THE STUDY OF GLYCOSYLATED PROTEINS IN DIABETES MELLITUS.

A thesis presented by
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SUMMARY.

The use of glycosylated haemoglobin measurement for monitoring the long-term glycaemic control of diabetic patients is now well established. To enable this material to be measured routinely at the Royal Sussex County Hospital, a method based on affinity chromatography using an m-aminophenylboronate agarose was developed. This technique provides a simple, reproducible assay, which is inexpensive and allows the assay of up to 60 samples per day. A "between-batch" CV of 2% was achieved.

Affinity chromatography was also used for the separation of glycosylated plasma proteins with a "between-batch" CV of 5%. Measurement of the protein content in the fractions collected from the gel was performed by the Coomassie Brilliant Blue method of Bradford. In this way both glycosylated haemoglobin and glycosylated plasma proteins could be measured in the same sample and their individual usefulness in any clinical situation assessed. Investigation of patients admitted to hospital after a period of poor glycaemic control confirmed that glycosylated plasma proteins with their shorter half-life, decreased at a far quicker rate following the onset of treatment, than did glycosylated haemoglobins.

Part of the clinical work showed that both glycosylated haemoglobins and glycosylated plasma proteins could be used to detect glucose intolerance, although glycosylated haemoglobin measurement proved more useful as a screening test for diabetes mellitus possibly due to its better precision. A two-year study
of 35 pregnant diabetic patients established that both glycosylated haemoglobin and glycosylated plasma proteins measured in the third trimester of pregnancy could be used with a high frequency (73.5% and 68.8%, respectively) to correctly predict macrosomia, and with a higher frequency (85.3% and 84.4%) to predict problems at delivery.

Investigation of both measurements in association with miscarriages to diabetic patients showed abnormal glycosylated plasma protein results in 3 cases. No correlation was found between birth weight ratios and glycosylated material in cord blood.

A two-year study of diabetic patients attending an out-patient clinic showed that their overall control, in terms of glycosylated haemoglobin measurement, improved significantly during the period of study. Initial investigations to establish exactly what the affinity gel separates for both the glycosylated haemoglobin and glycosylated plasma protein assay are described.
PREFACE

This thesis has not previously been submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree. All of the investigations in this thesis are the original work of the author, except where specifically stated otherwise.

I should like to thank all of my colleagues at the Royal Sussex County Hospital who have given their help and advice in the production of this work and to Dr. J.G.H. Cook for being my external supervisor and assisting in the preparation of this thesis.

I should particularly like to thank Dr. B.J. Gould for his constant encouragement and advice as my internal supervisor at the University of Surrey during the course of my work. I should like to acknowledge his assistance in the method of development of the assays for both the glycosylated haemoglobins and glycosylated plasma proteins, during the period of his sabbatical year at the Royal Sussex County Hospital. The results of this joint practical work are discussed in Chapter 2 and part of Chapter 3.

I should also like to mention Dr. G. Cawdell for his clinical assistance in obtaining suitable specimens, and to acknowledge Mr. R.A. Sherwood for his advice and enthusiastic support and Mrs. L. Mitchell for typing this thesis.

Finally I would like to extend my greatest thanks to mother-in-law, my mother and to my husband Geoff for taking care of my baby, Stephen, so that I could write this thesis; without their help this would not have been possible.
For Geoff and Stephen,
Phyllis and Stuart, Mum and Dad,
Barry and John

with love
ABBREVIATIONS.

CV    coefficient of variation
SD    standard deviation
IDDM  insulin-dependent diabetes mellitus
OGTT  oral glucose tolerance test
LGA   large-for-gestational-age
RDS   respiratory distress syndrome
TBA   thiobarbituric acid
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Values of glycosylated haemoglobins in cases of aspirin poisoning

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CHAPTER 1.

GENERAL INTRODUCTION.
HISTORICAL BACKGROUND.

The symptoms of diabetes mellitus have been known for many centuries. The term diabetes is, in fact, of Greek origin and derives from the word meaning "passer-through", thus describing the polydipsic and polyuric characteristics of this syndrome. Likewise, the word mellitus is Latin for honey and thus expresses the association of the disease with glucose metabolism.

Many books outline the history of the investigations to show the association between insulin and diabetes mellitus (Faris, 1982, Ireland, Thompson and Williamson, 1980, Bloom, 1978). As quoted, in 1682 Brunner reported that the surgical removal of the pancreas produced extreme thirst and polyuria, but not until 1889 did Mering and Minkowski demonstrate the relationship between diabetes and pancreatectomy. In 1869 Paul Langerhans presented his doctoral thesis noting the presence of previously unreported cell structures in the pancreas. But it was 1893 before the French histologist Edward Laguesse suggested a physiological role for these cell groups, which he named "Ilots de Langerhans". He proposed that they were sites of "an internal secretion which continues throughout existence". This was confirmed by Hedon in the same year, as he was able to prevent the occurrence of diabetes in pancreatectomised animals by grafting pancreatic tissue under their skin.
By the turn of the century there was a general awareness of the association between pancreatic tissue and the control of blood sugar levels. In 1909 Jean de Meyer named the hypoglycaemic secretion from the pancreas "insuline". However, due to proteolytic destruction and the problems this presented to scientists, it was 1922 before Banting and Best obtained their active extract. This preparation was successful in the treatment of a patient in diabetic coma and represents one of the most important advances in medical history.

A more recent article (1982) by Marliss acknowledged the contribution by Banting and Best to medicine, but noted that sixty years after the discovery of insulin, it has not proved a total "cure" for diabetes mellitus. Indeed, although insulin administration prolongs the survival of diabetic individuals, many develop degenerative complications, despite different therapeutic regimes.

**INSULIN.**

Today it is generally accepted that insulin, a pancreatic hormone, occupies a central role in the co-ordination and utilisation of available energy sources and precursors, to provide efficient homeostatic control in any individual (Montague, 1983). In order to understand where this mechanism fails in diabetes mellitus, and the various consequences of that failure, it is pertinent to describe the production and action of insulin in a non-diabetic individual.
Structure, Synthesis and Storage of Insulin.

Human insulin consists of two polypeptide chains, denoted A and B, which are joined by two disulphide bridges. There is an additional interchain disulphide bridge on the A chain. Steiner (1977) noted that these chains are found within proinsulin, a larger, relatively inactive precursor molecule. A complex pathway, involving proteolytic cleavage of this molecule is required to synthesise insulin, which is then packaged within the β-granules (in the β-cells) of the Islets of Langerhans, ready for secretion. The rate of insulin biosynthesis is largely dependent on the extracellular glucose concentration, and when this rises above the fasting level there is a dramatic increase in the rate of proinsulin biosynthesis.

Secretion of Insulin.

There is a wide range of physiological agents which have been shown to affect insulin secretion, suggesting that the β-cell is equipped with a variety of distinct sensor systems which are able to detect and quantify each of these possible factors. (Table 1.1). In addition, the coupling between such sensor systems which results in the exocytosis of insulin molecules is likely to involve a variety of signal molecules. Two such molecules have been identified as playing an important role in linking stimulus to secretion;
Table 1.1  Physiological agents which affect insulin secretion.

**Primary Stimuli.**

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<tr>
<td>Mannose</td>
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<td>Arginine</td>
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<tr>
<td>Lysine</td>
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<tr>
<td>Short-chain fatty acids</td>
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<td>Long-chain fatty acids</td>
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<tr>
<td>Acetoacetate</td>
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<td>B-hydroxybutyrate</td>
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**Secondary Stimuli.**

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<th>Glucagon</th>
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<tr>
<td>Secretin</td>
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<tr>
<td>Pancreozymin</td>
</tr>
<tr>
<td>Gastrin</td>
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<tr>
<td>Acetylcholine</td>
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<tr>
<td>Prostaglandin $E_1$ and $E_2$</td>
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**Inhibitors.**

<table>
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<th>Somatostatin</th>
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<tr>
<td>Adrenaline</td>
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<td>Noradrenaline</td>
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calcium and cyclic AMP. Calcium appears to be the primary signal response for activating the secretory process, and cyclic AMP appears to modulate the size of this secretory response.

As blood glucose concentrations increase above 4 to 5 mmolar the β-cells of the pancreas respond by the biphasic release of insulin. Initially those granules near the plasma membrane of the cell are released, then there is a delay while other granules containing insulin are moved to the surface of the cell via the microtubular system. This secretory response is specific, as galactose and fructose are both without effect. The β-cell must therefore have a specific recognition unit which both senses and responds to changes in extracellular glucose concentration. This unit is likely to be a protein which binds glucose with a low affinity causing the initiation of a series of events leading to depolarisation of the cell and calcium entry. The rate-limiting enzymes of glucose metabolism have been suggested as these units (Montague, 1983A); glucokinase and phosphofructokinase.

The secretory response of insulin is modified by both the circulating levels of D-glucose, certain amino acids, fatty acids and ketone bodies, and the extracellular concentrations of a variety of hormones. Glucagon, pancreozymin and secretin are all produced locally in other cells in the pancreatic islets, and all potentiate the response of the β-cells to glucose. This effect is mediated by the cyclic AMP system of the β-cell. Glucagon is produced by the α-cells, and has metabolic effects opposite to those of insulin and serves to raise blood
glucose. Its production is also stimulated by some L-amino acids and fatty acids, but D-glucose acts as a physiological inhibitor. In this way the Islets of Langerhans play an essential role in regulating nutrient homeostasis in man. The β-cell threshold for the response to D-glucose is about 5 mmolar, but with α-cells suppression is not relieved until below 4 mmolar. This results in a very narrow limit of blood glucose concentration in a normal individual, caused by this very fine hormonal balance. In combination, the α-cells and the β-cells of the pancreas are well designed as an efficient fuel homeostat.

A further important role in the fuel homeostatic system is also played by the hypothalamus, anterior pituitary and adrenal cortex. Here the chemoreceptor functions are apparently confined to the central nervous system and interpreted by the endocrine glands as increased corticosteroid or catecholamine secretion. Both of these act as inhibitors, and have converse effects to those of insulin, serving to raise blood glucose. Inhibitors, such as catecholamines, have the ability to activate α-receptors on the β-cell which causes inhibition of adenyl cyclase and lowers intracellular cyclic AMP levels. As a consequence the secretory response to any stimulus is reduced.
**Insulin Receptors.**

Insulin receptor sites have been demonstrated in both the classic target cells of insulin action (adipose, muscle and liver) and several other cell types with no recognised insulin response (lymphocytes and monocytes). These receptors reversibly bind insulin with high affinity and specificity. The binding of insulin molecules is saturable, occurring rapidly until a steady state is reached. Dissociation also occurs rapidly. Insulin receptors are localised as an integral part of the plasma membranes of these cells. When a receptor binds insulin, together they become internalised by the process of endocytosis. Insulin is then rapidly degraded but the more resistant receptor may be cycled back to the cell membrane for reuse. The mechanism as to how the interaction between insulin and its receptor initiates the response is not yet understood (Houslay and Heyworth, 1983), although a second messenger has been proposed. Again calcium and cyclic AMP are likely to be involved to alter protein kinase activity and thereby to alter enzyme phosphorylation. Regulation of the number of receptors may allow a cell to alter its response to insulin.
Insulin Action.

The physiological effects of insulin in man occur at plasma concentrations of about $10^{-10}$ molar and affect carbohydrate, protein and lipid metabolism. When such fuels are in excess, following a meal, insulin promotes their rapid uptake and storage. When these fuels are subsequently required, insulin promotes their controlled release. In this way insulin controls homeostasis of fuel molecules maintaining them at levels suitable for tissue requirements, at all times. This is of particular importance for some tissues, such as erythrocytes, the retina, the kidney, and to a lesser extent the brain, which are obligatory glucose users. Other tissues can adapt to different nutritional states by altering their energy source, but these tissues have a constant demand for glucose.

In the fed state insulin ensures that glucose is rapidly removed from the blood and utilised by the tissues, keeping to a minimum the period of hyperglycaemia. It promotes the storage and utilisation of glucose by muscle, adipose tissue and the liver. The storage of glucose by both the liver and muscle is achieved by the formation of glycogen, as insulin increases glycogen synthetase activity and inhibits glycogenolysis by reducing phosphorylase activity. When the glycogen stores are full, excess glucose is then either
Fig. 1.1 To illustrate the conversion of glucose to ATP via glycolysis and the Kreb's cycle.

GLYCOGEN → ATP

GLUCOSE → GLUCOSE-1-PHOSPHATE → GLUCOSE-6-PHOSPHATE → FRUCTOSE-6-PHOSPHATE

ATP

GLYCERALDEHYDE 3-PHOSPHATE → NAD → NADH₂

1,3, DIPHOSPHOGLYCERATE → ADP → ATP

3, PHOSPHOGLYCERATE

2, PHOSPHOGLYCERATE

OXALOACETATE → GTP

PHOSPHOENOLPYRUVATE

PYRUVATE → ATP

MITOCHONDRIAL WALL

MALATE

FUMARATE

ACETYL CoA

OXALOACETATE

CITRATE

NADH₂, FADH₂ and GDP can be all used in the production of ATP for energy via the process of oxidative phosphorylation.
oxidised for ATP synthesis via the Krebs cycle (Fig. 1.1), or converted via lipogenesis into fatty acids which are then stored in adipose tissue as triglycerides. In general, muscles oxidise glucose whereas liver and adipose tissue are sites for lipogenesis. In order to promote glucose utilisation via glycolysis, insulin increases the activity of the rate-limiting enzymes, pyruvate kinase, pyruvate dehydrogenase and possibly 6-phosphofructo-1-kinase. Insulin also encourages lipogenesis from acetyl CoA via the activation of acetyl CoA carboxylase, and promotes the esterification of newly formed fatty acids by increasing the availability of glycerol phosphate. In addition, it may stimulate the activities of diacylglycerol acyltransferase and glycerolphosphate acyltransferase. In the fed state insulin also ensures the more rapid uptake of dietary triglycerides by increasing the activity of lipoprotein lipase. In addition fatty acids are not released as insulin inhibits triglyceride lipase, and ketone production by the liver is kept to a minimum. Dietary protein is also taken up by tissues and insulin has a general anabolic effect.

**Insulin Degradation.**

Insulin is removed from the circulation by degradation. This occurs primarily in the liver, but most peripheral tissues have the specific enzymes to degrade insulin; insulin protease and insulin glutathione transhydrogenase. These enzymes are located within cells and degrade insulin
after it enters the cell by endocytosis. Insulin degrading activity in the liver changes in the fed and fasted state.

**DIABETES MELLITUS.**

As the Islets of Langerhans regulate nutrient homeostasis, it is not surprising that when pancreatic endocrine function is derranged this causes profound effects on blood nutrient content. In most cases there is a reduction in the hormone produced and this is most commonly recognised in the major clinical condition of diabetes mellitus. Although primary diabetes mellitus is a heterogeneous syndrome resulting from any one or combination of factors altering insulin production, it is usually considered as two major disease types; type 1 (juvenile-onset); type 2 (maturity-onset). However it is worth noting that these two categories are far from separate (Fajans, Cloutier and Crowther, 1978) even though they are often considered as such. Secondary diabetes mellitus can occur when other metabolic factors alter, which oppose the action of insulin, such as high levels of growth hormone.

**Type 1 Diabetes Mellitus.**

Type 1, or insulin-dependent diabetes mellitus is characterised by an absolute deficiency of insulin and massive β-cell lesion and necrosis. In human diabetics there must be 80% or more loss of functional β-cells before the fasting insulin levels decline. As insulin is not
adequately produced by the pancreas these patients are treated by the administration of insulin, usually intra-muscularly. If insulin is not present in sufficient quantities other hormones can act unopposed with dramatic consequences. Fasting hyperglycaemia is a major metabolic feature, and this is used as one criteria for diagnosis of this disease (WHO.2nd.Report, 1980). Fasting blood glucose rises when insulin is not available to facilitate the uptake of glucose by such tissues as muscle, liver and adipose tissue. In addition other hormones such as glucagon act unopposed to increase glycogenolysis and gluconeogenesis but inhibit glycogen synthesis, glycolysis and lipogenesis. The liver produces rather than utilises glucose, so blood glucose levels rise, and when these exceed the renal threshold (about 10mmolar) glycosuria results. Loss of glucose in the urine causes polyuria due to the osmotic effects of glucose. Liquid intake is increased to accommodate frequent urination.

There are also dramatic changes in lipid and protein metabolism seen with untreated type 1 diabetes mellitus. Lipolysis, proteolysis and ketogenesis all increase, with consequent rise of fatty acids, amino acids and ketones in the blood. Amino acids provide the substrates for gluconeogenesis, the fatty acids for ketogenesis. Clinically, as muscle and adipose tissue are lost, body wasting may be noted. As the concentration of ketones continues to rise in the blood these exceed the renal threshold and ketonuria occurs. Acetone may be smelt on the breath. The major ketone
bodies are strong acids, and so as their concentration increases they cause the buffering capacity of the blood to fail and cause acidosis. Unless insulin is administered to a patient in this condition, the patient will die, due to both dehydration and acidosis which affect the central nervous system.

**Type 2 Diabetes Mellitus.**

Type 2 diabetes mellitus is usually less severe than type 1, usually not requiring insulin but treatment by diet or oral glycaemic agents alone. Fasting plasma insulin levels may be normal, or even increased, although there is a relative lack of insulin efficacy under certain circumstances, such as after a meal. This is due to either the β-cell secretory system failing to respond rapidly enough to stimuli, or the insulin sensitive tissues having reduced sensitivity to insulin. This target-organ resistance to insulin is exacerbated by diet and obesity as well as by genetic, endocrine and nervous system factors. If the response or the production of insulin is altered then persistent hyperglycaemia may result.

In chronic hyperglycaemia, due to insufficient insulin administration or poor dietary control in both type 1 and type 2 diabetes mellitus there is a less dramatic rise in blood glucose levels than the acute situation described earlier, but increases are observed above those found in normal individuals. Although no immediate effects of this high blood glucose are apparent, it has been shown (Engerman et al. 1982,
Chobanian et al. 1982) that patients who consistently have a high level of blood glucose are more at risk later in life of having "diabetic complications", such as retinopathy, neuropathy and cardiovascular disease. Aspects of the importance of good control will be discussed in detail later. The administration and availability of insulin has therefore overcome the problems of an acute, possibly fatal disease, but potentiated a chronic disorder with degenerative complications.

MONITORING OF CONTROL IN DIABETES MELLITUS.

Historically, the efficacy of treatment of diabetic control by insulin or diet has been measured using both urine and blood samples. Initially reducing substances in urine were measured by the ability of a urine sample to reduce a test substance (Varley, Gowenlock and Bell, 1980) and cause a detectable colour change. Benedict's solution was usually used for this purpose, changing from green to a reddish-brown according to the amount of reducing substance present. These tests could therefore be either qualitative or quantitative depending on the degree of technical accuracy employed. However reducing substances other than glucose, such as uric acid or ascorbic acid may be present in sufficient quantities in a urine sample to cause interference. Reducing substances in blood were also measured after precipitation of blood proteins. The ability of the sample to reduce either
alkaline cupric copper solution to cuprous oxide, or alkaline potassium ferricyanide to ferrocyanide, was then measured either iodometrically or colorimetrically.

As methodology improved, the specific measurement of glucose in both blood and urine could be achieved. 'Clinistix' are marketed by Ames Co. Ltd. Each stick has an area impregnated with the enzymes glucose oxidase and peroxidase, together with o-tolidine and a pink dye. When dipped in urine, any glucose present is oxidised to gluconic acid and hydrogen peroxide. O-tolidine is then oxidised to give a blue colour which, in combination with the pink dye present, produces various shades of purple, the depth of colour indicating the concentration of glucose in the urine sample. Blood glucose could also be measured using similar biochemistry.

These were laboratory tests which were, until fairly recently the only ones available to the clinician to aid the clinical assessment of long-term diabetic control. They are also used in the acute situation for the management of diabetes mellitus together with the measurement of urinary ketones. For the past decade this test repertoire has increased in many laboratories to include the measurement of glycosylated haemoglobins.
Discovery of Glycosylated Haemoglobins.

In 1955 Kunkel and Wallerius reported that haemoglobin could be separated using starch gel electrophoresis, into both a slow (HbA₀) and a fast moving band (HbA₁). Allen, Schroeder and Balog in 1958 achieved similar separations using a cation-exchange column, and they were further able to resolve the fast moving band (HbA₁) into three components designated HbA₁a, HbA₁b, and HbA₁c according to their order of elution from the column. Further studies by McDonald et al. (1978) have shown HbA₁a may be further separated into two peaks, HbA₁a₁ and HbA₁a₂ (Fig. 1.2), and more recently Castagnogla et al. (1983) separated HbA₁b into two peaks, HbA₁b₁ and HbA₁b₂.

The clinical significance of these faster moving minor haemoglobin fractions was not understood until 1968 when Rahbar noted their presence in the blood of certain diabetic patients at levels much higher than those found in the blood of non-diabetic individuals. Since this study there has been considerable work both to identify the chemical nature of these peaks and to examine their clinical relevance for the monitoring of diabetic treatment.
Elution pattern of normal and diabetic hemolysates chromatographed on Bio-Rex 70. The concentration of haemoglobin is shown by --- shows the presence of ketoamine-linked sugar detected by the TBA test. Haemoglobin-bound sugar was detected in the leading edge of the Hb A_o peak as well as in Hb A_1c.

Bunn et al. (1979) JBC. 254, 3892.
The Chemical Nature of Glycosylated Haemoglobins.

The major component of normal human red cells is HbA, which consists of two \( \alpha \) chains and two \( \beta \) chains \((\alpha_2 \beta_2)\) and comprises about 97% of the total haemoglobin. The remainder of the haemoglobin molecules are classed as HbA2 \((\alpha_2 \delta_2)\) which represents about 2% of the total, and HbF \((\alpha_2 \gamma_2)\) which represents less than 1%. These haemoglobins differ genetically from the bulk of the haemoglobin (HbA).

The minor haemoglobins (HbA\(_{a1}\), HbA\(_{a2}\), HbA\(_{lb}\) and HbA\(_{lc}\)) are post-translational modifications of HbA (Bunn, 1981). Recently Tegos and Beutler (1980) have shown that HbA\(_{a1}\) also has components analogous to those in HbA\(_{a1}\), but since most haemoglobin in human adults is in the HbA form, the post-translational forms of HbA\(_{a2}\) are usually considered insignificant and ignored. HbA\(_{lc}\) is the most abundant of the minor post-translational modified forms of haemoglobin (comprising about 4% of the total haemoglobins in non-diabetic individuals) (Bunn, 1981), and was thus extensively studied to elucidate the nature of this chemical modification.

1. The chemical nature of HbA\(_{lc}\), synthesis and kinetics.

Holmquist and Schroeder (1966) showed that the N-terminal valines of the \( \beta \)-chains are blocked by an unidentified residue containing a linkage capable of being reduced by sodium borohydride, the \( pK \) of this group being about 6.65. Their results indicated that a Schiff base was formed. They demonstrated that the blocking group had a mass spectrum comparable with a hexose linked to the N-terminal valine, although they could provide no information about its
identity. Dixon (1972) reacted glucose with peptides to show that the glycosylating agent in HbA\textsubscript{1c} could be glucose, and that glucose could theoretically react with many other amino groups, although at a slower rate. However it was Flückiger and Winterhalter (1976) who provided evidence using D-(2-\textsuperscript{3}H) glucose incubation experiments that the initial product of glucose and haemoglobin undergoes an Amadori rearrangement (Fig. 1.3). Tritium is lost from the second carbon in an Amadori rearrangement and so, by measuring the ratio between \textsuperscript{3}H and \textsuperscript{14}C, it can be determined if this rearrangement has occurred, and to what extent. Such studies indicated that about 91\% of the initial material had undergone the rearrangement after 22 days and that glucose had been thus converted to 1-deoxy-D-fructose attached to the haemoglobin molecule.

The term "glycosylated haemoglobin" was coined as a result of all of these investigations to indicate that initially a condensation reaction occurs between glucose and haemoglobin (Bunn, 1981) (Fig. 1.3). Although this is the terminology used in this thesis, a recent letter by Roth (1983) drew attention to the fact that glycosylated haemoglobin should more correctly be called glycated haemoglobin, as this more accurately describes the amino-linked 1-deoxyfructose derivative of haemoglobin. In the case of HbA\textsubscript{1c} this reaction occurs at the N-terminal valine of the \textbeta-chains of haemoglobin molecules. Originally an aldimine linkage is formed which is labile, as the process
Fig. 1.3 To illustrate the reaction of glucose with haemoglobin to form $\text{HbA}_{1c}$. 

Glucose $\rightarrow$ (pre-$\text{A}_{1c}$) Aldimine $\rightarrow$ Stable

Unstable Schiff base $\rightarrow$ Ketoamine

$K_1$ $0.3 \times 10^{-3}$ mM$^{-1}$ h$^{-1}$

$K_2$ $0.0055 \pm 0.0010$ h$^{-1}$

$K_{-1}$ $0.33$ h$^{-1}$
is reversible (Fig. 1.3). However if the availability of glucose remains then, by the law of mass action, a percentage of this Schiff base will undergo an Amadori rearrangement to the stable ketoamine form previously described (Fig. 1.3), a process which is essentially irreversible.

The kinetics of this two-part reaction have been studied using various in vitro and in vivo experiments. In 1975 Koenig and Cerami showed that a haemoglobin variant existed in diabetic mice which could be shown to be very similar to the HbA\textsubscript{lc} moiety found in humans. By injecting labelled (Na\textsubscript{3}H\textsubscript{4}) reticulocytes into both normal and diabetic mice they showed that the rate of synthesis of HbA\textsubscript{lc} in the diabetic animals was much faster, concurring with Rahbar's association of the molecule with diabetes. In vitro synthesis experiments incubating suspensions of reticulocytes and bone marrow cells (Bunn et al., 1976) with (\textsuperscript{3}H) leucine have shown that there is a slower increase in the specific activity incorporated into HbA\textsubscript{lc} than in HbA\textsubscript{o}, strongly suggesting that the addition of glucose to haemoglobin occurs as a post-translational modification. Also in vivo work by Koenig and Cerami where they injected (\textsuperscript{59}Fe) transferrin into two individuals, showed that the specific activity of HbA\textsubscript{o} rose rapidly to a maximum in the first week and remained at this level, whereas the specific activity of HbA\textsubscript{lc}, HbA\textsubscript{la} and HbA\textsubscript{lb} increased gradually over a period of about 60 days. This reflects the slow formation of these haemoglobins continuously and nearly irreversibly from HbA during the life span of the red cell. Studies separating
young and old erythrocytes using density gradient techniques support this idea as they show higher concentrations of HbA\textsubscript{lc} in the old cells (Fitzgibbons, Koler and Jones, 1976), although the efficiency of this type of separation has been questioned (Svendsen \textit{et al.} 1981).

The \textit{in vivo} synthesis of HbA\textsubscript{lc} after an infusion of \textsuperscript{59}Fe-bound transferrin (Svendsen \textit{et al.} 1981) was shown to be slow, this being consistent with the non-enzymic nature of the process as postulated by Bunn \textit{et al.} (1975).

Mathematical models of both the \textit{in vitro} and \textit{in vivo} work described indicate that the stable ketoamine form of HbA\textsubscript{lc} after the Amadori rearrangement forms at a rate of about 0.006\% of total Hb/ mmol glucose /24hr. (Svendsen \textit{et al.} 1981).

The kinetics of both the condensation reaction (reaction rate constant \(k_1\)) to form the Schiff base, and the rearrangement reaction (reaction rate constant \(k_2\)) to form the stable ketoamine have been studied in great detail (Bunn \textit{et al.} 1976, Gabbay \textit{et al.} 1977, Higgins and Bunn, 1981). Cyanoborohydride was introduced into the reaction mixture of haemoglobin and D-\textsuperscript{14}C glucose to ensure that the Schiff base was totally converted to the stable compound. Cyanoborohydride reduces the labile component (Fig. 1.3) but does not affect the C=O in the stable molecule. In this way the reaction rate constant \(k_1\) could be measured. The reverse reaction rate constant \(k_{-1}\) was derived by incubating the same components but excluding the cyanoborohydride. The reaction to form the stable ketoamine was allowed to proceed for 6-21 days and then HbA\textsubscript{lc} was separated and quantified. These rate constants describe the chemical mechanism for the formation of HbA\textsubscript{lc} and can also be used to assess the distribution of both the labile Schiff base
and the stable ketoamine in both normals and diabetics. Most importantly they indicate that the rate of formation of stable HbA$_{1c}$ should be directly proportional to the time-averaged concentration of glucose within the red cell. For this reason a non-diabetic individual having blood glucose within narrow limits of about 3-5mmol/l will still have a percentage of their haemoglobin converted to HbA$_{1c}$. This percentage though, will be considerably lower than for a diabetic patient who consistently has a higher blood glucose concentration. In vitro experiments incubating erythrocytes from both patients with IDDM and non-diabetic controls with identical glucose concentrations (Spicer et al. 1979) show that HbA$_{1c}$ is synthesised at the same rate in both cases. They confirm that the production of HbA$_{1c}$ is related to time, pH, glucose concentration and temperature as it is increased from 4°C to 37°C.

Once the stable HbA$_{1c}$ is formed in the red cell it remains within that cell until it is removed by the reticuloendothelial system. The average life-span of an erythrocyte is about 120 days and within any cell there will be a gradual accumulation of HbA$_{1c}$ over this period. Since the removal and synthesis of erythrocytes is constantly occurring, any single measurement of stable HbA$_{1c}$ at any point in time will reflect the time-averaged accumulation of these molecules throughout the whole population of red cells. As the half-life of an erythrocyte is about 50 days therefore,
a single HbA\textsubscript{lc} measurement should provide information about a patient's glycaemic history over the previous 3-10 weeks. This retrospective capability of the HbA\textsubscript{lc} measurement is supported by two observations. The life-span of red cells in some species of animals is shorter and likewise the percentage of HbA\textsubscript{lc} in the non-diabetic members of these species is decreased. Similarly if a human patient has haemolytic anaemia their level of glycosylated haemoglobin will be lower, and so all interpretation of HbA\textsubscript{lc} results should be made with the realisation that any abnormality of an individual's red cells affecting their life-span will alter the results.

(2) The chemical nature of HbA\textsubscript{la1}, HbA\textsubscript{la2} and HbA\textsubscript{lb}.

Haney and Bunn (1976) investigated the reaction of the sugar phosphates found in erythrocytes with HbA\textsubscript{o} and discovered the glycosylation rate to be about 20 times faster than associated with glucose. Carbon labelling experiments of glucose 6-phosphate showed that the incorporation of this molecule occurred at the N-terminal of the $\beta$-chain. Stevens et al. (1977) showed that many sugar phosphates react more rapidly than glucose with haemoglobin. These include fructose 6-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate and ribulose 5-phosphate, although glucose 1-phosphate and glucose 1,6-diphosphate have been shown not to react (Dolhofer and Wieland, 1978). This would suggest that the rapidly reacting sugars have an aldehyde or ketone group.
which is separated from a weakly charged negative group (Bunn, Gabbay and Gallop, 1978).

Co-chromatography work (Bunn, Gabbay and Gallop, 1978, McDonald et al. 1978) of these synthetic post-translational haemoglobins indicates that the attached molecules of HbA_{1a} and HbA_{1a_2} are fructose 1,6-diphosphate and glucose 6-phosphate respectively. Although these sugar phosphates react inherently faster than glucose with haemoglobin, since their concentrations within the red cell are considerably lower this is reflected in the smaller peaks observed. (Fig. 1.2). As neither fructose 1,6-diphosphate or glucose 6-phosphate are elevated in diabetes mellitus it is not surprising that no increase has been observed in HbA_{1a_1} or HbA_{1a_2} (Bunn, Gabbay and Gallop, 1978).

The true nature of HbA_{1b} has yet to be determined. Krishnamoorthy, Gacon and Labie (1977) showed acidification of the modified \( \beta \)-chain, and a dissociation constant of the group responsible for this acidification which suggested deamination had occurred. This is in direct conflict with Garrick et al. (1980) who show HbA_{1b} to be negatively charged, reducible with borohydride and producing a positive test with thiobarbituric acid.

(3) The chemical nature of glycosylated A_0.

As well as the HbA_{1c} peak which can be separated by chromatographic means from HbA_0 (Fig. 1.2), Bunn et al. (1979) found that the leading edge of HbA_0 also contained carbohydrate as detected by the thiobarbituric acid test. This "glycosylated haemoglobin" (GHB_A_0) which did not separate from the main peak represented about 8-10% of the total haemoglobin found in non-diabetic individuals. Bunn et al.
(1979) also showed, using parallel incubation studies with $^3$H glucose (bound to the second carbon atom of the glucose molecule), that both synthetic HbA$_{1c}$ and GHbA$_o$ have undergone Amadori rearrangements to the more stable ketoamine linkage. Further work shows that the glucose in GHbA$_o$ is attached to the amino terminus of the $\alpha$-chains, and via $\varepsilon$ amino groups of lysines on either the $\alpha$ or $\beta$-chains. In all cases the charge characteristics are not sufficiently altered by the attachments to generate chromatographically distinct species and they remain within the main peak HbA$_o$ (Fig. 1.2).

(4) **Additional factors which may affect the glycosylation of haemoglobin.**

Recently two other factors which may, or may not affect the glycosylation of haemoglobin have been discussed; notably the role of red cell permeability to glucose, and the presence, if any, of enzymes which affect the glycosylation process.

Higgins, Garlick and Bunn (1982) showed that non-enzymic glycosylation of haemoglobin in mammals was determined by three main variables; mean plasma glucose, red cell life span and the red cell permeability to glucose. They were able to study this last characteristic which varies in different species of animals. *In vitro* experiments where glucose has been incubated with extracts of either old or young red blood cells (Gillery et al. 1982) have shown a
significant increase in the amount of HbA\textsubscript{lc} formed in the young cells in contrast to the very small amounts formed in the older cells. They suggested the presence in the young erythrocyte membranes of a factor responsible for the transfer of glucose, which is lost in the older cells. Whether or not this is the case, the passage of glucose into the red cell either by passive or active transport appears to be important in the formation of glycosylated haemoglobin. Although the nature of the factors influencing this transfer of glucose is not yet clear, it has been suggested that a glycoconjugate, of still unknown structure and under enzymic control, is involved (Gillery et al. 1982).

The presence of other enzymes which cleave the carbohydrate from the stable form of glycosylated haemoglobin (Fig. 1.3) has also been suggested (Lubec et al. 1983). In 1976 Bunn et al. developed equations encompassing the rate of synthesis and net accumulation of HbA\textsubscript{lc} under various conditions. He used three possible mathematical models to describe the process, termed "reversible," "irreversible" and "slightly reversible" and showed that his experimental data best fitted the latter category. This indicated that there was possibly an enzyme present which could reverse the transfer of the labile to the stable form. Recently Lubec et al. (1983) have produced evidence indicating that there are enzymes present in diabetic lysosomes which can cleave the bond between protein
and carbohydrate molecules. If present, these enzymes are N-1-deoxyfructofuranosyl-glucohydrolases and, in contrast to glucosidases, only a few enzymes hydrolysing glycosyl compounds have previously been described in mammalian systems. The effect and significance of that effect, if any, of these enzymes has yet to be investigated, but it would seem that although other enzymic pathways may influence glycosylation of haemoglobin, the non-enzymic pathway as postulated by Bunn et al. (1979) still remains of major importance. Monnier (1983) questioned the evidence presented by Lubec et al. (1983) suggesting that their data was not sufficient to support the existence of an enzyme which can hydrolyse ketoamine-linked glucose. If this enzyme does not exist, then presumably the enzymes found in lysosomes by Lubec et al. (1983) are associated with another catabolic pathway which does not affect sound red cells.

(5) **Lability.**

Now that the chemical nature of glycosylation has been elucidated, it is widely accepted that there are two steps to the non-enzymic addition of glucose to a haemoglobin molecule (Fig. 1.4); with the initial formation of the labile aldimine before the second step to the stable ketoamine product. The presence of these two glycosylated haemoglobin fractions, together with the wide multitude of techniques used for their measurement has lead to conflicting results and confusion in the literature (Svendsen et al. 1979) when
Fig. 1.4 To show HbA$_{1c}$ when a glucose molecule is attached to the terminus of the $\beta$-chains of the normal adult haemoglobin molecule, HbA.

from: Jovanovic L, Peterson CM. (1978) Hemoglobin A$_{1c}$ - the key to diabetic control. LAB 78; July/Aug. : p.12.
interpreting glycosylated haemoglobin results in terms of clinical findings. To ensure that the usefulness of the glycosylated haemoglobin measurement is not brought into question, many workers recommend the routine removal of the labile adduct (Daneman, Luley and Becker, 1982, Compagnucci et al. 1981).

GLYCOsylation OF PROTEINS OTHER THAN HAEMOGLOBIN.

As early as 1912 Maillard (quoted by Monnier and Cerami, 1982) suggested that chemical reactions known as "browning" reactions occur, and that they may play an important role in the secondary complications observed with diabetes mellitus. In these reactions reducing sugars attach to amino groups of proteins forming Schiff bases which polymerise to give a brown product, hence the terminology. Although the food industry has long recognised the importance of these reactions in the way they influence the availability of essential amino acids, or toxicity of food products, the metabolic implications have until quite recently been virtually ignored. However, since the awareness of the non-enzymic glycosylation of haemoglobin in vivo, this has led to investigation of glycosylation in other proteins. Subsequently interest has been shown in assessing both the effect of glycosylation on the biological function of different proteins, and also determining whether their measurement is of any value in monitoring glycaemic control.
Glycosylation of Proteins - Possible Pathophysiological Complications.

Perhaps the most far reaching and challenging aspect of non-enzymic glycosylation is its possible role in the long term complications of diabetes mellitus. This post-translational modification appears to be widespread, affecting many diverse tissues and proteins. The type and longevity of the protein studied should be crucial as to the effects observed.

The first tissue to be investigated to try and link non-enzymic glycosylation and the pathological sequelae of diabetes mellitus was the ocular lens, and therefore lens protein. In vitro studies showed that lens protein could become glycosylated to form protein aggregates which diffracted light (Monnier and Cerami, 1983). This observation mimicked the in vivo findings of the protein within the diabetic lens (Kasai et al. 1983). Further in vitro glycosylation of the protein caused "browning" to occur. If glycosylation is occurring within the lens this will be essentially an irreversible effect as lens crystallin has remarkable longevity. Glycosylation therefore, may be implicated in the development of diabetic cataracts and retinopathy (Kennedy and Baynes, 1984).

As glycosylation of haemoglobin is well studied, the investigation of possible glycosylation of the proteins of the red cell membrane was a logical choice. McMillan and Brooks (1982) studied spectrin, which is the major protein
of the inner surface of red blood cell membranes. This protein is incorporated into a network which allows the erythrocyte to deform and return to its original shape. This flexibility of form is reduced in diabetics, but as yet there is no evidence to suggest that this is a direct consequence of glycosylation. It is possible however that glycosylation may be indirectly implicated through its inhibition of membrane protein transglutamidation which is responsible for the removal of aged erythrocytes.

Arteriosclerosis occurs at an accelerated rate in diabetes mellitus and so the role of glycosylation of lipoproteins was studied to see if any connection could be produced. In vitro and in vivo glycosylation of both LDL and HDL has been demonstrated (Schleicher, 1981) using the furosine method. Glycosylation of lipoproteins was higher in diabetics than in the control group. In vitro studies of $^{14}$C glucose uptake showed that the rate of glycosylation of lipoproteins was about 25% less than that observed for albumin. These workers argued that as the chemical modification of apo-B lysine residues by acetylation increases the cellular uptake of LDL and cholesterol via scavenger receptors in peripheral cells, it follows that glycosylated modifications of lysine residues may produce a similar effect. Gonen et al. (1981) found that in vitro glycosylated LDL became enclosed in human fibroblasts and so was degraded less than the control LDL. Glycosylation of LDL also impairs its ability to stimulate cholesteryl synthetase (Witztum et al. 1982). The implications of all of
these findings are that glycosylation may alter the function of lipoproteins and so contribute to the problems of arteriosclerosis associated with diabetes mellitus. Similarly Brownlee, Vlassara and Cerami (1983) demonstrated that the glycosylation of fibrin occurs and that this modification reduces the susceptibility of fibrin to plasmin degradation. Acetylation or carbamylation at the lysine amino groups may cause a similar effect. The accumulation of fibrin within a diabetic patient may well contribute to vascular problems.

Chavers, Etzwiler and Michael (1981) used immunofluorescent techniques to show that there was increased deposition of albumin in dermal capillary membranes associated with diabetes mellitus, (IDDM). In vitro studies of the incorporation of $^{14}$C-glucose into purified rat glomerular basement membranes (Cohen et al. 1981) indicated that increasing glucose alters the chemistry of this, and other basement membranes. This suggests that glycosylation may be the cause of altered protein metabolism leading to its accumulation, and that it may relate to the various microangiopathic sequelae associated with diabetes mellitus. Kohn and Schnider (1982) found that glycosylation of collagen occurs at the $\alpha$-amino group of lysine. They (Kohn, Cerami and Monnier, 1984) later showed that there was an association between glycosylation and the cross-linking of collagen as found in the ageing processes. This work was supported by Eble, Thorpe and Baynes (1983). The cross-linking causes decreased solubility of collagen in
a similar manner to that observed when albumin (Kennedy et al. 1981) or lens protein (Monnier and Cerami, 1982) are glycosylated. For this reason Kohn and Schnider recommended control of glycaemia, particularly in periods of collagen synthesis. Similarly Yue et al. (1983) demonstrated glycosylation of collagen but related it to increased thermal rupture, or collagen stability. Bassiouny, Rosenberg and McDonald (1983) found that collagen altered by glycosylation became antigenic. Myelin, both peripheral myelin and that of the central nervous system, have been shown to be subject to excessive glycosylation in diabetes. This work was done by borohydride reduction followed by separation on a m-aminophenylboronic acid gel, Biogel P6, which selectively retains glycosylated amino acids. The appropriate aliquots of amino acids were then analysed. The glycosylation of myelin may contribute to the functional abnormality of myelinated neurons associated with diabetes mellitus. (Vlassara, Brownlee and Cerami, 1983).

Short-lived proteins (eg. peptide hormones) may also become glycosylated. However as the process is non-enzymic it is relatively slow and the corresponding glycosylation of these short-half-life-molecules is likely to be so small as to have an insignificant effect. Glycosylation of peptides and amino acids as investigated by affinity separation of the different sugar groups (Brownlee, Vlassara and Cerami, 1980) followed by specific chemical identification may reveal more about any alterations in their biological properties. Other aldose sugars may also react significantly with protein amino groups. Urbanowski et al. (1982) demonstrated galactosylation of albumin in association with a child that
had galactosaemia. This modification of the protein is very similar to that of glycosylation with an Amadori rearrangement and may contribute to the complications of the disease.

Although there is evidence for glycosylation of many proteins which must have functional abnormalities to cause the observed pathological defects observed with diabetes, the evidence of the modification actually causing these effects is poor. Most of the work has been performed in vitro. It is known that the binding of glucose to the end of the $\beta$ chain of haemoglobin ($\text{HbA}_1\text{c}$) decreases the binding of 2,3-DPG at that site. As binding of 2,3-DPG serves to shift the oxyhaemoglobin dissociation curve thereby reducing the affinity of haemoglobin for oxygen, it would follow that high levels of $\text{HbA}_1\text{c}$ in vivo would result in higher oxygen affinity. This is not observed as there is a corresponding increase of 2,3-DPG in diabetic red cells to compensate for this effect (Arturson et al. 1974).

The effect of reductive alkylation on the properties of proteins has been noted by Means and Chang (1982) and only a few proteins have as yet been identified as having their function altered by modification. These include HDL and LDL in humans. The overall evidence suggests that these are simply the early investigations into this problem and as yet no proven connection between glycosylation and its effect on the function of proteins has been conclusively shown. If in the future this were to be the case then the argument for stringent diabetic control would be irrefutable.
Chemistry and Kinetics of Protein Glycosylation.

Reactions between reducing sugars and the amino groups of proteins are now widely recognised, as is the natural occurrence of many non-enzymically glycosylated proteins. The rate and extent of the reaction will depend on temperature, concentration and time of contact (Yue et al. 1980), as well as the basicity of the carbonyl group and its steric accessibility (Means and Chang, 1982). The amine basicity appears to have little significance but its pKa value does. For this reason the $\alpha$-amino groups which usually have lower pKa values than the $\varepsilon$-amino groups should be more susceptible to non-enzymic glycosylation. However, the $\varepsilon$-amino lysine groups are generally more accessible in proteins for glycosylation, haemoglobin being a special case. The importance of steric hindrance can be demonstrated by the fact that the addition of acyclic amines to formaldehyde is considerably less favoured than the addition of the simple primary amines (Means and Chang, 1982). After the attachment of the carbonyl group to the amine an Amadori rearrangement occurs, probably followed by cyclization for stability of the product. Evidence for the Amadori rearrangement was provided by Bunn et al. (1975) who demonstrated that on hydrolysis albumin yielded glucose and mannose in the ratio of 4:1. The amino portions of proteins are susceptible to modification by many different aldehydes; formaldehyde; acetaldehyde and glucose, all of which cause "browning" at
high levels (Fantyl, Stevens and Peterson, 1982). Consequently the studies of glycosylation in association with diabetes may involve other molecules which bind to proteins. The attachment of acetaldehyde in alcoholics or the carbamylation of proteins in association with uremia may also contribute to the various pathological effects observed.

Yue et al. (1980) showed that plasma proteins of all sizes could undergo non-enzymic glycosylation, and that there was no difference between normal and diabetic plasma proteins. The elevation of glycosylated plasma proteins (GPP) was directly proportional to that of glycosylated albumin (GAlb) (McFarland et al. 1979) but only 60-70% of the total glycosylated material was attributable to albumin. Studies of glycosylated proteins other than haemoglobin (Day et al. 1979) by incubating human serum with tracer amounts of labelled glucose showed a linear accumulation of non-dialysable label. Further investigation indicated that the radioactivity was associated with three major molecular weight proteins: albumin, IgG and IgM.

When assessing the use of glycosylation as a tool with which to monitor glycaemic control, both GPP and GAlb as well as glycosylated haemoglobin have been used. Dolhofer and Wieland (1980) showed a good correlation of GAlb with glycosylated haemoglobin (r=0.88, n=74) and Day et al. (1980) with fasting glucose (r=0.91), illustrating the interrelationships between these various components. Since
the survival of albumin in the circulation is about one-sixth that of haemoglobin, it presents an opportunity to measure diabetic control over a much shorter time-span; of 1-2 weeks rather than 8-10 weeks. This is consistent with the half-life of albumin being approximately 17 days (Schultze and Heremans, 1966). The advantage of this approach of assessing medium-term control is particularly relevant in the more acute situations such as where control is being regained after coma. It could also be applicable to the monitoring of diabetic pregnancy where small deviations from optimum control should be detected at the earliest opportunity.

Several workers have shown that there are higher levels of GA1b (n=107) in the diabetics studied than in the controls (n=82). Different studies confirm the faster turnover time of GA1b and GPP compared to glycosylated haemoglobin. Dolhofer and Wieland (1980) found a 50% decrease in GA1b over 20 days compared to a 15% decrease in glycosylated haemoglobin, and similarly Kennedy et al. showed a mean decline of 35% in GPP in their studies whereas the glycosylated haemoglobin value only fell 8%. Dolhofer, Renner and Wieland (1981), studying patients with either severe diabetic ketoacidosis or non-acidotic coma found, as they recovered, a marked decrease in GA1b (40% in 17 days) yet no observable change in glycosylated haemoglobin values over the same period. Day et al. (1980) induced diabetes in rats with varying severity using alloxan. Following withdrawal of insulin the half-times to reach a new steady state for glucose, GPP and glycosylated haemoglobin were 2, 3 and 8 days respectively.
On reinstatement of treatment the times were 2, 3.5 and 15 days, again emphasising the different turnover times of the different components.

Not only is the rate of change increased, but the absolute rate of change in concentration of glycosylated protein has been shown to exceed that observed for haemoglobin in the same amounts. With increases of up to 30% occurring for GAlb (Guthrow et al. 1979) whereas only 20% as a maximum for glycosylated haemoglobins, this suggests that the measurement of GAlb may also be a more sensitive indicator of integrated blood glucose. This increased sensitivity is also indicated by the observation that many diabetics that appear to be controlled in terms of glycosylated haemoglobin measurement have less than satisfactory results for GPP (McFarland et al. 1979). As well as presenting information over a shorter time scale and possibly being a more sensitive constituent than HbA, the measurement of glycosylated proteins may circumvent problems associated with HbF or haemolytic disease which affect the glycosylated haemoglobin measurement.
METHODOLOGY USED FOR THE MEASUREMENT OF GLYCOXYLATED HAEMOGLOBINS.

There have been some excellent reviews by Bunn (1981), Garel et al. (1979) and, more recently Mayer and Freedman (1983), of the various methods employed for the measurement of glycosylated haemoglobins. The methods have been described and their various shortcomings outlined. Most techniques exploit the inherent small charge differences between HbA_0 and HbA_1 ("fast haemoglobins") to achieve separation. These techniques include ion-exchange column chromatography, high-pressure liquid chromatography (HPLC), agar gel electrophoresis and isoelectric focussing, and since they have the same underlying principle many of these methods experience similar problems. Other methods are based, not on charge differences but on the chemical removal of the attached carbohydrate molecule from the haemoglobin and its quantitation via a colour reaction (Flückiger and Winterhalter, 1976). These latter techniques therefore measure the actual glycosylated material rather than just a separated peak or set of peaks, but are still prone to problems albeit different ones. This last category also includes immunological methods which are as yet, not proving suitable for routine use.

As these current methods measure different moieties of the process of nonenzymic post-translational modification of haemoglobin, then different methodological parameters and interfering agents bring these differences into perspective.
At present this is probably the most widely used technique in clinical laboratories for the measurement of glycosylated haemoglobins. This is probably for two reasons. Firstly the technique is simple, relatively easy to do and does not require expensive equipment, secondly it was historically one of the first methods available which was suitable for routine use. Although Allen, Schroeder and Balog (1958) used a cation-exchange column to resolve the faster moving haemoglobins into their constituent components (HbA\textsubscript{1a}, HbA\textsubscript{1b} and HbA\textsubscript{lc}) this technique was very time-consuming as it involved extremely long columns. Trivelli, Ranney and Lai (1971) produced a faster long column procedure using a Biorex 70 cation-exchange resin (sodium form) of 100 to 200 mesh size, on which most of the subsequent methods have been based. They were able to separate HbA\textsubscript{lc} from the other "fast haemoglobins" in a suitable time scale (approximately 3hr.) so that the routine measurement of HbA\textsubscript{lc} became feasible.

With the growing awareness of the importance of the assay for routine diabetic monitoring there arose a need for simpler and more rapid techniques. These were frequently based on the original method of Trivelli, Ranney and Lai (1971). Invariably the column length was shortened and although this increased the speed of separation it was at the expense of resolution, the process separating only the total "fast haemoglobin" or HbA\textsubscript{l} and not its components. Kynoch and Lehmann (1977) first
reported a short column method which completed the separation in only two and a half hours. They used a 20ml syringe barrel (i.d.2cm) to hold a 5ml column of resin, which was equilibrated prior to the run with a phosphate buffer at pH6.8. Cyanide was included in the solution used to prepare the haemolysate prior to loading onto the column. The same equilibration buffer was used (100ml) to remove and collect the "faster moving haemoglobins" from the column. Then a further 100ml of a second phosphate buffer at pH 6.4 was used to collect the remainder of the haemoglobin (HbA).

After suitable dilutions, the absorbance at 414nm of both fractions was measured and the proportion of material in the "fast fraction" calculated. The columns were then re-equilibrated with the first buffer before reuse. The main attraction of such a technique is its simplicity, requiring only a spectrophotometer, and a group of columns which can be run simultaneously. However as large quantities of buffer are used and it is important that the columns do not run dry, a buffer reservoir must be set up for each column.

Other methods claimed to reduce the assay time to forty minutes (Chou, Robinson and Siegal, 1978) and twenty minutes (Welch and Boucher, 1978). As well as speed these methods have additional advantages; requiring smaller samples and less cyanide for haemolysis, thereby reducing hazard to the operator. Nowadays several short column methods using disposable columns and resin, are commercially available.
These include the "Fast Haemoglobins Test System" as marketed by Isolab Inc. and assessed by Dix et al. (1978), and the commonly used Biorad Kit. Comparisons have been made between the collective measurements for all of the fast moving components as measured by long columns, and the HbA1c results achieved for the same samples using the shorter columns. Abraham et al. (1978) showed these to be clinically equivalent, although Arnquist et al. (1981) indicated that the shorter columns gave lower values for glycosylation. Recently Castagnogla et al. (1983) pointed out that the results from the two methods are only comparable when the temperature is maintained at precisely 20°C. Higher temperatures cause overestimation with the shorter Biorad columns, and vice versa. Close attention to temperature control has been considered very important when ion-exchange chromatography methods are used for the measurement of glycosylated haemoglobins (Schellekens et al. 1981, Simon and Eissler, 1980). Rosenthal (1979) reported that coefficients of variation, between-batch, could be reduced to below 2% with commercial kits when rigid temperature control was adhered to. Schellekens et al. (1981) stated that poor temperature control affected both the results obtained from long and short columns.

Conditions for optimum chromatographic separation have been extensively studied and the effect of altered buffer composition and pH on the elution characteristics has been reported (Jongeel and van Wissen, 1979, Boucher et al. 1983). Schellekens et al. (1981) recommended attention to buffer composition, and a relatively constant sample loading (1.3 to
2.0 mg Hb per ml of resin). Simon and Eissler (1980) investigated the effects of chromatographic conditions on the separation of glycosylated haemoglobins by comparing purified HbA_1c recovery and pool results when each parameter was independently varied. They suggested that the temperature should be kept within 19 to 21°C, the pH of the eluting phosphate buffer between 6.70-6.72 and its ionicity at 0.06±0.005mM/l to ensure a good recovery of HbA_1c with the minimal contamination from HbA_0. They also found that the molarity of the equilibration buffer was important and recommended that this should be 0.07M. The precision was also shown to improve when the absorbances were recorded at 414nm rather than 540nm. Other workers have carried out similar studies; Jongeneel and van Wissen (1979) also recommended close limits for the elution buffer, namely pH6.85 and a concentration of 87mOsM/kg. To correct for possible variable recoveries due to small variations in these parameters at least one commercial kit (Biorad) employs a reference HbA_1 material for use with each batch of tests.

Factors other than those described above have been shown to cause problems with ion-exchange column procedures. Artificial increases in the glycosylated fraction due to sample ageing (Gruber and Koets, 1979) and uremic carbamylation (Flückiger et al. 1981) have been reported. Samples containing high levels of foetal haemoglobin (HbF), for example from children under 4 years of age (Kaplan et al. 1982) also yield
falsely high results, as the HbF coelutes with HbA\textsubscript{1}. Other haemoglobinopathies in which there is a predominance of HbS or HbC do not affect the recovery if they are in the heterozygous state, although HbA must be assessed independently (Aleyassine, 1979). Column chromatography cannot be used at all with homozygous haemoglobinopathies. Other factors which have been shown to give errant results with ion-exchange chromatography include severe jaundice (Simon and Cuan, 1980), and severe lactescence (Dix et al. 1979).

(1) Further modifications of ion-exchange chromatography.

There have been a progression of modifications of the original method described by Trivelli, Ranney and Lai (1971) to achieve faster methods with better resolution, however none avoided the need for stringent control of the assay conditions. A natural progression was the use of HPLC. This was developed (Davis, McDonald and Jarett, 1978) using small mesh Biorex 70 columns and a 3 \mu l sample size to separate and measure HbA\textsubscript{1c}. Each run took 20 minutes and coefficients of variation of 5-10\% were claimed. By automating HPLC Gruber and Koets, (1979) and Dunn, Cole and Soeldner (1979) were able to obtain superior precision figures for the assay of less than 2\%. Although in his review Bunn (1981) favours column chromatography, particularly HPLC for the measurement of glycosylated haemoglobins, there are some inherent problems for many clinical laboratories. Most obviously many laboratories do not have
the expensive equipment required, and there still remains a need for stringent control of the exact operating conditions. Also only a single sample may be analysed at any one time, which is prohibitive for use with large clinics. The technique therefore is not ideal for routine use. The main benefit of superior resolution probably proves more useful for research purposes rather than in the clinical situation (James et al. 1981).

Other techniques to increase the speed and specificity of the separation have been tried. Davis and Nicol (1978) used centrifugation to increase the speed of elution of material to 5 minutes thereby making outpatient results at the clinic a reality. Rosenthal, Vazquez and Seckinger (1981) put haemolysate into a slurry of resin which selectively absorbs HbA$_o$. By subsequently using a cell separator to isolate the HbA$_1$ fraction this could then be quantitated using a spectrophotometer. A further method (Maquart et al. 1980) used three sequential buffers of differing saline concentration, rather than the conventional two phosphate buffer system, to increase the specificity and so separate HbA$_{lc}$ from the column. Very recently a letter by Rowe and Goodland (1984) mentioned a commercial kit based on ion-exchange chromatography, from Biorad Laboratories, which offers increased specificity for HbA$_{lc}$, with prior elution of HbA$_{la+b}$, but at the expense of a slightly longer elution time (an additional 30 min.).
Although there are various problems, short column ion-exchange methods or various modifications thereof will probably continue to find wide acceptance in clinical laboratories.

(2) **Labile material as measured by ion-exchange chromatography.**

There have been various recommendations of how best to dissociate the labile material from the stable glycosylated haemoglobin fraction, prior to measurement with ion-exchange chromatography. Most workers recommend a preincubation step with the erythrocytes in isotonic saline. The temperature and duration of the incubation remains in dispute with 37°C for 6 hours (Compagnucci et al. 1981) and 4°C for 48 hours (Daneman, Luley and Becker, 1982) both advised. To increase the speed of the preincubation procedure Nathan, Avezzano and Palmer (1981) developed a 30 minute incubation step where erythrocytes were suspended in 30mM semicarbazide and 12mM aniline at pH5.0 and 38°C. Other workers have shown that the requirement for the rapid removal of labile material is less complex and can be achieved by adjusting the glucose-free media to pH5 at 37°C. (Bannon 1982, Bisse, Berger and Flügiger, 1982). Goldstein (1982) compared dialysis and preincubation as alternative methods for the removal of labile material. He found that while both techniques were satisfactory to eliminate that material which reflects the recent changes in blood glucose, dialysis did not give an accurate measurement of the true ketoamine form as it tended to underestimate the HbA₁c form and overestimate the HbA₁a+b fractions.
Compagnucci et al. (1981) studied day-to-day variation in both labile and stable material in two groups of patients; unstable diabetics (n=7) and well-controlled diabetics (n=15). They calculated the labile fraction as the total minus the stable results where these were achieved before and after saline incubation respectively. They found that both groups did not exhibit any significant day-to-day variation in their stable glycosylated haemoglobin results, but there was marked variation of the amount of labile material associated with the unstable diabetic group. This was not seen with the well-controlled diabetics. On this basis, Compagnucci et al. recommended the removal of labile material routinely for all situations where both unstable and well-controlled diabetics are being monitored clinically. Daneman, Luley and Becker (1982) also highlighted the possible relationship of labile fraction with diabetic instability. Arnquist et al. (1981), using ion-exchange showed that with 9 patients changing from very poor control the initial rapid decrease in HbA1 values was due to labile glycosylated haemoglobin and then a slower decrease due to the stable fraction was observed. Many workers have also noted the association between blood glucose of a sample and its labile fraction. (Compagnucci et al. 1981, Trovati et al. 1981). When Bolli et al. (1981) incubated samples in vitro with concentrations of glucose upto 21.5 mmol/l over 2 to 12 hours, the labile fraction fluctuated 1.47% whereas the stable fraction did not significantly alter. Other workers have discussed the influence of a glucose load, such as in the oral glucose tolerance test, on the HbA1 values of labile and stable HbA1 and there has been some disagreement.
about their findings. Goebel et al. (1981) found severe hyperglycaemia over a period in excess of 6 hours was required in healthy subjects to increase their stable HbA₁, whereas Scobie et al. (1981) found an increase in values of HbA₁ 10 to 30 days after an oral glucose load of 50g. These discrepancies are possibly due to the labile material, and the removal of this fraction before the determination of stable glycosylated haemoglobin should be performed to circumvent similar misunderstandings.

Agar Gel Electrophoresis.

In the early work of separating the "faster moving" haemoglobins Rahbar (1968) employed an electrophoretic technique. Allen et al. (1979) were the first to describe the use of agar gel electrophoresis for their separation and quantitation, a system which exploits both the chromatographic and electrophoretic characteristics of the gel. As HbA₀ carries a more positive charge than HbA₁c it interacts more strongly with the fixed negative charges in
the gel leaving \( HbA_\perp \) to be swept at a faster rate towards the cathode by the electro-endosmotic flow of the buffer. The whole electrophoretic system is designed to emphasise the endosmotic features present and so achieve a good separation. Indeed the wide separation between the \( HbA_\circ \) and \( HbA_\perp \) makes agar gel electrophoresis an attractive alternative to column chromatography. Only a small sample of a few \( \mu l \) is required (Menard et al. 1980, Ambler, Janik and Walker, 1983), and several samples can be run at one time. Usually eight samples are run together as this is the capacity of the commercially available gels as marketed by Corning Medical Ltd. (Thornton, Schellekens and Sanders, 1981). The recommended conditions are set out by Thornton, Schellekens and Sanders using a citrate buffer of pH 6.3, prefabricated agar plates and a 40 minute electrophoretic run. With this system the percentage of haemoglobin in the \( HbA_\perp \) peak is determined by using the Corning M-720 densitometer, which scans both peaks at 420nm and automatically integrates the area enclosed by each. However the inclusion of additional "background" material (due to poor electrophoretic technique) within the \( HbA_\circ \) peak has been known to cause slightly low results (Thornton, Schellekens and Sanders, 1981).

Agar gel electrophoresis has been shown to correlate well with both HPLC (Hayes et al. 1981) \( r=0.977 \), and ion-exchange column chromatography (Aleyassine, 1981) \( r=0.931 \) mini-columns and \( r=0.92 \) (macro-columns) (Thornton, Schellekens and Sanders, 1981). The between-batch variation however, is
not as precise as by HPLC (normal sample CV = 9.0%, high diabetic sample CV = 4.6%) (Hayes et al., 1981). Unlike column chromatography it is insensitive to changes in buffer pH between pH 5.8 and 6.5 (Menard et al., 1980). Similarly changing the temperature between 4°C and 30°C has been shown to have little effect (Aleyassine, 1981). High levels of triglycerides in the sample apparently do not cause any interference (Aleyassine, 1981). The technique can be used to detect HbS and HbC, unlike column chromatography which underestimates these samples, although the presence of HbF still causes problems. Agar gel electrophoresis cannot be used to distinguish between the intermediate labile aldimine and the stable ketoamine product of glycosylation. To remove labile material a pre-incubation step of either 22°C for 12 hours (Nathan, 1981) or 37°C for 4 hours (Tibi, Young and Smith, 1982) have been recommended. Using these techniques, Tibi, Young and Smith (1982) were able to show both an association between blood glucose and labile material in the same sample, and an intra-individual variation of labile material within the patients studied, between -4.4% and +15.6% of the total glycosylated material.

The major disadvantage of the technique is that to achieve a reasonable rate of analysis the system relies on the use of a purpose built densitometer, preferably with integration facilities, and the purchase of commercially prepared agar plates. These requirements although not mandatory, are desirable but expensive. However the high throughput potential, together with the relative simplicity of the method and production of a visual record of the results
all combine to make this technique attractive for routine use in clinical laboratory, particularly with a large throughput of diabetic samples.

**Isoelectric focussing.**

Isoelectric focussing on polyacrylamide gels was first described by Drysdale, Righetti and Bunn, (1971). Schoos, Schoos-Barbette and Lambotte (1978) applied this technique to thin layer gels using a high voltage and a pH gradient to achieve a good separation of HbA\textsubscript{lc} from HbA\textsubscript{o}. Stickland, Perkins and Wales (1982) used isoelectric focussing for the separation of glycosylated haemoglobin and showed that using 20 watts at a constant temperature of 15\(^{\circ}\)C for one and a half hours, an intermediate band of "labile" material could also be detected. This observation was supported by Mortensen (1980) who found this new band, which lies closely anodic to the HbA\textsubscript{lc} band, to be related to the blood glucose concentration. This suggests that the technique can be used to resolve labile material and the more stable ketoamine.

To improve the separation of glycosylated haemoglobin from HbA\textsubscript{o} still further, amphoteric substances were added to transform the linear pH gradient into a non-linear one. By "flattening out" the gradient in this fashion in the correct region, superior separation of the desired materials, in this case haemoglobins, can be achieved. Beccaria et al. (1978) used dipeptide histidyl-glycine (pI 6.8; pI-pK=1) whereas
Jeppson, Fránzen and Nilsson, (1978) used β-alanine for this purpose. The improved resolution gave correspondingly better sensitivity with inter-assay CV=4.8% and intra-assay CV=5.4% (Jeppson, Fránzen and Nilsson, 1978).

Once separation of HbA₁c from HbA₀ has been achieved then the problem remains of how to quantify the material in the two bands and thereby express the result as a percentage glycosylation. A commercial system known as the Multiphor system is marketed by LKB Ltd. This contains all of the equipment necessary to achieve the desired separation; ready-prepared plates, a tank and power pack, as well as a scanning densitometer. As an alternative to densitometry, Jeppson, Fránzen and Gaal (1980) showed that it was possible to cut out the two bands and solubilise the haemoglobin before reading their absorbances in a spectrophotometer at 414nm. This removes the necessity for the expensive densitometric equipment. This technique was shown to give equivalent results to that of the scanning method (Mortensen, 1980).

Used correctly, isoelectric focussing surpasses the other techniques based on either charge or size-dependent electrophoresis in its ability to resolve similar materials. It can resolve HbA₁c from the other haemoglobin components HbA₁a, HbA₁b, HbA₂, HbS and HbF, and with the correct conditions it allows the separate detection of labile material (Stickland, Perkins and Wales, 1982). However the acetylated HbF migrates with HbA₁c (Simon and Cuan, 1982) and remains a problem. The interference by acetylated materials is further
exemplified by samples from patients having high dosage aspirin therapy (Bridges et al. 1975) where the extensive acetylation causes falsely high results. The major drawbacks with the technique are the need for expensive equipment, expensive materials and a high degree of technical skill to achieve good results. For these reasons isoelectric focussing probably remains more suited for research purposes in the investigation of glycosylated material, rather than as a tool for their routine measurement.

**Immuoassay.**

Javid et al. (1978) purified HbA1c using the column method of Trivelli, Ranney and Lai, (1971), and injected this material with suitable adjuvant into sheep to develop an antiserum. Using this antiserum they were able to develop a radioimmunoassay which was specific and linear throughout the range studied (0-16% glycosylation). There was partial cross-reactivity observed with HbA1 and HbA1b although HbF did not interfere. The work suggests that this technique will provide a suitable method of assay for the measurement of different glycosylated haemoglobins using similar immunological systems. At present however, there are difficulties with the development of such radioimmunoassay methods, as the antibody titres are very low.
Colorimetric Techniques.

The most commonly described colorimetric method is that based on the original concept by Flückiger and Winterhalter (1976). In this method the ketoamine-linked hexose is hydrolysed with a weak (oxalic) acid (Fig. 1.5) to generate 5-hydroxy methylfurfural, (5-HMF). This product is then condensed with thiobarbituric acid to form a coloured adduct with an absorbance maximum at 443nm. Both stages require incubation. Subramanium, Radhakrishnamurthy and Berenson (1980) optimised the conditions for hydrolysis and colour development for the manual assay for both glycosylated haemoglobin and glycosylated albumin. They recommended that a 16 hour hydrolysis at 100°C in 10mol/l acetic acid should be used and that the maximal colour development with thiobarbituric acid could be achieved after a 50min incubation at 40°C. The results are reported as a 5-HMF index expressing the number of micromoles of 5-HMF generated for each gm. of globin protein. For the calculation, the original content of haemoglobin or protein in the sample must be known and standardised. This is usually done prior to the assay using the method of Van Kampen and Kijlstra (1961), and commonly the original haemoglobin content is adjusted to 5% (W/v).

To increase the speed and therefore the usefulness of the technique certain adaptations of the basic method have been made. Parker et al. (1981) recommended the use of an
Fig. 1.5  **Simplified scheme of the reaction sequence involved in the formation of HbA\textsubscript{lc} and the subsequent Amadori rearrangement, as described by Flückiger and Winterhalter in Febs. Letts. (1976); 71 : p359.**
autoclave at 124 °C for 60 minutes at 181b/in² to accelerate the rate of hydrolysis, and claims high reproducibility (CV=3%). Similarly Koch, Sidloï and Tonks (1982) used a pressure cooker for 20 minutes at 15 lb/in² for this purpose. For the second stage of their method they used the semi-automated technique of Menez et al. (1981) incorporating an autoanalyser system. Burrin et al. (1980) also used automated analysis. The precise standardisation of both parts of the reaction is critical. For example, the conditions of the initial incubation affect both the formation and destruction of 5-HMF. This affects the method of standardisation; only reference standards of human haemoglobin or pooled haemolysate are suitable (Postmes et al. 1981). The time allowed for the formation of the coloured complex is also critical for not only does it affect the absorbance maximum due to the glycosylated fraction, but it also alters the blank value in any individual sample (Bunn, 1981). To overcome this problem the inclusion of individual haemolysate blanks without colour reagent is recommended. The sensitivity of the test for different sites of origin (HbA₁c or GHBₐₒ) is also low, causing problems for standardisation. The yield of HMF from HbA₁c is only 30% (Bunn et al. 1979) and is probably considerably less from GHBₐₒ.

As the assay requires very precise control of the experimental conditions its use as a routine technique is questionable. Although it is relatively simple, requiring no expensive equipment or reagents, many of the reagents employed are poisonous and can cause potential hazard. High
levels of glucose within the samples have been shown to interfere with they assay (Bunn, 1981). This interference has been overcome by the use of a modified method (Ma, Naughton and Cameron, 1981) which includes a trichloroacetic acid precipitation step. Alternatively the inclusion of dialysis equipment in the automated systems have been recommended (Koch, Sidloi and Tonks, 1982). This though, serves to further reduce the sensitivity of the method which may be a disadvantage. Problems with individual blanks and standardisation have already been mentioned.

The thiobarbituric acid test has several advantages over the chromatographic and electrophoretic techniques. Because it is specific for the ketoamine-linked glucose it is not affected by the presence of HbF, haemoglobin variants, other post-translational modifications such as acetylated haemoglobin and, most importantly, it measures only the stable glycosylated material (Parker et al. 1981), and not the labile intermediate (pre-$\text{Alc}$) (Postmes et al. 1981). It is also extremely useful as it has the capability of measuring not only glycosylated haemoglobin, but other glycosylated proteins as well. (Subramaniun, Radhakrishnamurthy and Berenson, 1980, Parker et al. 1981, Menez et al. 1981).

Nayak and Pattabiraman (1981) followed the hydrolysis and precipitation procedures, but used phenol followed by concentrated sulphuric acid for colour development, with an absorbance maximum at 480nm.
A totally different type of colorimetric technique was recently described by Gallop et al. (1981) for measuring protein glycosylation. This was based on the fluorimetric measurement of formaldehyde released from the molecule after periodate oxidation. The oxidation was carried out for 30 minutes at room temperature with sodium periodate. Any excess iodate was then removed by zinc sulphate precipitation. The fluorimetric determination of the resultant formaldehyde was in combination with ammonium acetate and acetylacetone, using an excitation wavelength of 410nm and an emission wavelength of 510nm. This method gave a good correlation with both the traditional thiobarbituric acid method ($r=0.87$) and mini ion-exchange columns ($r=0.95$). However unlike the method of Flückiger and Winterhalter (1976) both labile and stable glycosylated material are measured. As a fructose standard curve is used the results are defined as the absolute glycosylcl group content of the sample rather than as a percentage. This method shows promise of being a potential alternative to the thiobarbituric acid method for the measurement of glycosylation in proteins other than haemoglobin.

Yet another colorimetric technique, marketed as the Glycospec Spectrophotometric kit has been developed (Wallinder, Ronquist and Fager, 1982). This relies on the fact that certain organophosphorus compounds like inositol hexaphosphate (phytic acid) bind to the N-terminal of haemoglobin chains (the 2,3-diphosphoglycerate sites) causing spectrophotometric changes which can be recorded. However when these sites are
already occupied with glycosylated molecules then this binding cannot occur. If sufficient phytic acid is added to haemolysate to fill all the available sites then the change in absorbance will be inversly proportional to the glycosylation of the haemoglobin. The differences in absorbance at 560nm and 633nm is patients samples are recorded and interpreted using a graph prepared from standards with known HbA1 values. The contribution of exogenous 2,3-diphosphoglycerate is very small (0.5%) and does not constitute a problem. However as the standards should contain only HbA1, they differ in their content of 2,3-diphosphoglycerate from the tests, and therefore cause a small false increase in results. In spite of this a good correlation has been shown to exist between this, and the ion-exchange methods \((r=0.89)\). Storage of samples is a problem with the spectrophotometric method since falsely elevated results are obtained; possibly due to the failure of phytic acid to bind completely to denatured haemoglobin. Like the ion-exchange methods the presence of HbF causes interference with the spectrophotometric technique.
METHODOLOGY FOR THE ASSAY OF GLYCOSYLATED PROTEINS.

In order to investigate the effects of protein glycosylation or their clinical use, suitable methods of assay had to be developed. The methods for the measurement of glycosylated haemoglobin have already been discussed at length.

Methods for the determination of glycosylated plasma proteins usually fall into one of two categories; those which measure glycosylated albumin (GAlb) which has suitable longevity to become glycosylated; those which measure the glycosylation of all proteins found in human serum or plasma (GPP). Most of the methodology used is based on the colorimetric technique where 5-HMF is generated by hydrolysis of plasma proteins and the subsequent colour reaction of this with thiobarbituric acid provides a quantitative estimate of the glycosylation of the protein in the sample. Ney, Colley and Pizzo (1981) have optimised the conditions of this reaction.

The measurement of glycosylated albumin is slightly more complex as it requires the initial separation and purification of albumin. Dolhöfer, Renner and Wieland (1981) achieved this by using DEAE cellulose chromatography and Affi-gel blue prior to the TBA reaction. Using various modifications of the colorimetric reaction, different coefficients of variations have been claimed for GPP analysis varying from less than 2.5% (McFarland et al. 1979) to 6.3% (Yue et al. 1980), and 9.3% (Johnson, Metcalf and Baker, 1982).
Column chromatography has also been employed to separate glycosylated from non-glycosylated albumin (Flückiger and Winterhalter 1976), using a column of carboxymethyl cellulose eluted with a gradient of 0.01M to 0.5M sodium acetate. The normal range quoted using this technique is 6-15% of the total albumin is glycosylated. Guthrow et al. (1979) compared the column and colorimetric techniques for the measurement of glycosylated albumin. They found that although the two techniques gave an excellent correlation (r=0.99) more material was detected by the colorimetric technique. They concluded that it was likely that some material was not being separated chromatographically, an analogous situation to the finding of GHbA.

Day et al. (1979) showed that the extent of protein glycosylation was a function of glucose concentration, time of exposure and temperature. In 1980 Kennedy, Mehl and Merimee hypothesised that additional rapid glycosylation of serum protein could occur in vitro at the high temperature of hydrolysis, used in the colorimetric technique. They demonstrated a 241% increase in protein glycosylation after adding 300mg/dl glucose to the sample immediately before hydrolysis and concluded that glucose levels in the sera may seriously alter the results. Their observations were supported by Ney, Colley and Pizzo (1981) who also noted spuriously high results related to blood glucose concentration in the sample. In order to overcome this problem Kennedy, Mehl and Merimee (1980) employed dialysis with inter- and intra-batch variation of 8% and 12% respectively. Ma, Naughton and Cameron (1981) recommended an alternative
technique using trichloroacetic acid to precipitate proteins prior to analysis, with claimed coefficients of variation of below 2%.

Details of other methods not based on the thiobarbituric acid reaction have also been published. Schleicher and Wieland (1981) have developed a specific and sensitive method to measure the fructose-lysine linkage in non-enzymically glycosylated albumin and other proteins. In this, furosine, which is the specific degradation product of the fructose-lysine link, is released by hydrolysis in 6M-HCL for 18 hours at 95°C. The furosine is then separated on HPLC and its UV absorbance compared with that of a series of fructose-lysine standards. From this, the degree of glycosylation is calculated. Another approach is to measure serum-bound hexose first using perchloric acid to precipitate protein, and then making alkaline with NaOH before the colour reagent is added. The resultant colour is read after 40 minutes at 490nm and the concentration determined using a hexose standard curve, (Kennedy, Kandell and Merimee, 1979). Johnson, Metcalf and Baker (1982) use a method which relies on the ability of ketoamines (fructosamines) to act as reducing agents in alkaline solutions, to measure serum glycosylprotein. By adding serum to a solution of nitrobluetetrazolium in carbonate buffer a colour develops after 15 minutes at 37°C with an absorbance maximum at 530nm. Lipaemic and haemolysed sera do not affect the results unless there is gross contamination. The method produces a significant but poor correlation with the TBA colorimetric
technique \( r=0.58 \), but the authors claim that this is due to the poorer reproducibility of the traditional method \( CV=9.3\% \) compared to their own \( CV=6.5\% \). The colorimetric technique which uses fluorimetric measurement of formaldehyde released after periodate oxidation (Gallop et al. 1981) for the measurement of either glycosylated haemoglobins or glycosylated plasma proteins was discussed earlier in this chapter.

**Affinity Chromatography.**

Most recently a totally new methodology has been developed for the separation and quantitation of glycosylated haemoglobins, using affinity chromatography. Mallia et al. (1981) produced a cross-linked agarose gel activated with carbonyl diimidazole and subsequently coupled to aminophenyl boronic acid, to form the affinity matrix. The affinity gel, so formed, was pipetted into small chromatography columns. After loading a sample of haemolysate onto the column of gel they washed off and collected the non-glycosylated material using a buffer containing sodium acetate and magnesium chloride at pH8.5. The glycosylated material was subsequently removed with a second buffer containing 0.2M sorbitol; this was collected. This, then is essentially the reverse of the ion-exchange separation where the glycosylated material is eluted from the column first. The absorbances of the separated fractions were measured at 414nm. and the percentage of the haemoglobin contained in the sorbitol-eluted, "bound" fraction was calculated.
Bouriotis et al. (1981) showed that immobilised amino-phenyl boronic acid binds selectively to cis-diols and can therefore be used to separate glycosylated haemoglobins. They used a different buffer of 50mmol/l morpholine -HCl and 10mmol/l KCN for this purpose and only added 0.1mol/l sorbitol for elution.

These initial publications suggested that this technique would be a promising new approach for the measurement, not only of glycosylated haemoglobins, but also of other glycosylated proteins. At the start of the work for this thesis the affinity gel was not available commercially and was obtained for the earlier studies from the research laboratories of Pierce Chemical Co. in the USA. As the work progressed the affinity gel was marketed in the UK.

AIMS OF THIS THESIS.

It was the aim of the work for this thesis; to examine the analytical conditions of the affinity chromatography in detail and develop a reliable, inexpensive and simple method for the measurement of both glycosylated haemoglobins and glycosylated plasma proteins in the routine laboratory; to compare the newly developed method with those previously established; to assess its usefulness in monitoring diabetic control in a variety of clinical situations; to assess any additional advantages that the measurement of glycosylated plasma proteins may confer.
CHAPTER 2.

OPTIMIZATION OF AFFINITY CHROMATOGRAPHY METHODS FOR THE MEASUREMENT OF BOTH GLYCOSYLATED HAEMOGLOBINS AND GLYCOSYLATED PLASMA PROTEINS.
INTRODUCTION.

The affinity gel used in this work consists of an m-aminophenylboronate ligand attached to an agarose, uncharged, gel support. This gel selectively condenses with cis-diol groups, such as are present in the glucose molecule. In this way any protein which is attached to glucose may also condense with the gel, via the glucose molecule, with the elimination of two molecules of water (Fig. 2.1). If a solution containing glycosylated and non-glycosylated proteins is introduced onto a small column of the gel, and this is followed by a buffer solution, glycosylated proteins will be retained and the non-glycosylated washed through. For the subsequent removal of the glycosylated material, a buffered solution of sorbitol is employed. This competes for the glucose binding site thereby dislodging the glycosylated molecule. By measuring the quantity of protein in the two fractions thus separated, the percentage of glycosylated material in the original clinical sample can be calculated. This principle forms the basis for both the measurement of glycosylated haemoglobin and glycosylated plasma proteins by affinity chromatography.

This chapter provides an account of the experimental techniques used throughout this thesis. Initially the details of both techniques for the measurement of glycosylated haemoglobin and glycosylated plasma proteins by affinity chromatography are described. The reasons for choosing the experimental conditions are then outlined, together with the statistical analysis of the results. Further details relating
Fig. 2.1 Schematic representation of the chemical reaction between glycosylated protein and the m-aminophenyl boronate agarose, Glycogel B.
to different methods or specific samples are described separately in the relevant chapters.

The work described in this chapter has been published (Gould, Hall and Cook, 1982; Hall, Cook and Gould, 1983; Gould, Hall and Cook, 1984).
SECTION 1.
ROUTINE AFFINITY METHODS FOR THE MEASUREMENT OF GLYOSYLATED HAEMOGLOBINS AND GLYOSYLATED PLASMA PROTEINS.

SAMPLES.

Clinical Material.

Samples of whole blood were collected from non-diabetic and diabetic individuals into lithium heparin tubes. These samples were stored at 4°C until use, up to a maximum of 7 days.

Preparation of Samples.

(1) For glycosylated haemoglobins analysis (test haemolysate). Erythrocytes were separated from plasma by centrifugation at 1200g for 10 minutes. Approximately 100 μl of packed cells were added to 2ml of deionised water, and thorough haemolysis was achieved using a vortex mixer. This diluted haemolysate was routinely incubated for 5 hours at 37°C before the determination of stable glycosylated haemoglobins.
For glycosylated plasma proteins analysis.

Plasma was separated from erythrocytes by centrifugation at 1200g for 10 minutes. 100 μl of plasma was added to 2ml of deionised water and mixed using a vortex mixer. Care was taken to avoid contamination of the diluted sample with material from the "buffy" layer. The diluted plasma was incubated for 5 hours at 37 °C prior to the determination of stable glycosylated plasma proteins.

APPARATUS.

Columns used for affinity chromatography were supplied by Uniscience Ltd., Cambridge, U.K. The dimensions of these polystyrene columns were as follows;

- internal diameter = 0.7cm
- column length = 6.0cm
- total column volume = 2.3ml
- reservoir volume = 5.3ml

Each column had a 7μ filter support and a cap for the tip of the column. They were claimed to have good chemical resistance to aqueous solutions of mild acids or weak bases. The columns were clipped into a purpose-built rack, capable of holding a total of 30 columns.

The wash (glycosylated) fractions eluted from the columns were collected in 25ml measuring cylinders. These, and all other glassware were originally acid-washed, and then thoroughly washed with deionised water after every use.
The elution (non-glycosylated) fractions were collected into 5ml plain glass bottles. (Seward Laboratories., UAC House, Blackfriars Road, London, U.K.).

The absorbances of the fractions were measured with a SP1800 Spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.), using disposable cuvettes (Sarstedt Ltd., Leicester, U.K.). The buffer pH was measured and adjusted using a Biotech PHM6 pH meter (V.A. Howe and Co. Ltd., London, U.K.).

All pH measurements were done at 20 ± 1 °C.

REAGENTS.

Anticoagulant tubes containing 75IU lithium heparin, and preservative tubes containing 2.0mg sodium fluoride, were obtained from Seward Laboratories, UAC House, Blackfriars Road, London, U.K.

Glycogel B, the affinity gel, an m-aminophenylboronate agarose, was supplied by Pierce and Warriner, Chester, Cheshire, U.K.

Biorad Protein Assay Reagent was obtained from Biorad Laboratories, Watford, Herts., U.K.

All other chemicals were purchased from British Drug Houses, Poole, Dorset, U.K.

All water used was double deionised to a level of conductivity below 5Mho per ml.

All reagents used were Analar grade.
Preparation of Reagents.

(1) **Wash buffer.**
38.54g ammonium acetate (250mmol/l) and 20.33g magnesium chloride (50mmol/l) were dissolved in water. The pH was adjusted to either pH8.3 for the glycosylated haemoglobin analysis or to pH 8.9 for the glycosylated plasma protein measurement, by the addition of 1M NaOH or 1M-HCl. The buffer was then made up to 2l with water. For the earliest work, pH8.5 was used for glycosylated haemoglobin measurement.

(2) **Elution buffer.**
6.058g tris (hydroxymethyl) methylamine (100mmol/l) and 9.306g disodium ethylenediaminetetra-acetic acid (50mmol/l) and 18.22g sorbitol (200mmol/l) were dissolved in water. The pH was adjusted to either pH 8.3 for the glycosylated haemoglobin analysis, or to pH 8.9 for the glycosylated plasma protein measurement by the addition of 1M NaOH or 1M-HCl. The buffer was then made up to 500ml with water. For the earliest work, pH8.5 was used for glycosylated haemoglobin measurement.

(3) **Regeneration solution.**
3ml of glacial acetic acid was made up to approximately 500ml with water (~ 0.01M acetic acid).
(4) **Protein assay reagent.**

The Coomassie Brilliant Blue protein reagent was diluted with water and glacial acetic acid in the ratio 4:5:1 respectively; the quantity prepared depending on the number of samples tested. The solution was mixed and filtered before use. As the solution stained glassware, a designated bottle was used for the reagent. The diluted acidified reagent was prepared fresh for each assay. All other prepared reagents were stored at 4°C, and used at 20°C ± 1°C.

(5) **Saline.**

9.0g of sodium chloride was dissolved in deionised water and made up to 1 litre. This isotonic solution (154mmol/l) was used whenever saline was required.

(6) **Cyanide solution.**

0.189g of potassium cyanide was dissolved in deionised water and made up to 500ml to provide a solution of 0.58mmol/l KCN. This was used in the investigation of storage conditions.
INVESTIGATION OF COOMASSIE BRILLIANT BLUE DYE FOR PROTEIN MEASUREMENT IN THE ASSAY FOR GLYCOXYLATED PLASMA PROTEINS.

Choice of Coomassie Brilliant Blue Dye.

At the beginning of this work there were no publications using affinity chromatography for the measurement of glycosylated plasma proteins, and the Glycogel Test Kit from Pierce Chemical Co. Ltd., was not marketed. Early work to achieve separation of this material on m-aminophenylboronate gels was attempted using similar techniques to those that had been established for the separation of glycosylated haemoglobins. However, both the primary dilution of the sample to add to the column, and the separation of "bound" from "unbound" material with different buffers, caused considerable dilution of the original plasma sample. The use of a very sensitive technique was required to measure the protein content in the collected fractions containing material which did, or did not, bind to the gel. The biuret method was found to be too insensitive for this purpose, even when the test:dye ratio was increased. Similarly, the direct measurement of absorbance at 280nm was found to be inadequate at these low protein concentrations. Coomassie Brilliant Blue dye was also investigated. This dye, recommended by Bradford (1976) for the quantitation of microgram quantities of protein, appeared to be ideal for the purpose, as it was sensitive to protein in very small amounts and had previously been used for
this purpose with cerebrospinal fluid (Hische et al., 1982) and in homogenates of tumour tissue (Sherwood, 1980). Following enquiries, it was found that Biorad Ltd. marketed a protein reagent containing Coomassie Brilliant Blue G-250 dye in phosphoric acid (55%) and methanol. Initial experiments using this reagent showed that it was sensitive enough to detect protein in the fractions collected after affinity chromatography.

**Acidification of the Biorad Reagent.**

To compensate for the alkaline nature of the buffers used for the separation of "bound" and "unbound" material, the Biorad Protein Assay Reagent was acidified before use. It was found that by the addition of glacial acetic acid, as described in the preparation of reagents, this could be suitably achieved.

**Regeneration of Gels.**

In the original literature associated with the affinity gel, Glycogel B, there was no indication that the affinity gel could be regenerated for further use. However, instruction leaflets supplied from another manufacturer utilising agarose-boronate gels for another purpose, showed that a weak acid could successfully regenerate the ligand. This was successfully applied to Glycogel B which was regenerated.
using 5ml of a weak acetic acid solution. Further study showed that regeneration was even more successful if the columns were washed with 5ml of deionised water before the addition of the acid, presumably as this removes the remaining alkaline buffer retained in the gel so that it does not neutralise the acid solution. Results obtained when columns were reused are given later in this chapter.

**Linearity of the Protein Concentration Measurement.**

To ascertain the linearity of the protein method in the test for glycosylated plasma proteins, a series of plasma dilutions ranging from 0.1mg/ml to 0.5mg/ml were prepared, these concentrations being consistent with those to be found in the column eluates. At a wavelength of 595nm, this range of protein concentrations gave a linear plot against absorbance (Fig. 2.2) up to an absorbance of 0.6. To adjust the level of wash-fraction protein into the linear range of this concentration plot, it was necessary to dilute this column eluate to 24ml with wash buffer.

**Colour Development.**

To measure the colour development of the Coomassie Brilliant Blue reagent with a protein sample, 500μl of an elution fraction was mixed with 500μl of acidified reagent in a disposable plastic cuvette, and mixed by inversion. The cuvette was immediately placed in a spectrophotometer
Fig. 2.2

To illustrate that the modified Coomassie Brilliant Blue protein reagent behaves linearly with increasing protein concentration.
and the absorbance at 595nm, monitored against air. The colour development was very rapid in the first few seconds and then gradually increased until a plateau was reached at about 25 minutes, after which the absorbance was stable for nearly 60 minutes before starting to decrease. This was also true when a wash fraction was tested. For this reason the measurement of the absorbance at 595nm. was made at exactly 30 minutes after mixing. The absorbance of the wash and elution fractions were read concurrently for each test.

The absorbance maxima of 595nm. was verified by measuring the absorbance of a sample mixed with reagent between 400nm. and 700nm. (Fig. 2.3).

AFFINITY CHROMATOGRAPHY

Preparation of Affinity Columns.

The affinity gel, Glycogel B, was supplied as a 50% slurry. This slurry was mixed and 2ml transferred to each column, which were mounted vertically on a purpose-built rack. When allowed to drain, a 1ml gel column was thus achieved. Before use, each column was washed with 5mL of deionised water followed by 5mL of 0.01M acetic acid (regeneration solution). The columns were then primed with 5mL of wash buffer at the correct temperature. When not in use, the columns were stored vertically in the dark at 4°C, in 0.01M acetic acid.
Fig. 2.3 To illustrate that the maximum absorbance for the modified Bradford Brilliant Blue dye reagent occurs at 595nm.
(1) **For glycosylated haemoglobins.**
A volume of 100μl of test haemolysate was pipetted onto the top of a column and allowed to soak into the gel. One ml of wash buffer was also added and allowed to drain, to ensure that all of the haemolysate had soaked into the gel. This was followed by a further 7ml of wash buffer. Both elutions of wash buffer were collected in a 25ml measuring cylinder and made up to exactly 15ml by the addition of further wash buffer. The column was then transferred to a supporting rack, positioned over a plain glass bottle and labelled to identify both the sample and wash fraction number. Exactly 3ml of elution buffer was allowed to run through the column and this too, was collected. A portion of wash sample and a portion of elution sample were poured into separate cuvettes and their absorbances measured at 414nm, reading both tests against a wash buffer blank. The absorbance of the elution buffer blank was noted.

(2) **For glycosylated plasma proteins.**
The separation of glycosylated plasma proteins by affinity chromatography was performed in an almost identical manner with the exception that 150μL of diluted plasma was added to the gel, and the wash
fraction was made up to 24ml before the protein concentration was assessed.
For the measurement of the protein concentration in both the wash and elution fractions for each test, a portion of each was well mixed with an equal volume (0.5ml) of the acidified Coomassie Brilliant Blue reagent in disposable microcuvettes. The blank was prepared by adding 0.5ml of wash buffer to 0.5ml of the acidified Coomassie Brilliant Blue reagent. After exactly 30min the absorbances of both the wash and elution fractions for each sample were measured at 595nm against the wash buffer blank. An elution buffer blank was also prepared from a mixture of 0.5ml of elution buffer and 0.5ml of reagent, and its absorbance measured against the wash buffer blank.
To ensure that there was no residual interfering material on the gel columns, water instead of diluted plasma was added to one column in each batch and the eluates treated with reagent.

Blank Corrections.

In the measurement of both glycosylated haemoglobins and glycosylated plasma proteins a correction was made to compensate for the different effects of the two buffers used. The basis of the correction depends on the fact that the gel bed for every column was 1ml, and that the addition of 3ml of elution buffer served to displace 1ml of residual wash
buffer into the 3ml of collected eluate. To compensate for differences between the buffers, a value of two-thirds of the absorbance obtained for the elution buffer blank was subtracted from all of the tests elution absorbances before calculations were made.

Calculations.

The "bound" material for each method was calculated using the following equations:

\[
\% \text{ Glycosylated haemoglobins} = \frac{\text{elution}}{\text{wash elution}} \times 100
\]

\[
\text{elution} = \frac{A}{414 x 5 + 414}
\]

\[
\% \text{ Glycosylated plasma proteins} = \frac{\text{elution}}{\text{wash elution}} \times 100
\]

\[
\text{elution} = \frac{A}{595 x 8 + 595}
\]

In each case the absorbances for both the wash and elution fractions of the separation are used.

A computer programme (Fig. 2.4) was devised under instruction from M.P. Bailey, for the calculation of the corrected values for each method. The input required for the correction was simply the wash and elution absorbances for each test, and the absorbance of the elution buffer blank.
Fig. 2.4. A computer programme to calculate the %
glycosylated haemoglobins or % glycosylated
plasma proteins from the abosorbance values.

5 @CHR$(27);"+"
7 DIM A$(40),B$(40)
10 @"ENTER VOLUME LOADED";
20 INPUT V
30 @"ENTER BLANK ABSORBANCE";
40 INPUT B
42 @ : @
45 OPEN\l+$LP"
46 ON ESC GOTO 225
50 B=2*B/3
60 @"ENTER ABSORBANCE OF WASH";
70 INPUT A1
80 A$="Aw = ###.###" : @\l+ USING A$,A1
90 @"ENTER ABSORBANCE OF ELUATE";
100 INPUT A2
110 A2=A2-B
120 A$="Ae = ###.###" : @\l+ USING A$,A2
130 @\l+
140 K=5
150 IF V<>150 THEN 170
160 K=8
170 A3=A2+K*A1
180 A$="TOTAL ABSORBANCE = ###.###" : @\l+ USING A$,A3
190 R$="% GLYCOXYLATION = ###.###" : @\l+ USING B$,100*A2/A3
200 @\l+ : @\l+
205 @
210 GOTO 60
225 CLOSE\l+
230 END

When calculating % glycosylated haemoglobins, enter
100 as the volume loaded.

When calculating % glycosylated plasma proteins, enter
150 as the volume loaded.
To standardise the affinity method for the measurement of both glycosylated haemoglobins and glycosylated plasma proteins, various aspects of the method were examined.

Unless stated otherwise, the methods used were exactly as specified in Section 1 of this chapter. The columns were eluted at gravitational pressure, producing a flow-rate of 0.46 ml/min for the wash buffer and 0.30 ml/min for the elution buffer, with the 1ml gel columns used.

Statistics.

Linear correlations were calculated by the least squares method, and statistical comparisons were performed by Students 't' test.
Elution Profiles.

Mallia et al. (1981) described the preparation of a boronic acid affinity support for the separation and quantitation of glycosylated haemoglobins on 1ml columns of gel. They recommended removal of "unbound" material with 5ml of wash buffer followed by removal of the "bound" material with 2ml of elution buffer. The work for this thesis was started on the basis of this short publication, and initially the pH of the buffers used for glycosylated haemoglobins was pH8.5, as recommended by these pioneer workers. The buffers used for the glycosylated plasma protein assay were at pH8.9. Both the assay for glycosylated haemoglobins and that for glycosylated plasma proteins were carried out at room temperature. The quantities of wash buffer and elution buffer used, in each case, were greater than recommended by Mallia et al. (1981), to ensure good separation of "bound" from "unbound" material.

A 1ml gel column of the affinity gel, Glycogel B was prepared and equilibrated with wash buffer. Blood taken from a diabetic patient was diluted and incubated in accordance with earlier instructions to provide a haemolysate of stable glycosylated haemoglobin; 100 \( \mu l \) was added to the column and allowed to soak in. Nine 1ml volumes of wash buffer were then added to the gel and each fraction was collected separately. Four 1ml volumes of elution buffer were added
Fig. 2.5A  Elution profile of haemoglobin from a 1ml affinity column (Glycogel B).

Fig. 2.5B  Elution profile of plasma proteins from a 1ml affinity column (Glycogel B).
and collected similarly. The absorbance of each fraction at 
414nm was recorded and an elution profile plotted (Fig. 2.5A).

The colour of haemoglobin provides a clear visual 
separation of the "unbound" material being washed from the 
column, leaving a small red band of "bound" material. When 
the buffers are changed, this band can be seen to be removed 
from the column. This is clearly shown in Fig. 2.6.

To determine the optimum conditions for the separation 
of glycosylated proteins diluted plasma from both diabetic 
and non-diabetic individuals was prepared and 150μl added to 
a column. An elution profile (Fig. 2.5B) was prepared for 
each by collecting 1ml fractions of wash and elution buffer 
in an identical manner to that outlined above. The protein 
concentration of each fraction was measured at 595nm after 
the addition of acidified Coomassie Brilliant Blue reagent. 
As the bulk of the material was in the first wash fractions 
these were diluted with wash buffer to ensure an absorbance 
within the linear range of the reagent (Fig. 2.2). The 
results were calculated accordingly.

Blank values were obtained using an elution profile 
scheme for a column to which water rather than diluted plasma 
had been added. This produced a horizontal line (i.e. no 
profile) with absorbances at 595nm between 0.001 and 0.002.

On the basis of the elution profile studies for both 
methods, the wash volume was increased to 8ml to ensure the 
removal of all of the "unbound" material from the column. The 
elution buffer volume was also increased to 3ml to ensure 
that likewise all of the "bound" material was removed. These
Fig. 2.6 Visual account of the separation of "bound" from "unbound" haemoglobin, using the affinity gel, Glycogel B.
volumes are not in agreement with those of Mallia et al. (1981), whose recommendations would not seem to provide such a clear-cut separation.

**pH.**

Mallia et al. (1981) recommended the use of both the wash and elution buffers at pH 8.5 for the separation of glycosylated haemoglobins using affinity chromatography.

To investigate the optimum pH to use for the measurement of glycosylated haemoglobins or glycosylated plasma proteins, a large quantity of both wash and elution buffers were prepared, at 20 ± 1°C. Each buffer was then subdivided and each aliquot was adjusted to a different pH value varying from pH 7.5 to pH 10.0 by the addition of either 1M-HCl or 1M NaOH. For each pH studied, a group of six columns was primed before the assay with the wash buffer of the appropriate pH.

Haemolysate and diluted plasma were prepared from a sample of blood taken from a diabetic individual, and these were incubated at 37°C for 5 hours before use. Aliquots of either the haemolysate (100 μl) or the diluted plasma (150 μl) were added to each column in a six column group. The assays were run as previously detailed, the elution buffer used for each group being at the same pH as the wash buffer. Initially both the glycosylated haemoglobin and glycosylated plasma protein assays were studied over the range pH 7.5 to pH 10.0 in order to gauge the area of optimum pH for each assay. It was found that the pH optimum for glycosylated haemoglobins
Fig. 2 The effect of pH on the % glycosylated haemoglobins (■) and % glycosylated plasma proteins (▲) bound to the affinity gel. Each point shows the mean (± SD) of the results from 6 columns.
was between pH 8.0 and pH 8.5, and that the optimum for glycosylated plasma proteins was nearer to pH 9.0. On the basis of these early studies the pH range studied in greater detail for glycosylated haemoglobins was pH 7.5 to pH 8.5, whereas for glycosylated plasma proteins it was pH 8.5 to pH 10.0; the results are shown in Fig. 2.7. For each pH studied a value was obtained for both the wash buffer blank and the elution buffer blank. Compensation for a blank value was particularly important with the glycosylated plasma protein study as varying the pH altered the behaviour of the acidified Coomassie Brilliant Blue reagent, and thereby the protein measurement. Blank corrections cancelled this effect.

Fig. 2.7 shows that there is a plateau between pH 8.0 and pH 8.5 for glycosylated haemoglobins, and for the glycosylated plasma protein measurement there was a maximum at about pH 8.9. On the basis of this work and later studies on storage the pH values routinely adopted for the two methods were pH 8.3 and pH 8.9 respectively.
Temperature.

To investigate the effect of temperature on the assay for glycosylated haemoglobins a series of six column groups were prepared and positioned in temperature controlled incubators at 7°C, 10°C, 12.5°C, 15°C, 17.5°C, 19.5°C, 25°C, 30°C and 37°C. A large quantity of both wash and elution buffers were prepared, divided, and these too were incubated at the temperatures indicated. Before the test each column group was primed with 5ml of wash buffer at the temperature to be studied.

Haemolysate was prepared from a blood sample taken from a diabetic individual, incubated to remove labile material and 100µl was added to each column. The assay was performed as outlined previously, except that the columns and buffers were kept at the required temperatures throughout. The effect of temperature on volume, if any, was calculated and corrected for by weighing the elution sample. The whole study was repeated using haemolysate prepared from the blood of a non-diabetic individual. The effect of temperature was shown for both samples by the differences obtained in % glycosylated haemoglobin calculated for the six column groups at each temperature (Fig. 2.8).

The effect of varying the temperature on the glycosylated plasma protein assay was similarly studied by keeping the column separation stage of the assay at 10°C, 20°C or 37°C. All eluates were brought to room temperature before their protein content was measured with the acidified Coomassie Brilliant Blue Protein reagent.
Fig. 2.8 To illustrate the influence of altering temperature on the affinity chromatography methods for glycosylated haemoglobins (A) and glycosylated plasma proteins (B).
Fig. 2.8 shows that altering the temperature affects the values obtained for either glycosylated haemoglobins or glycosylated plasma proteins. In both cases as the temperature is increased there is a decline in the percentage of "glycosylated" material bound to the column. The extent of this decline, represented by the slopes of the lines, is similar for both methods, and in both cases the effect on the sample from a diabetic patient was more marked than that for the sample from the non-diabetic individual.

The experimental results show that most "glycosylated" material was retained on the column at the lowest temperature used. However, one of the principle aims of this project was to develop a simple, inexpensive and precise technique for routine use in most laboratories, and there are enormous practical difficulties in accurately maintaining low temperature conditions without a cold room. As the laboratory did not have such facilities, and many of the clinical laboratories wishing to adapt this method will be similarly placed, it was decided to proceed with all future work at room temperature. By priming the columns with wash buffer before each run it is possible to accurately maintain $20^\circ C - 1^\circ C$ against a room temperature background which varied between $17^\circ C$ and $24^\circ C$ throughout the year. This degree of temperature control could not be achieved by simply priming the columns with buffer at $7^\circ C$ before use at room temperature because the temperature differential which caused a significant increase in column temperature during the run. This was shown experimentally by thrusting a thermometer into the gel bed immediately after
Fig. 2.9 To illustrate the effect of temperature on the separation of glycosylated haemoglobins by affinity chromatography (▲) and mini-ion-exchange columns (■). From: Glycogel Test Kit: Pierce bio-research products technical bulletin (1981).
the run, and obtaining results between 10°C and 13.5°C.

By maintaining narrow temperature limits (20°C ± 1°C) the changes in results encountered for affinity chromatography with temperature were minimal, representing changes in reproducibility in the order of 2%. These precision figures look most favorable when compared to the effect of similar temperature limits on ion-exchange chromatography methods where similar temperature variation can cause alterations in values of up to 13% (Fig. 2.9).

**Loading.**

For glycosylated haemoglobin measurement Mallia et al. (1981) recommended that 100μl of a 1 in 20 dilution of packed red cells should be added to each column. The Glycogel test kit recommends the addition of 200μl of neat plasma to each column for the measurement of glycosylated plasma proteins, and the final measurement of protein concentration in the two eluates by monitoring absorbance at 280nm. The recommendations of both of these publications were assessed.

(1) **Effect of loading different amounts of haemolysate.**

Erythrocytes collected from normal and diabetic individuals were incubated at 37°C for 5 hours in 20 volumes of isotonic saline (154mmol/l NaCl) to remove labile material, and recovered by centrifugation. These red cells were then haemolysed by freezing and thawing, and their haemoglobin
<table>
<thead>
<tr>
<th>Amount of Haemoglobin Loaded (mg)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>7.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL % Glycosylated Haemoglobins</td>
<td>6.43</td>
<td>6.59</td>
<td>6.49</td>
<td>5.73</td>
<td>5.29</td>
<td>4.91</td>
</tr>
<tr>
<td>SD</td>
<td>0.31</td>
<td>0.13</td>
<td>0.11</td>
<td>0.18</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>DIABETIC % Glycosylated Haemoglobins</td>
<td>13.64</td>
<td>13.90</td>
<td>13.63</td>
<td>14.34</td>
<td>12.49</td>
<td>-</td>
</tr>
<tr>
<td>SD</td>
<td>0.23</td>
<td>0.14</td>
<td>0.44</td>
<td>0.45</td>
<td>0.31</td>
<td>-</td>
</tr>
</tbody>
</table>
concentration was measured using a Coulter Counter (Coulter Electronics Ltd., Luton, Beds.). The normal haemolysate was then adjusted with water to contain 10g/dl haemoglobin and likewise the diabetic sample to contain 7g/dl haemoglobin. 100μl of each haemolysate was added to a gel column and analysed, each different haemolysate being tested four times. For those samples containing 4g/dl haemoglobin or above, an extra 3ml of wash buffer and an extra 3ml of elution buffer were used to ensure the complete removal of the "unbound" and "bound" fractions respectively from the columns. The absorbances of these extra fractions at 414nm were added to the values obtained for the original wash and elution samples.

Table 2.1 shows that the value obtained for % glycosylated haemoglobin for both the normal and the diabetic samples studied, was independent of loading between 0.5mg and 2.0mg of haemoglobin onto the column. When in excess of 4.0mg for the normal sample and 7.0mg for the diabetic, was applied, the columns became overloaded. In excess of these values the binding of the glycosylated haemoglobin is impaired with significantly lower results (p < 0.001).

(2) Effect of loading different amounts of plasma.

Plasma from a diabetic patient was incubated and dialysed against saline for 5 hours to remove any labile glycosylated material.
The concentration of protein in this sample was measured using a standard Biuret method whereby 50μl of the original sample was added to 3ml of Biuret reagent, mixed and incubated at room temperature for 15 minutes before the absorbance was read at 560nm. Quantitation was achieved using protein standards of known value.

Once the protein concentration of the sample was known, the desired dilutions were prepared in deionised water. Then 150μl of each dilution was added to each of a set of four prepared columns, which was equivalent to adding 2.0, 1.0, 0.5, and 0.25mg protein respectively. Similarly 100μl and 200μl of undiluted plasma was applied to two groups of four columns. This was consistent with the addition of 6.7mg and 13.4mg of protein to each column. All of the column groups were analysed using the standard procedure, but to ensure that all of the "bound" and "unbound" material was recovered, two additional 3ml volumes of wash buffer were collected and one additional 3ml volume of elution buffer. To enable all of the eluates to be measured within the linear range established for the protein reagent, appropriate dilutions in wash or elution buffer were made before the addition of the acidified Coomassie Brilliant Blue Protein reagent.

An identical protein-loading investigation was repeated using plasma from a non-diabetic individual. This plasma was diluted so as to provide the same loading of protein on to the columns as with the diabetic sample. For both the non-diabetic and the diabetic samples, where neat plasma was loaded
the absorbances of the eluates at 280nm were noted before any secondary dilutions were made or acidified Coomassie Brilliant Blue Protein reagent was added. Wash buffer or elution buffer, as appropriate, were used as blanks.

Table 2.2 shows the % of glycosylated plasma proteins for the non-diabetic and diabetic samples when different amounts of plasma were added to the affinity columns. The values obtained were independent of loading when the amount of protein loaded was between 0.25 and 2.0mg per one ml column and when protein was measured by the acidified Coomassie Brilliant Blue Protein reagent. When larger amounts of neat plasma were loaded onto the columns and the proteins were measured using the acidified Coomassie Brilliant Blue Protein reagent there was a significant decrease in the percentage of the glycosylated fraction. (\( p < 0.001 \)). At a loading of 13.4mg protein per column the decrease was 41% and 33% for normal and diabetic samples, respectively. To achieve the optimum concentration to permit the use of the acidified Coomassie Brilliant Blue Protein reagent with high column loading it was necessary to dilute the eluates very considerably. This almost certainly accounts for the wider standard deviation of results. When, with neat plasma loading, the protein was measured at 280nm instead of with the acidified Coomassie Brilliant Blue Protein reagent, the results were significantly higher and had a smaller standard deviation. However, the trend of decreasing values with increasing load from 6.7mg to 13.4mg per ml of
Table 2.2  Effect of loading different amounts of plasma protein onto 1ml affinity gel columns.

<table>
<thead>
<tr>
<th>Amount of Plasma Protein Loaded (mg)</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>6.7</th>
<th>13.4</th>
<th>6.7*</th>
<th>13.4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%GPP</td>
<td>6.95</td>
<td>6.93</td>
<td>6.83</td>
<td>7.16</td>
<td>5.83</td>
<td>4.20</td>
<td>7.15</td>
<td>6.06</td>
</tr>
<tr>
<td>SD</td>
<td>0.25</td>
<td>0.33</td>
<td>0.30</td>
<td>0.22</td>
<td>0.19</td>
<td>0.73</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>DIABETIC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.34</td>
<td>0.48</td>
<td>0.50</td>
<td>0.19</td>
<td>0.61</td>
<td>0.28</td>
<td>0.17</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Key:

%GPP is percentage glycosylated plasma proteins

* Value determined at 280nm
column was still observed for both the non-diabetic (p < 0.001) and the diabetic (p < 0.01) samples. This is important to note for as the test sheet supplied with the Glycogel test kit for the measurement of glycosylated plasma proteins recommends the addition of neat plasma to the columns, then different amounts of protein will be added depending on the original protein concentration in the patients sample. These observations are discussed more fully at the end of this chapter, and in chapter 7.

Recovery of Protein from Column.

Recovery values for both glycosylated haemoglobin and the glycosylated plasma protein assays were made. The appropriate absorbance in the original sample and the volume of material added to each column was noted. The absorbances of all of the wash and elution fractions were totalled after making corrections to compensate for the fact that they were collected into different volumes. Since the amount of material added to any column could be interpreted in terms of absorbance value per ml, and the recovered material could be similarly assessed then a % recovery could be calculated. Recovery values between 95% and 98% were obtained.
Lability.

Fig. 1.3 shows that before undergoing an Amadori rearrangement to produce stable glycosylated haemoglobin, glucose and haemoglobin initially combine to form an unstable or labile compound. This labile glycosylated haemoglobin readily reverts to its original components, and is influenced by the glucose concentration at the time of sampling. For this reason, if the labile fraction is measured in addition to the stable form a higher value will result and this will be biased towards recent blood glucose concentrations. Thus the removal of the labile material is theoretically desirable, to provide an accurate measurement of glycaemic history. By diluting a sample in water the equilibrium (Fig. 1.3) is shifted as the free glucose concentration can be made so low as to be virtually removed from the system. This effect causes the reversion of any labile material to the original components, although stable glycosylated haemoglobin will be unaffected as the Amadori rearrangement is essentially an irreversible process. The method of removal of labile material by dilution and incubation, as recommended by Compagnucci et al. (1981) was investigated.

A sample of haemolysate was prepared and immediately tested in accordance with the recommended method. The remainder was divided, and one portion was incubated at 37°C, the other at room temperature (20°C). At intervals of 1, 2.5, 5, 16 and 24 hours respectively aliquots of the incubated haemolysates were removed and analysed, each being tested five times.
Fig. 2.10 The effect of different incubation times and temperatures on the % haemoglobin bound by the affinity method.

The diagram shows the results for incubating a normal haemolysate at 20°C (■) and 37°C (●) for various time intervals up to 24 hours. Each point represents the mean (± SD) of the results from 5 columns.
Fig. 2.10 shows that the values of glycosylated haemoglobin decrease from that achieved with the fresh sample by incubation at both 37°C and 20°C, this would be consistent with removal of labile material. After 5 hours at 37°C a plateau was obtained. This lower value took 24 hours when incubation was at 20°C.

When incubation was continued at 37°C overnight the value for % glycosylated haemoglobin apparently increased. This was originally assumed to be due to the formation of met-haemoglobin interfering with the method, but the work with the storage experiments have shown this not to be the case. This phenomenon, if real, remains as yet unexplained.

Other experiments were done which showed similar trends to those outlined; an initial decrease occurring by incubating at 37°C which gave a plateau after 5 hours and then if left still longer was seen to increase again. However many of these observations with other diabetic and non-diabetic samples, although showing similar trends, were not so marked as in the given sample. As the amount of labile material during these investigations seemed to vary greatly from individual to individual a larger study was devised to see if this was the case and if there was any relation between labile material and the glucose at the time of sampling.

Blood was taken from patients at a diabetic clinic. Part was put into lithium heparin tubes which were stored at 4°C until required, up to a maximum of one hour, and the remainder was put into sodium fluoride tubes for the
Glycosylated plasma proteins and haemoglobins were measured before and after (total) incubation at 37°C for 5 hours.
measurement of glucose. This was measured within 10 minutes of sampling using the Yellow Springs Glucose Analyser, Model 23 AM (Yellow Springs Instrument Co., Yellow Springs, OH 45387, USA). The whole blood samples were centrifuged and both haemolysate and diluted plasma were prepared in accordance with the standard method instructions. These were then tested immediately and after a 5 hour incubation at 37°C by the affinity chromatography method outlined.

Of the 12 patients studied, all had lower glycosylated haemoglobin (Fig. 2.11) values after the incubation, but there was no significant correlation with blood glucose ($r = 0.235$). Again, a large intra-individual variation was observed, with the labile material representing between 2.5% and 14.5% of the total value obtained for the patient concerned. The results of studying lability in glycosylated plasma proteins were less clear-cut as 7 patients had decreased values after the incubation but 5 gave increased results (Fig. 2.11). This may be due to the fact that the method is less precise than that for glycosylated haemoglobins and so the removal of any labile material may not be clearly seen. However, other factors should not be discounted which may be influencing the results. It may be that the protein molecules gradually unwind when diluted in water and this may alter their binding characteristics, to the column, with glucose, or even with the Coomassie Brilliant Blue reagent. For all of these reasons it was decided for the sake of consistency that diluted plasma should be incubated for 5 hours at 37°C prior to the measurement of glycosylated plasma proteins.
Similarly all haemolysates were routinely incubated for 5 hours at 37°C before the glycosylated haemoglobin assay.

**Storage.**

As the methods described were to be routinely used for the clinical assessment of diabetic patients, it was necessary to determine the effect of storage on samples, many of which originated at evening clinics or in general practitioner's surgeries where the immediate measurements of glycosylated materials were impractical.

A diabetic haemolysate was prepared and incubated as recommended in section 1 of this chapter. Some of this haemolysate was tested immediately, and some aliquots were stored at 4°C for analysis after one week. Similarly, a 20 to 1 dilution of the same sample was prepared using cyanide solution instead of water to haemolyse the cells. This was treated in an identical manner.

The remainder of the red cells from the sample were stored undiluted at 4°C, and the haemolysate was prepared for analysis after a period of 7 days.

All samples were analysed not only at pH 8.3 but also at 8.5, 8.1, 7.8 and 7.5. The pH of the appropriate wash and elution buffers was adjusted with 1M-HCl. Four samples of each different haemolysate were analysed.
Table 2.3 shows that different storage conditions affect glycosylated haemoglobin values over the pH range 7.5 to 8.5. For fresh haemolysate the values obtained for pH 7.8 to 8.5 are equivalent. Storage of haemolysate at 4°C for seven days causes a decrease in value for pH 7.5 and 8.5 compared to that at 8.1 and 8.3. A similar trend is found when erythrocytes are kept for seven days at 4°C before the preparation of the haemolysate.

Cyanide increases the values of glycosylated haemoglobin at all of the pHs studied, and these values increased further when the haemolysate containing cyanide was stored for seven days at 4°C. This is discussed later in the chapter.

The effect of storage on the estimation of glycosylated plasma proteins was also investigated. A 20 to 1 dilution of plasma from a diabetic patient was prepared and incubated. The glycosylated plasma proteins were determined on the same day and the remainder of the sample was split into five portions. Two of these fractions were stored at 4°C, one to be analysed after 4 days and the other after a 7-day incubation. The remaining material was all stored frozen (-10°C) for analysis after 1, 4 and 7 days respectively. All the samples were analysed over a range of pH values; pH 8.5, 8.9, 9.2, 9.5, 10.0. The pH of the appropriate wash and elution buffers being adjusted with 1M NaOH. Each different fraction of the diluted plasma was analysed four times.

Table 2.4 shows how different storage conditions affect the glycosylated plasma protein values over the pH range from 8.5 to 10.0. The values obtained at pH 8.5, 8.9, and 9.2 were similar for all samples, both fresh and stored at 4°C for up to 7 days. However freezing the plasma for up to 7
Table 2.3  **Effect of sample storage on % glycosylated haemoglobin**

<table>
<thead>
<tr>
<th>pH of buffers</th>
<th>7.5</th>
<th>7.8</th>
<th>8.1</th>
<th>8.3</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysate</td>
<td>Mean</td>
<td>16.74</td>
<td>17.57</td>
<td>17.09</td>
<td>17.34</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.12</td>
<td>0.35</td>
<td>0.31</td>
<td>0.49</td>
</tr>
<tr>
<td>Haemolysate</td>
<td>Mean</td>
<td>15.50* * *</td>
<td>16.70* * *</td>
<td>17.14 NS</td>
<td>17.37 NS</td>
</tr>
<tr>
<td>stored 7 days</td>
<td>SD</td>
<td>0.45</td>
<td>0.15</td>
<td>0.30</td>
<td>0.13</td>
</tr>
<tr>
<td>at 4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Mean</td>
<td>15.70* * *</td>
<td>16.50* * *</td>
<td>16.65*</td>
<td>17.14 NS</td>
</tr>
<tr>
<td>stored 7 days</td>
<td>SD</td>
<td>0.41</td>
<td>0.09</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>at 4°C then</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>haemolysed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemolysate</td>
<td>Mean</td>
<td>16.77 NS</td>
<td>17.81 NS</td>
<td>18.24* * *</td>
<td>18.24* * *</td>
</tr>
<tr>
<td>containing</td>
<td>SD</td>
<td>0.49</td>
<td>0.17</td>
<td>0.31</td>
<td>0.12</td>
</tr>
<tr>
<td>cyanide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemolysate</td>
<td>Mean</td>
<td>17.26* * *</td>
<td>18.69* * *</td>
<td>18.63* * * *</td>
<td>19.13* * * *</td>
</tr>
<tr>
<td>containing</td>
<td>SD</td>
<td>0.16</td>
<td>0.29</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>cyanide stored</td>
<td>7 days at 4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results for stored samples were compared to those for fresh haemolysate at the appropriate pH.

Key:

- **NS**  no significant difference
- *  significantly different at p = 0.05
- **  significantly different at p = 0.02
- ***  significantly different at p = 0.01
- ****  significantly different at p = 0.001
days causes a significant decrease in values at pH's 8.5 (p = 0.01) and 8.9 (p = 0.05), but not at pH 9.2. Freezing the diluted plasma sample serves to alter the values, possibly by the precipitation of fibrinogen, or by denaturing the protein and thereby altering its binding properties.

The results of these investigations show that for both assays the sample material can be stored in diluted form for up to seven days at 4°C without deterioration, providing the pH of the buffers is correctly adjusted. Red cells can be similarly stored before the haemolysate is prepared. This simplifies the practical difficulties of dealing with samples taken at times when immediate assay is not feasible. This work further substantiates the original experiments in which the optimum pH for the glycosylated haemoglobin and glycosylated plasma protein assays was found to be pH 8.3 and 8.9, respectively.
Table 2.4  The effect of sample storage on the % glycosylated plasma proteins.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh plasma</td>
<td>18.77</td>
<td>19.01</td>
<td>18.43</td>
<td>16.77</td>
<td>14.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>0.66</td>
<td>0.55</td>
<td>0.33</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Plasma stored for 4 days at 4°C</td>
<td>17.80NS</td>
<td>18.82NS</td>
<td>18.67NS</td>
<td>17.47NS</td>
<td>15.10NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.92</td>
<td>0.23</td>
<td>0.66</td>
<td>0.54</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Plasma stored for 7 days at 4°C</td>
<td>18.51NS</td>
<td>19.48NS</td>
<td>19.24NS</td>
<td>18.48***</td>
<td>15.39**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.51</td>
<td>0.57</td>
<td>0.97</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Plasma stored for 24 hr at -10°C</td>
<td>18.30NS</td>
<td>18.41NS</td>
<td>19.03NS</td>
<td>17.74NS</td>
<td>15.09NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.32</td>
<td>0.71</td>
<td>0.97</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Plasma stored for 4 days at -10°C</td>
<td>18.16*</td>
<td>19.36NS</td>
<td>19.08NS</td>
<td>17.49NS</td>
<td>15.20*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>0.78</td>
<td>0.23</td>
<td>0.74</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Plasma stored for 7 days at -10°C</td>
<td>17.96***</td>
<td>18.13*</td>
<td>18.34NS</td>
<td>18.06*</td>
<td>15.01NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.29</td>
<td>0.11</td>
<td>0.45</td>
<td>0.79</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

The results for stored samples were compared to those for fresh plasma tested at the appropriate pH.

Key:
- **NS** no significant difference
- * significantly different at p = 0.05
- ** significantly different at p = 0.02
- *** significantly different at p = 0.01
Effect of Met-haemoglobin.

As met-haemoglobin forms during sample storage it was considered important to investigate its influence on the affinity method for glycosylated haemoglobin measurement.

Sodium nitrite solution was prepared by dissolving 7.6mg of NaNO$_3$ in 10ml of isotonic saline.

Method.

Haemolysates containing a high proportion of met-haemoglobin were prepared by the method of Van Assendelft and Zijlstra (1965). Red blood cells (0.5ml) from both a normal and a diabetic individual were dialysed against isotonic saline at 37°C for 5 hours. After centrifugation the saline was removed and the packed red cells resuspended in an equal volume of fresh saline. This suspension was divided into two 1ml portions. To the first, or control portion, was added 2ml of saline. To the second, was added 2ml of saline containing the sodium nitrite. Both were then incubated at 37°C for 15 minutes, and it was noted that the fraction containing the nitrite went very dark brown. Both fractions were then centrifuged and washed three times with saline to remove any excess nitrite, after which 100μl of packed red cells from both the test and control fractions were added to 2ml of deionised water. After vortexing, these haemolysates the percentage of glycosylated haemoglobin was measured as previously described.
Table 2.5  The effect of met-haemoglobin on the
% haemoglobin bound by the affinity method.

<table>
<thead>
<tr>
<th>% met-haemoglobin</th>
<th>% Glycosylated haemoglobin (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.22 + 0.12</td>
</tr>
<tr>
<td>5</td>
<td>11.17 + 0.62</td>
</tr>
<tr>
<td>10</td>
<td>10.47 + 0.10</td>
</tr>
<tr>
<td>20</td>
<td>10.28 + 0.11</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16.63 + 0.17</td>
</tr>
<tr>
<td>5</td>
<td>16.24 + 0.18</td>
</tr>
<tr>
<td>10</td>
<td>16.23 + 0.26</td>
</tr>
</tbody>
</table>
| 20                | 16.22 + 0.08                         **

All quoted values are the results of triplicate analysis.

** This was the only value which was significantly different (p < .02) from the result at 0%.
It was shown (Table 2.5) that there was no significant difference in values obtained for the untreated samples or the samples containing varying amounts of met-haemoglobin. Only the diabetic sample containing 20% met-haemoglobin had values significantly different from the untreated sample. This was probably due to the very small deviation found in this particular test group (Table 2.5), and it was concluded that met-haemoglobin did not interfere with the method. It was therefore unnecessary to include cyanide in the diluent for the preparation of the haemolysate.

**Effect of Anticoagulant.**

Blood was collected from a diabetic patient and 5ml was put into a tube containing 75IU of lithium heparin, and mixed. Aliquots of this blood were transferred to other tubes to achieve concentrations of 3 times and 6 times the recommended level of 15 IU/ml. Blood was added to tubes containing sodium fluoride, and in a similar manner different multiples of the recommended concentration of 1.0mg/ml were achieved. Blood was collected from a non-diabetic patient and prepared in an identical manner.

All of the prepared material was then haemolysed and incubated in accordance with the routine method for glycosylated haemoglobin measurement and four samples of each haemolysate were analysed.
There was no significant difference between the two anticoagulants used (Table 2.6) and so these tubes could be used interchangeably for the preservation of blood samples for this test. Furthermore, the concentration of anticoagulant, in both cases, did not appear to be critical. Only the non-diabetic sample at 6 times the recommended concentration of sodium fluoride or lithium heparin differed significantly ($p < 0.05$, $p < 0.05$). No such observations were made for the diabetic sample (Table 2.6). Although the use of sodium fluoride tubes offered the advantage that blood from the same tube could be tested for blood glucose concentration, this material proved to be less efficient than lithium heparin as an anticoagulant and so the latter was used routinely for the preservation of samples.
Table 2.6  The effect of different anticoagulants, at varying concentrations, on the % glycosylated haemoglobins.

<table>
<thead>
<tr>
<th>M</th>
<th>Non-diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.42 ± 0.12</td>
<td>13.52 ± 0.45</td>
</tr>
<tr>
<td>3</td>
<td>7.19 ± 0.93 NS</td>
<td>13.51 ± 0.12 NS</td>
</tr>
<tr>
<td>6</td>
<td>6.74 ± 0.23 *</td>
<td>13.34 ± 0.35 NS</td>
</tr>
<tr>
<td>Lithium heparin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.66 ± 0.19</td>
<td>13.33 ± 0.53</td>
</tr>
<tr>
<td>3</td>
<td>6.61 ± 0.54 NS</td>
<td>13.38 ± 0.14 NS</td>
</tr>
<tr>
<td>6</td>
<td>6.26 ± 0.24 *</td>
<td>13.51 ± 0.44 NS</td>
</tr>
</tbody>
</table>

Each level of anticoagulant used was tested against the recommended level to see if the results differed significantly.

Key:
- NS  non-significant difference
- * differed at $p = 0.05$
- M is the multiple of the recommended level of anticoagulant.
Column Reuse.

Five groups of columns were prepared, with five columns in each group. Each group was used a different number of times for the measurement of glycosylated haemoglobin. The number of times used were 3, 7, 12, 16 and 19 for each group, respectively. When not in use the columns were stored in the dark at 4°C. After each use the gel was regenerated with acetic acid (0.01M).

When all of the columns had been used for the specified number of times (within a period of four weeks), all of them were then used for the replicate measurement of glycosylated haemoglobins in a single diabetic haemolysate. Each group was statistically compared to a fresh set of five columns. A small decrease in the amount of "bound" material was apparent with increasing usage (Table 2.7). However, when compared to fresh columns this decrease was less than 2% for all groups of columns, except those that had been used more than 16 times. The columns that had been used 19 times showed a significant decrease from 20.24 \pm 39\% to 19.72 \pm 0.12\% (p < 0.05) (Table 2.7). The columns gradually became darker with re-use, particularly at the surface of the gel. A single column was prepared of unused gel which was then stored at room temperature and in the light for three weeks. The percent bound haemoglobin for this column was compared to unused columns stored at 4°C and in the dark for the same period of time. The column kept in the light went a dark purple colour, this is well illustrated in Fig. 2.12. Although not previously
Fig. 2.12 To illustrate the darkening of the affinity gel when exposed to light at room temperature.
used to analyse samples, this column was shown to have reduced capacity to bind glycosylated haemoglobin, and a value of 6.22% was observed, compared to 12.11% for the same sample using fresh columns.

Similar investigations were done to see how many times the gel could be regenerated between use, for glycosylated plasma protein measurements. Again five column groups were prepared and each group was used 3, 6 and 9 times for the measurement of glycosylated plasma proteins. When not in use the columns were stored in the dark at 4°C. After each use the gel was regenerated with acetic acid (0.01M).

When the same diabetic sample was then loaded onto all the columns only those columns used nine times previously showed a significant decrease (p < 0.001) from 15.68 ± 0.33% to 14.09 ± 0.32%. Again the columns became darker with increasing use.

As a result of these investigations the columns were regenerated and reused no more than 12 times for the measurement of glycosylated haemoglobins, and no more than 6 times for the measurement of glycosylated plasma proteins.
Table 2.7 To illustrate the effect of gel reuse on the measurement of glycosylated haemoglobin and glycosylated plasma proteins by affinity chromatography.

<table>
<thead>
<tr>
<th>Number of uses</th>
<th>% glycosylated haemoglobins</th>
<th>% glycosylated plasma proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>fresh</td>
<td>20.24 ± 0.39</td>
<td>15.68 ± 0.33</td>
</tr>
<tr>
<td>3</td>
<td>19.85 ± 0.24</td>
<td>15.43 ± 0.29</td>
</tr>
<tr>
<td>6</td>
<td>20.16 ± 0.27</td>
<td>15.51 ± 0.27</td>
</tr>
<tr>
<td>9</td>
<td>19.92 ± 0.20</td>
<td>14.09 ± 0.32 **</td>
</tr>
<tr>
<td>12</td>
<td>18.95 ± 1.02</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>19.89 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>19.72 ± 0.12 **</td>
<td></td>
</tr>
</tbody>
</table>

Results are the mean result from 5 analysis, together with the standard deviation.

Key:

- **NS** non-significant
- **differed at p = 0.02**
- **differed at p = 0.001**
The work in this section was done using the conditions outlined in section 1 of this chapter. All assays were carried out at 20°C ± 1°C. The pH of the buffers used was pH8.3 for glycosylated haemoglobins and pH8.9 for glycosylated plasma proteins, except for the precision studies when pH8.5 was employed for the glycosylated haemoglobin assay. All samples were collected in lithium heparin and were assayed within 24 hours of sampling, unless otherwise stated. Labile material was removed routinely by incubation of the diluted sample for 5 hours at 37°C. The volume of diluted sample (100μl of sample +2ml deionised water) added to the column was 100μl and 150μl for glycosylated haemoglobins and glycosylated plasma proteins, respectively.
Precision.

Using the recommended conditions, experiments were carried out to determine the precision of the measurement for both glycosylated haemoglobins and glycosylated plasma proteins.

Haemolysates were made from blood of both a normal and a diabetic individual, and incubated. Within-batch precision was determined by preparing a series of 20 fresh columns and adding 100μl of the haemolysate to each. The results for both normal and diabetic samples are shown in Table 2.8. The same samples were stored at 4°C and run on different columns over a period of a week; a total of 15 runs for each. These values were used to calculate the between-batch precision of the assay (Table 2.8). Table 2.8 shows that the method for the assay of glycosylated haemoglobins is highly reproducible, with coefficients of variation of 1.5% to 2.0%.

Values for within-batch and between-batch precision were obtained for glycosylated plasma proteins by doing identical studies with diluted plasma instead of haemolysate and using the affinity method described earlier in this chapter for this measurement. (Table 2.8). It was necessary to assess the contribution to the observed error made by the additional step of adding the acidified Coomassie Brilliant Blue protein reagent. A sample of diluted plasma was therefore further diluted with wash buffer to 27ml, and 500μl of this solution was put into 20 cuvettes. Fresh acidified protein reagent
Table 2.8  **Precision of the affinity method for glycosylated haemoglobins and glycosylated plasma proteins.**

<table>
<thead>
<tr>
<th></th>
<th>Within-batch (n = 20)</th>
<th>Between-batch (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% C.V.%</td>
<td>% C.V.%</td>
</tr>
<tr>
<td><strong>GLYCOSYLATED HAEMOGLOBINS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>mean 6.81 2.0</td>
<td>mean 7.00 1.8</td>
</tr>
<tr>
<td></td>
<td>S.D. 0.14</td>
<td>S.D. 0.13</td>
</tr>
<tr>
<td>Diabetic</td>
<td>mean 20.04 1.5</td>
<td>mean 19.73 1.5</td>
</tr>
<tr>
<td></td>
<td>S.D. 0.30</td>
<td>S.D. 0.30</td>
</tr>
<tr>
<td><strong>GLYCOSYLATED PLASMA PROTEINS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>mean 5.83 4.1</td>
<td>mean 5.87 7.6</td>
</tr>
<tr>
<td></td>
<td>S.D. 0.24</td>
<td>S.D. 0.45</td>
</tr>
<tr>
<td>Diabetic</td>
<td>mean 13.11 3.9</td>
<td>mean 12.37 5.2</td>
</tr>
<tr>
<td></td>
<td>S.D. 0.51</td>
<td>S.D. 0.65</td>
</tr>
</tbody>
</table>
was then added to each, mixed and the absorbance recorded after exactly 30 minutes at 595 nm against air.

The mean absorbance value for these 20 assays was 0.351 with a standard deviation of 0.010, giving a coefficient of variation of 2.72%. From these investigations therefore it is likely that the wider variation (Table 2.8) found with the glycosylated plasma protein measurement, as compared with the glycosylated haemoglobins, is due almost entirely to the additional step required for the protein measurement. The contribution of this additional step to the total method error can be calculated.

Calculation of the contribution of the Coomassie Brilliant Blue dye measurement to the total error in the measurement of glycosylated plasma proteins using affinity chromatography.

The variation that individual components (x₁, x₂, etc.) contribute to the total method error can be calculated through the sum of squares rule:

\[ \text{Total method error} = x_1^2 + x_2^2 + \ldots \]

The total method error for glycosylated plasma proteins of a non-diabetic was + 4.1%, and of a diabetic was + 3.9%.

The error calculated for the Coomassie Brilliant Blue dye measurement was 2.72%.
By substitution the variation of the affinity step can be calculated to be 3.06% and 2.8% for the non-diabetic and diabetic groups, respectively. These figures are only slightly higher than those for the glycosylated haemoglobin assay (1.5% and 2.0%).

Normal Range.

The range of stable glycosylated haemoglobins and glycosylated plasma proteins found in 62 non-diabetics is shown in Table 2.9. The range in a group of 138 insulin-treated diabetic patients is also shown (Table 2.9). These diabetic patients were all seen as out-patients and were considered to be clinically in glycaemic control. However, only 15 of the patients had values within the non-diabetic range and there was a considerable spread of results with values as high as 21.5% observed. This is clearly seen in Fig. 2.13. The limitations of a single blood glucose measurement are clearly illustrated by the fact that 5 patients with glycosylated haemoglobins values above 15% had simultaneous glucose below 10mmol/l, and conversely 3 patients with high blood glucose levels had glycosylated haemoglobin values below 10%. (Fig. 2.13). The normal range was narrower and differed significantly from the diabetic group (p < 0.001). The range for non-diabetic individuals encompassed two different populations; normal subjects being healthy laboratory workers (6.51 ± 0.52%) and non-diabetic patients (7.45 ± 0.90%), which differed significantly (p < 0.001).
Fig. 2.13 Histogram showing the distribution of % glycosylated haemoglobins for diabetic and non-diabetic individuals. The results include those for individual laboratory staff (▲). The diabetic patients with blood glucose above 10mmol/l are noted (●).
The ranges shown for 58 non-diabetic and 153 insulin-treated, supposedly stabilised diabetic patients for the stable glycosylated plasma protein measurement are also given in Table 2.9. Again a wide spread of values for diabetic patients was observed (Fig. 2.14), with only 38 patients having values within the calculated normal range. Of these, only 4 had high glucose values. Similar to the findings with the measurement of glycosylated haemoglobins, of the 35 patients with glycosylated plasma protein values of 15% or above, only 14 had simultaneous blood glucose levels below 10mmol/l.

Again the normal range was narrower, having within it the two populations, namely laboratory staff (5.73 ± 0.99%) and non-diabetic patients (6.43 ± 2.03%). However, this time these groups did not differ significantly. The results from non-diabetic and diabetic groups of individuals did differ significantly ($p < 0.001$).
Fig. 2.14  Histogram showing the distribution of % glycosylated plasma proteins for diabetic and non-diabetic individuals. The results include those for individual laboratory staff (▲). The diabetic patients with blood glucose above 10mmol/l are noted (●).
Table 2.9  To illustrate the ranges obtained for glycosylated haemoglobins and glycosylated plasma proteins with non-diabetic and diabetic patients.

<table>
<thead>
<tr>
<th></th>
<th>GLYCOSYLATED HAEMOGLOBINS</th>
<th>GLYCOSYLATED PLASMA PROTEINS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NON-DIABETIC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 62)</td>
<td>7.31 ± 0.92%</td>
<td>(n = 58) 6.29 ± 1.87%</td>
</tr>
<tr>
<td>observed range</td>
<td>5.25% to 9.70%</td>
<td>2.95% to 10.27%</td>
</tr>
<tr>
<td><strong>DIABETICS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 138)</td>
<td>12.70 ± 2.88%</td>
<td>(n = 153) 12.62 ± 3.36%</td>
</tr>
<tr>
<td>observed range</td>
<td>6.50% to 21.5%</td>
<td>6.05% to 23.31%</td>
</tr>
</tbody>
</table>
Quality Control.

The work for this study was begun in January 1982 and quality control measurements were employed from July 1982, for both the glycosylated haemoglobin and glycosylated plasma protein assays.

1) Glycosylated haemoglobins.

A large pool of diabetic haemolysate was prepared by diluting 5ml of red blood cells from a poorly controlled diabetic in 1 litre of deionised water. After thorough mixing, aliquots (0.5ml) of this haemolysate were stored in liquid nitrogen until required. Before use, a sample was brought to room temperature and then incubated at 37°C for 5 hours to remove labile material. A sample of this quality control material was run with every batch of columns for the glycosylated haemoglobin assay.

The results for the first 20 runs using this quality control material were noted and the mean and between-batch variation was calculated (18.96 ± 0.89%). Subsequent quality control results were compared against these values and each had to be within these limits for the results of the rest of the batch to be reported.
(2) **Glycosylated plasma proteins.**

The quality control employed for the glycosylated plasma protein assay was the Wellcomtrol standard (Wellcome Diagnostics, Dartford, Kent. batch no. K1619), which was prepared daily from lyophilised material for use throughout the laboratory. After reconstitution, 100μl of the material was diluted with 2ml of water and incubated for 5 hours at 37°C before use. As with the glycosylated haemoglobins, the first 20 results were used to calculate the mean value and between-batch variation (17.47 ± 1.33%) and all subsequent quality control values for the assay had to be within these limits to be acceptable.

(3) **Changing between different batches of affinity gel.**

Gel of various different batch numbers was supplied by Pierce Chemical Co. Ltd. for this affinity chromatography work. As well as the quality control scheme, when changing between batches, the last batch of 20 freshly poured columns of the older batch number were run in tandem with 20 fresh columns from the new batch and the results were compared to check that there was no significant difference. Only one batch of gel was rejected on this basis.
DISCUSSION.

Affinity chromatography provides a very reproducible and sensitive technique for the measurement of both glycosylated haemoglobins (CV. 1 to 2%) and glycosylated plasma proteins (CV. about 5%). There are only two critical volume measurements; the 3ml of elution buffer and the adjustment of the wash fraction to 15ml or 24ml, as appropriate. As the values are expressed in terms of percentages, standards are not required, which may partly account for the high reproducibility of the technique. The slightly wider variation (5%) found with the measurement of glycosylated plasma proteins can be accounted for by the additional step required to measure the protein content in each fraction.

The methods both give good discrimination between diabetics (IDDM) and non-diabetic individuals (Figs 2.13 and 2.14). The glucose results in both figures show that a diabetic patient with either a high glycosylated haemoglobin or glycosylated plasma protein, does not necessarily have a high simultaneous blood glucose, and vice versa. This implies that the measurement of glycosylated material is more useful for monitoring long-term glycaemic control. For the studies using glycosylated haemoglobins, the normal range had within it two significantly different populations: normal subjects (the laboratory workers) and the non-diabetic patients. This is possibly age-related as the laboratory workers were generally between the second and third decades, while the patient population were predominantly of middle to older age. However other studies described later in this
thesis can show no association between age and control in our diabetic clinic. Maybe the difference is due to another factor such as the reduced mobility of many of the patients.

The removal of labile material is recommended. This is necessary as the percentage of labile material varies considerably between individuals (Fig 2.11) and is not apparently related to blood glucose concentration in the same sample. These findings do not agree with those of Abraham, Perry and Stallings (1983) who suggest that only the stable ketoamine binds to the affinity gel. However, theoretically labile material should bind to the gel as the glucose attached to haemoglobin has cis-diol groups. The wide intra-individual variation may be due to dissociation of labile material during chromatography causing variable results in the separation.

The method is very versatile. Since either lithium heparin or sodium fluoride may be used as anti-coagulants, it is possible to do the test on most of the blood received routinely. The use of sodium fluoride allows the simultaneous measurement of glucose and glycosylated haemoglobin on the same sample. However as lithium heparin proved to be a much better anti-coagulant, this was recommended for use in the diabetic clinics studied. Very little sample is required, enabling glycosylated haemoglobin to be measured in blood taken from a fingerprick. Theoretically the measurement of glycosylated plasma protein should also require only a small sample of plasma, but in practice it was difficult to avoid extensive haemolysis in small plasma samples.
Binding to the gel column appears to be independent of the amount of haemoglobin between 0.5mg and 2.0mg loaded per column. This is equivalent to a haemoglobin concentration between 5.0g/dl and 20g/dl in the original sample, assuming a packed cell volume of 50%. This means that the method is applicable to almost the whole range of concentrations encountered in non-diabetic and diabetic patients. It must be remembered however, that other features such as reduced red cell survival time may affect the glycosylated haemoglobin result. Similar results were found with the measurement of glycosylated plasma proteins, where the results were independent of loading between 0.25mg and 2.0mg of protein per ml gel column.

Storage of both the samples and the gel is important. The results (Table 2.3) indicate that immediate analysis of the stable haemolysate is preferable, but storage at 4°C for up to a week still enables 96% of the original result to be obtained. This is not as long as the storage allowed by Little et al. (1983) of upto 21 days. The pH of both buffers for the measurement of glycosylated haemoglobins as recommended by Mallia et al. (1981) was 8.5, but the results in this thesis (Table 2.3) show that the pH of 8.3 may be preferable since it appears to nullify the difference in percent glycosylated haemoglobin in fresh and stored samples. Similarly, plasma can be stored successfully at 4°C for up to a week with little effect on the results. The use of both the wash and elution buffers at pH8.9 seems to be optimum for this assay (Fig. 2.7, Table 2.4).
Met-haemoglobin was shown not to interfere with the method (Table 2.5) and so it was considered unnecessary to include cyanide in the diluent for the preparation of haemolysate to stop its formation. This avoided any potential hazard clue to the inclusion of cyanide in the reagent. Also, Middle et al. (1983) have indicated that the ionicity of the buffers used with this gel is important as increased hydrophobic conditions affects the boron-diol interaction. Therefore the inclusion of material such as cyanide, or indeed saline, in any sample added to the column was avoided, as this could potentially alter the column characteristics. This effect was shown in the storage experiments (Table 2.3) where the presence of cyanide with a fresh sample caused an increased percentage of haemoglobin binding to the column, and this value increased still further with storage.

At the beginning of this work no satisfactory QC scheme suitable for general use had been found and the use of 'in-house' quality control was recommended (Boucher et al. 1983) for monitoring the performance of any individual assay. During the work for this thesis various companies supplied QC material for use with their particular methodology. However, these proved unsuccessful for use with the affinity method, presumably as the affinity method measures different material (including GHbA0), and so the values for methods measuring other components were not directly applicable. At this time Pierce Chemical Co. Ltd. did not supply QC material for use with Glycogel B. The use of a pool of haemolysate stored at -20°C proved to be satisfactory as a means of quality control.
for glycosylated haemoglobins, as did the use of freshly prepared wellcomtrol standard for the glycosylated plasma protein assay. When changing from one batch of affinity gel to another, the consistency of the results was also checked. This, and the quality control schemes were important considerations as some of the clinical studies were relatively long-term (18 months to 2 years) and so it was necessary to ensure that observed effects were not due to differences in the gel manufacture.

As described, the method is eminently suitable for routine use, as the work may be carried out at room temperature on the laboratory bench. There is no requirement of stringent control of either temperature or pH as with the ion-exchange methods, although the columns must be suitably primed before use. Positioning the columns in direct sunlight should be avoided to prevent a more rapid ageing of the gels. The technique is simple to perform, and the affinity gels are very robust. They may be left to drain for several hours without drying out and so 30 samples may be handled in a run, the main limiting factor being bench space. Since the measurement of glycosylated haemoglobin only take 40 minutes, it is convenient to test 60 samples in a single working day. The measurement of glycosylated plasma proteins takes about an hour longer to perform as there is an additional step required for the protein measurement. However with good organisation 50 to 60 plasma samples may, alternatively, be measured within a working day. All the equipment required for either assay should be available in any hospital laboratory, and by using this method the need for expensive equipment is obviated.
Since the gel can be successfully reused, the price of the gel is kept to about 33p per assay, which is highly competitive with other techniques such as that marketed by Corning (53p per test) and the mini-ion-exchange columns from Biorad (97p per test). These costs are calculated at the time of writing this thesis, and do not include V.A.T.

At the beginning of the work for this thesis the major aim was to develop an affinity method to provide the measurement of glycosylated haemoglobins for the clinicians at a reasonable cost. Ideally this method should technically be within the capability of the most junior of laboratory staff. At the time no suitable inexpensive method was marketed and the colorimetric technique proved difficult to do on a day-to-day basis. The method described in this chapter fulfils all of the original aims. It provides good discrimination between diabetics and non-diabetics, and has been used to monitor the progress of diabetic patients over a two year period (see later, Chapter 5). Affinity chromatography confers the additional advantage that glycosylated plasma proteins may also be measured using the same material. As these are also expressed in terms of a percentage value this measurement may be directly compared to that of glycosylated haemoglobin, and the usefulness of each in different clinical situations assessed (see later Chapters 4, 5 and 6).
CHAPTER 3.

ASSESSMENT OF THE AFFINITY METHODS FOR THE MEASUREMENT OF GLYCOSYLATED HAEMOGLOBINS AND GLYCOSYLATED PLASMA PROTEINS.
INTRODUCTION.

In 1981 Bunn reviewed the techniques then available for the measurement of glycosylated haemoglobins. The most commonly used method was cation-exchange chromatography using Biorex 70, generally packaged in the form of disposable columns. As already discussed (Chapter 1) this technique has several disadvantages, a serious one being that it is very sensitive to small variations in temperature and pH (Rosenthal, 1979). Also the presence of haemoglobin variants can either cause a false increase, e.g. HbF (Simon and Eissler, 1980) or a false decrease e.g. HbS, HbC (Aleyassine, 1979). Falsely high values for HbA₁ have been found in patients with uraemia (Flückiger et al. 1981), possibly in alcoholics (Stevens et al. 1981) or those on high doses of aspirin (Spicer et al. 1979), and may be expected in some young children with lead poisoning (Charache and Weatherall, 1966). Lactescent plasma also causes falsely high values, but only if whole blood is analysed by a rapid assay (Dix et al. 1979). Some of these interferences are caused by other components attaching themselves to the haemoglobin molecule; uraemic patients yield elevated levels of carbamylated haemoglobin, and aspirin poisoning produces acetylated haemoglobin. These changes alter the change/mass ratio of haemoglobin A₁, causing it to behave like glycosylated material when using those methods which are charge-dependent for separation. However, the principle of separation with affinity chromatography depends on the presence of the glucose
moiety and therefore it should not be affected by these other species. This work investigates this claim and compares the affinity technique with another method recently gaining in popularity, that of agar gel electrophoresis (Menard et al. 1980), which depends on the charge/mass ratio for separation.

In this chapter the affinity technique for the measurement of both glycosylated haemoglobins and glycosylated plasma proteins has been compared with established techniques; agar gel electrophoresis and the colorimetric method respectively. The possible effects of haemolysed samples on glycosylated plasma protein measurement was also investigated. Preliminary studies of the nature of the material binding to the gel for both the assay of glycosylated haemoglobins and glycosylated plasma proteins, are described.
MATERIALS AND METHODS.

SAMPLES.

Maternal and cord bloods were taken immediately after birth from 7 non-diabetic and 3 IDDM mothers. Blood was also obtained from 8 uraemic patients, 5 patients with elevated \( \gamma \)GT, 3 cases of aspirin poisoning, and 2 children with lead poisoning. Lithium heparin was used as an anticoagulant for all blood samples, and each sample was tested using both agar gel electrophoresis and affinity chromatography for the measurement of glycosylated haemoglobins.

Samples used for the measurement of glycosylated haemoglobins by both agar gel electrophoresis and affinity chromatography, and for the comparison of glycosylated plasma protein measurement by the colorimetric method and affinity chromatography, were all obtained from the out-patient diabetic clinic at the Royal Sussex County Hospital. Non-diabetic samples used were obtained from laboratory staff. These were also the sources of samples for the comparison of glycosylated haemoglobins and glycosylated plasma proteins both measured on the same sample using the affinity techniques. Blood glucose was routinely measured for these patients at the time of blood sampling.
(1) **Preparation of samples containing different amounts of fetal haemoglobin (HbF).**

To investigate the interference of HbF, cord blood was mixed in various proportions with either blood from a diabetic or a non-diabetic. The samples used contained 100%, 80%, 65%, 50%, 35%, 20% and 0% of cord blood in each case. Each sample was mixed and 100μl of packed red cells were diluted in 2ml of deionised water and incubated at 37°C for 5 hours to remove labile material. Each sample was then analysed in triplicate by the affinity method described in Chapter 2 for glycosylated haemoglobin measurement. An identical investigation was carried out using a sample containing 48% HbS and 48% HbC, instead of cord blood.

(2) **Preparation of plasma samples containing different amounts of haemoglobin.**

A sample of blood from a poorly controlled diabetic patient, preserved in a lithium heparin container, was chosen to assess the effect of haemolysis on the measurement of glycosylated plasma proteins. Before these investigations the sample was analysed by the usual affinity methods to give a glycosylated haemoglobin value of 22.0% and a glycosylated plasma protein value of 23.1%. The whole blood sample was then shaken gently and then re-centrifuged to reveal plasma of a pale pink colour. One aliquot was removed
for the estimation of haemoglobin concentration and others
for mixing with plasma from a well-controlled diabetic
patient which was apparently free from haemolysis. The
proportions of haemolysed plasma used were 100%, 80%, 65%,
50%, 35%, 20% and 0%. The original sample was then remixed
and shaken more vigorously to further contaminate the plasma
with haemoglobin. After centrifugation plasma of a clear,
red appearance was obtained. Some of this was also removed
to mix with non-contaminated plasma in quantities between 0%
and 100%. Again the haemoglobin concentration was measured
and noted. Finally the original blood sample was remixed
and further haemolysis achieved using a vortex. After
centrifugation the plasma was then found to be grossly haemolysed
and it was difficult to distinguish between the plasma and the
red cell layer. Aliquots were removed from the top of this
sample to prepare another experiment as described before, but
using a more grossly contaminated sample. Haemoglobin
concentration was noted. All of the prepared plasma samples
were then analysed in triplicate using the affinity method
for glycosylated plasma proteins.

(3) Preparation of samples for immunoelectrophoresis.

Blood was taken from 44 non-diabetic patients and
13 healthy laboratory staff into lithium heparin tubes. After
centrifugation the plasma (100 \mu l) from each was diluted in
deionised water (2ml) and incubated at 37°C for 5 hours to
remove labile material. Each sample was then separated using
affinity chromatography in a total of 4 runs, the first on
freshly poured gels. The fractions containing material which did not bind to the gel ("unbound") in wash buffer, were pooled into a large flask and stored at 4°C. Similarly the fractions containing material which was retained by the gel ("bound") collected in elution buffer, were pooled in a separate flask and stored at 4°C. Blood from 91 diabetic patients was separated and a pool of "unbound" and "bound" material from this source was prepared in an identical manner. This took a total of 5 runs, again the first run was on freshly poured gel. The whole collection took 11 days. All samples were stored at 4°C and were then transported to the University of Surrey where they were ultrafiltered using a stirred Diaflo ultrafiltration cell with a membrane (UM05) with a cut off at 500 daltons. Each ultrafiltrate was washed several times with deionised water to remove excess buffer constituents before lyophilisation. This part of the preparative work was carried out by Dr. B. J. Gould.

Approximately 1mg of each sample was reconstituted with 15μl of tris/barbitone buffer before immunoelectrophoresis.

(4) Preparation of samples for rechromatography studies.

This is described in the methods section of this chapter.
REAGENTS.

For the colorimetric measurement of glycosylated plasma proteins.

0.15 mol/l sodium chloride (NaCl), 2.5 mol/l trichloroacetic acid (TCA) and 1.0 mol/l oxalic acid were prepared. A saturated solution of thiobarbituric acid (TBA) was prepared by adding 0.721g to 100ml of water and incubating at 40°C for 1 hour. The solution was filtered before use.

Care was taken with the use of the poisonous reagents oxalic acid and thiobarbituric acid.

For immunoelectrophoresis and crossed immunoelectrophoresis.

0.05M tris/barbitone buffer was prepared and adjusted to pH8.6. Agarose (HEEO grade) was obtained from FMC Corporation, Marine Colloids Division, Rockland, ME 04841, USA, and 1% agarose was prepared in buffer for immunoelectrophoresis.

Agarose (Type HSA) was from Litex, Denmark, and 1% agarose was prepared in buffer and used for the crossed immunoelectrophoresis.

Antisera;

Rabbit-anti-\(\alpha_2\) macroglobulin was from Behringwerke AG, Marburg-Lahn, Germany.

Rabbit-anti-human haptoglobin was from Dako Immunoglobulins a/s, Denmark.

Sheep-anti-whole-human; sheep-anti-human albumin; sheep-anti-human transferrin; sheep-anti-human \(\alpha_1\)-antitrypsin; sheep-anti-human \(\alpha_1\)-acid glycoprotein; sheep-anti-human IgG, IgA, and IgM; were all obtained from Guildhay Antisera, Guildford, Surrey.
Coomassie Brilliant Blue Stain was prepared by adding 5g of Coomassie Brilliant Blue R250 to one litre of water and leaving overnight at room temperature. This solution was filtered and mixed with ethanol (96%), water, and glacial acetic acid in the ratio 1:9:9:2, respectively, before use. Destainer was prepared using ethanol (96%), water and glacial acetic acid mixed in the proportions, 9:9:2.

0.15 mol/l saline was also prepared.
METHODS.

Measurement of Blood Glucose.

Whole blood glucose was measured using a Yellow Springs Glucose Analyser Model 23AM (Yellow Springs Instruments, Yellow Springs, Ohio, USA).


Glycosylated haemoglobins and glycosylated plasma proteins were measured by the affinity methods described in Chapter 2, buffering at pH 8.3 and 8.9, respectively. Labile material was routinely removed by incubation of the diluted sample at 37°C for 5 hours prior to analysis.

Measurement of HbA_1c by Agar Gel Electrophoresis.

Packed red cells (0.5ml) were added to 10ml of isotonic (0.15 mol/l) saline and incubated at 37°C for 5 hours. Prior to electrophoresis, the erythrocytes were collected by centrifugation at 1200g for 10 min. Haemolysis, electrophoresis and quantitation were carried out according to the instructions provided by Corning Medical Ltd. (Halstead, Essex, U.K.).
Measurement of Glycosylated Plasma Proteins by the Colorimetric Technique.

The manual method as described by Ma, Naughton and Cameron (1981) was chosen for the colorimetric estimation of glycosylated plasma proteins, with slight modifications to the volumes used for hydrolysis and colour development.

Plasma (0.5ml) was diluted with ice-cold 0.15 mol/l NaCl (4.5ml) and the protein precipitated by the addition of ice-cold 2.5 mol/l TCA (0.5ml). This was followed by centrifugation at ⁹⁰°C and 1500 g for 15 minutes. The supernatant was discarded and the precipitate allowed to drain for 5 min. The protein was then resuspended in ice-cold 0.15 mol/l NaCl (4.5ml) by sonication with a microprobe (Dawes Instrument Ltd., London W3, U.K.). These protein preparations (3.0ml) were mixed with 1.0 mol/l oxalic acid (1.5ml) in 15ml stoppered glass centrifuge tubes, and heated in a boiling water bath for 4 hours. To prevent the glass stoppers from becoming jammed, each tube had a piece of card inserted between it and the stopper. The tubes were cooled in ice for 10 min. Ice-cold 2.5 mol/l TCA (1.5ml) was added and vortex mixed for 30 seconds. The samples were then centrifuged at ⁹⁰°C and 1500g for 15 min. Colour was developed by the addition of a freshly prepared saturated solution of TBA (0.5ml) to the supernatant (2.0ml) followed by incubation at ⁴⁰°C for exactly 30 min. Individual blanks were prepared for each sample by adding 0.5ml of water instead of TBA solution to 2ml of the appropriate supernatant, and
incubating alongside the test sample at 40°C for 30 min. The absorbance of each sample was measured against its own blank at 443nm after allowing 15 min. for equilibration to room temperature.

Protein was determined in the original sonicated preparations by taking aliquots (0.5ml) of each, adding 3ml of Biuret reagent and measuring absorbance at 560nm after 15 min. The results were then calculated using a standard curve prepared from standards of known protein content.

A fructose standard curve was prepared by dissolving 9mg of fructose in 200ml of deionised water (0.25mmol/l) and diluting this appropriately to provide standards of 0, 0.1, 0.15, 0.2 and 0.25 mmol/l. These were treated in an identical manner to the samples (although protein precipitation was unnecessary) to check for variation particularly due to variations in time and temperature during hydrolysis.

Measurement of Urea.

Urea was measured on the Vickers 300 multichannel analyser (Vickers Ltd., Medical Engineering, Basingstoke, Hants, U.K.) using a method adapted from that described by Gutmann and Bergmeyer (1974).
Measurement of $\gamma$GT.

$\gamma$GT was measured using a Centrifichem method as described in a manual supplied by the manufacturers (Bakers Instruments Ltd., Egham, Surrey, U.K.), based on a kinetic photometric method by Szasz (1969).

Measurement of Salicylate.

Salicylate measurement was performed manually as described by Trinder (1954).

Measurement of Lead.

Blood lead concentrations were measured using graphite furnace atomic absorption with standards of known value, on an Atomspek H1550 (Rank Hilger, Margate, U.K.).

Measurement of Haemoglobin.

Haemoglobin concentrations were measured using a Coulter Counter (Coulter Electronics Ltd., Luton, Beds.).

Measurement of HbS and HbC.

Samples of blood from non-diabetic patients with sickle cell anaemia were received from Kings' College Hospital, London. The percentage of HbS and HbC in these samples was measured in the Haematology department of Kings' College.
Rechromatography of the Haemoglobin Fractions Previously Analysed by Affinity Chromatography.

Three different diabetic samples were each analysed 25 times, using freshly prepared columns of Glycogel B and the recommended method for glycosylated haemoglobins (Chapter 2), with mean results of 10.5%, 12.5% and 19.5%, respectively. For each sample, after chromatography, all of the haemoglobin which had not bound to the column ("unbound") was collected and pooled. Similarly, all of the haemoglobin which had been bound to the column and removed using a buffer containing sorbitol ("bound") was pooled and collected in a separate container. Using a Diaflo stirred ultrafiltration cell and a filter (UM 05) (Amicon Co., Massachusetts, U.S.A.) with a cut-off at 500 daltons, each quantity of "bound" or "unbound" material was concentrated, as the filter retained the haemoglobin and allowed free passage to water and small molecules. After concentrating, each ultrafiltrate was washed several times with deionised water to ensure all the buffer constituents were removed. When the 6 ultrafiltrates from the original 3 samples had been prepared as described, each was freeze-dried using an Edwards EF03 freeze drier (Edwards High Vacuum Inc., Grand Island, New York, U.S.A.).

In the described method for glycosylated haemoglobins approximately 1.0mg of haemoglobin was added in the 100μl of haemolysate added to each column. To be consistent with
this loading, the total amount of haemoglobin collected from 25 columns was diluted in 2500μl of deionised water before rechromatography. The distribution of this dilution between the "bound" and "unbound" material was calculated directly in accordance with the percentage of haemoglobin in each fraction. After the addition of the water, each fraction was thoroughly reconstituted by vortex mixing the samples.

After reconstitution each sample was checked using the method of Van Kampden and Zjilstra (1961) to ensure that the haemoglobin concentrations were comparable to the original loading, to avoid overloading in the rechromatography studies. The quantities of haemoglobin in the reconstituted samples were found to be between 53mg/100ml and 92mg/100ml, compared with the target values of about 100mg/100ml.

Once the material had been reconstituted, samples were analysed in triplicate with the quantities of reconstituted material being added to the column varying between 50μl and 200μl (Table 3.4), to be consistent with the sort of levels of haemoglobin typically loaded onto the affinity gel. To allow for the very small quantities of haemoglobin in the wash fractions of the rechromatography experiments of samples which had previously bound to the gel, these fractions were only diluted to 10ml rather than the recommended 15ml. The absorbances were multiplied by two-thirds before they were incorporated in the usual calculations to obtain percentage values for "bound" material. Alongside these rechromatography experiments two columns were tested adding water, rather than reconstituted haemoglobin.
Immunoelectrophoresis.

Immunoelectrophoresis was performed as described by Grabar and Williams (1952). Moulten agarose (HEEO grade) (10ml) was poured onto clean glass plates (8 x 8 cm) and allowed to solidify. Wells (2mm diameter) were then punched, and troughs cut in the gel as required. Each well was filled with ~5μl of sample in tris/barbitone buffer. To some wells a trace of bromophenol blue was added as a tracking dye. The gels were placed in an electrophoresis tank (Immunoelectrophoresis Tank, Medical and Biological Instruments Ltd., Ashford, Kent), on a water-cooled platform in contact with filter paper (3MM) wicks. The power pack was switched on and adjusted to approximately 10V/cm., and this was maintained until the bromophenyl blue-albumin tracking spot had travelled a suitable distance. The gel plate was removed from the tank and the gel in the troughs removed. Antiserum was added to the troughs and the gels were then left for 24-48 hours in a moist box at room temperature to allow diffusion to occur.

The gel plates were then washed in saline and their surface was pressed with absorbent paper to remove non-precipitated proteins. This process was repeated and then the gel was washed with distilled water, before drying in a current of warm air. The gel was then stained and destained and allowed to dry before the immunodiffusion arcs were examined.
Crossed Immunoelectrophoresis.

Agarose plates were prepared as described for the immunodiffusion work but this time low endosmotic agarose was used. Wells were punched about 1.5cm from one corner of the gel and filled with sample diluted in buffer. Again electrophoresis was carried out as previously described until the tracking dye had covered a suitable distance. The gel was then removed from the tank and cut along a line about 2cm from the edge. Excess gel was removed. Agarose (8ml) containing antiserum at 57° C was poured to fill the space on the glass plate. The plate was then returned to the tank overnight for electrophoresis at right-angles to the original run, at a voltage of about 2V/cm.

The gels were then washed, dried and stained as previously described. The pattern of "rockets" on the gel was examined.

Both the immunoelectrophoresis and the crossed immunoelectrophoresis were done by Dr. B. J. Gould.
RESULTS.

Comparison of Affinity Chromatography with Agar Gel Electrophoresis for the Measurement of Glycosylated Haemoglobins.

Samples were analysed from 16 non-diabetics and 42 diabetics by affinity chromatography and the well-established method of agar gel electrophoresis. For both methods the labile fraction was removed before analysis. Fig. 3.1 shows that there is a good correlation \((r = 0.95)\) between the results obtained by the two methods. Fig. 3.1 also shows that there are only two diabetics with values in the range observed for non-diabetics when the affinity technique is used. The equivalent figure for the agar gel electrophoretic method was nine. The regression line with an intercept of \(-0.43\) on the \(y\)-axis and a slope of \(1.20\) indicates that the two methods are measuring similar species, but that the affinity method measures additional glycosylated material.


Samples were analysed from 10 non-diabetics and 28 diabetics by affinity chromatography and the well-established colorimetric method for the measurement of glycosylated plasma proteins. For the affinity method, the labile fraction was removed before analysis by incubation. Fig. 3.2 shows that there is quite a good correlation \((r = 0.69)\) between the results obtained by the two methods.
Fig. 3.1 Correlation between glycosylated haemoglobins measured by agar gel electrophoresis and by the affinity chromatography method. Normals (■); diabetic patients (○).
Fig. 3.2 Correlation between the glycosylated plasma proteins measured by the colorimetric method and by the affinity chromatography method. Normals (▼); diabetic patients (●).

Note: Correlation line plotted by computer.
Relationship Between Glucose with Glycosylated Haemoglobins and Glycosylated Plasma Proteins Measured by Affinity Chromatography.

The glucose concentration of whole blood samples taken from 153 diabetic individuals was determined. There was a significant correlation between these values and glycosylated haemoglobin ($r = 0.43$) in the same samples. There was a slightly poorer correlation between glucose and glycosylated plasma proteins ($r = 0.33$).

Relationship Between Glycosylated Haemoglobins and Glycosylated Plasma Proteins Both Measured by Affinity Chromatography.

Glycosylated haemoglobins and glycosylated plasma proteins were measured by affinity chromatography on blood samples from 16 normals and 153 diabetic patients. Fig. 3.3 shows that there is a good correlation ($r = 0.82$), with the line passing close to the origin and having a slope close to unity (0.94).
Fig. 3.3 Correlation between glycosylated haemoglobins and glycosylated plasma proteins, both measured by the affinity chromatography method. Normals (▲); diabetic patients (●).

Note: Correlation line plotted by computer.
INTERFERENCES.

Two types of possible interference with the glycosylated haemoglobin method are described. The first experiments show the effects, if any, that the genetic make-up of the haemoglobin molecule has on the method. The investigation of the post-translational changes of the haemoglobin molecule and their effects are then described. In most of these experiments both affinity chromatography and agar gel electrophoresis were used to measure "glycosylated haemoglobins" in samples.

The interference of haemoglobin with the affinity assay for glycosylated plasma proteins was also investigated, as many of the samples received for analysis were haemolysed.

Effect of Fetal Haemoglobin on Methods for Measuring Glycosylated Haemoglobins.

Fig. 3.4 shows that when cord blood was proportionally mixed with blood either from a normal mother, or from a diabetic, between 0% and 100% and measured using affinity chromatography, straight lines were obtained. This indicates that haemoglobin F (HbF) and the normal adult haemoglobin are given equal weighting by the affinity method. However, when samples of cord blood were tested by the agar gel electrophoretic method, there was gross interference by HbF (Table 3.1). The overall mean value for the maternal samples using the affinity method was 5.8%, and for the cord samples was 4.0%. The corresponding values obtained by the agar gel electrophoretic method were 6.7% and 81.5%, respectively. The values for the
Fig. 3.4 Effect on the value of glycosylated haemoglobin of mixing cord blood with non-diabetic maternal blood and blood from a diabetic. Increasing amounts of cord blood were added to non-diabetic maternal blood (▲) and diabetic blood (●) in the proportions indicated. The glycosylated haemoglobins were then measured by affinity chromatography.
the maternal samples of non-diabetic mothers obtained by electrophoresis are significantly higher (p < 0.01) than the corresponding values for the affinity method.

**Effect of HbS and HbC on the Affinity Method for Measuring Glycosylated Haemoglobins.**

Fig. 3.5 shows that when blood containing 48% HbS and 48% HbC was mixed in varying proportions with blood from a diabetic patient without sickle cell anaemia, a straight line was obtained, indicating that HbS and HbC and normal adult haemoglobin are all given equal weighting by the affinity method. This is further substantiated by the results listed in Table 3.2, showing non-diabetic patients with different quantities of both HbS and HbC, have glycosylated haemoglobin results within the normal range using the affinity method.

Analysis of the sample containing HbS which had a value of 6.16% by affinity chromatography, gave 8.9% by agar gel electrophoresis.
Table 3.1  Values for glycosylated haemoglobin in maternal and cord blood at delivery.

<table>
<thead>
<tr>
<th></th>
<th>% Fast Fraction (Electrophoretic Method)</th>
<th>% Glycosylated Hb (Affinity Method)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non Diabetic Mothers (n = 7)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Blood</td>
<td>6.34 $\pm$ 0.94</td>
<td>4.92 $\pm$ 0.70</td>
</tr>
<tr>
<td>Cord Blood</td>
<td>79.23 $\pm$ 4.47</td>
<td>4.17 $\pm$ 0.92</td>
</tr>
<tr>
<td><strong>Diabetic Mothers (n = 3)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Blood</td>
<td>7.66 $\pm$ 0.58</td>
<td>7.75 $\pm$ 3.03</td>
</tr>
<tr>
<td>Cord Blood</td>
<td>86.67 $\pm$ 1.5</td>
<td>3.58 $\pm$ 0.91</td>
</tr>
</tbody>
</table>
Fig. 3.5 Effect on the value of glycosylated haemoglobin of mixing blood containing HbS and HbC with blood from a poorly controlled diabetic patient. The glycosylated haemoglobins were then measured by affinity chromatography.
Table 3.2  Values for glycosylated haemoglobins in samples containing HbS and, or HbC.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>% glycosylated haemoglobins</th>
</tr>
</thead>
<tbody>
<tr>
<td>48%HbS, 48%HbC</td>
<td>5.23</td>
</tr>
<tr>
<td>49%HbS, 48%HbC</td>
<td>8.78</td>
</tr>
<tr>
<td>39%HbS, 39%HbC</td>
<td>4.92</td>
</tr>
<tr>
<td>49%HbS</td>
<td>8.14</td>
</tr>
<tr>
<td>38%HbS</td>
<td>6.80</td>
</tr>
<tr>
<td>41%HbS</td>
<td>6.10</td>
</tr>
<tr>
<td>45%HbS</td>
<td>5.90</td>
</tr>
<tr>
<td>HbS &amp; HbC (% not known)</td>
<td>5.44</td>
</tr>
<tr>
<td>HbS &amp; HbC (% not known)</td>
<td>6.07</td>
</tr>
<tr>
<td>HbS (% not known)</td>
<td>6.16</td>
</tr>
<tr>
<td>HbS (% not known)</td>
<td>5.93</td>
</tr>
<tr>
<td>Mean</td>
<td>6.32</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.18</td>
</tr>
</tbody>
</table>

None of the patients in this survey were known to be diabetic.

Normal range found for glycosylated haemoglobins by affinity chromatography is 5.5% - 9.1%.

Range for these patients is 3.9% - 8.7%.
Post-translational changes to the haemoglobin molecule, and their effects are now considered;

Values of Glycosylated Haemoglobins in Uraemic Patients

The values for uraemics are plotted against their blood urea nitrogen (BUN) in Fig. 3.6(a). In each case the electrophoretic method gives a higher value than the corresponding value by the electrophoretic method, but neither method gives a good correlation against BUN. Some patients have values below the normal range for the affinity method and half have values above the normal range for the electrophoretic method.

Values of Glycosylated Haemoglobins in Alcoholics.

Fig. 3.6(b) shows the values for both methods plotted against the \( \gamma \)-GT level. Three patients have values above the normal range for the electrophoretic method. The only value outside the normal range for the affinity method is low. There is no obvious correlation between glycosylated haemoglobins and \( \gamma \)-GT.
Fig. 3.6 The % glycosylated haemoglobin measured by agar gel electrophoresis and affinity chromatography in (a) uraemic patients, (b) alcoholic patients, (c) cases of aspirin poisoning, and (d) children with lead poisoning.

▲ % Fast haemoglobin by agar gel electrophoresis. △ % Bound haemoglobin by affinity chromatography.

The limits for both methods are shown (mean ± 2 s.d.). For agar gel electrophoresis (mean 6.5%), lower limit (---, 5.5%) and upper limit (---, 7.5%). For affinity chromatography (mean 7.3%), lower limit (---, 5.5%) and upper limit (---, 9.1%).
Values of Glycosylated Haemoglobin in Cases of Aspirin Poisoning.

The values for the electrophoretic method are well above the normal range and this also applies to two of the samples with the affinity method (Fig. 3.6(c)). There may be some correlation between % glycosylated haemoglobin and salicylate concentration.

Values of Glycosylated Haemoglobin in Cases of Lead Poisoning.

One value was found outside the normal ranges, this was a high value by the electrophoretic method (Fig. 3.6(d)).
Interference in the glycosylated plasma protein assay is now considered;

**Effect of haemolysis on the measurement of glycosylated plasma proteins by affinity chromatography.**

When slightly haemolysed plasma containing 0.9g/dl haemoglobin was mixed with plasma containing virtually no haemoglobin (0.2g/dl) a straight line was obtained (Fig. 3.7). However, as haemolysis within the test sample increased to 9.8g/dl, and 17.3g/dl in the grossly haemolysed sample this linear relationship was no longer found. Increased haemolysis caused a reduction in the percentage of glycosylated plasma proteins and a tendency to plateau (Fig. 3.7). This indicates that the presence of excess haemoglobin within a sample alters the results obtained by affinity chromatography using the method as described. However samples which are only mildly haemolysed (i.e. a pale pink colour) can be used.
Fig. 3.7 Effect on the value of glycosylated plasma proteins of haemolysis, shown by mixing haemolysed plasma with non-haemolysed plasma. Increasing amounts of a diabetic sample with slight haemolysis (●), haemolysis of 9.8g/l (▲) and gross haemolysis of 17.3g/l (▼) were added in the proportions indicated. The glycosylated plasma proteins were then measured by affinity chromatography.
Preliminary investigations of material binding to Glycogel B.

Preliminary studies were made to elucidate more fully the nature of the binding of either glycosylated haemoglobins or glycosylated plasma proteins to the affinity gel, Glycogel B. Mallia et al. (1981) had used isoelectric focussing to analyse the haemoglobin fractions which were retained by the affinity gel, and they reported that the "bound" fraction contained all the HbA$\text{_{1c}}$, and also portions of HbA$_1$, HbA$_2$ and methaemoglobins. The first experiments to be described in this thesis show rechromatography of "bound" and "unbound" haemoglobin through the affinity gel, to test the efficiency of the primary separation. The other experiments described in this section studied the nature of the plasma proteins which did, or did not, bind to the gel, by the techniques of immunoelectrophoresis and crossed immunoelectrophoresis.

Rechromatography of the haemoglobin fractions previously analysed by affinity chromatography.

The results for the rechromatography of "bound" and "unbound" material originating from 3 diabetic samples are shown in Table 3.3. It is clear from these results that the "unbound" fraction is contaminated in every case with about 2 - 4% of "bound" material. The "bound" material, which should give results of 100%, gave values between 58% and 89%. These reduced values may be in part caused by contamination of the "bound" fraction with "unbound" material, and indeed
Table 3.3 Values obtained for rechromatography of haemoglobin obtained in the "bound" and "unbound" fractions from the affinity gel, Glycogel B.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unbound</strong> fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.05 ± 1.1%</td>
<td>2.90 ± 0.12%</td>
<td>2.94 ± 0.39%</td>
</tr>
<tr>
<td>100</td>
<td>1.81 ± 0.04%</td>
<td>5.52 ± 0.03%</td>
<td>3.13 ± 0.18%</td>
</tr>
<tr>
<td>200</td>
<td>2.14 ± 0.12%</td>
<td>4.02 ± 0.19%</td>
<td>2.90 ± 0.20%</td>
</tr>
<tr>
<td><strong>Bound</strong> fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>61.20 ± 3.12%</td>
<td>62.95 ± 0.39%</td>
<td>86.57 ± 0.75%</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>60.37 ± 0.59%</td>
<td>86.13 ± 0.79%</td>
</tr>
</tbody>
</table>

The values shown are the mean results (± SD) from triplicate assays of rechromatography of material collected after affinity chromatography which had ("bound") and had not ("unbound") been retained by the gel. The original haemoglobins tested had been obtained from three diabetics A, B and C, with glycosylated haemoglobin results 10.5%, 12.5% and 19.5% respectively.
(corrected) absorbances of 0.01 and 0.02 obtained for the "unbound" fraction with the absorbances of the "bound" fraction between 0.57 and 0.65 show this is so. However, the loss of material in the preparation of the material for rechromatography may also be part of the explanation. Interestingly, the "bound" material studied from a poorly-controlled diabetic with the highest original glycosylated haemoglobin result of 19.5%, gave the highest recovery result on rechromatography. The "bound" fraction was more difficult to study than the "unbound" material, as it was reconstituted with much smaller volumes of water after freeze-drying. The columns used, loading only water, gave maximum absorbances of 0.001 for both "unbound" and "bound" fractions.

**Immunoelectrophoresis.**

Fig. 3.8 shows the immunodiffusion arcs obtained with material from plasma which did not bind to the affinity gel (W) and that which did bind (E). Both pooled sample material from non-diabetics (N) and diabetics (D) were used. Fig. 3.8 shows the results obtained when the antiserum used was anti-whole human. The "unbound" (W) fractions show more arcs than do the "bound" fractions (E). Both the "bound" fractions (E) show 4 arcs. There was no obvious qualitative difference between the "bound" material obtained from non-diabetics and diabetics.
Fig. 3.8 Photograph to illustrate the patterns obtained using immunoelectrophoresis of samples of material collected which did (E) and did not (W) bind to the affinity gel, Glycogel B. Material from both non-diabetics (N) and diabetics (D) was examined.
Further studies were also done using specific antisera. The results are described in Table 3.4 as the background for these gels proved too dark to photograph successfully although the arcs were clearly visible. Again the "unbound" material was found to be a more complex mixture of proteins, with all but IgA and IgM being present. The four major arcs in the "bound" fraction were shown to be albumin, α₂-macroglobulin, transferrin and IgG. A very faint arc for haptoglobin was also apparent in the same region as that for α₂-macroglobulin. These results were interpreted using the guidelines reported by Turner and Hulme (1971).

**Crossed Immunoelectrophoresis.**

The results for the crossed immunoelectrophoresis are shown in Fig. 3.9. "Unbound" material from normal and diabetic samples are shown in Fig. 3.9A and C respectively, and "bound" material from normals and diabetics in Fig. 3.9B and D. The results confirm those shown with the initial immunoelectrophoresis work. Again the "unbound" (W) fraction is more complex than the "bound" fraction (E). When the various 'rockets' are examined there are three clear peaks seen with the "bound" material; albumin, transferrin and α₂-macroglobulin. IgG does not produce a peak in crossed immunoelectrophoresis. These results were interpreted using the criteria and examples illustrated by Moody and Thomas (1975). The height of the α₂-macroglobulin peak relative to albumin as shown in Fig. 3.9 appears to be higher for the diabetic sample (D) than with the normal sample (B).
Fig. 3.9  Photograph to illustrate the patterns obtained when performing crossed-immunoelectrophoresis of samples of material collected which did (E) and did not (W) bind to the affinity gel, Glycogel B. Material from both non-diabetics (N) and diabetics (D) was examined.
Table 3.4  *Immunodiffusion arcs present with specific antisera for material which did, and did not bind to the affinity gel, Glycogel B.*

<table>
<thead>
<tr>
<th>Specific antisera</th>
<th>&quot;unbound&quot;</th>
<th>&quot;bound&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-albumin</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>(\alpha_1)-glycoprotein</td>
<td>/</td>
<td>X</td>
</tr>
<tr>
<td>(\alpha_1)-antitrypsin</td>
<td>/</td>
<td>X</td>
</tr>
<tr>
<td>(\alpha_2)-macroglobulin</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>haptoglobin</td>
<td>/</td>
<td>/ (very faint)</td>
</tr>
<tr>
<td>transferrin</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>IgA</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IgM</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IgG</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

The presence (/) or absence (X) of arcs is noted when material collected after affinity chromatography which either did ("bound") and did not ("unbound") bind to the affinity gel was allowed to diffuse into specific antisera.
DISCUSSION.

The two methods compared in this study for the measurement of glycosylated haemoglobins differ in their analytical principle which causes the separation between glycosylated and non-glycosylated material. The agar gel electrophoretic method, like ion-exchange chromatography, separates these haemoglobins on the basis of small charge differences. The affinity method relies on the glycosylated portion of the molecule, and in principle should not be influenced by the composition of the protein. There was a good correlation ($r = 0.95$) between the agar gel electrophoretic method and the affinity technique. The present work shows that there is less overlap between the diabetic and the non-diabetic populations using the affinity method when compared with the electrophoretic technique. This suggests that the affinity method may be more selective, but the numbers studied would need to be increased for this to be verified. The slope of the regression line (Fig. 3.1) indicates that affinity chromatography measures material in addition to $\text{HbA}_1$. This is in accord with Mallia et al. (1981) who showed using isoelectric focussing, that the "bound" fraction contained all the $\text{HbA}_{1c}$ and also portions of $\text{HbA}$, $\text{HbA}_2$ and methaemoglobins. It is known that a small proportion of $\text{HbA}$, $\text{HbA}_2$ and methaemoglobin are glycosylated (Bunn, 1981), and presumably it is these glycosylated molecules which account for the higher values observed when using the affinity method. This
increased sensitivity however, is not reflected in the two normal ranges which are surprisingly similar (agar gel electrophoresis 5.5%-7.5%, affinity chromatography 5.5%-9.1%). This may be related to the preliminary evidence presented in this chapter (Table 3.3) using rechromatography, supported by the subsequent publication by Flückiger, Woodti and Berger (1984), which suggests that using a 1ml affinity gel column produces results which are falsely low. The details and implications of these findings have been discussed fully in Chapter 7.

The results for cord blood illustrate the importance of the difference in the principle for separation for the two different methods studied, and shows that in the electrophoretic method HbF runs with the fast fraction, HbA\textsubscript{1}. This probably invalidates the use of this method for the measurement of HbA\textsubscript{1} during diabetic pregnancy since about 17% of the pregnant population have concentrations of HbF in excess of 1% of total haemoglobin (Pembrey, 1973). This may explain why the non-diabetic maternal samples had significantly higher values (p < 0.01) when measured with agar gel electrophoresis compared to the affinity method. Fig. 3.4 clearly illustrates that fetal haemoglobin does not interfere with the affinity method.

The result for HbS sample measured by the electrophoretic method (8.90%) is higher than when measured by affinity chromatography (6.16%), unlike the low values associated with these samples when measured using ion-exchange chromatography (Aleyassine, 1979). On the electrophoretic method 4 peaks
Fig. 3.10 Illustration of 4 peaks obtained with a sample from a sickle cell patient, using agar gel electrophoresis.
(S₀, A₀, S₁ and A₁) are obtained rather than the normal two (A₀, A₁) (Fig. 3.10), (Meynard et al. 1980), and the glycosylated haemoglobin, which is the sum of S₁ and A₁, should be normal. However, the separation between A₀ and S₁ is poor after the recommended 40 min. electrophoresis and is therefore subject to error. Menard et al. (1980) continued their electrophoresis of these samples for 50 minutes, but this change in methodology, unless applied to all samples, would inevitably complicate batch handling and possibly lead to extra expense.

The results for investigations of the post-translational modifications of haemoglobin are also important. In uraemia carbamylated haemoglobins are formed, most of which run in advance of the main haemoglobin peak on ion-exchange chromatography. The results presented here indicate that carbamylated haemoglobins may be present in the fast fraction in the electrophoretic method (Fig. 3.6(a)). The affinity method gave low values for some uraemic patients. This may be explained by the shortened red cell survival time associated with uraemia (Bruns and Wills, 1984).

Alcoholic patients have been found to have elevated amounts of total HbA₁c, as measured by ion-exchange chromatography (Hoberman, 1979), probably due to the formation of adducts with acetaldehyde. The three high results (Fig. 3.6(b)) obtained by the electrophoretic method are in agreement with these findings. The single low value obtained with the affinity method is in agreement with the low values for HbA₁c in alcoholics reported by Stevens et al. (1981) who used
radioimmunoassay, since the acetaldehyde-haemoglobin adduct should not bind to the affinity ligand.

A fast haemoglobin fraction (HbPb) has been observed in about 40% of pre-school children with lead poisoning (Charache and Weatherall, 1966) using starch gel electrophoresis at pH 8.6. Therefore some children with lead poisoning would be expected to have an elevated HbA\textsubscript{1a} by the electrophoretic method but HbPb would not be expected to alter the results of the affinity method. The limited results obtained (Fig. 3.6(d)) in this work agree with these statements.

Bridges et al. (1975) reported that aspirin, acetyl salicylate, which is rapidly converted to acetate and salicylate \textit{in vivo}, causes the acetylation of HbA \textit{in vivo} and \textit{in vitro}. Spicer et al. (1979) using isoelectric focussing showed that acetylated haemoglobin focussed near HbA\textsubscript{1b}. Therefore patients on high doses of aspirin may be expected to have elevated HbA\textsubscript{1a}, as was found with the electrophoretic method (Fig. 3.6(c). However the affinity method would not be expected to sense acetylated haemoglobin, so the increased levels found warrant further investigation. It may be that alterations in body pH due to the salicylate overdose affect glycosylation.

Until now the colorimetric method using thiobarbituric acid (TBA) has been the method of choice for the measurement of glycosylated plasma proteins. Affinity chromatography provides a very attractive alternative as it does not require the removal of glucose, use of poisonous reagents such as TBA or oxalic acid, or sample blanks. In addition the colorimetric method requires much larger samples since 5-hydroxymethylfurfural is formed, even after prolonged acid treatment, in only small
non-stoichiometric amounts (Goldstein et al. 1982A). Furthermore, the colour yield of this method is not the same for all proteins (Bunn et al. 1979). Although protein-to-protein variation also occurs with the Bradford method employed with affinity chromatography in this work, it is known to have a greater colour yield with albumin than with most other proteins (Pierce and Suelter, 1977). The preliminary investigations shown here (Figs. 3.8 and 3.9) indicate that the bulk of the material in both the "glycosylated" and "non-glycosylated" fractions, as separated by the affinity gel, is albumin. Since this is the case, this source of error is minimal.

There was a good correlation \( r = 0.7 \) between the colorimetric technique and the affinity method for the measurement of glycosylated plasma proteins, indicating that the two methods are measuring similar moieties. The slope of the graph (2.93) may be an indication of the greater sensitivity of the affinity method, but it should be noted that the units of the two axes are quite different (Fig. 3.2). The cause of the intercept (Fig. 3.2) is not clear, since the colorimetric method used takes into account known interferences by glucose and the need for each sample to have an individual blank. Maybe other material interferes with the colorimetric method which has yet to be identified. The multiplication factor require to produce the colorimetric values however, is very large and may obscure the picture. In contrast with these results, Fig. 3.7 shows an excellent correlation between glycosylated haemoglobins and glycosylated plasma proteins.
both measured by affinity chromatography \( r = 0.82 \), with a slope close to unity and the line passing close to the origin. The relatively poor correlation of both glycosylated haemoglobin \( r = 0.43 \) and glycosylated plasma proteins \( r = 0.38 \) with simultaneous blood glucose is similar to other reports (Gonen et al. 1977, Ma, Naughton and Cameron, 1981).

In this chapter the interference of haemolysed samples with the glycosylated plasma protein assay using affinity chromatography has been examined. These are important studies as many of the samples routinely received are, to some degree, haemolysed, and yet there have been no publications to date as to whether these samples may be used without affecting the results. This work suggests that grossly haemolysed samples cause reduced values (Fig. 3.7). There are two possible explanations. Firstly the affinity gel sites for the attachment to the glycosylated molecules may become overloaded when both glycosylated haemoglobin and glycosylated plasma proteins are present in the sample material in high amounts. However, a more likely explanation is that the Coomassie Brilliant Blue method for protein measurement does not give the same emphasis to haemoglobin as to albumin and the other plasma proteins.

The study of plasma proteins which bound or did not bind to the affinity gel, has not previously been reported. The \( \kappa_1 \)-acid glycoprotein, despite having 41.4% glyco-material (Schultze and Heremans, 1966) was only present in the "unbound" (W) fraction (Table 3.4). Haptoglobin produced
only a very faint band versus specific antisera in the immunoelectrophoresis studies of the "bound" (E) fraction, despite having 19.3% glycoprotein content (Schultze and Heremans, 1966). Similarly, \( \alpha_1 \)-antitrypsin has 12.4% glycoprotein content (Schultze and Heremans, 1966A) yet does not appear in the "bound" fraction. These results strongly suggest that enzymically formed glycoproteins do not bind to the affinity gel, Glycogel B. This is supported by the earlier observation (Fig. 3.3) that when glycosylated haemoglobins and glycosylated plasma proteins, as measured by affinity chromatography are correlated, the line goes almost through the origin. If glycoproteins had bound to the gel a significant intercept on the glycosylated plasma proteins axis would be expected. Presumably glycoproteins do not bind as they are highly branched structures and so their cis-diol groups are not accessible for the binding sites on the gel. Also many of their chains end in negatively charged groups such as sialic acid, which would prohibit binding.

The proteins which are found in the "bound" fraction include albumin, IgG and transferrin, all of which have longer half-lives than the other proteins investigated (Scultze and Heremans, 1966B). This is to be expected as non-enzymic glycosylation is proportional to both the glucose concentration and the half-life of the protein (Beach, 1979). The most abundant of the immunoglobulins, IgG, with the longest half-life is found with the "bound"
material. IgA and IgM were not detected in either fraction using specific antisera, possibly due to lack of stability of these proteins with ultrafiltration and storage. The height of the macroglobulin peak relative to albumin appears to be higher in the "bound" fraction from diabetics than from non-diabetics, although the transferrin peaks are of comparable heights. The reasons for these findings are unclear.
CHAPTER 4.

GLYCOSYLATED HAEMOGLOBINS AND GLYCOSYLATED PLASMA PROTEINS IN THE DIAGNOSIS OF DIABETES MELLITUS AND IMPAIRED GLUCOSE TOLERANCE.
INTRODUCTION.

Keen, Jarrett and McCartney (1982) drew attention to the limited ability of clinicians to predict metabolic deterioration in subjects with impaired glucose tolerance. The extreme ends of the glucose regulatory spectrum are easily defined; there are diabetic patients with characteristic symptoms and unequivocally high blood glucose concentrations, and non-diabetics with blood glucose levels universally recognised as normal. However, between these extremes there is a range of considerable diagnostic uncertainty with no natural cut-off point between normal and abnormal glycaemia.

The Oral Glucose Tolerance Test.

In spite of all the advances in research and technology which have lead to a much deeper understanding of the disease diabetes mellitus, the clinician still relies largely on the measurement of blood glucose for diagnostic purposes. The concept that early diabetes might be discovered by exposing the patient to a glucose challenge was first introduced by Jacobsen in 1913, according to Siperstein (1975). Since then the oral glucose tolerance test (OGTT), based on this concept, has been widely used as a diagnostic aid for the diagnosis of diabetes in cases where the clinical features or blood glucose concentration were equivocal. However, because of the lack of uniformity in both the procedure and interpretation, there is still sometimes difficulty in interpreting results.
In general the OGTT is performed by giving an oral dose of glucose to a fasting individual. The blood glucose concentration is measured immediately before, and at intervals after the administration of the dose for periods of two to six hours. Many factors influence the glycaemic profile such as; the pre-test diet of the patient; the quality, strength and even the flavour of the glucose solution; the position of the patient during the administration of the dose and the degree of movement allowed during the test; the source of the blood sample (capillary or venous) and the type of sample used (whole-blood, plasma or serum). Even the many different methods for measuring glucose influence the results. It is not surprising therefore to find wide differences in the results and their interpretation.

Recognising these problems numerous workers have published detailed protocols and interpretations. Mosenthal and Barry in 1950, recommending a 100g glucose load concluded that a peak glucose exceeding 150 mg/100ml (8.3 mmol/l) and a value of 100mg/100mL (5.6 mmol/l) 2 hours after the dose was abnormal. Since this work other recommendations have been published by Fajans and Conn (1954), Unger (1957) and Wilkerson et al. (1960). This last group of workers devised a points system for interpreting the test profile allocating either whole or half-points when various time-points of the test had glucose values exceeding recommended levels. When a patient had a score of 2 or above, diabetes mellitus was diagnosed. Various organisations have also made recommendations; The U.S. Public Health Service (Sherwin, 1977), the National Diabetes Data Group (1979) and the University Group Diabetes Program (Cooper et al. 1969). Although the test format in most of these publications
was similar, opinions varied as to the normality of the results at different time intervals. Most recently the World Health Organisation (WHO) (1980) have published a report to try and overcome these problems. A dose of 75g was chosen as a compromise between the 100g dose favoured by the Americans and the 50g dose of the Europeans. The WHO guidelines for interpretation are given later in this chapter. In the guidelines is reference to a new category of impaired glucose tolerance consisting of those individuals whose results lie in the borderline region between non-diabetic and diabetic. The rationale of this category is to detect people who are at risk of becoming diabetic, but sparing them the social, economic and psychological stigma attached to the label of diabetes (Keen, Jarrett and Alberti, 1979). However, there has been considerable discussion (Mutch and Stowers, 1982) as to whether these individuals can be considered to be "prediabetic." Jarrett et al. (1979) noted that many patients with a single OGTT showing impaired glucose tolerance revert to normal tolerance when retested. However, Fajans (1981) reported on asymptomatic relatives of maturity onset diabetics, both with normal and impaired glucose tolerance. Of the latter group, 80% progressed towards diabetes over the 24 years studied. Kadowaki et al. (1984) studied 288 subjects with impaired tolerance and showed that after 15 years 48 patients had worsened to overt Type 2 diabetes mellitus with elevated fasting blood glucose levels. Fuller et al. (1980) studied 18403 civil servants and showed that mortality with coronary heart disease approximately doubled in those individuals with impaired glucose tolerance. They therefore emphasised the importance of this
category and defined impaired glucose tolerance as involving large vessel disease but without the risk of small vessel disease as occurs in overt diabetes mellitus. These findings are supported by Zammit Maempel (1981) who states that macrovascular disease significantly increases in incidence when glucose levels are found beyond a mere 5.3 mmol/l at 2 hours after a 50g oral glucose load.

The controversy regarding the significance of impaired glucose tolerance continues, as absolute conclusions are clouded by the numerous problems which beset the OGTT. Siperstein (1975) and Sherwin (1977) are both major critics of the OGTT and have detailed many of the problems, some of which were mentioned earlier in this chapter. The diet of the individual in the days preceding the test must be regulated, 200 to 300g of carbohydrate a day for 3 days is usually necessary. Posture is important, and the patient should be ambulatory. Diurnal rhythms have been demonstrated with the OGTT and so the test is usually started between 9 and 10 am. Drugs may alter results. Dilantin and β adrenergic drugs produce glucose intolerance by direct inhibition of insulin secretion, and diuretics reduce insulin release indirectly by producing potassium depletion. It may not be practical however to discontinue the drug for the purposes of an OGTT. There are also many medical disorders which affect the test (Table 4.1). Unger (1957) noted that a repeated OGTT done in the same individual under similar conditions with respect to all controllable factors could still yield dissimilar results. This may be due to the effect of stress and emotion on the test (Sherwin, 1977).
<table>
<thead>
<tr>
<th>Medical Disorders Which May Alter Glucose Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>chronic renal failure</td>
</tr>
<tr>
<td>cirrhosis</td>
</tr>
<tr>
<td>chronic pancreatitis</td>
</tr>
<tr>
<td>starvation</td>
</tr>
<tr>
<td>hypokalaemia/hyperaldosteronism</td>
</tr>
<tr>
<td>Cushing's Syndrome</td>
</tr>
<tr>
<td>Acromegaly</td>
</tr>
<tr>
<td>pheochromocytoma</td>
</tr>
<tr>
<td>insulinoma</td>
</tr>
<tr>
<td>glucagonoma</td>
</tr>
<tr>
<td>acute infection</td>
</tr>
<tr>
<td>myocardial infarction</td>
</tr>
<tr>
<td>post-surgery</td>
</tr>
</tbody>
</table>
The Measurement of Glycosylated Haemoglobin to Detect Diabetes Mellitus.

Since glycosylated haemoglobin measures the cumulative effect of glycaemia over the previous 6 to 8 weeks (Koenig et al. 1976), it is consequently not as prone to environmental influences as is the OGTT, and so many of the sources of error are avoided. For this reason several workers have investigated the possibility of using glycosylated haemoglobin as an indicator of glucose intolerance. Most published work uses methods based on ion-exchange chromatography. These include measurements of HbA\textsubscript{lc} by high-performance liquid chromatography (Santiago, Davis and Fisher, 1978), measurement of HbA\textsubscript{lc} by the method of Trivelli (Koenig et al. 1976), or the widely available mini-columns to measure HbA\textsubscript{lc} (Dods and Bolmey, 1979, Boucher, Welch and Beer, 1981). Koenig et al. (1976) found a correlation between OGTT and the corresponding glycosylated haemoglobin results ($r=0.82$), but there was considerable overlap between the non-diabetic (3.97% to 5.04%) and overt diabetic (4.49% to 11.20%) groups studied. Howard (1982) used macaca nigra monkeys to study the relationship between diabetes mellitus and glycosylated haemoglobin values. These monkeys gradually progress towards to diabetes mellitus and their diabetic status can be assessed by hormones and metabolites. Although he was able to show three distinct groups of diabetics, borderline diabetics and normals using both these and glycosylated haemoglobin measurements, the numbers he used were very small with 6 or less in each group. Orchard et al. (1982) tried to assess the value of glycosylated haemoglobin for screening early diabetes in 400 relatives of
diabetic patients using ion-exchange micro-columns in comparison with the OGTT. Using the National Diabetic Data Group's criteria to interpret the OGTT, they found only 25% of those diagnosed as either diabetic or glucose intolerant had abnormal glycosylated haemoglobin values. They concluded that the glycosylated haemoglobin measurement was too insensitive. However, Boucher, Welch and Beer, (1981) reported good correlation between summed glucose values in OGTT and HbA\textsubscript{1} values for both diabetic and non-diabetic subjects. This encouraging paper was supported by Saibene et al. (1978), who used ion-exchange methods and 100g glucose load for their OGTT, to produce a normal range of 4.2% to 7.8% and a range for carbohydrate intolerance of 3.8% to 13.6%. Although there was overlap between these two groups, they suggested that HbA\textsubscript{1} measurement would be useful to improve the specificity of OGTT.

In this chapter the usefulness of the affinity technique in the measurement of glycosylated haemoglobins and glycosylated plasma proteins on blood collected during oral glucose tolerance tests, is assessed for its ability to detect patients with glucose intolerance.
MATERIALS AND METHODS.

Glucose Tolerance Test.

Capillary blood samples were collected via a fingerprick from 53 patients who were referred to the hospital as outpatients for glucose tolerance tests. Each patient had been on a 300g carbohydrate diet for each of the previous 3 days and had fasted for at least 12 hours (overnight) prior to the blood being collected before the glucose load was given. This blood was collected into sodium fluoride preservative and the fasting glucose was measured. If this exceeded 10mmol/l then the test was discontinued. All OGTT were started between 9 and 10am.

An oral glucose load of 50g was given to each patient using the prepared glucose mixture 'Dextran' (Lab Sales UK Ltd., Rochdale, Lancs., UK.) made up in 200 ml of water. Repeated blood samples were taken via fingerprick at half hourly intervals up to 2 hours after the glucose dose. All of the samples were collected into tubes containing sodium fluoride preservative and the glucose content was measured within 30 minutes.

Urine samples were collected while the patient was fasted and at hourly intervals after the glucose load until the test was complete.
Glucose Measurement.

Whole blood glucose was measured in each sample using the Yellow Springs Glucose Analyser Model 23AM (Yellow Springs Instrument Co., Yellow Springs, OH 45387, U.S.A.).

A qualitative assessment of the glucose content of each urine sample was made using Clinistix (Ames Co., Miles Laboratories Ltd., Stoke Poges, Slough, UK).

INTERPRETATION OF ORAL GLUCOSE TOLERANCE TESTS.

The criteria proposed by the WHO Second Report on Diabetes (1980) were for a 75g glucose load. It was suggested that values obtained using a 50g glucose load should be decreased by 1mmol/l at 1 and 2 hours (h) after the load. On this basis the diagnostic values for the OGTT studied would be;

(1.) Normal.

At Oh and 2h capillary blood glucose concentration less than 7.0mmol/l was considered as normal. A fasting value below 6.0mmol/l excluded the diagnosis of diabetes.

(2.) Impaired glucose tolerance.

Impaired glucose tolerance was defined as a Oh capillary blood glucose concentration lower than 7.0mmol/l with a concentration at 2h between 7.0 and 10.0mmol/l.
(3.) **Diabetes mellitus.**

Overt diabetes mellitus was defined in this study as a 0h capillary blood glucose concentration of 7.0mmol/l or above, and/or a value of 10mmol/l or above at 2h. In the absence of any clinical symptoms of diabetes at least one additional abnormal blood glucose concentration in the OGTT profile confirmed the diagnosis.

**Measurement of Glycosylated Haemoglobins and Glycosylated Plasma Proteins.**

After glucose analysis the blood sample taken from a fasting patient was centrifuged at 1200g for 10 minutes. The erythrocytes were used for the measurement of total and stable glycosylated haemoglobin as described in Chapter 2. Similarly the plasma was prepared and used for the measurement of both stable and total glycosylated plasma proteins.
RESULTS.

Total and stable glycosylated haemoglobins and glycosylated plasma proteins were determined on patients who were referred for an OGTT. There were several significant correlations between these values and fasting blood glucose, the 2h glucose, and the area under the glucose tolerance curve (Table 4.2). For both glycosylated haemoglobins and glycosylated plasma proteins the best correlations were found with fasting blood glucose. The labile (total minus stable) glycosylated haemoglobins and glycosylated plasma proteins were calculated for each patient, but perhaps surprisingly these values did not correlate significantly with fasting glucose ($r < 0.3$). Also, urinary glucose did not produce a consistent pattern. Glycosuria was demonstrated in 3 patients who had normal glucose tolerance, but was absent for the duration of the test from 5 patients with diabetes mellitus.

Of the 53 patients tested, 31 had normal oral glucose tolerance tests, 17 had diabetes mellitus and 5 were classified as having impaired glucose tolerance according to the criteria previously defined (Table 4.3). In 5 cases the fasting blood glucose was greater than 10mmol/l and so the glucose dose was not given. For these patients the range of stable glycosylated haemoglobin values was from 15.6% to 25.6%, and their values for glycosylated plasma proteins ranged from 13.9% to 24.4%. Comparison of the glycosylated protein values showed that in all cases these measurements in those with impaired glucose tolerance and diabetes mellitus were significantly greater ($p < 0.001$) than in normals.
The discrimination between the three classes of patient and their values of stable glycosylated proteins are illustrated in Figure 4.1. The diabetic patients had stable glycosylated haemoglobin values between 9.4% and 24.4%. The 5 patients with impaired glucose tolerance had stable glycosylated haemoglobin values between 8.6% and 10.0%, which were all greater than the highest values for the 31 patients with normal tests (range 5.0% to 8.5%). Figure 4.1(b) shows that with stable glycosylated plasma proteins one diabetic patient and two with impaired glucose tolerance had values within the normal range. The patients where overlap occurred were the same when total glycosylated plasma proteins were studied.
Fig. 4.1 Distribution of individual values of stable glycosylated haemoglobins (A) and stable glycosylated plasma proteins (B) according to the results of an oral glucose tolerance test.
Table 4.2  **Summary of the relationship between the results**  
**for the glucose tolerance tests and glycosylated**  
**haemoglobins and glycosylated plasma protein values**

<table>
<thead>
<tr>
<th></th>
<th>Fasting glucose</th>
<th>2h glucose</th>
<th>Area under curve (0-2h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated haemoglobins (total)</td>
<td>n = 53</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>r = 0.89</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>slope = 1.34</td>
<td>0.74</td>
<td>0.54</td>
</tr>
<tr>
<td>Glycosylated haemoglobins (stable)</td>
<td>n = 53</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>r = 0.89</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>slope = 1.28</td>
<td>0.74</td>
<td>0.52</td>
</tr>
<tr>
<td>Glycosylated plasma proteins (total)</td>
<td>n = 52</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>r = 0.92</td>
<td>0.72</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>slope = 1.35</td>
<td>0.57</td>
<td>0.38</td>
</tr>
<tr>
<td>Glycosylated plasma proteins (stable)</td>
<td>n = 52</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>r = 0.92</td>
<td>0.69</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>slope = 1.27</td>
<td>0.54</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Although 53 patients were studied, for 5 patients the glucose load was not given as they had a fasting blood glucose $\geq 10$mmol/l. In one case the glycosylated plasma proteins were not measured.
Table 4.3  **Summary of the glycosylated haemoglobins and glycosylated plasma protein values when patient results were classified by the WHO criteria (1980) for a 50g oral glucose load.**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Impaired glucose tolerance</th>
<th>Diabetes mellitus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated haemoglobins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>7.2%</td>
<td>9.7%</td>
<td>15.9%</td>
</tr>
<tr>
<td>SD</td>
<td>1.3%</td>
<td>0.4%</td>
<td>4.1%</td>
</tr>
<tr>
<td>(total) n</td>
<td>31</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>Glycosylated (stable) n</td>
<td>6.6%</td>
<td>9.3%</td>
<td>15.1%</td>
</tr>
<tr>
<td>SD</td>
<td>1.0%</td>
<td>0.6%</td>
<td>3.9%</td>
</tr>
<tr>
<td>Glycosylated plasma proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>6.2%</td>
<td>8.4%</td>
<td>13.9%</td>
</tr>
<tr>
<td>SD</td>
<td>1.0%</td>
<td>0.7%</td>
<td>5.3%</td>
</tr>
<tr>
<td>(total) n</td>
<td>31</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Glycosylated (stable) n</td>
<td>5.9%</td>
<td>8.3%</td>
<td>13.3%</td>
</tr>
<tr>
<td>SD</td>
<td>1.1%</td>
<td>0.9%</td>
<td>4.3%</td>
</tr>
<tr>
<td>(stable) n</td>
<td>31</td>
<td>5</td>
<td>16</td>
</tr>
</tbody>
</table>
DISCUSSION.

The OGTT, as well as being subject to alteration by numerous factors, as mentioned earlier, is also inconvenient to the patient and time-consuming for laboratory staff. Glycosylated haemoglobin is not as prone to environmental influence, and has the additional advantage that only a single blood sample is required with no patient preparation.

Numerous workers have assessed the usefulness of the measurement of glycosylated haemoglobin alongside the more traditionally recognised OGTT. Two main factors are responsible for the wide discrepancies between different results and recommendations. The first of these relates to the differences in criteria for the interpretation of OGTT already mentioned in the introduction to the chapter. Secondly, the glycosylated haemoglobin measurements may be either HbA or HbA \(_1c\). This readily explains why Bolli et al. (1980) using a 40g OGTT and the Fajans-Conn criteria for interpretation obtained a normal range of 5.2% to 7.2% and a diabetic range of 6.3% to 18% for glycosylated haemoglobin. They concluded that the OGTT appeared more sensitive than HbA \(_1\) for measuring patients with reduced carbohydrate tolerance, and yet the Fajans-Conn criteria has been shown to overestimate the diagnosis of diabetes (Unger, 1957) and so this is more likely to be responsible for the large overlap between the ranges. On the basis of this and many other studies, Siperstein (1975) stated "over the past 20 years almost every study using OGTT has reported a prevalence of
'diabetes' that is obviously unrealistically high." He recommended that diagnosis could only be made on the grounds of persistent hyperglycaemia. However, if this approach was adopted then many patients with impaired glucose tolerance who would later require expensive health care may not be recognised, and yet early recognition may allow successful intervention (Sartor et al. 1980).

The initial part of this dilemma has been in part solved by the WHO 2nd. Report (1980) which clearly sets out a logically based criteria for the interpretation of OGTT for universal use. The problem of how to measure glycosylated haemoglobin remains. In a recent paper Kesson et al. (1982) compared the usefulness of HbA and HbA measurement as a screening procedure for diabetes mellitus with the conventional OGTT. They found in agreement with others (Santiago, Davis and Fisher, 1978, Koenig et al. 1976) that HbA was not sufficiently sensitive in the screening of diabetes. However, the HbA measurements gave a more consistent comparison with the OGTT, which is also consistent with the findings of others (Clipson et al. 1981, Dods and Bolmey, 1979, Boucher, Welch and Beer, 1981). Nevertheless Kesson et al. (1982) stressed the methodological problems common to all these groups of workers who were using methods based on ion-exchange chromatography. Verrillo et al. (1983) classified patients according to the WHO criteria (1980) and compared their results with HbA measurement and yet they found marked overlap between normals and those with impaired glucose tolerance.
Affinity chromatography provides a simple, sensitive and very precise (CV < 2%) method for the measurement of glycosylated haemoglobins in the routine clinical laboratory (Hall, Cook and Gould, 1983), and has none of the methodological problems associated with ion-exchange chromatography. It measures mainly HbA and glycosylated HbA (Klenk et al. 1982, Flückiger, Woodtli and Berger, 1984); both fractions are elevated in diabetes (McDonald et al. 1978), but the latter fraction may be a more sensitive indicator of hyperglycaemia (Klenk et al. 1982). As shown with this work, a single measurement using affinity chromatography appears to distinguish between patients with diabetes mellitus (stable glycosylated haemoglobin > 10%) and normals (stable glycosylated haemoglobin < 8.5%). There were no false positives recorded in the non-diabetic population, and the two groups were significantly different. This clear-cut separation (Fig 4.1 (a)) is possibly due to increased sensitivity of the affinity method by the capability of the technique to measure glycosylated HbA (usually overlooked by other methods) in addition to HbA0, and this is a substantial proportion of the total glycosylated material. Furthermore, recent work by Flückiger, Woodtli and Berger (1984) suggests that this sensitivity can be further enhanced by modifying the affinity technique to use larger columns and a reduced flow rate.
Those patients who showed impaired glucose tolerance also differed significantly from both the normal and diabetic groups. However there was a small overlap between patients with impaired glucose tolerance and the overt diabetic population, as the highest value for the former was 10%. This could simply be a reflection of the errors in the glucose tolerance test for the reasons outlined earlier.

Very similar results were also evident with the total glycosylated haemoglobin measurement (Tables 4.2 and 4.3). This would imply that the incubation stage for the removal of labile material is not strictly required for glycosylated haemoglobin to be used as a successful screening technique for diabetes mellitus. However, since the proportion of labile material is large (mean = 8.5% of the stable fraction), and the relationship between fasting blood glucose and labile glycosylated haemoglobin shows no consistent pattern from patient to patient, its removal is recommended.

Glycosylated plasma proteins were measured in these investigations since it has been shown that they are more sensitive to hyperglycaemia (Kennedy et al. 1981), and their usefulness in this context had not previously been assessed. Tables 4.2 and 4.3 also show that similar conclusions can be drawn from the results of both the glycosylated plasma proteins (total and stable). With these measurements however, most of the differences between the different populations as interpreted by the OGTT are slightly less distinct. This may be due to the glycosylated plasma protein results being unexpectedly
generally lower than those for glycosylated haemoglobins, or due to the fact that the method for glycosylated plasma proteins is less precise (CV ~ 5%). In some cases haemolysed plasma may yield falsely low values (see Chapter 3), and haemolysis did occur in some samples. Sodium fluoride was less suitable than lithium heparin as an anticoagulant and the small fingerprick samples obtained were easily haemolysed.

The results in this study, although limited, are very encouraging and have been published in full (Hall et al. 1984). They suggest that the affinity technique is more suitable for the detection of carbohydrate intolerance than other methods using ion-exchange chromatography, as workers using other techniques do not show such clear-cut separation of the different groups. It would be valuable to extend this work, with standardised methodology, to several centres. If the encouraging results obtained in this limited study are more generally applicable, it would be appropriate for the measurement of glycosylated haemoglobin by this method to be included in future discussion on the diagnostic criteria for diabetes. The early detection of subject 'at risk' with carbohydrate intolerance, as well as those likely to be especially at risk in pregnancy, would seem to be both possible and desirable using this technique.
CHAPTER 5.

THE UTILISATION OF GLYCOSYLATED HAEMOGLOBINS AND GLYCOSYLATED PLASMA PROTEINS FOR MONITORING BOTH ACUTE AND CHRONIC CHANGES IN GLYCAEMIC CONTROL.
INTRODUCTION.

Insulin-dependent diabetes mellitus (IDDM) is a well recognised disease. It is a universal health problem affecting all human societies, affecting at least 30 million people throughout the world (WHO, 1980). Nowadays, treatment is well regulated with numerous clinical facilities, although the major emphasis is on the patient himself to maintain good glycaemic control. In fact diabetes is an excellent example of a chronic disorder where the affected person must take the major responsibility for his own health. Self-monitoring as well as clinical monitoring are both an integral part of any diabetic care.

Although there is debate regarding the direct association of poor glycaemic control and diabetic complications, the general medical opinion is that good control is beneficial. Disabilities such as ischaemic heart disease, retinopathy, glomerulosclerosis, nephropathy, neuropathy, strokes and cataracts are 2-3 times higher in diabetics than in the general population. Blindness is 20 times higher, and gangrene is 20-30 times higher than in the general population. However, there is evidence which challenges the necessity of close glycaemic control. For example, microangiopathic complications may be the presenting feature in patients with only mildly impaired glucose tolerance, (Pyke and Roberts, 1959), but conversely it has been reported (Mur, 1954) that 20% of the ketosis-prone patients studied did not develop the usual complications of retinopathy and neuropathy even after 25 years. In spite of these observations, however, there is an
impressive array of evidence to support those who favour the view that good glycaemic control is beneficial. Lesions in the kidney, retina and muscle can be shown by chemically inducing diabetes mellitus in rats (Mauer et al. 1975). These lesions were prevented in smaller groups when using insulin therapy good glycaemic control was achieved over a period of 5 years. A later publication by these workers (Mauer et al. 1976) showed that diabetic vascular lesions developed in normal kidneys after they were transplanted into human patients with diabetes mellitus.

The balance of evidence suggests that there is more likely to be a better prognosis for any diabetic patient who maintains good glycaemic control, and therefore it is pertinent to consider the criteria for good control and how it can be achieved. The definition of diabetic control is not clear-cut. Ideally a well-controlled diabetic patient should be as similar to a non-diabetic as possible. In practice this degree of control is virtually impossible to achieve by insulin injections, and compromises are made. There are also inherent problems with blood glucose monitoring, as the concentration will vary with glucose intake, not just daily, but from hour to hour. In the past few years glycosylated haemoglobin measurement has been used routinely to monitor patients with IDDM. As this measurement provides cumulative information of a patients glycaemic history, the periodicity of the change in concentration is much longer than for blood glucose.
However, unless the availability of the glycosylated haemoglobin measurement actually influences the attitude of the patient to his condition, or alters the clinical management, it does not prove itself as a worthwhile test. Using affinity chromatography (as described in Chapter 2) to measure glycosylated haemoglobin in this study, the progress of diabetic patients was followed (Section A). Those patients attending an outpatient clinic at the Royal Sussex County Hospital, were monitored over a period of about 18 months, starting when the test was first introduced. Trends within this patient group have been examined, regarding age and sex. As well as this relatively long term study of diabetic control, the combined measurement of glycosylated haemoglobins and glycosylated plasma proteins in the acute situation have been assessed (Section B).
SECTION 1.
MONITORING CHRONIC CHANGES IN GLYCAEMIA.

MATERIALS AND METHODS.

Subjects.

This study began in March 1981 and continued until September 1983. All of the patients included in this study have insulin-dependent diabetes mellitus, and attend an out-patient clinic for clinical assessment, approximately once every three months. The age range of those attending the clinic was 14 to 71, with a mean age of 36 years and 5 months. Sequential samples were taken, usually at 3 monthly intervals, over a duration of about 18 months to 2 years after the glycosylated haemoglobin assay was introduced.

Samples.

Venous blood was collected for glycosylated haemoglobin estimation at each visit. The blood was preserved in lithium heparin containers and stored at 4°C until measurement, within 48 hours.
Measurement of Glycosylated Haemoglobins.

Erythrocytes were separated and used for the measurement of glycosylated haemoglobins as described in Chapter 2. For some of the earlier samples the pH of the buffers used was pH8.5, rather than pH8.3. This should cause slightly lower initial results so will not affect observations where control had improved. Labile material was removed by incubation prior to analysis in all cases.

Statistics.

Statistical comparisons were performed by Student's t-test.

RESULTS. INVESTIGATION OF CHANGES IN CONTROL

Overall Control Changes.

Assessment of patient control was made using glycosylated haemoglobin results which were close to 12 months apart. Overall control for the whole group of 120 patients, as measured by the paired t-test ($t=3.121$) showed significant improvement ($p < 0.01$).

Since the method has a between-batch C.V. of 2% (Chapter 2), individual change was defined as when the two
values (-2\%) did not overlap. Using this criteria 65 patients
within the group showed improvement, 15 showed no change and
40 had poorer control.

(1) **Control changes in 3 sub-groups, classified according to original control.**

To determine which type of patient was altering its control, the main group was divided into sub-groups according to the level of control of the individual patients when they were first tested. Those patients who had initial levels below 9.7\% (the top of the normal range) were termed to be in good control. Patients with an initial value between 9.7\% and 14\% were considered as an intermediate control group, and those exceeding 14\% were considered as a group exhibiting poor control. These values were considered to match best the clinical assessment of diabetic patients attending an outpatient clinic (Fig. 2.13).

In the well-controlled group of 13 patients there was no significant overall change in their control \((t = -1.9)\). Similarly for the 69 patients in the intermediate group there was overall no significant change in control \((t = -0.12)\). However, for those patients classed as poorly controlled there was
significant improvement over the year of the study ($p < 0.001$, $t=5.262$). These results are summarised in Table 5.1. At the beginning of the year 13 patients had values below 9.7% and at the end of a year monitoring using glycosylated haemoglobin results 17 patients could now be included in this group, although 4 of the original patients included were relegated to the intermediate group. Of the 38 patients originally defined as poor control ($> 14\%$), after 12 months only 17 could still warrant this definition. Seven others however, were relegated from the intermediate group to poor control.

(2) **Effect of duration of diabetes on control.**

To exclude the effect of new diabetics on the poor control group, who should be undergoing a period of improved control, any patients (2) who were diagnosed as IDDM in the preceding 3 years were removed from the poor control group. The remaining 36 patients however, still show a significant improvement in their control ($p < 0.001$, $t=5.39$). These patients have had diabetes for a mean duration of $14.7 \pm 8.8$ years, with an observed range of $3 - 39$ years.
(3) **Effect of attendance at the clinic on control.**

To exclude any effect that could be attributed to attendance at the outpatient clinic at the Royal Sussex County after different clinical management elsewhere, any patients who had attended the clinic in the study for less than 3 years were excluded from the poor control group. The remaining 28 patients still showed a significant improvement in their control \((p < 0.001, t=4.378)\) with their mean value of \(16.25 \pm 1.87\%\) decreasing to \(14.03 \pm 2.72\%\).

(4) **The relationship between age and sex of patient on diabetic control.**

The relationship between age and diabetic control is illustrated in Table 5.2, with the group under study being divided according to the different decades. The values used in this case were the last values obtained in the 12 - 15 month period that the patient was studied. There is no significant difference between the diabetic control as assessed by glycosylated haemoglobin in the different age groups.
When each age group is divided according to sex (Table 5.3), it is clear that the females in this study are less well-controlled than the males for every age group. 51 women (mean age 35 years and 2 months) and 69 men (mean age 37 years and 5 months) were included in this study. As there was no significant difference between the age distribution of the two sexes as shown by 'f' and t-tests, the two groups could be compared statistically. The overall mean figures at the beginning of the 12 month period, disregarding age, of 13.55 ± 3.02% for females and 12.37 ± 2.86% for males support this observation. This difference is more marked in the overall figures after 12 months when the females have a mean glycosylated haemoglobin value of 13.29 ± 3.04% and the males 11.28 ± 1.93%. The first values show a significant difference at p < 0.05 (t=2.16) while the difference after 12 months is p < 0.001 (t=4.14). Table 5.4 shows the proportions of males and females with improved control, poorer control or unchanged over the 12 month period, depending on their original placement in the various groups.
Table 5.1. **Trends in diabetic control in relation to time of monitoring using GHb.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control Level</th>
<th>Initial Result</th>
<th>Result after 1 Year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GROUP 1</strong></td>
<td>Good Control (mean)</td>
<td>8.42%</td>
<td>9.35% NS</td>
</tr>
<tr>
<td></td>
<td>below 9.7% (n=13) (SD)</td>
<td>1.08%</td>
<td>1.78%</td>
</tr>
<tr>
<td><strong>GROUP 2</strong></td>
<td>Intermediate Control (mean)</td>
<td>11.86%</td>
<td>11.88% NS</td>
</tr>
<tr>
<td></td>
<td>9.7% - 14% (n=69) (SD)</td>
<td>1.24%</td>
<td>1.77%</td>
</tr>
<tr>
<td><strong>GROUP 3</strong></td>
<td>Poor Control (mean)</td>
<td>16.33%</td>
<td>13.88% ***</td>
</tr>
<tr>
<td></td>
<td>above 14% (n=38) (SD)</td>
<td>1.86%</td>
<td>2.80%</td>
</tr>
</tbody>
</table>

**Key**
- NS: not statistically significant
- ***: p < 0.001
Table 5.2.  The relationship between age of patient and diabetic control.

<table>
<thead>
<tr>
<th>AGE (years)</th>
<th>&lt;20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>&gt;60</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>27</td>
<td>22</td>
<td>19</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>mean %</td>
<td>12.68</td>
<td>12.39</td>
<td>11.88</td>
<td>11.62</td>
<td>11.96</td>
<td>12.13</td>
</tr>
<tr>
<td>SD %</td>
<td>2.69</td>
<td>3.06</td>
<td>2.04</td>
<td>2.47</td>
<td>2.69</td>
<td>2.44</td>
</tr>
</tbody>
</table>
Table 5.3. The relationship between age and sex of patient and their diabetic control.

<table>
<thead>
<tr>
<th>AGE (years)</th>
<th>&lt;20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>&gt;60</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>13</td>
<td>13</td>
<td>9</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>mean %</td>
<td>12.02</td>
<td>10.94</td>
<td>11.07</td>
<td>11.11</td>
<td>11.35</td>
<td>11.13 MALES</td>
</tr>
<tr>
<td>SD %</td>
<td>2.52</td>
<td>1.67</td>
<td>1.39</td>
<td>2.04</td>
<td>2.25</td>
<td>1.29</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>14</td>
<td>9</td>
<td>10</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>mean %</td>
<td>13.68</td>
<td>13.74</td>
<td>13.05</td>
<td>12.25</td>
<td>13.35</td>
<td>14.15 FEMALES</td>
</tr>
<tr>
<td>SD %</td>
<td>3.53</td>
<td>3.47</td>
<td>2.35</td>
<td>2.85</td>
<td>3.28</td>
<td>3.24</td>
</tr>
</tbody>
</table>
Table 5.4. To show the relationship between sex of patient and diabetic control.

<table>
<thead>
<tr>
<th></th>
<th>% Improved control</th>
<th>% Poorer control</th>
<th>% No change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n=69)</td>
<td>56.5</td>
<td>29.0</td>
<td>14.5</td>
</tr>
<tr>
<td>Females (n=51)</td>
<td>51.0</td>
<td>39.2</td>
<td>9.8</td>
</tr>
<tr>
<td>TOTAL</td>
<td>54.2</td>
<td>33.3</td>
<td>12.5</td>
</tr>
</tbody>
</table>
DISCUSSION.

The major observation to be made from this study is that the patients monitored in this clinic over a period of 18 months to 2 years have improved their diabetic control in terms of glycosylated haemoglobin measurement. Since the observation was true for patients who had been diabetic and attended the clinic for a period in excess of 3 years, then the introduction of this test would appear to be directly responsible for this change. Ideally, however, these patients would need to be followed over several years, to see if the trend for better control continued.

These findings may be due to increased patient awareness or as a direct result of the diabetician altering treatment in response to these results. The measurement of glycosylated haemoglobin should provide the clinician with additional information to a single blood glucose concentration, as it provides a cumulative index of glycaemic control over a period of several weeks (Bunn, 1981). Theoretically this should lead to a more accurate interpretation of diabetic control and consequently improved clinical management. This trend is observed in the results presented in this thesis. Although there are several publications (Gonen et al. 1977, Dahlquist et al. 1982, Frazer et al. 1982) showing a significant correlation between glycosylated haemoglobin results and the physician's rating of diabetic control, Citrin, Ellis and Skyler (1980) indicated that the glycosylated haemoglobin measurement identified many patients who were erroneously considered to be in good control as they were falsely claiming negative results for urinary glucose between clinic visits.
Similarly, Campbell et al. (1983) measuring HbA1 by agar gel electrophoresis, studied 264 IDDM patients attending a diabetic clinic at a district hospital and found 73 of those patients whose control was considered satisfactory had high HbA1 results. They also showed that only 6 of the patients considered to have poor control had HbA1 values within the normal range. The evidence supporting the argument for introducing the glycosylated haemoglobin assay into the repertoire of biochemical tests for diabetic control is strong. Affinity chromatography provides a simple, reliable, inexpensive and reproducible method (Chapter 2) of providing this service. The method is suitable for use in most laboratories.

The second observation to be made from this study is that the females proved to have significantly worse control than the males (Table 5.3). Oestrogen levels are known to cause higher insulin resistance in pregnancy (Ciba Foundation Symposium 63, 1979), and initially it was thought that these results might be due to rising oestrogen levels in the menstrual cycle causing injected insulin to be less effective. However, since this poorer control in the females is present in every age group this cannot be the underlying cause, as when menopause is reached this effect should be negated. If 40 years is chosen as an arbitrary time for the onset of the menopause there is no significant difference between those patients under 40 years of age (mean = 13.59, SD = 3.14) and those who are older (mean = 12.86, SD = 2.92). Oral contraceptives have been shown to decrease glycosylated haemoglobin levels (Oakley and Rostron, 1982) so this does not provide the answer. Possibly the explanation could lie
in the awareness of the males and the females to their treatment. Most of the men in the survey visited the evening clinic as they worked and so they had to be well-motivated to attend. Many of the women attended the evening clinic due to social circumstances rather than day-time employment. It is possible that the awareness of the women to the diabetic condition and their self-motivation was not as positive as the males studied; or alternatively their carbohydrate intake was high due to poor diet as a result of low income.

There are numerous arguments in the literature regarding the desirability of diabetic control. Unger (1982A) outlined how meticulous management should delay or prevent the complications of diabetes. However, there have been studies using subcutaneous, continuous insulin infusions to achieve better control which have been shown to accelerate the deterioration of retinal function (Lauritzen et al. 1983, Drash, Daneman and Travis, 1980), although these findings followed a period of very poor glycaemic control in these patients. These workers note however that there is difficulty in accurately defining retinal function and retinal morphology. Although these limited studies cast doubt upon the need for close control of blood glucose, the weight of evidence supports the view that glycaemic control is important. There seems to be no justifiable reason to withhold a therapeutic option which requires no new drug, but merely the careful matching of insulin does to the analogue presentation of a varying
glucose profile, as achieved with the glycosylated haemoglobin measurement. However, it is important to note that patients may be at increased risk of hypoglycaemia if the dose of insulin is not correctly matched to the patients' requirements. This has been demonstrated when devices for continuous intravenous insulin infusion therapy (White et al. 1983) have been used for the meticulous metabolic control of diabetes mellitus. Unger (1982B) drew attention to the possibility of hypoglycaemia, caused by this means of control, causing permanent brain damage.
SECTION 2.
MONITORING ACUTE CHANGES IN GLYCAEMIA.

MATERIALS AND METHODS.

Subjects.

Eight in-patients (A-H) of the Royal Sussex County Hospital were included in this study. Five (A, B, C, D and E) were admitted with ketoacidosis, and three (F, G and H) were admitted for stabilisation of their diabetes. Their relevant clinical details are recorded in Table 5.5. Permission was obtained from the consultant diabetician for these samples to be taken and studied.

Samples.

Several samples of venous blood were collected into lithium heparin from each patient over the period of their hospitalisation; this varied from 4 to 15 days. The first sample in the series was taken on admission to hospital, or for those patients admitted in coma, as soon as emergency treatment was completed.
### Table 5.5. Clinical details of those patients (A-H) admitted to hospital with poor diabetic control

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Admitted in coma</th>
<th>Type of coma</th>
<th>Infusions</th>
<th>Mode of control</th>
<th>Other relevant details</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>56</td>
<td>F</td>
<td>Y</td>
<td>acute</td>
<td>S, D</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>15</td>
<td>F</td>
<td>Y</td>
<td>acute, severe</td>
<td>S, D, B</td>
<td>I</td>
<td>cataracts</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
<td>M</td>
<td>Y</td>
<td>acute</td>
<td>S, D</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>41</td>
<td>M</td>
<td>Y</td>
<td>acute</td>
<td>S, D</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>17</td>
<td>M</td>
<td>Y</td>
<td>sub-acute</td>
<td>S, D</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>48</td>
<td>F</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>D, I</td>
<td>obese, hypothyroid</td>
</tr>
<tr>
<td>G</td>
<td>68</td>
<td>F</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>D, I</td>
<td>recent cerebrovascular accident, spreading cerebral thrombosis</td>
</tr>
<tr>
<td>H</td>
<td>29</td>
<td>M</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>D, I, O</td>
<td>recent infection</td>
</tr>
</tbody>
</table>

**Key:**

**Infusions:**

- **S**: normal saline given with KCl
- **D**: 5% dextrose
- **B**: sodium bicarbonate

**Mode of control:**

- **D**: diet
- **I**: Insulin
- **O**: Oral hypoglycaemic agents
Methods.

The treatment of samples and measurement of glycosylated haemoglobins and glycosylated plasma proteins was performed as outlined in chapter 2. Labile material was removed by incubation prior to measurement in every case.

RESULTS.

The changes of glycosylated haemoglobins and glycosylated plasma proteins in the eight patients (A-H) studied are shown in Fig. 5.1. In one instance (Patient H) glycosylated haemoglobin was still rising during the first day after admission, whereas the glycosylated plasma protein values had already started to decline. The remainder of the patients showed that both sets of results started to decline immediately after admission to hospital.

In most cases (A, B, C, D, E, G and H) glycosylated plasma proteins have a faster decline than glycosylated haemoglobins, and are often higher (A, C, D, E, G and H) on admission. Table 5.6 shows the first and last result taken for each patient during their hospital stay. The percentage decline per day for both glycosylated haemoglobin and glycosylated plasma proteins is recorded. The daily percentage decline required for each to halve the original result has been calculated for each patient and noted. A half-life of 60 days and 20 days (Jones et al., 1983) was assumed for glycosylated haemoglobin and glycosylated plasma protein respectively. It can be seen that although many of the results are in broad agreement with these theoretical values (Table 5.6), many (marked with an *) decline at a much
and glycosylated plasma proteins in patients (A-H) admitted to hospital for stabilization of their diabetes.

Patient A.

Patient B.
Patient C.

Patient D.

Patient E.

Patient F.
For all patients;

x-axis represents days after admission.

y-axis represents % glycosylation.

**Patient G.**

**Key:**

For all patients;

- ● glycosylated haemoglobins
- ▲ glycosylated plasma proteins

**Patient H.**
quicker rate.

The calculations were in agreement with those outlined by Beach (1979), who showed that any change in glycosylated haemoglobin concentration in a red blood cell was proportional to glucose concentration, haemoglobin concentration, and the life-span of that red blood cell.

Calculations.

(1) **For theoretical decrease in glycosylated haemoglobins.**

\[
\frac{\text{initial GHb result}}{2} \times \frac{1}{60} = \% \text{ decrease per day}
\]

(2) **For theoretical decrease in glycosylated plasma proteins.**

\[
\frac{\text{initial GPP result}}{2} \times \frac{1}{20} = \% \text{ decrease per day}
\]

(3) **For actual decrease in glycosylated haemoglobins.**

\[
\frac{\text{initial} - \text{final GHb results}}{\text{number of day studied}} = \% \text{ decrease per day}
\]

(4) **For actual decrease in glycosylated plasma proteins.**

\[
\frac{\text{initial} - \text{final GPP results}}{\text{number of days studied}} = \% \text{ decrease per day}
\]
Table 5.6. To compare the rate of decline of glycosylated haemoglobins and glycosylated plasma proteins observed in patients following poor control, with the theoretical rate.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>GHb(1)</th>
<th>GHb(2)</th>
<th>GPP(1)</th>
<th>GPP(2)</th>
<th>T</th>
<th>GHb(C)</th>
<th>GHb(A)</th>
<th>GPP(C)</th>
<th>GPP(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20.3</td>
<td>18.6</td>
<td>22.8</td>
<td>15.5</td>
<td>10</td>
<td>0.169</td>
<td>0.155</td>
<td>0.570</td>
<td>0.664</td>
</tr>
<tr>
<td>B</td>
<td>17.1</td>
<td>15.1</td>
<td>13.8</td>
<td>13.0</td>
<td>4</td>
<td>0.142</td>
<td>0.500*</td>
<td>0.345</td>
<td>0.200</td>
</tr>
<tr>
<td>C</td>
<td>15.8</td>
<td>14.7</td>
<td>17.9</td>
<td>12.5</td>
<td>9</td>
<td>0.132</td>
<td>0.122</td>
<td>0.270</td>
<td>0.600*</td>
</tr>
<tr>
<td>D</td>
<td>11.3</td>
<td>12.3</td>
<td>13.0</td>
<td>11.4</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>0.325</td>
<td>0.229</td>
</tr>
<tr>
<td>E</td>
<td>27.4</td>
<td>20.8</td>
<td>26.8</td>
<td>17.0</td>
<td>15</td>
<td>0.228</td>
<td>0.440*</td>
<td>0.670</td>
<td>0.653</td>
</tr>
<tr>
<td>F</td>
<td>18.8</td>
<td>17.4</td>
<td>22.2</td>
<td>17.6</td>
<td>6</td>
<td>0.157</td>
<td>0.233</td>
<td>0.555</td>
<td>0.767</td>
</tr>
<tr>
<td>G</td>
<td>13.5</td>
<td>12.8</td>
<td>15.7</td>
<td>13.0</td>
<td>7</td>
<td>0.113</td>
<td>0.100</td>
<td>0.393*</td>
<td>0.386</td>
</tr>
<tr>
<td>H</td>
<td>27.6</td>
<td>21.7</td>
<td>40.6</td>
<td>23.3</td>
<td>5</td>
<td>0.230</td>
<td>1.18*</td>
<td>1.015</td>
<td>3.460*</td>
</tr>
</tbody>
</table>

**Key:**

- **GHb(1)** represents the first glycosylated haemoglobin result in the series
- **GHb(2)** represents the last glycosylated haemoglobin result in the series
- **GPP(1)** represents the first glycosylated plasma protein result in the series
- **GPP(2)** represents the last glycosylated plasma protein result in the series
- **GHb(C)** is the theoretical % decrease in glycosylated haemoglobin required per day to achieve half the original value after 60 days
- **GHb(A)** is the actual % decrease in glycosylated haemoglobin per day
- **GPP(C)** is the theoretical % decrease in glycosylated plasma protein required per day to achieve half the original value after 20 days
- **GPP(A)** is the actual % decrease in glycosylated plasma protein per day
- **T** is days

* indicates those results where the % decline in the actual results is very much quicker than the theoretical, expected % decline
DISCUSSION.

For nearly all of the patients (except H) both glycosylated haemoglobins and glycosylated plasma proteins decreased immediately after admission to hospital. Other studies have shown the decline of glycosylated haemoglobins after a period of poor control. Trovati et al. (1981) studied new juvenile diabetics, and found reductions in $\text{HbA}_1$ values within 7 to 10 days after the start of insulin treatment. When labile material was not removed prior to measurement the decrease was observed after only one day. However, as labile material in this study was removed by incubation (Chapter 2) this cannot be the explanation for the immediate decrease observed. Wettre et al. (1983) induced poor glycaemic control by withdrawing insulin, and then studied patients through the measurement of glycosylated haemoglobin once insulin treatment was resumed. They did this using ion-exchange methods but were unable to show a significant decrease in stable $\text{HbA}_1c$ within 2 weeks of the improved glycaemia. A similar study by Brooks, Nairn and Baird (1980) followed the progress of 7 IDDM patients after a week of poor glycaemic control. They used both colorimetric and ion-exchange techniques to do this, and found considerable variation in the pattern and degree of change in percentage glycosylation. The results in this work show that glycosylated haemoglobins decrease significantly within a few days of treatment, if the between-batch variation for the affinity method of about 2% (Chapter 2) is considered. This may be due to the sensitivity of the affinity technique. Arnquist et al. (1982) using ion-exchange chromatography, showed that the initial decline
in HbA\textsubscript{1a+b} was faster than HbA\textsubscript{1c} after a period of poor control; these components decreasing in a biphasic fashion. Since affinity chromatography should measure all glycosylated material, it may be detecting this decrease in HbA\textsubscript{1a+b} in the early days of treatment.

The severity of the ketoacidotic coma for these individuals did not relate directly to the extent of their recent poor control. Patient E had very high values of both glycosylated haemoglobins and glycosylated plasma proteins and yet was admitted in a coma considered to be 'sub-acute'. In contrast patient B had a very severe comatose condition and yet her results, though high, were not as high as with patient E. These findings support those noted by Wieland \textit{et al.} (1979).

In this study glycosylated plasma proteins decreased at a far quicker rate than glycosylated haemoglobins for most of the patients, an observation which is not unexpected as they have a far shorter half-life. Jones \textit{et al.} (1983) compared glycosylated haemoglobin change as measured by ion-exchange chromatography, with that of glycosylated albumin measured by the colorimetric technique, following a period of poor glycaemic control. They found a significant change in glycosylated albumin after 4 weeks, but no observable change in glycosylated haemoglobin in this time. A similar study by Dolhofer, Renner and Wieland (1981), again using ion-exchange chromatography to measure glycosylated haemoglobins, and the colorimetric method for glycosylated albumins, studied patients recovering from ketoacidotic coma. They showed a significant decrease in glycosylated albumins, as measured
by 5-HMF, within the first week of treatment, but no such change was noted with the glycosylated haemoglobins. Affinity chromatography provides an advantage over these techniques since it allows the comparison of glycosylated haemoglobins and glycosylated plasma proteins (or glycosylated albumins) using the same technique and expressing results in the same units. Other workers have tried to create indices of recent changes in control by combining results of fasting blood glucose and glycosylated haemoglobins on a single occasion (Pecoraro, Chen and Porte, 1982) but these calculations are now shown to be an unnecessary complication, as the single measurement of glycosylated plasma proteins by affinity chromatography can provide this information.

Table 5.6 shows that process of glycosylation of haemoglobin or protein may not be a simple one. Although many of the results decrease at the expected rate, some decrease far quicker. The precise temporal relationship of \( \text{HbA}_1 \) to plasma glucose has been questioned in the literature (Borsey et al. 1982, Brooks, Nairn and Baird, 1980). This study, although limited, confirms that there is no place for complacency in the belief that the true nature of glycosylated haemoglobin has fully been elucidated.
CHAPTER 6.

MONITORING NORMAL AND DIABETIC PREGNANCIES USING GLYCOSYLATED HAEMOGLOBINS AND GLYCOSYLATED PLASMA PROTEINS.
INTRODUCTION.

Pregnancy is a diabetogenic condition which confers additional problems for the clinical management of the diabetic patient. To understand the complications and interactions promoted by these two conditions, it is first necessary to consider the metabolic changes that occur in normal pregnancy. In this way the influence of pregnancy on carbohydrate metabolism can be defined, the associated problems for IDDM mothers clarified, and the circumstances which cause some patients to become diabetic in pregnancy (gestational diabetics) elucidated.

Metabolic Changes in Normal, Uncomplicated Pregnancy.

Fasting plasma glucose levels decline during pregnancy (Bleicher, O'Sullivan and Frienkel, 1964) whereas there is a significant rise in circulating insulin. The glucose decline is evident by the tenth week of gestation (Lind, Billewicz and Brown, 1973) but the rise in insulin continues to a plateau at the 36th week of pregnancy (Cowett et al. 1983), so the two cannot be considered in terms of cause and effect. The explanation for both observations is the influence of the growing conceptus, namely the fetus and the placenta.
In the non-pregnant condition an individual eats intermittently and can be considered at any point in time to be in one of two broad categories; the fed or fasted state. In the fed state the nutrients are maximally used for prevailing oxidative needs or catabolic repair. Excesses are stored. These resources can then be used for the production of fuel while the fasted condition prevails. However, in pregnancy, the fetus functions as a continuously feeding boarder within an intermittently eating host. Indeed the fetus has been shown to continue growth even in periods of total maternal deprivation (Frienkel et al. 1970) and persistently abstracts glucose for its needs. This is consistent with the reduced maternal glucose levels seen in pregnancy. To do this the fetus must modify the maternal response to food deprivation and this influence must increase as gestation progresses. After a brief overnight fast, a pregnant woman will demonstrate raised plasma ketones and free fatty acids which are characteristic of prolonged starvation in the non-pregnant state. This tendency has been labelled "accelerated starvation" (Frienkel, 1965) and is thought to be needed to "spare" circulating maternal glucose for the demands of the fetus. Glucose utilisation is 2 to 3 times greater in the fetus than the glucose turnover rate in adults (Page, 1969).

Insulin levels have been shown to increase during pregnancy (Cowett et al. 1983) yet tissue resistance to insulin increases. The main protagonist of this effect is
human placental lactogen (HPL), which is a polypeptide hormone produced by the placenta. This has been shown to induce insulin resistance \textit{in vitro} (Beck and Daughaday 1967) and to exert direct lipolytic effects (Turtle and Kipnis, 1967). However as some pregnancies have progressed successfully despite very low levels of HPL, the exact role of HPL pertaining to the maintenance of normal pregnancy is not clear. Other hormones of pregnancy, progesterone and oestrogen cause exaggerated insulin response to stimuli. Administration of progesterone to non-pregnant individuals has been shown to enhance their basal and stimulated levels of insulin secretion (Kalkhoff, Jacobson and Lemper, 1970). Oestrogens are thought to cause pancreatic islet cell hyperplasia (Redman and Hockaday, 1983). Hence the combined effects of all of these hormones is to increase circulating insulin whilst reducing its efficacy, and their concentration and effect is in direct proportion to the growing placental mass. The overall effect is to produce heightened oxidation of fat and changes in gluconeogenesis. This is well illustrated by Cowett et al. (1983) who used D-(U-\textsuperscript{13}C) glucose in constant infusion studies to investigate the glucose turnover rate in pregnancy. They were able to show that when fasted, both non-diabetic and diabetic subjects accelerated their glucose turnover rate in pregnancy to provide for the increased maternal and fetoplacental metabolic requirements.
Nutrients are transferred from the mother to the growing fetus, although some are used to maintain the placenta. Glucose crosses the placenta by a system of facilitated diffusion (Holmberg et al. 1956), and changes in fetal blood glucose lag about ten minutes behind maternal changes (Redman and Hockaday, 1983). The transfer system becomes saturated when maternal levels reach about 15mmol/l. To maintain this diffusion system the fetal glucose has to be lower than maternal levels, and part of this gradient is accounted for by the use of glucose by the placenta. Amino acids are transferred by an active process, lipids are transferred as free fatty acids, and ketones diffuse freely to the fetus. However, neither maternal nor fetal insulin crosses the placenta, so the two can be considered as separate compartments. As fetal insulin response is relatively sluggish to plasma glucose the main determinant of fetal glucose at all stages of pregnancy is circulating maternal glucose. Fetal insulin does have a key role in promoting intra-uterine growth, therefore maternal hyperglycaemia causes fetal hyperglycaemia with fetal islet cell hyperplasia and hyperinsulinaemia, resulting in excessive fetal growth. There is a whole multitude of problems associated with maternal hyperglycaemia affecting fetal development and neonatal life. These are discussed at length later in this chapter.
Diabetes in Pregnancy.

(1) **Gestational diabetes.**

The definition of diabetes, even in the non-pregnant state is controversial and in the pregnant state is far from clear-cut. In pregnancy circulating insulin levels increase and this puts extra demands on the β-cells of the pancreas. Any potential inability of these cells to meet the additional requirements for insulin will be unmasked in these circumstances. For this reason diabetes may be diagnosed for the first time in pregnancy. If it remits afterwards then it can be correctly termed "gestational diabetes". If it does not, then it is simply a diagnosis made concurrently with pregnancy. Gestational diabetes cannot therefore be diagnosed with certainty until a postpartum assessment has been made.

Gestational diabetes is a subset of the category "chemical diabetes of pregnancy", which is usually defined by an abnormal glucose tolerance test. The effect of chemical diabetes upon the fetus is to increase the supply of many classes of nutrients and to provoke a relative degree of hyperinsulinism which may cause macrosomia. Management may be by dietary control alone or together with insulin administration. The main problem with gestational diabetes however is that it may go unrecognised and untreated, resulting in an unexplained stillbirth or abnormally large baby.
2) **Insulin-dependent diabetes in pregnancy.**

Prior to the discovery of insulin in 1921, the association of diabetes mellitus and pregnancy was almost non-existent. With the advent of insulin, the lifespan and fertility of the diabetic patient increased, and pregnancy became feasible, presenting a whole range of problems for the obstetrician. The management of pregnancy of overt diabetics as well as the supervision of diabetes unmasked by pregnancy, now had to be considered. Before the availability of insulin, maternal mortality was about 45%. This decreased to about 2% with the use of insulin (Gugliucci et al. 1976), but perinatal mortality was still very frequent. Many of the babies died in utero and were noticeably larger than average size. This led Peel (1972) to suggest that these babies were maturing earlier, and he therefore recommended pre-term delivery. This, together with hospitalisation from 32 weeks gestation (Pedersen, 1954) decreased perinatal mortality to 25% by the mid 1950's (Gugliucci et al. 1976). Nowadays this figure has decreased to about 10-15% due to advances in monitoring fetal wellbeing and the availability of better hospital facilities. Wheeler, Gollmar and Deeb (1982) in South Carolina, USA. showed 182 perinatal deaths in every 1000 deliveries to IDDM women, compared to 25 in every 1000 for non-diabetic women. This figure was slightly lower (102/1000) when all forms of diabetes in pregnancy were considered. These figures are all unacceptably high, especially as many deaths no longer occur in the uterus but shortly after delivery and are not included in these results.
Effect of maternal hyperglycaemia on the growing fetus.

There is a wide range of problems for the fetus or newborn thought to be associated with poor diabetic control in pregnancy. Congenital abnormalities occur far more frequently. Kučera (1971) suggested 4.8% of births to diabetic women had congenital anomalies compared with 1.6% of births to non-diabetic women. These results were supported by Watkins (1982) who reported the frequency in diabetics to be 2 to 3 times higher than in non-diabetics, and noted that these anomalies accounted for about half of the observed fetal deaths. The types of congenital malformations observed included caudal regression, anencephaly, spina bifida as well as various heart defects such as transposition of the main vessels, and kidney defects like the ureter duplex. Mills, Baker and Goldman (1979) suggested that the insult causing these malformations occurs before the seventh week of gestation. It is unlikely that it is insulin itself which causes these effects as maternal insulin does not cross the placenta and fetal insulin is not produced until about the eighth week of gestation, after the critical period of teratogenesis. There are two biochemical effects which suggest that it is maternal hyperglycaemia and not other causative factors, which is responsible for the high incidence of anomalies observed. Hyperglycaemia induces low intracellular ascorbic acid and high plasma dehydroascorbic acid, both of which inhibit mitosis (Edgar, 1970).
Interference with mitosis at the very early stages of fetal development would be expected to cause defects. This association between maternal hyperglycaemia early in pregnancy and defects, has been shown using glycosylated haemoglobin for the retrospective assessment of glycaemic control. Miller et al. (1981) used HPLC to measure HbA₁C in the first trimester of pregnancy for 116 IDDM subjects. Fifteen infants were delivered in this study with major congenital abnormalities, defined as abnormality causing death or severe handicap. Eight infants had congenital heart defects, three of which were fatal, and four infants had malformations of the brain, three of which were fatal. When compared to the group delivering normal infants, this group with congenital anomalies had significantly higher HbA₁C results early in pregnancy. Leslie et al. (1978) also showed the association, using ion-exchange chromatography to measure HbA₁C. Of the five diabetic women in their study with high values early in pregnancy, three produced children with fatal congenital abnormalities. No abnormalities were detected in children born to diabetic mothers who were well controlled early in pregnancy. The implications are that good management of diabetes in the crucial first weeks of pregnancy can decrease the incidence of congenital malformations, and pre-pregnancy counselling is advocated (Pedersen, 1977). Steel et al. (1982) reported the results of a pre-pregnancy clinic over a 5 year period and noted that of the 5 births of abnormal babies to diabetic women during this time at their hospital, 4 did not attend the clinic. The fifth birth was to a lady who attended the clinic infrequently and had persistent hyperglycaemia.
There are various ante-natal complications observed more frequently with diabetic women. These include polyhydramnios, pre-eclampsia, and infection (Redman and Hockaday, 1983); and their incidence can be reduced by adequate glycaemic control. These conditions have adverse effects on the growing fetus, contributing to the perinatal mortality and compromising the fetus in terms of nourishment and oxygen availability. In these circumstances often abnormally small infants are born.

Abnormally large infants are thought to result from maternal hyperglycaemia causing fetal hyperglycaemia and hyperinsulinaemia, with the resultant excessive growth. Such macrosomic neonates have typically fat round faces with sunken eyes, a short neck, and a lot of head hair. Many are longer than normal as well as being heavier. Macrosomia is seen as a consequence of poor diabetic control unless a second pathology such as placental ischaemia intervenes. Macrosomia may mean that vaginal delivery is impossible and many such infants are born by Caesarian section, which confers certain disadvantages. Also the large size of these babies in utero may hinder the accurate assessment of gestational age by ultrasound techniques (Ogata et al. 1979).

Sudden intra-uterine deaths are seen more frequently in diabetic pregnancy. Typically these occur suddenly between 36 and 38 weeks and are associated with macrosomia. The actual cause of death is not known but it has been postulated (Redman and Hockaday, 1983) that the fetus dies from lactic acidosis which results from an influx of
maternal glucose which exceeds the fetal capacity for its oxidative metabolism. Again the implication is that sudden fetal death can be reduced by meticulous maternal glycaemic control. Madsen and Ditzel (1982) also associated poor maternal glycaemic control in terms of HbA1c with reduced arterial oxygen saturation and reduced oxygen affinity (P02 at 50% oxygen saturation). They postulated that poor glycaemic control may contribute to fetal hypoxia and intrauterine death.

There are several potential causes of death in the neonatal period for infants of diabetic mothers. A frequent complication in these infants is respiratory distress syndrome (RDS). This syndrome was discussed by Stubbs and Stubbs (1978) who suggest that hyperinsulinaemia reduces glycerol 3-phosphate and dihydroxyacetone phosphate (DHAP) production, and this impairs phospholipid synthesis and surfactant production in the lung (Fig. 6.1). A decrease of surfactant has been implicated with RDS (Avery, 1973). RDS is a major cause of neonatal mortality. In addition many babies are born to diabetic mothers by caesarian section, which itself augments the risks of developing RDS (Redman and Hockaday, 1983). Other problems such as jaundice, hypocalcaemia, cardiomyopathy and polycythaemia have all been seen to increase in diabetic pregnancies (Wright et al., 1982). Polycythaemia seems to result from increased erythropoiesis in the liver, and causes increased blood viscosity and poor peripheral perfusion. This predisposes to multiple organ thrombosis, of which renal vein thrombosis is the most important. Neonatal hypoglycaemia
Fig. 6.1 Glycolysis and the possible effects of hyperinsulinaemia on the production of surfactant in the lung.

GLUCOSE

GLUCOSE-6-PHOSPHATE

FRUCTOSE 1,6- BISPHOSPHATE

DIHYDROXYACETONE ↔ GLYCEROL-3-PHOSPHATE

PHOSPHOLIPID

PYUVATE

ACETYL CoA

KREB's CYCLE

Pyruvate Dehydrogenase * activated by insulin increases the conversion of glucose to acetyl CoA. Hyperinsulinaemia would decrease intermediary metabolites required for the production of surfactant in the lung. This hypothesis is outlined by Stubbs and Stubbs (1978) Lancet; 2 : 308-309.
is another complication frequently observed. This is thought to result from fetal islet cell hyperplasia, which produces excessive fetal insulin in utero to manage the high levels of maternal glucose supplied. When, at birth, this glucose supply is abruptly terminated, hypoglycaemia occurs, as the neonate is still producing insulin in excess. In addition these neonates seem to have impaired mechanisms for compensating for this hypoglycaemia; in particular the secretion of catecholamines and glucagon remains inappropriately low, and the infants can not use their increased reserves.

(4) Management of glycaemic control in diabetic pregnancy.

The management of pregnancy in IDDM women requires great care and attention to detail, although in most cases the mother is well motivated. The clinician must gradually alter the insulin dosage to allow for the progressive insulin resistance that occurs. Between 16 and 36 weeks gestation the insulin given is, in general, increased by 2/3, but for some women insulin requirements will increase by two or threefold. The dietary requirements of pregnancy needs to be carefully balanced or ketosis and acidosis may rapidly develop, adversely affecting the fetus. Problems due to sickness in early pregnancy, or infection in later pregnancy may upset the delicate balance between carbohydrate intake and insulin administration.
The ultimate aim of management of diabetic pregnancy is to improve perinatal survival and prevent both maternal and neonatal complications. The evidence supporting the need for good diabetic control is strong, but metabolic control is only one factor in the whole spectrum of clinical management of the pregnancy. However as control is important, its assessment in biochemical terms aids the obstetrician in his supervision of a successful pregnancy. Because of the physiological glycosuria that occurs in normal pregnancy the usefulness of routine urine checks for glucose is limited (O'Shaunessy, Russ and Zuspan, 1979). Frequent blood glucose measurements are used instead, but as blood glucose fluctuates from hour to hour hyperglycaemia may easily be missed. For this reason the cumulative assessment of glycaemia by glycosylated haemoglobin was investigated by many workers. Also in gestational diabetes, by definition, glucose tolerance returns to normal after delivery and so it is not possible to confirm the diagnosis retrospectively by the more traditional methods for evaluating carbohydrate metabolism. Glycosylated haemoglobin measurement, by its nature, may provide retrospective information and may help in the understanding of, for example, sudden stillbirths or the birth of large babies.
Glycosylated haemoglobin measurement in pregnancy.

There have been many studies on the measurement of HbA or HbA during pregnancy, most workers using ion-exchange chromatography. For these values to be useful to the obstetrician, normal ranges in pregnancy are essential, because the physiological and biochemical changes seen in pregnancy may affect the usual mechanism of glycosylation and result in different values. However, there has been a good deal of discrepancy in the published ranges. Some workers have reported no significant change between non-pregnant and pregnant non-diabetic individuals (Kjaegaard and Ditzel, 1979, Ylinen, Helaki and Teramo, 1981, Fadel et al. 1979), whereas others have noted both higher (Vintzileos and Thompson, 1980, Davies and Welborn, 1980) and lower ranges (O'Shaunessy, Russ and Zuspan, 1979, McFarland et al. 1981, Lind and Cheyne, 1979). Schwartz et al. (1976) used Amberlite IRC-50 resin and showed that HbA levels were elevated slightly in normal pregnancy (from 5.74 ± 0.42% to 6.97 ± 0.69%). However in a later publication (Schwartz, Widness and Schwartz, 1981) they refute their earlier evidence. Lind and Cheyne (1979) found a progressive decrease in percentage glycosylation to about 20 weeks gestation from when this lower value was maintained. As fasting blood glucose concentrations fall by about 0.3mmol/l at 10 weeks gestation (Lind, Billewicz and Brown, 1973) these observations would be consistent with new erythrocytes being
exposed to lower time-averaged glucose levels. Also red
cell volume increases progressively throughout pregnancy due
to increased fluid volume and iron stimulation (Taylor and
Lind, 1976). This dilutional effect, due to increased
numbers of young erythrocytes would be expected to reduce
the overall percentage glycosylation. Recently Phelps
et al. (1983) used ion-exchange chromatography to study
377 non-diabetic pregnancies and suggested that the changes
in \( \text{HbA}_1c \) during pregnancy were biphasic, reaching a minimum
value at about 24 weeks gestation and rising again towards
term. They indicated that this reflects (with the
appropriate displacement in time) the biphasic alterations
of mean blood sugar concentrations that occur in normal
pregnancy due to sequential changes in glucoregulation.

Most workers are now in agreement that in IDDM, pregnancy
is accompanied by reduced glycosylated haemoglobin values,
but that these ranges are still higher than observed for
non-diabetic pregnancies. However, the association of high
glycosylated haemoglobin values and macrosomia is in dispute.
No retrospective correlation between the two using either
maternal or cord samples could be shown by workers using
ion-exchange chromatography (O'Shaunessy, Russ and Zuspan,
focussing (Poon, Turner and Gillmer, 1981) or the
thiobarbituric acid colorimetric technique (Sosenko et al.
1982, Worth et al. 1983). In contrast, other studies have
shown the association. Widness et al. (1978) using cation-exchange chromatography presented data on 12 diabetic women without vascular disease, and found a strong correlation between infant birth weight and HbA₁c in the third trimester (p < 0.05). This correlation improved still further when adjustment was made for gestational age (p < 0.01). Burke et al. (1981) used the Flückiger colorimetric technique to measure glycosylated haemoglobin in the third trimester and found it had some predictive capability; detecting 4 out of the 6 macrosomic babies in their study. Vintzileos and Thompson (1980) also showed the relationship between birth weight and HbA₁c and noted the additional association between the latter and placental weight. Mansani et al. (1982) found an association between HbA₁c with both maternal weight gain and the size of the infants as assessed by their thoracic circumference. Recently Feldman et al. (1984) have used affinity chromatography to measure glycosylated haemoglobin in cord samples. They showed that the birth weight ratios (calculated by dividing the birth weight by the 50th percentile weight) were markedly increased in the cord bloods of the 15 diabetic mothers studied, and eleven of the infants born were above the 90th percentile for weight.

Several workers (Steel et al. 1981, Pollak et al. 1981, Coen et al. 1980) have used the measurement of glycosylated haemoglobin to investigate retrospectively the cause for babies being born who are large-for-gestational-age (LGA),
as this tests for abnormal carbohydrate tolerance in pregnancy. As 1-2% of pregnant woman develop gestational diabetes, with the associated risks of neonatal morbidity, their identification is important for any future pregnancies (Shah et al. 1982). Steel et al. (1981) used ion-exchange chromatography to study 50 mothers who had produced large babies and 50 who had not. They demonstrated raised HbA₁ levels immediately after delivery in 9 of the mothers with LGA babies and in only one mother with a normal-sized child. All patients studied had a normal OGTT and HbA₁ value 6 weeks after delivery. Pollak et al. (1981) showed that samples from a group of 50 mothers with LGA babies produced significantly higher TBA colour (measured in haemoglobin) than in a corresponding control group. Coen et al. (1980) found that when the 19 women in their study who produced LGA babies were considered as a group, their mean glycosylated haemoglobin value did not vary significantly from a group with normal births, however 3 of the women with LGA offspring had very elevated levels of glycosylated haemoglobin.

Many of these discrepancies in results and conclusions may be attributable to the methodology, in particular the ion-exchange techniques where minor adult haemoglobins have been difficult to distinguish from the fetal haemoglobins, due to coelution. This of course may prove significant when investigating maternal and cord blood samples. The importance of HbF is stressed differently by different workers, depending on the actual samples being analysed.
However, although the major problem occurs when measurement of glycosylated material in cord blood is considered, HbF levels of 2-3% have been shown which might have serious interference effects on maternal samples when glycosylated haemoglobin values of about 6 to 8% are being considered. Svendsen and Søegaard (1982) suggest that as most patients have HbF values below 1% this problem is of minor importance. However, Krause, Stolc and Campbell (1982) stated that the relationship between HbF and HbA\textsubscript{1} in ion-exchange chromatography was not a simple one and followed the relationship as expressed by the equation \[ y = 6.03 + 1.24x. \] Furthermore, they found that 1.5% of the samples with elevated HbA\textsubscript{1} levels had high (> 2%) HbF levels. Schwartz, Widness and Schwartz (1980A) circumvented the coelution problems by utilising isoelectric focussing and ion-exchange chromatography using DEAE cellulose, which allowed for the separation of the minor haemoglobins in cord blood, in the presence of HbF. However they were unable to demonstrate any difference in either acetylation or glycosylation in the cords of diabetics or non-diabetics. HbF can be acetylated as well as glycosylated (Abraham et al. 1979) and many methods are unable to distinguish between these two types of changes to the haemoglobin molecule, and report a combined result. Only methods such as the TBA colorimetric technique or isoelectric focussing with suitable spacers (Fitzgerald and Cauchi, 1980) can distinguish between them.
The ratio of the percentages of glycosylated haemoglobin in maternal and cord samples has been calculated by several workers. Caangary, Curran and Malone (1979) used the thiobarbituric acid method to measure this ratio. They assessed maternal samples to be 1.3 times higher than their corresponding cord samples at non-diabetic deliveries, whereas a higher ratio of 1.6 was recorded for diabetics. Zeller et al. (1983), again using the colorimetric technique found that the cord samples for both diabetics and non-diabetics represented levels only 60% of the corresponding maternal values. Similarly Worth et al. (1983) used this method and found a lower percentage of glycosylated haemoglobin in cord samples. Fitzgerald and Cauchi (1980) using isoelectric focussing demonstrated that there was no difference in the in vitro glycosylation rate between fetal and adult blood. However there may be many factors which account for the lower percentage glycosylation of fetal blood observed. Fetal red cell survival is known to be slightly decreased (Bratteby et al. 1968), which would decrease the duration of haemoglobin exposure to glucose. Also the lower plasma glucose concentration in the fetus when compared to the mother, would similarly affect the result. (Crawford, 1965). As about 15-20% of fetal haemoglobin is acetylated at the N-terminal amino group of the \( \beta \) chain, this blocks the glycosylation site (Schwartz et al. 1980, Garlick et al. 1981). Decreased fetal red cell membrane permeability to glucose could be another contributing factor (Widdas, 1971).
The aim of the work in this chapter is to use the affinity technique to measure glycosylated haemoglobins and glycosylated plasma proteins both throughout pregnancy, and at delivery in maternal and cord samples. Both diabetic and non-diabetic pregnancies will be considered. The predictive capability of these tests regarding the outcome of the pregnancy can be assessed. The role of the measurement of glycosylated plasma proteins in the monitoring of diabetic pregnancy has never previously been evaluated.
MATERIALS AND METHODS.

Subjects.

Patients were enrolled for this study from March 1982 to September 1983. All patients gave their informed consent to this investigation. Non-diabetic women had no family history of diabetes and were free from glycosuria during pregnancy. All mothers received routine ante-natal care, although diabetic mothers were seen weekly in the last trimester.

(1) Non-diabetics.

110 non-diabetic women were studied at one time-point during their pregnancy. 18 had samples taken in the first trimester, 37 in the second trimester and 55 in the third trimester. The gestational age of the fetus was estimated by the mothers menstrual history, clinical examination, and ultrasound techniques. Each patient was tested for the possibility of anaemia, by routine haematological tests.

Fifty live births to non-diabetic women were monitored, 19 of the mothers were primigravidae. Most of the births were normal vaginal deliveries, but there were 4 forceps deliveries and 3 by Caesarian section. No transfusions were given at any of the births. Again the gestational age of each fetus was based on the mothers menstrual history, clinical and ultrasound examinations. All of the births were within 2.5 weeks of the predicted date of delivery. The mean age of the mothers was 28 years (range 19 to 42 years). Delivery details, antepartum complications, placental and birth weights were all noted.
Diabetics.

48 diabetics were studied at some time-point during their pregnancy, many having ante-natal samples taken at monthly intervals over several months and more frequently towards term. Of these, only 35 had additional maternal and cord samples taken at birth; 12 were primigravidae. A further 13 diabetics were studied only at delivery.

For the major part of this study only the 35 diabetics with both full pre-term and delivery assessment and full clinical details will be considered. This was a two-center study with 19 patients being treated at the Royal Sussex County Hospital in Brighton, and the remaining 16 at Kings College Hospital in London. The group included 28 diabetics who were established on insulin before pregnancy, one diagnosed before pregnancy but maintained on diet alone, and 6 who were gestational diabetics. The diagnosis of gestational diabetes was confirmed by the standard criteria for oral glucose tolerance tests in pregnancy (O'Sullivan and Mahan, 1964). The average maternal age of these patients was 27 years and 8 months (range 21 to 36 years).

Four samples were taken from insulin-dependent diabetics whose pregnancies had resulted in intra-uterine death. The samples were taken at the time, or very shortly after the miscarriage.
Statistics.

Statistical comparisons of either pairs or means were performed by Students t-test.

Samples.

Samples of venous blood were taken in pregnancy, whether diabetic or non-diabetic, usually at routine ante-natal visits. One part of the blood was put into a standard lithium heparin tube for glycosylated haemoglobin and glycosylated plasma protein analysis. The remainder of the blood was preserved in a tube containing fluoride oxalate for the measurement of glucose. Non-diabetic patients also had blood taken and preserved in sodium EDTA for routine haematology.

Maternal and cord samples were taken from both non-diabetic and diabetic mothers immediately after birth, and treated in an identical manner. In two instances the cord sample was not available, one due to an unexpected still-birth and the other when the cord snapped during delivery. In one diabetic patient the maternal and cord samples were not taken at delivery, but a sample was taken from the mother and infant on the following day.

As this was a two center study, samples from Kings' College Hospital were sent in vacutainer tubes containing lithium heparin, by first class post for analysis the next day. Any
samples which were haemolysed were excluded from the study.

Methods.

(1) Glycosylated haemoglobins and glycosylated plasma proteins.

Measurement of both glycosylated haemoglobins and glycosylated plasma proteins were done in accordance with the techniques outlined in Chapter 2. Labile material was removed by incubating the sample at 37°C for 5 hours. The stable sample was then stored at 4°C until analysis, in most cases up to a maximum of 48 hours. The pH of the buffers used for the chromatography was 8.3 and 8.9 respectively. Three different batches of gel were used in the study, and when a new batch of gel was to be used, it was first run in tandem with the old batch of gel to ensure that the results obtained were not significantly different. By the middle of the study quality control was introduced, as outlined in Chapter 2.

(2) Blood glucose.

Whole blood glucose was measured using the Yellow Springs Glucose Analyser Model 23AM (Yellow Springs Instruments, Yellow Springs, Ohio, USA). This was usually carried out within 30 minutes of sampling, but when this was not possible the sample was stored at 4°C.
RESULTS.


Table 6.1 shows the mean values (±SD) of glycosylated haemoglobins, glycosylated plasma proteins, and whole blood glucose for the various non-diabetic patients studied during the different trimesters of pregnancy. The trimesters were defined as three sequential groups, each of 13 weeks, and the gestational age of the fetus determined which trimester, or group, that a result was considered in. There was a significant decline in glycosylated haemoglobins from 7.31 ± 0.90% for non-pregnant, non-diabetics, to 6.50 ± 1.06% (p < 0.001) at the third trimester (Table 6.1) and 6.49 ± 1.22% (p < 0.001) for the non-diabetics at term (Table 6.3). Table 6.1 well illustrates how the values of both glycosylated haemoglobins and glycosylated plasma proteins decrease with the progress of pregnancy. Glycosylated plasma proteins also decrease significantly (p < 0.02) from 6.29 ± 1.90% for non-pregnant, non-diabetics, to 5.61 ± 0.93% for non-diabetic patients at term (Table 6.3), although this decrease was not significant by the third trimester when values of 5.79 ± 0.95% were obtained. Blood glucose concentrations were significantly lower (p < 0.001) at every stage of pregnancy (Table 6.1) and at birth (Table 6.3) when compared to the non-pregnant condition. However, during the progress of pregnancy the levels rose significantly (p < 0.001) from 3.48 ± 0.43% in the first trimester to 4.11 ± 0.62% in the third.
Table 6.2 shows the mean values (± SD) of glycosylated haemoglobins, glycosylated plasma proteins and whole blood glucose, for the various diabetic patients studied in the different trimesters of pregnancy, again defined as 13 week periods. When a diabetic mother had more than one result for any of these measurements in a particular trimester, the mean value for the trimester was calculated and considered as the result for that trimester in this, and later studies. The diabetics considered in this investigation included the 35 with a complete set of clinical details, and a further 13 who only had samples taken during pregnancy, most of whom had not delivered before the end of this study. Thirteen diabetics were also studied who only had results at delivery. Again there was a significant decline (p < 0.001) observed during pregnancy, from values of glycosylated haemoglobins and glycosylated plasma proteins in non-pregnant diabetics of 12.70 ± 2.88% and 12.62 ± 3.36% to 9.43 ± 2.25% and 8.15 ± 2.09% in the third trimester (Table 6.2), and 9.67 ± 2.57% and 7.67 ± 1.85% (Table 6.3) at term in these patients. Table 6.2 shows that both glycosylated haemoglobins and glycosylated plasma proteins decline with the progress of pregnancy. For the diabetics in this study the glucose values were significantly lower throughout pregnancy when compared to the ranges observed for the non-pregnant diabetics (Chapter 2). However, although glucose values at the third trimester (6.13 ± 2.28%) were higher than in the first (5.61 ± 2.51%) (Table 6.2), this rise was not significant.
The results obtained for the diabetics in this study were compared to the non-diabetic values in the different trimesters. The diabetic patients had significantly higher (p < 0.001) values for glycosylated haemoglobins, glycosylated plasma proteins and glucose at every instance.

**Relationship Between Maternal and Cord Samples.**

Table 6.3 shows that glycosylated haemoglobins in maternal and cord blood were significantly higher (p < 0.001) at diabetic births in this study. The same was true for glycosylated plasma proteins in these samples (p < 0.001 for maternal blood, p < 0.01 for cord blood), but no significant difference could be shown for blood glucose, possibly due to the wide range of values obtained (Table 6.3).

There was a positive correlation between maternal and cord samples for both glycosylated haemoglobins (r = 0.614) and glycosylated plasma proteins (r = 0.424) at deliveries to non-diabetic patients. For non-diabetic deliveries glycosylated haemoglobin values were greater than glycosylated plasma protein values (p < 0.001) in maternal blood, and significantly lower (p < 0.001) in cord blood (Table 6.3), as assessed using the paired t-test. Only 10 out of the 50 patients studied had lower glycosylated haemoglobins than glycosylated plasma proteins in maternal blood, and only 6 of the 50 had higher values in cord blood.
Table 6.1  Values for glycosylated haemoglobins, glycosylated plasma proteins and glucose before, and during non-diabetic pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>% Glycosylated haemoglobins</th>
<th>% Glycosylated plasma proteins</th>
<th>Whole blood glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant</td>
<td>7.31 ± 0.90 (n = 62)</td>
<td>6.29 ± 1.90 (n = 58)</td>
<td>6.49 ± 0.51 (n = 13)</td>
</tr>
<tr>
<td>1st trimester</td>
<td>7.01 ± 1.17 (n = 18) NS</td>
<td>5.93 ± 0.67 (n = 18) NS</td>
<td>3.48 ± 0.43 (n = 18) ****</td>
</tr>
<tr>
<td>2nd trimester</td>
<td>6.25 ± 1.14 (n = 37) ****</td>
<td>5.84 ± 0.76 (n = 37) NS</td>
<td>3.94 ± 0.78 (n = 37) ****</td>
</tr>
<tr>
<td>3rd trimester</td>
<td>6.50 ± 1.06 (n = 55) ****</td>
<td>5.79 ± 0.95 (n = 55) NS</td>
<td>4.11 ± 0.62 (n = 55) ****</td>
</tr>
</tbody>
</table>

All results are expressed as mean ± SD; the results for each trimester and the non-pregnant results are compared.

Key:

NS  no significant difference

****  significant difference at p = 0.001
Table 6.2 Values for glycosylated haemoglobins, glycosylated plasma proteins and glucose, before, and during diabetic pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>% Glycosylated haemoglobins</th>
<th>% Glycosylated plasma proteins</th>
<th>Whole blood glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-pregnant diabetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.70 ± 2.88 (n = 138)</td>
<td>12.62 ± 3.36 (n = 153)</td>
<td>9.24 ± 4.83 (n = 143)</td>
</tr>
<tr>
<td></td>
<td>1st trimester</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.57 ± 2.43 (n = 28) ****</td>
<td>9.59 ± 2.91 (n = 26) ****</td>
<td>5.61 ± 2.51 (n = 20) ****</td>
</tr>
<tr>
<td></td>
<td>2nd trimester</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.58 ± 1.71 (n = 38) ****</td>
<td>8.65 ± 1.79 (n = 37) ****</td>
<td>6.14 ± 2.35 (n = 35) ****</td>
</tr>
<tr>
<td></td>
<td>3rd trimester</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.43 ± 2.25 (n = 46) ****</td>
<td>8.15 ± 2.09 (n = 43) ****</td>
<td>6.13 ± 2.28 (n = 35) ****</td>
</tr>
</tbody>
</table>

All results are expressed as mean ± SD; the results for each trimester and the non-pregnant results are compared.

Key:

**** significant difference at p = 0.001
Table 6.3 Values for glycosylated haemoglobins, glycosylated plasma proteins and glucose in maternal and cord blood of non-diabetic and diabetic mothers at delivery.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic Births</th>
<th>Diabetic Births</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>maternal</td>
<td>cord</td>
</tr>
<tr>
<td>GHb (%)</td>
<td>6.49±1.22</td>
<td>3.85±1.01</td>
</tr>
<tr>
<td>n=50</td>
<td>(3.33-9.13)</td>
<td>(1.94-7.18)</td>
</tr>
<tr>
<td>GPP (%)</td>
<td>5.61±0.93</td>
<td>4.75±0.62</td>
</tr>
<tr>
<td>n=50</td>
<td>(3.47-7.79)</td>
<td>(3.25-6.08)</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>4.53±0.99</td>
<td>3.59±0.82</td>
</tr>
<tr>
<td>n=50</td>
<td>(2.4-8.7)</td>
<td>(1.4-5.6)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD; observed range in parentheses.

Results for non-diabetic births and diabetic births are compared.

Key:

- GHb is glycosylated haemoglobins
- GPP is glycosylated plasma proteins
- NS no significant difference
- *** significant difference at p = 0.01
- **** significant difference at p = 0.001
For the diabetics studied, there was also a significant positive correlation between maternal and cord samples, for both glycosylated haemoglobins (n = 46, r = 0.641) and glycosylated plasma proteins (n = 37, r = 0.771) (Figs. 6.2A and B). These results include the 35 diabetic patients with full clinical details, and a further 13 births to diabetic patients who had only blood taken at, and not prior to, delivery. This was because either they were delivered very early in the study, or were treated elsewhere during pregnancy and only sent to the Royal Sussex County Hospital or Kings' College Hospital for delivery. Again for these deliveries to diabetic patients glycosylated haemoglobin values were significantly greater than those for glycosylated plasma proteins (p < .001) in maternal blood, but not significantly lower (p > .05) in cord blood (Table 6.3), as assessed by the paired t-test. Only 6 out of the 48 patients studied had lower glycosylated haemoglobins than glycosylated plasma proteins in maternal blood, and only 8 of the 48 had higher values in cord blood.


The mean birth weight (± SD) for infants of non-diabetic pregnancies was 3.317 kg ± 0.518 kg, whereas that of the diabetic pregnancies was 3.522 kg ± 0.713 kg. In the non-diabetic group there was 23 male and 27 female offspring, and in the diabetic group there were 19 male and 16 female infants
Fig. 6.2 Relationship between % glycosylated haemoglobins (A) and % glycosylated plasma proteins (B) in maternal and cord blood of diabetic patients.

Both lines were drawn by computer.
Fig. 6.3 Interpretation of birth weights as percentile values for first pregnancies (A) and second and subsequent pregnancies (B), allowing for gestation.

Key: —————— males
     —————— females
delivered. Only the 35 diabetic patients who were studied both during pregnancy and at delivery were considered. The observed range of infant birthweight for the non-diabetic deliveries was 2.280 kg to 4.360 kg (this baby arrived 2 weeks late), whereas for the deliveries to the diabetic mothers the range was 2.211 kg to 5.180 kg, and none of these mothers exceeded their date of delivery.

The mean placental weights (± SD) for the non-diabetic pregnancies was 661.33g ± 146.94g, and for the diabetic pregnancies was 624.17g ± 184.60g. The placental weight was not recorded at 5 non-diabetic births and at 11 diabetic births.

An infant was considered to be macrosomic when their birth weight exceeded the 90th centile. (Brans et al. 1982). This was determined using the figures from Thompson, Billewicz, and Nyten (1968), who assessed the distribution of fetal weight in terms of centiles, where 90% of normal infants have a birth weight below the 90th centile. Their figures also allow for the effect of sex of the infant and parity (Figs. 6.3A and B) on the birth weight. The infants of each of the 35 diabetic patients considered in this study had their birth weights interpreted as a centile value using these graphs and 15 infants were defined as macrosomic (5 males, 10 females). These values were then plotted against the glycosylated haemoglobin or glycosylated plasma protein result in the third trimester or in the maternal and cord samples, providing 6 centile graphs (Figs. 6.4, 6.5, 6.6, 6.7, 6.8, 6.9). In this
Fig. 6.4
Relationship between the birth weights of infants born to diabetic mothers (expressed as percentile values), and the corresponding maternal glycosylated haemoglobins during the third trimester.

Key:
- ▲ male infants
- ▼ female infants

The x-axis represents % glycosylated haemoglobins
Fig. 6.5

Relationship between the birth weights of infants born to diabetic mothers (expressed as percentile values), and the corresponding maternal glycosylated plasma proteins during the third trimester.

Key:
- △ male infants
- ● female infants

X-axis represents % glycosylated plasma protein

Y-axis: Birth Weight Centiles

X-axis: Normal Range (±2SD)
way the association between macrosomia and these values could be assessed.

Fig. 6.4 shows the relationship between the birth weight, expressed as a percentile value, and the corresponding maternal glycosylated haemoglobin value in the third trimester. Of the 35 patients, 15 had infants which had birth weight in excess of the ninetieth centile as defined by Thompson, Billewicz and Hytten (1968); of these 14 had values for glycosylated haemoglobins in the third trimester above the normal range, as defined by the previous studies of non-diabetic pregnancies. Of the remaining 20 diabetics studied, 11 had values within this normal range, 8 above and one patient had no sample taken at this time. Of these 8 patients, 2 (patients D and B) had abnormally small babies with birth weights below the tenth percentile value. This was suggestive of a secondary pathology superceding that of macrosomia yet still possibly linked to diabetes mellitus and reflected in the high glycosylated haemoglobin values. Indeed patient B has very high results throughout the whole period studied, and this child had to be delivered by an emergency Caesarian section. Of the remaining 6 results which were falsely positive both patients T and V have birth weights very close to the ninetieth percentile, and Patient K had a baby whose weight was only just above the eightieth percentile line, yet this infant clearly had the features of a macrosomic child. It was excessively fat with a short neck and a lot of head hair. The parents of this child were both short, under 5 ft tall, possibly
Fig. 6.6
Relationship between the
birth weights of infants
born to diabetic mothers
(expressed as percentile
values), and the
corresponding maternal
glycosylated haemoglobins
at delivery.

Key:
△ male infant
● female infant
x axis represents
% glycosylated haemoglobins
Fig. 6.7 Relationship between the birth weights of infants born to diabetic mothers (expressed as percentile values), and the corresponding maternal glycosylated plasma proteins at delivery.

Key:
- △ male infant
- ● female infant

The x-axis represents % glycosylated plasma proteins.
explaining this result. Fig. 6.5 shows the relationship between birth weight, expressed as a percentile value, and the corresponding maternal glycosylated plasma protein value in the third trimester. Of the 15 macrosomic babies 11 had glycosylated plasma protein results above the normal range, one in the normal range and 3 patients did not have results for this period. Of the remaining 20 non-macrosomic neonates 10 had values within the normal range, 1 below and 9 above. Again patients B, D and K were included in this group of false positives. Patients V and S had birth weights only just below the ninetieth percentile line.

Fig. 6.6 shows the relationship between birth weight, expressed as a percentile value, and the corresponding maternal glycosylated haemoglobin result at delivery. Of the 15 macrosomic infants, 13 had glycosylated haemoglobin results above the normal range. Patients Y and R have macrosomic infants yet have results within the normal range. Of the 20 infants which were not macrosomic, 14 were within the normal range and 6 had glycosylated haemoglobin values above the normal range; these include patients B and K. Again patients S and V had birth weights very close to the ninetieth centile. Similarly, Fig. 6.7 shows the relationship between birth weight, expressed as a percentile value, and the corresponding maternal glycosylated plasma protein result at delivery. This time the discrimination between the non-macrosomic and macrosomic groups is not as clear-cut with 6 of the macrosomic infants having
Fig. 6.8

Relationship between the birth weights of infants born to diabetic mothers (expressed as percentile values), and the corresponding glycosylated haemoglobins in cord blood.

Key:
- ▲ male infant
- ● female infant

The x-axis represents % glycosylated haemoglobins.
values within the normal range (patients Y, A, L, N, G and Z) and 6 of the non-macrosomic patients having values above this range (patients T, S, V, K, W and B). In this latter group again patients S, V, B and K are included.

Fig. 6.8 shows the relationship between birth weight, expressed as a percentile value, and the corresponding glycosylated haemoglobin result in cord blood. In this instance discrimination between the macrosomic and non-macrosomic individuals in terms of percentage glycosylation is very poor. Only 6 of the macrosomic infants have values which are above the normal range and 9 have values within it (patients Y, A, L, E, R, P, J, N and Z). One patient (N) has a result right at the top of the normal range. Similarly 3 of the non-macrosomic group have high values (T, K and B), although again these include patients K and B. One result was not available. When birth weight is similarly assessed in terms of the glycosylated plasma protein result from cord blood (Fig. 6.9), again there is very poor discrimination. Of the macrosomic group, 2 have results above the normal range, 4 have no result due to obstetric problems or haemolysis of the sample, and the remainder have values within the normal range. Six of the non-macrosomic group have values which exceed the normal range, although these again include patients B, D and K. Patients V and T have birthweights very close to the ninetieth centile line. There was no result for one patient in this latter group.
Fig. 6.9

Relationship between the birth weights of infants born to diabetic mothers (expressed as percentile values), and the corresponding glycosylated plasma proteins in cord blood.

Key:

- ▲ male infant
- • female infant

x axis represents % glycosylated plasma proteins
The actual birth weight of each infant was divided by
the appropriate 50th centile value for the corresponding
week of gestation, as calculated from the intra-uterine
growth graphs (Figs. 6.3A and B) to provide a birth weight
ratio. These birth weight ratios were plotted against the
glycosylated haemoglobin or glycosylated plasma protein
values for the third trimester, or in the maternal and cord
samples. Fig. 6.10 shows as an example, the graph of
glycosylated haemoglobin in the third trimester, to illustrate
the association between this and birth weight ratio. The
remaining results are not plotted individually, but summarised
in Table 6.4. There was a positive correlation between these
birth weight ratios and the appropriate maternal and third
trimester values of glycosylated haemoglobins and glycosylated
plasma proteins (Table 6.4). There was a poor positive
correlation between glycosylated haemoglobin and birth weight
ratio in cord blood, and a poor, negative correlation between
glycosylated plasma proteins and birth weight ratio in cord
blood (Table 6.4).
Relationship between birth weight ratios of infants born to diabetic mothers, and the corresponding glycosylated haemoglobins in the third trimester.

Note: This graph was plotted by computer.

\[ n = 34 \]
\[ r = 0.46 \]
\[ y = 0.044x + 0.71 \]
Table 6.4  Correlation between glycosylated haemoglobins or glycosylated plasma proteins in the third trimester of pregnancy and at birth, with birth weight ratio.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>correlation(r)</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third trimester</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% glycosylated haemoglobins</td>
<td>34</td>
<td>.463</td>
<td>.0435</td>
</tr>
<tr>
<td>% glycosylated plasma proteins</td>
<td>31</td>
<td>.341</td>
<td>.0334</td>
</tr>
<tr>
<td>Birth, maternal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% glycosylated haemoglobins</td>
<td>35</td>
<td>.348</td>
<td>.030</td>
</tr>
<tr>
<td>% glycosylated plasma proteins</td>
<td>35</td>
<td>.194</td>
<td>.029</td>
</tr>
<tr>
<td>Cord</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% glycosylated haemoglobins</td>
<td>33</td>
<td>.086</td>
<td>.013</td>
</tr>
<tr>
<td>% glycosylated plasma proteins</td>
<td>30</td>
<td>-.019</td>
<td>-.032</td>
</tr>
</tbody>
</table>
Of the 35 patients who were studied in this survey, 15 produced macrosomic babies. The glycosylated haemoglobin and glycosylated plasma proteins of this group were compared to the group of 20 diabetic mothers whose offsprings were not macrosomic. (Table 6.5). Although the mothers of macrosomic babies had higher results in all categories except for glycosylated plasma proteins in cord blood, only glycosylated haemoglobins in maternal blood in the third trimester or in cord blood proved to be significantly higher.

When patients D, B and K were included in the "macrosomic" group (the first two as they had abnormally small babies, and patient K as the baby born was classically macrosomic in features, although not above the ninetieth centile) then the differences between the two groups becomes clear (Table 6.6). For the group containing babies of abnormal birth weight, all of the results were significantly higher than in the group of non-macrosomic offspring, with the exception of the glycosylated plasma proteins in cord blood.

**Combined Measurement of Glycosylated Haemoglobins and Glycosylated Plasma Proteins for the Detection of Macrosomia.**

Table 6.7 shows both the false positive and the false negative predictions in the third trimester of macrosomia, and the retrospective assessment in maternal and cord samples using glycosylated haemoglobin and glycosylated plasma protein measurements. These results, when combined, show that the number of incorrect predictions or assessments simultaneously indicated by both results decreases. In the third trimester
false positives using the combined measurements still includes patients B, D and K, all of whom could arguably be included with the macrosomic group.

**Relationship between haemoglobin concentration and % glycosylated haemoglobins in non-diabetic pregnancy.**

Of the 110 non-diabetic women who were studied at one time-point during their pregnancy, 93 had their haemoglobin concentration measured by routine haematological tests. For these patients the mean haemoglobin value was 11.69 g/dl with an observed range of 8.6 to 14.1 g/dl. There was no significant correlation ($r = 0.053$, slope $= 0.042$) between these values and their corresponding glycosylated haemoglobin results.
### Table 6.5

Values for glycosylated haemoglobins and glycosylated plasma proteins during the third trimester and at delivery (maternal and cord) for pregnancies resulting in non-macroscopic and macroscopic (weight above 90th centile) babies.

<table>
<thead>
<tr>
<th></th>
<th>Non-macroscopic</th>
<th>Macrosomic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THIRD TRIMESTER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>8.82 ± 2.04 (n=20)</td>
<td>11.20 ± 2.25 (n=14) **</td>
</tr>
<tr>
<td>% GPP</td>
<td>7.41 ± 1.81 (n=20)</td>
<td>9.34 ± 2.31 (n=10) NS</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>8.75 ± 2.17 (n=20)</td>
<td>10.90 ± 2.61 (n=15) NS</td>
</tr>
<tr>
<td>% GPP</td>
<td>6.98 ± 7.73 (n=20)</td>
<td>7.73 ± 1.40 (n=15) NS</td>
</tr>
<tr>
<td><strong>CORD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>4.56 ± 1.32 (n=19)</td>
<td>5.52 ± 1.69 (n=14) ***</td>
</tr>
<tr>
<td>% GPP</td>
<td>5.46 ± 1.40 (n=19)</td>
<td>5.21 ± 0.79 (n=11) *</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD; the non-macroscopic and macroscopic groups are compared.

**Key:**

- **NS** no significant difference
- * significant difference at p = 0.05
- ** significant difference at p = 0.02
- *** significant difference at p = 0.01

%GHb is % glycosylated haemoglobins

%GPP is % glycosylated plasma proteins
Table 6.6  Values for glycosylated haemoglobins and glycosylated plasma proteins during the third trimester and at delivery (maternal and cord) for pregnancies resulting in babies of abnormal size.

<table>
<thead>
<tr>
<th></th>
<th>Non-macrosonic</th>
<th>Macrosomic (+ Patients D, B, &amp; K)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THIRD TRIMESTER</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>+ 8.40 - 1.78 (n=17)</td>
<td>11.20 ± 2.15 (n=17) ***</td>
</tr>
<tr>
<td>% GPP</td>
<td>+ 7.14 - 1.82 (n=17)</td>
<td>9.61 - 2.15 (n=14) ***</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>+ 8.36 - 1.91 (n=18)</td>
<td>10.91 + 2.54 (n=18) ***</td>
</tr>
<tr>
<td>% GPP</td>
<td>+ 6.73 - 1.49 (n=17)</td>
<td>7.84 ± 1.33 (n=18) *</td>
</tr>
<tr>
<td><strong>CORD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% GHb</td>
<td>+ 4.28 - 1.14 (n=16)</td>
<td>5.42 ± 1.52 (n=17) **</td>
</tr>
<tr>
<td>% GPP</td>
<td>+ 5.12 - 1.25 (n=16)</td>
<td>5.63 ± 1.09 (n=15) NS</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, the non-macrosonic and "macrosonic" groups are compared.

**Key:**

- **NS** no significant difference
- **★** significant difference at $p = 0.05$
- **★★** significant difference at $p = 0.02$
- **★★★** significant difference at $p = 0.01$
- **★★★★** significant difference at $p = 0.001$

%GHb is % glycosylated haemoglobins
%GPP is % glycosylated plasma proteins
Table 6.7  Combined measurement of glycosylated haemoglobins and glycosylated plasma proteins for the detection of macrosomia.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>FALSE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third trimester</td>
<td>8</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>POSITIVES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>cord</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>FALSE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third trimester</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NEGATIVES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>cord</td>
<td>9</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Column A shows results using only % glycosylated haemoglobins
Column B shows results using only % glycosylated plasma proteins
Column C shows results using the combined measurements of % glycosylated haemoglobins and % glycosylated plasma proteins.
Differences in Glycosylation Values for Gestational Diabetics and Diabetics Insulin-Dependent Before Pregnancy.

Of the 35 diabetic patients studied, 6 were gestational diabetics and 29 were insulin-dependent before, and during pregnancy. The glycosylated haemoglobin and glycosylated plasma protein results for each of these two groups was assessed and compared (Table 6.8). In all cases the values for the gestationally diabetic group were lower, but only glycosylated haemoglobins and glycosylated plasma proteins in cord blood, and glycosylated haemoglobins in maternal blood were significantly lower (p < 0.02).

Comparison of Gestational Diabetics with Non-diabetics During Pregnancy and at Delivery.

Figure 6.11 shows the results for the 6 gestational diabetics for glycosylated haemoglobins, glycosylated plasma proteins and glucose, during pregnancy and at delivery, compared against the appropriate normal ranges (Table 6.9). Three of the patients had values within or below the normal ranges throughout pregnancy and at term. One patient (c), only had a high glycosylated haemoglobin result in the second trimester. Patient d had high glycosylated haemoglobin at delivery, and produced a macrosomic baby. Patient b had high results for all three measurements during pregnancy and in maternal blood at delivery, and produced a child whose weight was only just below the ninetieth centile. Two patients (d and e) were diagnosed during the second trimester.
Fig. 6.11 Progress of the glycosylated haemoglobin, glycosylated plasma protein and glucose results for 6 gestational diabetics (a-f) through pregnancy and at delivery. Each result is compared to the appropriate normal range.

Key: T represents trimester, M represents maternal results, C represents results for cord blood.
Table 6.8  Values of glycosylated haemoglobins and glycosylated plasma proteins in maternal and cord blood of insulin-dependent diabetic mothers (IDDM) and gestational diabetics.

<table>
<thead>
<tr>
<th></th>
<th>Insulin-dependent diabetics.</th>
<th>Gestational diabetics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>% GHb maternal</td>
<td>10.06 ± 2.57 (n=29)</td>
<td>7.82 ± 1.75 (n=6) **</td>
</tr>
<tr>
<td>% GHb cord</td>
<td>5.00 ± 1.48 (n=27)</td>
<td>4.26 ± 1.23 (n=6) NS</td>
</tr>
<tr>
<td>% GPP maternal</td>
<td>7.64 ± 1.22 (n=27)</td>
<td>5.66 ± 1.77 (n=6) **</td>
</tr>
<tr>
<td>% GPP cord</td>
<td>5.57 ± 1.12 (n=25)</td>
<td>4.30 ± 1.04 (n=6) **</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD; the results for insulin-dependent mothers and gestationally diabetic mothers are compared.

Key:

- **NS**  no significant difference
- **NS**  significant difference at p = 0.02

%GHb  is % glycosylated haemoglobins
%GPP  is % glycosylated plasma proteins
Table 6.9  Normal ranges for glycosylated haemoglobins, glycosylated plasma proteins and whole blood glucose during the different trimesters of pregnancy and at delivery.

<table>
<thead>
<tr>
<th></th>
<th>Glycosylated Haemoglobins (%)</th>
<th>Glycosylated Plasma Proteins (%)</th>
<th>Whole blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester</td>
<td>4.67 to 9.35</td>
<td>4.59 to 7.27</td>
<td>2.62 to 4.34</td>
</tr>
<tr>
<td>Second trimester</td>
<td>3.97 to 8.53</td>
<td>4.32 to 7.36</td>
<td>2.38 to 5.50</td>
</tr>
<tr>
<td>Third trimester</td>
<td>4.38 to 8.62</td>
<td>3.89 to 7.69</td>
<td>2.87 to 5.35</td>
</tr>
<tr>
<td>Maternal</td>
<td>4.05 to 8.93</td>
<td>3.75 to 7.47</td>
<td>2.55 to 6.51</td>
</tr>
<tr>
<td>Cord</td>
<td>1.83 to 5.87</td>
<td>3.51 to 5.99</td>
<td>1.95 to 5.23</td>
</tr>
</tbody>
</table>

Ranges are calculated from the mean values - 2SD of the different trimesters (Table 6.1) and at birth (Table 6.3) for non-diabetic pregnancies.
Abnormalities of Babies Born to Diabetic Mothers in This Study.

Of the 35 diabetic pregnancies in this study, 2 of the insulin-dependent diabetic patients produced abnormal babies (patients B and G). In both instances the nature of the abnormality was only specified as "heart defect". One of the other 13 diabetics studied only at delivery (patient DW) also produced an abnormal offspring, this time with spina bifida. The results for all these patients at delivery, and for patients B and G early in pregnancy are given in Table 6.10, together with some details of the births. The normal ranges against which these results are compared are given in Table 6.9 and are a summary of the results in Tables 6.1, and 6.3. All three patients have maternal glycosylated haemoglobin values which exceed the normal range, although only patients B and DW have high glycosylated haemoglobins in cord blood. Only patient B had glycosylated plasma proteins in maternal and cord blood above the normal range. Interestingly both patients B and G had high values for glycosylated haemoglobins and glycosylated plasma proteins in the first trimester. None of the patients had glucose results at delivery, but both patients B and G had high glucose values in the first trimester with average values of 7.7 and 8.9 mmol/l, respectively.
Values Obtained for Diabetic Patients With Intra-Uterine Deaths.

Four diabetics during the period of this study had intra-uterine deaths, and had their glycosylated haemoglobins and glycosylated plasma proteins measured. These patients were not included in the group of 35 diabetics which are considered throughout this chapter. The results of these four patients were 7.18% and 5.36%, 8.93% and 8.63%, 11.73% and 15.79%, and 9.10% and 10.18% for glycosylated haemoglobins and glycosylated plasma proteins, respectively. The last two patients had glycosylated haemoglobin values above the normal range, and the last three patients had high glycosylated plasma proteins. In the last two patients the glycosylated plasma proteins were higher than the glycosylated haemoglobins.

All of the deaths occurred between 10 and 16 weeks of gestation, and a sample was taken within 24 hours of the patient being admitted to hospital after miscarriage.
Table 6.10 Glycosylated haemoglobin and glycosylated plasma protein results at delivery and in the first trimester of pregnancy for those diabetic patients who delivered babies with abnormalities.

<table>
<thead>
<tr>
<th>%GHb</th>
<th>%GPP</th>
<th>Weight and sex of infant</th>
<th>Mode of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient B.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal</td>
<td>10.99</td>
<td>9.85</td>
<td></td>
</tr>
<tr>
<td>cord</td>
<td>6.95</td>
<td>7.30</td>
<td>2.170kg Male</td>
</tr>
<tr>
<td>first trimester</td>
<td>13.53</td>
<td>12.38</td>
<td></td>
</tr>
</tbody>
</table>

Patient G.

<table>
<thead>
<tr>
<th>%GHb</th>
<th>%GPP</th>
<th>Weight and sex of infant</th>
<th>Mode of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal</td>
<td>16.65</td>
<td>6.83</td>
<td></td>
</tr>
<tr>
<td>cord</td>
<td>4.87</td>
<td>3.54</td>
<td>4.400kg Female</td>
</tr>
<tr>
<td>first trimester</td>
<td>11.82</td>
<td>9.59</td>
<td></td>
</tr>
</tbody>
</table>

Patient DW.

<table>
<thead>
<tr>
<th>%GHb</th>
<th>%GPP</th>
<th>Weight and sex of infant</th>
<th>Mode of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal</td>
<td>12.07</td>
<td>6.93</td>
<td></td>
</tr>
<tr>
<td>cord</td>
<td>9.21</td>
<td>5.78</td>
<td>4.030kg Male</td>
</tr>
</tbody>
</table>

Results for the third trimester are the mean values for each patient during that period. Since patient DW was only studied at delivery, there were no results for this patient earlier in pregnancy.
DISCUSSION.

In order to assess any relationship between glycosylation and the outcome of diabetic pregnancy it is first necessary to study non-diabetic pregnancy. Previous studies have shown that glycosylated haemoglobin is either unchanged (Ylinen, Helaki and Teramo, 1981, Leslie et al. 1978, Kjaegaard and Ditzel, 1979) or slightly decreased (Lind and Cheyne, 1979) during normal pregnancy. This study is in agreement with these findings, since there is a significant decrease in maternal glycosylated haemoglobin during non-diabetic pregnancy (\( p < 0.001 \)) (Table 6.1). This decrease may be caused by the increase in red cell volume and decrease in fasting blood glucose concentration that occurs during normal pregnancy (Lind and Cheyne, 1979). Measurement of glycosylated plasma proteins during pregnancy and at term has not been reported previously. In this study the values, not surprisingly, follow a similar pattern to the glycosylated haemoglobins, but with a smaller though significant decrease (\( p < 0.02 \)) between the normal value and that for pregnant women at delivery. This is consistent with the decrease in plasma glucose concentration observed in this study when values during pregnancy are compared to those of non-pregnant patients. Glucose levels were shown to increase as pregnancy progressed, but in many cases the samples were taken from patients who were not fasted, and this may obscure the results. The concentration of plasma proteins also decreases during pregnancy (Haram, Augensen and Elsayed, 1983) but the decrease is very small, and should not affect the glycosylated plasma protein results.
It is also interesting that there is an inverse ratio between the percentage of glycosylated plasma proteins and glycosylated haemoglobins in maternal and cord blood. In cord blood the percentage of glycosylated plasma proteins is greater than glycosylated haemoglobins, and in maternal blood the opposite is true. This is also the case with the diabetic pregnancies studied. In maternal blood the concentration of plasma proteins decreases during the last stages of pregnancy due to increased body fluids (Lind, 1980). This combined with the decrease in glucose and the fact that glycosylated plasma proteins reflect a shorter time period than glycosylated haemoglobins, probably explains the observation. In cord blood however, there is an increased concentration of haemoglobin compared with mother's blood which probably alters the balance so that the concentration of glycosylated plasma proteins is greater than that of glycosylated haemoglobins in cord blood.

Only a few previous studies have attempted to measure glycosylated haemoglobins in both maternal and cord blood. One group using ion-exchange chromatography (Fitzgerald and Cauchi, 1980) obtained a value for HbA₁ in maternal blood of $6.4 \pm 0.7\%$ and in cord blood of $12.4 \pm 3.6\%$. However, two different chromatography procedures were used to determine these values. Using radioimmunoassay it was found that the maternal HbA₁c values were $3.96 \pm 0.7\%$ compared with $4.56 \pm 1.8\%$ in cord blood expressed as a percentage of haemoglobin A (Peterson et al. 1979). Another study, (Poon, Turner and Gillmer, 1981) this time using isoelectric focusing, quotes maternal HbA₁c as $7.8 \pm 1.9\%$ and glycosylated haemoglobin F which probably includes acetylated haemoglobin F, as $7.8 \pm 1.5\%$. 
Contrary to the findings outlined above, Sosenko et al. (1982), using a colorimetric method, found a significantly lower value (by 36%) for glycosylated haemoglobin in cord blood compared with maternal blood. The significant decrease reported here from 6.49 ± 1.2% for maternal blood in non-diabetic pregnancies to 3.85 ± 1.0% for cord blood (Table 6.3), is in general agreement with their findings. A similar trend is shown with the diabetic pregnancies, with a significant decrease from 9.67 ± 2.57% in maternal blood to 5.03 ± 1.64% in cord blood (Table 6.3). These decreases are consistent with the lower blood glucose in cord blood. The results for the non-diabetic births show a consistent trend with lower glucose, glycosylated haemoglobins and glycosylated plasma proteins in the fetus when compared with the corresponding maternal blood. It has been shown in studies in vitro that fetal haemoglobin becomes glycosylated at the same rate and to the same extent as adult haemoglobin (Fitzgerald and Cauchi, 1980). It seems likely therefore that these lower values in the fetal blood reflect the rapid utilisation of glucose by the fetus (Holmberg et al. 1956). Indeed, although there is a significant correlation (r = 0.641) between the individual values of maternal and cord blood for the diabetics studied, the slope of the graph comparing maternal and cord values (Fig. 6.2A) is 0.418, suggesting that the glucose levels circulating on the fetal side of the placenta are only about half those on the maternal side. This is probably due at
at least in part, to the rapid utilisation of glucose by the fetus, as well as glucose uptake by the placenta itself. This is also reflected with the glycosylated plasma proteins. Again there is a good correlation between maternal and cord blood (r = 0.771), but this time the slope of the graph (Fig. 6.25) is slightly higher (0.736). This may reflect the increased glucose utilisation, but presumably other factors such as altered protein concentration or turnover rates in either fetal or maternal plasma influence the result causing the slope to be closer to unity.

Of the diabetic pregnancies studied, a group of 35 were considered for most of the work as results were obtained for these patients in pregnancy and at delivery, and their full clinical details were available. Of these, 6 were gestational diabetics and, as a group, most of their results for both glycosylated haemoglobin or glycosylated plasma proteins in the third trimester or at birth were lower (p < 0.02) than for the group comprising of insulin-dependent diabetic patients (Table 6.8). The results for the gestational diabetics were also compared against those ranges obtained for non-diabetic pregnancies (Fig. 6.11). Three of these gestational diabetics did not have a single high result throughout pregnancy. One patient had a single high glycosylated haemoglobin value in the third trimester, and two of the others had many high results during their pregnancy, all of which may have allowed the detection of their gestational diabetes if this had not previously been suspected. Of these two patients with high
results, one produced a macrosomic infant and the other, a child whose birth weight was only just below the ninetieth centile. These results suggest that diabetes is more manageable in gestational diabetics providing it is recognised, as was the case obviously with all of these patients. However, since only 6 patients were studied, a larger survey would be required before any such observations could be confirmed. Most of the gestationally diabetic patients were maintained on insulin throughout pregnancy, but one was controlled with diet restriction alone.

Glycosylated haemoglobins and glycosylated plasma proteins, as measured by affinity chromatography, can both be used in the third trimester to predict macrosomia. As shown in Table 6.4 glycosylated haemoglobin \( r = 0.463 \) and glycosylated plasma proteins \( r = 0.341 \) both correlated with birth weight ratio. Glycosylated haemoglobin measured in the third trimester predicted correctly 11 out of the non-macrosomic and 14 out of the 15 macrosomic births (Fig. 6.4). This represents a sensitivity of 25 correct results out of 34 (73.5%). Indeed at least 3 of the patients in the non-macrosomic group could be considered to be correctly predicted as abnormal (D, B and K) as two had very low birth weights (D and B) and one (K) had a baby with classical macrosomic features. These patients could arguably be considered with the macrosomic group, as glycosylated haemoglobins and glycosylated plasma proteins predicted that these patients too were 'at risk'. This would
give an improved sensitivity (85.3%). Patient K had a very
difficult birth for her baby, although not statistically
macrosomic was very large for the mother, a very small woman,
to deliver. Two other patients (T and V) were false positives
with birth weights just below the ninetieth centile line. All
of the diabetic patients had ultrasound scans for dating
purposes early in their pregnancy, usually at 12 - 15 weeks
gestation. Ultrasound can date the age of the fetus plus or
minus about one week (Dr. Cawdell, personal communication).
This being the case, patient V would also be included in the
macrosomic group if her dating was a week adrift, increasing
the sensitivity of the test to 85.3%.

Glycosylated plasma proteins can also be used in the
third trimester to predict the outcome, showing only one false
negative and nine false positive results (Fig. 6.5). Again
patients V, K, B and D are included in this latter group.
The sensitivity in this instance is 22 correct predictions
out of a total of 32 (68.8%). When patient S is re-evaluated
assuming a possible misdating by ultrasound of one week, this
result too can be included in the macrosomic group. When
patients V, K, B, D and S are also considered to be correctly
predicted to be 'at risk' then the sensitivity increases to
84.4%.

The retrospective analysis of maternal and cord blood
shows a slightly different picture. Maternal glycosylated
haemoglobin valued are in agreement with 14 of the 20 non-
macrosomic births and 13 of the 15 macrosomic births (Fig.
6.6). In this case the sensitivity is still good (77.1%).
although there is a poorer correlation with maternal
glycosylated haemoglobin and macrosomia (Table 6.4, $r = 0.348$)
in terms of birth weight ratio. Maternal glycosylated plasma
proteins at delivery only agreed with 8 of the macrosomic
births and again there was 6 false negative values (Fig. 6.7).
Patients W and T remain unexplained as false positives. The
clinical details on patient W indicated that several scans
showed an abnormally large baby, yet the child was not
macrosomic. There was a suggestion that the gestational dating
may have been muddled. The infant had several severe bouts of
hypoglycaemia immediately after birth.

The relationship between glycosylated haemoglobin in
cord blood was even less selective for macrosomia, agreeing
with only 6 of the macrosomic (LGA) births (Fig. 6.8).
Glycosylated plasma proteins in cord blood for the macrosomic
infants only had two values outside the normal range. This
is a reasonable finding in light of the fetal hyperglycaemia-
hyperinsulinaemia-macrosomia theory. If the macrosomic infants
are producing large amounts of insulin in response to high
maternal glucose levels, their glucose turnover and
utilisation will be very rapid and glycosylation may not
occur. This is also reflected in the slopes (0.418, 0.736)
of the graphs (Figs. 6.2A and B) correlating the results from
maternal and cord samples from the 35 patients in this study,
which suggests a more rapid turnover of glucose in fetal blood.
Results for maternal samples at birth show better correlation
with LGA babies (Table 6.4, $r = 0.348$), but these are less
satisfactory than the maternal results in the third trimester.
(r = 0.463). This is presumably as the maximum growth spurt of the fetus occurs at the end of the second trimester (Ogata et al. 1979) so, allowing for the retrospective nature of glycosylated haemoglobins this would be best reflected in the results of the third trimester. This growth may cause macrosomia due to hyperglycaemia in the second trimester, and yet glycaemic control may revert to normal near term when these patients are more closely supervised, consequently producing normal glycosylation in the birth samples.

The prediction of macrosomia, or indeed abnormally light weight is important as it allows for the better treatment of the associated complications (Pettit et al. 1983) both at delivery and during the neonatal period. This work shows that the measurement of either glycosylated haemoglobins or glycosylated plasma proteins in the third trimester, can be used to predict these conditions. However, the third trimester is 13 weeks long and future work to ascertain the time within this trimester when these measurements are most useful clinically, is recommended. The role of glycosylated plasma proteins in this study does not appear to confer any additional advantage for this purpose, possibly due to the method being less sensitive than that for glycosylated haemoglobins, with a between-batch variation of about 5% (Chapter 2). However, the combined assessment of glycosylated haemoglobins and glycosylated plasma proteins as an indicator of possible impending obstetric problems does prove to be better than their individual consideration (Table 6.7).
It is interesting and important that with two of the four cases of intra-uterine death, the glycosylated plasma proteins were considerably higher than the glycosylated haemoglobins, and in one other case the glycosylated plasma protein result was abnormal, yet the glycosylated haemoglobin result was within the normal range. Glycosylated haemoglobin measurement is possibly inadequate in these cases to assess the rapidly changing glycaemic conditions which may be associated with intra-uterine death. Recently, Ashby, Deacon and Rinsler (1984) recommended that glycosylated plasma proteins should be investigated in relation to diabetic pregnancy, as they have a faster turnover time than glycosylated haemoglobins. This work suggests that although glycosylated plasma proteins do not appear to show any additional information regarding macrosomia, they may be of use to detect fetal distress due to sudden changes in diabetic control. However, since only four such patients were considered in this study it is not possible to draw any major conclusions, as the numbers are too small. Also it must be noted that intra-uterine death may occur as a result of sudden transient maternal and then fetal hypoglycaemia. This would not affect the glycosylated plasma protein result.

In this study, 3 insulin-dependent diabetic patients were included who produced abnormal babies. All of these patients had maternal glycosylated haemoglobins above the normal range; two had macrosomic babies and one had a baby whose birth weight was below the 5th centile. The data available was limited but for 2 of these patients studied in the first trimester glycosylated haemoglobins, glycosylated plasma proteins and glucose at this time were all shown to be well above the
appropriate normal range. These few observations are in agreement with those of other workers (Miller et al. 1981, Leslie et al. 1979) who were mentioned earlier in this chapter for showing the association between poor glycaemic control early in pregnancy and abnormalities in the fetus.

The maintenance of normal glucose profiles in pregnancy for IDDM individuals has been shown to be possible with good education and regular self-monitoring (Jovanovic et al. 1980). However, in this study of 35 patients who were all carefully supervised throughout pregnancy, 15 produced macrosomic babies, and a further 2 had babies who were 'at risk'. Several of these babies had hypoglycaemia at birth and were put into special care units. Glycosylated haemoglobins and glycosylated plasma protein measurement may help to predict these problems. In this work these results were available to the clinicians at the Royal Sussex County Hospital, but not to those at Kings' College Hospital. Interestingly, 12 of the 15 patients producing macrosomic offspring were seen at Kings' College Hospital, as was one patient producing an abnormally small baby. Of these patients, 3 had emergency Caesarian sections. These findings may, or may not be related to the absence of this biochemical information. All of the 3 births of abnormal babies were also seen at Kings' College Hospital. Of these, 2 of the mothers had high glycosylated haemoglobins, glycosylated plasma proteins and glucose early in pregnancy. However, it is important to note that of the other diabetic mothers studied, 11 had high glycosylated haemoglobins, 13 had high glycosylated plasma proteins and 9 had high glucoses in their first trimester.
Affinity chromatography provides a very sensitive technique for the measurement of both glycosylated haemoglobins and glycosylated plasma proteins on the same sample, by the same principle, and both expressed in the same units (percentages). Fetal haemoglobin (HbF) has been shown not to interfere with the results (Hall et al. 1983, Chapter 3). Unlike ion-exchange chromatography, elaborate combined techniques do not have to be employed to make the method feasible. Affinity chromatography makes the routine measurement of both glycosylated haemoglobins and glycosylated plasma proteins available to the clinician managing a diabetic pregnancy.

The ranges at birth for non-diabetics measured by affinity chromatography have been published (Hall et al. 1983). Talwar et al. (1983) reported the use of affinity chromatography for the measurement of glycosylated haemoglobins in cord blood. Their values were 5.2 ± 0.3% (range 4.8 to 5.8%), which is significantly higher than our reported range (3.85 ± 1.01%). They also used the colorimetric technique to show that the extent of glycosylation in cord blood is lower than in maternal blood. Since this work, Feldman et al. (1984) have published details relating glycosylated haemoglobins in cord blood as measured by affinity chromatography, to macrosomia. They used 1ml columns of Glycogel B for this purpose. They too, noted that glycosylated haemoglobin in cord blood was only half the percentage found in adult red cells, and they used in vitro
incubation studies to show that HbA and HbF were glycosylated at the same rate. They found a significant correlation between the birth weight ratio and glycosylated haemoglobins in cord blood ($r = 0.66$) with 11 of the 15 diabetic mothers studied producing LGA babies. This is not in agreement with the findings in this thesis which showed this correlation to be very poor ($r = 0.086$), and the results of the Worth et al. (1983) who failed to correlate birth weight ratio with cord or maternal glycosylated haemoglobins using the thiobarbituric acid method. This discrepancy may be due to the relatively low numbers of diabetic mothers studied by Feldman et al. (1984).

It is apparent in diabetic pregnancy that nurture, in terms of metabolic fuels, affects nature in terms of intrinsic genetic expression; and this change can be reflected in the glycosylated values obtained. There is evidence that lesions at this stage in development, due to improper fuels supplied, can persist into later life. A study of Pima Indians by Pettit et al. (1981) revealed that macrosomia at birth predisposed obesity in later life. Frienkel (1981) wrote "the consequent implications for fuel-mediated teratogenesis may have ramifications for developmental biology that extend far beyond the traditional and more parochial considerations of diabetes in pregnancy".
CHAPTER 7.

GENERAL DISCUSSION.
Binding of Glycosylated Proteins to the Affinity Gel, Glycogel B.

Originally Mallia et al. (1981) produced a cross-linked agarose gel activated with carbonyl diimidazole, and subsequently coupled this to m-aminophenyl boronic acid to form the affinity matrix. At least three commercial companies now market versions of this gel; Pierce Chemical Company, Isolab inc. and Amicon, with the respective trade names of Glycogel B, Glyc-Affin and Matrix Gel PBA. All three gels are similar, and the separation of glycosylated material is based on their ability to selectively retain cis-diol groups (Fig. 2.1). They therefore bind molecules containing carbohydrate groups, such as glycosylated haemoglobins. The work in this thesis shows that other glycosylated proteins may also be separated in this way (Gould, Hall and Cook, 1983). The affinity gel is loaded into small chromatography columns and either haemolysate or diluted plasma allowed to soak in. As the non-glycosylated material should not be retained by the gel, it may be washed through and collected. The 'bound' glycosylated material can be subsequently removed with a second buffer containing 0.2M sorbitol, and this may be also collected. Excess sorbitol is added to the elution buffer to compete with the carbohydrate groups for the binding sites, and so these molecules are removed from the column.
Extensive work has been done by Middle et al. (1983) to investigate the effect of different parameters on the separation process. They used a Matrex Gel PBA, and particularly emphasised the importance of precisely regulating the ligand concentration of the gel. This standardisation is critical, as two-fold increases of "glycosylated haemoglobin" could be achieved by altering the ligand concentration between 30 and 40mmol/l. Pierce Chemical company claim to standardise the manufacturing process so that the affinity chromatographic support is lot-to-lot reproducible. The sample size was also shown to be important (Hall, Cook and Gould, 1983, Middle et al. 1983) to avoid overloading or underloading the column. Middle et al. (1983) also recommended that magnesium chloride should be included in the elution buffer for optimum results. Several workers have shown that there is a small increase in the percentage of the glycosylated fraction with decreasing temperature (Gould, Hall and Cook, 1982, Mallia et al. 1981, Klenk et al. 1982) although the temperature dependancy is not nearly as marked as with ion-exchange chromatography. Clearly then, the separation of glycosylated from non-glycosylated material is dependent on the experimental conditions and, since recovery figures in the order of 98% (Chapter 2) are achieved, this dependence is probably due to the weak affinity of the boronate ligand for the glycosylated material rather than that the buffers are inadequate to remove it from the gel.
The schematic presentation illustrating the binding of cis-diol groups to the affinity gel, Glycogel B, as shown in Fig. 2.1 is oversimplified. There has recently been much discussion in the literature regarding the ring structure of the carbohydrate in glycosylated materials and its binding to boronate. Fischer and Winterhalter (1981) noted that the carbohydrate moiety in haemoglobin A\textsubscript{1c} is present in the ring form rather than as a linear, open-form attachment. They argue that the open-form would react with phenylhydrazine and the lack of detectable keto groups by this technique can be explained if the ketoamine is present as a ring-form. Also upon periodate oxidation of glycosylated haemoglobin (Gallop et al. 1981) only one mol formaldehyde/mol glyco group is formed. Periodate treatment of an open glucose would liberate two molecules of formaldehyde. Brownlee, Vlassara and Cerami (1980) also supported the ring formation of the 1-deoxyfructosyl derivatives of amino acids and peptides, although their illustrations suggest a slightly different ring formation to that of Fischer and Winterhalter (1981), (Fig. 7.1). Their ring contained four carbons, and an oxygen joining carbons 2 and 5, whereas that outlined by Fischer and Winterhalter contained five carbons, and an oxygen between carbons 2 and 6. Brownlee, Vlassara and Cerami (1980) also suggested the complex formation between phenylboronic acid in alkaline solution and the cis-diol groups to be via carbons 2 and 3. Ferrier (1978) concluded that this binding could occur via carbons 2 and 3, or carbons 4 and 5. A more recent study by Neglia et al. (1983) used RNase A as a model protein for $^{13}$C NMR studies to characterize the products of nonenzymic glycosylation. They showed that the initial aldimine
Fig. 7.1 *Ring formation of the carbohydrate moiety present in haemoglobin* $\text{A}_1c$.

Ring form described by Fischer and Winterhalter (1981).

OR

Ring form described by Brownlee, Vlassara and Cerami (1980).
(Schiff's base) cyclizes to a glycosylamine derivative and then undergoes a slow Amadori rearrangement to yield the ketoamine adduct. They show (Fig. 7.2) that the ketoamine can exist in various tautomeric forms, including both the 4 and 5 carbons within a ring-form. This is consistent with the binding of the boronate ligand to carbons 2 and 3, 3 and 4, 4 and 5 or 2 and 4 (B.J. Gould. personal communication).
Fig. 7.2  Tautomeric equilibria of Amadori adducts to protein.

Glycosylated Material Measured by Affinity Chromatography

At the beginning of this work there had been only a single publication by Mallia et al. (1981) who had made a m-aminoboronate affinity gel and had performed limited experiments suggesting its use for the measurement of glycosylated haemoglobins. The work in this thesis, much of which has been published, substantiates the initial claims of these workers, showing affinity chromatography can be very successfully used to measure glycosylated haemoglobins in a routine laboratory. The consistently low coefficients of variation achieved with this technique, unusual for column methods, probably explain a lot of the data described in the clinical sections of this work, where the distinction between diabetics and non-diabetics was well-defined. A further reason for this clear-cut distinction is that, due to the principle of separation underlying affinity chromatography, it should be possible to separate all glycosylated material. Cation-exchange chromatography resolves the haemoglobins glycosylated at the amino terminus of the beta-chains (HbA\textsubscript{1a-c}), but affinity chromatography should, in addition to these, detect haemoglobin glycosylated at all other sites (GHB\textsubscript{A\textsigma}) but which does not normally separate from the bulk of haemoglobin with ion-exchange chromatography. Estimates of the amount of glycosylation which is not measured by ion-exchange have been obtained by incorporating radioactivity from reduction of the ketoamine bond with tritiated sodium borohydride (Bookchin and Gallop, 1968) and the release of carbohydrate moieties to be measured as 5-hydroxylfurfural (Gabbay et al. 1979). These results
suggest that the additional contribution of this type of glycosylated haemoglobin ("non"-beta-terminal glycosylation) is approximately the same as that of HbA₁c, although it may be even higher (Bunn et al. 1979). However the normal ranges achieved with the described affinity method were similar to, and not significantly higher than those quoted for ion-exchange methods. Also this discrepancy was investigated by rechromatography of the original fractions containing material which had bound, or not bound, to the affinity gel. It was shown that there was contamination of bound material with unbound material, and vice versa. This finding was also noted by Middle et al. (1983) and in a recent publication by Flückiger, Woodtli and Berger (1984), who used larger columns of gel (14 x 130mm) rather than the gel columns used in this work (7 x 25mm), and overcame this problem. Indeed with these larger columns and a reduced flow rate (0.5 ml/min) they were able to demonstrate that there was no contamination of "bound" with "unbound" material in the final fractions, or vice versa. They also found that the levels of "non"-beta-terminal glycosylated haemoglobins were about the same as those for HbA₁c, as measured by ion-exchange chromatography. The affinity gel is expensive to buy, although once it had been established that it was possible to regenerate and reuse the gel several times (Hall, Cook and Gould, 1983), the technique became competitive with other more established methods. However, the use of a very large column for this work on a routine basis would be prohibitively expensive for most laboratories. Production of the gel was difficult as the content of ligand in the gel had to be strictly controlled
(Middle et al. 1983). In addition the recommendation by Flückiger, Woodtli and Berger (1984) for working at 4°C may also not be practical. The work published from this thesis showed that there was increased amounts of "glycosylated molecules" to be found at lower temperatures. The loss of this material was considered an acceptable error for the convenience of working at room temperature. Recovery from the columns was not affected. Until the affinity method can be suitably modified to circumvent these problems, affinity chromatography as used in this thesis appears to provide the best alternative for the monitoring of all diabetic patients on a routine basis. The aim of this work to establish a simple, reproducible, reliable and inexpensive technique for the routine monitoring of diabetic patients has therefore been achieved.

Garlick et al. (1983) emphasised that problems regarding accuracy also exist with other methods. Peaks isolated by ion-exchange chromatography or electrophoresis were shown to be likely to be contaminated by non-glycosylated proteins. Furthermore, both the thiobarbituric acid test and the borohydride reduction show variable reactivity depending on the site of the ketoamine-linked glucose. Therefore, although the affinity method as described in this thesis has been shown not to be accurate (Flückiger, Woodtli and Berger, 1984), other methods can also be shown to be at fault in this respect. However, the preliminary work of rechromatography in Chapter 3 shows that affinity chromatography using a 1ml gel column at
gravitational pressure, appears to produce a constant percentage of the total glycosylated material binding to the column. There was only a very small percentage (approximately 2%) of "bound" material collected with the "unbound" material in each experiment.

Since the work described in this thesis was first published (Gould, Hall and Cook, 1982), affinity chromatography has been recognised and used by several other workers (Yue et al. 1982, Klenk et al. 1982) for the measurement of glycosylated haemoglobins. Very good correlations with other methods have been found. Correlations of $r = 0.97$ with the thiobarbituric acid method, $r = 0.98$ with the mini ion-exchange columns (Klenk et al. 1982), and $r = 0.97$ with high pressure liquid chromatography (Little et al. 1983) have all been claimed. A good correlation ($r = 0.95$) with the agar gel electrophoretic method has been shown in this thesis.

**Measurement of Protein in Glycosylated Plasma Protein Assay.**

Glycosylated plasma proteins can also be measured using affinity chromatography (Gould, Hall and Cook, 1983), but problems arise when measuring the protein content in the wash and elution fractions, since very low levels of protein are loaded onto the gel. The recommended modified Bradford method used here, utilises Coomassie Blue dye which is very sensitive to the presence of protein. This affinity method gave a good correlation ($r = 0.69$) with the established
colorimetric method for glycosylated protein estimation.

Since this work began, Glycogel B has become available in kit form for the measurement of glycosylated plasma proteins and glycosylated albumin. According to the instructions supplied with each kit, a column loading of 200\(\mu\)l of neat plasma is used for the assay. This represents an addition of 10-15mg of protein per ml of gel, an amount needed because of the insensitivity of the direct method for protein measurement at 280nm that they recommend. The results in this thesis (Table 2.2) show that protein in excess of 2mg per ml of gel causes overloading, with correspondingly lower results for the percentage of glycosylated plasma proteins. The recovery of protein is not altered by different loadings. However, as the loading increases, the binding sites available to glycosylated molecules presumably become saturated. Those in excess are therefore collected in the unbound fraction, causing the decrease in the percentage of the bound material that was observed when more than 2mg of protein per column was used. This may be an over-simplification in view of the relatively small decreases found with much higher loadings, and will require further study.

The method for measuring protein is critical. The modified Bradford method is not only more sensitive than measuring the absorbance at 280nm, thus allowing a lower loading, but also more specific since many compounds other than protein absorb at 280nm. There is significant protein-to-protein variation with the direct method and with the Bradford method. The latter is known to have a greater colour yield with albumin than with most other proteins (Pierce, 1977). This protein-to-protein variation presumably
accounts for the different answers obtained by the two methods in the investigations regarding loading of protein onto the gel columns (Chapter 2). The variation observed with the Coomassie blue dye may be counteracted by the addition of sodium dodecyl sulphate to the reagent, which Macart and Gerbaut (1982) claim equalises the reactivity of many of the proteins in plasma. Further work would be needed to see if this could be usefully applied in the measurement of glycosylated plasma proteins.

Further Investigation Recommended for the Glycosylated Plasma Protein Assay.

The main objective of this thesis was to set up methods using affinity chromatography for the measurement of glycosylated haemoglobins and glycosylated plasma proteins, and to compare their clinical usefulness. The underlying principle for the separation of glycosylated from non-glycosylated material should be the same for both assays. The main outstanding problem when considering the glycosylated plasma protein assay is to establish what exactly the method measures. Only recently has this aspect of glycosylated haemoglobin measurement using affinity chromatography been investigated (Garlick et al., 1983, Flückiger, Woodtli and Berger, 1984).

This thesis has covered several aspects of the measurement of glycosylated plasma proteins including lability and buffer pH, as well as the precision figures and clinical ranges. The study of lability (Chapter 2) produced some interesting data showing that, in contrast to the findings with glycosylated haemoglobins, incubation of the protein in water caused both
decreases and increases in the results. This serves to emphasise the increased complexity of working with plasma rather than haemoglobin. Buffer pH was shown to be optimum at pH 8.9 for the glycosylated plasma protein assay, rather than at 8.3, as for glycosylated haemoglobins. The reasons for this are not clear.

As with all research, each avenue of investigation indicates many more that need to be studied. Preliminary investigations pertaining to glycosylated plasma proteins have been reported in Chapter 3, using immunoelectrophoresis of protein material which bound, or did not bind, to the affinity gel Glycogel B. These studies attempted to find out which proteins were glycosylated and which were not. They showed that enzymically formed glycoproteins did not bind to the affinity gel. The implications of this are discussed fully in Chapter 3. The other major finding was, not surprisingly, that the glycosylated proteins were those that had the longest half-lives.

More detailed future work is indicated by these initial investigations. Studies regarding the efficacy of the separation could be achieved by applying the thiobarbituric acid treatment or rechromatography to both the "bound" and "unbound" material. Problems for both the lines of studies suggested may occur however, in obtaining enough material to study since both the "bound" and "unbound" fractions are very dilute and difficulties may occur when trying to concentrate them. Perhaps a more fruitful investigation would be to add tracer amounts of $^{14}$C-glucose labelled serum protein and follow the progress.
of the label through the separation procedure. Unfortunately this, and other similar experiments using radioactive labelling techniques were not possible for this thesis, as the necessary facilities were not readily available. Future investigation using the fructosamine method, which is gaining in popularity, might be possible.

The method as described in Chapter 2 gave a good correlation with the established colorimetric technique. The affinity method for glycosylated plasma proteins also gave a very good correlation with that for glycosylated haemoglobins, as measured with the same affinity gel, with a line which, somewhat surprisingly, went close to the origin. This observation implied that the method discriminates between enzymically and non-enzymically glycosylated protein for if enzymically glycosylated protein was separated with this technique this should give a positive intercept on the glycosylated plasma protein axis. This was in agreement with the studies using immunoelectrophoresis.

Another development of affinity chromatography for the measurement of glycosylated plasma proteins has been suggested (Candiano et al. 1983) using lectins combined with A-sepharose for the gel matrix.
Clinical Information from Glycosylated Haemoglobin and Glycosylated Plasma Protein Measurements, Using Affinity Chromatography.

The measurement of glycosylated haemoglobins and glycosylated plasma proteins was assessed in a variety of clinical situations; the detection of glucose intolerance; the monitoring of glycaemic control both in the acute situation and long-term; the monitoring of diabetic pregnancy.

Very clear-cut discrimination was shown between diabetic and non-diabetic subjects in Chapter 4, with a small, well-defined group of subjects who were glucose intolerant. This study, although small, indicates that this measurement should be used in a larger population for the detection of glucose intolerance. However, it should be noted that the patients in this particular study were to some extent pre-selected for glucose intolerance as they were sent to the laboratory for investigation.

Glycosylated haemoglobins were measured on all samples from a diabetic out-patient clinic during the study period. The results shown in Chapter 5 show that since the test was introduced the overall control of the patients attending the clinic improved significantly. This suggests that the availability of this measurement aids the clinician in the assessment of diabetic control. This is consistent with the findings of Nathan et al. (1984) who, measuring HbA₁ by HPLC, concluded that their assay provided information about the degree of long-term glucose control that was not otherwise available in the clinical setting. The study of diabetic
out-patients also found significantly higher glycosylated haemoglobin results in women compared with men. This is in agreement with the findings of Stickland, Paton and Wales (1984) who used isoelectric focussing in their investigations. Glycosylated haemoglobins and glycosylated plasma proteins were both measured in the acute situation (Chapter 5), after the admission of patients for stabilisation following a period of poor glycaemic control. Most of these patients were admitted in coma. As expected, the percentage of glycosylated plasma proteins decreased at a faster rate than the percentage of glycosylated haemoglobins, presumably as the half-life of glycosylated plasma proteins is much shorter (Kennedy et al. 1981). In this situation neither measurement appears to offer, in terms of clinical information, any advantage to regular blood glucose measurements. However, as a research tool they may prove a convenient way of showing improved control in patients during both short and long-term studies. As discussed in Chapter 5 the results presented in this thesis show that for some of the patients either glycosylated haemoglobin or glycosylated plasma proteins decreased at a far quicker rate than expected. These investigations, suggest that the nature of the process of non-enzymic glycosylation may not yet have been fully elucidated. A further interesting observation was that some of the patients who were admitted had higher glycosylated plasma protein than glycosylated haemoglobin results on their visit to the clinic less than a month before admission. This aspect too, may be worth investigating more
thoroughly in future work, as disparity between the results in this way may be able to predict future clinical problems.

In Chapter 6 a two-year, fairly detailed study of diabetic patients and non-diabetic individuals both during pregnancy and at term is described. Ashby, Deacon and Rinsler (1984) recently suggested that affinity chromatography would be suitable for this purpose and the work in this thesis illustrates that this is the case. The results shown in Chapter 3 indicate that fetal haemoglobin does not interfere with the method, although gross interference with the agar gel electrophoretic method was demonstrated (Hall et al., 1983). This has been similarly noted by Talwar et al. (1983) and is not a surprising finding as the separation process using affinity chromatography should not be affected by the nature of the haemoglobin molecule. This lack of interference is of particular importance when studying glycosylation in cord blood to retrospectively assess the glycaemic history of the neonate. However, in this study the retrospective analysis did not correlate well with the outcome of the births in terms of birth weight. This is contrary to the recent findings of Feldman et al. (1984). They also used boronate agarose affinity chromatography to measure glycosylated fetal haemoglobin and showed a significant relationship between this and macrosomia, as reflected in the birth weight ratios. They noted that glycosylated fetal haemoglobin in umbilical cord blood was consistently lower than adult values, a finding consistent with the results in this thesis. The reasons for this discrepancy are, as yet, unclear, although it is important to note that both Sosenko et al. (1982) and Worth et al. (1983) using the thiobarbituric acid assay, also failed to find a good correlation between
birth weight ratios and either cord or maternal glycosylated haemoglobin levels. Also, Feldman et al. (1984) studied relatively few diabetic pregnancies (n = 15) on which to base their conclusions.

Although the results of the study of the diabetic pregnancies are discussed in detail in Chapter 6, there are two particular findings worthy of emphasis. Firstly, the measurement of glycosylated haemoglobins and glycosylated plasma proteins appear to be good predictors of macrosomia when assessed in blood taken from the mother in the last trimester of pregnancy. Considering the many factors which must influence the outcome of any pregnancy, this predictive capability of the test is very encouraging; although the inclusion of skin-fold thickness measurement in future studies should be considered (Sheridan-Pereira et al. 1983). Other studies have shown how the retrospective assessment at birth can be achieved, but this work suggests that using affinity chromatography the biochemist can provide a measurement which may alert a clinician managing a diabetic pregnancy to future birth difficulties. Such information should lead to the improved outcome of diabetic pregnancies in general. Further studies involving closer monitoring of patients in the third trimester of pregnancy may pinpoint more accurately when fetal problems in terms of glycaemic control are occurring. This is an exciting possibility.

The second point worthy of mention is that the glycosylated plasma protein assay appears to offer an early warning of threatened abortion or miscarriage in diabetic pregnancy. In
four of the cases of intrauterine death occurring in this study, three had abnormal glycosylated plasma protein results. However, since the numbers were very small, for any significant conclusions a larger study would seem mandatory. It is important to note though, that miscarriage may be due to fetal distress due to hyperglycaemia, but also may be due to fetal abnormality.

Recently there has been a review by Kennedy and Baynes (1984) which evaluated the speculation that the "browning" reactions of proteins caused by hyperglycaemia could have a role in the pathogenesis of diabetes mellitus. Much of the evidence supporting these speculations was outlined in Chapter 1 of this thesis. More recently a study by the KROC Collaborative Study Group (1984) implied by their findings that improved control can reverse the progress of a patient towards overt nephropathy. This was substantiated by Ghiggeri et al. (1984) who produced data which showed that for both normal and diabetic patients with normal excretion rates of albumin, the passage of glycosylated albumin was facilitated through the glomerular wall of the kidney, in contrast to non-glycosylated albumin. Their results indicate that the glycosylation of albumin plays an important role in the development of functional nephropathy. These findings suggest that the measurement of glycosylated albumin or other glycosylated plasma proteins may prove more relevant than glycosylated haemoglobin measurement in terms of diabetic complications; especially if the alteration of albumin could be shown to compromise its physiological functions.
Quality Control.

The glycosylated haemoglobin measurement in this thesis has been used to assess long-term changes in both the control of diabetic patients attending an out-patient clinic, and for the study of diabetic pregnancies. Glycosylated plasma proteins were also used in the latter investigation. For any conclusions to be drawn from these results it was necessary to check on between-batch precision, especially when the batch number of the gel changed. At the beginning of this work no satisfactory quality control (QC) scheme was available, and following the recommendations of Boucher et al. (1983) an "in-house" QC scheme was set up. This proved very satisfactory for the day-to-day check on precision. Over the two years of the research, five different batches of gel were ordered from Pierce Chemical Co., two with the same batch number. When changing from one batch to another, a set of 20 columns were prepared from each of the different gels and 20 diabetic samples were analysed concurrently on each set. The results were calculated to check that there was no significant difference between the two batches of gel. On this basis one batch of gel was rejected, and it was noticed that this particular gel was pink rather than white in appearance, suggesting prolonged storage at the manufacturers. Precision of batch-to-batch variation of the affinity gel has very recently been brought into question by Fielden (1984) who found quality control values increased by about 16% when changing from one batch of gel to another.
This was not our finding; although one batch of gel was rejected in this study. Perhaps Fielden also received gel which had been stored too long for his first batch of gel, which would explain his observations. This being the case, it is strongly suggested that Pierce Chemical Co. should investigate this now, to ensure that an otherwise excellent method should not be brought into disrepute. Apart from this single exception there was no significant variation when changing from one batch to another, and this was also found by John (personal communication, 1984) who calculated not greater than 0.4% variation between the different batches of gel received for use in his laboratory.

Precision figures within batches from a single gel batch number, have also been questioned. A variation of 5.6% was quoted by Fielden (1984) and 13% by Peterson et al. (1984). In the past year W. G. John has set up a quality control scheme for glycosylated haemoglobins with 19 participating laboratories, using affinity chromatography. All of these laboratories find similar precision figures to those quoted in this thesis (personal communication, John, 1984).
Future of Glycosylated Proteins.

The results in this thesis indicate that glycosylated haemoglobins are useful for monitoring diabetic patients in numerous clinical situations, and that affinity chromatography is an extremely promising method for achieving this measurement. Future developments may follow the guidelines outlined by Flückiger, Woodtli and Berger (1984) as to the improvement of the affinity gel column technique. Other workers (Middle, Longstaff and Dean, 1983) have tried to immobilise phenylboronic acid onto paper. This would have obvious advantages, allowing the assay to be done "at the bedside". However, problems of distributing the affinity material evenly over the paper will have to be overcome before this mode of assay is acceptable. Capillary blood samples have been used successfully with ion-exchange chromatography (Ferrell et al. 1984) and have been suggested for use with affinity chromatography (Little et al. 1983). This may prove useful in the future.

With these, and similar improvements, affinity chromatography could offer in the future an accurate and sensitive technique for the measurement of glycosylated haemoglobins, and in some instances glycosylated plasma proteins, which should lead to a significant improvement in diabetic control. If, as appears likely, glycosylation is responsible for many of the pathophysiological complications of diabetes mellitus, then improved management should ultimately lead to a better prognosis for the patient.
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Erratum;

Additional reference.

Measurement of glycosylated haemoglobins using an affinity chromatography method

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Summary

After removal of the labile material, we have measured the stable glycosylated fraction of haemoglobin with a new, commercially available, phenylboronic acid affinity gel, Glycogel B. The mean value was established for 61 non-diabetics as 7.31 (SD ± 0.92)% and for 108 diabetics as 12.70 (SD ± 2.88)%.

The method is highly reproducible with a coefficient of variation below 2.0%. The effect of changing the temperature from 7°C to 37°C, and pH from 8.1 to 8.9 was investigated. For accurate results the temperature should be maintained between 20°C ± 1°C, and the pH between 8.6 ± 0.1. A poor, but significant correlation (r = 0.43) between glycosylated haemoglobin and simultaneous blood glucose was shown. There was a good correlation with the agar gel electrophoretic method (r = 0.95). The slope of the regression line was 1.20 which indicates that this affinity method measures more than just HbA1c. The affinity method appears to offer greater selectivity for diabetics than the electrophoretic method.

Introduction

Several methods are used to estimate the different glycosylated fractions of haemoglobin that are elevated in diabetics. The most abundant single glycosylated fraction, HbA1c, has been estimated using high pressure liquid chromatography [1], ion-exchange chromatography [2] and isoelectric focusing [3]. Alternatively the fast fraction, or HbA1, can be measured by agar gel electrophoresis [4], high pressure liquid chromatography [5] or with mini ion-exchange columns [6]. All of these methods measure both the labile and stable forms of glycosylated haemoglobin. It has been suggested that the labile form should be removed [7] prior to measurement. Only the colorimetric method, in which the reaction is predominantly with the HbA1c fraction [8], measures the stable glycosylated haemoglobin without the need
for any pre-treatment of the haemolysate. All of the methods mentioned above have been reviewed [9] and their relative merits and disadvantages discussed.

Preliminary work on an alternative method based on affinity chromatography is claimed to have advantages over other methods [10,11]. Both groups used phenylboronic acid affinity supports which bind diols selectively and thus separate glycosylated haemoglobins from non-glycosylated haemoglobins. The technique is claimed to be highly reproducible and is suggested to be relatively unaffected by changes in temperature and pH. We have investigated the effect of these, and other parameters on the measurement of glycosylated haemoglobin in the clinical laboratory, using a commercially available affinity gel, Glycogel B.

Methods

Sample collection

Samples were collected in lithium heparin tubes from non-diabetic and diabetic individuals. These samples were stored at 4°C until use, up to a maximum of 5 days.

Preparation of the haemolysate

Erythrocytes were separated from plasma by centrifugation at 1200 x g for 10 min. Approximately 100 µl of packed cells were added to 2 ml of deionised water, and thorough haemolysis was achieved using a vortex mixer. This diluted haemolysate was incubated at 37°C for 5 h.

Affinity chromatography

The method used was that described by Mallia et al [10] with minor modifications. Microcolumns, to which 1 ml of Glycogel B (Pierce and Warriner, Chester, Cheshire, UK) was added, were equilibrated with wash buffer containing 250 mmol/l ammonium acetate, 50 mmol/l magnesium chloride and 3 mmol/l sodium azide. This buffer was adjusted to pH 8.5. Haemolysate (0.1 ml) was added to the gel and allowed to soak in. The unbound haemoglobin fraction was collected by passing 8 ml of wash buffer through the column. The haemoglobin having affinity for Glycogel B, the bound fraction, was then eluted with 3 ml of elution buffer which contained 200 mmol/l sorbitol, 50 mmol/l EDTA and 3 mmol/l sodium azide in a 100 mmol/l Tris buffer, final pH 8.5. Elution from all the columns was carried out at room temperature, 20°C ± 1°C, unless otherwise stated. The unbound fraction, containing the majority of the haemoglobin was diluted to 15 ml with wash buffer. The absorbances of this, and the bound fraction, were measured at 414 nm and the % haemoglobin bound calculated.

Removal of labile glycosylated haemoglobin

Haemolysate was prepared as described above. Ten minutes after preparation samples were loaded onto five columns. Part of the haemolysate was incubated at 37°C, while the remainder was maintained at room temperature, 20°C. Samples were removed and analysed at the times indicated.
Effect of temperature and pH

To investigate the effect of temperature, the columns and both buffers were allowed to equilibrate to the temperature indicated, either in the laboratory working area or in temperature controlled incubators. The columns were rinsed with 5 ml of wash buffer at the required temperature before the run. When necessary the effect of temperature on the volume of elution buffer was calculated and corrected for by weighing the elution sample.

To study the effect of altering pH, both buffers were adjusted to the required pH by the addition of either 1 mol/l HCl or 1 mol/l NaOH. The columns were equilibrated with 5 ml of the appropriate wash buffer before loading the haemolysate.

Normally six columns were run at each temperature and pH.

Measurement of glucose

Whole blood glucose was measured within 10 min of sampling using the Yellow Springs Glucose Analyser Model 23AM (Yellow Springs Instrument Co., Yellow Springs, OH 45387, USA).

Estimation of HbA1c by agar gel electrophoresis

0.5 ml of packed red cells were added to 10 ml of isotonic (154 mmol/l) NaCl solution and incubated at 37°C for 5 h. Prior to electrophoresis, the erythrocytes were collected by centrifugation at 1200 × g for 10 min. Haemolysis, electrophoresis and quantitation were carried out according to instructions provided by Corning Medical Ltd. (Halstead, Essex, UK).

Statistics

Linear correlations were calculated by the least squares method. Statistical comparisons were performed by Student's t test.

Results

Removal of labile glycosylated haemoglobin

The total glycosylated haemoglobin is the sum of two forms termed labile and stable. We used the method of Compagnucci et al to remove the labile fraction [7]. The results in Fig. 1 show that there is a decrease in the bound fraction of haemoglobin after incubation at both 20°C and 37°C. The greatest decrease observed was between 2.5 and 5 h at 37°C. These results were both significantly different from the original value (p < 0.001), but not from each other. However, when incubation was continued at this temperature to 24 h a significant increase (p < 0.01) was observed, possibly due to the formation of methaemoglobin.

The results of incubation at room temperature show a slower rate of change, and the value at 24 h is not significantly different from the value obtained for the same sample incubated at 37°C for 5 h.

The results from this particular haemolysate of normal blood were selected as they provide such a clear illustration of the presence and effective removal of the
labile fraction of glycosylated haemoglobin. Other experiments with normal and diabetic haemolysates showed similar trends but these were not so marked. From these investigations we decided that the labile fraction was best removed by incubation of haemolysates for 5 h at 37°C before estimation of the stable glycosylated haemoglobin fraction.

**Effect of temperature on affinity chromatography**

An increase in the temperature used for chromatography from 7°C to 37°C, caused a decrease in the percentage of haemoglobin that remained bound to the

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**Fig. 1.** The effect of different incubation times on the % haemoglobin bound by the affinity method. The diagram shows the results for incubating a normal haemolysate at 20°C (■) and 37°C (●) for various time intervals. Each point represents the mean (±SD) of the results from 5 columns.

**Fig. 2.** The effect of temperature on the % haemoglobin bound to the affinity gel. The diagram shows results from normal (■) and diabetic (●) samples. Each point represents the mean (±SD) of the results from 6 columns.
affinity gel (Fig. 2). With the diabetic sample this value dropped almost linearly from 18.7% to 13.3%, reaching a value of 15.3% at 19.5°C. The values for the normal haemolysate show a similar trend. There was a marked and significant decrease from the 9.6% bound at 7°C to the 7.6% bound at 19.5°C, but further increase in temperature caused no further change in binding. We decided to work in the temperature range 20°C ± 1°C, since we found that it was possible to maintain the column within this range despite small fluctuations in ambient room temperature, without special apparatus. Furthermore the variation in % haemoglobin bound is within the coefficient of variation for the method over this chosen range.

Effect of pH on affinity chromatography

Fig. 3 shows the effect of using different pH buffers for separating the unbound and bound fractions of haemoglobin. As the pH was increased from 8.1 to 8.9 there was a significant decrease in the percentage of haemoglobin that remained bound to the affinity gel. For both the normal and diabetic haemolysates there was no significant change in the range pH 8.6 ± 0.1.

Normal range and precision

The affinity chromatography method is highly reproducible, as shown by the results in Table I. Both within- and between-batch precision were determined by analysing replicates of haemolysates prepared from normal and diabetic individuals. The coefficient of variation ranged from 1.5% to 2.0%. For the between-batch study the haemolysates were stored at 4°C and were run on different columns over a period of a week. The recovery of haemoglobin from the columns was about 98%.

We have studied haemolysates from 61 non-diabetic subjects and 108 diabetic patients. The mean percentage of glycosylated haemoglobin for the non-diabetic group was 7.31 (SD ± 0.92)% (range 5.25% to 9.70%). In contrast, the value for

![Graph showing effect of pH on haemoglobin bound](image)
TABLE I
PRECISION OF THE AFFINITY METHOD FOR GLYCOXYLATED HAEMOGLOBIN

<table>
<thead>
<tr>
<th></th>
<th>Within-batch ($n = 20$)</th>
<th>Between-batch ($n = 15$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>CV%</td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>6.81</td>
<td>2.0</td>
</tr>
<tr>
<td>SD</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td><strong>Diabetic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>20.04</td>
<td>1.5</td>
</tr>
<tr>
<td>SD</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

diabetic patients was 12.70 (SD ± 2.88%) (range 6.5% to 22.5%). The results for these two groups are significantly different ($p < 0.001$) with only 14 of the diabetic patients having values within the range quoted for healthy persons.

**Correlation of glycosylated haemoglobin with glucose concentration**

The glucose concentration of whole blood samples taken from 143 diabetic individuals was determined. There was a significant correlation between these values and glycosylated haemoglobin in the same samples as measured by the affinity technique ($r = 0.43$).

Fig. 4. Correlation between HbA1 measured by agar gel electrophoresis and % glycosylated haemoglobin determined by the affinity method. Normals (■); diabetics (●). 

% HbA1 (Agar Gel Electrophoretic Method) 
% Glycosylated Haemoglobin (Affinity Method)
Comparison of affinity chromatography with agar gel electrophoresis

We have analysed samples from 16 non-diabetics and 42 diabetics by affinity chromatography and the well-established method of agar gel electrophoresis. For both methods the labile fraction was removed before analysis by incubation at a 20 to 1 dilution for 5 h at 37°C. Fig. 4 shows that there is a good correlation ($r = 0.95$) between the results obtained by the two methods. Fig. 4 also shows that there are only two diabetics with values in the range observed for the non-diabetics when the affinity technique is used. The equivalent figure for the agar gel electrophoretic method was nine. The regression line with an intercept of $-0.43$ on the $y$-axis and a slope of $1.20$ indicates that the two methods are measuring similar species, but that the affinity method measures additional glycosylated material.

Discussion

We have used an affinity chromatography method similar to that described by Mallia et al [10]. The major differences are that in our studies labile glycosylated haemoglobin was removed by incubation, and a greater volume of wash buffer was used. These changes readily explain why the mean values of glycosylated haemoglobin obtained in this work for normal and diabetic individuals (7.31% and 12.70%) are lower than theirs (7.65% and 15.5%), although this difference could also be due to the smaller number of samples analysed by these workers. The mean values quoted by Bouriotis et al [11] for non-diabetics and diabetic patients are 5.4% and 11.0%, respectively. We assume that this difference is due to the different gels used. We used Glycogel B which is an aminophenyl boronic acid derivative of agarose, whereas they used Matrex Gel PBA-30 which is a similar gel produced by different suppliers.

The method described in this paper is highly reproducible with a coefficient of variation between 1.5% and 2.0%. It is simple to perform, and allows analysis of as many as 60 samples a day without difficulty.

Contrary to the statement of Mallia et al [10], the method is susceptible to changes in pH and temperature; an increase in either causes a decrease in the percentage of haemoglobin bound to the column. However, this affinity method is considerably less sensitive to such changes than is the method using mini ion-exchange columns. For instance, the correction factor for a temperature change from 22°C to 20°C for the ion-exchange method is 1.33 (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK). Under similar conditions the appropriate correction factor for the affinity method is about 1.04. The very low coefficient of variation found with this method requires that the pH and temperature are maintained within the limits selected; for pH, 8.6 ± 0.1 and for temperature, 20°C ± 1°C.

The correlation between the percentage glycosylated haemoglobin and the simultaneous blood glucose readings of 143 diabetics is similar to that found with other methods which measure different fractions of glycosylated haemoglobin [12]. There was a good correlation ($r = 0.95$) between the Corning agar gel electrophoretic method and our affinity method. The present work shows there is less overlap between the diabetic and non-diabetic populations using the affinity method.
compared with the electrophoretic technique. This suggests that the affinity method may be more selective for diabetics.

The main outstanding problem with this affinity method is to determine exactly what is being measured. The slope of the regression line in Fig. 4 indicates that we are measuring glycosylated material additional to HbA1. This is in accord with Mallia et al [10] who showed, using isoelectric focusing, that the bound fraction contained all the HbA1c and also portions of HbA, HbA2 and various methaemoglobins. It is known that a small proportion of HbA, HbA2 and methaemoglobin is glycosylated [9] and presumably it is these glycosylated molecules which account for the higher values observed when using the affinity method. However, our results investigating the effect of temperature and pH indicate that some molecules of glycosylated haemoglobin are not retained by the column as the temperature or pH are raised. We intend to investigate these changes in more detail.

Acknowledgements

We are indebted to Corning Medical Ltd., (Halstead, Essex, UK) for the loan of equipment and supplies of materials for agar gel electrophoresis. The authors wish to thank the School of Biological Sciences of the University of Sussex for the use of their temperature controlled incubators.

References

6 Welch SG, Boucher BJ. A rapid micro-scale method for the measurement of haemoglobin A1c(a+b+c). Diabetologia 1978; 14: 209–211.
An inexpensive, rapid and precise affinity chromatography method for the measurement of glycosylated haemoglobins

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SUMMARY We have assessed an affinity chromatography technique, using commercially available materials, for the estimation of total glycosylated haemoglobin in the routine clinical chemistry laboratory. The method gives good discrimination between normals (7-31±0.92%) and diabetics (12-70±2.88%) and has excellent precision (CV 1.5-2.0%). Labile glycosylated haemoglobin is normally removed as it is so variable. There is no significant correlation between labile glycosylated haemoglobin and blood glucose. Immediate analysis of incubated haemolysates is preferable to storage of haemolysates or erythrocytes. The affinity gel can be reused about 16 times, but oxidation must be reduced by keeping the gel at 4°C in the dark when not in use. The cost of the gel is about 7p a test and 60 samples can be analysed in a working day. The method is not affected by the presence of up to 20% met-haemoglobin and should also give correct values for samples containing genetic variants of haemoglobin.

Measurement of glycosylated haemoglobin is well documented as a technique for the retrospective assessment of carbohydrate metabolism in diabetics. However, in recent years doubts have arisen as to its usefulness. This is due partly to the large number of different techniques available for the measurement of different fractions of haemoglobin and partly to the inclusion of the more variable labile glycosylated haemoglobin in a lot of the earlier work. Since Rahbar isolated by electrophoresis a fast running band which was elevated in diabetic individuals, it has been shown that this fraction, designated HbA1, is composed of a number of components: HbA1a, HbA1b, HbA1c, and HbA2. The identity of the various carbohydrates that react with HbA to produce these components has been partially elucidated, and fractions HbA1b and HbA1c, although present in non-diabetics, have been shown to increase in diabetes mellitus. Separation, either of the fast fraction from HbA or of the different minor components (HbA1b, HbA1c) from each other, has been achieved mainly by exploiting small charge differences between them. Isoelectric focusing, HPLC, ion-exchange chromatography, and agar gel electrophoresis have all been used. Bunn et al. have shown that as well as the fast fraction or HbA1, a portion of the HbA is also glycosylated but does not separate from the rest, despite being chemically different. This portion is also increased in diabetes yet it is not included by the techniques previously mentioned. The colorimetric technique assesses all the glycosylated material but does not give equal weighting to each type of glycosylated haemoglobin, has poor sensitivity, and needs a correction for haemoglobin concentration.

Affinity chromatography has many research applications but few routine clinical uses. However its use for the separation of total glycosylated haemoglobins by exploiting the presence of the carbohydrate moiety (the main point of difference between them and the non-glycosylated molecules) should have advantages over the techniques already mentioned. Mallia et al. synthesised an affinity gel and claimed that it should be useful for this separation. We have confirmed this and have shown that it correlates well with the agar gel electrophoretic method but is simpler to perform. The proposed method is relatively insensitive to changes in pH and temperature, which are important considerations for any column method. In this paper we have assessed this technique for use in a clinical laboratory.

Materials and methods

SAMPLE COLLECTION

Samples were collected into lithium heparin tubes.
from normal and diabetic individuals. These samples were stored at 4°C until assayed, up to a maximum of seven days.

**PREPARATION OF HAEMOLYSATE FOR THE DETERMINATION OF STABLE, TOTAL, AND LABILE GLYCOSYLATED HAEMOGLOBIN**

Erythrocytes were separated from plasma by centrifugation at 1200 g for 10 minutes. Approximately 100 µl of packed red cells were added to 2 ml of deionised water, and thorough haemolysis was achieved using a vortex mixer. This diluted haemolysate was divided, and one fraction was incubated at 37°C for 5 hours before the determination of stable glycosylated haemoglobin. The total glycosylated haemoglobin was determined by analysing the unincubated fraction, and the labile material was calculated as the difference between the total and stable results.

**BUFFER COMPOSITION**
Wash buffer and elution buffer were prepared as shown in Table 1.

**ELUTION PROFILE AND AFFINITY CHROMATOGRAPHY**
Chromatography columns (Uniscience Ltd, Cambridge, UK) containing 1 ml of affinity gel, Glycogel B (Pierce and Warriner, Chester, Cheshire, UK), were prepared, and 100 µl of haemolysate was added to the surface and allowed to soak in. Nine 1-ml volumes of wash buffer were then added to the gel and each fraction was collected separately. Four 1-ml volumes of elution buffer were added and collected similarly. This gave the elution profile (Fig. 1), which shows that the unbound fraction is completely removed from the column before the elution buffer is used.

After this study the standard procedure adopted for clinical samples was to apply 100 µl of the haemolysate to the column and allow it to soak in. One millilitre of wash buffer was then added to the column and allowed to drain in. This was followed by a further 7 ml of wash buffer, and the combined eluates were collected and made up to 15 ml with buffer. The bound material was then collected after elution with 3 ml of elution buffer. The absorbances of both eluates were measured at 414 nm ($A_{414}^{wash}$ and $A_{414}^{elution}$) using the appropriate buffers as blanks.

**CALCULATIONS**
The per cent bound material was calculated using the following equation:

$$\text{Glycosylated haemoglobin} = \frac{A_{414}^{elution}}{A_{414}^{wash} \times 5 + A_{414}^{elution}} \times 100\%$$

**REGENERATION OF THE GELS**
After use, each column was washed with 5 ml of deionised water followed by 5 ml of 0·1 M acetic acid and then stored in the dark at 4°C.

Before use, each column was washed with 5 ml wash buffer, the temperature of which was 20 ± 1°C.

**EFFECT OF ANTICOAGULANT**
Five millilitres of blood was collected and mixed in a tube containing 75 IU lithium heparin, the anticoagulant concentration recommended by Seward Laboratory (UAC House, Blackfriars Rd, London, UK). Aliquots of this blood were transferred to other similar tubes to achieve concentrations of 3 x and 6 x the recommended level. Blood was also added to tubes containing sodium fluoride, and in a similar manner different multiples of the recommended concentration of 1·0 mg/ml were achieved.

All samples were haemolysed and incubated to
remove the labile fraction before measurement.
Four samples of each haemolysate were analysed.

**EFFECT OF REUSING THE COLUMNS**

Five groups of columns were prepared, with five columns in each group. Each group was used a different number of times for the measurement of glycosylated haemoglobin. The number of times used were 3, 7, 12, 16, and 19 for each group, respectively. While not in use the columns were stored in the dark at 4°C.

When all the columns had been used for the specified number of times (within a period of four weeks), all of them were then used for the replicate measurement of glycosylated haemoglobin in a single diabetic haemolysate. Each group was statistically compared with a fresh set of five columns.

A single column was prepared of unused gel which had been stored at room temperature and in the light for about three weeks. The percent bound haemoglobin for this column was compared to unused columns stored at 4°C and in the dark for the same period of time.

**EFFECT OF LOADING DIFFERENT AMOUNTS OF HAEMOLYSATE**

Erythrocytes collected from normal and diabetic individuals were incubated at 37°C for 5 hours in 20 volumes of isotonic saline (154 mmol/l NaCl) to remove labile material and recovered by centrifugation. These red cells were then haemolysed by freezing and thawing, and their haemoglobin concentration was measured using a Coulter Counter (Coulter Electronics Ltd, Luton, Beds). The normal haemolysate was then adjusted with water to contain 10 g/dl haemoglobin and likewise the diabetic haemolysate to contain 7 g/dl haemoglobin. Each was further diluted to provide haemolysates containing 7-0, 4-0, 2-0, 1-0, and 0-5 g/dl haemoglobin.

Four samples of each haemolysate were analysed. For those samples containing 4 g/dl haemoglobin or more, an extra 3 ml of wash buffer and an extra 3 ml of elution buffer were used to ensure complete removal of the unbound and bound fractions from the columns.

**EFFECT OF STORAGE OF HAEMOGLOBIN**

A 20 to 1 dilution of a diabetic haemolysate was prepared and incubated. Some of this haemolysate was tested immediately, and some aliquots were kept at 4°C for analysis after storage for one week. Similarly, a 20 to 1 dilution of the same sample was prepared using 0-58 mmol/l KCN instead of water to lyse the cells. This was treated in an identical manner.

The remainder of the red cells from the sample were stored undiluted at 4°C, and the haemolysate was prepared for analysis after a period of seven days.

All samples were analysed not only at pH 8-5 but also at 8-3, 8-1, 7-8, and 7-5. The pH of the appropriate wash and elution buffers being adjusted with 1m HCl. Four samples of each different haemolysate were analysed.

**EFFECT OF MET-HAEMOGLOBIN CONCENTRATION**

Erythrocytes were collected, incubated at 37°C for 5 hours in 20 volumes of isotonic saline, and recovered by centrifugation at 1200 g for 10 minutes. Part of the sample was converted to met-haemoglobin by incubation with sodium nitrite solution.14 Haemolysates containing haemoglobin and methaemoglobin were prepared and mixed together in known proportions. Four columns were tested at each met-haemoglobin concentration.

**STATISTICS**

Statistical comparisons were performed by Students t test. Linear correlations were calculated by the least squares method.
Table 2  Stable and labile glycosylated haemoglobin in normals and diabetics

<table>
<thead>
<tr>
<th></th>
<th>Stable glycosylated haemoglobin (%)</th>
<th>Labile glycosylated haemoglobin (%)</th>
<th>Labile (%) of stable (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 12)</td>
<td>Mean 6-51 ± 0-52</td>
<td>−0-34 ± 0-44</td>
<td>−5-65 ± 7-32</td>
</tr>
<tr>
<td></td>
<td>Observed range 5-25 to 7-17</td>
<td>+ 0-48 to −1-04</td>
<td>+ 7-22 to −17-06</td>
</tr>
<tr>
<td>Diabetic (n = 12)</td>
<td>Mean 12-97 ± 2-94</td>
<td>−0-71 ± 0-30</td>
<td>−5-90 ± 3-40</td>
</tr>
<tr>
<td></td>
<td>Observed range 9-81 to 17-28</td>
<td>−0-31 to −1-42</td>
<td>−2-52 to −14-50</td>
</tr>
</tbody>
</table>

**Results**

**NORMAL RANGE AND PRECISION**

Figure 2 shows the results for 62 normal and 138 insulin-treated, supposedly stabilised diabetics. There is a wide spread of values for glycosylated haemoglobin in the diabetic patients, but only 15 had values within the normal range. Five patients with glycosylated haemoglobin values above 15% had a simultaneous blood glucose below 10 mmol/l, while three patients with high blood glucose had glycosylated haemoglobin values below 10%. The normal range is more compact but has within it two different populations: normal subjects, the laboratory workers (6-51 ± 0-52%), and the non-diabetic patients (7-45 ± 0-90%). The two groups differ significantly ($p < 0-001$).

The mean value for 62 normals for glycosylated haemoglobin was 7-31 ± 0-92% (observed range 5-25-9-70%) and for 138 diabetics 12-70 ± 2-88% (observed range 6-50-21-5%). The two groups are significantly different ($p < 0-001$).

The method was very precise. Twenty replicate estimations using a normal sample (mean value 6-81 ± 0-14%) and a diabetic sample (mean value 20-04 ± 0-30%) gave coefficients of variation of 2-0% and 1-5%, respectively. The recovery of haemoglobin, as judged by absorbance at 414 nm, was about 98%.

**LABILE GLYCOSYLATED HAEMOGLOBIN**

The results of measurement of labile haemoglobin in 12 normals and 12 diabetics are summarised in Table 2. Although the labile glycosylated haemoglobin is subject to greater change in diabetics than in normals, when this change is expressed as a percentage of the stable form, both groups show changes between 5% and 6%. There are large variations in the amount of labile material between different individuals in both groups, and indeed

Table 3  Effect of loading different amounts of haemoglobin onto affinity columns

<table>
<thead>
<tr>
<th>Amount of haemoglobin loaded (mg)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>7.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Glycosylated haemoglobin</td>
<td>6-43</td>
<td>6-59</td>
<td>6-49</td>
<td>5-73</td>
<td>5-29</td>
<td>4-91</td>
</tr>
<tr>
<td>SD</td>
<td>0-31</td>
<td>0-13</td>
<td>0-11</td>
<td>0-18</td>
<td>0-17</td>
<td>0-07</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Glycosylated haemoglobin</td>
<td>13-64</td>
<td>13-90</td>
<td>13-63</td>
<td>14-34</td>
<td>12-49</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0-23</td>
<td>0-14</td>
<td>0-44</td>
<td>0-45</td>
<td>0-31</td>
<td></td>
</tr>
</tbody>
</table>

Table 4  Effect of sample storage on percent glycosylated haemoglobin

<table>
<thead>
<tr>
<th>pH of buffers</th>
<th>7.5</th>
<th>7.8</th>
<th>8.1</th>
<th>8.3</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysate</td>
<td>Mean</td>
<td>16-74</td>
<td>17-57</td>
<td>17-09</td>
<td>17-34</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0-12</td>
<td>0-25</td>
<td>0-31</td>
<td>0-49</td>
</tr>
<tr>
<td>Haemolysate stored 7 days at 4°C</td>
<td>Mean</td>
<td>15-50</td>
<td>16-70</td>
<td>17-14</td>
<td>17-37</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0-45</td>
<td>0-15</td>
<td>0-30</td>
<td>0-13</td>
</tr>
<tr>
<td>Erythrocytes stored 7 days at 4°C then haemolysed</td>
<td>Mean</td>
<td>15-70</td>
<td>16-50</td>
<td>16-65</td>
<td>17-14</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0-41</td>
<td>0-09</td>
<td>0-10</td>
<td>0-30</td>
</tr>
<tr>
<td>Haemolysate containing cyanide</td>
<td>Mean</td>
<td>16-77</td>
<td>17-81</td>
<td>18-24</td>
<td>18-24</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0-49</td>
<td>0-17</td>
<td>0-31</td>
<td>0-12</td>
</tr>
<tr>
<td>Haemolysate containing cyanide stored 7 days at 4°C</td>
<td>Mean</td>
<td>15-26</td>
<td>18-69</td>
<td>18-63</td>
<td>19-13</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0-16</td>
<td>0-29</td>
<td>0-20</td>
<td>0-21</td>
</tr>
</tbody>
</table>
two of the normals showed increases rather than decreases in the stable glycosylated haemoglobin after incubation, although one of these was within the variation of the method. There is not a significant correlation between labile glycosylated haemoglobin and glucose.

**EFFECT OF ANTICOAGULANT**

There was no significant difference between the two anticoagulants, lithium heparin and sodium fluoride, when used in blood in which glycosylated haemoglobin is to be measured. There is no significant effect when these two anticoagulants are used at their standard concentration or at concentrations up to six times the recommended levels.

**RE-USE OF AFFINITY COLUMNS**

Glycosylated haemoglobin was measured on groups of columns that had been prepared simultaneously but used a varying number of times. The same diabetic haemolysate was loaded on all the columns and this showed that there was a small decrease in the amount of bound material with increasing usage. However, when compared with fresh columns, this decrease was less than 2% for all groups of columns, except those that had been used more than 16 times. The columns that had been used 19 times showed a decrease from 20·24±0·39% to 19·72±0·12%; this is a significant difference (p < 0·05).

The columns gradually become darker with re-use, particularly at the surface of the gel. When fresh gel is kept in the light it becomes dark purple in colour. A column of this material, although not previously used to analyse samples, was shown to have a reduced capacity to bind glycosylated haemoglobin. A glycosylated haemoglobin value of 6·22% was observed compared to 12·11% for the same sample using fresh columns.

**EFFECT OF LOADING**

Table 3 shows that the value of percent glycosylated haemoglobin obtained for normals and diabetics is independent of loading between 0·5 and 2·0 mg of haemoglobin onto the column. When in excess of 4·0 mg for the normals and 7·0 mg for the diabetics is applied, the columns become overloaded. In excess of these values the binding of the glycosylated haemoglobin is impaired and causes significantly lower results (p < 0·001).

**EFFECT OF STORAGE**

Table 4 shows how different storage conditions affect glycosylated haemoglobin values over the pH range 7·5–8·5. For fresh haemolysate the values obtained for pH 7·8–8·5 are equivalent. Storage of haemolysate at 4°C for seven days causes a decrease in value for pH 7·8 and 8·5 compared to pH 8·1 and 8·3. A similar trend is found when erythrocytes are kept for seven days at 4°C before preparation of the haemolysate. In this case only the value at pH 8·3 is not different from the original value of fresh haemolysate.

Cyanoide increases the values of glycosylated haemoglobin at all pHs studied, and these values increased further when the haemolysate containing cyanide was stored for seven days at 4°C (Table 4).

**EFFECT OF MET-HAEMOGLOBIN**

Haemolysates prepared from normal and diabetic individuals containing up to 20% met-haemoglobin were tested. Despite the very high concentrations of met-haemoglobin present, there was no significant difference in percent glycosylated haemoglobin for any of the haemolysates.

**Discussion**

The methods for measuring glycosylated haemoglobin can be considered in two categories. There are those that separate parts of the glycosylated

![Fig. 3 Chemical reaction between glycosylated haemoglobin and the m-aminophenyl boronate agarose, Glycogel B.](image-url)
haemoglobin by means of their very small charge differences (these include the ion-exchange and electrophoretic methods) and there are those that make use of the carbohydrate portion of the glycosylated haemoglobin (these include the colorimetric and affinity chromatography methods). Of the two methods in this latter category, affinity chromatography has three important advantages over the colorimetric technique. First, it has greater sensitivity; second, all types of glycosylated haemoglobin are measured with equal weighting at 414 nm; and, third, the difficulties associated with blank absorbance correction are avoided.

The theoretical basis of the affinity method is illustrated in Figure 3. This shows that the m-aminophenyl boronate agarose (Glycogel B) has an affinity for cis-diol groups. These are found predominantly in carbohydrate or carbohydrate-containing molecules. These molecules combine with the gel while other molecules pass straight through (Fig. 1). The molecules that have combined with the gel can then be eluted using a competing ligand at high concentration, 200 mmol/l sorbitol. All forms of glycosylated haemoglobin, including HbA1 and the forms not normally separated from HbA1 should be retained by the gel. The specificity for haemoglobin and its glycosylated forms is due to measurement at 414 nm.

The affinity gel gives a clear-cut separation between the two fractions (Fig. 1), but if it was considered necessary this separation could be improved by using more wash buffer. The procedure recommended here takes about 40 minutes for a single column run, and 30 columns can be run by one operator at any one time. This is possible because the gel does not dry out, even if left to drain for several hours. This simplifies the change over from wash buffer to elution buffer. The gels can be regeneraged and re-used the same day, allowing 60 samples to be completed in a working day.

The method has high precision with coefficients of variation for within-batch and between-batch samples of 1-5 to 2-0%. This is possible because there are no critical small-volume measurements. The measurements that affect the precision are the adjustment of the wash sample to 15 ml, and the accurate pipetting of 3 ml of elution buffer for collection. The method is simplified by the fact that the red cells do not have to be washed, and the pipettings to produce the haemolysate and load the column do not affect the precision of the method since the final glycosylated haemoglobin concentration is expressed in proportional and not absolute terms. There is also no need for stringent control of temperature or pH as long as these are kept within the guidelines indicated.11

The method gives good discrimination between normals and diabetics (Fig. 2). The glucose results incorporated in Fig. 2 show that a diabetic with high glycosylated haemoglobin does not necessarily have a high simultaneous blood glucose, and vice versa. This implies that glycosylated haemoglobin is the more useful measurement for monitoring long-term diabetic control. The normal range has within it two significantly different populations: normal subjects (the laboratory workers) and the non-diabetic patients. This is possibly age-related as the laboratory workers were generally between the second and third decades while the patient population was predominantly middle to older age. Since only 12 laboratory workers were studied a more extensive survey is required to confirm this, although a comparable finding was reported by Boucher et al.15

The removal of the labile fraction by incubation is recommended. This is necessary since the percentage of labile material varies considerably between individuals and is not apparently related to blood glucose concentration in the same sample. The method is very versatile. Since either lithium heparin or sodium fluoride may be used as anticoagulants, it is possible to do the test on most of the blood received routinely. The use of sodium fluoride allows simultaneous measurement of blood glucose and glycosylated haemoglobin on a single sample. Very little sample is required, so that glycosylated haemoglobin can be measured in blood taken from a fingerprick. The results in Table 3 show that the binding is independent of the amount of haemoglobin between 0-5 mg and 2-0 mg loaded per column. This is equivalent to a haemoglobin concentration between 5-0 g/dl and 20 g/dl in the original sample, assuming a packed cell volume of 50%. This method is applicable to almost the entire range of concentrations encountered in normals and patients. It should be remembered, however, that other features such as reduced red cell survival time may affect the glycosylated haemoglobin result.

Storage of both the samples and the gel is important. The results (Table 4) indicate that immediate analysis of the stable haemolysate is preferable, but storage at 4°C for up to a week still enables 96% of the original result to be obtained. The pH of both buffers was 8-5 for most of the work, as this is recommended by Mallia et al.19; but the findings show that pH 8-3 may be preferable since it appears to nullify the difference in percent glycosylated haemoglobin in fresh and stored samples. The use of cyanide in the preparation of the haemolysate causes spurious increases in results and is not therefore recommended.

We have shown that the columns can be used a t
least 16 times by regeneration between use. This is important as it effectively reduces the cost of the gel to 7p for each test. A darkening of the gels with re-use coincides with a reduction in their binding capacity. This is a light-dependent oxidation process. For this reason we recommend that between use the columns are stored at 4°C and in the dark. To reverse this process we have tried various reducing substances, such as sodium borohydride and ascorbic acid, but as yet without success.

In conclusion, the affinity gel appears to fulfil the need for a simple, inexpensive, reproducible technique for the measurement of glycosylated haemoglobin in the routine clinical laboratory. It is a versatile method which is not affected by methaemoglobin and should give correct values even for samples containing genetic variants of haemoglobin.

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A sensitive method for the measurement of glycosylated plasma proteins using affinity chromatography

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SUMMARY. We describe a simple, sensitive affinity technique for the routine measurement of glycosylated plasma proteins in clinical laboratories. The commercially available phenylboronic acid gel used for the chromatography has recently been marketed as a kit for this purpose (Glycogel Test Kit, Pierce Chemical Co). The manufacturers of this kit recommend loading 200 µl neat plasma to each 1 ml gel column. This high loading is to enable the direct measurement of protein in the bound and unbound fractions at 280 nm. This loading is consistent with 10-15 mg protein being added per ml gel. Our results show that protein levels greater than 2 mg per ml gel overload the column. Therefore we used a modification of the more sensitive Bradford procedure to measure protein. The method discriminate between normals (6.29 ± 1.87%) and diabetic patients (12.62 ± 3.36%) and has good precision (CV 4-6%). The results obtained correlate with the colorimetric method using thiobarbituric acid (r = 0.70) and with glycosylated haemoglobin (r = 0.82).

The measurement of glycosylated haemoglobin is gaining increasing use in the management of patients with diabetes. Other proteins are also glycosylated non-enzymically, and it has been shown that both glycosylated albumin and glycosylated plasma proteins respond more quickly than glycosylated haemoglobin to improvement in glycaemic control. The original method for measuring glycosylated albumin, which involves isolation of the pure protein followed by estimation of the glycosylated fraction, is laborious. Glycosylated plasma proteins can be estimated directly by a colorimetric method or after protein precipitation. For accurate results, these methods also require the estimation of protein, the removal of glucose, and correction for background colour on each sample. A specific method for quantitation of the lysine-bound glucose in serum albumin and other glycosylated proteins is also available. This requires isolation of pure protein, followed by hydrolysis and then analysis by HPLC.

None of the available methods for measurement of glycosylated plasma proteins is suitable for routine analysis in the clinical laboratory. There have been several recent reports on the use of simple affinity column methods for the measurement of glycosylated haemoglobins. A similar chromatographic procedure should separate glycosylated plasma proteins from their non-glycosylated counterparts. The gel used is a m-aminophenylboronat agarose, which binds selectively to cis-diols. Therefore the bound fraction measured by this method should detect those proteins in plasma that have free cis-diol groups. These will include all proteins which have combined non-enzymically with glucose. It is this group, the glycosylated plasma proteins, which are known to be elevated in diabetes.

Materials and methods
SAMPLE COLLECTION, PREPARATION OF PLASMA
MEASUREMENT OF GLUCOSE
Blood samples were collected into lithium heparin tubes from normal and diabetic individuals. These samples were stored at 4°C until assayed up to maximum of seven days. Plasma was separated from erythrocytes by centrifugation at 1200 g for 1 minute; 100 µl plasma was added to 2 ml deionised water and mixed using a vortex mixer. The diluted plasma was incubated for 5 hours at 37°C before determination of glycosylated plasma proteins.
Whole blood glucose was measured within 10 minutes of sampling using the Yellow Springs glucose Analyser Model 23 AM (Yellow Springs Instrument Co, Yellow Springs, OH 45387, USA).

**Finitity Chromatography**

Chromatography columns (Uniscience Ltd, Cambridge, UK) containing 1 ml of affinity gel, lycogel B (Pierce and Warriner, Chester, Cheshire, K) were prepared. They were equilibrated with ml wash buffer (250 mmol/l ammonium acetate, 0 mmol/l magnesium chloride, and 3 mmol/l sodium azide, pH adjusted to 8.5) at 20 ± 1°C. Initially, 150 µl diluted plasma was applied to the top of the column and allowed to soak in. Then 1 ml ash buffer was added to the surface of the gel and the absorbances were measured at 595 nm. Using this modified reagent it was shown that Beer's Law was obeyed for increasing concentrations of protein in the bound and unbound fractions. The protein in the bound and unbound fractions was determined by a modification of the method of Bradford using the Biorad Protein Assay reagent (Bio-Rad Laboratories, Watford, Herts, UK). The reagent was diluted with water instead of plasma and acidified Biorad Protein A Assay reagent was added. Reusability of the columns was tested with columns, five in each group, that had been used three, six and nine times over a period of four weeks.

**Loading Different Amounts of Protein onto the Column**

Plasma from a diabetic patient was incubated and dialysed against saline at 37°C for 5 hours to remove any labile glycosylated material. The concentration of protein in this sample was then measured using a standard biuret method. Different dilutions of this plasma in deionised water were prepared. Then 150 µl of each dilution was added to a set of four prepared columns, which was equivalent to adding 6.7 and 13.4 mg protein to each column. Similarly, two groups of four columns had 100 µl and 200 µl undiluted plasma applied, which was consistent with adding 6.7 and 13.4 mg protein to each column. All of the column groups were analysed using the standard procedure, but to ensure that all of the bound and unbound material was recovered, two additional 3 ml volumes of wash buffer were collected and one additional 3 ml elution buffer. To enable all the eluates to be measured within the linear range of the protein reagent, appropriate dilutions in wash or elution buffer were made before the addition of the acidified Biorad Protein Assay reagent.

An identical protein-loading investigation was repeated using plasma obtained from a normal individual. This plasma was diluted so as to provide the same loading of protein on to the columns as with the diabetic sample.

For both the normal and the diabetic sample, where neat plasma was loaded, the absorbances of the eluates at 280 nm were noted before any secondary dilutions were made or acidified Biorad Protein Assay reagent was added. Wash buffer or elution buffer, as appropriate, were used as blanks.

**Measurement of Glycosylated Plasma Proteins Using the Flückiger and Winterhalter Reaction**

The method used was a slight modification of that...
recommended by Ma et al.17 0·5 ml plasma was diluted with 4·5 ml cold 0·15 mol/l NaCl, and the protein was precipitated with 0·5 ml 2·5 mol/l TCA followed by centrifugation at 1200 g for 10 minutes. The supernatant was discarded and the protein resuspended in 4·5 ml cold 0·15 mol/l NaCl by sonication with a microprobe (Dawes Instruments Ltd, London, UK). A translucent-to-clear stable preparation was obtained. Of this protein suspension 3·0 ml was put in a stoppered quick-fit tube, together with 1·5 ml 1 mol/l oxalic acid and heated in a boiling water bath for 4·5 hours. The tubes were cooled for 10 minutes before 1·5 ml cold TCA was added to each tube and mixed using a vortex mixer. The samples were centrifuged at 1200 g for 10 minutes and 2 ml supernatant was transferred to a clean tube containing 0·5 ml freshly prepared saturated thiobarbituric acid. After mixing, all samples were incubated at 40°C for exactly 30 minutes, and each sample was read at 443 nm against its own blank. A fructose standard curve (0·0-25 mmol/l final concentration) was put through procedures.

Protein was estimated in the original sonicated preparation by the biuret method, and the degree of glycosylation was expressed as absorbance at 443 nm per g protein.

**Statistics**

Linear correlations were calculated by the least squares method. Statistical comparisons were performed by Student's t test.

**Results**

**Reuse of Affinity Columns**

The same diabetic sample was loaded on all the columns, and this showed that only columns used nine times showed a significant decrease (p<0·001) from 15·68±0·33% to 14·09±0·32%. Therefore columns could safely be reused six times although they gradually became darker with reuse.

**Effect of Protein Loading**

Table I shows the percentage of glycosylated plasma proteins for normal and diabetic samples when different amounts of plasma were loaded on to the affinity columns. The values obtained were independent of loading when the amount of plasma protein loaded was between 0·25 and 2 mg per 1 ml column and when protein was estimated with acidified Biorad reagent. When larger amounts of neat plasma were loaded on to the columns and the proteins were measured using Biorad reagent there was a significant decrease in the percentage of glycosylated fraction (p<0·001). At a loading of 13·4 mg protein per column the decrease was 41% and 33% for normal and diabetic samples, respectively. To achieve the optimum concentration of protein to permit the use of the Biorad method with high column loading it was necessary to dilute the eluate very considerably. This almost certainly accounts for the wider standard deviation of the results. When, with neat plasma loading, the protein was measured directly at 280 nm instead of with Biorad reagent the results were significantly higher and had a smaller standard deviation. However, the trend of decreasing values with increasing load from 6·7 mg to 13·4 mg per ml of column is still observed for both normal (p<0·001) and diabetic (p<0·01) samples.

Recovery experiments gave in excess of 97% recovery of protein for both diluted and neat plasma

**Normal Range and Precision**

Figure 1 shows the results for 58 normal and 15 insulin-treated, supposedly stabilised diabetic patients. There is a wide spread of values for glycosylated plasma proteins in the diabetic patients and 38 had values within the observed normal range. Also, of the 35 patients with glycosylated plasma proteins above 15%, 14 had simultaneous blood glucose levels below 10 mmol/l. Out of 38 patients who had glycosylated plasma protein values of less than 10% only four had high blood glucose values. The normal range is more compact and has within it two populations: normal laboratory staff (5·73±0·90%) and non-diabetic patients (6·43±2·03%). However, the two groups do not differ significantly.

The mean value for the 58 normals for glycosylated plasma proteins was 6·29±1·87% (observed range 2·95-10·27%) and for the 153 diabetic patients it was 12·62±3·36% (observed range

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of plasma protein loaded (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0·25</td>
</tr>
<tr>
<td>Normal</td>
<td>% Glycosylated</td>
</tr>
<tr>
<td></td>
<td>Plasma protein</td>
</tr>
<tr>
<td></td>
<td>SD</td>
</tr>
<tr>
<td>Diabetic</td>
<td>% Glycosylated</td>
</tr>
<tr>
<td></td>
<td>Plasma protein</td>
</tr>
<tr>
<td></td>
<td>SD</td>
</tr>
</tbody>
</table>

*Value determined at 280 nm.
sensitive method for the measurement of glycosylated plasma proteins using affinity chromatography

Fig. 1 Histogram showing the distribution of % glycosylated plasma proteins for diabetic and normal individuals. ▲ Results for individual laboratory staff.
● Results for individual diabetics with blood glucose above 10 mmol/l.

-05-23-31%). The two groups differ significantly ($p<0.001$).

Replicate estimations were done on normal and diabetic samples; each gave a coefficient of variation of less than 4%. Between-batch variation for a diabetic sample gave a coefficient of variation of 5-3% and for a normal 6-2%.

CORRELATION OF GLYCOXYLATED PLASMA PROTEINS WITH GLUCOSE AND GLYCOXYLATED HAEMOGLOBIN

The glucose concentration of whole blood samples taken from 153 diabetic individuals was determined. There was a poor correlation between these values and glycosylated plasma proteins measured on the same samples ($r=0.38$).

Glycosylated plasma proteins and glycosylated haemoglobin were measured by the affinity method on blood samples from 16 normals and 153 diabetic patients. Figure 2 shows that there is a good correlation ($r=0.82$).

The line passes close to the origin and has a slope close to unity (0.94).

Fig. 2 Correlation between % glycosylated haemoglobin and % glycosylated plasma protein, both determined by the affinity method. Normals (▲); diabetic patients (●).

CORRELATION OF GLYCOXYLATED PLASMA PROTEIN MEASURED BY THE AFFINITY METHOD AND THE COLORIMETRIC METHOD

Figure 3 shows that there was a correlation ($r=0.7$) between glycosylated plasma proteins measured by the commonly used colorimetric method and the affinity method. The slope of the line was 2.93 and there was a positive intercept of 3.2 $A_{418}$ per g protein on the x-axis.

Fig. 3 Correlation between glycosylated plasma proteins measured by the colorimetric method and by the affinity method. Normals (▲); diabetic patients (●).

Discussion

The affinity method described in this paper provides a simple and sensitive assay for the measurement of glycosylated plasma proteins. The separation of non-glycosylated and glycosylated material is clear-cut and rapid, allowing up to 30 samples to be analysed in a working day. Affinity chromatography has three main advantages over the colorimetric method which has previously been the method of choice for the measurement of glycosylated plasma proteins. Firstly, it does not require removal of glucose; secondly, sample blanks are unnecessary; and, thirdly, it does not require poisonous reagents such
as oxalic acid and thiobarbituric acid. Also the
colorimetric method requires much larger samples
since 5-hydroxymethylfurfural is formed, even after
prolonged acid treatment, in only small non-
stoichiometric amounts, and the colour yield of this
method is not the same for all proteins. Since this work began, Glycocel B has become
available in kit form for the measurement of glyco-
sylated plasma proteins and glycosylated albumin.
According to the instructions supplied with each kit,
a column loading of 200 µl neat plasma is used for
the assay. This represents an addition of 10–15 mg
protein per ml gel, an amount that is needed because
of the insensitivity of the direct method for protein
measurement at 280 nm. Our results (Table 1) show
that protein in excess of 2 mg per ml gel causes over-
loading with correspondingly lower results for the
percentage of glycosylated plasma proteins. The
recovery of protein is not altered by different
loadings. However, as the loading increases, the
binding sites available to glycosylated molecules
presumably become saturated. Those in excess are
therefore collected in the unbound fraction, causing
the decrease in the percentage of the bound material
that was observed when more than 2 mg protein per
column was used. This may be an over-simplification
in view of the relatively small decreases found with
much higher loadings and will require further study.

The method for measuring protein is critical. The
modified Bradford method is not only more sensitive
than measuring the absorbance at 280 nm, thus
allowing a lower loading, but also more specific since many compounds other than protein absorb
at 280 nm. There is significant protein-to-protein
variation with the direct method and with the
Bradford method. The latter is known to have a
greater colour yield with albumin than most other
proteins. This protein-to-protein variation pre-
sumably accounts for the different answers obtained
by the two methods. It is essential for this method
using Bradford reagent that the colour yield of
plasma proteins is directly proportional to concen-
tration over the range used so that the calculation of
the ratio of bound to total protein is valid. We have
shown that direct proportionality exists up to
A=0·6. There is a small and slow decrease in this
absorbance with time so that accurate results require
a constant incubation, in this case 30 minutes.

The cost of the assay is decreased by the fact that
the affinity gel can be reused about six times although
for the estimation of glycosylated haemoglobin the
gels can be reused about 16 times. During reuse the
gel darkens due to an oxidative process. The method has good precision with a coefficient
of variation of about 4% within batch and 5–6%
between batch, although this is not as good as
reported for estimations of glycosylated haemoglo-
in† (CV<2%). Also the discrimination
between diabetic individuals and normals is not s
marked with glycosylated plasma proteins (Fig. 1
as with haemoglobins. This could be due to th
poorer precision or to the more rapid fluctuations o
amounts of glycosylated plasma proteins that have
been reported. However, the values for glycosylate
plasma proteins for non-diabetic subjects (mea
6·29±1·87%) and the range for diabetic subject
(6·05–23·31%) are in good agreement with th
values for glycosylated albumin obtained b
Guthrow et al. They found a mean level of glyco-
sylated albumin of 7·0±1·9% in non-diabeti
subjects and a range of 12·8–26·0% in 22 diabeti
subjects.

The relatively poor correlation of glycosylated plasma proteins with glucose (r=0·38) is similar to
that in other reports. The correlation with th
colorimetric method (r=0·7) indicates that the tw
methods are measuring similar moieties. The slop
of this graph (2·93) may be an indication of th
greater sensitivity of the affinity method but i
should be noted that the units for the two axes ar
quite different (Fig. 3). The cause for the intercept on
the x-axis is not clear since the colorimetric metho
takes into account the known interference b
glucose and the need for an individual blank. It ma
be that there is some other interfering material tha
is yet to be identified. The multiplication facto
required to produce the colorimetric values is ver
large and may obscure the picture. In contrast t
Fig. 3, the correlation shown in Fig. 2 betwee
glycosylated plasma proteins and glycosylate
haemoglobin, both measured by the affinity method
has a slope close to unity and goes virtually throug
the origin. These results were not expected since i
theory the plasma glycoproteins should bind to th
affinity gel and cause a significant positive intercept
value on the y-axis of Figure 2.

We have shown that glycosylated plasma protein
can be measured by a simple, sensitive affinity
procedure. The method has been used in a routin
laboratory, alongside the similar method fo
glycosylated haemoglobin, and is only slightly mor
complex. It should now be possible to assess whic
of these two measurements is most useful to th
clinician in particular situations, for example
diagnosis of diabetes mellitus, following the treat
ment of patients with hyperglycaemic coma, an
monitoring diabetic pregnancies. A minor advan
tage in this respect is that the affinity method unlik
the colorimetric procedure gives results directly as
percentage, as is the case with most methods fo
glycosylated haemoglobin. However, it is necessar
that the method recommended by the manufacturer
for the measurement of glycosylated plasma proteins
which appears to give incorrect results because th
columns are overloaded, be corrected as soon a
possible. Otherwise this potentially useful metho
sensitive method for the measurement of glycosylated plasma proteins using affinity chromatography

ay be disregarded because of doubts concerning its liability in the same way as has already happened ith the estimation of glycosylated haemoglobins here for a time problems with different methodologies masked its usefulness as a clinical test.23

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Summary. We have used a simple affinity chromatography method to measure total glycosylated haemoglobins and glycosylated plasma proteins in maternal and cord blood at 50 normal deliveries. The affinity method gives equal weighting to glycosylated haemoglobins including haemoglobin F in cord blood. The mean values for glycosylated haemoglobins in maternal blood (6.49 ± 1.2%) were significantly higher than those in cord blood (3.85 ± 1.0%; p < 0.001). The difference with glycosylated plasma proteins was less marked (maternal blood 5.61 ± 0.9% and cord blood 4.75 ± 0.6%; p < 0.001). A contributory factor to these differences was the decrease in glucose concentration from 4.53 ± 0.99 mmol/l in maternal blood to 3.59 ± 0.8 mmol/l in cord blood. The results obtained at the birth of six children to diabetic mothers showed the same trends although the mean values for glycosylated haemoglobins (maternal blood 9.27 ± 2.3%, cord blood 4.21 ± 0.9%), glycosylated plasma proteins (maternal blood 7.44 ± 1.6%, cord blood 5.45 ± 1.7%) and glucose (maternal blood 10.22 ± 7.3 mmol/l, cord blood 5.18 ± 3.4 mmol/l) were higher in all samples than for the deliveries to non-diabetic mothers.

Key words: Affinity chromatography, cord blood, diabetes, diabetic pregnancy, glycosylated fetal haemoglobin, glycosylated fetal plasma proteins, glycosylated haemoglobins, glycosylated plasma proteins.

The measurement of glycosylated haemoglobins (Gly-Hb) is well recognized as a retrospective assessment of glycaemic control in diabetic patients [1]. As a clinical tool, these haemoglobins are most useful in labile diabetes such as occurs in pregnancy where their concentration correlates with mean maternal glycaemia [2]. Despite improvements in the control of maternal diabetes during pregnancy, macrosomia, neonatal morbidity and congenital malformations are still frequent in centres with low perinatal mortality [3]. Several methods have been used to determine glycosylated haemoglobins during pregnancy. These include ion-exchange chromatography [3–9], high pressure liquid chromatography [10], and radioimmunoassay of HbA1c [11].

It is also possible to determine Gly-Hb in cord blood and this might be used as an index of fetal exposure to glucose during the last few weeks of pregnancy [12]. The measurement of glycosylated haemoglobin F has been carried out using ion-exchange chromatography [13, 14] and isoelectric focusing [12, 15] but both techniques probably measure the total of glycosylated haemoglobin F and acetylated haemoglobin F [12, 14]. HbA1c has also been measured in cord blood by radioimmunoassay [16] and may be useful in the future for the determination of gestational age in the third trimester.

A simple, sensitive affinity chromatography technique which can be used for the measurement of glycosylated haemoglobins [17, 18] is now available commercially. It is rapid and suitable for use in a routine clinical laboratory for monitoring all diabetic patients including those who are pregnant. In this paper we report the ranges for normal pregnancies at term, and show that this single technique, with minor modification, can be used to determine both glycosylated haemoglobins and glycosylated plasma proteins in adult and fetal blood.

Subjects and Methods

Subjects

Fifty live births to non-diabetic women were monitored; 19 of the mothers were primigravidae. Most births were normal vaginal deliveries, but there were four forceps deliveries and three by Caesarean sec-
tion. Gestational age of the fetus was based on the mother's menstrual history and ultrasound examinations. Maternal and cord bloods for analysis were taken immediately after birth. For the glucose estimation, the sample was preserved using fluoride oxalate; a further sample of each was taken into a standard lithium heparin tube to prevent coagulation. The mean age of the mothers was 28 years (range 19 to 42 years). The mean ± SD birth weight was 3.417 ± 0.518 kg with the observed range being 2.280-4.360 kg. All the births were within 2.5 weeks of the predicted date of delivery. Six live births to insulin-dependent diabetic patients were also monitored. Maternal and cord bloods for analysis were taken immediately after birth, and were treated in an identical manner to the samples from normal subjects.

Measurement of Blood Glucose
Whole blood glucose was measured using the Yellow Springs Glucose Analyser Model 23A (Yellow Springs Instruments, Yellow Springs, Ohio, USA). This was usually carried out within 30 min of sampling, but when this was not possible, the sample was stored at 4°C.

Measurement of Glycosylated Haemoglobins
Glycosylated haemoglobins were measured by the previously reported affinity chromatography technique [18]. This method uses an m-aminophenylboronate agarose gel, Glycogel B (Pierce & Warriner, Chester, UK) for the separation of glycosylated from non-glycosylated material. The pH of the buffers used for the chromatography was 8.3.

For each sample, plasma was separated from the erythrocytes by centrifugation at 1200 g for 10 min. Then 100 μl of packed red blood cells were diluted with 2 ml of deionised water and incubated at 37°C for 5 h to remove labile material [19]. The stable sample was then stored at 4°C until analysis, in most cases up to a maximum of 48 h.

Effect of Fetal Haemoglobin on Affinity Chromatography Method
Packed red blood cells obtained from cord blood were mixed in different proportions with either the appropriate maternal blood or with erythrocytes from a diabetic patient. The samples used contained 100%, 80%, 65%, 50%, 35%, 20% and 0% of cord blood in each case. Each sample was mixed and a 100 μl aliquot was diluted in 2 ml of deionized water and incubated at 37°C for 5 h to remove any labile material, and then analysed in triplicate using the method outlined for glycosylated haemoglobins.

Results
Effect of Fetal Haemoglobin on Methods for Measuring Glycosylated Haemoglobins
Figure 1 shows that when cord blood was mixed with either haemoglobin from a normal mother or haemoglobin from a diabetic mother in different proportions between 0% and 100%, straight lines were obtained. This indicates that the haemoglobin F and the normal adult haemoglobin are given equal weighting by the affinity method. However, when samples of cord blood were tested by the agar gel electrophoretic method there was gross interference by haemoglobin F (Table 1). The mean value for the maternal samples using the affinity method was 5.8%, and for the cord samples it was 4.0%. The corresponding values obtained by the agar gel electrophoretic method were 6.7% and 81.5%, respectively.

Values of Glycosylated Haemoglobins, Glycosylated Plasma Proteins and Glucose in Maternal and Cord Blood of Normal Pregnancies
Table 2 summarizes the results of glycosylated haemoglobins, glycosylated plasma proteins and glucose mea-
Table 1. Glycosylated haemoglobins in cord and maternal samples non-diabetic (A-H) and diabetic (I and J) mothers

<table>
<thead>
<tr>
<th>Maternal blood</th>
<th>Cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity method (%)</td>
<td>6.27</td>
</tr>
<tr>
<td>Agar gel electrophoresis (%)</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 2. Values for glycosylated haemoglobins, glycosylated plasma proteins and glucose in maternal and cord blood of non-diabetic mothers at delivery

<table>
<thead>
<tr>
<th>Glycosylated haemoglobins (%)</th>
<th>Glycosylated plasma proteins (%)</th>
<th>Glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal blood (n = 50)</td>
<td>6.49 ± 1.22 (3.33 - 9.13)</td>
<td>5.61 ± 0.93 (3.47 - 7.79)</td>
</tr>
<tr>
<td>Cord blood (n = 50)</td>
<td>3.85 ± 1.01 (1.94 - 7.18)</td>
<td>4.75 ± 0.62 (3.25 - 6.08)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD; observed range in parentheses

Table 3. Values for glycosylated haemoglobins, glycosylated plasma proteins and glucose in maternal and cord blood of six diabetic mothers at delivery

<table>
<thead>
<tr>
<th>Patient</th>
<th>Glycosylated haemoglobins (%)</th>
<th>Glycosylated plasma proteins (%)</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maternal 8.56</td>
<td>7.66</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Cord 3.33</td>
<td>4.63</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>Maternal 12.21</td>
<td>8.99</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>Cord 5.25</td>
<td>6.76</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>Maternal 6.43</td>
<td>7.76</td>
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<tr>
<td></td>
<td>Cord 4.76</td>
<td>5.32</td>
<td>11.3</td>
</tr>
<tr>
<td>4</td>
<td>Maternal 10.30</td>
<td>8.13</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>Cord 4.59</td>
<td>5.47</td>
<td>5.8</td>
</tr>
<tr>
<td>5</td>
<td>Maternal 7.02</td>
<td>4.35</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Cord 2.85</td>
<td>2.81</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>Maternal 11.12</td>
<td>7.75</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Cord 4.47</td>
<td>7.70</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Mean ± SD, Maternal 9.27 ± 2.31 | 7.44 ± 1.59 | 10.22 ± 7.25
Mean ± SD, Cord 4.21 ± 0.92 | 5.45 ± 1.70 | 5.18 ± 3.43

The Affinity Chromatography Method measures glycosylated haemoglobins (%) and agar gel electrophoresis gives the percentage in the fast fraction.

The mean percentage glycosylated plasma protein values for the maternal population were significantly lower than those for glycosylated haemoglobins (p < 0.001). In contrast, in cord blood the glycosylated plasma proteins were higher than the glycosylated haemoglobin values (p < 0.001).

Results from Diabetic Pregnancies

The values for glycosylated haemoglobins, glycosylated plasma proteins and blood glucose in maternal and cord blood samples from six diabetic mothers are shown in Table 3. As observed with the non-diabetic mothers, the results from cord blood are lower than those from maternal blood samples. Comparison of Tables 2 and 3 shows a higher mean value for each measurement in samples from diabetic subjects. Since only six results are at present available no further statistical analysis has been done.

Discussion

The affinity chromatography method provides a simple, sensitive and rapid method for measurement of both glycosylated haemoglobins and glycosylated plasma proteins. The separation technique depends on the affinity of the stationary phase (Glycogel B) for the carbohydrate moiety of either glycosylated haemoglobins or glycosylated plasma proteins. For this reason it should be independent of the haemoglobin variant and should measure the sum of the glycosylated haemoglobins including glycosylated haemoglobin F and haemoglobin A. Figure 1 clearly illustrates that fetal haemoglobin does not interfere with the affinity method. Other methods utilising small changes in charge to separate glycosylated and non-glycosylated material may be affect-
ed by the type of haemoglobin if this carries a different charge. The resulting interference is clearly shown by the results of the agar gel electrophoresis method where the fetal haemoglobin runs with the fast fraction of HbA₁c.

Glycosylated haemoglobins in cord blood have been measured in several ways with results which depend on the degree of separation and the component measured. The resulting literature is confusing and apparently inconsistent. Using isoelectric focusing, results for non-diabetic mothers have been reported by two groups as 7.8 ± 1.5% [12] and 6.12 ± 1.64% [15]. These values were claimed to represent the sum of glycosylated haemoglobin F, HbA₁c and acetylated haemoglobin F. However, Schwartz et al. [13], using a combination of isoelectric focusing and ion-exchange chromatography, separated glycosylated haemoglobin F (2.7 ± 0.5%) and acetylated haemoglobin F (14.6 ± 4.8%), which together represent more than twice the values mentioned above. With two different ion-exchange methods, Fadel et al. [14] reported the separation of two fractions; HbA₁ (2.1 ± 0.5%) and what they considered to be glycosylated haemoglobin F (14.4 ± 1.9%). The latter is more than five times greater than the percentage of glycosylated haemoglobin F reported by Schwartz et al. [13]. With all these methods, measurement of one or more glycosylated fractions is rendered inaccurate because of contamination with non-glycosylated haemoglobin. Radioimmunoassay has been used for the specific measurement of HbA₁c in cord blood and a value of 1.1 ± 0.3% was obtained for the babies of non-diabetic mothers [16]. The price of specificity in this case has been the loss of detection of a significant amount of glycosylated haemoglobin. The affinity method used for this work provides a means of isolating and measuring all glucose bound fractions of whatever haemoglobin species [18], the results (3.85 ± 1.01%) being understandably between the specific value for HbA₁c and the values obtained by isoelectric focusing which include acetylated haemoglobin F.

Previous studies have shown that Gly-Hb is either unchanged [3, 6, 9] or slightly decreased [22] during normal pregnancy. This study is in agreement with these findings, since the value obtained for maternal glycosylated haemoglobins was 6.49 ± 1.2% compared with the normal value of 7.31 ± 0.9% [18]. This significant decrease (p < 0.001) may be caused by the increase in red cell volume and decrease in fasting plasma glucose concentration that occurs during normal pregnancy [22]. Measurement of maternal glycosylated plasma proteins at term has not been reported previously. In this study we have found, not surprisingly, that it follows a similar pattern to the glycosylated haemoglobins but with a slightly smaller but significant decrease between the normal value (6.29 ± 1.9%; p < 0.02) (unpublished results) and that for pregnant women at delivery (5.61 ± 0.9%). This is consistent with the decrease in plasma glucose concentration. Although the concentration of plasma proteins also decreases during pregnancy [23] the decrease is very small.

It is also interesting that there is an inverse ratio between the percentage of glycosylated plasma proteins and glycosylated haemoglobins in maternal and cord blood. In cord blood, the percentage of glycosylated plasma proteins is greater than Gly-Hb, and in maternal blood the opposite is true. In maternal blood, the concentration of plasma proteins decreases during the last stages of pregnancy due to increased body fluids [23]. This, combined with the decrease in glucose and the fact that glycosylated plasma proteins reflect a shorter time period than glycosylated haemoglobins [24], probably explains the observations. In cord blood, however, there is an increased concentration of haemoglobin compared with the mother's blood which probably alters the balance so that the concentration of glycosylated plasma protein is greater than that of glycosylated haemoglobins in cord blood.

Only a few previous studies have attempted to measure glycosylated haemoglobins in both maternal and cord blood. One group using ion exchange chromatography [15] obtained a value for HbA₁ in maternal blood of 6.4 ± 0.7% and in cord blood of 12.4 ± 3.6%. However, two different chromatography procedures were used to determine these values. Using radioimmunoassay it was found that the maternal HbA₁c values were 3.96 ± 0.7% compared with 4.56 ± 1.8% in cord blood expressed as a percentage of haemoglobin A [16]. Another study, this time using isoelectric focusing [12], quotes maternal HbA₁c as 7.8 ± 1.9% and glycosylated haemoglobin F, which probably includes HbA₁c and acetylated haemoglobin F, as 7.8 ± 1.5%.

Contrary to the findings outlined above, Sosenko et al. [25], using a colorimetric method, found a significantly lower value (by 26%) for Gly-Hb in cord blood compared to maternal blood. The significant decrease reported here from 6.49 ± 1.2% for maternal blood, to 3.85 ± 1.0% for cord blood, is in general agreement with their findings. This decrease is also consistent with the lower blood glucose in cord blood. The results for normal births show a consistent trend of lower glucose, glycosylated haemoglobins and glycosylated plasma proteins in the fetus when compared with the corresponding maternal blood. A similar trend is apparent from the preliminary data for diabetic pregnancies. It has been shown in studies in vitro that fetal haemoglobin becomes glycosylated at the same rate and to the same extent as adult haemoglobin [15]. It seems likely therefore that these lower values in the fetal blood reflect the rapid utilisation of glucose by the fetus [26].

The affinity method can offer two measurements for any patient at birth, glycosylated haemoglobins and glycosylated plasma proteins. Each of these may be useful to the clinician since they reflect different time periods of glycaemic control in pregnancy, which is a rapidly changing situation both metabolically and physiologically. Glycosylated haemoglobins reflect the glucose...
history over the previous 6–8 weeks, whereas glycosylated plasma proteins have a much shorter half-life and can provide information over a shorter time scale [24]. We are at present investigating the predictive capabilities of these measurements in relation to the complications associated with diabetic pregnancy.

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Glycosylated Hemoglobins and Glycosylated Plasma Proteins in the Diagnosis of Diabetes Mellitus and Impaired Glucose Tolerance

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Total and stable glycosylated hemoglobins and glycosylated plasma proteins were determined on 53 patients referred for a glucose tolerance test. Significant correlations were found with fasting blood glucose ($r > 0.89$), 2-h glucose ($r > 0.69$), and area under the glucose tolerance curve ($r > 0.75$), but the correlations with labile glycosylated proteins were not significant. Thirty-one of the patients were normal, five had impaired glucose tolerance (IGT), and seventeen diabetes mellitus (DM) according to the WHO criteria. Comparison of the glycosylated protein values showed that, in all cases, the values for those with IGT and DM were significantly ($P < 0.001$) greater than the values for normals. The range of values of stable glycosylated hemoglobins for those with DM (9.4-24.4%), those with IGT (8.6-10.0%), and normals (5.0-8.5%) shows that there was no overlap between overt diabetic subjects and normal subjects. This was also found for total glycosylated hemoglobins. The results for glycosylated plasma proteins, total and stable, were comparable, but one patient with overt DM and two with IGT had values within the normal range. The measurement of glycosylated hemoglobins and glycosylated plasma proteins by the simple, precise, affinity-chromatography method is potentially a quick, accurate, and simple screening test for patients with DM and IGT and deserves consideration as criteria for their diagnosis.

For many years the oral glucose tolerance test (OGTT) has played an important role in the diagnosis of diabetes mellitus (DM), frequently after the detection of glycosuria. However, the interpretation of the test is hindered by lack of uniformity in procedures and by lack of agreement on the criteria for abnormality. Furthermore, there is day-to-day variation in individuals, despite standardization of methods, and the results are affected by age, many drugs, and diseases.

The WHO Expert Committee on Diabetes Mellitus, in its second report, recommended a standardized test and diagnostic criteria. Two classes of abnormal response were identified, DM and impaired glucose tolerance (IGT). The same criteria may be used for the diagnosis of gestational diabetes. The cut-off points remain somewhat arbitrary, and individuals near the limits may require further investigation.

Several workers have investigated the possibility of using glycosylated hemoglobin as an indicator in the diagnosis of glucose intolerance. All the published work uses methods based on ion-exchange chromatography. These include measurements of HbA1c by high-performance liquid chromatography, measurement of HbA1c by the method of Tri- velli, or the widely available mini-columns to measure HbA1c.

In this article, we report on the use of a simple affinity technique to measure glycosylated hemoglobins and glycosylated plasma proteins on blood collected during OGTTs. The measurement of glycosylated hemoglobins appears to provide a sensitive screening system for the detection of DM and IGT.

MATERIALS AND METHODS

Glucose tolerance test. Capillary blood samples were collected via a fingerprick from 53 patients who were referred to the hospital as outpatients for glucose tolerance tests (OGTTs). Each patient had been on a 300-g carbohydrate diet for each of the previous 3 days and had fasted for at least 12 h (overnight) prior to blood being collected before the glucose load was given. This blood was collected into sodium fluoride preservative and the fasting glucose was measured. If this exceeded 10 mmol/L then the test was discontinued.

An oral glucose load of 50 g was given to each patient.
using the prepared glucose mixture "Dextran" (Lab Sales UK Ltd., Rochdale, Lancashire, United Kingdom) made up in 200 ml of water. Repeated blood samples were taken via fingerprick at half-hourly intervals up to 2 h after the glucose dose. All of the samples were collected into sodium fluoride preservative bottles and kept at room temperature for a maximum of 30 min before the measurement of glucose.

Urines samples were collected while the patient was fasted, and at hourly intervals after the glucose load until the test was completed.

**Interpretation of OGTTs.** The criteria proposed by the WHO Second Report on Diabetes were for a 75-g glucose load. It was suggested that values obtained using a 50-g glucose load should be decreased by 1 mmol/L at 1 h and 2 h after the load. On this basis, the diagnostic values for our OGTT would be (1) Normal. At 0 h and 2 h capillary blood glucose concentration less than 7.0 mmol/L. A fasting value below 6 mmol/L excludes the diagnosis of diabetes. (2) Impaired Glucose Tolerance. At 0 h capillary blood glucose concentration less than 7.0 mmol/L and at 2 h between 7.0 and 10.0 mmol/L. (3) Diabetes Mellitus. At 0 h capillary blood glucose concentration 7.0 mmol/L or above and/or 10 mmol/L or above at 2 h. In the absence of clinical symptoms of diabetes, at least one additional abnormal blood glucose concentration confirms the diagnosis.

**Glucose measurement.** Whole blood glucose was measured in each sample using the Yellow Springs Glucose Analyzer Model 23AM (Yellow Springs Instrument Co., Yellow Springs, Ohio).

A qualitative assessment of the glucose content of each urine sample was made using Clinistix (Ames Co., Miles Laboratories Ltd., Stoke Poges, Slough, United Kingdom).

**Preparation of hemolysate for measurement of glycosylated hemoglobins.** After glucose analysis, the blood sample taken from a fasting patient was centrifuged at 1200 x g for 10 min, and a 1:20 dilution of the erythrocytes in water was prepared. This hemolysate was thoroughly mixed using a vortex mixer, and then divided. One aliquot was kept at 4°C for the measurement of total glycosylated hemoglobin while the remainder was incubated at 37°C for 5 h to remove labile material before the measurement of stable glycosylated hemoglobin. Both total and stable glycosylated hemoglobin were measured using an affinity-chromatography method at 20 ± 1°C and pH 8.3.

**Preparation of plasma for measurement of glycosylated plasma proteins.** After centrifugation, plasma was taken from the fasting blood sample, diluted 1:20 with water, and thoroughly mixed. As with the hemolysate, half of the diluted plasma was kept at 4°C for total glycosylated plasma protein measurement and the remainder incubated for 5 h at 37°C before the measurement of stable glycosylated plasma proteins. Both total and stable glycosylated plasma proteins were measured using an affinity-chromatography method at 20 ± 1°C and pH 8.9.

**Statistics.** Linear correlations were calculated by the least-squares' method. Statistical comparisons were performed by Student's t test.

**Results**

Total and stable glycosylated hemoglobins and glycosylated plasma proteins were determined on patients who were referred for an OGTT. The several significant correlations between these values and fasting blood glucose, the 2-h glucose, and the area under the glucose tolerance curve are given in Table 1. For both glycosylated hemoglobins and glycosylated plasma proteins, the best correlations were found with fasting blood glucose. The labile (total-stable) glycosylated hemoglobins and glycosylated plasma proteins were calculated for each patient, but these values did not correlate significantly (r < 0.3) with fasting glucose. Also, urinary glucose did not produce a consistent pattern. Glycosuria was demonstrated in 3 patients who had normal glucose tolerance tests but was absent for the duration of the test from 5 patients with DM.

**TABLE 1**

Summary of the relationship between the results for the OGTTs and glycosylated hemoglobin and glycosylated plasma protein values

<table>
<thead>
<tr>
<th>Glycosylated</th>
<th>Fasting glucose</th>
<th>2-h glucose</th>
<th>Area under curve (0–2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hemoglobins</td>
<td>N = 53</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>(total)</td>
<td>r = 0.89</td>
<td>0.75</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>slope = 1.34</td>
<td>0.74</td>
<td>0.54</td>
</tr>
<tr>
<td>Glycosylated</td>
<td>N = 53</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>hemoglobins</td>
<td>r = 0.89</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>(stable)</td>
<td>slope = 1.28</td>
<td>0.74</td>
<td>0.52</td>
</tr>
<tr>
<td>Glycosylated</td>
<td>N = 52</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>plasma proteins</td>
<td>r = 0.92</td>
<td>0.72</td>
<td>0.79</td>
</tr>
<tr>
<td>(total)</td>
<td>slope = 1.35</td>
<td>0.57</td>
<td>0.38</td>
</tr>
<tr>
<td>Glycosylated</td>
<td>N = 52</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>plasma proteins</td>
<td>r = 0.92</td>
<td>0.69</td>
<td>0.75</td>
</tr>
<tr>
<td>(stable)</td>
<td>slope = 1.27</td>
<td>0.54</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Although 53 patients were studied, for 5 patients the glucose load was not given, as they had a fasting blood glucose >10 mmol/L. In one case, the glycosylated plasma proteins were not measured.
In this article, we show that there is a clear-cut separation of the overt diabetic subjects from the nondiabetic subjects using glycosylated (stable) hemoglobin measurements (Figure 1A). Of the overt diabetic subjects, as interpreted by the OGTT, all had high glycosylated (stable) hemoglobin values exceeding 9.4%. Furthermore, there were no false positives recorded in the nondiabetic population, with the maximum value being 8.5%. The two groups were significantly different. Those patients who showed IGT tests also differed significantly from both the normal and diabetic groups. However, there was a small overlap between patients with IGT and the overt diabetic population, as the highest value for the former was 10.0%. This could simply be a reflection of the errors in the OGTT for the reasons outlined earlier. Identification of these patients with IGT is important, as they have been shown to be “at risk” of macrovascular disease (especially coronary heart disease) and in a significant minority this diagnosis equates with “early diabetes.”

Very similar results are also evident with the total glycosylated hemoglobin measurement (Tables 1 and 2). This would imply that the incubation stage for the removal of labile material is not strictly required for glycosylated hemoglobin to be used as a successful screening technique for DM. However, since the proportion of labile material is large (mean = 8.5% of the stable fraction), and the relationship between fasting blood glucose and labile glycosylated hemoglobin shows no consistent pattern from patient to patient, its removal is recommended.

Glycosylated plasma proteins were measured in this investigation, since it has been shown that they are more sensitive to hyperglycemia. Tables 1 and 2 also show that similar conclusions can be drawn from the results of both of the glycosylated plasma proteins (total and stable). With these measurements, however, most of the differences between the different populations, as interpreted by the OGTT, are slightly less distinct. This may be due to the glycosylated plasma protein results being unexpectedly, generally lower than those for glycosylated hemoglobins or due to the fact that the method for glycosylated plasma proteins is less precise (CV = 5%).8 and in some cases hemolyzed plasma may yield false values.

In a recent article, Kesson et al.12 compared the usefulness of HbA1c and HbA1 measurement as a screening procedure for DM with the conventional OGTT. They found, in agreement with others,13 that HbA1c is not sufficiently sensitive in the screening of diabetes. However, HbA1c measurements gave a more consistent comparison with the OGTT, which is also comparable to the findings of others.4,5 Nevertheless, Kesson et al.12 stressed the methodologic problems common to all these groups of workers who were using methods based on ion-exchange chromatography. Verrillo et al.13 classified patients according to the WHO criteria1 and compared their results with HbA1c measurements. They found marked overlap between normals and those with IGT.

Affinity chromatography provides a simple, sensitive, and very precise (CV < 2%) method for the measurement of glycosylated hemoglobins in the routine clinical laboratory7

**DISCUSSION**

The OGTT, although well established, is inconvenient to the patient and time consuming for staff. It is prone to environmental influences, as well as to intraindividual variation.1 Glycosylated hemoglobin measures the cumulative effect of glycemia over the previous 3–4 wk8 and is consequently not as prone to environmental influences. This readily accounts for the several significant correlations found between the variables measured in the OGTT, i.e., fasting blood glucose, 2-h glucose and area under the glucose tolerance curve, and the values for glycosylated proteins.
that is not affected by met-hemoglobin or hemoglobin F.\textsuperscript{14} It measures mainly HbA\textsubscript{k} and glycosylated HbA\textsubscript{0}; both fractions are elevated in diabetes,\textsuperscript{16} but the latter fraction may be a more sensitive indicator of hyperglycemia.\textsuperscript{15} We have now shown that a single measurement, using affinity chromatography, appears to distinguish between patients with DM (stable glycosylated hemoglobin > 10%) and normal subjects (stable glycosylated hemoglobin < 8.5%). Patients with values between 8% and 10% are most likely to have IGT and require further clinical investigation. It would be valuable to extend this work, with standardized methodology, to several centers. If the encouraging results obtained in this limited study are more generally applicable, it would be appropriate for the measurement of glycosylated hemoglobin by this method to be included in future discussions on the diagnostic criteria for diabetes.

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