Gly-Alb in non-diabetics was not available when this study began.

The assay system developed was simple to perform, with good sensitivity and precision, and was specific for Gly-Alb without the need to raise antisera to Gly-Alb. The addition of the affinity separation step to the ELISA system yielded an ideal combination of specificity for Gly-Alb from the affinity method, and sensitivity from the ELISA technique.

Since the development of this technique other sensitive albumin assays have been developed, some using ELISA systems (Mohamed et al. 1984; Chesham et al. 1986) or an immunoenzymometric method (Townsend 1986) or fluorimmunoassay and radioimmunoassay (Silver et al. 1986).

A method using the affinity separation method followed by rocket immunoelectrophoresis has been developed (John & Jones 1985), but this was only used for plasma samples. Ghiggheri et al. (1984) have also recently published a method for the measurement of Gly-Alb in urine but their technique is very
complex, using affinity purification of the albumin (on Sepharose gel) followed by
isoelectrofocusing, staining and then bidimensional immunoelectrophoresis to check
for purity, followed by the TBA colorimetric assay for Gly-Alb.

In conclusion, a useful method was developed to allow the measurement of
Gly-Alb at very low levels, such as those found in non-diabetics, to enable
comparison between normal and raised albumin excretion.
CHAPTER 3

INVESTIGATION OF PROTEINS AND GLYCOSYLATED PROTEINS IN DIABETES MELLITUS
3:1 Introduction to the Study

It has been suggested that increased capillary permeability to large molecular weight plasma proteins is an early phenomenon in diabetes mellitus, that is demonstrable before tissue and organ failure develop (Keen & Viberti 1981). It appears to be related to the metabolic disturbance of diabetes and associated haemodynamic changes and affects the microvasculature of the kidney, eye, brain and many peripheral tissues, such as muscle and skin. Correction of hyperglycaemia and increased blood flow may reduce and normalise the excessive leakage (Kroc 1984).

The development of nephropathy, with the detection of increased protein excretion in the urine as a predictor of the condition, has been studied in depth by groups such as Viberti et al. (1982a), the Kroc Collaborative Study Group (1984), Mogensen & Christensen (1984).

Keen & Chlouverakis (1963) developed a sensitive radioimmunoassay for human urinary albumin on the assumption that sequential measurement of urinary albumin excretion rates would provide a sensitive detector of the onset and rate of development of diabetic kidney disease. The two main sources of proteinuria are glomerular and tubular lesions (Sweny 1983). Glomerular proteinuria results from increased permeability of the glomerulus, resulting in albumin being present in the urine at higher levels than normal. In this case the proteinuria is described as 'selective', if the urine contains larger molecules, it is termed 'non-selective'.
Tubular proteinuria occurs in a variety of conditions in which there is tubular damage or interstitial inflammation. Proteins of tubular origin are predominantly α- and β-globulins of low molecular weight.

Peterson et al. (1969) compared the excretion of β₂-microglobulin, albumin and total protein between healthy subjects and subjects with clinical proteinuria. They showed that the predominant part of plasma proteins in urine arises from a process of glomerular filtration followed by tubular reabsorption. The glomerular permeability of a substance is greatly dependent on its size. Arturson & Wallenius (1964) demonstrated that in normal subjects dextran fractions of molecular weight below 10,000 - 15,000 pass across the glomerular membrane at the same rate as water. Larger dextran molecules are retained in increasing degrees with increasing molecular weight. Dextran of molecular weight 50,000 is practically excluded from the glomerular filtrate. These studies using dextran, however, take no account of the charge on plasma protein molecules as dextran is uncharged, or of the shape of the protein, since dextran is not a globular molecule.

The findings of Peterson et al. (1969) suggest that patients with glomerular proteinuria, i.e., increased permeability to relatively large plasma proteins, had a normal or only slightly increased excretion of β₂-microglobulin, despite a greatly increased excretion of albumin and total protein. A close correlation was found between serum levels of β₂-microglobulin and creatinine, also found in patients with chronic renal failure (Bernier et al. 1968) which suggests that β₂-microglobulin is handled by the glomerular membrane in a similar way to creatinine. Viberti et al. (1983) reported elevated albumin excretion in insulin-dependent diabetes, with normal levels of β₂-microglobulin excretion. They concluded that tubular function was normal in patients without clinical proteinuria (negative Albustix < 200mg/l).
These studies suggest that albumin measurement may be of use in the early
detection of nephropathy in diabetes. As described earlier, a lower molecular
weight protein measured in the urine allows more specific diagnosis of glomerular
proteinuria.

\(\alpha_1\)-microglobulin, a low molecular weight glycoprotein, has been detected in
human serum and urine, and found to be present in much higher concentrations in
subjects with renal failure (Takagi et al. 1980b). Increased serum levels were also
found in subjects with a variety of benign and malignant diseases (Takagi et al.
1980a). \(\alpha_1\)-microglobulin has a molecular weight of 25,000-30,000 and contains
approximately 20% carbohydrate (Takagi et al. 1981). A comparison of \(\alpha_1\)-
microglobulin concentrations to the behaviour of other indicators of renal tubular
disorders, \(\beta_2\)-microglobulin, retinol-binding protein and n-acetyl-\(\beta\)-D-
glucosaminidase (all with a molecular weight of <33,000) has been made by Yu et
al. (1983). They reported elevated concentrations of \(\alpha_1\)-microglobulin in patients
with tubular dysfunction and found that this glycoprotein was also more stable at
low pH than the other parameters. Urinary \(\alpha_1\)-microglobulin measurement has
been used to assess nephrotoxicity in subjects exposed to substances such as
cadmium (Nogawa et al. 1984; Kido et al. 1985) and found to be a useful indicator
of early renal tubular damage. As plasma proteins of low molecular weight are
eliminated mainly in the kidney by reabsorption and catabolism in the tubules,
raised serum and urinary levels indicate tubular disorders. Clinically, the levels of
serum and urinary \(\alpha_1\)-microglobulin are elevated in patients with reduced GFR
and/or tubular disorders. Kusano et al. (1985) showed that increased levels of
serum and urinary \(\alpha_1\)-microglobulin were found in subjects with a range of renal
diseases and confirmed that its measurement was indeed indicative of early tubular
damage.
The measurement of this protein was therefore chosen and the assay carried out as in Section 3.3.

A number of investigations have been made using urine collections over 24 hours (Kroc Collaborative Study Group 1984) and overnight, (Viberti et al. 1984). The Steno group (Mathiesen et al. 1984) compared 1-2 hour urine collections with 24-hour samples and found high correlations between the two collection procedures. Viberti et al. (1984) concluded that the overnight collection was the most convenient for the patients and found that the albumin excretion rates were reproducible. As albumin excretion increases after exercise (Viberti et al. 1984) random urine samples taken from patients at an outpatient clinic may give falsely high levels.

A protein creatinine index, defined as milligrams of albumin per millimole of creatinine, was suggested by Shaw et al. (1983) as a means of allowing the substitution of a random urine sample to replace the 24 hour collection. However, Wilkin et al. (1983) found that this index also increased after exercise, and that there was no significant correlation between random protein creatinine index and 24-hour albumin excretion.

It has been suggested that glycosylated albumin may have a role in the pathogenesis of diabetic nephropathy (Ghiggeri et al. 1984), associated with an altered renal handling of the glycosylated molecule. As a result of these findings, it was decided to measure plasma and urinary glycosylated albumin levels in the subjects and controls.
Glomerular filtration rate has been shown to be linked with diabetic nephropathy (Mauer et al. 1983) and so GFR was estimated using creatinine clearance measurements (Campbell et al. 1984).

A study was set up to investigate the prevalence of microalbuminuria among the population of a diabetic clinic. A study was also made on the short and long-term metabolic control of the subjects, and an investigation of the relationships between GHb, plasma and urinary Gly-Alb and AER. At each clinic attendance a random blood glucose measurement was made on each diabetic patient, but GHb was only routinely measured on those subjects who were under 35 years old. Urinary glucose and total protein are measured at each visit using Diastix and Albustix (Ames Division, Miles Laboratories Limited, Stoke Poges, UK) respectively. It was decided that a more detailed study of proteinuria, metabolic control, duration of diabetes and prevalence of retinopathy would provide useful information on the diabetic population of the clinic.
3:2 Subjects

All the diabetic subjects who took part in this study regularly attended the diabetic clinic at St Luke's Hospital, Guildford, Surrey. Names and addresses of all patients due to attend the clinic three weeks in advance were obtained, and all those between the ages of sixteen and sixty-five were sent a letter with a reply slip enclosed (see Appendix 1).

Each individual who replied was contacted by telephone and the details of the samples required were explained. A litre plastic bottle for the urine sample, was sent with the covering letter (see Appendix 2) giving further details. The subjects were asked to carry out a timed overnight urine collection which they were to bring with them to their next clinic appointment. An additional 7.5ml blood sample was also taken at the clinic.

The subjects were recruited from ten consecutive clinics and the recruitment rate can be seen in Table 3:1. There was a fifty percent success rate, which enabled a large number of subjects to be recruited in a short time.

Age-matched controls were recruited from the University staff and postgraduate students by personal approach. A total of thirty-nine non-diabetic subjects also took part in the study.

Table 3:2 shows a complete list of the details obtained on all the diabetic individuals.

These details and the values from the assays carried out as described in Section 3:3:2 were all analysed using the University of Surrey Prime computer and the Minitab statistical package.
### Table 3.1: Recruitment of Diabetic Subjects

<table>
<thead>
<tr>
<th>Description</th>
<th>Number of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic patients sent initial letter</td>
<td>166</td>
</tr>
<tr>
<td>Replies received</td>
<td>91</td>
</tr>
<tr>
<td>Subjects recruited by letter</td>
<td>83</td>
</tr>
<tr>
<td>Subjects recruited by interview</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>85</strong></td>
</tr>
</tbody>
</table>

### Table 3.2: Additional Information Obtained on the Diabetic Subjects

- **Age**
- **Body Weight**
- **Duration of Diabetes**
- **Type of Diabetes**
- **Treatment**
- **Concomitant Drug Therapy**
- **Retinopathy**
Glycosylated haemoglobin quality control materials were obtained from Ortho Diagnostics Systems Inc, New Jersey, USA.

Glucose and creatinine standards and reagents for use with the Cobas Bio Centrifugal Analyser (Roche Diagnostics, Welwyn Garden City, Herts, UK) were also obtained from Roche Diagnostics. The protein reagent was obtained from Bio-Rad, California, USA. The rabbit anti-α₁-microglobulin antiserum was obtained from Dako Limited, High Wycombe, Bucks, UK. Sheep anti-human albumin antiserum was obtained from Guildhay Antisera, Division of Clinical Biochemistry, University of Surrey, Guildford, Surrey, UK. All other chemicals were obtained from British Drug Houses Limited, Poole, Dorset. All chemicals were of the highest grade or purity available.

3:3:1 Methods

3:3:1:1 Affinity Method for the Measurement of GHb

Chromatography columns containing 1ml of the affinity gel were prepared. The gel was equilibrated by washing through with 5ml of buffer I (as described in Section 2:2) 250mM ammonium acetate (wash) buffer at 20 ± 1°C.

Erythrocytes were diluted 1:20 with distilled water, mixed thoroughly, and left to haemolyse for ten minutes.

100μl of the haemolysate, or 100μl of the diluted quality control material were applied to the top of each column and allowed to soak in. 1ml of wash buffer
was added and allowed to run into the gel. A further 7ml of wash buffer were added, the eluates collected in a measuring cylinder, and the volume made up to 15ml with wash buffer.

3ml of Buffer J (as described in Section 2:2) 200mm sorbitol (elution) buffer at 20 ±1°C were added to the gel and the eluate collected separately.

The absorbances of the two fractions were then read at 414nm in a CECIL 292 spectrophotometer (Cecil Instruments Limited, Milton, Cambridge) against the appropriate buffer blanks.

The percentage GHb was calculated from the following equation:

\[
\text{GHb\%} = \frac{A_{\text{elution}}}{A_{\text{wash} \times 5 + A_{\text{elution}}}} \times 100
\]

where \( A_{\text{elution}} \) = absorbance of the elution fraction at 414 nm

where \( A_{\text{wash}} \) = absorbance of the wash fraction at 414 nm
The between assay coefficient of variation for this method was 1.8%.

3:3:1:2 **Glucose Measurements in Plasma and Urine**

Plasma and urinary glucose were determined using the Cobas Bio Centrifugal Analyser with the settings as described in Table 3:3. The method uses the hexokinase/glucose-6-phosphate dehydrogenase method (Schmidt 1961) with the production of NADPH from NADP in direct proportion to the amount of glucose present, as the end point. The glucose standards were made up in saturated benzoic acid and stored at 4°C. The standards were loaded onto the sample plate followed by the specimens, the reagent, obtained from Roche Diagnostics, was poured into the reagent well. Quantitation was by comparison of the absorbance change of the unknowns versus the standards at 340nm, 5 minutes from the start of the reaction.

3:3:1:3 **Creatinine Measurement in Plasma**

Creatinine was measured using the Jaffe reaction and based on the method of Varley (1969). The Cobas Bio centrifugal analyser was used with the settings as described in Table 3:4.

The reagent used was picric acid (35mmol) and the diluent NaOH (0.32mol). The standards were made up in 10mM HCL.

The creatinine standards were loaded onto the sample plate followed by the specimens. Quantitation was by comparison of the absorbance change of the unknown versus the standards at 500nm between 30 seconds and 2 minutes from the start of the reaction.
Table 3:3

List of the Parameters Used for the Plasma Glucose Assay as Carried Out on the Cobas Bio Analyser

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Units</td>
<td>mmol/l</td>
</tr>
<tr>
<td>2</td>
<td>Calculation factor</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Standard 1 conc</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>Standard 2 conc</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Standard 3 conc</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Limit</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>Temperature (Deg C)</td>
<td>37.0</td>
</tr>
<tr>
<td>8</td>
<td>Type of analysis</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Wavelength (nm)</td>
<td>340</td>
</tr>
<tr>
<td>10</td>
<td>Sample volume (μl)</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>Diluent volume (μl)</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>Reagent volume (μl)</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>Incubation time (sec)</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>Start reagent volume (μl)</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Time of first reading (sec)</td>
<td>.5</td>
</tr>
<tr>
<td>16</td>
<td>Time interval (sec)</td>
<td>300</td>
</tr>
<tr>
<td>17</td>
<td>Number of readings</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>Blanking mode</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>Print-out mode</td>
<td>1</td>
</tr>
</tbody>
</table>

The standard values shown are those used for plasma glucose estimation. The following standards were used for urinary glucose estimation: 2.5, 5 and 10 mmol/l.
Table 3:4

List of the Parameters Used for the Plasma Creatinine Assay as Carried Out on the Cobas Bio Analyser

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Units</td>
<td>g/dl</td>
</tr>
<tr>
<td>2</td>
<td>Calculation factor</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Standard 1 conc</td>
<td>.5</td>
</tr>
<tr>
<td>4</td>
<td>Standard 2 conc</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>Standard 3 conc</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>Limit</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>Temperature (deg. C)</td>
<td>25.0</td>
</tr>
<tr>
<td>8</td>
<td>Type of analysis</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>Wavelength (nm)</td>
<td>520</td>
</tr>
<tr>
<td>10</td>
<td>Sample volume (μl)</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>Diluent volume (μl)</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Reagent volume (μl)</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>Incubation time (secs)</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>Start reagent volume (μl)</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Time of first reading (sec)</td>
<td>30.0</td>
</tr>
<tr>
<td>16</td>
<td>Time interval (sec)</td>
<td>120</td>
</tr>
<tr>
<td>17</td>
<td>Number of readings</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>Blanking mode</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>Printout mode</td>
<td>1</td>
</tr>
</tbody>
</table>
The following assays for urinary creatinine, total protein, $\alpha_1$-microglobulin and albumin were carried out in the Clinical Chemistry Department at St Luke's Hospital, Guildford, as part of a collaborative project. It was arranged to supply samples to both laboratories for separate research studies and also to assist in the validation of the new ELISA technique.

3:3:1:4 Creatinine Measurement in Urine

The method was based on the Jaffe reaction of creatinine with alkaline picric acid to yield a red/amber colour complex which can be read between 500nm and 520nm. Although many compounds yield chromogenic complexes with alkaline picrate a two-point method overcomes the interference.

Picric acid was mixed 2:1 with deionised water, 0.5M NaOH was mixed 1:1 with diluted picric acid to make the working solution. The sample volume was 25μl, reagent volume, 200μl and diluent volume 30μl. The assay was carried out using a Centrifi Chem 500 plus centrifugal analyser and P1000 sampler (Baker Instruments Limited, Surrey) at 30°C. Quantitation was by comparison of the absorbance change of the unknown versus the standards.

3:3:1:5 Measurement of Urinary Total Protein

The urinary total protein assay used the binding of Coomassie Brilliant Blue G-250 with protein to cause a shift in the absorption maximum of the dye from 465nm to 495nm. This increase in absorbance was monitored at 495nm. The dye binding process was complete in approximately 2 minutes with good colour stability for one hour (Bradford 1976).
Samples were diluted to contain 10µg to 100µg protein in a volume up to 100µl. 5ml Bio Rad protein reagent were added and the contents mixed thoroughly. The absorbance at 595nm was measured after 2 minutes and before 1 hour against a reagent blank prepared from 0.1ml water and 5ml protein reagent. Standards, also supplied by Bio Rad Inc, were run in parallel and their values plotted against the corresponding absorbance to produce a standard curve. This was used to determine the protein content of the unknown values.

3:3:1:6 Measurement of Urinary \( \alpha_1 \)-Microglobulin

An exact volume of solution containing antigen was applied in an Agarose gel of uniform thickness containing the anti-\( \alpha_1 \)-microglobulin antiserum. An electric field induced migration of antigen and antibody molecules, which react with one another to form precipitation zones like ascending rockets. Unbound antigen within the conical head migrated into the zone of antigen-antibody precipitate and redissolved the complexes in the front. The leading precipitate zone was displaced towards one of the electrodes at a rate decreasing with that of the disappearance of free antigen. The final position of the precipitation frontier of each antigen at a given antibody concentration varied with the amount of antigen applied (Laurell 1966).

Anti-\( \alpha_1 \)-microglobulin antiserum was added to the agarose gel, and 10µl of antigen-containing solution applied to holes cut in the gel. Dilutions of the standard antigen obtained from Behringwerke (Hoechst UK Limited, Hounslow, Middlesex) were used for calibration.

The electrophoresis was run with 10 volts/cm for 2-10 hours. The height of the various peaks formed was measured directly under dark-field illumination. The peak height corresponded to the distance found between peak and the centre of the application hole.
3:3:1:7 Measurement of Urinary Albumin

A similar electrophoretic technique was used for measuring urinary albumin and was calibrated using a reference preparation of albumin (SPS-01) supplied by the Sheffield Protein Reference Unit.

3:3:2 Sample Collection

The subjects participating in the study brought the urine sample, which had been passed that morning, with them to the clinic. 7.5ml of blood were taken; 5ml collected in lithium heparin tubes and 2.5ml in fluoride oxalate tubes.

The blood samples were centrifuged for 10 minutes at 1200 g and the plasma stored in four aliquots at -40°C. The red cells were stored separately at -40°C, and plasma from the fluoride oxalate tubes was also kept and stored at -40°C.

The volume of each urine sample was measured, and three plastic universal bottles were each filled (approximate volume 20mls), sodium azide was added as a preservative (0.1%), and stored at -40°C. One universal bottle was sent to St Luke's Hospital laboratory for overnight storage at 4°C prior to measurement.
3:4 Results

3:4:1 Parameters for Diabetic Subjects: Sex Differences

Table 3:5 shows the data for the diabetic subjects (excluding those with proteinuria) split into male and female groups. Only two of the parameters measured show a significant difference as calculated by the Mann-Whitney test. These are glomerular filtration rate ($p<0.01$) and body weight ($p<0.001$) where sex differences would be expected. Thus in future treatment of the data, male and female subjects have been grouped together.

3:4:2 Age and Sex Comparison of Diabetic and Control Groups

The histogram, Figure 3:1 with the age distribution of diabetic and control groups shows that there was an age and sex matched control group for each group of diabetic subjects.

3:4:3 Parameters for Diabetic Subjects: Disease Duration

The data for the diabetic subjects were sub-divided by duration of diabetes, groups 1, 2 and 3 comprise, respectively, individuals diagnosed as diabetic less than ten, eleven to twenty and over twenty years prior to the study. The results can be seen in Appendix 3 and show no statistically significant differences between the groups as measured by the Mann-Whitney test. However, non-significant trends can be seen for the results of urinary protein measurements, i.e. AER, urinary total protein and urinary $\alpha_1$-microglobulin. Group 3 has the highest mean AER, whereas group 2 has the highest mean value for total protein and group 1 for $\alpha_1$-microglobulin. Group 3 consists entirely of IDD subjects, whereas groups 1 and 2 have lower proportions of IDD subjects.
### Table 3:5

**Diabetic Subjects: Male Compared with Female**

<table>
<thead>
<tr>
<th></th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>Albumin Excretion</td>
<td>8.9 (14.72)</td>
<td>5.64 (7.72)</td>
</tr>
<tr>
<td>rate µg/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>122.6 (66)(a)</td>
<td>86.8 (43.42)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.76 (4.43)</td>
<td>8.00 (2.51)</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>12.42 (3.85)</td>
<td>13.74 (3.31)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>9.27 (8.59)</td>
<td>10.19 (6.53)</td>
</tr>
<tr>
<td>Urinary α-1-M (mg/l)</td>
<td>5.44 (4.88)</td>
<td>4.9 (7.86)</td>
</tr>
<tr>
<td>Urinary Total Protein (g/l)</td>
<td>84.1 (62.38)</td>
<td>88.8 (65.12)</td>
</tr>
<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>14.04 (6.52)</td>
<td>15.71 (6.95)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.1 (12.80)</td>
<td>40.1 (14.47)</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>8.43 (9.12)</td>
<td>11.05 (9.01)</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>76.8 (12.4)(b)</td>
<td>65.9 (15.2)</td>
</tr>
<tr>
<td>Urinary Glucose (mmol/l)</td>
<td>10.06 (7.88)</td>
<td>9.76 (9.08)</td>
</tr>
<tr>
<td>IDDM (%)</td>
<td>70%</td>
<td>66%</td>
</tr>
<tr>
<td>NIDDM (%)</td>
<td>30%</td>
<td>33%</td>
</tr>
</tbody>
</table>

**All values shown are means with SD in parentheses**

- a represents p<0.01
- b represents p<0.001
Figure 3.1 Age distribution of diabetic and control subjects

- **CONTROL SUBJECTS**
  - MALE: 12, 8, 6, 4, 2
  - FEMALE: 12, 8, 6, 4, 2

- **DIABETIC SUBJECTS**
  - MALE: 28, 26, 24, 22, 20
  - FEMALE: 16, 14, 12, 10, 8

**Age in Years**
- 16-25
- 26-35
- 36-45
- 46-55
- 56-65
Parameters for Diabetic Subjects: IDDM and NIDDM

The data for the diabetic subjects was divided into treatment groups, with an extra group comprising those IDDM patients whose duration of diabetes fell within 1-13 years (mean ± 2SD of the duration of the NIDDM group). Body weight and age show significant differences (p<.001) between IDDM and NIDDM groups, and the data can be seen in Appendix 4.
The same differences remain when the IDD group of shorter duration is compared with the NIDD group. The results for the other parameters, i.e. AER, urinary $\alpha_1$-microglobulin and total protein and plasma and urinary glucose show differences between NIDD subjects and both the short duration and the complete IDD groups. Plasma and urinary GAib and GHb show no significant differences.

Table 3:6 shows the p values for this data. Not all of the parameters show significant differences due to the great inter-individual variation.

Parameters for Diabetic and Control Subjects: Age Differences

The data for diabetic and non-diabetic subjects was divided into five ten year age bands. Where statistically significant differences exist between diabetic and control groups compared as a whole as shown in Table 3:1, the difference remain when the groups are divided into the age ranges. AER, GHb, plasma GAib, plasma and urine glucose, urinary $\alpha_1$-microglobulin and total protein all show significant differences when the groups are sub-divided. Figures 3:2, 3:3 and 3:4 illustrate the most striking differences, for $\alpha_1$-microglobulin, urinary total protein and AER.

However, the 46-55 years old group of both diabetic individuals show lower values for AER, GHb, urinary $\alpha_1$-microglobulin and total protein, and plasma and urinary glucose levels than the age groups immediately younger and older.

Parameters for Diabetic and Control Subjects: Grouped by AER

Table 3:7 shows the data for the control group compared with the diabetic group sub-divided by AER. Group A, B and C comprise, respectively, subjects with an AER below 10$\mu$g/min, 10-100$\mu$g/min and greater than 100$\mu$g/min. When only the IDDM results were grouped in a similar way only urinary total protein showed a significant difference between groups A and C. These results can be seen in Appendix 5.
Table 3:6

Statistical Difference for Data Grouped by Type of Diabetes

<table>
<thead>
<tr>
<th></th>
<th>Significance between</th>
<th>P value</th>
<th>Significance between</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IDDM and NIDDM</td>
<td></td>
<td>IDDM and NIDDM matched</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Duration of Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>0.053</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary α1-M (mg/l)</td>
<td>0.022</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary Total Protein (g/l)</td>
<td>0.017</td>
<td>0.086</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>0.013</td>
<td>0.0086</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>0.0001</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>0.0003</td>
<td>0.0003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>0.05</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1: Urinary \( \alpha_1 \)-microglobulin concentration according to age group. Values are represented as mean with S.D.

- \( \square \) = non diabetic subjects
- \( \blacksquare \) = diabetic subjects
Urinary total protein (mg/l)

Values shown - mean with 1 S.D.

= non-diabetic subjects
= diabetic subjects
Albumin excretion rate (μg/min)

AGE IN YEARS

16-25 26-35 36-45 46-55 56-65

[Diagram showing excretion rates for different age groups, with bars indicating mean and error bars for standard deviation.]

= non-diabetic subjects
= diabetic subjects

Values shown - mean with 1 S.D.
<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>A (&lt;10μg/min)</th>
<th>B (10-100μg/min)</th>
<th>C (&gt;100μg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>39</td>
<td>64</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>AER (μg/min)</td>
<td>3.54 (2.67)</td>
<td>3.14 (2.26)</td>
<td>25.53 (17.9)</td>
<td>984.6 (810)</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>6.41 (0.51)(a)</td>
<td>12.71 (3.54)</td>
<td>15.23 (3.3)</td>
<td>13.67 (2.56)</td>
</tr>
<tr>
<td>Plasma Gly Alb (%)</td>
<td>3.34 (1.35)(a)</td>
<td>7.76 (3.33)</td>
<td>8.48 (4.33)</td>
<td>8.12 (2.69)</td>
</tr>
<tr>
<td>Urinary Gly Alb (%)</td>
<td>9.18 (4.48)</td>
<td>8.45 (4.98)</td>
<td>12.35 (7.9)</td>
<td>15.10 (6.28)</td>
</tr>
<tr>
<td>α₁-M (mg/l)</td>
<td>3.31 (1.82)(c)</td>
<td>4.96 (6.75)(c)</td>
<td>6.00 (6.2)</td>
<td>15.17 (15.7)</td>
</tr>
<tr>
<td>Total Protein (g/l)</td>
<td>83 (33)</td>
<td>73.7 (35.7)(c)</td>
<td>142.8 (114)</td>
<td>565.7 (392)</td>
</tr>
<tr>
<td>Plasma Glucose (mmol/l)</td>
<td>5.03 (1.20)(a)</td>
<td>14.1 (6.5)</td>
<td>19.0 (6.7)</td>
<td>13.4 (5.6)</td>
</tr>
<tr>
<td>Urinary Glucose (mmol/l)</td>
<td>0.20 (0.13)</td>
<td>9.05 (7.88)</td>
<td>13.75 (8.50)</td>
<td>8.68 (4.3)</td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>115.4 (5.99)</td>
<td>107.3 (6.04)</td>
<td>102.6 (6.78)</td>
<td>77.7 (4.6)</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>-</td>
<td>8.7 (8.4)</td>
<td>15.8 (12.4)</td>
<td>18.4 (15.3)</td>
</tr>
<tr>
<td>Urinary Flow (mls/min)</td>
<td>0.9 (0.59)</td>
<td>1.09 (.63)</td>
<td>1.07 (.99)</td>
<td>1.56 (4.7)</td>
</tr>
<tr>
<td>α₁-M/μg/min</td>
<td>2.81 (2.2)</td>
<td>5.4 (11.5)</td>
<td>4.36 (3.1)</td>
<td>24.9 (26.2)</td>
</tr>
<tr>
<td>Total Protein/ (mg/min)</td>
<td>64.2 (39.1)</td>
<td>66.3 (31.3)</td>
<td>87.6 (28.8)</td>
<td>949 (767)</td>
</tr>
<tr>
<td>Urinary Glucose (mmol/min)</td>
<td>0.16 (.16)</td>
<td>9.4 (11.9)</td>
<td>14.1 (19.2)</td>
<td>16.3 (17.6)</td>
</tr>
<tr>
<td>% IDDM</td>
<td>-</td>
<td>59%</td>
<td>86%</td>
<td>82%</td>
</tr>
<tr>
<td>% Retinopathy</td>
<td>-</td>
<td>18%</td>
<td>30%</td>
<td>71%</td>
</tr>
</tbody>
</table>

All values shown are means with SD in parentheses
(a)= Significant difference from groups A, B and C
(b)= Significant difference from groups B and C
(c)= Significant difference from group C
GHb, plasma Gly-Alb, α₁-microglobulin, plasma glucose, urinary glucose and α₁-microglobulin/min show statistically significant differences between the control group and all three diabetic groups (p<.05). However, none of these parameters show a statistically significant difference between the diabetic groups, due to the wide range of results within each group, indicated by the high standard deviation values. Total protein excretion, however, shows a difference between groups A and C (p<0.001).

The mean GFR value falls across the three diabetic groups, but the change is not significant. Duration of diabetes, percentage IDDM and prevalence of retinopathy all show an increase with increasing AER. Percentage with IDDM and prevalence of retinopathy are significantly different between group A and group C (p<0.01).

As these results showed few significant differences and the numbers in the groups small, groups B and C were joined as one group and compared with group A. The results can be seen in Table 3:8.

AER, GHb, urinary Gly-Alb, urinary total protein, duration, percentage with retinopathy, α₁-microglobulin/μg/min, total protein mg/min, and urinary glucose mmol/min all show significant differences between group A with AER <10μg/min and group B plus C with AER >10μg/min.

Table 3:9 shows the results for urinary:plasma Gly-Alb (U/PGly-Alb) ratio for the groups divided by AER. The ratio has been correlated with overnight albuminuria (mg albumin) for each of the groups, and the correlation coefficients are given in the Table. Urinary Gly-Alb has also been correlated with albuminuria
### Table 3:8

**Data for all Diabetic Subjects: Divided at 10µg/min**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AER &lt;10µg/min</th>
<th>P values</th>
<th>AER &gt;10µg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=64</td>
<td>P values</td>
<td>n=21</td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>3.14 (2.26)</td>
<td>&lt;0.0001</td>
<td>345 (624)</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>12.71 (3.54)</td>
<td>&lt;0.05</td>
<td>14.68 (3.08)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.76 (3.33)</td>
<td>NS</td>
<td>8.36 (3.79)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>9.20 (7.37)</td>
<td>&lt;0.05</td>
<td>13.27 (7.36)</td>
</tr>
<tr>
<td>α₁-M (mg/l)</td>
<td>4.96 (6.75)</td>
<td>NS</td>
<td>9.1 (10.9)</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>73.8 (35.7)</td>
<td>&lt;0.01</td>
<td>284 (3.0)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>14.1 (6.5)</td>
<td>NS</td>
<td>17.03 (6.8)</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>9.05 (7.88)</td>
<td>NS</td>
<td>12.06 (7.59)</td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>107.3 (60.4)</td>
<td>NS</td>
<td>94.3 (61.3)</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>8.7 (8.4)</td>
<td>&lt;0.05</td>
<td>16.2 (12.5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.4 (13.6)</td>
<td>NS</td>
<td>40.6 (13.9)</td>
</tr>
<tr>
<td>% with retinopathy</td>
<td>18%</td>
<td></td>
<td>43%</td>
</tr>
<tr>
<td>Urinary flow (mls/min)</td>
<td>1.09 (0.63)</td>
<td>NS</td>
<td>1.24 (0.87)</td>
</tr>
<tr>
<td>α₁-M/min</td>
<td>5.4 (11.5)</td>
<td>&lt;0.05</td>
<td>11.2 (17.6)</td>
</tr>
<tr>
<td>Total protein/min</td>
<td>66.3 (31.3)</td>
<td>&lt;0.01</td>
<td>375 (592)</td>
</tr>
<tr>
<td>Urinary glucose/min</td>
<td>9.4 (11.9)</td>
<td>&lt;0.01</td>
<td>14.8 (18.3)</td>
</tr>
</tbody>
</table>

All values are means with SD in parentheses.
Table 3:9

**Urinary/Plasma Gly-Alb Ratios and Correlation Data**

<table>
<thead>
<tr>
<th>Group</th>
<th>U/P Gly-Alb</th>
<th>U/P VS mg Albumin</th>
<th>Urinary Gly-Alb VS mg Albumin</th>
<th>Urinary Gly-Alb VS GHb</th>
<th>Plasma Gly-Alb VS GHb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>All subjects</td>
<td>124</td>
<td>2.00 (1.91)</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>39</td>
<td>3.37 (2.64)</td>
<td>-0.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic</td>
<td>85</td>
<td>1.38 (0.97)</td>
<td>0.27</td>
<td>0.31 (c)</td>
<td>0.22</td>
</tr>
<tr>
<td>AER &lt;10 µg/min</td>
<td>64</td>
<td>1.26 (0.93)</td>
<td>-0.14</td>
<td>-0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>AER 10-100 µg/min</td>
<td>14</td>
<td>1.62 (1.1)</td>
<td>-0.06</td>
<td>-0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>AER &gt;100 µg/min</td>
<td>7</td>
<td>1.98 (0.94)</td>
<td>0.79 (c)</td>
<td>0.517 (c)</td>
<td>0.14</td>
</tr>
<tr>
<td>AER &gt;10 µg/min</td>
<td>21</td>
<td>1.71 (1.0)</td>
<td>0.39 (c)</td>
<td>0.293 (c)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a = Significantly different from control group
b = Significantly different from group AER <10 µg/min
c = Statistically significant correlation
and with GHb. Plasma Gly-Alb has been correlated with GHb also, and the results are shown on the same table. When all the results are grouped together a significant correlation is found, as illustrated in Figure 3:5 \((p<0.001)\). Blood glucose levels showed a significant relationship \((p<0.001)\) with both GHb (Figure 3:6) and Gly-Alb (Figure 3:7).

\[\text{U/P Gly-Alb did not correlate with albuminuria for the control group, all the diabetic subjects, group A or group B treated separately. However, when group B and C or group C alone were considered, statistically significant correlations were found.}\]

Significant correlations were found between plasma Gly-Alb and GHb for all subjects, all diabetic subjects, and group A. Groups B and C treated separately and together showed no correlation between these two parameters. Urinary Gly-Alb did not correlate with GHb for any of the groups.

A clearance value for Gly-Alb was calculated which allowed for the different albumin concentrations in urine and plasma and for the different urine volumes and sample collection times. Using the percentage of Gly-Alb and the concentration of albumin in urine and plasma, the concentration of Gly-Alb in both urine and plasma was calculated. As the plasma albumin concentration had not been measured, the mean normal value was taken as 40g/l (normal range 34-48g/l) (Zilva and Pannall 1975). The following calculation was made: \(U \times V/P \times T\), where \(U\) = urinary Gly-Alb concentration, \(V\) = urine volume, \(P\) = plasma Gly-Alb concentration and \(T\) = time over which the urine sample collection was made. The clearance values were compared with AERs and a significant relationship \((p<0.0001)\) was found, as shown in Figure 3:8.
Figure 3.5 Graph to show the relationship between GHb and Gly-Alb values

\[ y = 0.887 + 0.495x, \quad r = 0.636, \quad p<0.001 \]

\( n = 122 \)
Figure 3.6  Graph showing the relationship between blood glucose and GHb

n = 122, r = 0.78
Figure 3.7 Graph showing the relationship between blood glucose and Gly-Alb.

n = 122, r = 0.56
Figure 3:8 Graph to show the relationship between Gly-Alb clearance and AER

\[ y = 1.60 + 0.911x, \ r = 0.80, \ p < 0.001, \ n = 121 \]
An AER of 30μg/min has also been considered as an upper limit of the normal range, and Table 3:10 shows the data grouped in this way. AER, urinary Gly-Alb, urinary α₁-microglobulin, urinary total protein, duration and total protein mg/min show significant differences between the two groups.

3:4:7 Parameters for Diabetic and Control Subjects: Diabetic Compared with Control Groups

Table 3:11 shows the results for the complete diabetic and non-diabetic groups, showing significant differences for all the parameters measured except for GFR, urinary Gly-Alb and α₁-microglobulin.
Table 3:10

Data for Diabetic Subjects: Divided at 30µg/min

<table>
<thead>
<tr>
<th></th>
<th>AER &lt;30µg/min</th>
<th>AER &gt;30µg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=75</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>5.29 (6.07)</td>
<td>** 705 (800)</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>12.96 (3.48)</td>
<td>NS 14.82 (3.58)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.93 (3.57)</td>
<td>NS 7.75 (2.33)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>9.69 (7.55)</td>
<td>* 14.07 (6.55)</td>
</tr>
<tr>
<td>α₁-M (mg/l)</td>
<td>5.05 (6.61)</td>
<td>* 12.9 (13.8)</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>84.0 (61.8)</td>
<td>* 441 (380)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>14.59 (6.66)</td>
<td>NS 16.34 (6.78)</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>9.76 (7.89)</td>
<td>NS 10.07 (8.14)</td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>105.8 (5.9)</td>
<td>NS 91.2 (7.0)</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>9.4 (8.5)</td>
<td>* 18.8 (15.5)</td>
</tr>
<tr>
<td>Urinary flow (mls/min)</td>
<td>1.10 (.71)</td>
<td>NS 1.31 (.61)</td>
</tr>
<tr>
<td>α₁-M/min</td>
<td>5.2 (10.7)</td>
<td>NS 18.8 (23.6)</td>
</tr>
<tr>
<td>Total protein/min</td>
<td>69.6 (31.8)</td>
<td>* 690 (752)</td>
</tr>
<tr>
<td>Urinary glucose/min</td>
<td>10.4 (13.7)</td>
<td>NS 13.4 (15.3)</td>
</tr>
</tbody>
</table>

All values shown are means with SD in parentheses

* = p<0.05

** = p<0.0001
Table 3:11

Data for all Diabetic and Control Subjects

Showing Statistical Differences

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Subjects</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>85</td>
<td>39</td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>88.0 (34.7)</td>
<td>** 3.54 (2.67)</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>13.2 (3.52)</td>
<td>*** 6.41 (.52)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.91 (3.44)</td>
<td>*** 3.34 (1.35)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>10.21 (7.54)</td>
<td>NS 9.83 (6.65)</td>
</tr>
<tr>
<td>Urinary α₁-M (mg/l)</td>
<td>5.99 (8.12)</td>
<td>NS 3.1 (1.82)</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>127 (182)</td>
<td>*** 83 (33.4)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>14.8 (6.66)</td>
<td>*** 5.03 (1.20)</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>9.8 (7.87)</td>
<td>*** 0.203 (0.13)</td>
</tr>
<tr>
<td>GFR (mls/min)</td>
<td>104.1 (6.04)</td>
<td>NS 115.4 (5.9)</td>
</tr>
<tr>
<td>Albumin/creatinine (mg/mmol)</td>
<td>12.2 (3.8)</td>
<td>*** 0.372 (0.06)</td>
</tr>
</tbody>
</table>

All values shown are means with SD in parentheses

** p<.001

*** p<.0001
Discussion

3:5:1 Comparison Between Male and Female Subjects

The results indicate that the only parameters showing a difference between male and female subjects are body weight and GFR. Differences would be expected in these two cases, but no differences have been found in the assessment of metabolic control or proteinuria. Male and female subjects have, therefore, been treated as one group. Other studies involving either IDDM (Mathiesen et al. 1984) or NIDDM (Mogensen 1984a) have also treated male and female subjects similarly. This study establishes that sex differences are limited and not important in the study of the complications of diabetes mellitus.

3:5:2 Duration of Diabetes

No statistically significant differences were observed when the data were considered by duration of the disease. However, AER shows a non-significant trend with increasing duration. The mean values for the three groups when grouped by disease duration indicate a considerable increase in AER, but the large variance for each group prevents the differences from showing a statistical significance.

Plasma Gly-Alb, urinary Gly-Alb, GHb, urinary total protein, plasma and urinary glucose show the highest mean value for the intermediate duration group. The most important difference between the groups is the type of diabetes of the individuals making up the groups. Any differences that can be seen may be due to either differences in duration of the disease or the type of diabetes.

Mathiesen et al. (1984) found no differences in AER between groups of diabetic subjects of increasing duration. However, when a long-term study was made of a group of diabetic subjects, the same authors found an increase of AER
after a six year follow-up period. Viberti et al. (1982a) also found an increase in
AER during a 14 year study. Thus, a long-term prospective study may show the
development of albuminuria and other complications in individuals, but a cross-
sectional study is not adequate to demonstrate these changes with duration of
diabetes.

3:5:3 Age Differences

The results suggest that age is not an important factor in the development of
albuminuria. Age does not correlate significantly with any of the parameters
measured.

It is, however, surprising that the 46-55 years old group do not follow the
trend of increasing AER, urinary total protein, urinary $\alpha_1$-microglobulin, GHb, Gly-
Alb, or plasma and urinary glucose. This age group appears to be in better short-
and long-term metabolic control than the younger groups. This group is made up of
50% IDD's and 50% NIDDM's, the same proportion as the 56-65 years old group.

There are therefore no obvious differences between the 36-45 years age
group and the 46-55 years group to explain the lower protein excretion levels found
in the older group, except for the lower percentage of IDDM subjects (84% for 36-
45 years age group and 50% for 46-55 years age group). Duration and age appear to
be factors associated with differences between the diabetic subjects. Thus far,
however, these factors have not been considered independently of differences
between IDDM and NIDDM, as it may be the higher proportion of NIDDM in the 46-
55 years age group that causes the lower AER and other results.
Comparison Between IDDM and NIDDM

The parameters associated with urinary protein excretion all show differences between IDDM's and NIDDM's suggesting more serious disruption of kidney function in the IDDM group. When matched for duration of diabetes, these differences remain except for \( \alpha_1 \)-microglobulin. This suggests that both glomerular and tubular functions are impaired in short- and long-term IDDM subjects, but only glomerular function in NIDDM subjects, and to a lesser degree.

The glycosylated protein levels show very small, non-significant differences between the three groups, the NIDDM group having the lowest mean value for GHb, plasma and urinary Gly-Alb.

However, plasma and urinary glucose levels are significantly \((p<0.05)\) lower in the NIDDM group than the other two groups. These samples were, however, random samples and not fasted, and as such, any significant differences must be treated with caution.

These results suggest that there is a greater differences in short-term glycaemic control than long-term control between the groups. Stable GHb levels are not affected by rapid changes in blood glucose (Svendsen et al. 1980; and Compagnucci et al. 1981) providing that the GHb measurement has been carried out by a method that only measures the stable form. It has been shown that mean blood glucose levels (Svendsen et al. 1982) correlate well with GHb levels, but in this study only one random non-fasted glucose measurement was made. Thus, the fact that there is a difference between the blood glucose levels of the groups, but not the glycosylated protein levels, is not a contradictory finding.
This study was the first to include the measurement of $\alpha_1$-microglobulin as an index of tubular function in diabetes. Table 3:7 shows the increased excretion of this protein in the groups with elevated AER. Only the group with an AER above 100$\mu$g/min shows a significant increase in $\alpha_1$-microglobulin excretion when compared with the control group, or with the diabetic group with normal AER. These results suggest that the highly elevated albumin excretion is of tubular as well as glomerular origin.

The significant relationship found between Gly-Alb clearance and AER, in addition to the urinary/plasma Gly-Alb ratios, show that as AER is increased, there is an even greater excretion of Gly-Alb, suggesting a preferential loss of the glycosylated molecules.

As discussed in Chapter 1, there are several different ways of defining microalbuminuria. Groups who have used 24-hour urine collections have set two levels as the upper limit of normal; 15$\mu$g/min (Mogensen et al. 1983) and 10$\mu$g/min (Ghiggeri et al. 1984). Those who have used overnight urine collections have set the upper limit of normal as 30$\mu$g/min (Viberti et al. 1982a; Gatling et al. 1985). When an early morning urine sample only has been taken, 30-140mg/l (Mogensen 1984) was found to be predictive of proteinuria. Protein:creatinine ratios have also been used to classify levels of protein loss. Gatling et al. (1985) found that a ratio greater than 3.5mg albumin/mmol creatinine demonstrated individuals at risk of future nephropathy. Gragnoli et al. (1984) compared those diabetic subjects with an AER within 2 SD of their mean normal level, with those above 2 SD of the mean normal level. This method of grouping the diabetic subjects results in the same individuals falling into the normal and micro-proteinuric groups as Groups A and B.
The diabetic subjects have therefore been grouped in two ways. Firstly, with the upper limit of normal AER set at 10µg/min and secondly at 30µg/min.

With the upper limit of normal AER set at 30µg/min the results are shown in Table 3:10. The GHb levels are not significantly different in this instance, although there is a small increase in the second group. Urinary glycosylated albumin $\alpha_1$-microglobulin and duration show statistically significant differences between the groups ($p<0.05$).

Viberti et al. (1982) showed that subjects with an AER, measured on an overnight sample, between 30 and 140µg/min were more likely to suffer from nephropathy in later years. When the subjects in this study are grouped in this way, only three individuals fall into this group and so all those with an AER above 30µg/min have been treated as one group.

Gatling et al. (1985) also took 30µg/ml as the upper limit of normal AER on an overnight sample. They correlated this measurement with an albumin:creatinine ratio and found a highly significant correlation between the two parameters ($r=0.91$). The correlation between these two parameters found in this study was also significant ($r=0.86$, $p<0.001$). However, as the two measurements were made on the same urine sample in both studies, a significant correlation would be expected.

AER appears to be the most informative way of grouping diabetic subjects, as clear differences then appear between these groups. GHb and plasma Gly-Alb show differences, as do duration and prevalence of retinopathy and urinary $\alpha_1$-microglobulin.
Previous studies have only considered either IDDM or NIDDM subjects, but not both, with measurements of glycosylated proteins and urinary proteins. IDDMs and NIDDMs showed no differences in the levels of glycosylated proteins, but urinary protein loss was greater in the IDDMs than in the NIDDMs.

When the subjects are divided into three groups by AER, as in Table 3:7, only plasma and urinary glucose levels show significant differences between groups A and B, and B and C. There is also a higher incidence of retinopathy in groups B and C than in Group A. This latter group comprises only 59% IDDM subjects, compared with 86% and 82% for groups B and C respectively. Appendix 5 shows the data for IDDM subjects only, which can be compared with the results of Ghiggeri et al. (1984). The main differences between results in the two studies are that Ghiggeri's subjects had a much shorter duration of diabetes (5.5 ± 1.8 years, 5.3 ± 1.7 years, and 9.8 ± 1.6 years for groups A, B and C respectively) and yet were much older (37.4 ± 5.1 years, 44.6 ± 3.8 years and 51.4 ± 4.1 years for groups A, B and C respectively). Thus, the two groups studied by Ghiggeri et al. who have microalbuminuria comprise older individuals who were diagnosed as diabetic up to ten years previously. However, the subjects in groups B and C in this study had been diabetic on average, up to 21 years out of an average 40 years of age. These workers found an increase in urinary $\beta_2$-microglobulin excretion with increasing AER, and a decrease in creatinine clearance in Group C. Their results are in agreement with the results of this study, as $\alpha_1$-microglobulin indicates the same type of kidney dysfunction as $\beta_2$-microglobulin.

However, Ghiggeri and co-workers found a decrease in urinary Gly-Alb concentration with increasing AER. This is the reverse of the results of this study, which showed an increase in urinary Gly-Alb percentage with increasing AER. Groups A showed a similar percentage Gly-Alb to the control group.
The difference in urinary Gly-Alb levels between the group with normal AER and with elevated AER is statistically significant (p<0.05). The ratio of U/P Gly-Alb also shows a statistically significant difference between groups A and B and C.

Group A had a similar mean urinary Gly-Alb level to the control group, but a raised mean plasma Gly-Alb level. This would cause the ratio of U/P Gly-Alb to be considerably lower in group A than in the normal group, group B had an elevated mean urinary Gly-Alb level, but a similar plasma Gly-Alb level to group A. This led to the U/P ratio being higher than that of group A, but lower than the control group. Group C had a higher mean urinary Gly-Alb level still, but a similar plasma Gly-Alb level to groups A and B, resulting in a higher U/P ratio than groups A and B, but lower than the control group. All the diabetic groups have a lower mean ratio than the control group, but the ratio increases with increasing AER.

Gragnoli et al. (1984) however, found that the ratio was highest in group A (AER <10µg/min), lower in the control group, decreasing to group B (AER 10-100µg/min) and lowest in group C (AER >100µg/min). Their results suggest that group A had elevated urinary excretion of Gly-Alb, whereas groups B and C had lower urinary levels, as the serum Gly-Alb levels were similar. Thus in groups B and C there was a progressive decrease in the excretion of glycosylated proteins/mg proteinuria, in spite of their overlapping serum levels.

In the present study, only the groups with microalbuminuria or proteinuria demonstrated a relationship between urinary Gly-Alb and albuminuria. U/P ratio also indicated a significant correlation with albuminuria in these groups. These results indicate that where the albumin excretion is elevated, more Gly-Alb is present in the urine, and the ratio of urinary/plasma Gly-Alb is also increased.
Gragnoli and co-workers however, found negative correlations between urinary Gly-Alb (nmol urinary 5HMF) and levels of 24-hour proteinuria in control and diabetic groups. This would appear to suggest that there was no preferential excretion of glycosylated proteins in their patients. Gragnoli and co-workers and Ghiggeri and co-workers both found lower levels of glycosylated protein in the urine of diabetic subjects with elevated protein excretion, and yet have both concluded that glycosylated proteins are preferentially excreted, from the Gly-Alb clearance values that they calculated.

The raised U/P ratio in groups B and C in this study, and the positive correlations between urinary Gly-Alb, the U/P ratio and the Gly-Alb clearance with the level of albuminuria would all seem to suggest preferential excretion of the glycosylated molecule.

Ghiggeri and co-workers have studied extensively the glycosylation process, particularly with respect to albumin (Candiano et al. 1983; Candiano et al. 1984; Ghiggeri et al. 1985a; Ghiggeri et al. 1985b; Candiano et al. 1985). They have investigated albumin and Gly-Alb from serum and urine of diabetic subjects using ultrathin-layer isoelectric focusing and silver staining (Candiano et al. 1984) and found Gly-Alb to be micro-heterogeneous. There were, in the case of the serum Gly-Alb, several more positively charged bands with a pH (isoelectric point, i.e., the pH at which the molecule has no net charge) below 7. However, urinary albumin showed a different result, there was a similar main unmodified albumin band (pH 4.8), but in the case of the urinary Gly-Alb the other bands had a lower pH (4.0 - 4.7) and were more negatively charged.
These results are the reverse of those found by Purtell et al. (1979) who prepared more positively charged (or raised pI) human albumins. When these were infused into rats the albumins with the highest pI exhibited higher clearance rates compared with unmodified albumins. The two groups of workers have, therefore, found opposing results. Candiano and co-workers have postulated that the more negatively charged albumin molecules, which they have shown with the colorimetric assay of McFarland et al. (1979) to be glycosylated, have been preferentially excreted into the urine. When these Gly-Alb molecules have been studied in detail (Ghiggeri et al. 1985c) the carbohydrate moieties attached were found to be glucose, mannose and fructose.

The differences between the results of the two Italian groups and those presented here are probably due to different methodologies being used. Both groups used chromatography systems for separating Gly-Alb from the urine and serum samples, based on the charge differences between glycosylated and non-glycosylated molecules. This is a less specific separation technique than the affinity method used in this study, which separates samples on the basis of cis-diol groups only. As a result of these differences of methodology, it is not possible to define precisely which protein adducts have been measured by the Italian groups, making comparison of the results more difficult. They then measured the quantity of glucose present in the sample attached to the protein using the colorimetric method. The process involves treatment of the glycosylated protein with oxalic acid, which induces re-arrangement of the Schiff base (Gabbay et al. 1979). The colorimetric method is highly dependent on sample size (Klenk et al. 1982) and has a higher non-specific reactivity (Rendell et al. 1985). As the affinity chromatography method was used in this study the two methods do not measure exactly the same parameter. Direct comparisons between results are not
straightforward because of this difference in specificity. The level of glycosylation of a protein as estimated by affinity chromatography is expressed as a percentage, whereas the units quoted for the colourimetric method are nmoles HMF per mg protein. This also complicates comparison of the results.

The results of these two groups of workers differ from the results of this study most significantly in the degree of glycosylation of urinary protein. However, they have also concluded that gly-protein is preferentially excreted, despite negative correlations between levels of 5HMF/mg proteinuria versus proteinuria. In this study, however, the groups with raised albumin excretion have higher levels of urinary Gly-Alb. Also positive correlations were found between U/P Gly-Alb and albuminuria, between urinary Gly-Alb and albuminuria and between Gly-Alb clearance and AER. These findings strongly suggest that elevated AER is linked with increased urinary gly-alb (Taylor et al. 1986).

Another group (Pagano et al. 1986) have investigated the effect of exercise on AER and Gly-Alb excretion in six IDDM subjects. They found a positive correlation between urinary Gly-Alb levels and AER, but did not compare a urinary:plasma Gly-Alb ratio or clearance value with AER. They concluded that Gly-Alb was not preferentially excreted, but without further analysis of the data, this is not conclusive.

There are therefore contradictory findings on the preferential excretion of Gly-Alb, and this complex area of the complications of diabetes will not be clarified until the mechanisms involved have been fully explained.
The Italian workers separated the albumin initially on the basis of charge, and then measured the glucose content of the more negatively charged molecules using the colorimetric method. They found that there was less glucose present per mg protein in the group with higher protein excretion, and yet their Gly-Alb clearance calculations led them to conclude that Gly-Alb was preferentially excreted. The results of this study show increased Gly-Alb present in the urine of subjects with increased AER. This suggests that separation based on charge is not a sufficiently specific technique for separating Gly-Alb and that increased negative charge is not the only reason for Gly-Alb being excreted in greater quantities in subjects with elevated AER. Further investigations are therefore needed, with prospective studies, so that the development of kidney changes, with the concomitant increases in glycosylated and native albumin excretion can be followed.
CHAPTER 4

AN ASSESSMENT OF THE AFFINITY CHROMATOGRAPHY GLYCOXYLATED HAEMOGLOBIN ASSAY AS A SCREENING TEST FOR DIABETES,
AS PART OF THE ISLINGTON SURVEY
The OGTT is still the standard diagnostic test for diabetes, where the clinical signs are unclear, or the blood glucose levels are borderline. The criteria described in Table 1:2 have been based on the findings of long-term follow-up studies such as the Whitehall Survey (Reid et al. 1974) and the Bedford Survey (Sharp et al. 1964). These studies indicated the predictive ability of a 2-hour BG level in an OGTT for the development of diabetic vascular disease (Keen et al. 1982). The diagnostic test used in these studies was a 2-hour BG level, which has resulted in this single measurement being given the most diagnostic weighting. However, fasting blood glucose has not been investigated as a diagnostic test as thoroughly as the 2-hour blood glucose level, and where comparisons have been made (Modan et al. 1984) the fasting blood glucose has been shown to be only 45% sensitive compared to the 2-hour blood glucose level's 95% sensitivity. It is only the small number of unclassifiable cases that prevents the 2-hour blood glucose value from reaching 100% sensitivity.

An effective method for population screening should be sensitive enough to identify most of the affected population, and be relatively cheap, quick and simple to perform. As discussed in Chapter One, GHb has been suggested as a possible diagnostic test for diabetes (Santiago et al. 1978). GHb has been thoroughly evaluated as a means of monitoring metabolic control and several methods have been compared (Mortensen et al. 1983; Boucher et al. 1983; Peterson et al. 1984). However, most studies which have attempted to assess the value of GHb for diagnostic purposes have been incomplete, lacking large subject groups (Hall et al. 1984) and sufficient comparison of sensitivity and specificity with the values obtained from the OGTT (Ferrell et al. 1984; Mortensen et al. 1983). Several
methods are available for the measurement of GHb, but as yet no one method has been shown to be more sensitive than the others. However, methods which measure only the stable GHb and are not affected by the labile form may be more accurate for diagnostic use (Forrest et al. in press, b).

The Islington Diabetes Survey was designed so that the sensitivity, specificity and predictive value of a positive test for GHb could be compared with those of the 2-hour blood glucose levels as screening and diagnostic tests for diabetes.

The survey was carried out in two phases. The first phase was a screening study to ascertain the prevalence of diabetes, hypertension and anaemia in a group practice population. The second phase involved the recall of a stratified sample of these individuals, so that more detailed work could be carried out with a section of the population. At this second, recall phase, three different GHb assays were used, to compare sensitivity, specificity and predictive value of the different techniques with classification based on the 2-hour blood glucose value following a 75g oral glucose load. Electroendosmosis, iso-electric focusing and affinity chromatography were used to measure GHb in the recall phase.

Our involvement in this study was the measurement of GHb, by affinity chromatography, on the 2-hour post-glucose load blood sample of 222 subjects from the screening phase, and on fasting and 2-hour post load samples of all the recalled subjects. The affinity chromatography method was then assessed for specificity, sensitivity and positive predictive value compared with the fasting and 2-hour blood glucose levels.
The screening phase of the Islington Diabetes Survey involved 1084 subjects selected at random from a single group practice over the age of 40 who took a 75g oral glucose load in their own homes. Two hours later in the hospital a finger-prick capillary blood sample was taken for estimation of 2-hour, blood glucose, (BG) by an automated glucose oxidase method (Technicon 1, Technicon Instrument Company, Basingstoke, Hampshire, UK). A portion of the sample was collected into an EDTA tube (Sarstedt Limited, Leicester, UK) for analysis of GHb by electroendosmosis at the Whittington Hospital and by affinity chromatography at the University of Surrey. The affinity chromatography method was carried out as previously described in Chapter 3, Section 3:3. The within assay coefficients of variation for the affinity chromatography method were 2.0% and 1.5% for the non-diabetic and diabetic ranges respectively. The between assay c.v. were 1.8% and 1.5% for the same ranges.

The degree of glucose tolerance of the subjects was assessed according to modified WHO criteria and subjects were classified as having normal tolerance (2-hour BG <8.0mmol/l), IGT (2-hour BG 8.0-10.9mmol/l) or DM (2-hour BG >11.0mmol). The subjects were ranked according to their 2-hour BG level and GHb level as shown in Table 4:1. All those with either level above the 90th centile, with 50% of subjects with levels of either or both between the 80th and 90th centiles, and 10% of those with lower levels were selected to take part in a further test. This comprised a formal OGTT with measurement of BG at each time point, i.e., fasting, 1-hour and 2-hour and measurement of GHb fasting and at 2-hours.
Table 4.1: Centile Cut-Off Levels for Screening 2-Hour Post-Load Blood Glucose (2-Hour BG) Levels and GHb Levels Measured by Electroendosmosis and Affinity Chromatography

<table>
<thead>
<tr>
<th>Centile</th>
<th>2-Hour BG (mmol/l)</th>
<th>Electroendosmosis (%)</th>
<th>Affinity Chromatography (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>22.6</td>
<td>15.5</td>
<td>17.4</td>
</tr>
<tr>
<td>90</td>
<td>7.6</td>
<td>8.4</td>
<td>8.3</td>
</tr>
<tr>
<td>80</td>
<td>6.6</td>
<td>7.9</td>
<td>7.7</td>
</tr>
<tr>
<td>70</td>
<td>6.0</td>
<td>7.7</td>
<td>7.5</td>
</tr>
<tr>
<td>60</td>
<td>5.6</td>
<td>7.4</td>
<td>7.3</td>
</tr>
<tr>
<td>50</td>
<td>5.2</td>
<td>7.2</td>
<td>7.0</td>
</tr>
<tr>
<td>40</td>
<td>4.8</td>
<td>7.0</td>
<td>6.8</td>
</tr>
<tr>
<td>30</td>
<td>4.5</td>
<td>6.8</td>
<td>6.5</td>
</tr>
<tr>
<td>20</td>
<td>4.1</td>
<td>6.5</td>
<td>6.3</td>
</tr>
<tr>
<td>10</td>
<td>3.6</td>
<td>6.2</td>
<td>5.9</td>
</tr>
</tbody>
</table>
On this repeat test, blood glucose estimations were made on venous whole blood by the same method as before and samples were taken for GHb estimation by electroendosmosis and iso-electric focusing at the Whittington Hospital and by affinity chromatography at the University of Surrey.
Data Analysis

Discrimination measures, i.e. sensitivity, specificity and predictive value for a positive test have been calculated (Alderson 1983) for segregation of diabetic from non-diabetic individuals, for screening 2-hour GHb values, and recall fasting blood glucose, fasting and 2-hour GHb levels compared with the recall 2-hour blood glucose level used for classification as defined by the WHO.

The sensitivity of a test is defined as the per cent having a test variable above a selected cut-off point of the total group with diabetes as shown in Table 4:2. The specificity of a test is the per cent of diabetic individuals having a test variable below the selected cut-off point among the total group of non-diabetic individuals. The predictive value (positive test) expresses the percentage of true diabetic subjects among those with the test value above a selected cut-off point.
Table 4.2  
**Definition of the Test Characteristics**  
*Used in the Data Analysis*

<table>
<thead>
<tr>
<th>Test result</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>True Positives</td>
<td>False Positives</td>
</tr>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>-</td>
<td>False Negatives</td>
<td>True Negatives</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>(d)</td>
</tr>
</tbody>
</table>

Sensitivity of a test = \( \frac{a}{a + c} \times 100\% \)

Specificity of a test = \( \frac{d}{d + b} \times 100\% \)

Predictive value (positive test) = \( \frac{a}{a + b} \times 100\% \)
Results

4.4:1 Analysis of the Screening Data

2-hour post-load blood samples were received by us from 222 of the subjects in the screening phase for measurement of GHb by the affinity chromatography method. Figure 4:1 shows the GHb levels divided by the glucose tolerance class of the subjects, as defined by the WHO (1980). The distribution of the GHb levels can be seen in Figure 4:2. Table 4:3 shows the mean GHb values in the three groups, demonstrating that, despite the considerable overlap of GHb levels between the three groups, there are statistically significant differences between normal tolerance and IGT groups ($p<0.001$) and between IGT and DM tolerance groups ($p<0.001$). There was a significant correlation between the GHb levels and 2-hour blood glucose levels ($r=0.58$, $p<0.001$, $n=222$) as shown in Figure 4:3.

The test characteristics (sensitivity, specificity and positive predictive value) at a range of GHb cut-off values of the GHb assay as a screening test for AGT (abnormal glucose tolerance; IGT + DM) and DM can be seen in Figures 4:4 and 4:5 respectively. The specificity and predictive value (positive test) of the 2-hour GHb level as a screening test for DM and AGT at a fixed sensitivity of 90% have been summarised in Table 4:4.
Figure 4:1 Screening glycosylated haemoglobin values grouped by glucose tolerance class.
Figure 4.2 Distribution of the glycosylated haemoglobin values at the screening phase. Mean value 7.13%, mode 7.54% S.D. 1.40, n = 222
Table 4:3  GHb Levels in 222 Subjects in the Screening Phase, Showing Mean and Standard Deviations

<table>
<thead>
<tr>
<th>Glucose Tolerance Class</th>
<th>Normal</th>
<th>IGT</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 210</td>
<td>n=5</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>Mean %</td>
<td>6.9</td>
<td>7.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.9</td>
<td>0.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Significance

(p<) 0.001  0.001  0.001
Figure 4:3 Glycosylated haemoglobin levels plotted against blood glucose concentration at the screening phase.
Figure 4.4 Test characteristics (sensitivity, specificity and positive predictive value) at a range of GHb cut-off values for 'abnormal glucose tolerance' at the screening phase.
Figure 4:5 Test characteristics (sensitivity, specificity and positive predictive value) at a range of cut-off levels for diabetes at the screening phase.
<table>
<thead>
<tr>
<th>Glucose Tolerance Class</th>
<th>Specificity</th>
<th>PV+</th>
<th>Cut-Off Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>68.0</td>
<td>8.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Abnormal Glucose Tolerance</td>
<td>68.8</td>
<td>14.1</td>
<td>7.4</td>
</tr>
</tbody>
</table>
Analysis of the Recall Data

The stratified sample of subjects recalled from the screening study underwent a full OGTT: 223 subjects were included, and complete data are available on 201 of these individuals. They were again classified into glucose tolerance classes according to the WHO criteria and Table 4:5 shows the classification at recall compared with the classification at screening. This Table includes the individuals who could not be classified, most of whom were later excluded due to incomplete data, and who have been classified as IGT in subsequent data analysis.

The 2-hour GHb values from the recall phase of those subjects whose GHb levels were measured in the screening phase (n=44) have been shown as a percentage of the screening value (Figure 4:6). The relationship between screening and recall GHb levels, both fasting and post-load samples, are significant and can be seen in Table 4:6.
Table 4:5  Comparison of Glucose Tolerance Class at Screening with Glucose Tolerance Class at Recall Testing of all Subjects at Follow-Up (n = 223)

Classification at Screening

<table>
<thead>
<tr>
<th>Classification at Recall</th>
<th>Normal</th>
<th>IGT</th>
<th>DM</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>135</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>IGT</td>
<td>31</td>
<td>11</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>DM</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Unclassified</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Totals</td>
<td>170</td>
<td>27</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 4.6  Recall phase glycosylated haemoglobin values shown as a percentage of screening phase values. Mean 99.6%, mode 105%, S.D. 16.9, n = 44
### Table 4.6: Relationship of Screening GHb Levels to Recall Fasting and 2-Hour GHb Levels

<table>
<thead>
<tr>
<th>Screening GHb</th>
<th>r</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>with fasting recall GHb</td>
<td>0.41</td>
<td>&lt;0.003</td>
<td>44</td>
</tr>
<tr>
<td>with 2-hour recall GHb</td>
<td>0.43</td>
<td>&lt;0.002</td>
<td>44</td>
</tr>
</tbody>
</table>
Table 4:7 shows the results for all the subjects divided into three groups, according to their response to the 75g glucose load. No statistical difference was found between fasting and 2-hour GHb values for any group. There was considerable overlap in the GHb values for the three groups, as shown in Figure 4:7. The range of fasting GHb values for the normal group was 3.16-10.02% for the IGT group it was 5.60-7.95% and for the diabetic group the range was 7.39-19.36%. There were however, significant differences between the IGT group and control or diabetic group, and highly significant differences (p<0.0001) between diabetic and control groups for all the measurements.

Table 4:8 shows the regression analysis of fasting and 2-hour GHb values with the area under the glucose tolerance curve (AUC). The three glucose tolerance groups have been treated separately and combined. When all the results were taken together, a significant correlation (p<0.001) was found. However, when normal, IGT and diabetic groups were considered separately, only the diabetic group demonstrated a significant relationship (p<0.05). Also shown are the correlation coefficients for the GHb versus blood glucose levels for the fasting and 2-hour time points for the normal, IGT and diabetic subjects 2-hour GHb levels have been plotted against the 2-hour BG levels for the whole group (r = 0.55, p<0.001, n = 201), showing a significant relationship (Figure 4:8). The normal group showed no correlation between GHb and BG levels at either fasting or 2-hour samples. The IGT group showed a statistically significant correlation (p<0.01) between the two parameters in the fasting state and correlation in the 2-hour sample (p<0.02). The results for the diabetic subjects demonstrate a significant correlation between the two parameters for both time points, with greater significance in the fasting sample.
<table>
<thead>
<tr>
<th>Glucose tolerance</th>
<th>n</th>
<th>Mean GHb%</th>
<th>Mean blood glucose mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasting 2-hour</td>
<td>Fasting 2-hour</td>
</tr>
<tr>
<td>Normal</td>
<td>140</td>
<td>6.86 ± 1.0 (a)</td>
<td>7.03 ± 1.01 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.16 - 10.02)</td>
<td>(3.13 - 9.37)</td>
</tr>
<tr>
<td>Impaired</td>
<td>48</td>
<td>7.46 ± 1.4</td>
<td>7.7 ± 1.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.6 - 7.95)</td>
<td>(5.37 - 8.52)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>13</td>
<td>9.77 ± 3.16 (b)(d)</td>
<td>9.70 ± 3.00 (b)(d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.39 -19.36)</td>
<td>(6.59 -18.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.45 ± 0.56 (c)</td>
<td>4.73 ± 1.02 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.20 - 6.68)</td>
<td>(2.10 - 6.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.84 ± 0.62</td>
<td>8.30 ± 1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.60 - 6.50)</td>
<td>(6.70 - 9.90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.81 ± 1.96 (c)(d)</td>
<td>12.79 ± 3.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.71 -12.00)</td>
<td>(10.00 - 19.20)</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD with observed range in parentheses.

(a) Significantly different from IGT group p<0.05
(b) Significantly different from IGT group p<0.0002
(c) Significantly different from IGT group p<0.0001
(d) Significantly different from normal group p<0.0001
Figure 1: Fasting phase glycated haemoglobin values grouped by glucose tolerance class.
Table 4:8  Relationship of Fasting and 2-Hour GHb To Area Under the Glucose Tolerance Curve (AUC) and Blood Glucose Levels

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th>NORMAL</th>
<th>IGT</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting GHb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- with AUC</td>
<td>r= 0.10</td>
<td>0.37 (b)</td>
<td>0.72 (b)</td>
<td>0.54 (a)</td>
</tr>
<tr>
<td>- with fasting BG</td>
<td>r= 0.06</td>
<td>0.32 (c)</td>
<td>0.88 (a)</td>
<td>0.59 (a)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th>NORMAL</th>
<th>IGT</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hour GHb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- with AUC</td>
<td>r= 0.20</td>
<td>0.30 (c)</td>
<td>0.62 (c)</td>
<td>0.49 (c)</td>
</tr>
<tr>
<td>- with 2-Hour BG</td>
<td>r= 0.15</td>
<td>0.33 (b)</td>
<td>0.67 (b)</td>
<td>0.54 (c)</td>
</tr>
</tbody>
</table>

Significance values for correlation coefficients

(a) represents p<0.001
(b) represents p<0.02
(c) represents p<0.05
Figure 4:8 Glycosylated haemoglobin levels plotted against blood glucose concentrations at the recall phase.
The subjects were initially divided into normal, impaired and diabetic responses to the OGTT on the basis of the 2-hour blood glucose level, as described in Table 1:2. This resulted in 13 subjects (6.4%) being classified as diabetic, 48 (23.8%) as having impaired glucose tolerance and 140 (69.6%) as having normal glucose tolerance.

The fasting GHb value was then compared as a screening test with blood glucose measurements. The sensitivity, specificity and predictive value (positive test) of the fasting GHb and 2-hour GHb concentrations at a range of cut-off levels can be seen in Figures 4:9 and 4:10 respectively. The sensitivity, specificity and predictive value (positive test) of the fasting BG value at a range of cut-off levels have been plotted in Figure 4:11. These results are summarised in Table 4:9 showing specificity and predictive value at a fixed sensitivity of 90%.
Figure 4.9 Test characteristics (sensitivity, specificity and positive predictive value) at a range of fasting GHb cut-off levels (recall phase) for diabetes.
Figure 4: Test characteristics (sensitivity, specificity and positive predictive value) at a range of 2-hour GHb cut-off levels (recall phase) for diabetes.
Figure 4:11 Test characteristics (sensitivity, specificity and positive predictive value) of fasting blood glucose as a test for diabetes at a range of cut-off levels.
Table 4:9  Characteristics (Specificity and Positive Predictive Value, PV+) of the Fasting GHb and 2-Hour GHb Value and Fasting Blood Glucose Level as Tests for DM at a Fixed Sensitivity of 90%

<table>
<thead>
<tr>
<th>Test</th>
<th>Specificity</th>
<th>PV+</th>
<th>Cut-Off Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting GHb</td>
<td>81.3%</td>
<td>25.5%</td>
<td>7.7%</td>
</tr>
<tr>
<td>2-hour GHb</td>
<td>81.4%</td>
<td>25.5%</td>
<td>8.0%</td>
</tr>
<tr>
<td>Fasting Blood Glucose</td>
<td>78.7%</td>
<td>23.0%</td>
<td>5.0 mmol/l</td>
</tr>
</tbody>
</table>
4:5 Discussion

When the diagnosis of diabetes is not obvious from clinical signs or measurement of random and fasting blood glucose levels, a test is needed that will facilitate classification of individuals. The OGTT has become widely accepted as standard criteria, but variations in dose, timing, type of specimen and analytical methods have led to difficulties in interpretation. The development of precise methods for measuring blood glucose and the recent recommendations of the WHO for the performance and interpretation of the test have led to a more uniform approach, but inherent variations in the OGTT remain. It is also an inconvenient and time-consuming test to carry out. For these reasons, GHb has been considered as an additional test for the diagnosis of diabetes. The test is quick and easy to carry out and is practical for use in screening large populations. GHb measures the cumulative effect of glycaemia over the preceding 3-4 weeks (Koenig et al. 1976) and so is not affected by many of the environmental factors that make the OGTT so variable. In order to be of use as a screening test, it must discriminate between normal and diabetic glucose tolerance. The results of this study show that there is considerable overlap as illustrated in Figure 4:1 for the screening phase and Figure 4:7 for the recall phase.

There are significant correlations between both GHb measurements and the blood glucose levels and area under the curve of an OGTT when all subjects were considered. The relationship between 2-hour GHb levels and the corresponding 2-hour BG levels for both screening and recall samples are both significant (p<0.001) for the whole subject group, as illustrated in Figures 4:3 and 4:8. These results confirm a close relationship between GHb and the results of the OGTT. However, when the subjects are divided into glucose tolerance groups, in Table 4:8, the
normal tolerance group showed no correlation between area under the curve and fasting or 2-hour GHb levels. The IGT and diabetic groups showed significant correlations despite smaller numbers in the groups (n = 13 and 48 respectively). The highest correlation was found for the diabetic group. This suggests that the relationship found between GHb levels and area under the curve for the complete subject group is mainly due to the highly significant correlation found for the diabetic subjects.

Lester et al. (1985) found similar results, with a highly significant correlation between 2-hour GHb levels and area under the glucose tolerance curve in a group of 47 diabetic patients. They measured HbA1c using the Corning Glytrac electrophoretic procedure, and found a significant difference (p<0.05) between fasting and 2-hour HbA1c levels in the diabetic group only. This difference was also found by Forrest et al. (in press, b) when labile GHb had not been removed by dialysis prior to measurement by the Corning method.

In the present study, however, using affinity chromatography no difference was found between fasting and 2-hour GHb values for any of the three groups which suggests that the labile GHb interferes with the Corning method but not with the affinity technique. Lester et al. found that there was no correlation between GHb levels and area under the glucose tolerance curve for normal or impaired tolerance subjects. Our results are in agreement with this and the reason is probably due to the much narrower range of values for GHb and blood glucose values in these two groups than was found in the diabetic group. The significance of the relationship between GHb and area under the glucose tolerance curve for the diabetic group suggests that there is a close link between the parameters.
At the screening stage of the study, 24 subjects (2%) were classified as diabetic and 40 (4%) as having IGT, based on the two hour BG level (Forrest et al. 1984). The stratified sample then selected to take part in the recall phase included all the individuals with either 2-hour BG or GHb levels above the 90th centile, which should have included all the diabetic subjects. The sensitivity, specificity and predictive value (positive test) at a cut-off point of 11.0 mmol/l of the screening test compared with the recall 2-hour diagnosis were 58.3%, 97.2% and 54.2% respectively. The specificity of the screening 2-hour BG is excellent, with sensitivity and predictive value also good. The balance between sensitivity and specificity required in a particular test depends on the purpose of the survey. In general, methods with a low specificity are to be avoided, as the incorrect inclusion of large numbers of non-diabetic individuals in a population screen would result in falsely large numbers being diagnosed as diabetic or recalled needlessly for further tests.

At a cut-off point of 6.7 mmol/l the fasting BG from the recall OGTT shows a sensitivity, specificity and predictive value of 39.2%, 99.1% and 84.3% respectively (Figure 4:11). These figures show that fasting BG misses the majority of diabetic individuals, but there are virtually no false positive diagnoses. A lower BG cut-off level would improve sensitivity but lower specificity and predictive value, thus including more false positives.

An assessment has been made of the value of GHb measurement as a screening and diagnostic test. Figures 4:4 and 4:5 show respectively the test characteristics for a range of GHb cut-off levels as a screening test for DM and IGT, from the screening phase of the study. The graphs are very similar, and Table 4:9 confirms that there is only a small difference in predictive value at a fixed
sensitivity of 90%. A cut-off level of 7.4% would yield a sensitivity of 90% and a specificity of 68.8%, but an improved specificity could be achieved (ie, fewer false positives) at a higher cut-off level. A range of cut-off points from 5.0% to 10.0% have been considered for both fasting and 2-hour GHb levels from the recall phase. The two graphs (Figures 4:9 and 4:10) showing sensitivity, specificity and predictive value (positive test) indicate similar results for the two GHb measurements, whether fasting or two hours after the oral glucose load. Both graphs show that the best values for sensitivity and specificity were obtained at a cut-off level of 8.0% GHb. At this GHb level, the specificity, sensitivity and predictive value values are, respectively, 84.6%, 87.2% and 31.4% for the fasting measurement and 92.3%, 81.4% and 25.5% for the 2-hour measurement. This level does, however, pick out many false positives. In the normal glucose tolerance group 8.5% have a fasting GHb above 8.0%, and 13.5% of the subjects have a 2-hour GHb above 8.0%. The IGT group would yield 22.9% of its subjects with a fasting GHb value over 8.0%, and 37.5% with a 2-hour GHb value over 8.0%. Raising the cut-off level means that more diabetic subjects are missed. At a cut-off level of 8.5% GHb the fasting measurement gives a sensitivity, specificity and predictive value of 61.5%, 93.1% and 38.1% respectively with corresponding values of 69.2%, 89.8% and 33.3% for the 2-hour measurement.

Thus the lower cut-off level of 8% GHb results in greater sensitivity but lower specificity. Forrest et al. (in press, a) used the screening and follow-up data from this study and calculated sensitivity, specificity and predictive value for a range of HbA, levels in the screening samples. The screening HbA, values were measured by electroendosmosis, which include labile GHb in the measurement and gives a higher GHb value than affinity chromatography, which we used for the recall samples. Fasting GHb values have also been used to calculate sensitivity,
specificity and predictive value. There is little difference between fasting and two-hour GHb levels in the recall samples. Forrest et al. (1984) conclude that 8.5% may be useful as an upper limit of normal, but that a higher value such as 9.0% or 10.5% may be more useful as a screening value. The recall data yield lower sensitivity and predictive value figures, but similar specificity figures to the screening data. This is due to the smaller number of both diabetic and non-diabetic individuals included in the recall than the screening calculations. In the screening data there were approximately twice as many diabetic and five times as many non-diabetic individuals (but similar numbers of false positives and negatives) than in the recall data. This means that specificity, a measure of the false positive diagnoses, remains virtually the same, but the other two calculations are lowered by the reduction in numbers of true positives and negatives diagnosed.

The stratification of the recall subjects, with a bias towards those with higher 2-hour BG and GHb levels, meant that a higher proportion of the recall subjects were diabetic, i.e. 6% compared with 2% of the screening sample. This emphasis on the subjects with high 2-hour BG and GHb levels affects the cut-off levels for diagnosis by GHb measurement, as the group is no longer normally distributed and contains few individuals with low GHb levels.

Santiago et al. (1978) and Koenig et al. (1976) found that the measurement of HbA1c was not sufficiently sensitive for the screening of diabetes. Kesson et al. (1982) stressed the methodologic problems common to these groups of workers who had all used the ion-exchange technique. Verillo et al. (1983) classified patients using the WHO criteria and found considerable overlap in the HbA1c measurements between the groups, as was also found in this study. Hall et al. (1984) however, showed good discrimination between the glucose tolerance groups and suggested
that the use of the affinity chromatography technique had overcome these difficulties and enabled clear distinction to be made between normal, IGT and diabetic responses to the OGTT. They also favoured the affinity method because it avoided known interferences and had a lower c.v. than other techniques.

This study, however, indicates that using the affinity method does not sufficiently improve the diagnostic ability of the measurement of GHb. The subjects investigated by Hall et al. had been referred for an OGTT, and so were not a normal sample of the population. This study comprised a much larger number of subjects and considerable overlap was found both in the screening and recall phases. This resulted in a wide range of GHb values in the normal tolerance group, which reflects the inter-individual differences in GHb levels between subjects with a normal response to the OGTT. The confirmation of this variation, also found by Verillo et al. (1983), has implications for the use of GHb as a diagnostic tool and requires further study. The relationship between the screening phase and recall phase 2-hour GHb levels is significant (see Table 4:6) but Figure 4:6 illustrates the variation between the two measurements. Although a significant correlation was found between the screening 2-hour sample and both fasting and 2-hour recall samples ($p<0.003$ and $p<0.002$ respectively) the correlation coefficients were 0.41 and 0.43 respectively, where values closer to 1.0 would have been expected due to the stability of GHb levels.

More recent investigation of 535 consecutive patients having an OGTT by Albutt et al. (1985) found GHb did not differentiate clearly between glucose tolerance groups, and concluded that fasting blood glucose concentration, supplemented with information of the GHb level, might be sufficient in most individuals. There would still remain some subjects for whom the complete OGTT would remain necessary.
Both OGTT and GHb results must be interpreted with caution, especially in the areas where diagnosis is least clear. It is apparent that GHb measurement cannot make the OGTT redundant. Therefore GHb only has a role in the diagnosis of diabetes if it can be used by itself or with a screening sample (fasting or 2-hour blood glucose) to indicate those who are clearly normal or diabetic, and to help select a relatively small group for the full OGTT. If the cut-off point was set, say, at 8.0% GHb, on a fasted or random sample, over 90% of diabetic subjects would be found, after confirmation by the OGTT.

The sensitivity, specificity and predictive value curves indicate that fasting and 2-hour GHb measurements yield similar results, with acceptable levels of specificity but poor predictive value. Fasting BG however, at a cut-off level of 6.7mmol/l showed excellent specificity of 99.1% and very good predictive value of 83.3%, with lower sensitivity of 39.4%. In other words, fasting BG measurements miss a number of diabetic subjects, but pick out few false positives. GHb, conversely, picks out more of the diabetic subjects, but many false positive diagnoses are made as a result of the wide range of values encountered in the normal glucose tolerance group.

Thus a combination of fasting BG and GHb measurements might make a useful preliminary screen, to enable a high proportion of diabetic subjects to be identified, who could then undergo the full OGTT. Forrest et al. (in press, c) concluded that GHb measurement did not add any extra predictive ability to the two hour BG as a diagnostic test. However, as a population screening device it may be useful to reduce the number of OGTT’s required for diagnosis. Fasting BG, fasting and 2-hour GHb levels all gave similar specificities at a fixed sensitivity of 90% and all gave a very low predictive value (Table 4:9), confirming that none of these measurements has any advantage over the others.
In conclusion, although GHb values have been demonstrated to correlate well with the results of an OGTT both blood glucose levels and AUC, the measurement of GHb alone cannot replace the OGTT. However, used in conjunction with a single blood glucose level, it would be useful as a pre-screening assessment, to reduce the number of OGTT's required in a population.
CHAPTER 5
GENERAL DISCUSSION
This thesis describes, in Chapter 2, the development of a sensitive immunoassay system for the measurement of Gly-Alb. The technique finally used was an adaption of a sandwich ELISA method, preceded by sample separation using the affinity chromatography method for glycosylated proteins. The method was used to measure albumin in the urine and Gly-Alb in the plasma and urine of diabetic and non-diabetic subjects.

The other major piece of work was carried out as part of the Islington Diabetes Survey. The work arose because of a poster presented to the British Diabetic Association in 1983, which indicated that use of the affinity chromatography method to separate and quantify GHb appeared to have several advantages as a technique for screening for diabetes. The work described in Chapter 4 was done on a limited number of individuals at the initial screen and on almost all the individuals at follow-up.

In this final Chapter, the usefulness of the assays for glycosylated proteins will be considered, together with their clinical relevance and the role of glycosylated proteins in the development of diabetic complications.
Glycosylated Haemoglobin Measurements

As a Screening Test for Diabetes

The OGTT is the standard diagnostic test for diabetes, but the interpretation of results has been complicated in the past by lack of standardisation of the methodology or of the levels of blood glucose which define normal and abnormal glucose tolerance. These complications have been largely removed by standardisation of the test and diagnostic criteria (National Diabetes Data group 1979; WHO 1980; WHO 1985), but the OGTT remains a time-consuming test to carry out. This is of particular relevance in large-scale screening studies where large numbers of tests are required. Various short-cuts to the OGTT have been considered (Modan et al. 1984) but no satisfactory replacement has yet been found. GHb measurement has been suggested as a diagnostic technique; the idea that a simple blood test using a single sample obtained at any time of day could replace a time-consuming and unpleasant procedure, requiring dietary preparation and multiple blood samples, is attractive. However, initial speculation that measurement of GHb could be employed as a more sensitive or specific diagnostic test than the OGTT has not been entirely corroborated in clinical studies (Dunn et al. 1979; Flock et al. 1979; Dods and Bolmey 1979). The GHb level, however, correlates highly significantly with the blood glucose concentrations observed during the OGTT and with the area under the glucose tolerance curve in diabetic patients (Koenig et al. 1976).

The Islington Diabetes Survey was designed with the aim of assessing the sensitivity, specificity and positive predictive value for three different GHb assays and 2-hour blood glucose levels as screening and diagnostic tests for diabetes (Forrest et al. In press a). Isoelectric focusing, electroendosmosis (with and
without the labile fraction) and affinity chromatography were compared as a screening test, and the affinity chromatography showed the best specificity and predictive value at a fixed sensitivity of all the methods, whether the fasting or 2-hour post-load samples were used (Forrest et al. In press a). In the screening phase of the study electroendosmosis was carried out without prior removal of the labile fraction, and the results from this phase suggested that GHb measurements did not improve the predictive ability of a 2-hour post-load blood glucose concentration (Forrest et al. In press c). However, during the recall phase, the results from the OGTT showed clearly that the 75g glucose load significantly increased the total GHb level when measured by electroendosmosis without prior removal of the labile fraction. The isoelectric focusing and affinity methods showed no difference resulting from the oral glucose load (Forrest et al. In press b).

Other studies have assessed the use of the GHb assay as a screening (Ferrel et al. 1984; Modan et al. 1984; Verillo et al. 1983) or diagnostic test (Albutt et al. 1985; Lester et al. 1985) for diabetes. The GHb assay was shown in these studies to have poorer test characteristics than either fasting or 2-hour blood glucose concentrations. However, all of these studies used assay methods that included the labile fraction, and the results from the Islington study have since shown that GHb assay methods that measure only the stable fraction are more accurate as screening tests for diabetes (Forrest et al. In press a).

The comparison of isoelectric focusing, electroendosmosis (stable fraction only) and affinity chromatography GHb assay as screening tests suggested that the affinity chromatography assay was superior to the others (Forrest et al. In press a), which confirms the original reason for including this method in the study (Hall et al. 1984). Although the method does not show complete separation of the
glucose tolerance classes, it yields good test characteristics and may provide an acceptable screening test for diabetes. The use of the GHb assay to screen a population for the prevalence of diabetes would enable large numbers of subjects to be assessed quickly and then an OGTT could subsequently be carried out on a smaller number of subjects for accurate diagnosis if required. The affinity chromatography assay for GHb is quick, cheap and easy to perform. Sample preparation to remove the labile fraction is not required, nor is rigorous temperature control (Klenk et al. 1982) nor control of the haemoglobin concentration. Haemoglobin variants, such as HbF and HbS interfere with the results of the ion exchange chromatography method (Bunn 1981b) but do not affect the affinity technique (Yue et al. 1982b). These factors combined make the affinity chromatography method a potentially useful tool for screening for diabetes.
The assay system that was developed was sufficiently sensitive to measure the very low levels of albumin and even lower levels of Gly-Alb found in the urine of non-diabetic individuals.

The original assay systems that were attempted using APBA to replace a specific antibody for Gly-Alb would have resulted in a simple, specific and convenient assay. It would also have been possible to use the assay to measure any glycosylated protein, without the normal additional step in immunoassay systems of having to raise specific antibodies to the glycosylated portion of each protein to be measured. Unfortunately the affinity ligand was unsuitable for use in a solid-phase assay. Initially APBA was attached to the walls of the wells using poly-L-lysine as a spacer molecule, so that the boronate moiety of the APBA molecule would be kept at a distance from the wall and available for binding by the cis-diol groups of the Gly-Alb molecule. The well coating stage was probably successful as poly-L-lysine is frequently used in such circumstances, but for a number of possible reasons the Gly-Alb did not remain bound to the APBA for the rest of the assay procedure.

The other assay systems that were tested used anti-albumin IgG to coat the solid phase, with APBA conjugated to either horseradish peroxidase or alkaline phosphatase as the enzyme label. In the final successful system that was used effectively, the only difference was the replacement of APBA with anti-albumin IgG, which suggests that it was inadequate binding of APBA to Gly-Alb which prevented the assay system from functioning. The affinity of APBA for glycosylated molecules is unknown, but it is likely to be much lower than the very
strong affinity of a specific antibody for its antigen. The APBA/Gly-Alb bond was probably broken in the subsequent washing steps of the assay which were essential in an attempt to lower the background binding. The later steps of the assay also had to be carried out in an environment (i.e., phosphate buffered saline at pH 7.4) which was required for the subsequent processes of the assay, but was unsuitable for the binding of APBA and Gly-Alb to take place (i.e., ammonium acetate buffer at pH 8.5).

The systems which used an APBA enzyme labelled conjugate should have been more likely to be successful, as the solid phase was coated with anti-albumin IgG as is frequently used in ELISA methods (Voller et al 1979). In addition, there was only one washing step following the incubation of enzyme-labelled APBA with the coated, sample-containing wells. It is possible, therefore, that stearic hindrance prevented the APBA moiety from binding with the Gly-Alb molecules. The enzyme molecule may have obstructed the boronate from becoming attached to the Gly-Alb.

A combination of these potential difficulties may have prevented the adapted ELISA system from working effectively. This was unfortunate as a lot of time and effort was put into trying to develop a system using APBA that would work. A system utilising the specificity of APBA for the cis-diol groups of glycosylated proteins would have been simple, cheap, specific and versatile. It would have been possible to measure samples without prior separation using affinity chromatography, and so would have been more convenient than the assay system that was developed finally.
An assay method in which phenyl boronic acid was used has since been reported (Hayashi & Makino 1985). They made use of the spectral change undergone by dansylated phenyl boronic acid (N-(5-dimethyl amino-1-naphthalene sulphonyl)-3-aminobenzene boronic acid) which it reacted with human serum albumin. However, as only a one in four dilution of serum was used in the assay, it would not have had sufficient sensitivity to measure Gly-Alb in the urine of diabetics or non-diabetics.

As the APBA ligand could not be used to replace a specific antibody in an adapted ELISA system, it was decided to develop an ELISA for albumin. The affinity chromatography method was used to separate Gly-Alb from non-Gly-Alb prior to measurement of the albumin concentration of each fraction and calculation of the percentage glycosylated.

The complete method could be carried out in under 24 hours and up to 16 urine samples could be measured at one time, which entailed measuring in triplicate, two dilutions of unseparated urine, and dilutions of both the Gly-Alb and non-Gly-Alb fractions from the affinity separation. Each urine sample generated four albumin measurements, requiring twelve wells in a microplate. The plasma samples only had Gly-Alb measured by this method, as it was too sensitive, and required too high dilution factors for the accurate measurement of plasma albumin.

The assay was used to measure urinary albumin, and Gly-Alb in the plasma and urine of all the diabetic and non-diabetic subjects who took part in the study.
The Role of Protein Glycosylation in the Development of Diabetic Complications

Most proteins, some intimately involved in the complications of diabetes, may undergo non-enzymatic glycosylation (Kennedy & Baynes 1984). Correlative analyses of the extent of glycosylation of tissue proteins are scant. This is unfortunate because such data would help to address the issue of increased glycosylation as a contributing factor to clinical disease. A strong case exists for glycosylation as a potential source of collagen abnormalities in diabetes, based on observation of non-enzymatic browning and fluorescence of diabetic collagen investigated in autopsy samples (Monnier et al. 1984). Excessive non-enzymatic glycosylation has also been demonstrated in lens crystallins in vivo (Cerami et al. 1979); and in in vitro studies in peripheral and central nervous system myelin (Vlassara et al. 1983) and more recently, in ribonuclease A (Watkins et al. 1985) and DNA (Bucala et al. 1984) but the mechanisms by which these changes might cause the sequelae of diabetes have yet to be fully elucidated.

The products of non-enzymatic glycosylation have been found in several human tissues at autopsy, including tendon, artery, nerve and glomerular basement membrane (Vogt et al. 1982). More recently, advanced glycosylation end-products (AGE) have been found in human globin and albumin (Chang et al. 1985) and the structure of one of the AGEs has been fully described (Pongor et al. 1984). Exposure to raised glucose levels alters the chemistry of glomerular and other basement membranes. Accumulation of basement membranes, including that of the glomerulus, characterises several of the microangiopathic sequelae of diabetes, and is associated with increased permeability (Cohen et al. 1981). The glycosylation of ε-amino groups on lysine and hydroxylysine residues decreases the
availability of these amino groups for normal collagen cross-linking. Le Pape et al. (1981) showed that the amount of ketoamine-linked hexoses present, indicating an increased level of glycosylation was closely related to a lowered amount of collagen cross-links in the glomerular basement membrane. The AGEs form intramolecular cross-links and collagen with elevated AGEs which can be found as a normal consequence of ageing, has reduced solubility, which may lead to arterial stiffening, decreased joint mobility and microvascular complications (Monnier et al. 1984).

Brownlee et al. (1984) showed that glucose-derived cross-links form when reactive groups, formed as a result of glycosylation of long-lived structural proteins, trap non-glycosylated soluble proteins in the blood. These serum proteins (albumin and IgG) have been shown to be trapped by glycosylated collagen in the glomerular basement membrane, and their accumulation may lead to the thickening of the membrane. Cohen et al. (1981) suggested that the increased level of glycosylation of the glomerular basement membrane could alter the electrochemical properties of the membrane. These changes may compromise the molecular or electrical integrity of the filtration barrier, leading to increased microvascular permeability.

The exact mechanism by which the thickening of the glomerular basement membrane leads to elevated urinary albumin levels and preferential excretion of Gly-Alb has not yet been explained. One theory suggests that there is an alteration in charge selectivity of the glomerular basement membrane (Gragnoli et al. 1984). Their studies, however, measured Gly-Alb using a method which separated the albumin molecules on a charge basis, selecting the more negatively charged molecules, and then the carbohydrate content of the selected fraction was measured. The results of Gragnoli and co-workers and of Candiano et al. (1984) therefore, only takes account of the most negatively charged albumin species, rather than selectively separating those molecules with a glucose adduct.
The results of this study show that there is an association between increased glycosylation of haemoglobin and presence of retinopathy and microalbuminuria. The increase in Gly-Alb excretion in the diabetic subjects with raised albumin excretion suggests an enhanced passage of Gly-Alb across the glomerular filtration barrier. The relationship between the Gly-Alb clearance values, (which are a ratio of urinary to plasma levels of Gly-Alb corrected for urine volume and sample time), and AER, confirms the preferential excretion of Gly-Alb at raised AER.

A reduction of the charge selectivity of the glomerular basement membrane would not necessarily result in an enhanced filtration of more negative molecules. A more likely reason for the preferential excretion of Gly-Alb was suggested by Williams et al. (1981). They demonstrated that there was a preferential uptake of Gly-Alb over native albumin by endothelial micropinocytic vesicles. Their results suggested that glycosylation of albumin resulted in extravasation of the molecules. This would explain the enhanced passage of Gly-Alb across the filtration barrier of the glomerulus. Alterations in the physical and electrical properties of the membrane may result in the overall increase in albumin excretion that occurs, while another mechanism, possibly a simple recognition system for glycosylated proteins causes the enhanced movement of Gly-Alb across the glomerular basement membrane.

A recent report from Brownlee et al. (1986) has contributed further evidence that AGEs are associated with glucose-derived cross-link formation which results in membrane thickening. Cross-linking of long-lived proteins such as collagen increases with age and is accelerated in diabetes (Cerami 1986). The AGEs accumulate on long-lived proteins. In collagen from diabetic subjects which has been exposed to elevated glucose concentrations over a long period of time, the
age-associated accumulation is accelerated (Monnier et al. 1984). Brownlee and co-workers suggested that glucose-derived protein cross-link formation could be prevented by pharmacologically blocking the reactive carbonyl groups on the early glycosylation products. They were able to show that AGE accumulation and glucose-derived cross-link formation could be prevented by aminoguanidine, a nucleophilic compound, in animal studies. The scheme for the formation of AGEs and the intervention of aminoguanidine can be seen in Figure 5:1.

The contribution of AGEs and the concomitant trapping of serum proteins to the dysfunction of the glomerular basement membrane has yet to be clearly demonstrated in human subjects, but the evidence in animal studies is increasing. The theory of Gragnoli et al. (1984) that the preferential excretion of Gly-Alb is due to a loss of the negative charge of the glomerular filtration barrier alone, seems unlikely. This reduction of electrical charge may be one of a number of factors contributing to the increased albumin excretion, but the trapping of non-glycosylated albumin by AGEs on the membrane, enhanced vesicle uptake of Gly-Alb or other as yet unknown processes, may contribute to the preferential loss of the glycosylated molecule over the native one.
Figure 5.1 Suggested scheme of non-enzymatic glycosylation leading to glucose-derived cross-link formation and its prevention by aminoguanidine.

[Diagram of chemical reactions involving glucose, protein, Schiff base, ketoamine, aminoguanidine, substituted Amadori product, and glucose-derived cross-link (FFI).]
The association between glycosylation of proteins and the development of nephropathy still requires much further investigation. The source of the slightly elevated urinary albumin in the group with microalbuminuria was shown to be glomerular and not tubular, by the measurement of $\alpha_1$-microglobulin in this study (Nogawa et al. 1984).

Previous workers have used the presence of raised urinary levels of $\beta_2$-microglobulin to differentiate between glomerular and tubular damage (Viberti et al. 1979; Viberti et al. 1982) but Yue et al. (1983) have shown that $\alpha_1$-microglobulin is more stable at low pH than $\beta_2$-microglobulin, and should therefore be a more reliable index. This is the first study of microalbuminuria in diabetes which incorporates measurement of $\alpha_1$-microglobulin and the results showing a 5-fold increase in $\alpha_1$-microglobulin excretion in the patients with the highest AER confirm that the highly elevated urinary albumin levels are indicative of tubular damage in addition to glomerular damage. Further investigation of $\alpha_1$-microglobulin excretion with relation to albumin and Gly-Alb excretion over a period of time would be valuable.

This study, due to the very limited time that was available, had of necessity to be a cross-sectional one. A follow-up investigation of all the subjects would have yielded much useful information on the development of early nephropathy. There is great variability in individual AER and so further urine collections would have confirmed the classification of subjects according to their AER (Rowe et al. 1985). Causal relationships can only be suggested from a cross-sectional study; a prospective study, following the diabetic and control subjects over a number of years, would be needed to demonstrate conclusively the role of glycosylation in the development of nephropathy.
Breakdown products of the Amadori adduct, carboxymethyllysine and erythronic acid, have recently been reported (Ahmed et al. 1986), and the measurement of these would be a useful marker for assessing the cumulative exposure of tissue proteins to glucose. Comparison of the excretion of these markers with GHb and Gly-Alb levels over a period of time would give additional information on glycaemic control. Measurement of AGEs would also help to complete this information.

This study was a random screen of a diabetic clinic as patient selection was not possible in the time available. There was no computerised system for obtaining patient details, which meant that, due to the short time available, no selection of patients could be attempted. In a follow-up study it would be useful to have a more even distribution of patients with different AERs. The small number of subjects with raised AER coupled with large inter-individual differences, meant that statistical analysis of the data was often inconclusive, despite clear differences in mean values for the groups.

Therefore, larger numbers of subjects in each AER category and age range, studied over a period of time with additional parameters measured would yield more information on the pathogenesis of diabetic complications. Blood pressure data, for instance, was available for so few subjects that it could not be included in the study. More detailed information on the degree of retinopathy and its development would also be desirable.

The role of macrophage uptake of AGEs as a possible mechanism for the removal of senescent molecules in normal ageing and the accelerated ageing of diabetes is also an area requiring further study (Vlassara et al. 1985b). AGEs may be involved in the development of glomerular basement membrane thickening in a number of ways, but the mechanisms are far from being fully understood.
Non-enzymatic glycosylation of proteins not only results in accelerated glucose-derived cross-link formation, but also interferes with the normal function of proteins by blocking the lysine and hydroxylysine groups (Cerami et al. 1986). This aspect of non-enzymatic glycosylation also requires further investigation.

This study helped to confirm an association between protein glycosylation and microalbuminuria. It also indicated that $\alpha_1$-microglobulin might be a suitable index of glomerular/tubular function, and despite the short time available, provided some information on the prevalence of retinopathy and microalbuminuria in an outpatient diabetic clinic population.
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APPENDICES
Dear

I am writing to you to ask for your help with our research involving using some new methods which will give us,

(1) an indication of recent control of diabetes
and (2) an additional test for changes in the urine which can occasionally be associated with a leakage of protein from the kidney.

For this work two samples are required, the first, a timed urine specimen and the second, a blood sample.

If you agree to help, please return the enclosed form together with a telephone number where you can be contacted during the day or evening.

One of the researchers, Miss Julie Taylor will then contact you to arrange for the urine collection. We would suggest that you collect the urine sample during the night prior to your next appointment which is on and bring it with you to the clinic. A 5ml blood sample will also be taken at the clinic along with the normal Blood sugar.

We enclose an envelope for your reply which does NOT require a stamp and is already addressed.

Yours sincerely,

Dr M J Smith
Diabetic Clinic
Dear

Thank you for agreeing to take part in the diabetic urine study. For this study we would like an overnight urine sample to be collected in the bottle provided.

Please perform this test the night before your next clinic visit (that is).

On that evening we would like you
1) NOT to do any strenuous exercise
2) NOT to drink alcohol.

Collection of timed urine specimen.

i) Before going to bed, please empty your bladder down the toilet as normal AND NOTE THE TIME on the label on the bottle.

ii) Please collect ALL of the first urine passed the following morning (that is,) in the bottle provided and again NOTE THE TIME on the label.

iii) Please bring the sample with you to the clinic on where a 5ml blood sample will also be taken.

If you have any further queries please do not hesitate to contact me on Guildford 571281 Extn. 857.

Thanking you for your cooperation.

Yours sincerely

[Signature]

Miss Julie Taylor
## Appendix 3

### Diabetic Subjects: Divided by Duration of Diabetes

<table>
<thead>
<tr>
<th>Duration</th>
<th>Male n</th>
<th>Female n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10 yrs</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>11-20 yrs</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>&gt;20 yrs</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0-10 yrs</th>
<th>11-20 yrs</th>
<th>&gt;20 yrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AER (μg/min)</td>
<td>4.71 (6.26)</td>
<td>9.45 (12.75)</td>
<td>14.5 (23.46)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.40 (3.53)</td>
<td>8.00 (2.05)</td>
<td>7.52 (2.42)</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>11.97 (3.4)</td>
<td>15.01 (3.13)</td>
<td>13.92 (3.32)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>8.93 (7.87)</td>
<td>10.95 (7.51)</td>
<td>10.33 (6.79)</td>
</tr>
<tr>
<td>Urinary α1-microglobulin (mg/l)</td>
<td>5.56 (8.28)</td>
<td>5.28 (3.25)</td>
<td>2.85 (0.88)</td>
</tr>
<tr>
<td>Urinary total protein (g/l)</td>
<td>84.67 (62.83)</td>
<td>103.33 (78.67)</td>
<td>67.78 (29.91)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>14.64 (7.05)</td>
<td>15.85 (6.05)</td>
<td>14.85 (8.34)</td>
</tr>
<tr>
<td>Urinary glucose (mmol)</td>
<td>9.21 (7.78)</td>
<td>11.03 (8.40)</td>
<td>9.36 (8.28)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>72.88 (16.23)</td>
<td>67.02 (13.61)</td>
<td>69.78 (8.44)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40.6 (14.6)</td>
<td>43.8 (14.3)</td>
<td>45.1 (8.1)</td>
</tr>
<tr>
<td>% IDDM</td>
<td>52%</td>
<td>85%</td>
<td>100%</td>
</tr>
</tbody>
</table>

All values shown are means with SD in parentheses.
## Diabetic Subjects Grouped by Type of Diabetes

<table>
<thead>
<tr>
<th></th>
<th>IDDM</th>
<th>IDDM</th>
<th>NIDDM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration Matched</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Duration 1-13 yrs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>56</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td><strong>AER(µg/min)</strong></td>
<td>126 (422)</td>
<td>62 (308)</td>
<td>13.6 (47.9)</td>
</tr>
<tr>
<td><strong>Plasma Gly-Alb(%)</strong></td>
<td>7.91 (2.85)</td>
<td>7.93 (3.24)</td>
<td>7.02 (3.34)</td>
</tr>
<tr>
<td><strong>GHb (%)</strong></td>
<td>13.54 (3.2)</td>
<td>12.89 (3.56)</td>
<td>12.05 (3.86)</td>
</tr>
<tr>
<td><strong>Urinary Gly-Alb (%)</strong></td>
<td>10.85 (8.41)</td>
<td>10.02 (9.42)</td>
<td>8.52 (5.42)</td>
</tr>
<tr>
<td><strong>Urinary αl-micro-globulin (mg/l)</strong></td>
<td>7.04 (9.74)</td>
<td>7.3 (10.01)</td>
<td>3.82 (1.88)</td>
</tr>
<tr>
<td><strong>Urinary Total Protein (g/l)</strong></td>
<td>154 (2.9)</td>
<td>137 (173)</td>
<td>77.6 (43.7)</td>
</tr>
<tr>
<td><strong>Plasma glucose (mmol/l)</strong></td>
<td>16.01 (7.06)</td>
<td>16.88 (7.04)</td>
<td>12.36 (5.47)</td>
</tr>
<tr>
<td><strong>Urinary Glucose (mmol/l)</strong></td>
<td>10.79 (7.77)</td>
<td>12.14 (7.57)</td>
<td>7.13 (7.65)</td>
</tr>
<tr>
<td><strong>Duration of Diabetes (years)</strong></td>
<td>13.6 (10.5)</td>
<td>6.4 (4.0)</td>
<td>4.1 (4.3)</td>
</tr>
<tr>
<td><strong>Body Weight (Kg)</strong></td>
<td>66.0 (10.2)</td>
<td>65.3 (11.0)</td>
<td>81.0 (17.5)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>38 (14)</td>
<td>35 (15)</td>
<td>51 (8.5)</td>
</tr>
</tbody>
</table>

All values shown are means with SD in parentheses.
All The Parameters for the IDDM Subjects Only Grouped by AER

<table>
<thead>
<tr>
<th></th>
<th>0-10 Group A</th>
<th>11-100 Group B</th>
<th>&gt;100 Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>AER (µg/min)</td>
<td>2.86 (2.2)</td>
<td>26.81 (19.1)</td>
<td>1107.38 (813.2)</td>
</tr>
<tr>
<td>Plasma Gly-Alb (%)</td>
<td>7.9 (3.2)</td>
<td>7.7 (1.4)</td>
<td>8.4 (2.8)</td>
</tr>
<tr>
<td>GHB (%)</td>
<td>12.7 (3.1)</td>
<td>15.7 (2.7)</td>
<td>14.2 (2.2)</td>
</tr>
<tr>
<td>Urinary Gly-Alb (%)</td>
<td>9.5 (8.5)</td>
<td>12.4 (8.3)</td>
<td>16.2 (6.0)</td>
</tr>
<tr>
<td>Urinary α₁-M (mg/l)</td>
<td>5.5 (8.6)</td>
<td>6.5 (6.5)</td>
<td>17.5 (15.8)</td>
</tr>
<tr>
<td>Urinary total protein (g/l)</td>
<td>76.4 (42.0)</td>
<td>154.2 (119.2)</td>
<td>(a) 616.6 (403.3)</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>15.1 (7.0)</td>
<td>20.3 (6.3)</td>
<td>13.6 (6.1)</td>
</tr>
<tr>
<td>Urinary glucose (mmol/l)</td>
<td>9.5 (7.8)</td>
<td>15.3 (6.4)</td>
<td>10.1 (7.3)</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>11.7 (9.0)</td>
<td>15.7 (10.8)</td>
<td>21.0 (15.8)</td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>66.1 (10.3)</td>
<td>65.4 (9.9)</td>
<td>66.8 (11.9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.9 (14.1)</td>
<td>37.4 (14.7)</td>
<td>39.5 (11.7)</td>
</tr>
</tbody>
</table>

*a* = significant difference between Group I and Group III

All values shown are means with SD in parentheses