# A STUDY OF GLYCATED PROTEINS

# AND PROTEINURIA

# IN DIABETES

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## DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of the Nottingham Trent University or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's individual contribution. The author has attended appropriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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# ABSTRACT

The extent to which haemoglobin is glycated has been examined by extracting globin from haemolysed red blood cells and measuring globin's ability to reduce the dye nitroblue tetrazolium in alkaline solution. This analytical principle has been developed into a new semi-automated assay for glycated haemoglobin. Results of a clinical evaluation of the assay suggest that it may be suitable for monitoring diabetes and could possibly join other established glycated proteins as pseudo outcome measurements for the clinical audit of diabetes care.

Novel fluoroimmunoassay and latex enhanced immunoturbidimetry assays for measuring urine albumin have been developed and compared with polyethylene glycol (PEG) enhanced immunoturbidimetry. Results suggested that in-house methods requiring antibody to solid phase binding were less reproducible and sensitive than PEG enhanced immunoturbidimetry.

Using polyethylene glycol enhanced immunoturbidimetry, diabetic microalbuminuria was detected in a large sample of over 1000 diabetic patients. The incidence of microalbuminuria was similar to published incidences in the medical literature. A small trial has been conducted which tests the effect of imparting knowledge of adverse health risks, as determined by the presence of microalbuminuria, upon the ability of a group of patients to improve glycaemic control. Glycaemic control has been assessed using the newly developed glycated globin assay, glycated haemoglobin and serum fructosamine. The results of the trial suggest that, surprisingly, the group of patients who were aware of their microalbuminuria did not significantly improve their glycaemic control. The median increment between measurements made on entry into the study and 12-18 months later for

the patients subjected to intensive support was -1.05 mmol/g Total Protein x $10^3$ (glycated globin), 0.2 mmol/L (fructosamine) and -0.15% (GHb). A negative sign indicates that the median glycated protein at the end of the study period was higher than at the beginning. These results are discussed alongside the results of the recent Diabetes Control and Complications Trial. The implication of this research on the Clinical Audit of Diabetes Care are presented.

## ABBREVIATIONS

| | |
|---|---|
| AMP | Adenosine monophosphate |
| BCPDA | 4, 7 bis (chlorosulfophenyl) 1, 10 phenanthroline 2, 9 dicarboxylic acid |
| CMB | p-chloromercuribenzoic acid |
| DCCT | Diabetic Control and Complication Trial |
| DMF | 1-deoxy-1-morpholino-D-fructose |
| EDTA | Ethylene diamine tetra acetic acid |
| FIA | Fluoroimmunoassay |
| GHb | A generic term for all glycated haemoglobin. In this thesis it is also used when referring to glycated haemoglobin measured by affinity chromatography |
| GPs | General Practitioners |
| GSI | Global Severity Index |
| GTT | Glucose tolerance test |
| HbA | Adult haemoglobin of normal genetic make-up |
| $HbA_1$ | A generic term for glycated haemoglobins which migrate more quickly than non-glycated haemoglobin when minor haemoglobins are separated by techniques based on differences of ionic charge |
| $HbA_{1c}$ | Haemoglobin A modified by glycation of the terminal valine residue on the β haemoglobin chain |
| HPLC | High pressure liquid chromatography |
| IDDM | Insulin independent diabetes mellitus |
| MODY | Maturity onset diabetes of youth |
| MRDM | Malnutrition-related diabetes |
| mRNA | Messenger RNA |
| NBT | Nitroblue tetrazolium |
| NHS LC Biotin | Sulfosuccinimidyl-6-(biotinamido) hexanoate |
| NIDDM | Non-insulin dependent diabetes mellitus |
| PEG | Polyethylene glycol |
| PETIA | Particle enhanced turbidimetric immunoassay |
| PSDI | Positive Symptom Distress Index |
| PST | Positive Symptom Total |
| rER | Rough endoplasmic reticulum |
| RER | Residual error of regression |
| RNA | Ribonucleic acid |
| UKNEQAS | United Kingdom National External Quality Assessment Scheme |
| v/v | A liquid mixture expressed as a ratio of volumes (%) |
| w/v | A solution expressed as weight per volume (%) as a concentration unit |
| WHO | World Health Organisation |

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# SECTION 1

# GENERAL INTRODUCTION

# 1.0 INTRODUCTION

## 1.1 Historical introduction and overview of diabetes mellitus

### 1.1.1 The discovery of the nature of diabetes mellitus

From antiquity the association between 'sweet urine' and diabetes mellitus has been recognised. The clinical syndrome of uncontrolled insulin dependent diabetes was described in the second century AD by Aretaeus of Coppadolia (Montague 1983). During the eighteenth century it was discovered that the substance which made urine sweet was glucose. The next landmark in the discovery of the aetiology of diabetes mellitus was probably the experimental work of Minkowski and van Mering (Haussay 1952). They were studying the effects on the dog's digestive tract of the first total pancreatectomy. Unexpectedly, the dog became polyuric and was discovered to have marked glycosuria. The identification of insulin as the hormone produced by the pancreas which could ameliorate the clinical syndrome of diabetes and its subsequent production for clinical use is the subject of a fascinating historical account (Bliss 1982). Diabetes is now understood to occur either because of a lack of insulin or because of the presence of factors which oppose the action of insulin (Watkins et al 1990). This modern definition recognises the classification of diabetes mellitus into two principal types: insulin dependent and non-insulin dependent as pioneered by Himsworth (1936).

Following the discovery that insulin therapy dramatically improved the survival times and quality of life of young diabetics with life threatening disease, diabetes occurring in obese individuals later in life was assumed to be merely a mild form of the same disease. However, the different clinical presentations and the difference in treatment led to a classification which implied two different diseases. This process culminated in the first WHO classification (WHO Study Group 1965). The important observation that maturity

onset (non-insulin dependent) diabetes tended to be insulin resistant, i.e. only respond to large doses of insulin, whilst juvenile subjects tended to be insulin sensitive (i.e. respond very well to small doses of insulin), was thought to imply two different disease processes (Himsworth and Kerr 1942).

### 1.1.2 Classification of diabetes

#### 1.1.2.1 Insulin dependent diabetes mellitus (IDDM)

Although the precise aetiology of IDDM is unknown, both genetic and environmental factors are known to be important in its initiation (Cudworth et al 1979, Tuomilheto-Wolf and Tuomilheto 1991). IDDM is currently considered to be an organ-specific disease affecting individuals with a greater susceptibility (Eisenbarth 1986). A relatively long prodromal period exists during which genetically susceptible individuals produce antibodies against a variety of islet cell autoantigens leading to failure of the islet cells to produce insulin (Betterle et al 1984). As with similar autoimmune conditions IDDM is associated with other diseases with an autoimmune basis. Adrenal and thryogastric autoimmune diseases occur more frequently than would be expected in a background population. (Drell and Notkins 1987).

Evidence of the importance of genetic influences comes from epidemiological studies. A recent worldwide study (Karvonen et al 1993) using standardised (and therefore comparable) methodology has demonstrated clear differences in incidence between the Northern and Southern hemisphere with no country below the equator having an incidence of greater than 15 per 100,000. In contrast, above the equator the disease is common. General trends between the continents show Europe to have the highest incidence followed by South and North America, the Antipodes and Asia. However, there are pockets of high

incidence among the areas of low incidence, an observation which is thought to reflect the distribution of ethnic populations and therefore a genetic influence.

#### 1.1.2.2 Non-insulin dependent diabetes mellitus (NIDDM)

The definition of non-insulin dependent diabetes is problematical because it is largely a disease of exclusion (Alberti 1993). The 1985 WHO definition of NIDDM classifies malnutrition-related diabetes (MRDM) and gestational diabetes as separate disease entities and classifies all remaining diabetes which are not insulin dependent into a single classification (WHO study group 1985).

However, it is likely that within this classification there are several aetiologically distinct conditions. Maturity onset diabetes of youth (MODY) is non-insulin dependent and now recognised as a separate autosomal dominant condition (Tattersall 1974). However, it would have been included as NIDDM before this discovery.

There is also a certain heterogeneity between NIDDM and the non-diabetic state. Mildly affected NIDDM patients treated by diet alone can control their 'diabetes' to such an extent that after a mean duration of 4 years, 27% of 27 NIDDM patients showed a normal glucose tolerance test (GTT) with a normal glycated haemoglobin. (Akinnoken et al 1992).

Furthermore, a varying proportion of NIDDM patients became insulin-dependent. In some non-European populations the prodromal period for insulin-dependent diabetes is unusually long in a subset of patients with glutamic acid decarboxylase antibodies (Tuomi et al 1993). During the prodromal period they are glucose intolerant but not insulin dependent. This heterogenicity between IDDM and NIDDM may increase as other aetiologies are discovered.

### 1.1.3 The pathophysiology of diabetes mellitus

#### 1.1.3.1 Glucose transport

All animal cells use glucose for energy and have one or more mechanism for the uptake of glucose (Elbrink and Bihler 1975). For the cells of most tissues, the extracellular concentrations of glucose is higher than the intracellular concentration, leading to a concentration gradient which tends to favour diffusion of glucose into cells. The diffusion of glucose is assisted by membrane located glucose transporters. The glucose transporters of erythrocytes, brain cells and hepatocytes possess low affinity but high capacity transporters which are constitutively active and are independent i.e. do not require insulin for activation. (Mueckler et al 1985; Jones and Nickson 1981). In contrast, muscle and fat cells contain a reservoir of intracellularly located glucose transporter molecules which, in response to human insulin, translocate to the cell membrane and thus facilitate glucose uptake (Cushran and Wardzala 1980; Watanabe et al 1984).

#### 1.1.3.2 Secretion of insulin

Insulin is synthesised by pancreatic β cells and stored in cytoplasmic granules. In response to hyperglycaemic stimuli, a series of regulated exocytotic events take place which result in the release of granule contents and insulin secretion (Guest and Hutton 1992). The cytoplasmic granules are complex structures containing over 100 polypeptides which are estimated in rats to be the products of 30 independent genes (Hutton et al 1982).

Insulin gene transcription is regulated by nutrients and endocrine factors (Steiner et al 1985). There is good evidence that glucose specifically increases rat islet cell mRNA synthesis (Neilsen et al 1985) possibly mediated by cyclic AMP. The product of translated insulin mRNA is pre-proinsulin which consists of an N-terminal signal peptide linked to

proinsulin. The signal sequence seems to interact with a protein associated with rough endoplasmic reticulum (rER) thus ensuring its passage across the rER membrane. The signal sequence is cleaved as it clears the rER (Patzeldt et al 1978). Proinsulin folds upon itself forming the three disulphide bridges of native insulin while within the rER (Tager et al 1980).

Following secretory granule formation, proinsulin is converted to insulin by the sequential degradation of endopeptidases and a carboxypeptidase (Docherty and Steiner 1982). Final secretion of insulin is by endocytosis, a process stimulated by a rise in cytoplasmic ionised calcium. Glucose promotes the entry of ionised calcium into pancreatic β cells by at least two mechanisms (Hellman et al 1992).

#### 1.1.3.3 Mechanisms of insulin action

Insulin secreted from the β cells of the pancreas exerts its action by binding to specific receptors on the surface of cells. The insulin receptor is a large transmembrane glycoprotein with a molecular weight of approximately 300-400 KDa (Collier and Gorden 1991). The number of receptors varies among tissues from as few as 40 per cell on erythrocytes to $3x10^5$ per cell on adipocytes and hepatocytes. The receptor protein is a tetramer of two α subunits and two β subunits linked by disulphide bonds (Kahn and White 1988). The α subunits pröject from the cell surface into the extracellular fluid and contain the insulin binding sites. The β unit is a transmembrane protein that contains the insulin regulated tyrosine kinase activity. Although the post-receptor signal transduction mechanisms are known to begin with activation of the tyrosine kinase, the molecular elements that link the receptor to various insulin sensitive cellular effects are not known. (White and Kahn 1993).

The major metabolic effects of insulin are to increase glucose uptake (muscle), to promote glycogen synthesis (muscle and liver), to promote fat synthesis (adipose tissue) and to promote protein synthesis (muscle and other tissues). In addition insulin decreases gluconeogenesis in liver and decreases lipolysis (Watkins et al 1983).

Recently three genetic defects have been described which account for less than 1% of NIDDM cases. Hager et al (1994) have shown that 56% of French families with MODY carry a mutation in the glucokinase gene. Glucokinase has been implicated as a glucose sensing mechanism for the pancreatic β cell. Mutations in the gene lead to a failure of pancreatic β cells to produce insulin in response to high concentrations of circulating blood glucose.

Van der Vorm et al (1994) have described a mutation in the α chain of the insulin receptor which prevents nascent insulin receptors from translocating to the cell surface. A defect in mitochondrial DNA leading to a syndrome of NIDDM and deafness has been described by Van den Ouweland et al (1994).

## 1.2 DIABETIC PROTEINURIA

### 1.2.1 Classification of Proteinuria

#### 1.2.1.1 Normal protein constituents of urine

Normal urine contains approximately 100 mg/24h of protein. A proportion of this protein is derived from renal tubular cells and is of high molecular weight. Epithelial cells in the distal tubule synthesise Tamm-Horsefall protein, a mucoprotein with anti viral properties. Other high molecular weight proteins include urokinase, an anti-fibrinolytic enzyme secreted by tubular cells into the luminal fluid and secretory IgA which is synthesised by renal tubular epithelial cells. (King and Boyce 1963). Most other proteins

which reach the tubules after passing through the glomerulus are reabsorbed by the proximal tubular cells, primarily by endocytosis. The resulting composition of urine represents a balance between proteins which have been filtered by the glomerulus but not reabsorbed, and secreted proteins. A normal 24 h urine specimens contains 70 mg of mucoprotein, 16 mg albumin, 6 mg immunoglobulins, 16 mg acid mucopolysaccharides, 35mg of blood group substances and trace amounts of other protein enzymes and hormones (King and Boyce 1963).

The site at which blood is filtered is the renal glomerular capillary wall (Chang et al 1975). The glomerulus allows the passage of water and small molecular weight substances while impeding the passage of high molecular weight substances such as proteins. The glomerular capillary wall is a structure composed of three component. The glomerular basement membrane, a semi-solid matrix composed of fibrils is surrounded by an inner layer of fenestrated epithelium and an outer epithelial cell layer containing interdigitating foot processes separated by filtration slits (Rennke and Verkatachalam 1977).

Increased urine protein may arise due to, a) contamination of normal urine by other protein containing fluids or urinary tract infection, b) renal tubular damage causing reduced reabsorption of filtered proteins, c) increased glomerular permeability, d) saturation of tubular reabsorption mechanisms by filtered low molecular weight proteins in abnormal quantities. Many disease states can be conveniently classified by these functional definitions based on pathological mechanisms, which are only partly understood.

#### 1.2.1.2 Tubular proteinuria

In conditions such as chronic pyelonephritis or the Fanconi syndrome, damage is sustained either by the tubular cells themselves or interstitial cells within the tubule.

Consequent reduction in the capacity for the tubules to reabsorb low molecular weight filtered proteins leads to increased amounts of protein in the urine. There is usually no detectable glomerular dysfunction. Moderate amounts of protein are excreted (less than 2 g/L) usually with little albumin (Abuelo 1983).

#### 1.2.1.3 Overload proteinuria

The renal tubules have a saturable capacity for reabsorbing low molecular weight proteins. When abnormally large amounts of such proteins are produced, e.g. Bence Jones proteins in myeloma patients, the renal absorptive capacity becomes overloaded and a proteinuria varying from 100 mg/24 h to 10 g/24 h may occur. (Abuelo 1983). Another example of overloaded proteinuria is lysozymuria in patients with monocytic and monomyelocytic leukaemia (Osserman and Lawlor 1966).

#### 1.2.1.4 Glomerular proteinuria

Increased passage of protein through the glomerular capillary wall leads to proteinuria in a variety of diseases which are associated with serious and often progressive manifestations of disease. Glomerular proteinuria may be either primary, where the disease process is initiated at the glomerulus e.g. membranous glomerulonephritis, or secondary where the kidney may not be the main focus of the disease process. Systemic illness such as diabetes mellitus or systemic lupus erythematosus can lead to a secondary glomerular proteinuria (Abuelo et al 1983). The mechanisms by which the glomerular proteinuria occurs is poorly understood, however the leading theories on the aetiology of diabetic albuminuria are discussed in Section 1.2.4.

#### 1.2.1.5 Benign proteinuria

Benign proteinuria may occur in approximately 10% of patients admitted to a general hospital (Reuben et al 1982) and is usually unassociated with other signs of renal disease, or with any significant morbidity or mortality (King and Gronbeck 1952).

Functional proteinuria is a transient phenomena associated with high fever (Hemmigsen and Skaarup 1977), strenuous exercise (Castenfors et al 1967), exposure to cold (Sargent and Johnson 1956) and emotional stress (King and Gronbeck 1952).

The third category of benign proteinuria is idiopathic transient proteinuria which is usually detected at routine health screening and is common in children and young adults. Wolman (1945) has reported that most healthy young men have occasional positive screening tests for proteinuria and that the condition is a physiological phenomena in young people.

### 1.2.2 Detection and measurement of proteinuria

Chemical methods for detecting proteinuria have traditionally exploited either the protein shift of pH indicators such as bromophenol blue and bromocresol green or colloid formation and hence turbidity following precipitation of protein by acids such as sulphosalicylic acid and trichloroacetic acid. These methods, which are in widespread routine clinical use, measure urine protein to a minimum detection level of approximately 200 mg/L. More sensitive immunological tests establish that the true reference interval for urine albumin is 2.5-26 mg/24h (Viberti and Wiseman 1986). Thus urine albumin excretion may be abnormally high in patients with diabetic nephropathy, yet remain undetectable by the routine methods used on hospital wards and in pathology laboratories. This situation has led to diabetic proteinuria being known as 'microalbuminuria', 'incipient diabetic

nephropathy' or even 'paucialbuminuria'. This thesis uses the term microalbuminuria to signify protein excretion which exceeds the upper limit of the reference interval established using a highly sensitive immunological method for urine albumin.

### 1.2.3 Diabetic Proteinuria as a predictor of nephropathy

Diabetic proteinuria has been shown to predict nephropathy in three prospective studies: Viberti et al, (1982a); Mathiesen et al, (1984); Mogensen and Christensen, (1984). In each of these three studies ambulant insulin-dependent diabetics were followed over extended periods (14 years or more) with measurements of albumin excretion rate. A timed overnight urine collection was made, the volume recorded and urine albumin was measured. Albumin excretion rate was calculated and albumin concentration (mg/L) divided by urine volume (L). In two other studies (Jarrett et al 1984, Mogensen 1984) the same relationship between nephropathy and albumin excretion was observed in non-insulin dependent diabetics. Indeed, in the Guy's study (Mattock et al 1988) albumin excretion predicted overall mortality (which was predominantly due to cardiovascular events). Schmitz and Vaeth (1988) and Mattock et al (1988) have confirmed that diabetic proteinuria is a major risk factor for coronary heart disease in non-insulin dependent diabetics. There is some evidence that microalbuminuria is a predictor of vascular diseases in non-diabetic subjects (Yudkin et al 1988).

### 1.2.4 Mechanisms of Diabetic Proteinuria

Non-renal causes of proteinuria are not considered here in detail but should be excluded when interpreting urine protein data. In addition to urine tract infections, drug effects (Walker et al 1989), mechanical problems of the ureter and perineal contamination in women can cause non-renal proteinuria.

The glomerulus has been the target of much research and theory aimed at explaining the pathophysiological basis of diabetic proteinuria. The glomerulus is thought to act as a negatively charged membrane with pores of average size 5.5 nm. Any molecule which is in contact with the glomerular membrane will have a greater likelihood of passing through the pores into the glomerular filtrate if it is negatively charged, of small molecular weight (with a small Stokes radius) and is present while a high transglomerular pressure gradient is being exerted. Albumin with a Stokes radius of 3.6 nm can pass through the glomerular membrane and this is the source of albumin in the glomerular filtrate. Tubular reabsorption limits the concentration of albumin which is lost in urine but animal evidence suggests that this mechanism is nearly saturated in the normal individual (Bordeau and Corone 1974).

There is some debate in the literature about the mechanisms which lead to diabetic nephropathy. Since albumin secretion by the renal tubular cells is thought not to occur to any significant extent (Pesce and First 1979), the mechanism is likely to be either increased permeability of the glomerular basement membrane or decreased absorption by the kidney tubules. Most of the evidence in the literature favours the former mechanism and is based upon observations of the pattern of protein excretion in diabetic proteinuria. By measuring albumin and IgG (a marker protein of higher molecular weight, larger Stokes radius and neutral charge) during the progression of diabetic proteinuria from low rates of albumin excretion to higher rates of excretion, it is possible to observe any changes in the proportion of either molecule in the urine. Viberti et al (1982a) have demonstrated that, at albumin excretion rates which exceed 90 μg/min (abnormal threshold 30 μg/min), the clearance of albumin has increased by a significantly larger amount than that of IgG when compared with the clearance at 30 μg/min. This suggests that the selectivity of the glomerular basement membrane progressively changes as the disease process advances. Evidence of lack of

tubular involvement comes from studies of microglobulin excretion in diabetic patients with proteinuria. The appearance of ß-2 microglobulin in urine is used as a marker of tubular damage. Viberti and Wiseman (1986) have reported that ß-2 microglobulin is not increased in the presence of increased albumin excretion. However, it appears that renal tubular damage may precede glomerular damage and proteinuria. Lopes Virrela (1979), Teppo and Groop (1985), Holm et al (1987) and Walton et al (1988) have described excretion of a variety of different tubular function markers in juvenile diabetics without microalbuminuria.

Only 35% of insulin-dependent diabetics are susceptible to diabetic nephropathy. In an attempt to reconcile this observation with a biochemical mechanism for glomerular basement membrane damage, it has been suggested that a derangement in the synthesis of heparan sulphate occurs in genetically susceptible individuals with poor glucose control. Heparan sulphate is a component of glomerulus basement membrane and largely accounts for the fixed negative charge which affects albumin passage into the filtrate. Enzymes involved in the metabolism of heparan sulphate may, in genetically affected individuals and in the presence of high prevailing glucose levels, become functionally impaired leading to failure of heparan sulphate synthesis (Deckert et al 1989).

However, the more conventional explanation of the progression of diabetic nephropathy is that as a consequence of microvascular disease a number of morphological and functional abnormalities develop. Glomerular hypertrophy occurs early in diabetes and leads to an increased glomerular filtration rate with hyperfiltration and microalbuminuria (Olefsky 1992).

Viberti (1991) has put forward a hypothesis which incorporates the observation that elevation of arterial pressure is present in microalbuminuric diabetic patients without renal hypofunction and that long term uncomplicated diabetic survivors have low arterial

pressures. (Oakley et al 1984). One explanation of this observation is that hypertension, perhaps genetically determined, imparts a predisposition to renal damage when diabetes supervenes. An additional link between hypertension and renal damage is the physiologically important sodium hydrogen antiport. This system is crucial in the control of cell growth and replication and in the reabsorption of sodium and bicarbonate. Furthermore there is experimental evidence that overactivity of this system is associated with both hypertension and the risk of nephropathy during diabetes (Hasstedt et al 1988). The sequence of events in a patient with dysregulation of the mechanisms for controlling growth, hypertrophy and intracellular haemostasis is as follows:

Glomerular hypertrophy mesangial expansion, tubular hypertrophy and hyperplasia occur. Increased sodium reabsorption would tend to raise renal perfusion pressure which would in turn result in increased intra glomerular pressure. Increased glomerular pressure may be responsible for generating proteinuria (Viberti 1991).

## 1.3 GLYCATED PROTEINS

The recognition that better control of blood glucose was likely to lead to a reduction in the morbidity of diabetes led to the Diabetes Control and Complications Trial (DCCT) which was set up in 1986 (DCCT Research group 1986) and reported in 1993, (DCCT Research Group 1993). The trial concluded that, in insulin-dependent diabetes, the incidence and severity of retinopathy, nephropathy and neuropathy were reduced by intensive insulin regimes aimed at reducing mean blood glucose concentration. Even before this report was able to indicate a definite benefit, diabetic patients were managed with the aim of keeping their mean blood glucose within a target range which was as close as possible to the reference range for non-diabetic subjects.

This aim required frequent monitoring of blood glucose which became considerably more feasible with the introduction of home blood glucose monitoring. However, individual measurements in a blood glucose monitoring schedule represent only measurements at single points in time selected from a constantly changing continuum of blood glucose control (Nathan et al 1984).

### 1.3.1 Glycated haemoglobin

A chance observation of an unusual band appearing in haemoglobin electrophoresis (Rahbar 1968) in diabetic patients, led to the discovery of minor glycated fractions of haemoglobin. Bunn et al (1975) demonstrated that glycation of haemoglobin was a non-enzymatic post translational event. The glycation process is slow, continuous and irreversible, and proceeds at a rate which is dependent on the concentration of blood glucose in the red cell. Because the red cell is freely permeable to blood glucose, the proportion of haemoglobin which becomes glycated reflects blood glucose concentration over the lifespan of the red blood cells (Goldstein et al 1986). Although circulating red blood cells are a mixture of cells of varying ages, and therefore of different degrees of glycation, the mathematical relationship between the % glycation of haemoglobin in a haemolysed blood specimen and glucose is such that the glycated haemoglobin assay is a retrospective index of the integrated mean blood glucose concentration during the preceding six to eight weeks. (Svendsen et al 1982; Nathan et al 1984).

The glycation process takes place at several sites on the haemoglobin molecule. Glucose potentially forms ketoamine adducts with all amino ($NH_2$) groups on haemoglobin and other plasma proteins. The nomenclature of glycated haemoglobins is based on the mobility of a particular subset of glycated proteins during chromatography or

electrophoresis. Differing mobilities are produced by the change in ionic charge which occurs when glucose binds to charged amino groups on haemoglobin. Haemoglobin $A_{1C}$ is a haemoglobin molecule with a glycated terminal valine residue on the β chain and is thus structurally well defined. The generic term Haemoglobin $A_1$ refers to charge modified glycated haemoglobins which migrate more quickly than other fractions through ion exchange resins or various support media during electrophoresis. Methods which do not rely on charge effects measure varying proportions of the glycated fractions of haemoglobin. Approximately 50% of glycated haemoglobin consists of haemoglobin molecules which are glycated at non-valine amino acid residues e.g. ε-lysine (Shapiro et al 1980).

Although each method of measuring $HbA_1$ is associated with its own reference range, a typical reference range is 3-6% (Goldstein et al 1986).

The early methods which detected minor fractions of haemoglobin were based on ion exchange chromatography and electrophoresis (Allen et al 1958; Huisman and Horton 1965). With the recognition that glycated haemoglobin was one of the minor haemoglobins and that its proportion of total HbA was raised in diabetic patients both techniques were further developed to allow quantitation of $HbA_1$ and later $HbA_{1C}$.

Ion exchange methods based on disposable mini-columns were developed for measuring $HbA_1$ routinely (Dix et al 1978; Arnquist et al 1981). These were superseded by higher resolution columns which measured $HbA_{1C}$ (Marquart et al 1980). A number of analytical problems were identified with ion exchange methods. A Schiff-base intermediate in the glycated reaction is formed as the first step prior to molecular rearrangement to form the stable ketoamine (Bunn 1981). This intermediate, which co-chromatographs with $HbA_1$, tends to give falsely raised results. A pre-treatment step in the assay is therefore required (Nathan et al 1981).

The assay conditions, particularly temperature, pH, ionic strength and column size need to be rigorously controlled. Lack of robustness was a problem before these factors were recognised (Simon and Eissler 1980). Patients with haemoglobinopathies e.g HbF variants, can give falsely raised and falsely low results (Sosenko et al 1980; Aleyassine 1979). Non-glucose intermediates which are adducts between haemoglobin and urea or salicylate have an affect on ion exchange assays in a variety of ways. (Holmquist and Schroeder 1966).

Electrophoretic methods have been described which rely on the interaction of negatively charged groups on agar gel with haemoglobin. Under the influence of an electric current $Hb_1$ is retarded less than $HbA_o$. $HbA_{1C}$ cannot be measured by this technique. HbF and labile intermediates interfere with results (Menard et al 1980). Isoelectric focusing which requires expensive equipment can be used to measure $HbA_{1C}$ and is free from interference by variant haemoglobins. Labile intermediates do interfere (Spicer et al 1978).

Affinity chromatography using cross-linked agarose with m-aminophenylboronate (Gould et al 1982) takes advantage of the fact that the boronic acid component of the column matrix has a specific affinity for the keto amine cis-diol groups glycated haemoglobin. Separation of glycated haemoglobin is thus not dependent on charge. Consequently the glycated fraction detected by this techniques differs from $HbA_1$ and $HbA_{1C}$. The abbreviation GHb is used in this thesis to indicate glycated haemoglobin separated by techniques not dependent on ionic charge. Labile Schiff base intermediates, haemoglobinopathies and non-glycated adducts of haemoglobin do not interfere with GHb measurement (Abraham et al 1983). Minor changes in the structure of the affinity gel such as m-phenylboronate concentration can affect results and may occur as a result of a change of batch of material by the commercial manufacturer (Garlick et al 1983). Recently, the

affinity chromatography method has been partially automated (Herold et al 1993). In another approach 3-aminophenylboronate has been covalently attached to agarose bead samples and encased within a cartridge which, upon addition of whole blood, facilitates haemolysis of red blood cells selective binding of glycated haemoglobin by the aminophenylboronate and quantitation of GHb by bichromatic absorbance measurements on the Abbot Vision analyser. % GHb values are converted to % $HbA_{1C}$ by reference to ion exchange HPLC method.

Several commercial systems for automated HPLC measurement of $HbA_{1C}$ using cation exchange columns have been developed from research techniques (Cole et al 1978, Davis et al 1978) and are capable of low levels of imprecision and handling high workloads. The ability of this technology to produce high quality $HbA_{1C}$ results with a minimum of technician time has led to its increasing use within the U.K.

A colorimetric assay has been described for glycated haemoglobin which is based on hydrolysing the haemoglobin molecule with hot acid and measuring the methylfurfural which results from any sugar moiety attached to haemoglobin by reaction with thiobarbituric acid (Parker et al 1981). However, the harsh reaction conditions make the method unsuitable for most modern autoanalysers and calibration problems have led to difficulties in comparing results between laboratories (Goldstein et al 1986).

Following the development of a monoclonal antibody to $HbA_{1C}$ (Zeuthen et al 1986) several immunological assays have been developed. An enzyme immunoassay in a microtitre plate format has become commercially available which is free from interference by labile ketoamines fetal haemoglobin and carbamylated haemoglobin. Calibration is achieved by reference to $HbA_{1C}$ measurements made on HPLC ion exchange chromatography. Immunoturbidimetry has also been reported recently in an assay format which allows its use on general automated laboratory equipment (Cully et al 1992). A

preliminary evaluation of this assay suggests that analytical imprecision is acceptable. the interference characteristics are similar to the enzyme immunoassay (Palfrey and Labib 1994).

A further development of the affinity chromatography method has been used to facilitate automation of GHb measurement on the Abbot IMX analyser (Wilson et al 1993). This method used an 'ion capture' technology to separate glycated globin complexed to dihydroxyboronate from non glycated haemoglobin and then fluorescence quenching to detect captured (glycated) haemoglobin. The technique has similar characteristics to affinity chromatography.

### 1.3.2 Glycated serum protein

In contrast to glycated haemoglobin, glycated albumin reflects blood glucose control over the preceding 2-4 weeks, correlating with daily multi-point glucose profiles taken over this period (Dolhofer and Wieland 1979; Winocour et al 1989). In patients with insulin dependent diabetes glycated albumin correlates with changes in the mean blood glucose at 2, 4 and 6 weeks after intensification of insulin therapy. The half-life of albumin within the vascular space is approximately 13-17 days which is much shorter than the average red cell (Cohen 1992). Clinical studies show that following improvement of glycaemic control after an acute hyperglycaemic episode, glycated albumin levels fall before changes in glycated haemoglobin become detectable (Dolhofer and Wieland 1981).

Most published methods for measuring glycated albumin rely on phenylboronate affinity chromatography to separate glycated proteins from non-glycated proteins (qv affinity chromatography for measuring glycated haemoglobin) followed by a more or less specific assay for albumin. Using an immunoassay for albumin following separation of

glycated albumin by phenylboronate affinity chromatography, Winocour et al concluded that glycated albumin is a better and more reliable indicator of short-term improvement or worsening of control in type I diabetes than haemoglobin $A_{1c}$ (Winocour et al 1989).

An HPLC technique for measuring glycated albumin relies on separating albumin from other proteins by an ion exchange column followed by phenylboronate affinity chromatography to separate glycated from non-glycated protein (Shima et al 1988). Albumin peaks measured by fluorescence detection of the effluent from both columns led to a reference range of 20 ± 1.6% for non-diabetic subjects. This is far higher than based on mini-column affinity chromatography (reference range 1.5-2.6%, Ziel and Davidson 1987) and may reflect binding of non-glycated albumin to non-specific binding sites on boronate affinity resins which occur when small amounts of protein are applied (Austin et al 1987).

Cohen and Hud have described production of an antibody which recognises the naturally occurring glycated epitopes on albumin and distinguish between glycated albumin and all other glycated protein. This antibody has been incorporated into an enzyme immunoassay (Cohen and Hud 1989). Preliminary evaluation of the assay suggests that the assay gives a reference range similar to affinity chromatography based methods and correlates well with multi-point glucose concentrations determined in the preceding 2-4 weeks (Hud et al 1989).

### 1.3.3 Fructosamine

The fructosamine test was first described by Johnson et al in 1983 and can be seen as one of a group of methods which measure total serum glycated proteins by chemical methods. The main contribution to serum glycated protein comes from glycated serum

albumin (Johnson et al 1983). The analytical principle of the assay is the generation of a highly coloured reduced form of the dye nitroblue tetrazolium (NBT) by taking advantage of the reducing properties of N-substituted ketoamine group in alkaline solution. Thus, any amine group on a serum protein which has been glycated becomes a potential site for reducing NBT. In addition to glycated amine groups, other serum constituents are able to reduce NBT in the original assay (Schlerche and Wieland 1988). By comparison with an HPLC method which measures hydrolysed glycated lysine residues as the furosine derivative it has been estimated that approximately 50% of the colour generated during the fructosamine assay is generated by substances other than glycated proteins. Hill (1990) reviewed the analytical aspects of the fructosamine assay with recommendations for optimising the assay.

More recently a modified fructosamine test has been described which incorporates a detergent and the enzyme uricase (Siedel et al 1988). The 'Fructosamine-plus' test has been evaluated (Cefalu et al 1991).

It is clear that the specificity of the assay has been improved such that the non-specific component of the colour development has been reduced from 50% to approximately 30% There is also some suggestion that the interference of lipids and urate has been reduced (Baker et al 1991). A new calibrant based on polylysine will probably improve the poor inter-laboratory agreement which Hill demonstrated was largely due to differences in calibration when DMF was used (Hill 1988).

The clinical studies which attempt to assess the utility of fructosamine as a marker of metabolic control in diabetes have placed a great emphasis on correlation studies with glycated haemoglobin. Correlation coefficients as high as 0.8 and as low as 0.2 have been reported. Consequently some authors have concluded that fructosamine is highly correlated

with glycated haemoglobin and therefore useful (e.g. Lim and Staley 1985; Baker et al 1987) whereas others have reported low correlation coefficients and conclude that the test has limited use (e.g. Jerntop et al 1988; Ross et al 1986), Such differences between studies must reflect differences in patient selection. Fructosamine might be expected to correlate well with glycated haemoglobin in patients with poor but stable glycaemic control whereas fructosamine is likely to correlate poorly with glycated haemoglobin in patients which are either relatively well controlled (due to the influence of non-specific NBT reductants) or which are unstable or poorly controlled (due to the inability of glycated haemoglobin to reflect short term glycaemic change).

There is also conflicting evidence on the relationship between fructosamine and the measurement of glycated proteins using phenylboronate affinity chromatography.

Winocour et al (1989) found that fructosamine followed changes in $HbA_{1C}$ better than changes in glycated albumin whereas Sobel and Abbassi (1991) measuring changes in short term glycaemic control in children described a correlation coefficient of $r=0.76$.

The plethora of contradictory information in the literature makes the assessment of the value of fructosamine difficult. Windeler and Kobberling reviewed the literature in 1990 and concluded that fructosamine was of limited use for monitoring diabetes. Nevertheless many laboratories continue to measure fructosamine because it can be easily automated, has a low analytical imprecision and is relatively inexpensive (Hindle et al 1985).

Most of the problems associated with fructosamine appear to revolve around attempting to measure a group of glycated proteins in serum which are ill-defined in terms of their relative contributions to the measured substances in the fructosamine assay and with respect to the changes which occur in their concentration over time as serum glucose concentrations change in diabetic patients. If the analytical principle of the fructosamine

assay could be combined with the better defined GHb class of glycated haemoglobin molecules, a highly useful assay might result. This is one of the principal goals of the work that follows.

## 1.4 PROJECT OBJECTIVES

### 1.4.1. Introduction

Significant resources have been deployed by introducing specialist Diabetes Centres at most units providing diabetes care. At least part of the aims of such centres is to assist diabetic patients to improve their glycaemic control. Although it is generally assumed that Diabetes Centres are effective in this regard, there is little audit data to support this view. A further problem is that there are insufficient resources available to provide a high level of support and advice to all diabetic patients.

Until recently it was difficult to target patients at particular risk of the complications of diabetes. With the possible exception of screening for blood lipid abnormalities most patients are not tested for susceptibility to the many sequelae of diabetes.

Testing for microalbuminuria provides a means of detecting patients at risk of developing renal failure at a stage, in insulin dependent diabetes, when treatment can considerably delay if not prevent the onset of the disease.

In non-insulin dependent diabetics, microalbuminuria provides an indication of a markedly increased risk of death from coronary heart disease (see Section 1.2.3). The group of diabetic patients with microalbuminuria are likely to become the target of considerable efforts to reduce blood pressure by drug therapy since anti-hypertensive treatment is known to reduce microalbuminuria. However, improving glycaemic control in insulin dependent diabetes also reduces microalbuminuria, as the recent DCCT trial

shows. The same may be true for non-insulin dependent diabetes. It is therefore important that both drug therapy and blood glucose control are used as tools to help reduce the incidence of the complications of diabetes.

There is therefore, a need for research which is aimed at discovering effective ways of helping diabetic patients with microalbuminuria to improve their glycaemic control (as measured by glycated haemoglobin). The work which follows attempts to focus on the effect of revealing to patients that they have microalbuminuria and are therefore at increased risk from specific sequelae of diabetes. The hypothesis put forward is that a diabetic patient's knowledge that they carry a particularly high risk of complications leads to clinically significant improvements in glycaemic control. Further, that such improvements are additional to any effect that increased attention from health professionals might have.

### 1.4.2 Summary of important project objectives

1. To develop a novel, very precise method for measuring glycated haemoglobin.
2. To develop a robust and reliable method for measuring urine albumin at very low concentrations.
3. To use the urine protein assay to select a population of diabetic patients with microalbuminuria.
4. To determine whether patients' awareness of their increased risk of diabetic complications has a favourable effect upon their achieving better glucose control (i.e. lower glycated haemoglobin values).
5. To identify possible problems which prevent the maximum health gain being obtained from strategies aimed at reducing the complications of diabetes in microalbuminuric patients.

# SECTION 2

# DEVELOPMENT OF A NEW AUTOMATED ASSAY FOR GLYCATED HAEMOGLOBIN

## 2.1 INTRODUCTION

Measuring glycated haemoglobin is widely accepted for evaluating control of glycaemia in diabetic patients. Manual methods such as affinity chromatography with boronate gels and electroendosmosis are popular but require disposable columns or electrophoresis gels and are relatively labour intensive. Methods based on immunoassay involve expensive reagents and most automated HPLC methods require sophisticated dedicated instrumentation. A method that involves simple chemicals and that can be performed with standard laboratory equipment would have significant advantages for a busy clinical laboratory.

Recently, a manual method has been described for measuring haemoglobin $A_1$ in which, after acetone extraction of haem, glycated globin reduces nitroblue tetrazolium (NBT) to its coloured formazan derivative (Somani et al 1989). This method has been investigated and then substantially modified to make it suitable for adaptation to an automated chemistry analyser. The Mira S analyser (Roche Instruments Switzerland) is a discrete automated spectrophotometer analyser with the facility for sample and multireagent additions to disposable plastic cuvettes. Flexible software controlling the analyser allows multiple absorbance readings for over 20 minutes after adding all assay components.

Reduction of NBT is also the analytical principle of the serum fructosamine assay (Johnson et al 1983). This method is not entirely specific for serum glycated proteins (see section 2.4). Specificity for serum glycated serum proteins is enhanced by delaying measurement of absorbance change until rapidly reacting reducing agents such as glucose, have completed their reaction. The specificity of the same reaction for glycated haemoglobin is further compromised by the presence of sulphydryl groups which reduce NBT. The reducing activity of haemoglobin can be suppressed in the presence of

p-chloromercuribenzoic acid (CMB) (Somani et al 1989), a compound which is included in the assay reagents for the measurement of glycated globin.

Introducing other components, such as detergents, to improve the assay's performance on automated chemistry equipment could affect the specificty of the assay. The specificity of the assay has been assessed by examining the intercept of the regression line which describes the relationship between GHb, measured by an affinity chromatography method, and glycated globin, measured by the assay under development.

The preparation of globin from whole blood has been simplified compared to that of Somani et al (1989). Consequently the washing step, the removal of haem and the stability of extracted globin were critically assessed.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Preparation of globin from whole blood

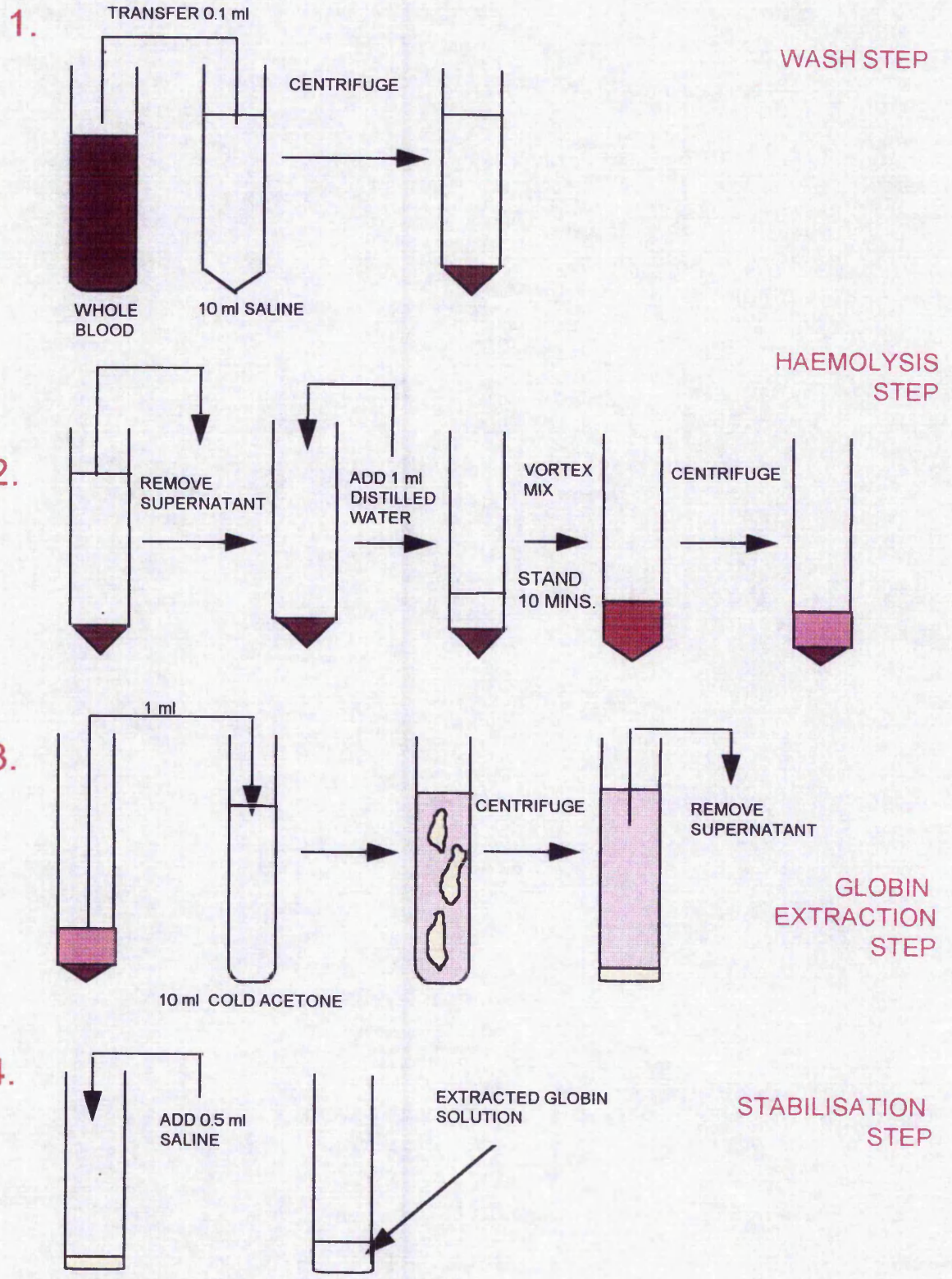
Samples of whole blood were collected into tubes containing ethylene diamine tetra-acetic acid by venepuncture and stored at 4°C. Whole blood (0.1 mL) was added to 10 mL of 0.9 g/L sodium chloride solution and mixed by inversion until the erythrocytes were suspended evenly in the saline. After centrifugation at 2500 g for 5 minutes the supernatant was discarded. To haemolyse the cells 1 mL of distilled water was added, followed by vigorous vortex mixing. After allowing the haemolysate to stand for 15 minutes it was centrifuged at 2500 g for 5 minutes. 1 mL of the supernatant was added dropwise to 10 mL ice cold acetone containing 50 µL 1 mol/L hydrochloric acid. After standing for 30 minutes the mixture of precipitated globin and haem solution was centrifuged for 10 minutes at 1000g. The supernatant was again discarded and the precipitated globin was immediately redissolved in 0.5 mL of 0.9 g/L sodium chloride solution. Figure 2.1 summarises the procedure.

**FIGURE 2.1**

Summary of the extraction of globin from whole blood

Whole blood is washed in 10 mL saline, haemolysed and then added to acidified acetone which precipitates globin.

# FIGURE 2.1 PREPARATION OF GLOBIN EXTRACT



### 2.2.2 Measurement of total globin

Globin concentration was measured by a Biuret method. 40 µl of globin extract prepared as in Section 2.2.1 was added to 250 µL of biuret reagent which contained 14.3 mmol/L copper sulphate, 38 mmol/L potassium sodium tartrate, 36 mmol/L potassium iodide and 700 mmol/L sodium hydroxide. The method was calibrated using commercial human serum (supplied by UK NEQAS, Wolfson Research Laboratories, Birmingham) to which consensus values for total protein had been assigned. After reconstituting the freeze-dried calibrant material in the recommended volume of distilled water, it was further diluted 1 in 5 before use.

### 2.2.3 Measurement of glycated globin

#### 2.2.3.1. Reagent preparation

The method of Somani et al (1989) was used as a starting point for modification. This reagent contained 0.2 mmol/L carbonate buffer, 0.5 mmol/L NBT and 2 mmol/L CMB. For the initial studies a diluent reagent and a dye reagent was prepared. The diluent reagent contained 75 mmol/L of sodium bicarbonate and 225 mmol/L sodium carbonate. The dye reagent was prepared by mixing equal volumes of solution A (NBT 3 mmol/L) and solution B (6 mmol/L CMB, 150 mmol/L sodium carbonate and 450 mmol/L sodium bicarbonate). However, Somani approximately halved the final concentration of his reagent by adding 9 g/L sodium chloride in a 1:1 ratio (v/v) Based on previous published recommendations (Hill et al 1990), on the fructosamine assay the NBT concentration was increased to a final concentration of 0.5 mmol/L NBT and the bicarbonate buffer was increased to 0.2 mmol/L. The concentration of CMB and detergent in the following experiments was varied by altering the concentration of a diluent reagent containing varying

concentrations of Triton X-100 and a dye reagent prepared by mixing equal volumes of solution A (NBT 3 mmol/L) and solution B containing varying concentrations of CMB. Preparation of NBT in unbuffered water prevents photo-oxidation of NBT to the coloured, aqueous insoluble coloured formazan. The diluent reagent, dye reagent and globin extract were mixed in equal volumes by the Mira S analyser at the beginning of the test.

#### 2.2.3.2 Preventing precipitation of globin during NBT reduction

Varying concentrations of Triton X-100 were added to the reaction mixture to prevent precipitation of globin. Triton X-100 was added to reagent B at final concentrations of 0, 7.5 and 15 g/L. The concentration at which protein precipitation was prevented was determined by observing the shape of the curve which results from plotting absorbance measurements at 550 nm against time. This experiment was conducted with the concentration of CMB set at 2 mmol/L.

The effect of detergent concentration upon the regression line describing the relation between glycated haemoglobin and glycated globin in a series of 32 specimens from a routine diabetic clinic was determined. The intercept and the residual error of regression of the glycated globin method were measured at 7.5 g and 15 g/L.

#### 2.2.3.3 Determination of Optimum CMB concentration

Optimum CMB concentration was determined by preparing reagent B (see Section 2.2.3.1) at four different concentrations (12, 6, 3 and 1.5 mmol/L CMB). This led to final concentrations in the reaction mixture of 2.0, 1.0, 0.5 and 0.25 mmol/L CMB). The kinetics of the absorbance change with time were recorded. The Triton X-100 concentration was kept constant at 7.5 g/L.

### 2.2.3.4 The effect of altering pre-incubation time

Forty five blood samples from a routine diabetic clinic were used to study the effect of varying the pre-incubation time of the assay. Pre-incubation time is defined as the time that elapses between mixing the reactants and taking the first absorbance reading. The same reagents and calibrants were used to analyse the globin extracts three times at pre-incubation times of 0, 5 and 10 minutes. The equation describing the regression line between affinity chromatography (Gould et al 1982) and glycated globin was calculated using the Deming modification of the standard least squares regression method. To check that the slope estimate was valid and not unduly influenced by outliers, the 'outlier resistant slope' was calculated using the statistical package 'Minitab' (Clecom Ltd., Birmingham). The outlier resistant slope method is based on the mean of multiple slope estimates of paired data.

### 2.2.3.5 Minimizing miscellaneous sources of interference and error in the preparation of globin from whole blood samples

Four possible sources of error and imprecision are likely to occur during the preparation of globin and its subsequent assay as glycated globin.

1. Failure to remove plasma adequately during the washing step.
2. Failure to remove extracted haem from globin prior to redissolving globin.
3. Interference from superoxide radicals contained in white blood cells.
4. Denaturation of preciptated globin on exposure to air.

The washing step is designed to remove plasma from whole blood. Plasma contains glycated proteins of which albumin is the most important (Johnson et al 1983). Serum must therefore be removed from the sample prior to haemolysing the erythrocytes. To determine

the contribution of plasma reducing agents to the glycated globin assay, 100 μL of whole blood from a poorly controlled diabetic was diluted in 1mL - 10mL of 154 mmol/L saline. After centrifugation 85 μL of supernatant was analysed for glycated globin according to the conditions for the optimised assay (Table 2.6).

After step 3 in Figure 2.1 extracted haem dissolved in acetone was removed from the precipitated globin by inverting the centrifuged mixture and discarding the haem/acetone supernatant. To determine whether residual haem/acetone interfered with glycated globin measurements, 10-100 μL of haem/acetone supernatent was added to 500 μL reconstituted globin prepared according to Figure 2.1. The globin/haem/acetone mixtures were assayed for glycated globin according to the conditions for the optimised assay (Table 2.6). As a control, 10-100 μL of 154 mmol/L saline was added in place of haem/acetone.

Denaturation of extracted globin was assessed by interrupting the assay between the last two steps of the stabilisation procedure (Figure 2.1) for increasing lengths of time.

The extent of denaturation was assessed by visually assessing the extent to which a brown discolouration developed and by assessing whether the globin dissolved in saline.

Assessing the effect of an increased population of white blood cells was achieved by measuring glycated globin on six non-diabetic individuals with blood leukocyte >11,000/μL before and after removing the buffy layer. Removal of the buffy layer reduced the leukocyte count to <4000/μL in all cases.

#### 2.2.3.6 Calibration

No standard preparations of glycated globin are available commercially. Consequently, the values assigned to the assay calibrators are arbitrary. Consensus values for serum fructosamine had been established for human serum lot HIQC13 (Wolfson

Research Laboratories, Birmingham). These were based on values assigned using the synthetic compound 1-Deoxy-1-morpholino-D-fructose (DMF) as a calibrator and are therefore expressed as concentration units of DMF.

The reducing activity of glycated globin is therefore related to the reducing activity of DMF in the serum fructosamine assay. HIQC13 was diluted 1 in 10 and assigned a value of 0.38 mmol/L.

#### 2.2.3.7 Assessment of assay imprecision

Imprecision was estimated by obtaining 10 mL of blood from two diabetic patients and one non-diabetic patient. Each blood specimen was put through the extraction procedure as far as the end of the haemolysis step (see Figure 2.1). To assess within batch imprecision, 30 samples at a time (at each level of GHb) were put through the extraction and stabilisation steps and analysed with a single batch for glycated globin. To assess between batch imprecision, samples were frozen after haemolysis and stored until a batch could be performed on the Cobas Mira autoanalyser. At the beginning of each batch, one haemolysate at each level was thawed then put through the final two stages of the extraction procedure. Batches were calibrated freshly against diluted pooled serum (see Section 2.2.3.6).

## 2.3 RESULTS

### 2.3.1 Preventing precipitation of globin

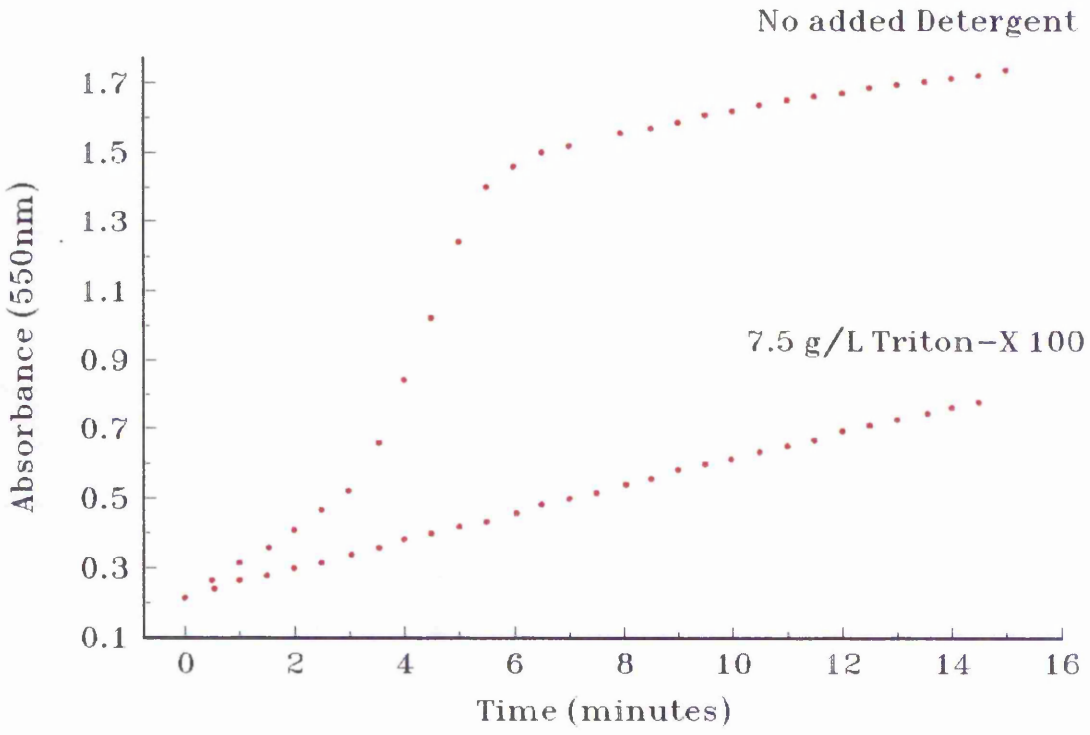
When performed according to the reaction conditions described in section 2.2.3.2, in the absence of detergent, the absorbance change was at first rapid, reaching a point after 5 minutes where it decelerated markedly (Figure 2.2). Examination of the reaction cuvettes

after 20 minutes revealed that a thick precipitate had formed. Adding Triton X-100 detergent and mixing caused the precipitate to re-dissolve.

The regression data describing the relationship between affinity chromatography and glycated globin with Triton X-100 at 7.5 and 15 g/L are summarised in Table 2.1 on Page 41.

**FIGURE 2.2 Effect of detergent on reaction kinetics**

A typical reaction course for glycated globin mediated reduction of NBT with and without Triton-X detergent, using the same glycated globin extract. The slope of the curves varied with glycated globin concentration. Where no detergent was added a steep increase in the rate of absorption at 550 nm always occurred. This coincided with the observation of turbidity in the reaction cuvette.



### 2.3.2 Determination of Optimum CMB concentration

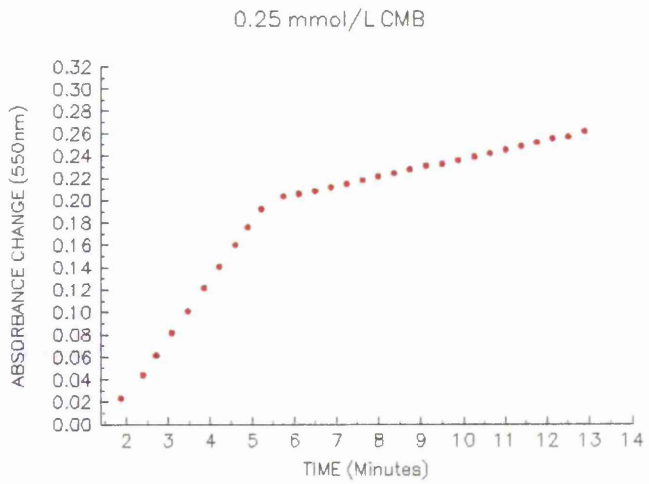
Prevention of globin precipitation did not lead to a continuous monotonic increased in absorbance. Figure 2.3 shows that at CMB concentrations below 2 mmol/L a change of slope occurs at approximately 3.5 minutes after monitoring the reaction has commenced. (13.5 minutes after the reaction started). At 2.0 mmol/L CMB the reaction kinetics became monotonic. Figure 2.3 shows the changes in absorbance.

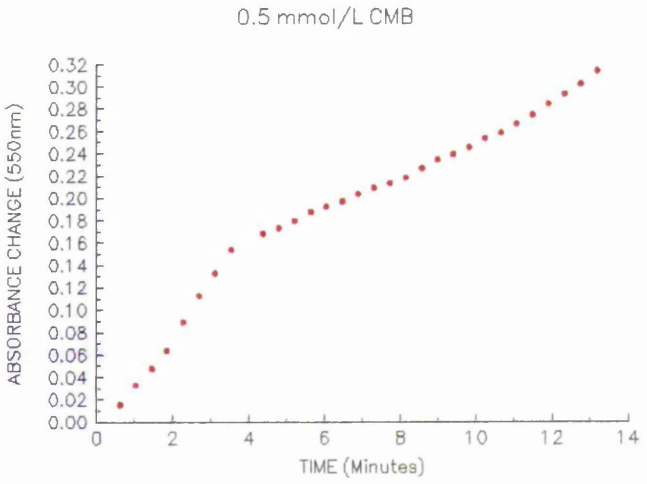
### 2.3.3 The effect of altering pre-incubation time

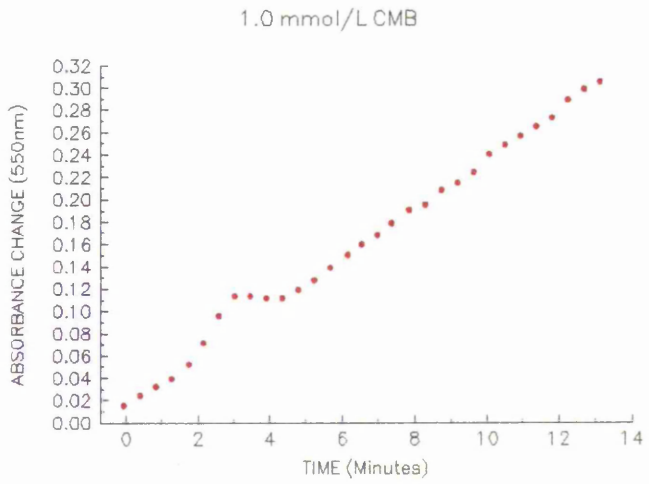
The time which elapsed between mixing the reactants and taking the first absorbance reading had a profound effect on the regression equation which described the relationship between GHb, measured by affinity chromatography (Gould et al 1982), and glycated globin. The regression data for pre-incubation times of 0, 5 and 10 minutes are shown in Table 2.2. Otherwise the conditions of reaction were as for the optimised assay (Table 2.6). The correlation coefficient is significantly lower, the slope is smaller and the scatter around the regression line is considerably greater when no pre-incubation time is used in the assay compared to 5 minutes pre-incubation. Increasing the pre-incubation time beyond 5 minutes has little effect on the regression slope or intercept. As a result of these findings 5 minutes was chosen as the pre-incubation time for the total assay. Figure 2.4 shows a scattergram of the relationship between GHb and glycated globin under optimised assay conditions (2 mmol/L CMB, 7.5 g/L Triton X-100 and 5 minutes pre-incubation time).

**FIGURE 2.3 Effect of CMB concentration on reaction kinetics**

The time course of absorbance change of glycated globin mediated NBT reduction at sub-optimal (0.25-1.0 mmol/L) and optimal (2.0 mmol/L) CMB. These results were obtained on the same globin extract. All patients showed a typical 'hump' between 3-5 minutes at 1.0 mmol/L CMB.


0.25 mmol/L CMB
ABSORBANCE CHANGE (550nm)
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
2 3 4 5 6 7 8 9 10 11 12 13 14
TIME (Minutes)


0.5 mmol/L CMB
ABSORBANCE CHANGE (550nm)
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0 2 4 6 8 10 12 14
TIME (Minutes)


1.0 mmol/L CMB
ABSORBANCE CHANGE (550nm)
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0 2 4 6 8 10 12 14
TIME (Minutes)

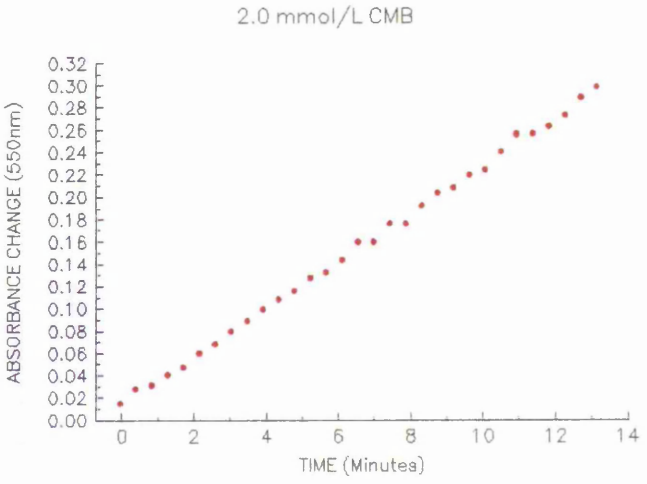

2.0 mmol/L CMB
ABSORBANCE CHANGE (550nm)
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0 2 4 6 8 10 12 14
TIME (Minutes)

**FIGURE 2.4 Scattergram showing the relationship between glycated globin and GHb measured by affinity chromatography**

Data is taken from a single batch of both assays performed on the same day.

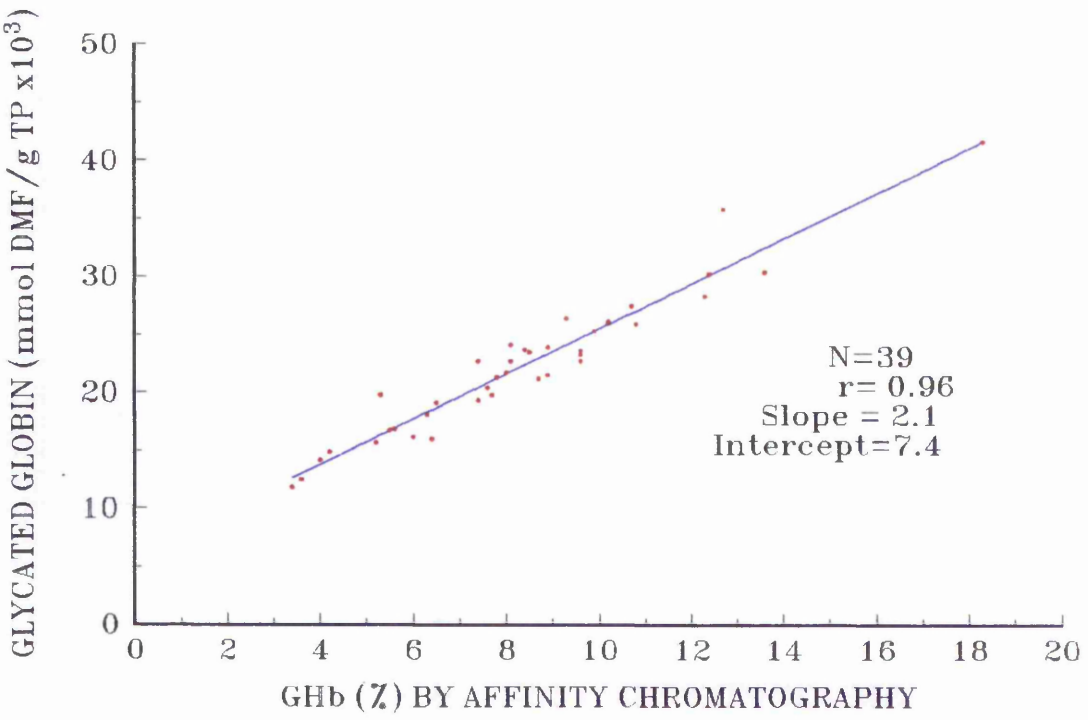


**TABLE 2.1**

Regression data for GHb (affinity chromatography) v glycated globin at 7.5 g/L and 15 g/L Triton X-100 (n=32)

| Triton-X-100 g/L | Slope | Intercept (% GHb) | RER | r | $r^2$ |
|---|---|---|---|---|---|
| 7.5 | 1.92 | +6.0 | 2.5 | 0.94 | 0.88 |
| 15 | 1.89 | +5.9 | 3.8 | 0.81 | 0.66 |

**TABLE 2.2**

Regression data for GHb (affinity chromatography) v glycated globin at 0, 5 and 10 minutes pre-incubation (n=45)

| Pre-incubation time (mins) | Slope | Intercept (% GHb) | RER | r | $r^2$ |
|---|---|---|---|---|---|
| 0 | 1.2 | 6.3 | 3.0 | 0.78 | 0.61 |
| 5 | 2.1 | 7.4 | 1.9 | 0.96 | 0.92 |
| 10 | 2.0 | 5.9 | 1.6 | 0.97 | 0.94 |

RER Residual Error of Regression
r Correlation Coefficient

### 2.3.4 Minimising miscellaneous sources of interference and error in the preparation of globin from whole blood samples

Interference from plasma-derived glycated proteins was measurable in the supernatant when 100 μL blood was diluted in 1 mL 154 mmol/L saline. When 10 mL saline was used the effect of plasma became undetectable (Table 2.3). The effect of residual haem/acetone was minimal at all dilutions (paired t test $t = 0.85$ $p = 0.31$, Table 2.4). Measurement of samples with raised white cell counts before and after removing the buffy layer showed no significant difference (paired t test $t = 1.14$ $p = 0.31$, Table 2.5). Removal of white cells was therefore unnecessary.

**TABLE 2.3 The effect of adding various volumes of saline wash solution prior to haemolysis of erythrocytes during the glycated globin assay.**

After adding a variable volume of saline to 100 µL of whole blood, the sample was centrifuged and an aliquot of the supernatent containing diluted serum protein was assayed for glycated globin.

| Volume saline wash (mL) | Glycated globin (mmol/g Total Protein) $x10^3$ |
|---|---|
| 1 | 2.5 |
| 2 | 1.5 |
| 3 | 1 |
| 4 | 0.2 |
| 5 | -0.1 |
| 10 | 0.2 |
| 30 | -0.1 |

**TABLE 2.4 The effect of adding haem-acetone to extracted globin upon the glycated globin assay**

Glycated globin was measured after adding various volumes of haem-acetone to a 500 μL extract of globin. As a control, the same volume of saline was added to another 500 μL sample of the same extract.

| | GLYCATED GLOBIN (mmol/g Total Protein x $10^3$) | |
|---|---|---|
| **mL added** | **haem/acetone** | **saline** |
| 10 | 25.4 | 24.8 |
| 20 | 23.2 | 24.3 |
| 30 | 24.2 | 23.9 |
| 40 | 24 | 23.5 |
| 50 | 23.2 | 22.8 |
| 100 | 22.2 | 21.6 |

**TABLE 2.5**

**The effect of the presence of pathological numbers of white cells on the glycated globin assay**

| GLYCATED GLOBIN (mmol/g Total Protein x $10^{3)}$ | | |
|---|---|---|
| **Sample number** | **Buffy layer intact** | **Buffy layer removed** |
| 1 | 17.9 | 17.7 |
| 2 | 16.2 | 17.5 |
| 3 | 16.8 | 16.5 |
| 4 | 16.3 | 16.2 |
| 5 | 15.2 | 15.8 |
| 6 | 15.8 | 16.2 |

### 2.3.5 Assessment of assay imprecision

Table 2.4 presents the imprecision data. Imprecision has been estimated at three different concentrations of glycated globin. The GHb result for each patient was measured by affinity chromatography in duplicate.

**TABLE 2.6**

**Summary of reaction conditions for the optimised glycated globin assay**

| **Reagent Concentrations** | |
|---|---|
| p-chloromercuribenzoic acid | 2 mmol/L |
| Nitroblue tetrazolium | 0.5 mmol/L |
| Triton X-100 | 7.5 g/L |
| Bicarbonate buffer pH 10.35 | 0.2 mmol/L |
| **Temperature** | 37°C |
| **Preincubation Time** | 5 minutes |

**TABLE 2.7**

**Imprecision data for the optimised glycated globin assay (n=30)**

| **GHb (%)** | **Mean glycated globin (mmol/g Total Protein x $10^{3}$)** | **CV(%) for determination of glycated globin** | |
|---|---|---|---|
| | | **Within batch** | **Between batch** |
| 5.0 | 16.7 | 7.6 | 8.5 |
| 10.2 | 25.7 | 3.1 | 4.2 |
| 12.8 | 32.9 | 2.0 | 3.5 |

## 2.4. DISCUSSION

Preparation of a globin extract in the manner described in Section 2.2.1 was intended to remove all interfering compounds present in plasma by washing centrifuged erythrocytes with saline. After haemolysis, globin was selectively precipitated facilitating the removal of low molecular weight compounds and other non-globin proteins which were present within the erythrocyte. The purity of the globin precipitation will affect the specificity of the glycated globin assay. Any other proteins which are either co-precipitated with globin or are associated with erythrocyte membrane ghosts will contribute to the NBT reducing activity. Two possible problems in using this approach were tested. Firstly, traces of the haem/acetone extract undoubtedly persisted in the final globin preparation. Table 2.4 demonstrates that the presence of haem/acetone in small quantities was unlikely to affect the glycated globin results. Secondly it was possible that superoxide radicals known to be generated by phagocytes (Taussig 1979) might persist after washing. Superoxide radicals are known to reduce NBT at alkaline pH (Jones et al 1987). Non-diabetic patients with high white counts showed similar results whether or not the buffy layer was removed prior to extraction (Table 2.5).

The patient numbers in both these experiments were small. Consequently quite large differences would be required before statistical significance was achieved. Nonetheless the differences observed in the above comparisons are small so it is reasonable to assume that the conclusions are valid.

Addition of detergent to NBT reducing systems has been advocated by Siedel et al (1988) and forms part of the most commonly used reagent formulation for serum fructosamine (Roche Ltd). The precise nature of the detergent mixture used by Siedel et

al has not yet been published. There is one report of Triton X-100 being used in NBT reduction systems (Phillipou et al 1988). Precipitation of globin during the NBT reaction was recognised as a problem by Somani et al (1989) who pre-warmed reagents and observed that despite precipitation of globin when all the reactants were initially mixed, the precipitate re-dissolved after a short time interval. During automated analysis, precipitation of reagent adds a variable which is difficult to control and is unacceptable. The correlation between GHb (affinity chromatography) and glycated globin was better at 7.5 g/L detergent than at 15 g/L ($r = 0.94$ and $r = 0.81$ respectively). Calculation of the coefficient of determination ($r^2$) gives an indication of the extent to which the variation in glycated globin is directly related to the variation in GHb. Thus at 15 g/L detergent where $r^2 = 0.65$, 65% of the variation in glycated globin is accounted for by the variation in GHb which implies that 35% of the variation in glycated globin is caused by other factors. These other factors include analytical imprecision, intra-individual variance and analytical non-specificity. At 7.5 g/L the glycated globin assay performance improves ($r^2 = 0.88$). At 7.5 g/L detergent the contribution of non GHb factors is reduced to 17%.

A pre-incubation period increases the specificity of the fructosamine assay (Johnson et al 1983). The data collected in Section 2.3.3 suggest that increasing the pre-incubation time beyond 5 minutes does not confer significant extra benefit to the assay. ($r^2$ is not greatly increased).

Determining assay specificity by comparison with another (presumed specific) assay for glycated haemoglobin is based on the assumption that the presence of a significant intercept on the regression line indicates non-specificity. This approach was used by Schleicher et al (1988) to demonstrate that serum fructosamine was not entirely specific for

glycated proteins. They hydrolysed serum proteins in a series of diabetic patients then measured hydrolysed glycated lysine by high performance liquid chromatography as a reference technique. Results for this technique were then compared with fructosamine and the regression line was calculated. By comparing the intercept value (i.e. the value of fructosamine when glycated lysine was theoretically at zero concentration) with fructosamine values in patient samples they showed that up to one third of the NBT reducing activity of non-diabetic patients was not due to glycated proteins. This finding led to a criticism of the assay and a reformulation of the reagents which included detergents (Siedel et al 1988). The new fructosamine reagent system reduced non-specific interference to approximately one quarter of the NBT reducing activity of non-diabetic patients. The optimised glycated globin assay demonstrates an intercept of 6.0 when a patient with GHb of 7.0% shows a glycated globin of approximately 20. Thus the non-specific component of the glycated globin assay comprises approximately one third of the glycated globin of a non-diabetic subject.

Davie et al (1993) have shown that the non specific component of fructosamine is constant and varies very little between individuals. This observation, if confirmed, raises the possibility of blanking both the fructosamine assay and the glycated globin assay and thus subtracting the non-specific components. Kricka et al (1991) demonstrated that boronates inhibit the reaction between glycated proteins and NBT. On theoretical grounds they have argued that this inhibition is due to specific binding of the boronate to the ketosamine cis 1, 2, diol groups. This observation may lead to the development of a sample blank procedure which could enhance the specificity of NBT based assays for glycated globin. Unfortunately, this publication did not appear until after the glycated globin

analyses had been started. Because the long term stability of samples stored for glycated globin analysis had not been tested, it was considered that it was better to continue with the method without a blank procedure.

The nature of the non-specific reducing activity of serum fructosamine is unknown. It is clear that the source of the non specific reducing activity in the glycated globin assay must be the globin molecule itself or a high molecular weight contaminant which has co-precipitated with globin. All low molecular weight compounds will have been removed by the washing and precipitation steps. The globin extraction technique is moderately labour intensive. Automated sample processing equipment would reduce the labour involved and might enhance the precision of the assay. The potential advantages of the glycated globin assay however, are that it should be highly reproducible and only needs equipment which is commonly available in a well-equipped clinical chemistry laboratory.

Using a 5 minute incubation period with a 10 mL saline wash, the effects of residual haem/acetone and pathologically increased white cell numbers are minimal. The small numbers used in the statistical analysis of the data presented in Tables 2.4 and 2.5 limit the size of any effect that can be detected. With such small numbers only relatively large differences can be shown to be statistically significant. However, the raw data suggest that within the limits tested the interference encountered from these factors will be small. A report on these findings has been published (Hill 1990).

## 2.5 CONCLUSION

The presence of a significant intercept on the regression line describing the relationship between glycated globin and GHb (affinity chromatography) is in contrast to an earlier report of a manual glycated globin method based on NBT reduction (Somani

et al 1989). It is difficult to know whether this is due to the modifications made to the glycated globin technique or to factors relating to the affinity chromatography method. Nevertheless, this observation suggests that the non-specific component of glycated globin may limit the technique's ability to detect small changes in GHb by increasing the intra-individual variation of measurement. It is possible to measure the intra-individual variation of the technique but this would require multiple venepunctures of around 20 individuals (Fraser 1983).

To avoid the possibility that the clinical trial might be affected by an insensitive outcome measurement, it seemed prudent to include GHb (measured by affinity chromatography) and fructosamine as additional outcome measures.

# SECTION 3

# DEVELOPMENT OF METHODS FOR MEASURING URINE ALBUMIN

## 3.1 GENERAL CONSIDERATIONS

The diversity of methodology for measuring urine albumin allowed a choice to be made on the grounds of both analytical performance and aspects such as cost, simplicity of operation and convenience. The instrumentation available included a gamma counter (NE1612, N E Technology, Reading), a time resolved fluorometer (Cyberfluor 615, Cyberfluor Inc, Toronto) and an automated spectrophotometer-based kinetic analyser (Cobas Mira S, Roche Diagnostics, Welwyn Garden City). These instruments made available radio-immunoassay, time-resolved fluoroimmunoassay and kinetic immuno turbidimetry techniques.

## 3.2 DEVELOPMENT OF A COMPETITIVE FIA FOR URINE ALBUMIN

### 3.2.1 Introduction

The principle of time-resolved immunofluorescence is similar to that of other competitive and non-competitive immunoassay systems. While immuno-assays have traditionally used radioactive nuclides, fluoroimmunoassays use non-isotopic fluorophores as labels. In the past fluoroimmunoassays failed to achieve the same sensitivities as radioimmunoassays (Soini and Hemmila 1979; Hemmila 1985).

Although fluorescent labels are detectable at extremely low concentrations, sensitivity of fluorescent assay systems is limited by non-specific background fluorescence caused by overlap of the label emission and the various spectra of interfering substances in the sample matrix. The use of lanthanide chelates as fluorophores largely overcomes these problems. The favourable characteristics of lanthanide chelates include a long lived fluorescent time, a wide Stoke's shift and a narrow emission band. These characteristics are exploited by the design of the fluorometer used to measure the signal generating during the

assay. The Cyberfluor 615 immunoanalyser uses a nitrogen laser to flash excite lanthanide chelate labelled antigen every 50 milliseconds. Fluorescence counting is delayed until 200 microseconds after excitation thus allowing non-specific fluorescence, which typically has a short lived fluorescence time (<200 microseconds), to dissipate. The chelate's large Stoke's shift is exploited by exciting the fluorophore at 337.1 nm and counting fluorescence at 615 nm. Most compounds which produce background fluorescence will emit at much lower wavelengths than 615 nm when excited at 337.1 nm. The narrow emission band allows the detection systems to operate over a narrow wavelength 'window' thus excluding emission from other compounds at other wavelengths.

Labelling antigen or antibody is the main production problem with immunoassay reagents. The phenanthroline derivative 4,7 bis (chlorosulfophenyl) 1,10 phenanthroline 2,9 dicarboxylic acid (BCPDA) is a suitable chelate for the lanthanide ion europium. The two carboxyl groups and the two heteroaromatic nitrogen atoms on BCPDA form a high affinity chelating site for europium ions. The two sulphonyl chloride groups provide a covalent attachment point for streptavidin. Biotinylated antibodies will readily bind the BCPDA-Europium-streptavidin complex thus forming fluorescence-labelled antibody.

To develop a competitive immunoassay, microtitre plates were coated with antigen at varying concentrations. Labelled antibody at concentrations varying over a wide range was added to the coated microtitre plate in the absence of competing antigen to establish the maximum change in fluorescence which was obtained on the different coating concentrations. An antigen coating concentration which gave an appropriate slope response was chosen. The antibody concentration at half the maximum response on the chosen curve was used as the limiting antibody concentration, i.e. the antibody concentration chosen for the assay.

### 3.2.2 Materials and methods

#### 3.2.2.1 Preparation of human albumin-coated microtitre plates

Albumin solution obtained from Elstree Blood Products Ltd (45 g/L) was diluted in 0.01 M Tris-buffered saline pH 7.8 to a concentration of 0.1 mg/L (1 in 45000). 200 µL of well coating solution was added to each one and incubated at ambient temperature (23°C) overnight. After washing the wells four times with 0.05 M Tris-buffered saline pH 7.8 containing 0.05% (w/v) Tween 20 detergent, 200 µl of blocking buffer was added to each well to saturate the remaining active sites. The blocking buffer was 0.05M Tris-buffered saline pH 7.8 containing 1% (w/v) caseine. The plate was stored overnight at 4°C and used the following morning.

#### 3.2.2.2 Preparation of biotin-labelled anti-human albumin antibody

Nephelometry grade anti-human albumin (PN 032 The Binding Site, Birmingham) was diluted to a concentration of 3 g/L with 0.15 M sodium chloride . The solution was dialysed twice against 4 L of saline solution. To 5 mL dialysed antibody solution, 1.7 mL of 1.0 M sodium carbonate buffer pH 9.1 was added and stirred for 10 minutes. The pH of the mixture was adjusted to lie within the range 8.5-9.1. 200 µl of dimethyl sulphoxide containing 30 mg of sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin) was added and stirred continuously for 1 hour at room temperature. After dialysis of the biotinylated antibody, as described above, the biotinylated antibody was diluted 1 in 1000 in 0.01 M Tris-buffered saline pH 7.8.

### 3.2.2.3 Optimisation of microtitre plate coating with albumin

Human albumin was bound to microtitre plate wells by incubating buffered albumin (0.05, 0.01, 0.001 g/L) as described in Section 3.2.2.1. Biotinylated anti-human albumin antibody was added at a range of dilution sufficient to determine the maximum signal that could be generated at each of the three concentrations of well-bound albumin. The plate was incubated on a microtitre plate shaker for 60 minutes. At the end of the incubation period the plates were washed in Tris-buffered saline pH 7.8 containing 0.5 mL/L Tween 20. Following this, 100 µl of Eurofluor-S Tracer solution (Cyberflour Inc, Toronto, Canada) were added to each well and incubated with shaking for 30 minutes. After incubation the wells were air dried then read in a time resolved fluorimeter.

### 3.2.2.4 Optimisation of antibody concentration

Having chosen a suitable albumin coating concentration, the antibody concentration for the assay was taken as that which gave 50% maximum signal. The characteristics of the assay were then assessed. Dilutions of albumin were made to cover the ranges 0-1000 mg/L. 20 µL of each dilution was applied in duplicate to the albumin coated microtitre plate wells. 100 µL of biotinylated antibody diluted 1 in 630 was added to each well. The microtitre plate was incubated on a shaker at ambient temperature for 60 minutes. After washing three times with Tris buffered saline, 100 µL of Eurofluor-S was added to each well. After a further incubation of one hour the wells were washed again with Tris-buffered saline and dried before reading in the time-resolved fluorimeter.

### 3.2.3 Results

#### 3.2.3.1 Optimisation of microtitre plate coating with albumin

At a concentration of 0.05 mg/L coating solution a maximal fluorescence signal was obtained in the range of antibody dilution used (1 in 10 to 1 in $10^7$). This compares favourably with other competitive assays which are commercially available as FIAs. There is a pronounced variation between duplicates (Figure 3.1) which leads to the possibility of considerable error both in determining optimum assay conditions and when the assay is used to measure urine albumin concentration. However, there is a discernable maximum plateau which allows estimation of the 100% binding signal for each bound antigen concentration. The less steep curves produced at 0.01 and 0.001 mg/L make these concentrations of bound antigen less suitable for assay.

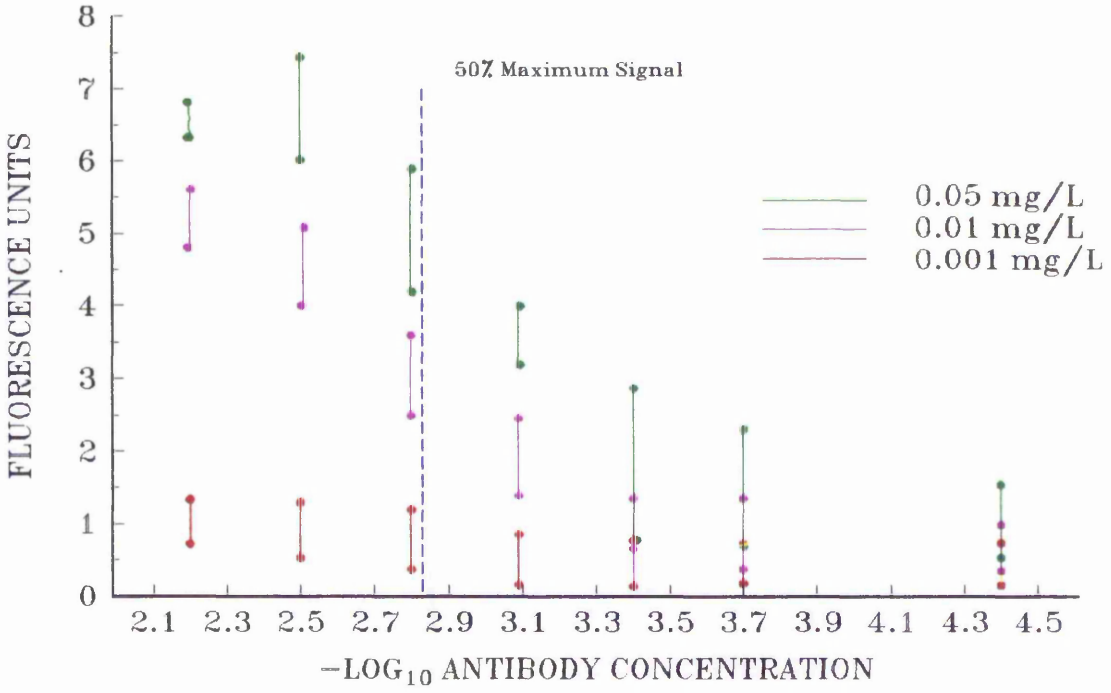
A dilution of 1 in 10 produced approximately 50% saturation of albumin using microtitre plates which had been coated with a solution containing 0.05 mg/L albumin.

#### 3.2.3.2 Optimisation of antibody concentration

A dilution of 1 in $10^{2.8}$ (1 in 631) produced approximately 50% saturation of albumin using microtitre plates which had been coated with a solution containing 0.01mg/L albumin (Figure 3.1). Higher dilutions of antibody (producing 40% and 30% saturation) did not produce curves which were significantly superior. The curve at 50% saturation was approximately linear between 100-1000 µg/L (Figure 3.2).

**FIG 3.1 Titration of albumin coated wells with antibody**

Microtitre plate wells were coated with albumin at three different concentrations. In three separate experiments the fluorescence signal generated by biotinylated antibody in the range (1 in 150 to 1 in 25,000) was recorded. Fluorescence was generated by adding streptavidin bound to the fluorophore Eurofluor-S. The tips of the error bars represent the two individual fluorescence measurements made at each antibody concentration.



### 3.2.3.3. Characteristics of the optimised assay

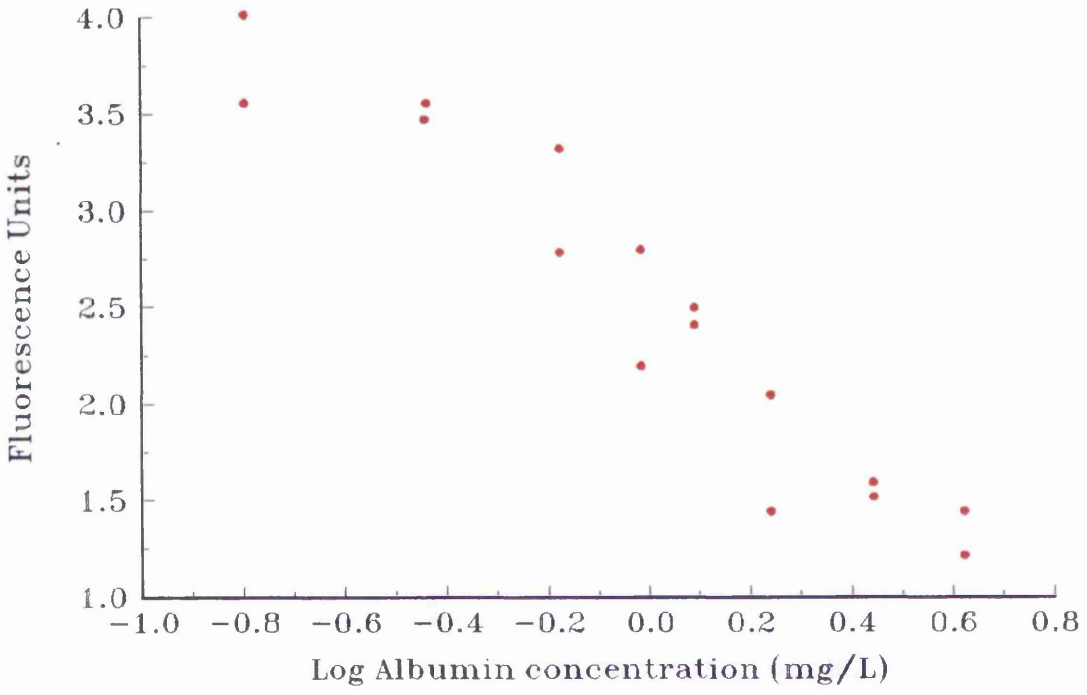
Figure 3.2 shows the dose response curve for the assay over the range 0-3.5 mg/L using the volumes described. By pre-diluting specimens 1 in 40, samples in the range 10-80 mg/L could be measured. This is a fairly narrow working range, but is comparable to the range obtained for radioimmunoassay. However, there is poor agreement between replicates which will lead to unacceptably poor precision of measurement. This method is discussed further in Section 3.5.

### 3.2.4. Discussion

The data presented here suggest that although time resolved fluoroimmunoassay has potential as a measurement system for assessing microalbuminuria, there was an inherent imprecision as illustrated in Figure 3.1. This may have been due to several factors including inconsistent antigen coating of the microtitre plate, inadequate removal of blocking agents followed by interference during measurement, sampling imprecision or reading imprecision. At this stage of the development it would be usual practice to examine each step of the assay in detail and to determine the step or steps which caused the problem. Unfortunately, the time resolved fluorimeter which had been in routine use within the laboratory was returned to the manufacturer because of imprecision in a TSH assay which was based on time resolved fluorimetry. This prevented further development of the assay. It was then necessary to develop an alternative method for urine albumin. After this work was completed and the equipment was returned, a two site sandwich immunoassay based on time-resolved fluor immunoassay was published using monoclonal antibodies (Diamandis and Ogilvie 1990). A competitive assay for urine albumin using Europium labelled albumin and time-resolved fluoroimmunoassay was described in 1993 with an assay working range of 10-200 mg/L (Nisbet et al 1993).

**FIG 3.2 Dose response curve for urine albumin FIA**

A typical dose response curve for the optimised assay. The plots represent two individual fluorescence measurements made at each albumin concentration



## 3.3 POLYETHYLENE GLYCOL (PEG) ENHANCED IMMUNO-TURBIDIMETRY

### 3.3.1 Introduction

Measurement of the turbidity of the suspension which results from mixing an antigen and antibody in suitable proportions can be used to monitor the process of immunoprecipitation. When the antibody concentration is held constant at a suitable level, the rate of immunoprecipitation becomes a function of antigen concentration. This principle forms the basis of light scattering techniques for measuring antigen concentration using specific antibodies. Turbidity may be measured by either nephelometry (measuring the intensity of scattered light) or turbidimetry (measuring the intensity of transmitted light). Measuring turbidimetry is often the favoured detection method in clinical chemistry laboratories because sophisticated spectrophotometers are usually readily available.

Polyethylene glycol (PEG) can be used to increase the rate at which insoluble antigen-antibody complexes form. PEG is an inert polymer which is readily hydrated. When mixed with an antibody/antigen mixture, PEG limits the proportion of aqueous phase which is available to render antigen-antibody complexes stable. This has the effect of increasing the effective concentration of both antibody and antigen giving rise to an accelerated reaction. An accelerated reaction leads to an enhanced sensitivity and a higher throughput of assays.

### 3.3.2. Materials and methods

#### 3.3.2.1 Preparation of Reagents

The final reaction solution consisted of a 13 mmol/L phosphate buffer pH 7.3 containing 50 g/L PEG 6000 and 0.1% (v/v) Tween 20 sodium chloride anti-human albumin antibody at a dilution of 2.5% (v/v). The anti-human albumin antibody was supplied by Dako, High Wycome, Bucks. as a nephelometric grade antibody (Q328). This method is described in an application note from the antibody manufacturer.

A diluent solution contained 13 mmol/L phosphate buffer pH 7.3 containing 154 mmol/L sodium chloride.

#### 3.3.2.2 Application of the method to the Miras automated chemistry analyser

The Mira S analyser pre-diluted a single calibrator in diluent reagent, then pipetted diluted calibrator into a cuvette. Calibrations of 0, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00 mg/L were prepared in this manner.

40 µL of urine or diluted calibrator was then aspirated with 30 µL of diluent and placed in a cuvette. 225 µL of a reagent containing PEG 6000 in phosphate buffer was added, followed by 25 µL of antibody solution in phosphate buffer, Final concentrations of the reaction solution were as stated above. Addition of the antibody solution started the reactions and triggered the instrument to commence absorbance measurements.

`The first absorbance reading was taken one second after adding antibody solution. Thereafter 15 readings were taken at twenty second intervals.

### 3.3.2.3. Data Evaluation

The absorbance change during the measurement interval was non-linear. Data reduction was performed by a multiparameter mathematical algorithm which related the rate measurement to the concentration of the human standard (Hoffman la Roche and Co 1989):

$$R = \dot{R}_O + K_C \frac{1}{1+\exp[-(a+b \cdot \ln C+cC)]}$$

R = Rate

$R_o$ = The predicted rate for a standard with zero concentration

$K_c$ = The predicted difference between the rate for a standard with infinite concentration and RO (a scale parameter)

C = Concentration of standard (mg/L)

a b c = Various parameters which define the non-linear elements of each mathematical function.

### 3.3.2.4 Calibration

Behring OTFO 02/03 human serum standard (assigned albumin value 3.24 g/L) and calibration serum obtained from the Protein Reference Unit at the Hallamshire Hospital (assigned albumin value 37.4 g/L) were diluted 1 in 30 and 1 in 300. These materials were assayed using a 1 in 200 dilution of human albumin fraction supplied by the Blood Transfusion Centre (Elstree, Herts) as the calibrator for the assay. A nominal value of 41.5g/L had been assigned to the calibrator based on the stated albumin concentration of the preparation. The result obtained from assaying the reference calibrators was used to assign an accurate value to the Blood Transfusion Centre human albumin which was used as the working calibrator in all subsequent assays.

Reference calibrators could be assayed at regular intervals in this way, to check on the assay's calibration point.

### 3.3.2.5. Optimisation of antibody concentration

Dilutions of antibody varying from 1 in 20 to 1 in 60 were compared with a view to determining the range of albumin concentrations which could be measured without encountering conditions of antigen excess. To define the working assay range, a urine containing less than 30 mg/L albumin was spiked with a solution of albumin in saline to produce a range of albumin values between 10 mg/L and 900 mg/L.

### 3.3.2.6 Assessment of assay imprecision

The within run imprecision of the assay was determined by analysing 20 replicates of urine containing albumin at three different levels arranged around the proposed cut-off point of 30 mg/L. Controls were prepared by spiking a normal urine (albumin <30 mg/L) with a solution of human albumin.

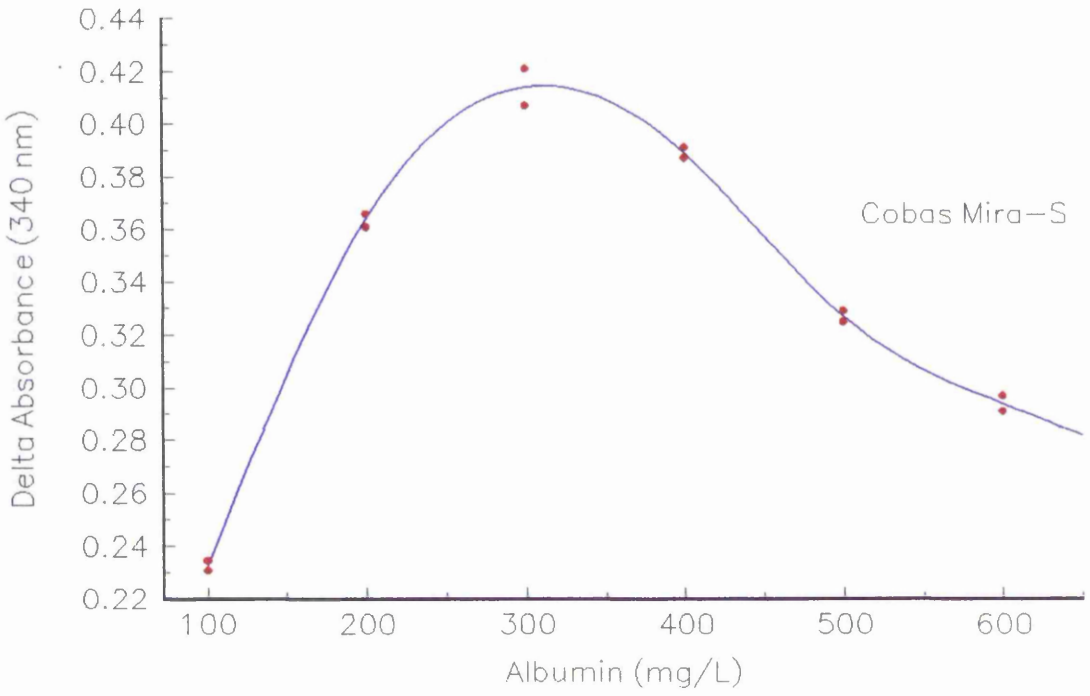
## 3.3.3. Results

### 3.3.3.1 Assessment of an appropriate working range for the assay.

Figure 3.3 shows that at a 1 in 50 dilution of antibody, antigen excess occurs at approximately 300 mg/L. Above 300 mg/L urine samples will test positive with a conventional urine dipstick. By testing urines with a dipstick before testing for microalbuminuria, it is possible to avoid antigen excess conditions with the PEG enhanced assay.

**FIGURE 3.3 Urine albumin by PEG enhanced immunoturbidimetry assay response during antigen excess**

Duplicate measurements of the change in absorbance (assay response) at varying urine albumin concentrations. Antibody dilution 1:50



### 3.3.3.2 Assessment of assay imprecision

Table 3.1 indicates the imprecision calculated as coefficient of variation (%).

**TABLE 3.1 The imprecision of PEG enhanced immunoturbidimetry assay for the urine albumin assay**

| | Mean Albumin (mg/L) | Within run imprecision (CV%) | Between run imprecision (CV%) |
|---|---|---|---|
| Control 1 | 32 | 3.6 | 4.8 |
| Control 2 | 50 | 3.1 | 3.2 |
| Control 3 | 90 | 4.4 | 2.7 |

All values are below CV 5.0%

## 3.4 LATEX ENHANCED IMMUNOTURBIDIMETRY

### 3.4.1 Introduction

Immunoturbidimetry provides a quantitative or semi-quantitative method for assaying antigen in a homogeneous immunoassay. The technique has been applied to the measurement of albumin in urine (Teppo 1982; Harmoinen 1987) and more recently latex beads have been used to enhance the performance of the assay (Viberti and Vergani 1982; Medcalf et al 1990). Latex agglutination confers two main advantages. Firstly, greater absorbance changes confer increased sensitivity. Secondly, the presence of latex beads within the precipitating complex allows the use of lower antibody concentrations thus reducing the assay cost. Currently commercial preparations of antibody coated latex particles are available for only a limited range of analytes. Development of this assay thus required preparation of a stock solution of anti-albumin coated latex particles.

This method has been developed from a method described by Winkles et al (1987). A third advantage of enhanced latex accumulation assays are their claimed validity over a wide range of antigen concentration thus leading to an elimination of antigen excess problems. Antigen excess may be a particular problem when measuring albumin in the urine of diabetics due to the large range of albumin concentration that may be present. The assay must therefore be assessed to determine both the range over which samples can be accurately quantitated (measurement range) and the concentration above which misleadingly low results may arise (safety zone upper threshold). Measurement of the assay precision is necessary to define the lowest concentration of antigen which can be detected.

### 3.4.2. Materials and methods

#### 3.4.2.1. Coating latex particles with antibody

1 mL of latex particles (2.5% 'Polybead' polystyrene microspheres 0.05 μm diameter obtained from Polysciences Inc. Northampton) was added to 10 mL of 200 mmol/L glycine buffered saline pH 8.2. To this mixture 0.1-0.8 mL of 10.2 g/L rabbit anti-human albumin was added. The anti-serum was supplied by Dako Ltd. Particle aggregation occurred resulting in a turbid suspension which was stored at 4°C overnight. Following centrifugation at 4°C and 2500 rpm (2050g), the clear supernatant was discarded. 10 mL of 200 mmol/L glycine buffered saline pH 10.0 and 100 μL 20% (w/v) bovine serum albumin was added as a blocking agent to the latex particle pellet. After careful mixing by inversion the suspension was sonicated at medium power for several periods of 20 seconds until it had cleared. The resulting suspension was diluted to 60 mL with 200 mmol/L glycine buffer pH 10.0.

#### 3.4.2.2. Optimisation of antibody concentration

In order to establish the optimum concentration of antibody with which to coat the latex particles, varying volumes of 10.2 g/L antibody solution were added to a fixed volume of latex particles. The absorbance change at each antibody concentration was measured at two concentrations of albumin.

#### 3.4.2.3. Definition of the assay working range

The assay working range was assessed by preparing solutions of albumin varying from 0-1000 mg/L and examining the dose response curve. To determine the lower end of the working range, two urines with an albumin of 26.5 mg/L and 13.3 mg/L respectively were analysed 30 times on separate calibrator runs.

### 3.4.2.4. Calibration

To assess whether both assays were calibrated identically, four replicates of the Behring reference material OTFO 02/03 were analysed by radioimmunoassay. The radioimmunoassay was performed using the calibrators supplied with the kit. The albumin values obtained by the radioimmunoassay were compared with the values assigned to the Behring reference material.

### 3.4.2.5 Correlation of latex enhanced immunoturbidimetry (PETIA) with radioimmunoassay

38 patient urine samples which tested negative for albumin by albustix were assayed by the PETIA method and a commercial radioimmunoassay method. To these samples varying amounts of human albumin obtained from the Blood Transfusion Centre (Elstree, Herts) was added so that the concentration remained within the 0-100 mg/L concentration range. The range of values found is illustrated in Figure 3.6. The PETIA assay was calibrated using a commercial protein calibrator OTFO 02/03 supplied by Behring Ltd. The radioimmunoassay was supplied by Pharmacia Ltd in kit format which included calibrators. Aliquots of OTFO 02/03 were included in quadruplicate on the radioimmunoassay to allow comparison of calibration points.

### 3.4.3 Results

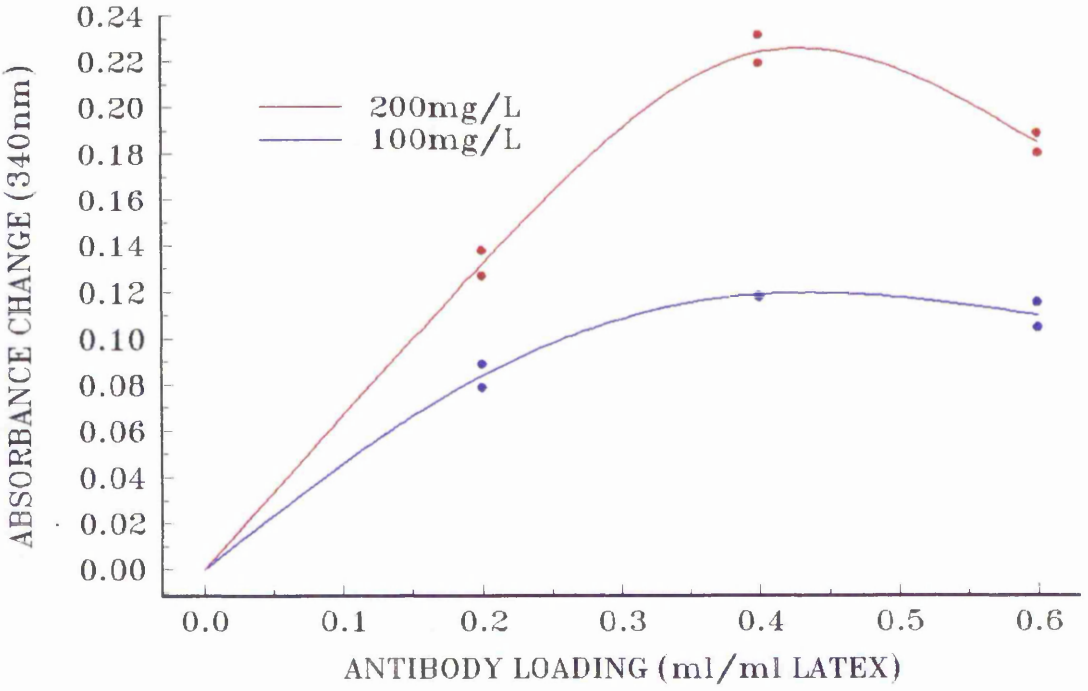
#### 3.4.3.1 Coating antibody concentration

The effect of coating antibody concentration on the assay response is illustrated in Figure 3.4. Increasing antibody concentration increases the response until antibody concentrations exceed 0.4 mL antibody/mL latex. Increasing antibody concentration beyond 0.6 mL antibody/mL latex resulted in an aggregated suspension which could not be resuspended by sonication. 0.4 mL was chosen as the optimal volume of antibody to add to 1 mL of latex.

**FIGURE 3.4 The effect of increasing antibody loading on latex particles**

Duplicate measurements of the change in absorbance at varying antibody loading concentrations and at albumin concentrations of 100 mg/L and 200 mg/L.

Increasing the antibody concentration of the solution used to coat the latex particles from 0.2-0.6 ml/ml latex increases the assay response.



### 3.4.3.2 Working range of the assay

Figure 3.5 suggests that conditions of antigen excess are marked above 500 mg/L. However the dose response curve becomes markedly less steep after 100 mg/L. The analytical variation measured as coefficient of variation was 8.3% and 10.8% at 26.5 mg/L and 13.3 mg/L respectively.

### 3.4.3.3 Comparison of latex enhanced immunoturbidimetry (PETIA) with radioimmunoassay

The mean of four replicates of OTFO 02/03 was 83 mg/L (assigned value to OTFO 02/03 was 81 mg/L). The original value of OTFO 02/03 was therefore accepted for the purposes of this comparison.

Figure 3.6 is a scatter diagram indicating the relationship between the PETIA albumin method and the albumin radioimmunoassay. The Deming slope line was also plotted. At very low concentrations the PETIA assay had a positive bias. Correlation is less good at higher concentrations where the samples were slightly above the assay's working range. The regression equation describes the relationship between the two albumin measurements using the Deming slope estimate is 1.05. The linear regression equation was $y = 1.05x - 0.76$.

However, an alternative outlier resistant method of estimating the slope gave a slope value of 0.90.

Eliminating samples above 100 mg/L reduced the number of outliers in the influential area towards the high concentration end of the sample distribution. This was justified on the grounds that the assay had been shown to be reliable only in the 0-100 mg/L

concentration range. Removal of these data points led to a Deming slope estimate of 0.899 which was closer to the outlier slope estimate (Figure 3.6). The regression equation based on this slope estimate was $y = 0.899x + 3.5$. The positive intercept was reflected in the high % bias observed at low albumin concentration by the difference plot (Figure 3.7). The plot suggests that there is little dose dependent bias over the concentration range 10-100 mg/L.

**FIGURE 3.5 The dose response curve for the latex enhanced immunoturbidimetry assay at 2-5 µL sample size**

The slope of the dose response curve changes as antigen dose is increased. High sample volumes lead to decreasing assay response when sample concentration exceeds 100 mg/L. Plotted points are the mean of duplicate measurements.

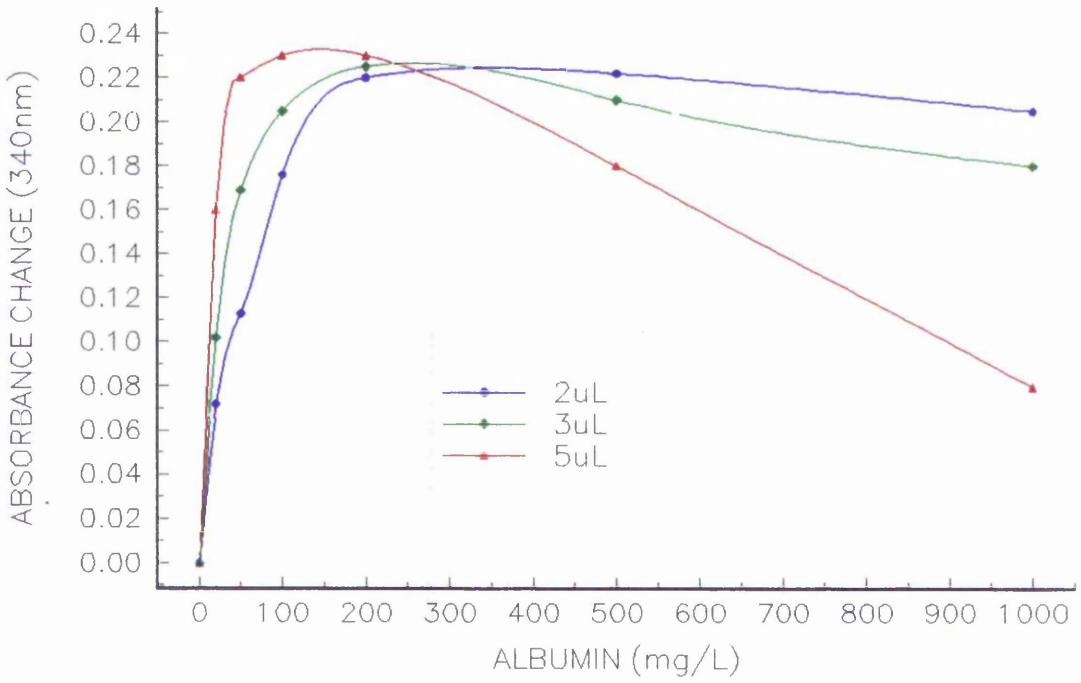


**FIGURE 3.6 Scatter diagram showing the relationship between urine albumin measured by radio-immunoassay and by latex enhanced immuno-turbidimetry and radioimmunoassay.**

No data have been excluded from the analysis. The regression line has been calculated by the Deming modification of the least squares method (Deming 1943)

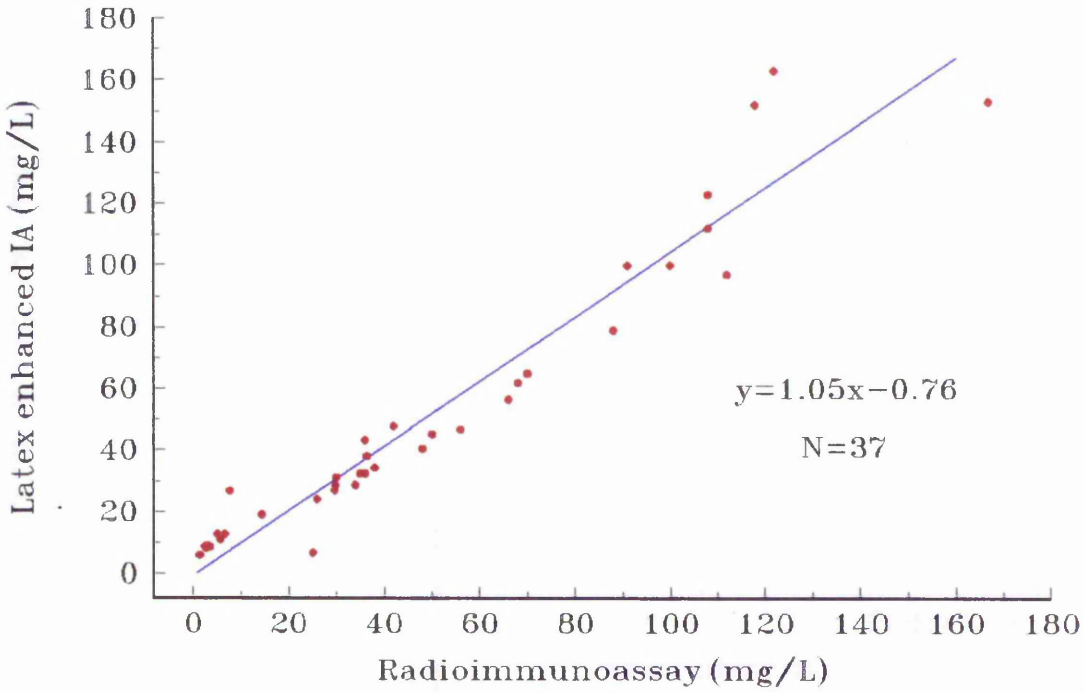
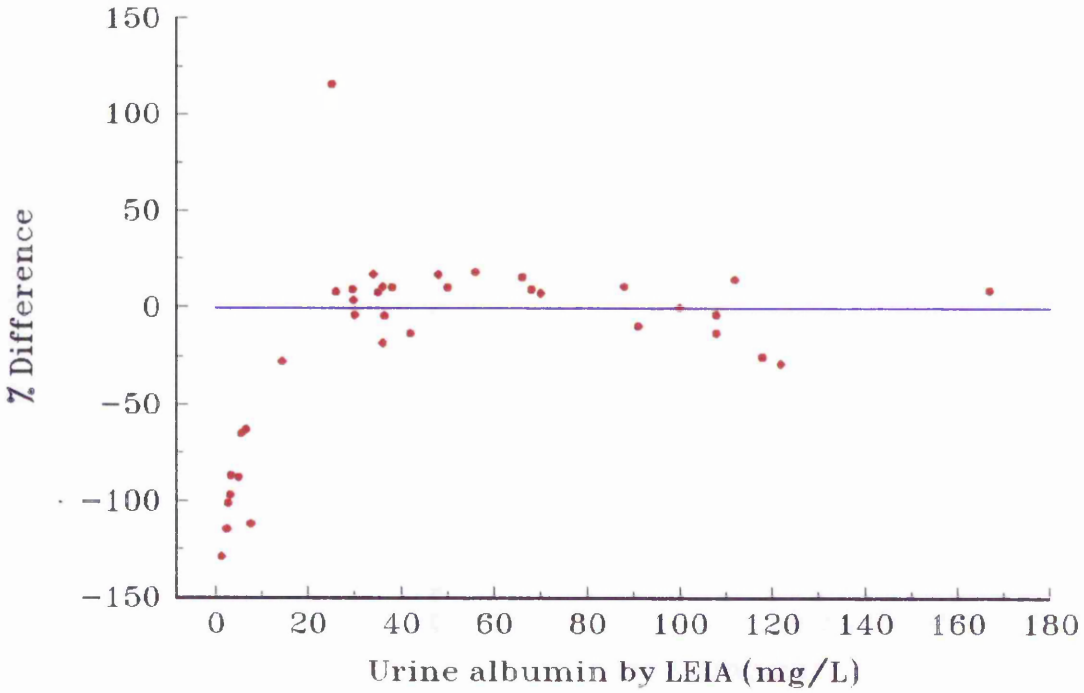


**FIGURE 3.7 Difference plot of urine albumin measured by latex enhanced immunoturbidimetry and radioimmunoassay**

Difference plot of urine albumin measured by RIA v urine albumin measured by latex enhanced immunoturbidimetry. The difference % was calculated as

$[(C_R - C_L)/(C_R + C_L)/2] \times 100$

(Pollock et al 1992)



### 3.4.3.4 Discussion

The above evaluation was carried out on a single batch of latex reagent. Subsequent batches showed a tendency to aggregation after one week of storage at 4°C. This caused logistical problems because there was no sonicator available.

Previous publications have shown optimum responses at pHs around 7.0 which reduce as pH is increased. However, Rheumatoid Factor is known to react with the Fc portion of rabbit IgG at pHs around 7.0. This effect can be reduced at little cost to assay response by performing the assay at pH 10 (Winkles et al 1989) . Although little Rheumatoid Factor would be expected in the urine, rabbit antibodies have been used in this assay, and so the pH has been chosen as pH10 as a precaution against interference from Rheumatoid Factor.

Although most of the analytical performance parameters suggested that the latex enhanced turbidimetry method was adequate for measuring microalbuminuria, the assay has a limited working range. This is surprising considering that a wide measurement range is theoretically one of the advantages of this technique. It is possible that one reason for this is that the reagent was partially aggregated before the assays were performed. Indeed, the optical clarity of a recently prepared batch of latex reagent was always absorbance >1.0 at 310 nm. Further sonification of the reagent did not result in a colloid of greater optical clarity.

In addition, the reagent was not sufficiently stable to allow reproducible results after more than a week of storage. It is likely that there would be considerable reagent wastage, largely cancelling out any cost advantage to using less antibody in the reagent preparation.

A stabilised latex enhanced immunoturbidimetric reagent has been described in which antibody is covalently attached rather than simply absorbed to latex particles (Price et al 1987). This reagent was shown to be stable for six months.

Imprecision of the assay is acceptable at a dose of 13.3 mg/L suggesting that the detection limit of the assay is less than 13.3 mg/L. A maximum of CV = 20% is generally accepted as the functional sensitivity of an immunoassay.

The positive bias of the PETIA assay at low concentrations may be due to a non-specific matrix effect. The differences between the Deming slope estimate and the outlier resistant slope estimate indicates that outliers are having a significant effect on the Deming slope.

## 3.5 CHOICE OF METHOD FOR URINE PROTEIN

The following criteria need to be met for a suitable test for microalbuminuria.

1. High sensitivity (i.e. low imprecision in the range 10-100 mg/L).
2. Freedom from antigen excess problems over the range 10-5000 mg/L.
3. Economical use of antibody to reduce assay cost.
4. Good between run precision leading to imprecision of less than 20% over the range 30-300 mg/L.

The highly sensitive detection system for time-resolved fluorimetry was largely nullified by the poor analytical precision of the assay. Experimentation with different blocking solutions and wash buffers may have resulted in an improved performance. However, imprecision problems with the instrument in other commercially developed assays led to it being returned to the manufacturer as an unsuitable laboratory instrument.

Particle enhanced turbidimetry theoretically combines the advantages of high analytical sensitivity with a wide dynamic range and economic use of antibody. However, for the assay described in Section 3.4.2 the assay dynamic range at maximal latex antibody

coating was 10-500 mg/L. The possible range of urine albumin concentration in diabetic patients is approximately 10 mL/L to 5 g/L (5000 mg/L). Because there remains a proportion of diabetic patients who will have urine albumin greater than the upper limit of the assay working range, the PETIA assay alone will not reliably measure all urines from diabetic patients. Another disadvantage of the PETIA assay is that the preparation of sonicated latex particles requires special equipment. Further, the high background absorbance of the reagent suggests that significant particle aggregation remains despite repeated sonication.

The PEG enhanced immunoturbidmetric assay uses relatively high concentrations of antibody but consists of a very simply prepared reagent. The assay is robust and reproducible. The assay dynamic range is easily extended by adding more antibody to the reagent. To limit the need for large amounts of antibody, a simple dip stick urine test was used to detect any urine samples with an albumin concentration of greater than 300 mg/L. The expense of dipsticks should be largely offset by the fact that each diabetic patient attending the diabetic clinic is screened for proteinuria as a matter of routine. The PEG enhanced immunoturbidmetric assay requires only equipment available in a well equipped clinical biochemistry laboratory. This has the advantage that all reagent preparation on analysis of samples can be undertaken in one laboratory on equipment which is likely to be available over the entire length of this project.

In conclusion the most appropriate urine albumin method for use in the following clinical studies was the PEG enhanced immunoturbidimetry method.

For the reasons discussed above it was decided that the PEG enhanced immunoturbidimetry method would be used to determine urine albumin in the clinical studies of the present project.

# SECTION 4

# CHARACTERISING THE DIABETIC POPULATION

## 4.1 INTRODUCTION

As a preliminary stage to the main clinical study the data collected from patients during the recruitment phase of the study were analysed. The aim was to characterise the diabetic population and to compare the blood pressure, glycated haemoglobin, glycated globin and the incidence of microalbuminuria with published data. Identification of large differences between this population and others would have indicated unusual selection factors operating in the study population which might influence the interpretation of results.

## 4.2 MATERIALS AND METHODS

Patients were recruited from 12 consecutive routine diabetic clinics at King's Mill Hospital. Paediatric patients (aged up to 16 years) and pregnant diabetics did not attend this clinic and were therefore not included in the audit. Patients were given two urine containers at the consultations and asked for two consecutive early morning urine collections which were returned within a week, either directly to the Diabetic Centre or via their General Practitioner. Clinical information was collected by using request forms which had been designed for the study (Appendix 1). In addition to demographic data, patients were classified into insulin-dependent (IDDM) or non-insulin dependent (NIDDM) diabetics based on their current therapy. The duration of each patient's diabetes, other concurrent diseases, grade of retinopathy, height and weight, blood pressure and drug therapy were recorded. Anti-hypertensive agents were recorded separately from other drugs. The above information was collected by one of six physicians involved in routine consultations over a period of three months. Blood pressure was measured using a manual sphygmanometer.

The data collection form used for all patients included a list of drugs known to cause albuminuria. Physicians were encouraged to test for urinary tract infection where

appropriate. Preliminary screening of the urine specimens detected subjects with frank proteinuria. Microalbuminuria was arbitrarily defined as a urine albumin of >30 mg/L in an early morning specimen for the purposes of this study.

Physicians were asked to record on the data collection form whether each subject suffered from non-diabetic renal disease, urinary tract infection or other concurrent systemic disease. A check list of systemic diseases known to cause proteinuria was included on the reverse of the data collection form. A list of drugs known to cause proteinuria was also included. In addition to making physicians aware of medical conditions and drugs which cause proteinuria, all urines were also tested for blood and nitrite in an attempt to exclude patients with urinary tract infections. Despite these precautions it was possible that some patients may have had a non-diabetic cause of microalbuminuria. This possibility was further reduced by requiring that at least two samples taken on different days should be above 30 mg/L before the patient was classified as having microalbuminuria.

All data collection forms were checked for completeness on the day of receipt. Missing information was collected from patients' medical records on the following day. A typical data collection form is shown in Appendix 1

Blood was taken for haemoglobin $A_1$, glycated globin and fructosamine. Haemoglobin $A_1$ and glycated globin were measured within three days of collection. Where this was not possible samples were rejected. Logistical problems during the study led to two rejected batches. A target of 500 patients with complete data sets was achieved within three months. Consequently, because urine proteins can be stored satisfactorily at 4°C (Osberg et al 1990), the number of patients with completed urine protein results exceeds the number of patients with completed glycated globin results. Table 4.1 shows the data collected.

**TABLE 4.1 Summary of data collected during recruitment**

| | Number | (% total) |
|---|---|---|
| Total number of patients seen by clinicians over the study period | 1030 | 100 |
| Completed urine protein measurements | 876 | 85 |
| Completed blood pressure measurements | 888 | 86 |
| Completed glycated globin | 560 | 54 |
| GHb (affinity chromatography) | 578 | 56 |
| fructosamine | 580 | 56 |

Comparison of two independent continuous sample distributions was done by the Mann-Whitney non-parametric test for unpaired data. A two sided P-value of $<0.05$ was regarded as statistically significant.

The Chi-squared test was used to assess the statistical significance of differences in the proportion of patients with microalbuminuria in insulin and non-insulin dependent diabetes.

## 4.3 RESULTS

Of the 900 completed urines, 24 had positive nitrite/blood tests and were rejected from the study. Of the 876 remaining urine collections received, 342 were from insulin

dependent diabetics, 144 were from non-insulin dependent diabetics on dietary treatment alone and 390 were from non-insulin dependent diabetics on oral pancreatic stimulating drugs. In all further analyses, the non-insulin dependent diabetic patient group comprised both patients treated with drugs and by diet alone.

19% of insulin dependent diabetics and 37% of non-insulin dependent diabetics excreted urine with an albumin concentration of 30-300 mg/L and were classified as microalbuminuric. The prevalence of microalbuminuria in these two groups was not significantly different at the $p = 0.05$ level. (Chi-squared = 5.366 $p<0.07$). Table 4.2 presents the results in tabular form. Figures 4.1, 4.2 show the distribution of albumin results in the two groups as a histogram. 3% of insulin-dependent and 5% of non-insulin dependent diabetes excreted urine with an albumin concentration of more than 300 mg/L. Including all this information on a 2 x 2 contingency table, the chi-squared test showed that the prevalence of proteinuria (when patients with albumin excretion of greater than 300 mg/L were considered as a single group) was significantly higher among non-insulin dependent diabetics. (chi-squared = 8.097 $p<0.02$)

**FIGURES 4.1 and 4.2**

**The distribution of urine albumin excretion between insulin dependent and non-insulin dependent diabetics amongst patients from the selected population**

The mean value of the first two samples received has been used to calculate urine albumin excretion.

Fig 4.1 DISTRIBUTION OF URINE ALBUMIN IN IDDM SUBJECTS

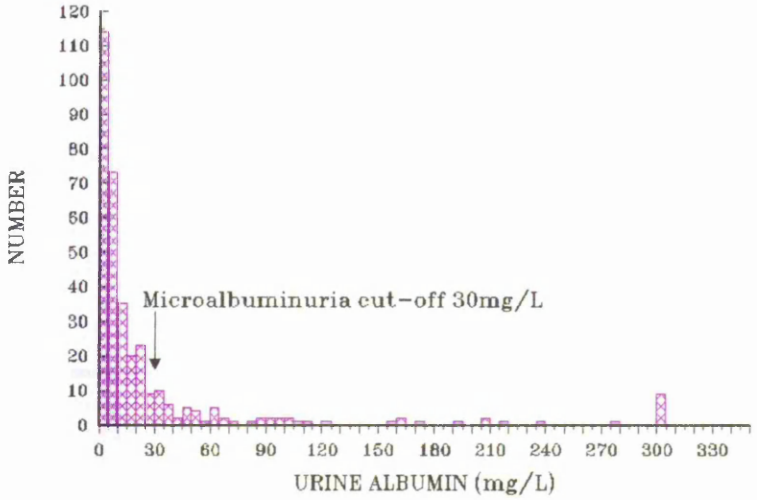



Fig 4.2 DISTRIBUTION OF URINE ALBUMIN IN NIDDM SUBJECTS

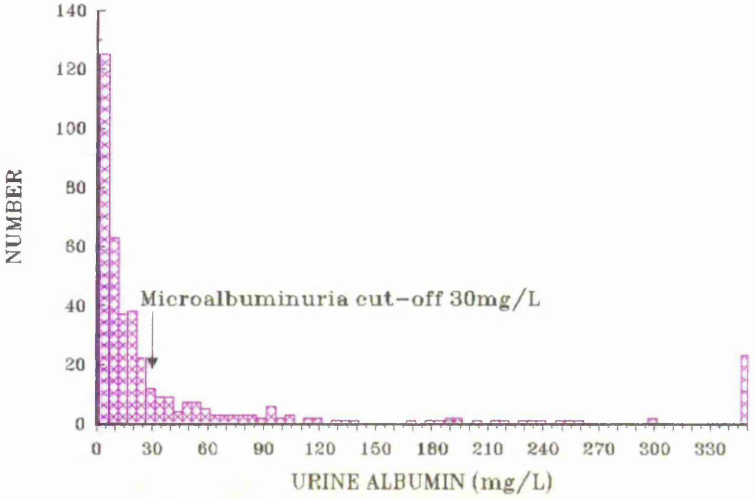


**TABLE 4.2 The distribution of urine albumin excretion between insulin and non-insulin dependent diabetics.**

| | Urine albumin <30 mg/L | Urine albumin 30-300 mg/L | Urine albumin >300 mg/L |
|---|---|---|---|
| Insulin dependent diabetes n = 342 | 266 | 66 | 10 |
| Non-insulin dependent diabetes n = 534 | 370 | 136 | 28 |

83 patients out of the 876 (9.5%) were hypertensive according to WHO criteria (systolic blood pressure greater than 160 mmHg and diastolic blood pressure greater than 95 mmHg). Of these, 34 were already on antihypertensive agents.

Other characteristics of the group were considered in two different ways: the distribution of systolic and diastolic blood pressure, GHb, glycated globin and fructosamine were considered firstly between non-insulin and insulin dependent diabetes and secondly between patients with and without microalbuminuria.

Figures 4.3 - 4.10 show the distribution of these parameters between insulin dependent and non-insulin dependent diabetics. Table 4.3 shows the median and range for each group. Insulin dependent diabetics showed significantly lower systolic blood pressure than non-insulin diabetics but significantly higher glycated globin, GHb and fructosamine. In contrast, patients with microalbuminuria showed no significant differences with respect to glycated proteins but did show a significant increase in systolic blood pressure (see Table 4.4 ).

**FIGURES 4.3 to 4.10**

**The distribution of glycated proteins and blood pressure in insulin dependent and non-insulin dependent diabetics. Data are presented to characterize the patient population in order to establish whether the study population is atypical of other diabetic populations.**

Fig 4.3 DISTRIBUTION OF GHb IN NIDDM SUBJECTS

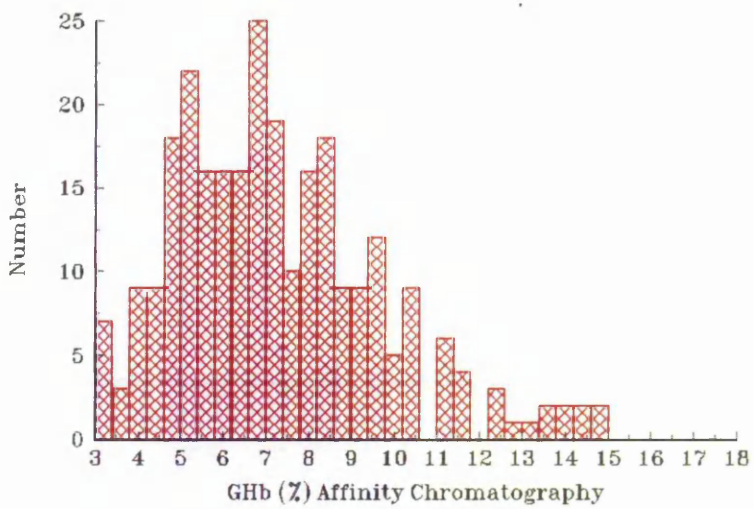



Fig 4.4 DISTRIBUTION OF GHb IN IDDM SUBJECTS

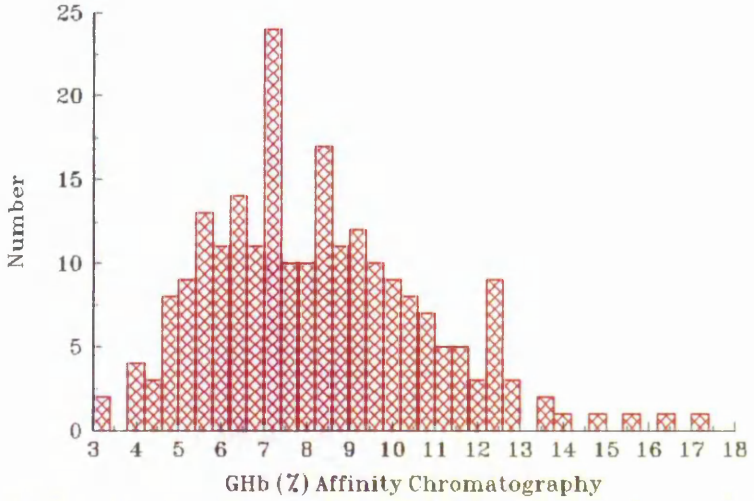


Fig 4.5 DISTRIBUTION OF GLYCATED GLOBIN IN NIDDM SUBJECTS

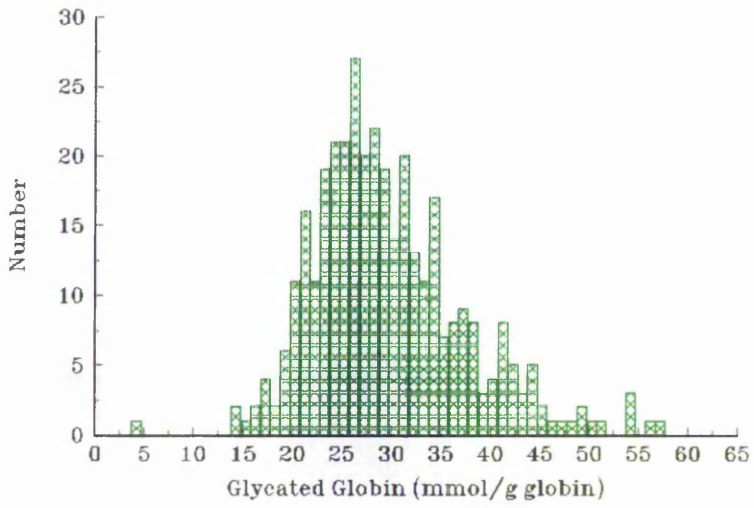





Fig 4.6 DISTRIBUTION OF GLYCATED GLOBIN IN IDDM SUBJECTS

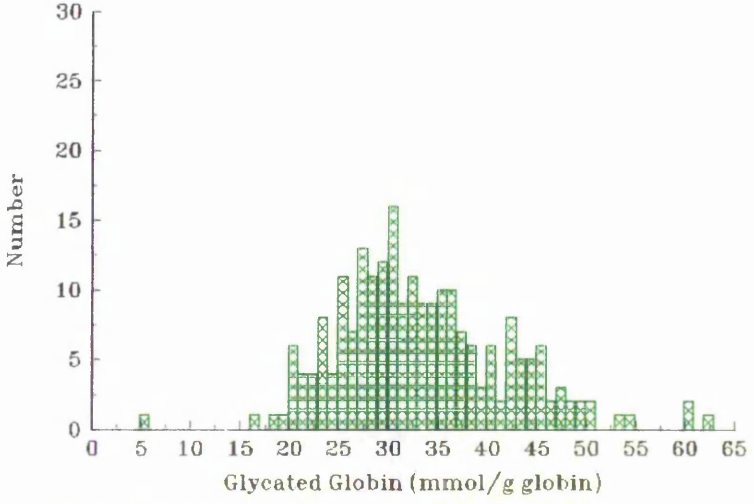


Fig 4.7 DISTRIBUTION OF SYSTOLIC BP IN NIDDM SUBJECTS

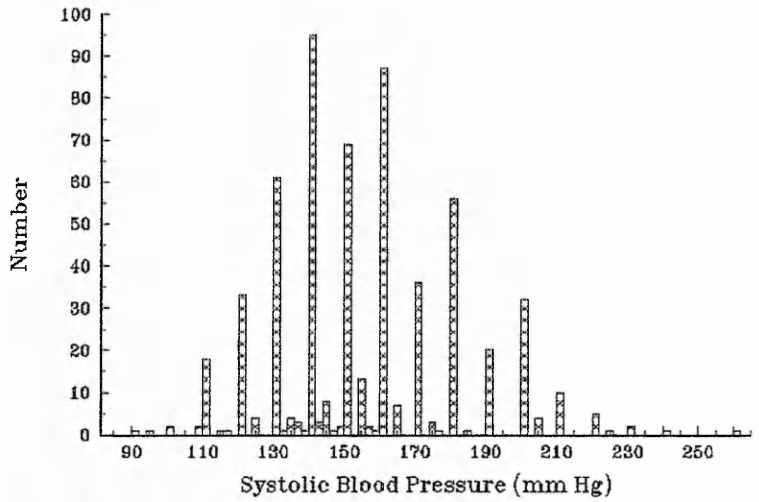



Fig 4.8 DISTRIBUTION OF SYSTOLIC BP IN IDDM SUBJECTS

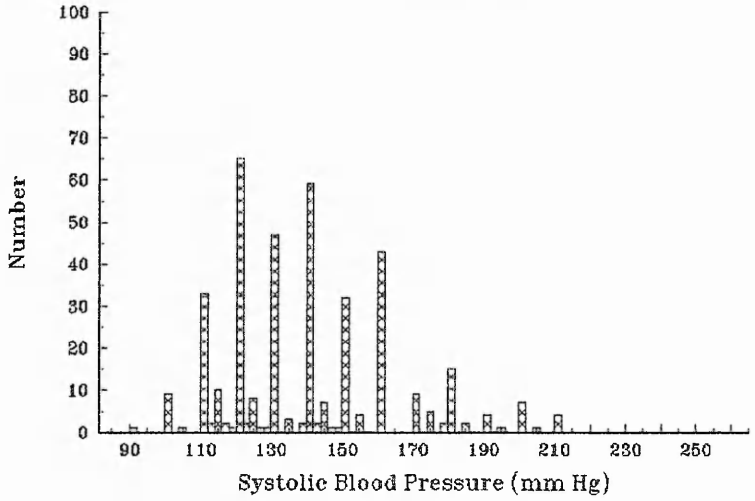


Fig 4.9 DISTRIBUTION OF DIASTOLIC BP IN NIDDM SUBJECTS

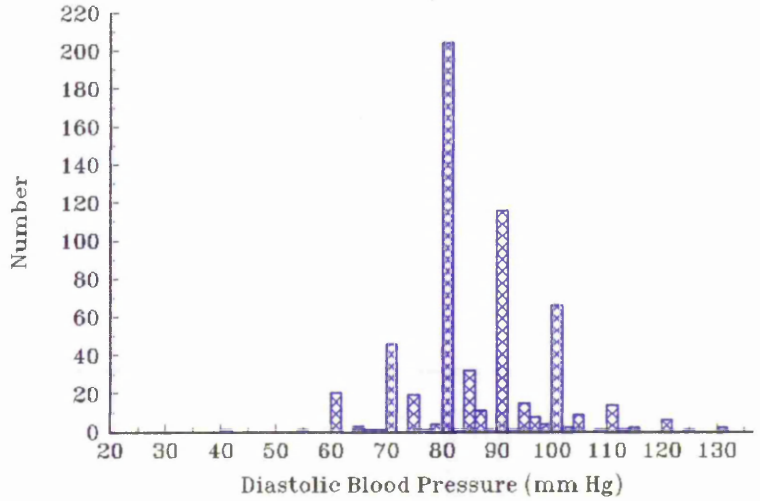



Fig 4.10 DISTRIBUTION OF DIASTOLIC BP IN IDDM SUBJECTS

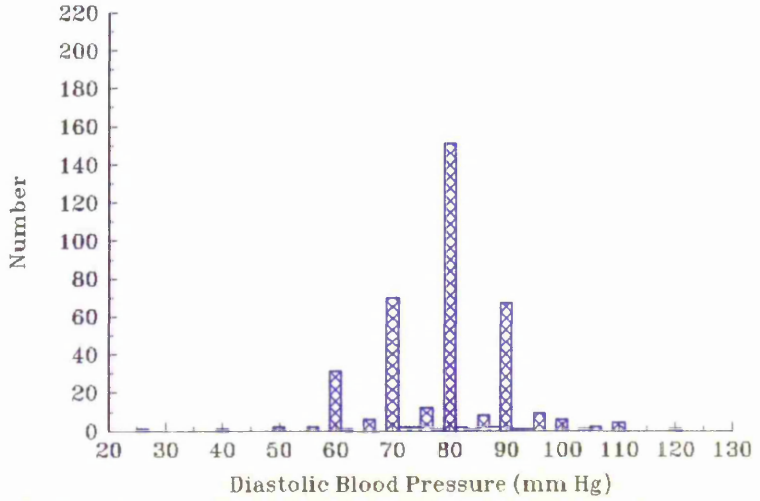


**TABLE 4.3**

The median value of blood pressure and glycated proteins in patients with insulin dependent and non-insulin dependent diabetes.

The range of data is shown in parenthesis

| | **Systolic BP (mm)** | **Diastolic BP (mm)** | **Glycated Globin (mmol/g)** | **$HbA_1$ (%)** | **Fructosamine (mmol/L)** |
|---|---|---|---|---|---|
| Insulin dependent diabetes n=342 | 140* (120-155) | 80 (70-85) | 32.1* (27-38) | 8.0* (6.2-9.8) | 3.1* (2.7-3.5) |
| Non-insulin dependent diabetes n=534 | 150 (140-170) | 80 (80-90) | 28.4 (24-34) | 6.9 (5.4-8.6) | 2.7 (2.4-3.1) |

* $p<0.05$ versus patients with non-insulin dependent diabetes

**TABLE 4.4**

The median value of blood pressure and glycated proteins in patients with insulin dependent and non-insulin dependent diabetes with and without microalbuminuria.

The range of data is shown in parenthesis

| | **Systolic BP (mm)** | **Diastolic BP (mm)** | **Glycated Globin (mmol/g)** | **$HbA_1$ (%)** | **Fructosamine (mmol/L)** |
|---|---|---|---|---|---|
| With microalbuminuria (urine albumin >30 mg/L) n=202 | 150* (130-170) | 80 (80-90) | 29.3 (25-35) | 6.9 (5.4-9.1) | 3.0 (2.6-3.3) |
| Without microalbuminuria (urine albumin <30 mg/L) n=636 | 142 (130-160) | 80 (80-90) | 28.4 (23.4-34.3) | 7.0 (5.5-8.8) | 2.8 (2.4-3.3) |

* $p<0.05$ versus patients without microalbuminuria

## 4.4 DISCUSSION

Although consecutive patients attending routine diabetic clinics were entered into the study, this sample of the diabetic population in North Nottinghamshire was not a truly random sample. Patients were referred to the hospital diabetic clinic by General Practitioners, but during and for some time before the study, GPs had been encouraged to monitor more of their diabetics without referral to the hospital. It is possible therefore that the hospital clinic contained diabetics with more complications and a higher proportion of patients with poor diabetic control.

Because patients attending diabetic clinics are a selected population, comparisons between diabetic clinics in different hospitals may detect differences (or similarities) which are due to the method of selection rather than genuine characteristics of the population under study.

Despite these difficulties it is useful to examine the characteristics of the population under study because it is important to establish whether this group is broadly similar or very different from other study populations.

In a study published in 1989 Marshall and Alberti reported significantly higher diastolic and systolic blood pressure in NIDDM as compared with IDDM in patients from a routine diabetic clinic in Newcastle upon Tyne. This study demonstrates a significantly higher median systolic blood pressure in NIDDM but no difference in diastolic blood pressure. The median diastolic blood pressure in patients with NIDDM (80 mmHg) is similar to the median blood pressure in the Newcastle study (83 mmHg).

The finding of a higher median GHb, fructosamine and glycated globin in IDDM patients than in NIDDM patients (median GHb for IDDMs 8.0 (reference range for GHb 4.3-6.3%) may reflect the inclusion of patients in the NIDDM group which were being

treated by diet alone and therefore may have mild diabetes. Even quite small differences in medians may be detected with statistical significance in these comparative studies because of the large numbers of patients in the sample. It is possible that these small differences are not clinically significant.

The prevalence of microalbuminuria amongst patients with NIDDM had been reported variously as 20% (Torfvitt et al 1991), 21% (Kikhava et al 1989), 20% (Mattock et al 1992), 26-29% (Gall et al 1991) and 38% (Friis et al 1994). The measured incidence in this study of 25% is therefore in good agreement with published results. The incidence of microalbuminuria in IDDM is reported as 15-20% (Schmitz and Vaeth 1988) and 18.9% (Adamson et al 1993).

The figures agreed well with the microalbuminuric IDDM patients in the current study (19%). Figures 4.3 - 4.7 indicate that glycated globin showed the same trend as GHb, when IDDMs are compared with NIDDMs. This concurrence was expected because the two measurements reflect different analytical approaches to the same analyte. However, it is important that glycated globin has been demonstrated to provide a similar discriminating power in a clinical setting.

The incidence of microalbuminuria in any study is dependent to a large extent on the cut-off points used to define 'microalbuminuria' Many studies do not report estimates of a range within which healthy individuals would be expected to fall with a defined probability (usually $p = 0.05$). An example is the study of Kumar et al (1993). When such a range is calculated it is clear that subjects in most studies could have an albumin excretion which is above the reference range but below the range designated as microalbuminuria. Timed overnight or 24 hour urine collections have been widely used in an attempt to standardise urine collections. The albumin excretion rate of albumin concentration which

is predictive of later clinical nephropathy in IDDM patients has been reported as 30 μg/min (Viberti et al 1982), 70 μg/min (Mathiesen et al 1984), 15 μg/min (Mogensen and Christiansen 1984).

For non-insulin dependent diabetes, the albumin excretion predictive for total mortality (chiefly cardiovascular disease) has been reported as 10 μg/min (Jarrett 1984) and 30 μg/min (Mogensen 1984).

A consensus has been reached (Mogensen et al 1985) which arbitrarily defines microalbuminuria as an albumin excretion rate of 20-200 μg/min in an overnight collection or 30-300mg/24hr)

This consensus allows research work to become standardised and facilitates comparison of studies by different research groups. Recently Adamson et al (1993) compared the predictive value of measuring albumin concentration with an albumin/creatinine ratio in early morning urine specimens compared with albumin excretion rate in the same specimen. They found that the predictive value of a negative result was 97.5% and 97.2% respectively.

This very small loss of predictive value when a measurement of albumin is made in an early morning specimen compared with a timed collection has to be set against the expected compliance rate in these two situations.

One previous study in which timed overnight urines were used in a population screen (Gatling et al 1985) found that only 69% of patients returned specimens. Discussions with medical and nursing staff locally suggested that compliance with timed urine testing was likely to be poor for a number of reasons. Additional support for using measurements of albumin concentration rather than albumin/creatinine ratios comes from

data on the biological variation of urine albumin excretion. (Howey et al 1987). The conclusion of this study was that adding a creatinine measurement increases the measured intra-individual variation in addition to adding random variation due to the measurement of a second analyte. As a consequence of these considerations the chosen specimen was an early morning urine and the measurement was albumin alone.

Although this would usually be a choice for routine clinic monitoring rather than research, it was felt that in order that an adequate number of specimens should be collected this compromise could be made.

The definition of insulin-dependent and non-insulin-dependent diabetes is not rigorous in this study. Several non-insulin dependent diabetics were reclassified as they were put onto insulin therapy. A formal C-peptide response to glucagon test is probably the best way to define non-insulin dependent diabetes. However, this was not possible for the study. In the hands of research workers 10% of NIDDMs may be unrecognised by this test (Hother Neilson et al 1988).

# SECTION 5

# CLINICAL EVALUATION OF THE GLYCATED GLOBIN ASSAY

## 5.1 INTRODUCTION

The glycated globin assay developed in Section 2 has been assessed as a clinical tool for monitoring the control of hyperglycaemia in diabetes. In attempting to assess the clinical value of the glycated globin assay it is necessary to consider how comparability of the test with established techniques for measuring glycated haemoglobin can be determined. Since the assay would be potentially used as an outcome measure both for individual diabetic patients and for auditing institutions which provide diabetic care, it is instructive to examine how existing measurements of glycated haemoglobin are used in this context.

The St Vincent Declaration (Anonymous 1990) is a series of goals agreed by consensus amongst physicians responsible for managing diabetes across Europe. The declaration specifically encourages the use of 'state of the art information technology for quality assurance of diabetes health care provision and for laboratory and technical procedures in diabetes diagnosis, treatment and self-management.' Arising out of the need to compare outcomes of care between diabetic services, a recommended data set for collecting information on personal computers has been published. (Wilson and Home 1993). Included on the information to be collected is $HbA_{1C}$. Recognising the difficulty of different methodology and the different forms of glycated haemoglobin which are being measured, Wilson and Home recommended classification of metabolic glucose control in terms of multiples of the standard deviation of a non-diabetic population. This approach improves comparability of different glycated haemoglobin methods. Nevertheless, most laboratories in the U.K. continue to report glycated haemoglobin leading to differences in results between centres. Currently, laboratories in the U.K. do not all measure $HbA_{1C}$. Amongst those which make returns to the United Kingdom National External Quality Assessment Scheme (UK NEQAS), 65 out of 218 laboratories (30%) currently measure $HbA_1$ (September 1993

report, UK NEQAS for Glycated Haemoglobins). The difference between $HbA_{1c}$ and $HbA_1$ has been discussed in Section 1.

24 laboratories in the U.K. measure fructosamine and an unknown number of these measure fructosamine alone i.e. without measuring glycated haemoglobin.

Even within the laboratories using $HbA_{1c}$ (the more tightly defined analytical quantity), a typical range of the mean value between methods for $HbA_{1c}$ is 8.1 - 9.8% for a single whole blood specimen. These differences make targets for $HbA_{1c}$ less useful when the methodology is not defined. An attempt to get around these difficulties has resulted in defining targets as multiples of standard deviations from the mean.

A further important feature of an analytical system used for monitoring purposes is that the analytical imprecision should not exceed half of the intra-individual biological variation (Fraser 1983).

The intra-individual biological variation for $HbA_{1c}$ has been estimated as CV = 1.8% (Godsland 1985) leading to an analytical goal of CV = 0.9%. Thus $HbA_{1c}$ measurements should have low imprecision.

The considerations above have led to the development of analytical techniques which have high specificity for $HbA_{1c}$ combined with high sample throughput and low analytical imprecision. Dedicated HPLC equipment is currently becoming increasingly popular. The high specificity for $HbA_{1c}$ results in a close agreement between laboratories using specific techniques but there are no clear advantages in terms of the test's clinical validity. Using dedicated HPLC equipment is expensive and only one system currently available in the U.K. is truly automated and suitable for use in a clinic situation remote from the laboratory (the Biomen HA8121 HPLC analyser, Biomen Ltd, Croydon, Surrey).

Development of a spectrophotometric method which is automated can be expected to display low imprecision. The fructosamine test which is based on a similar clinical reaction is highly reproducible. Serum fructosamine is performed directly on serum without the need for any sample pre-treatment. Extraction of globin is required in the glycated globin assay under development and this extra step may increase imprecision.

Fructosamine is known to measure reducing activity in serum which is not due to glycated proteins (Reviewed in Hill et al 1990). The glycated globin assay under consideration here involves a crude purification of globin prior to analysis (see Section 2), which should reduce interference from compounds other than glycated globin. However, the possibility remains that some non-specific reducing activity may be associated with the globin molecule itself. Any non-specific reducing activity may increase the intra or inter-individual variance of GHb.

A high intra-individual variance reduces the clinical usefulness of a test which is being used to monitor hyperglycaemia. This is because any observed change in the parameter may not be attributable to changes in glycaemia, but could be due to the inherent intra-individual variability. Comparison of such a method with a well established or reference method would be expected to produce a low correlation coefficient with a large scatter of points around the regression line.

To establish the intra-individual variability of glycated globin, several approaches are possible. Repeated measurements of diabetic individuals is problematical because even in stable diabetics it is difficult to be sure that glycaemia is not changing without another measurement of glycaemia as a control. This measurement will, itself, be associated with an intra-individual variability. Further, there are ethical problems in recruiting patients. A further possibility is to measure the intra-individual variability in non-diabetic volunteers as

advocated by Fraser (1983). However, it was difficult to persuade sufficient volunteers to allow multiple venepuncture.

A pragmatic approach which assesses how the glycated globin performs in its clinical setting is to monitor its performance in a clinical trial over an extended period. By using a well established technique for GHb in parallel with the glycated globin assay under evaluation, comparisons between the techniques can be used to generate scatter diagrams. (e.g. Figure 5.1). The scatter around the fitted line can then be analysed statistically to determine how much of it can be attributed to factors other than variation in GHb. This latter component will include the intra-individual variance of both techniques.

## 5.2 MATERIALS AND METHODS

The methods for glycated globin, total globin and glycated haemoglobin have been described in Section 2. The method for serum fructosamine (Johnson et al 1982) was used as described by these authors at a pH of 10.35.

The calculation of the regression line slope was made according to the method of Deming (1943). The intercept was then calculated from the equation $y = mx + c$ where $y$ = the mean of glycated globin measurements and $x$ = the mean of GHb measurements. The validity of the slope estimate was checked by a robust method based on the procedure described by Theil (1980) and which is available as 'rslope' on a Personal Computer Statistical Package (Minitab Clecom Ltd., Birmingham, U.K.)

Blood specimens for this study were taken from patients attending a routine diabetic clinic at King's Mill Hospital. Ethical approval was sought and given by the hospital Ethical Committee. Glycated globin, GHb and fructosamine were performed on each sample

obtained from the clinic until approximately 500 samples were collected. The samples were collected as part of the clinical study which determined entry into the trial.

All glycated globin and GHb analyses were performed within two days of sampling. The data used in the comparison exercise were collected over 5 months during which time 12 batches were performed of both GHb and glycated globin. To maintain the long term performance of both techniques, pooled haemolysates were prepared as quality control specimens to check imprecision. To 0.1 mL whole blood, 10 mL saline was added. After mixing and centrifugation the supernatent was discarded and the washed red cells were retained. The cells were haemolysed by adding 1 mL distilled water and then frozen in aliquots. Fructosamine imprecision was monitored using pooled human serum from non-diabetics (fructosamine 1) and diabetics (fructosamine 2).

## 5.3 RESULTS

The extended clinical evaluation of the glycated globin assay under routine working conditions gave coefficients of variation of 6.2% and 9.8% for whole blood pools with a mean glycated globin of 25.7 mmol/g total protein and 14.3 mmol/g total protein respectively.

Figure 5.2 shows the quality control data collected during the time over which patients were recruited into the main clinical trial (described in Sections 6 and 7). All analyses were performed in singlicate with quality control specimens being placed on each analytical run. An interval of one week elapsed between each run. The clinical evaluation thus extended over a 12 week period.

The data in Table 5.1 are plotted in Figure 5.2. There appears to be no discernible trends in the data shown in Figure 5.2.

**TABLE 5.1 Summary of glycated protein precision data**

| ANALYTE | QUALITY CONTROL MATERIAL | N | MEAN | STANDARD DEVIATION | RANGE | CV% |
|---|---|---|---|---|---|---|
| Glycated globin (mmol/g) | Whole Blood A | 12 | 25.8 | 1.6 | 23.9 - 29.1 | 6.1 |
| Glycated globin (mmol/g) | Whole Blood B | 12 | 14.3 | 1.4 | 11.7 - 17.2 | 9.8 |
| Glycated globin (mmol/g) | Roche Lo control (serum) | 12 | 26.4 | 1.0 | 24.5 - 28.5 | 3.7 |
| Glycated globin (mmol/g) | Roche Hi control (serum) | 12 | 55.7 | 1.6 | 53.1 - 58.3 | 2.9 |
| Glycated globin (mmol/g) | Roche fructosamine calibrator | 12 | 44.5 | 1.7 | 41.5 - 47.8 | 4.2 |
| $HbA_1$ (%) | Whole Blood C | 12 | 4.7 | 0.3 | 4.29- 5.14 | 5.6 |
| $HbA_1$ (%) | Whole Blood D | 12 | 16.1 | 0.5 | 15.4 - 17.1 | 3.2 |
| Fructosamine (mmol/L) | Fructosamine 1 (serum) | 12 | 2.4 | 0.1 | 2.31- 2.54 | 2.9 |
| Fructoasamine (mmol/L) | Fructosamine 2 (serum) | 12 | 3.3 | 0.1 | 3.12- 3.46 | 3.0 |

**FIGURE 5.1 Scattergram showing the relationship between glycated globin and glycated haemoglobin (affinity chromatography) during the recruitment phase of the study.**

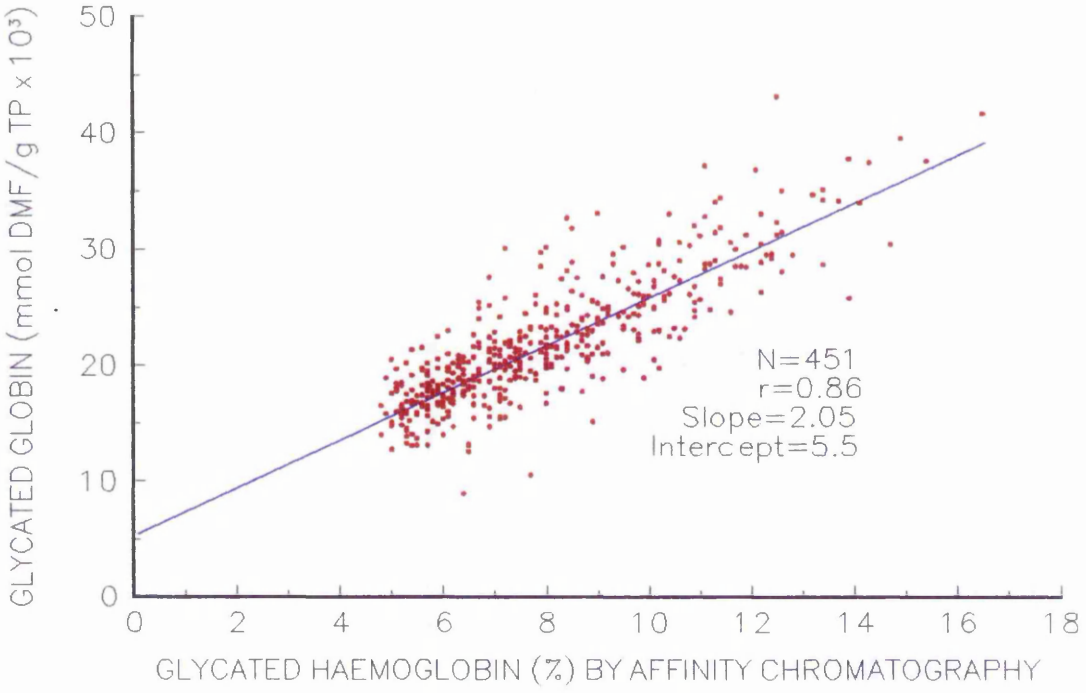


**FIGURE 5.2**

The variation of glycated globin measured in two pooled whole blood haemolysates used as quality control material during the 12 weeks in which the recruitment phase of the study was performed. The plotted values were single estimates of the pool.

Fig 5.2A Variation of Glycated Globin Measurement

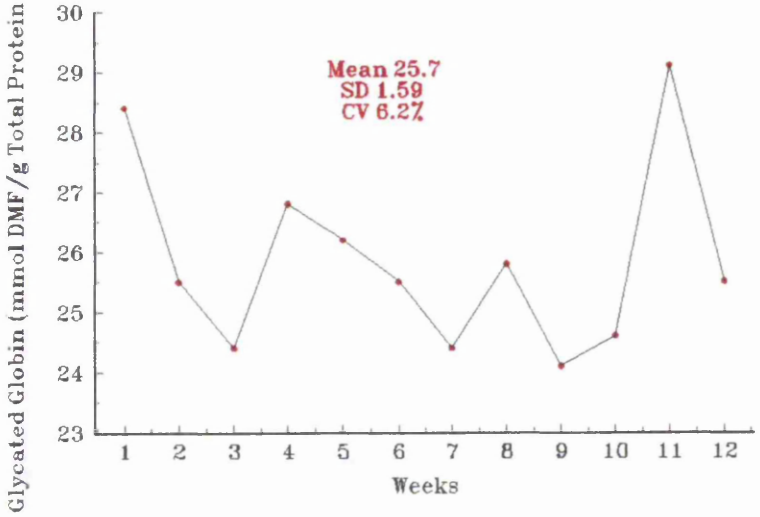
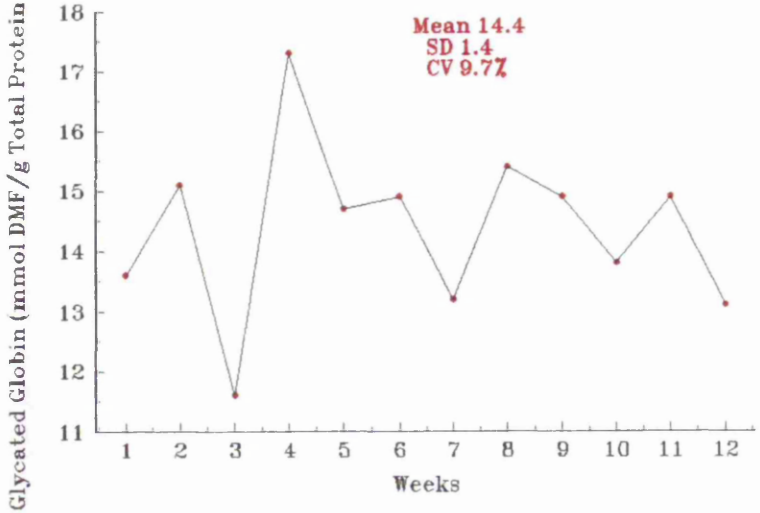



Fig 5.2B Variation of Glycated Globin Measurement



## 5.4 DISCUSSION

The correlation of glycated globin with GHb (Figure 5.1) (affinity chromatography) shows greater scatter than during the validation of the method ($r^2 = 0.895$ compared with $r^2 = 0.94$ (Section 2)). The between batch imprecision is also greater (see Table 5.1). The poorer correlation coefficient is almost certainly due to the higher analytical imprecision encountered during the clinical evaluation. Due to the lability of the control material it is possible that the calculated imprecision of the whole blood pools during the clinical evaluation does not represent the true imprecision of the assay when using fresh blood specimens.

However, deterioration of the stored material would have been expected to produce a gradual increase or decrease in glycated globin. There is no discernible trend in the data presented in Figure 5.2.

Imprecision as measured by serum controls (Table 5.1) suggest that the chemistry of the nitroblue tetrazolium reaction is acceptably precise. This observation may imply that imprecision is added at the stage of globin extraction.

# SECTION 6

# STUDY DESIGN AND SELECTION OF PATIENTS

## 6.1 INTRODUCTION

This section describes the study design and methods used to test the three patient groups which were selected. The method of selection and the characteristics of the selected population are described. Finally the results and conclusions of the study are discussed. The overall purpose of the study is to test the hypothesis that intervention by medical staff and nursing staff can improve blood glucose control in diabetic patients at particularly high risk for renal disease and early death from cardiovascular disease.

'High risk' diabetics are defined as these with urine albumin between 30-300 mg/L in an early morning urine specimen (see Section 1).

Blood glucose control has been chosen as an end point for this study because it is an outcome measure which is susceptible to change in a relatively short time. This allows a study to be performed within a reasonable time. Focusing on such a parameter can lead to changes in medical management or the delivery of nursing care (i.e. a change of process). In this sense the glycated proteins GHb, fructosamine and glycated globin become 'proxy' intermediate end points for a range of distressing diabetic complications (Vaughan 1994).

Measuring glycated proteins thus becomes pivotal in assessing the effectiveness of diabetic care and demands reproducible, transferable and cost effective analytical techniques. $HbA_{1C}$ has been used most extensively in the U.K. but many different techniques are used with differing reference ranges and differing costs. A method which can be performed on routine automated chemistry analysers is likely to improve both comparability (due to its likely popularity) and cost effectiveness. Accordingly, two established glycated proteins (GHb and fructosamine) have been used alongside a newly developed index (glycated globin).

In addition to checking that the groups were reasonably well matched for age, type of diabetes, initial blood pressure and glycated protein status, two psychometric methods were used to assess the psychological well-being of the patients in the study. Because the numbers of patients in each group are relatively small, it is possible that a few patients with an unusual psychological make-up may, by not responding to measures aimed at persuading them to improve glycaemic control, unduly influence the results. Another psychological influence on a patient's behaviour is likely to be any major cause of stress which they have been subjected to recently. For example, a patient who has recently lost a partner or has recently become unemployed may be less likely to respond positively to medical advice. Thus the purpose of collecting psychological data at the stage of entry into the study is to ensure that no group or groups scores particularly highly for psychological illness or stress. Psychological stress has been shown to lead to a lack of compliance with both insulin therapy and during therapy in diabetes mellitus (Hanson and Richert (1986), Hanson et al (1987)).

An information-giving questionnaire was designed for the purpose of checking that each patient had an adequate understanding of the information given to them immediately after the consultation.

Three patient groups were selected for the trial (see Section 6.2). All patients were poorly controlled diabetics with GHb of >8% (>5 SDs above the mean of a non-diabetic population).

Table 6.1 summarises the characteristics of the groups. Group B was intended to be a control group for Group A to test the effect of intervention. Group C was intended as a second control group to test whether selection on the basis of microalbuminuria produced patients who were more likely to worsen or improve their diabetic control regardless of intervention.

## 6.2 STUDY DESIGN

900 Diabetics were recruited to the study over a period of three months. All patients were accepted unless infirmity or social difficulties were thought to prohibit returns over the next year. Only 3 patients were excluded on these grounds. The first phase testing was aimed at collecting baseline data on the diabetic population and screening for microalbuminuria (see Figure 6.1). Patients with urine albumin concentrations greater than 300 mg/L were not included for retesting of diabetes but were included in the analysis of baseline characteristics. Of the 900 patients recruited, not all provided urine tests and glycated protein measurements were not performed on all specimens. Consequently, the numbers of patients which appear in the following data tables vary depending on the measurement considered.

At Phase 2 of the study, 21 patients with microalbuminuria and GHb >8% were allocated into each of two patient groups (A and B), which were matched for age, type of diabetes and GHb value. Urine samples from each patient were checked again for microalbuminuria. Patients with no microalbuminuria were rejected from the consideration for Groups A and B. A third group (Group C) was selected to have GHb >8% but with no microalbuminuria. Group A was an intervention group which was subjected to advice and support aimed at improving their blood glucose control and thus reducing their GHb. The patients were microalbuminuric and were informed of their increased risk for diabetic complications as part of their advice and support. The second group was also an intervention group, subjected to the same intensive advice and support but despite being microalbuminuric were not informed of their microalbuminuria. Group C patients did not have microalbuminuria and were conventionally treated. This conventional treatment included 6 or 12 monthly clinic visits, but without arranging interviews with the specialist

team. All groups (A-C) were comprised of patients with poor glucose control as defined by GHb >8%.

After allocation to patient groups, patients were tested 6 months later (phase 3) and 12 months later. Further measurements were taken of glycated proteins, blood pressure and weight at each visit.

**TABLE 6.1**

Table 6.1 summarises the age, glycated protein and blood pressure measurements on entry into the trial. This data indicates the extent to which the three groups were matched for the characteristics chosen.

**TABLE 6.1**

**General characteristics of the study groups on entry into the trial**

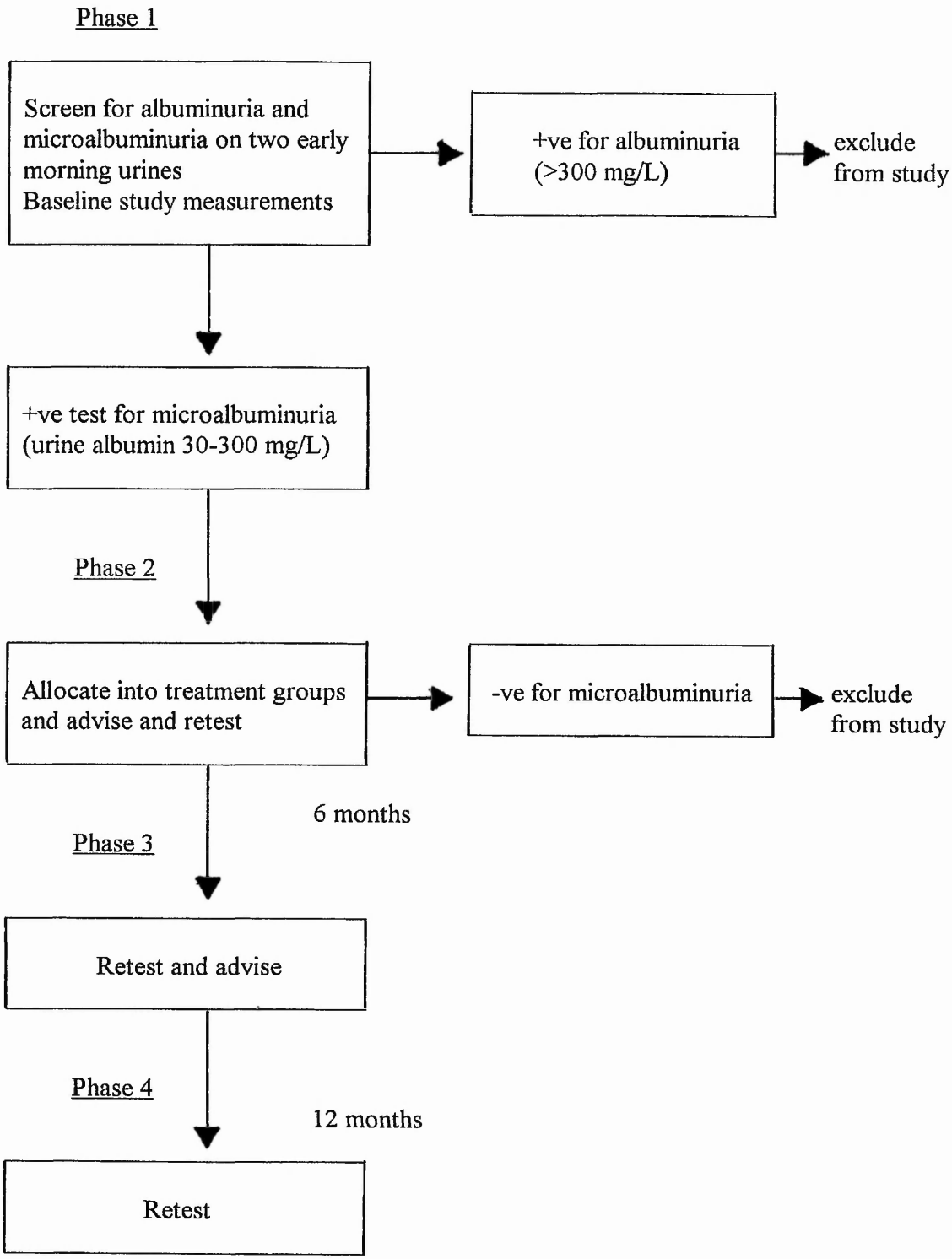
| STUDY GROUP | A | B | C |
|---|---|---|---|
| Median age yrs (range) | 64.0 (18-87) | 58.5 (36-79) | 58.5 (34-85) |
| NIDDM (n) | 11 | 8 | 7 |
| IDDM (n) | 8 | 10 | 8 |
| Median GHb (%) (range) | 9.9 (8.0-17.0) | 9.7 (8.0-15.4) | 9.9 (8.4-16.9) |
| Median fructosamine mmol/L (range) | 3.3 (2.5-5.4) | 3.2 (2.9-4.8) | 3.3 (2.7-4.7) |
| Median glycated globin mmol/g total protein x $10^3$ (range) | 36.0 (24.8-49.7) | 38.4 (29.4-54.8) | 40.4 (27.0-51.0) |
| Median systolic BP mmHg | 150 | 142 | 150 |
| Median diastolic BP mmHg | 80 | 85 | 90 |

Group A Intervention group with microalbuminuria
Aware of their increased risk factors

Group B Intervention group with microalbuminuria
Unaware of their increased risk factors

Group C Non-intervention group without microalbuminuria

**FIGURE 6.1 Flow diagram of the clinical study**



## 6.3 MATERIALS AND METHODS

The methods for glycated globin, GHb and fructosamine have been described in Section 2. Blood pressure was measured using a manual sphygmanometer. Two widely used psychometric methods were used to assess the mental health of groups A-C. The Brief Symptom Inventory (B.S.I.) is a self-report symptom inventory designed to reflect the psychological symptom patterns of psychiatric, medical and non-patient individuals (Derogatis and Melisarotos 1983). The psychological symptoms in the index used are self explanatory except for 'somatization' and 'interpersonal sensitivity' (see Table 6.2). Somatization is the tendency to channel anxiety into perceptions of ill health. Interpersonal sensitivity relates to exaggerated feelings of unpopularity or personal dislike from others as a results of stress or anxiety. After completing the questionnaires the indices were scored by the Clinical Psychology Department at Ransom Hospital, Rainworth, Nottinghamshire and three global indices were obtained which are devised from the weighted scores of the individual symptoms (Derogatis and Spencer 1982). The BSI scoring system is usually used to assess a group of individuals against published norms which are often based on data from U.S.A. populations. Francis et al (1990) have suggested that this may be inappropriate for a British population with a high incidence of unemployment. The norms used in this study are based on BSI scores from 376 questionnaires completed by households in Mansfield, Nottinghamshire which were targeted at 20 zones of human socio-economic status (as defined by Payne (1983)). All the patients in Groups A-C lived within 10 miles of Mansfield.

A copy of all the questionnaires discussed in this section appears in Appendix 1.

The life events inventory (Holmes and Rake 1967) is a commonly used assessment of events that may influence behaviour due to their intensity, traumatic motive or disruptive influence. The information giving questionnaire, the patient contract and the patient record were non-standard documents designed specifically for the study.

## 6.4 STATISTICS AND SAMPLE DESIGN

Estimating an appropriate sample size is a vital part of a research design. The following formula for estimating the minimum sample size is based on an adaptation of the work of Beal and Grieve (Beal 1989) (Grieve 1989) cited by Daly (1991).

$$n \geq \frac{2(z_{1-\alpha/2} + z_{1-\beta})^2 \sigma^2}{\Delta^2} \quad \ldots\ldots\ldots\ldots\ldots 1$$

where n = the minimum sample size needed to detect a minimum clinically significant difference of size $\Delta$ (as % GHb). See below for key to symbols.

EXPLANATION OF FORMULA FOR CALCULATING SAMPLE SIZE

The symbol $\sigma$ represents the population standard deviation of the population under study. $\sigma$ is a valid quantity in this calculation if the population shows a normal (gaussian) distribution for GHb. However, examination of the data for all glycated proteins indicates that the distribution of values is non-gaussian but becomes approximately normal following log transformation (Figures 6.2-6.4). The values for $\sigma$ and $\Delta$ are accordingly $\log_{10}$ values and the formula becomes:

calculated from log transformed data

$$n \geq \frac{2(z_{1-\alpha/2} + z_{1-\beta})^2 \sigma^2}{(\log_{10} \Delta)^2} \quad \ldots\ldots\ldots\ldots 2$$

$\alpha$ and $\beta$

The symbol $\alpha$ represents the confidence level expressed as $\alpha = 1$ - (confidence level (%) ÷ 100). Thus for a confidence level of 95%, $\alpha = 0.05$.

Similarly the symbol $\beta$ represents the power of the test expressed as $\beta = 1$ - (power (%) ÷ 100). Thus for a power of 80% $\beta = 0.20$.

This confidence limit and power have been chosen because these are conventional values in this situation (Daly 1991).

Calculation of $\eta$

The quantities $z_{1-\alpha/2}$ and $z_{1-\beta}$

became $z_{0.975}$ and $z_{0.80}$ respectively

Normal tables give $z_{0.975} = 1.96$ and $z_{0.80} = 0.84$

The value of $\sigma$ was found to be 0.33 from analysis of log transformed data for GHb. Since the minimum significant clinical difference was chosen as 2% GHb, $\log \Delta = 0.3$,

Equation 2 now becomes:

$$n \geq \frac{2\,(1.96 + 0.84)^2 \times 0.33^2}{0.3^2} \quad \ldots\ldots\ldots\ldots 3$$

which reduces to $18.97 \approx 19.0$.

Thus the sample size in each group for a two group comparison of GHb, of power 80% to detect a minimum difference of 2% GHb at 95% confidence is a minimum of 19.

## 6.5 RESULTS

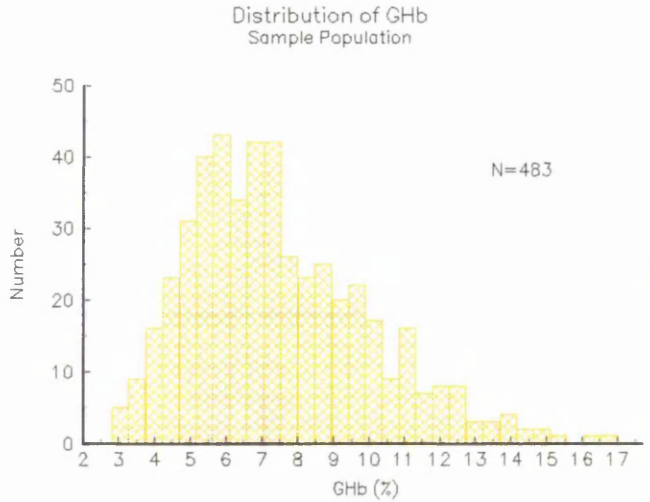
### 6.5.1 General characteristics of the study populations

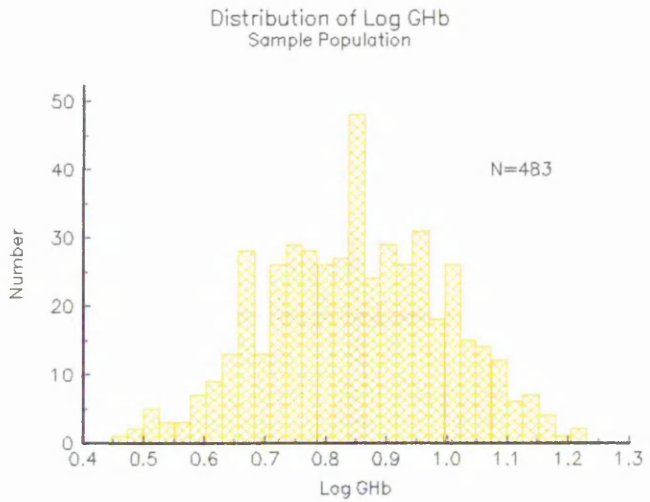
In selecting the patient groups greatest attention was paid to matching the groups for presenting GHb. Further matching took place to take into account the number of IDDM patients (compared to NIDDM patients) and age. Table 6.1 shows the summary characteristics of the three study groups. For age, GHb, fructosamine, systolic blood pressure and diastolic blood pressure, the median and the mean of each characteristic were reasonably close. The variances were also similar. For this reason the Mann-Whitney two sample test was used to test whether there were any statistically significant differences between the study groups.

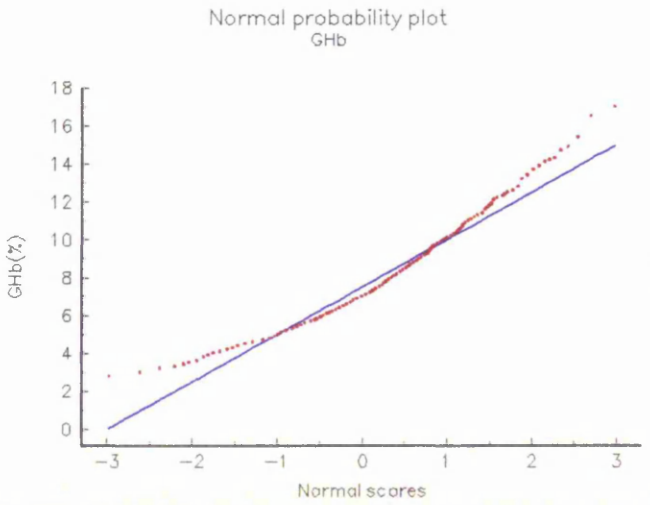
**FIGURES 6.2 - 6.4**

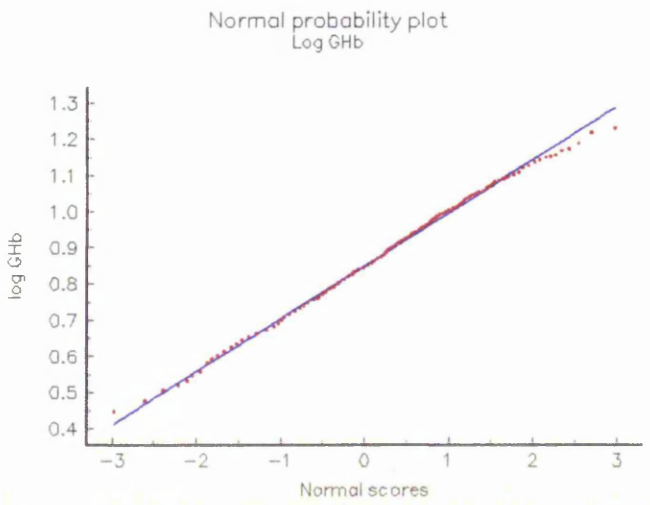
**Distribution of untransformed and log transformed glycated proteins in the study population. Normal probability plots test the normality of log transformed data. The closer to the normal distribution, the closer the data approximate a straight line.**

# LOG TRANSFORMATION OF GHb DATA

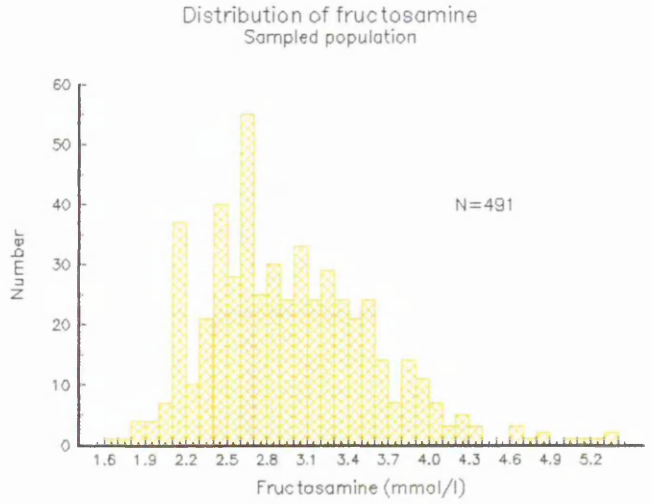


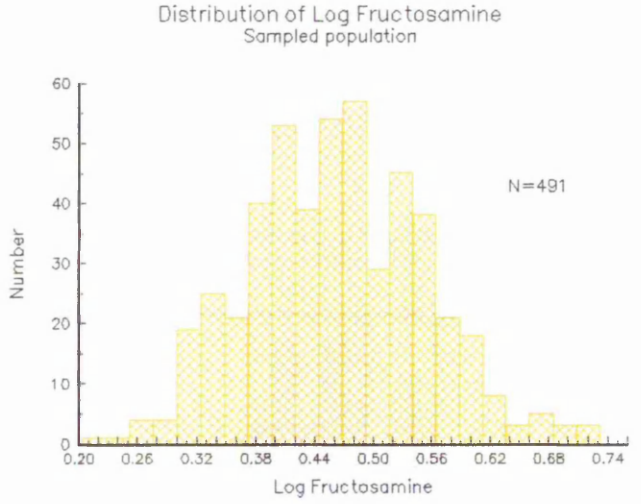



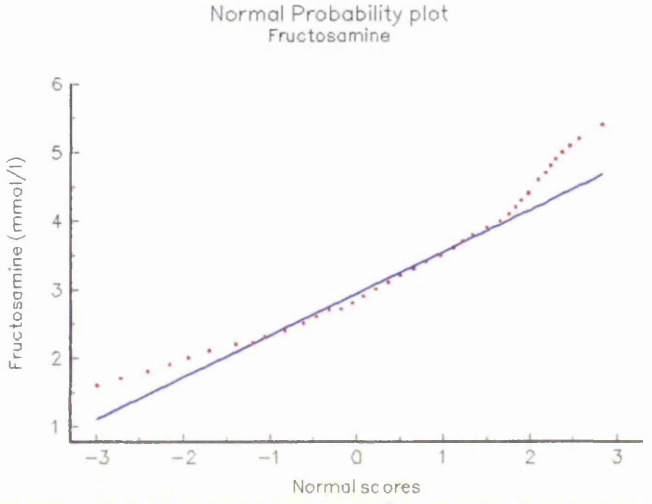



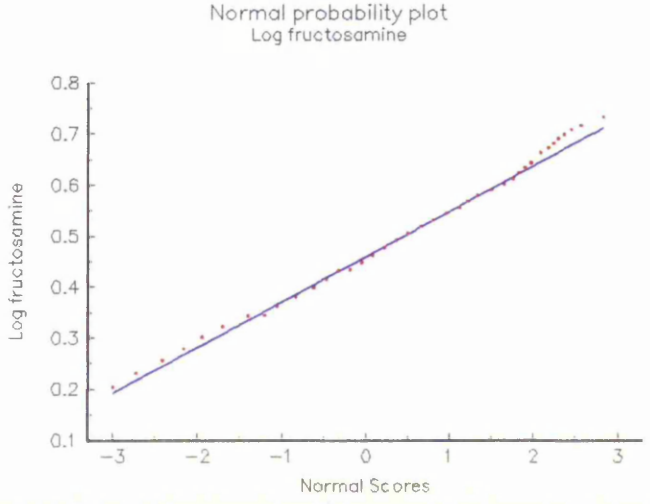



# LOG TRANSFORMATION OF FRUCTOSAMINE DATA

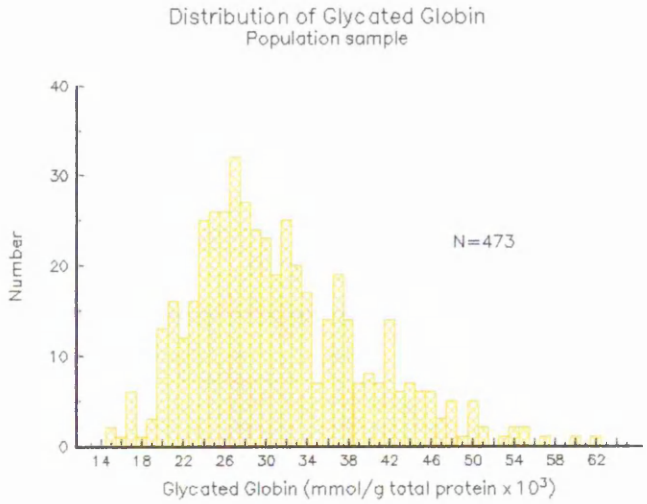


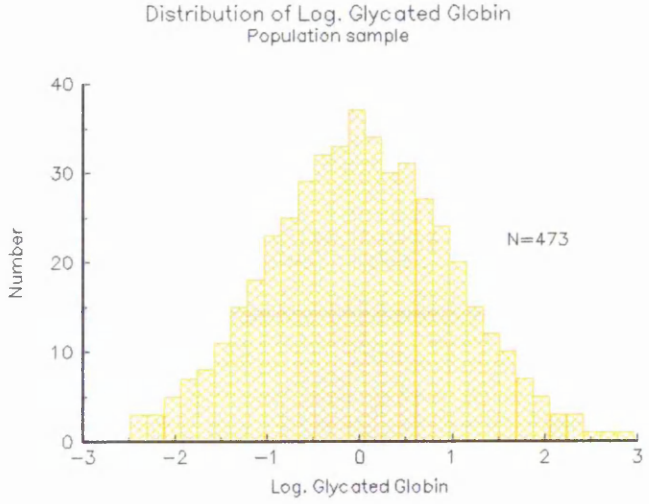



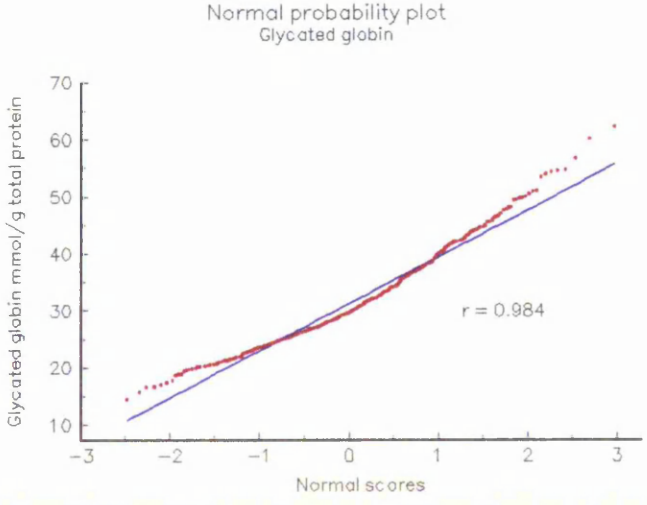



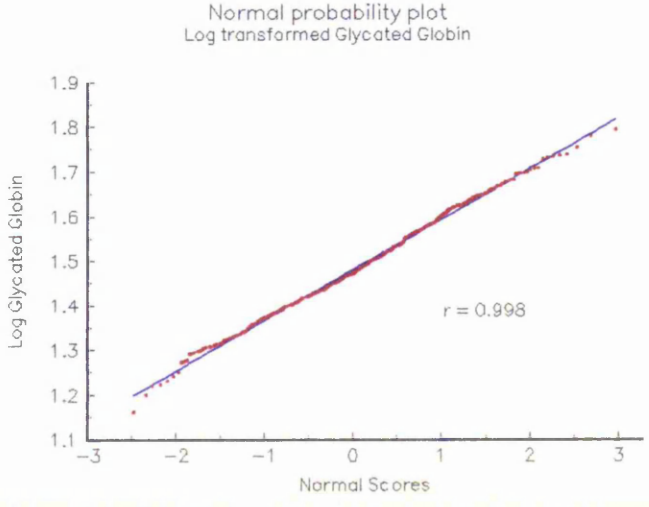



# LOG TRANSFORMATION OF GLYCATED GLOBIN DATA



In all cases using a three way comparison there were no differences detectable between the groups at a probability of <0.05.

The data in Table 6.1 suggest that there may be a difference in age between the groups with the median age of Group A being higher than Groups B and C. Similarly the median systolic blood pressure in Group B appears to be lower than Groups A and C. Although these differences did not reach statistical significance it is possible that small real differences did exist between the groups and that there were insufficient numbers to reach statistical significance.

### 6.5.2 Psychological characteristics of the study groups

Table 6.2 shows the mean scores obtained with the Brief Symptom Inventory. Using an analysis of variance across the groups for each psychological characteristic, there were no significant differences between the groups for any characteristic. However, the low numbers used in this sample and the high variance within the groups requires that only moderately large differences will acquire statistical significance. The largest differences between the groups is for somatization, interpersonal sensitivity and depression.

Group A may have a higher index of interpersonal sensitivity and depression and Group C may have a greater degree of somatization. A study with larger numbers would be required to confirm this.

Similarly the life events score for each group failed to achieve statistical significance.

**TABLE 6.2**

Mean scores on the Brief Symptom Inventory for Groups A-C compared with a British Community sample mean

| | **A Mean (SD)** | **B Mean (SD)** | **C Mean (SD)** | **British Community Sample Mean (SD)** |
|---|---|---|---|---|
| Somatization | 0.65 (0.59) | 0.61 (0.67) | 1.13 (0.79) | .43 (.57) |
| Obsessive/ compulsive | 0.56 (0.64) | 0.36 (0.60) | 0.72 (0.82) | .59 (.63) |
| Interperson sensitivity | 0.28 (0.48) | 0.15 (0.27) | 0.44 (0.5) | .58 (.72) |
| Depression | 0.47 (0.86) | 0.18 (0.24) | 0.31 (0.35) | .42 (.65) |
| Anxiety | 0.28 (0.50) | 0.33 (0.48) | 0.42 (0.44) | .45 (.60) |
| Hostility | 0.35 (0.50) | 0.60 (1.05) | 0.63 (0.62) | .44 (.60) |
| Phobic anxiety | 0.33 (0.56) | 0.16 (0.26) | 0.29 (0.39) | .24 (.50) |
| Paranoid ideation | 0.32 (0.66) | 0.2 (0.53) | 0.28 (0.37) | .54 (.65) |
| Psychoticism | 0.22 (0.42) | 0.23 (0.42) | 0.10 (0.16) | .27 (.48) |
| GSI | 0.29 (0.42) | 0.24 (0.31) | 0.43 (0.28) | .44 (.47) |
| PST | 9.0 (11.5) | 7 (7.8) | 12 (7.5) | 14.46 (10.43) |
| PSDI | 1.46 (0.6) | 1.46 (1.0) | 1.98 (0.91) | 1.38 (0.56) |

Key to summary indices:

GSI Global Severity Index
PSDI Positive Symptom Distress Index
PST Positive Symptom Total

See Materials and methods for an explanation of the psychological symptoms used.

# SECTION 7

# THE CLINICAL STUDY

## 7.1 INTRODUCTION

This section reports the results of the main study. The endpoint of the study was a change in the glycated proteins following either intervention to improve blood glucose control (Groups A and B) or no intervention (Group C). A full explanation of the study design and control groups is given in Section 6.

Although Groups A and B were designated as the intervention groups, the patients in Group C were attending hospital diabetic outpatient clinics on a 6-12 monthly basis and were therefore receiving some attention from health care professionals. Indeed, it is likely that they received advice on improving their blood glucose control. However, they were not exposed to the structured advice offered to patients in Groups A and B. Patients were informed of their high risk status due to microalbuminuria if they were in Group A but not if they were in Group B. The advice given to Group A was given by a team of diabetic nurse specialists who were familiar with the aims of the study. In discussions which preceded the study, the strategy for advising patients was formulated.

The approach to advising patients was based on the behavioural model of health care, defined by Schwartz and Weiss (1978) and advocated as suitable for application to diabetics by Surwit et al (1982). Shillitoe (1988) has assembled a list of methods which can be applied to diabetic patients when attempting to change their behaviour with respect to their disease:

*Specific assignments* which define what is to be done.

*Prompting and cueing* specific behaviours.

*Tailoring* the regimen to the particular needs of the patient.

*Contracts* which describe the role played by the patient and others.

*Graduated implementation* of the regimen or new behaviours of others.

*Modelling and imitation* to learn from the behaviours of others.

*Monitoring* behaviours and outcomes relevant to treatment.

*Skills training* to develop new behaviours.

*Reinforcement* of appropriate new behaviours.

*Counter-conditioning* to replace maladaptive behaviours with more appropriate ones.

In addition to explaining these concepts to nursing staff, patient contracts were designed which set self monitoring targets based on blood and urine glucose testing. Other targets were exercise and weight control. These contracts were intended to include realistic targets and to be mutually agreed between the patient and the nurse specialists.

After the first interview between the patient and diabetes nurses an 'information giving questionnaire' was completed by the patient if they were in Group A and had therefore been given information about their increased risk of complications due to microalbuminuria. This questionnaire established whether each patient had understood the information given to them (see Appendix 1). All patients except one indicated that they had understood the received information. The patient who had apparently not understood the information given to her, on further questioning had misunderstood the questionnaire.

## 7.2 MATERIALS AND METHODS

Methods for measuring glycated proteins have been described in Section 2. The groups were examined to determine whether the mean change in glycated protein was different between the groups. Because the numbers in each group were relatively small it was not possible to use a statistical procedure which adequately tested whether the differences in each group were approximately normally distributed. Visual inspection of

Figures 7.1-7.3 reveals that in each group there are some large differences in glycated proteins but the majority of differences are relatively small. This observation suggests that it would be unsafe to assume a normal distribution. Accordingly a non-parametric equivalent of the paired t test has been used - the Wilcoxon Signed Rank Test.

## 7.3 RESULTS

The change in glycated proteins achieved by each patient in Groups A-C is presented in Figures 7.1-7.3. Each patient is represented by two plots linked by a line. The left hand plot for each pair represents the glycated protein when the patient was recruited to the study. The right hand plot represents the glycated protein measured again after 12-18 months. Thus the slope of each line indicates whether and to what extent the glycated protein concentration has been decreased (indicating improved glycaemic control) or increased (indicating worsened glycaemic control). An overall impression of the data suggests that a few patients in each group show large differences between their two measurements of glycated protein. Some show dramatic improvements in glycaemic control, others show equally dramatic deterioration. One patient classified as NIDDM was changed to insulin therapy during the study period. The majority of patients however, show relatively modest changes.

The Wilcoxon Signed Rank Test shows no statistical differences between groups when the ranked differences of any of the glycated proteins are compared (see Tables 7.1-7.3).

**Changes in glycated proteins over the study period**

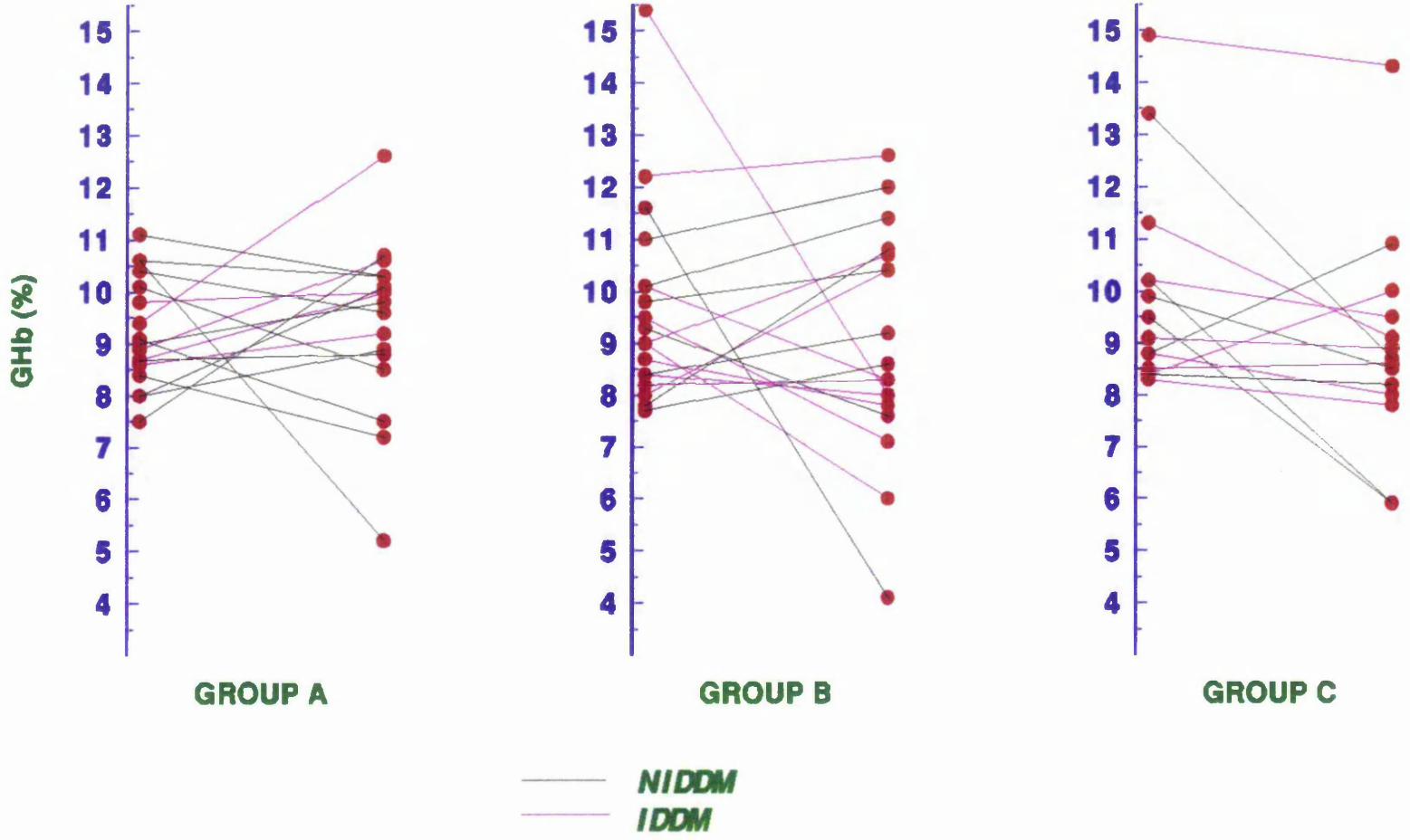
Tables 7.1-7.3 show the changes in glycated proteins which occurred during the study period. The data have been analysed by measuring the increment between measurements made on entry into the study and : a) the end of the study period (12-18 months later) or b) the lowest subsequent measurement.

There were statistically significant reductions in fructosamine in Groups B and C (Table 7.1) at the end of the study period. By including the lowest recorded fructosamine as the endpoint, all three groups showed significant reductions. For glycated globin (Table 7.3) only Group C showed a significant reduction at the end of the study.

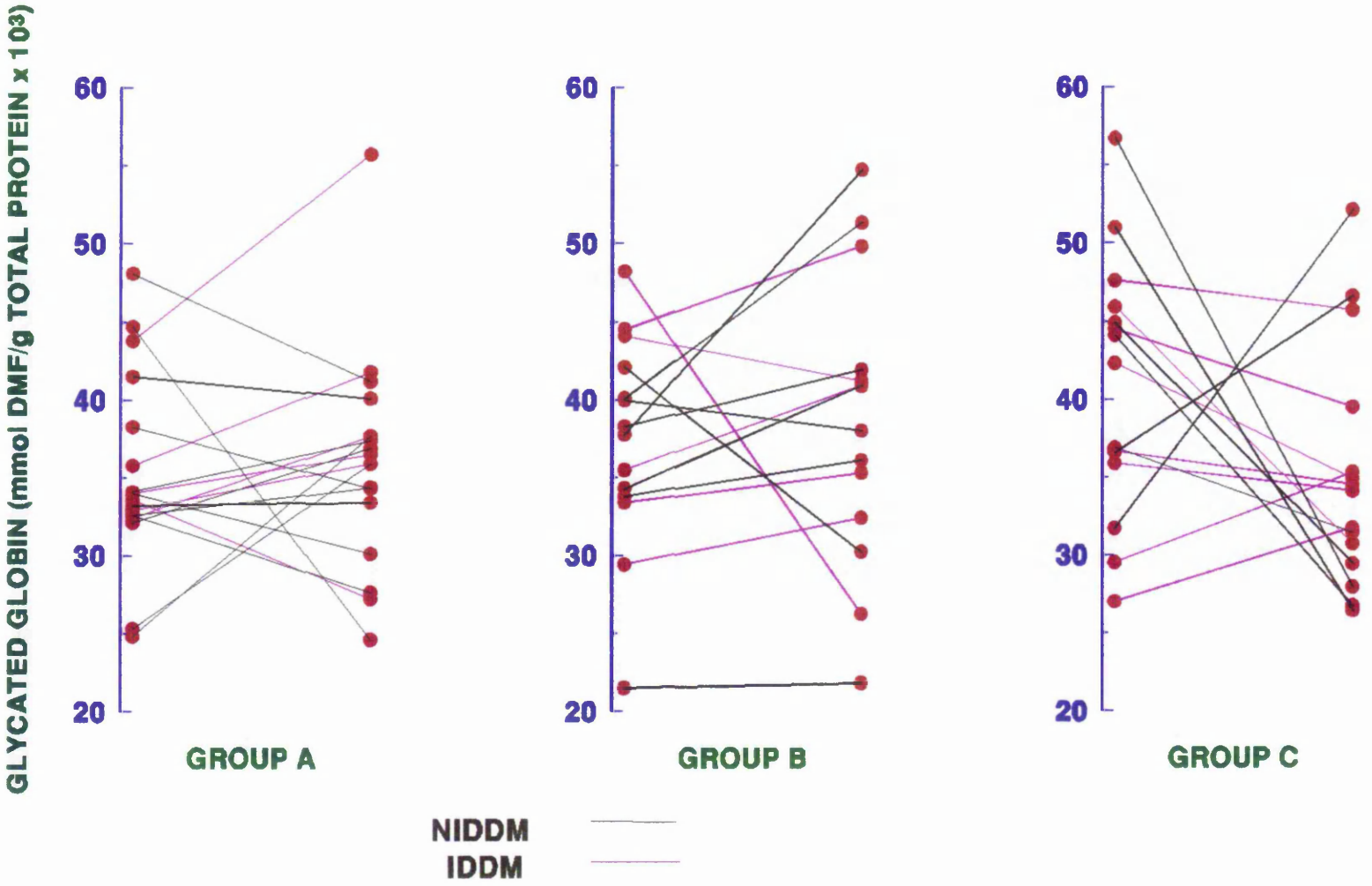
**FIGURES 7.1 - 7.3**

Changes in glycated proteins which occurred between the beginning and the end of the study period. The first plot of each pair represents the glycated protein measurement made on recruitment to the trial. Each plot is linked by a line to a second plot which represents the glycated protein measurement made at the end of the study period.

# CHANGES IN GHb BY TREATMENT GROUP



# CHANGES IN GLYCATED GLOBIN BY TREATMENT GROUP



# CHANGES IN FRUCTOSAMINE BY PATIENT GROUP

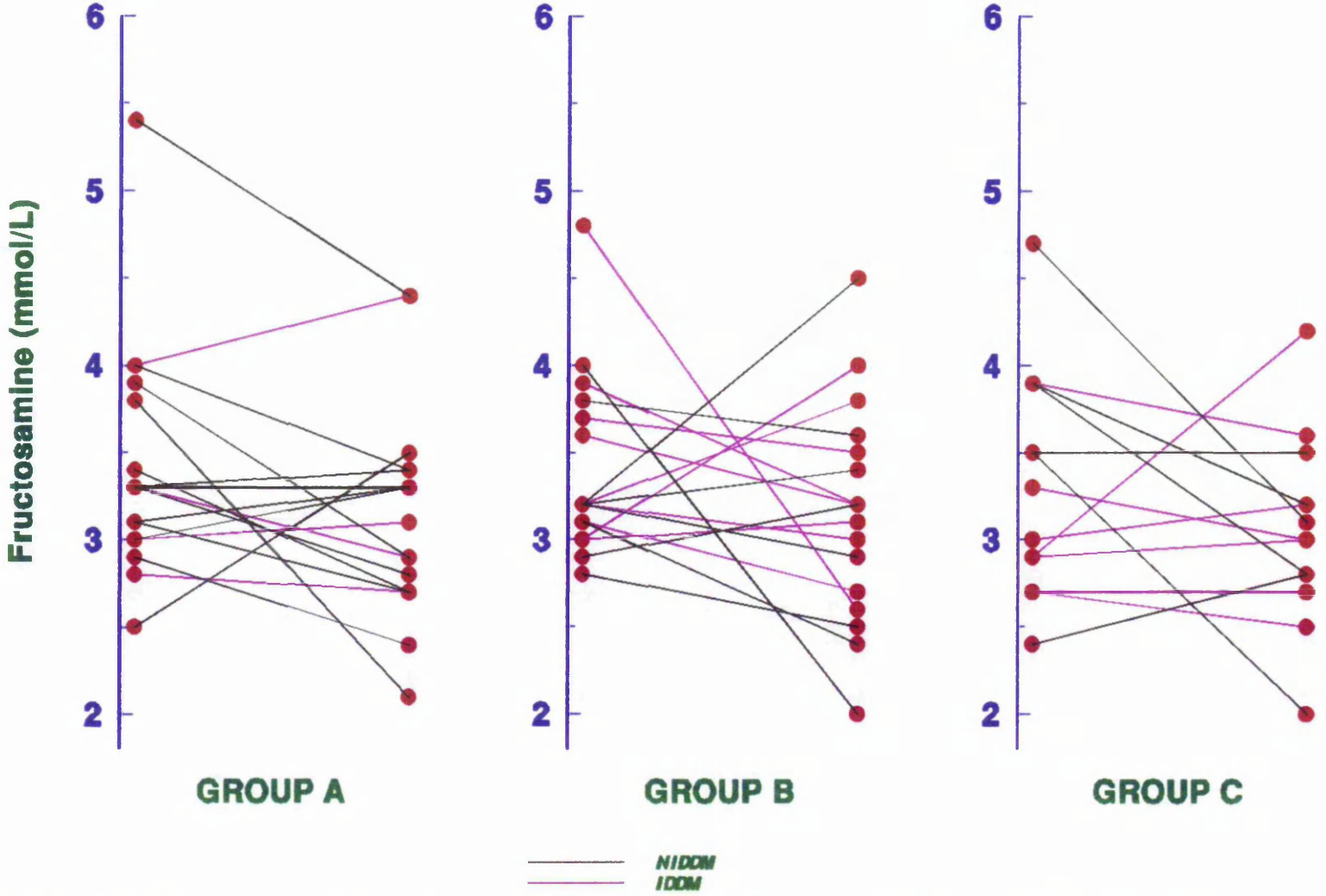


**TABLE 7.1 Results of the Wilcoxon signed rank test for differences in fructosamine**

| FRUCTOSAMINE | | | | | | |
|---|---|---|---|---|---|---|
| | **Baseline to end of study period** | | | **Largest difference from baseline** | | |
| | A | B | C | A | B | C |
| Probability (p) that the difference was due to chance | 0.18 | 0.05* | 0.05* | 0.05* | 0.02* | 0.01* |
| Estimated median (mmol/L) | 0.2 | 0.3 | 0.45 | 0.3 | 0.3 | 0.6 |

* statistically significant

**TABLE 7.2 Results of the Wilcoxon signed rank test for differences in glycated globin**

| GLYCATED GLOBIN | | | | | | |
|---|---|---|---|---|---|---|
| | **Baseline to end of study period** | | | **Largest difference from baseline** | | |
| | A | B | C | A | B | C |
| Probability (p) that the difference was due to chance | 0.57 | 0.72 | 0.1 | 1 | 0.43 | 0.03* |
| Estimated median (mmol/g Total Protein x $10^3$) | -1.05 | 0.6 | 0.1 | 0.05 | 1.65 | 9.6 |

* statistically significant

**TABLE 7.3 Results of the Wilcoxon signed rank test for differences in $HbA_1$**

| GHb | | | | | | |
|---|---|---|---|---|---|---|
| | Baseline to end of study period | | | Largest difference from baseline | | |
| | A | B | C | A | B | C |
| Probability (p) that the difference was due to chance | 0.73 | 0.68 | 0.03 | 0.49 | 0.68 | 0.03* |
| Estimated median (%) | -0.15 | 0.3 | 0.8 | 0.3 | 0.3 | 0.9 |

* statistically significant

## 7.4 DISCUSSION

Although statistically significant, the changes in serum fructosamine which occurred within Groups A, B and C are small and may not be clinically significant. The largest study in which insulin dependent diabetics were subjected to intensive insulin therapy is probably the Diabetes Control and Complications Trial (The Diabetes Control and Complications Research Group 1993). This trial achieved a median $HbA_{1C}$ change of approximately 1.8%. The maximum median change in GHb achieved in this study is 0.9% (Table 7.3). This degree of change could be clinically significant. However there are no trial data to support the conclusion that significant clinical benefit would accrue to patients who were able to sustain such a change in GHb of 0.9%. It is reasonable to suppose that patients who have improved their blood glucose control are more likely to avoid the serious complications of diabetes than those whose control deteriorates.

The most striking aspect of the data presented in Tables 7.1-7.3 is that in general, patients in Group C appeared to be more successful at improving their blood glucose

control than patients in Group A. Group A is the only group in which the estimated median fructosamine and GHb was negative indicating a general deterioration of blood glucose control over the study period. There are however, no statistically significant changes. Group C is the only group in which a statistically significant change in all three glycated proteins is achieved (although this was not sustained for the entire study period for glycated globin).

This finding is surprising and is indeed the opposite of what would be expected if knowledge of increased risk and exposure to support programmes form the Diabetic Centre affected patient's behaviour.

One explanation of these findings is that, in selecting patients with microalbuminuria, patients resistant to behavioural modification have been selected. Thus, Groups A and B, in which the patients all have microalbuminuria have done worse than Group C in which the patients do not. This finding implies that in a group of patients with microalbuminuria, an approach based on improvement of knowledge, feedback of monitoring data and structured interviews with the diabetic healthcare team is unlikely to be successful. Some insight into the likely reasons for this conclusion can be obtained from a very recent study designed to measure satisfaction in diabetes care (Jacobsen et al 1994). In this study a 21 page questionnaire was answered by 240 patients. Although the authors comment on problems which led to doubt about how representative this sample was, there was a clear indication that the severity of disease was the principal determinant of dissatisfaction. The influence of this factor was more powerful than the presence of a multi-disciplinary team, the site of the encounter between patient and health professional or the amount of time spent with the patient. It is possible that negative feelings of dissatisfaction have become exacerbated in Group A patients by finding out that their disease has

progressed to the stage that they are now albuminuric and require special support. Currently there is no evidence that feelings of dissatisfaction influence outcome measurements such as glycated haemoglobin or other glycated proteins. However, it would seem at least possible that a multi-disciplinary health team might be less effective when patients are dissatisfied with their health care. This is clearly an area for future research.

Because the effort put into interviewing and advising the patients in Group A does not seem to have produced significant benefits to the patients it is necessary to question the methods used. The questionnaires used in this study were not diabetes specific and at the beginning of the study no guidelines were available for their use in diabetic patients. Increasing interest in this area has led to the very recent development of a series of scales which are designed to measure diabetic patients' psychological outcome which will enable clinical audit of diabetes care (Bradley 1994). By focusing on psychological processes which are potentially modifiable it may be possible to intervene in the future by providing services such as stress management training to compliment the direct medical management of diabetes. Diabetes-specific scales have recently been recommended for use, which measure fear of hypoglycaemia and diabetes knowledge. Although a fear of hypoglycaemia scale has been published (Cox et al 1987) its role as a validated research tool has only recently been established (Irvine et al 1994). Similarly, several unvalidated questionnaires have been used for assessing diabetic knowledge with a view to maximising the impact of diabetic self care training. Rigorous testing of the groups in this study may have revealed differences in diabetic knowledge which would have affected patients ability to respond to the information given to them concerning their microalbuminuria. Recently, shortened validated diabetic knowledge (DKN) scales have become available (Beeny et al 1994) which can be used in the future for both research and audit.

The preceding argument assumes that adequate medical advice and treatment has been available to the patients in the study and that an important influence on outcome is the patient's attitude towards compliance with treatment and other aspects of self-care. It is difficult to measure the quality of treatment independently of outcome, except by auditing the processes which comprise health care delivery. In this study the processes were reviewed and arranged specifically for the groups of patients included. Thus perhaps the best indication that the study patients were receiving good quality treatment is their attendance record at clinics during the study. However, these patients are a self selected group since only patients which attended the clinics sufficiently frequently amassed enough data to be included.

This study included insulin dependent and non-insulin dependent diabetes and did not select on the basis of age except to attempt to obtain a similar age distribution between the groups. This may explain why little or no improvement in glycated protein occurred during the study period whereas considerable improvement was observed among microalbuminuric insulin dependent diabetics in the Diabetic Control and Complication Trial (DCCT) (The DCCT Research Group 1993).

A further reason is that the risk of symptomatic hypoglycaemia is a constraining factor on professionals who administer diabetes care. The DCCT showed a significantly raised level of hypoglycaemic attacks in patients on intensive insulin regimes. This degree of intensity may not have been achieved among the insulin dependent diabetics in Group A of the current study.

Choosing the most appropriate glycated protein measurements is very important in seeking to achieve good outcomes for diabetic patients (Benjamin and Sachs 1994). Accurate measurements of glycated protein which truly reflect integrated blood glucose

concentrations are especially important if patients are on intensive insulin therapy regimes as recommended by the American Diabetes Association (American Diabetes Association 1993). Glycated protein measurements which overestimate the integrated blood glucose may precipitate an unacceptable number of hypoglycaemic attacks which may have the effect of reducing patient compliance or even lead to significant morbidity. A recent External Quality Assessment Survey by the College of American Pathologists indicated that there were more than 15 different assays for glycated haemoglobin based on at least five completely different techniques in the U.S. The situation in the U.K. is similar with 4 method groups classified on the United Kingdom National External Quality Assessment Scheme (McKenzie 1993). These same reports indicate clearly that different methods produce widely differing results. Although each method's results are assessed against a different reference range, setting goals for glycated haemoglobin measurement is very difficult. This was recognised in the design of the DCCT Trial (The DCCT Research Group 1986) where all glycated haemoglobin measurements from a multi-centre trial were conducted in a single laboratory.

An attempt has been made to standardise glycated haemoglobin by using common calibrants (Little et al 1992) with values assigned by an HPLC procedure which measured $HbA_{1C}$. This approach significantly improved between laboratory agreement despite a non-linear relationship between $HbA_{1C}$ and glycated haemoglobin measured by affinity chromatography. (Bodor et al 1992). This approach could be used to overcome the calibration problems associated with the glycated globin assay. Unfortunately, most commercially available quality control materials do not give comparable results with all methods (Little et al 1991). In practice this would require access to an HPLC system which would assign values to crude haemolysates. Further development of the glycated globin

assay would seem justified. The clinical value of the assay should ideally be confirmed by including the assay in future trials of intensive therapy regimes such as the DCCT which contain sufficient numbers of patients to establish the relationship between changes in glycated proteins and regression of the complications of diabetes.

The fructosamine assay detected statistically significant changes in blood glucose control more frequently than GHb. Similarly the GHb assay detected statistically significant changes in blood glucose control more frequently than glycated globin.

One interpretation of these data is that fructosamine is a more sensitive test for detecting small changes in blood glucose control. The superior analytical precision of serum fructosamine may overcome the specificity problems discussed in Section 2.4. Similarly glycated globin appears to be the least sensitive test. It is difficult to give this conclusion any authority without a 'gold standard' of blood glucose control against which these glycated proteins can be judged. However, in so far as any conclusion can be drawn, the clinical performance of the glycated globin assay should be improved before it can be recommended as a routine assay.

The results of this study suggest that the measurement of glycated protein to assess blood glucose control may not be a simple matter of choosing a single parameter such as $HbA_{1C}$. If small changes in glycated proteins are anticipated, either very precise measurement of $HbA_{1C}$ is required (perhaps above the performance currently achieved in the U.K. for most methods) or a method with an intrinsically low analytical imprecision would be used e.g. serum fructosamine.

Improvements to the serum fructosamine assay used in this study have resulted in a 'second generation' assay for which a preliminary clinical evaluation has been carried out. $HbA_{1C}$ methods using high pressure liquid chromatography now approach fructosamine assays in terms of analytical imprecision.

Further improvements to the glycated globin assay are planned in this laboratory. Currently an evaluation is taking place of an alternative NBT based reaction which uses bichromatic spectrophotometry on crude haemolysates without removing haem (Fujita et al 1993).

# 8. CONCLUSION AND DISCUSSION

This thesis has examined a novel method for measuring glycated haemoglobin and used it in a study aimed at determining the effect of imparting detailed knowledge to patients with microalbuminuria who are at particular risk from the sequelae of diabetes. Several methods for measuring low concentrations of albumin in urine have been studied. Although there were significant theoretical advantages with fluoroimmunoassay and particle enhanced immunoturbidimetry, the lack of robustness shown by these methods led to the choice of polyethylene glycol enhanced immunoturbidimetry, despite limitations in the measurement range of the assay.

The clinical study failed to show any significant improvement in blood glucose control when patients were seen by a specialist nursing team and given knowledge concerning their microalbuminuria.

The large number of different methodologies used for measuring glycated haemoglobin prompts the question: Is there a need for another one based on an entirely different analytical principle? One line of argument would suggest that it is the heterogeneity of glycated haemoglobins which makes assay standardisation (in the sense of uniformity) and calibration difficult. Therefore, it would be logical to use methods which measure a single class of glycated haemoglobins. This is largely the argument used by those who advocate measuring $HbA_{1C}$ by HPLC as a reference method (Little et al 1992). The decision to measure $HbA_{1C}$ leads to a further choice of methodology. High performance liquid chromatography is able, to a greater or lesser extent depending on the precise method, to separate $HbA_{1C}$ from other glycated and non-glycated haemoglobins. HPLC can therefore quantitate $HbA_{1C}$ relative to the concentration of non-glycated haemoglobin without calibration. This is an analytically acceptable practice because the concentration of total haemoglobin is variable but not usually relevant to assessing glycaemic status

(Lester 1989). Specific monoclonal antibodies for the glycated terminal valine residue of $HbA_{1C}$ are the basis of several immunological methods, eg the Bayer DCA 2000 analyser, (Bayer Diagnostics, Basingstoke), and the Novoclone $HbA_{1C}$ assay (Novo Nordisk, Cambridge). However, unlike HPLC, immunological methods can only generate a signal from $HbA_{1C}$. Since no recognised reference materials exist for $HbA_{1C}$ there are effectively no calibrations for immunological methods. In practice, they are calibrated against HPLC techniques (Guthrie et al 1992).

Methods such as affinity chromatography detect glycated haemoglobins on the basis of interactions at several glycated amino acid sites on the haemoglobin molecule (Mallia et al 1981). While it is possible and common practice to quantitate such methods by relating the glycated fraction to total haemoglobin, the reference ranges are quite different to methods measuring $HbA_{1C}$. It is possible to relate the affinity chromatography measurement to $HbA_{1C}$ by calibrating the instrument using values derived from HPLC measurement of calibration material. This is the calibration system used by the Abbott Vision analyser (Fiechtner et al 1992).

The approach outlined above would be appropriate for the glycated globin assay developed here. If issues of specificity and analytical variation can be settled, there appears to be no reason why glycated globin assays should not provide information that is as useful as other methods for measuring glycated haemoglobin.

Polyethylene glycol enhanced immunoturbidimetry has been chosen as the method for detecting microalbuminuria. Dipstick methods based on specific immunological methods are now available which have the advantage of being able to generate results at the bedside or during a consultation in the outpatient clinic. Although currently rather expensive (approximately four times the cost of the laboratory procedures used in this

study), dipstick methods have been evaluated and found to be adequate for detecting and monitoring microalbuminuria (Marshall and Shearing 1991). Whichever method is used in the routine clinic situation an effective strategy is required to target microalbuminuria testing towards those diabetic patients who will benefit. The more expensive the test is, the less cost effective the procedure becomes if inappropriate patients are included. The results of this study suggest that there is at least some doubt that all patients will be able to improve their blood glucose control as part of a concerted effort to reduce the likelihood of suffering from the sequelae of diabetes. It may be logical to restrict testing for microalbuminuria to those diabetic patients most likely to benefit. Because good clinical trial evidence exists for insulin dependent diabetics, this group would probably be the most appropriate. However, the age at which to start monitoring for microalbuminuria is difficult to decide. The study of Marshall and Alberti (1989) suggested that very few patients with IDDM for less than five years are likely to have microalbuminuria. Thus duration of diabetes may be important when trying to target screening programmes for microalbuminuria amongst diabetic patients. The same study suggested that for NIDDM patients, due to the long silent phase of the illness before diagnosis, no such cut off could be used.

In non-insulin dependent diabetes identification of microalbuminuria indicates an increased risk of death from cardiovascular disease. It would therefore seem sensible to restrict testing for microalbuminuria to patients who are able or willing to take steps to reduce other risk factors for coronary heart disease, such as smoking, hyperlipidaemia and obesity.

An acceptable cost effective assay for a glycated protein is likely to become an audit tool which will be extensively used both for comparison of the effectiveness of diabetic

services between centres and for establishing the efficacy of treatment in individuals and clinical trails. This thesis has attempted to use glycated protein to indicate whether patients with microalbuminuria can be successfully encouraged to improve their glycaemic control. It addressed the question of whether microalbuminuric patients will benefit from the use of extra resources aimed at improving glycaemic control by education and information-giving. The conclusion was that this thesis provided no evidence that patients with microalbuminuria, unselected for the type of diabetes and age, benefited substantially from such measures. A more selective approach to patients with microalbuminuria is probably required. One possibility would be to repeat this study using selected groups such as insulin dependent diabetics with microalbuminuria and young patients with microalbuminuria.

The DCCT trial has shown that very intensive insulin therapy is associated with both an improvement in glycated protein status and a reduction in albumin excretion. A major problem which the DCCT trial cannot answer is the question of whether very significant improvements in glycaemic control can be achieved at centres involved in routine diabetic care rather than clinical trials.

Despite the problems, a concentration of resources on patients with microalbuminuria remains a reasonable way of targeting limited funding. The importance of detecting microalbuminuria which, if left untreated leads to frank proteinuria, has been highlighted recently in a WHO multi-national study which confirmed that proteinuria (urine protein excretion detectable by sulphosalicylic acid turbidimetry) is a common and important risk factor for morbidity and mortality in middle aged people with diabetes (Stephenson et al 1995) suggesting that microalbuminuria is simply part of the same continuum of disease. However, more work is required both to improve the way in which patients are motivated to improve glycaemic control and to identify those groups of microalbuminuric patients who are likely to benefit most from such efforts.

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# APPENDIX 1

This appendix includes copies of the psychometric questionnaires, contracts with patients, patient records, information-giving questionnaires and patient contracts.

INSTRUCTIONS:

Below is a list of problems people sometimes have. Please read each one carefully, and circle the number to the right that best describes HOW MUCH THAT PROBLEM HAS DISTRESSED OR BOTHERED YOU DURING THE PAST 7 DAYS INCLUDING TODAY. Circle only one number for each problem and do not skip any items. If you change your mind, erase your first mark carefully. Read the example below before beginning, and if you have any questions please ask about them.

SEX

MALE ○

FEMALE ○

NAME: _____

LOCATION: _____

EDUCATION: _____

MARITAL STATUS. MAR ___SEP ___DIV ___WID ___SING ___

| DATE | | | ID. NUMBER | AGE |
|---|---|---|---|---|
| MO | DAY | YEAR | | |
| | | | | |

VISIT NUMBER: _____

| EXAMPLE<br>HOW MUCH WERE YOU DISTRESSED BY: | NOT AT ALL | A LITTLE BIT | MODERATELY | QUITE A BIT | EXTREMELY |
|---|---|---|---|---|---|
| 1. Bodyaches | 0 | 1 | 2 | (3) | 4 |

| HOW MUCH WERE YOU DISTRESSED BY: | | NOT AT ALL | A LITTLE BIT | MODERATELY | QUITE A BIT | EXTREMELY |
|---|---|---|---|---|---|---|
| 1. Nervousness or shakiness inside | 1 | 0 | 1 | 2 | 3 | 4 |
| 2. Faintness or dizziness | 2 | 0 | 1 | 2 | 3 | 4 |
| 3. The idea that someone else can control your thoughts | 3 | 0 | 1 | 2 | 3 | 4 |
| 4. Feeling others are to blame for most of your troubles | 4 | 0 | 1 | 2 | 3 | 4 |
| 5. Trouble remembering things | 5 | 0 | 1 | 2 | 3 | 4 |
| 6. Feeling easily annoyed or irritated | 6 | 0 | 1 | 2 | 3 | 4 |
| 7. Pains in heart or chest | 7 | 0 | 1 | 2 | 3 | 4 |
| 8. Feeling afraid in open spaces | 8 | 0 | 1 | 2 | 3 | 4 |
| 9. Thoughts of ending your life | 9 | 0 | 1 | 2 | 3 | 4 |
| 10. Feeling that most people cannot be trusted | 10 | 0 | 1 | 2 | 3 | 4 |
| 11. Poor appetite | 11 | 0 | 1 | 2 | 3 | 4 |
| 12. Suddenly scared for no reason | 12 | 0 | 1 | 2 | 3 | 4 |
| 13. Temper outbursts that you could not control | 13 | 0 | 1 | 2 | 3 | 4 |
| 14. Feeling lonely even when you are with people | 14 | 0 | 1 | 2 | 3 | 4 |
| 15. Feeling blocked in getting things done | 15 | 0 | 1 | 2 | 3 | 4 |
| 16. Feeling lonely | 16 | 0 | 1 | 2 | 3 | 4 |
| 17. Feeling blue | 17 | 0 | 1 | 2 | 3 | 4 |
| 18. Feeling no interest in things | 18 | 0 | 1 | 2 | 3 | 4 |
| 19. Feeling fearful | 19 | 0 | 1 | 2 | 3 | 4 |
| 20. Your feelings being easily hurt | 20 | 0 | 1 | 2 | 3 | 4 |
| 21. Feeling that people are unfriendly or dislike you | 21 | 0 | 1 | 2 | 3 | 4 |
| 22. Feeling inferior to others | 22 | 0 | 1 | 2 | 3 | 4 |
| 23. Nausea or upset stomach | 23 | 0 | 1 | 2 | 3 | 4 |
| 24. Feeling that you are watched or talked about by others | 24 | 0 | 1 | 2 | 3 | 4 |
| 25. Trouble falling asleep | 25 | 0 | 1 | 2 | 3 | 4 |
| 26. Having to check and double check what you do | 26 | 0 | 1 | 2 | 3 | 4 |
| 27. Difficulty making decisions | 27 | 0 | 1 | 2 | 3 | 4 |
| 28. Feeling afraid to travel on buses, subways, or trains | 28 | 0 | 1 | 2 | 3 | 4 |
| 29. Trouble getting your breath | 29 | 0 | 1 | 2 | 3 | 4 |
| 30. Hot or cold spells | 30 | 0 | 1 | 2 | 3 | 4 |
| 31. Having to avoid certain things, places, or activities because they frighten you | 31 | 0 | 1 | 2 | 3 | 4 |
| 32. Your mind going blank | 32 | 0 | 1 | 2 | 3 | 4 |
| 33. Numbness or tingling in parts of your body | 33 | 0 | 1 | 2 | 3 | 4 |
| 34. The idea that you should be punished for your sins | 34 | 0 | 1 | 2 | 3 | 4 |
| 35. Feeling hopeless about the future | 35 | 0 | 1 | 2 | 3 | 4 |

Please continue on the following page ▶

| HOW MUCH WERE YOU DISTRESSED BY. | | NOT AT ALL | A LITTLE BIT | MODERATELY | QUITE A BIT | EXTREMELY |
|---|---|---|---|---|---|---|
| 36. Trouble concentrating | 36 | 0 | 1 | 2 | 3 | 4 |
| 37. Feeling weak in parts of your body | 37 | 0 | 1 | 2 | 3 | 4 |
| 38. Feeling tense or keyed up | 38 | 0 | 1 | 2 | 3 | 4 |
| 39. Thoughts of death or dying | 39 | 0 | 1 | 2 | 3 | 4 |
| 40. Having urges to beat, injure, or harm someone | 40 | 0 | 1 | 2 | 3 | 4 |
| 41. Having urges to break or smash things | 41 | 0 | 1 | 2 | 3 | 4 |
| 42. Feeling very self-conscious with others | 42 | 0 | 1 | 2 | 3 | 4 |
| 43. Feeling uneasy in crowds | 43 | 0 | 1 | 2 | 3 | 4 |
| 44. Never feeling close to another person | 44 | 0 | 1 | 2 | 3 | 4 |
| 45. Spells of terror or panic | 45 | 0 | 1 | 2 | 3 | 4 |
| 46. Getting into frequent arguments | 46 | 0 | 1 | 2 | 3 | 4 |
| 47. Feeling nervous when you are left alone | 47 | 0 | 1 | 2 | 3 | 4 |
| 48. Others not giving you proper credit for your achievements | 48 | 0 | 1 | 2 | 3 | 4 |
| 49. Feeling so restless you couldn't sit still | 49 | 0 | 1 | 2 | 3 | 4 |
| 50. Feelings of worthlessness | 50 | 0 | 1 | 2 | 3 | 4 |
| 51. Feeling that people will take advantage of you if you let them | 51 | 0 | 1 | 2 | 3 | 4 |
| 52. Feelings of guilt | 52 | 0 | 1 | 2 | 3 | 4 |
| 53. The idea that something is wrong with your mind | 53 | 0 | 1 | 2 | 3 | 4 |

**Life Events Inventory**

Please indicate whether any of these events has happened to you in the last two years.. Put a tick (√ ) beside each event you have experienced within this time.

Section 1. All

| | | |
|---|---|---|
| 1. | Unemployment (of head of household) | _____ (68) |
| 2. | Trouble with superiors at work | _____ (40) |
| 3. | New job in same line of work | _____ (31) |
| 4. | New job in new line of work | _____ (46) |
| 5. | Change in hours or conditions in present job | _____ (31) |
| 6. | Promotion or change of responsibilities at work | _____ (39) |
| 7. | Retirement | _____ (54) |
| 8. | Moving house | _____ (42) |
| 9. | Purchasing own house (taking out mortgage) | _____ (40) |
| 10. | New neighboours | _____ (18) |
| 11. | Quarrel with neighbours | _____ (26) |
| 12. | Income increased substantially (25%) | _____ (35) |
| 13. | Income decreased substantially (25%) | _____ (62) |
| 14. | Getting into debt beyond means of repayment | _____ (66) |
| 15. | Going on holiday | _____ (29) |
| 16. | Conviction for minor violition (eg speeding or drunkeness) | _____ (34) |
| 17. | Jail sentence | _____ (75) |
| 18. | Involvement in fight | _____ (38) |
| 19. | Immediate family member starts drinking heavily | _____ (65) |
| 20. | Immediate family member attempts suicide | _____ (66) |
| 21. | Immediate family member sent to prison | _____ (61) |
| 22. | Death of immediate family member | _____ (69) |
| 23. | Death of close friend | _____ (55) |
| 24. | Immediate family member seriously ill | _____ (59) |
| 25. | Gain of new family member (immediate) | _____ (43) |

| | | |
|---|---|---|
| 26. | Problems related to alcohol or drugs | _____ (59) |
| 27. | Serious restriction of social life | _____ (49) |
| 28. | Period of homeless ness (hostel or sleeping rough) | _____ (51) |
| 29. | Serious physical illness or injury requiring hospital treatment | _____ (65) |
| 30. | Prolonged ill health requiring treatment by own doctor | _____ (48) |
| 31. | Sudden and serious impariment of vision or hearing | _____ (59) |
| 32. | Unwanted pregnancy | _____ (70) |
| 33. | Miscarriage | _____ (65) |
| 34. | Abortion | _____ (63) |
| 35. | Sex difficulties | _____ (57) |

Section 2. Ever-married only

| | | |
|---|---|---|
| 36. | Marriage | _____ (50) |
| 37. | Pregnancy | _____ (49) |
| 38. | Increase in number of arguments with spouse | _____ (55) |
| 39. | Increase in number of agruments with other immediate family members (eg children) | _____ (43) |
| 40. | Trouble with other relatives (eg in-laws) | _____ (38) |
| 41. | Son or daughter left home | _____ (44) |
| 42. | Children in car of others | _____ (54) |
| 43. | Trouble or behaviour problems in own children | _____ (49) |
| 44. | Death of spouse | _____ (86) |
| 45. | Divorce | _____ (75) |
| 46. | Marital separation | _____ (70) |
| 47. | Extra-marital sexual affair | _____ (61) |
| 48. | Break up of affair | _____ (47) |
| 49. | Infidelity of spouse | _____ (68) |
| 50. | Marital reconciliation | _____ (53) |

Section 3. Never-married only

| | | |
|---|---|---|
| 51. | Break up with steady boy or girl friend | _____ (51) |
| 52. | Problems related to sexual relationship | _____ (54) |
| 53. | Increase in number of family arguments (eg with parents) | _____ (43) |
| 54. | Break up of family | _____ (77) |

**PATIENT RECORD**

NAME:

CLINIC VISIT DATE:

LENGTH OF TIME ON: BGM ☐ UGM ☐ CONTRACT ☐

| AGREED TARGETS | ACHIEVED % | POINTS |
|---|---|---|
| BGM | | |
| UGM | | |
| EXERCISE | | |
| WEIGHT | | |
| TRAINING | | |

---

THOUGHTS OF MOOD STATES ASASOCIATED WITH PROBLEM BEHAVIOUR EXPLORED ☐

OUTCOME:

CHANGES OF SOCIAL CIRCUMSTANCES: EXPLORED ☐

OUTCOME:

CONTACT:

## PATIENT CONTRACT

NAME: DATE:

After talking about ways to improve my diabetes control I have agreed to:

BLOOD SUGAR MEASUREMENT:

URINE SUGAR MEASUREMENT:

EXERCISE:

WEIGHT CONTROL:

TRAINING:

CONTACT:

## INFORMATION GIVING QUESTIONNAIRE

NAME:

1. Have you been given any **NEW** information about your diabetes?

| | |
|---|---|
| YES | |
| NO | |

2. If answer to Question 1 is YES, which part of your body is likely to be affected by this new information?

| | |
|---|---|
| Heart | |
| Lungs | |
| Kidney | |
| Feet | |
| Eyes | |
| Legs | |
| Can't remember | |

3. Have you been given any particular advice about how to reduce the risk of damage to this part of the body?

## PATIENT DATA COLLECTION FORM

DIABETES CENTRE

CONSULTANT:

DURATION OF DIABETES: DATE OF NEXT APPOINTMENT:

HEIGHT (m): TREATMENT: INSULIN / ORAL HYPOGLYCAEMIC / DIET ALONE

B.P.: WEIGHT (Kg): LEFT RIGHT

RETINOPATHY GRADE:

NUMBER OF DRUGS CURRENTLY ADMINISTERED:

NAME OF ANY ANTIHYPERTENSIVE USED:

DETAILS OF ANY ANTIHYPERTENSIVE DOSAGE CHANGE:

CIRCLE IF THIS PATIENT SUFFERS FROM:

NON-DIABETIC RENAL DISEASE, URINARY TRACT INFECTION, CONCURRENT SYSTEMIC DISEASE (PLEASE STATE, SEE OVER FOR CHECK LIST)

---

CLINICAL CHEMISTRY LABORATORY, KING'S MILL HOSPITAL

PLEASE INDICATE IF PRESENT:

SYSTEMIC DISEASE
- AMYLOIDOSIS
- CARCINOMA
- HODGKIN'S DISEASE
- LEUKAEMIA
- LYMPHOMA
- MEMBRANOUS GLOMERULONEPHRITIS (SECONDARY FORMS)
- MULTIPLE MYELOMA
- POLYARTERITIS NODOSA
- POST STREPTOCOCCAL AND OTHER FORMS OR PARA-INFECTIONS GLOMERULONEPHRITIS

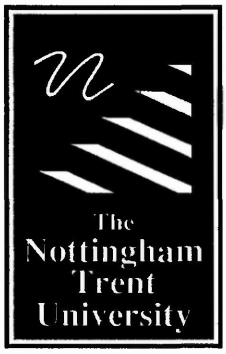
SCHONLEIN-HENOCH PURPURA
SICKLE CELL ANAEMIA
SYSTEMIC LUPUS ERYTHEMATOSUS
WEGENER'S AND OTHER FORM OF VASCULITIS

INFECTIOUS DISEASE
- HEPATITIS B
- MALARIA
- SUBACUTE BACTERIAL ENDOCARDITIS
- SYPHILIS

DRUGS AND TOXINS
- GOLD SALTS
- HEROIN
- LITHIUM
- MERCURY
- PENICILLAMINE

TRIMETHADIONE

MISCELLANEOUS
- CONGENITAL NEPHROTIC SYNDROME
- PREECLAMPSIA
- TRANSPLANT REJECTION



CLIN. CHEM. 36/12, 2131–2133 (1990)

# Semi-Automated Colorimetric Method for Measuring Glycohemoglobin, with Reduction of Nitroblue Tetrazolium, Evaluated

R. P. Hill

I evaluated a semi-automated method for measuring glycohemoglobin via reduction of nitroblue tetrazolium by acetone-extracted globin. The simultaneous measurement of glycated protein and total protein with the Roche Mira S analyzer showed good analytical precision (between-batch CV 3.5%–8.5%). Correlation of results ($y$) with those by a boronate affinity chromatography method ($x$) gave a regression line of $y = 2.0x + 5.9$ for samples from patients attending a diabetic outpatient clinic. I also studied reaction kinetics; chloromercuribenzoate concentration, sample volume, and detergent concentration affect the reaction, probably by modulating the reduction of dye by globin-thiol groups.

**Additional Keyphrases:** *globin protein · diabetes · boronate affinity chromatography compared*

Measuring glycohemoglobin is widely accepted for evaluating control of glycemia in diabetic patients. Manual methods such as affinity chromatography with boronate gels and electroendosmosis are popular but require disposable columns or electrophoresis gels and are relatively labor intensive. Methods based on immunoassay involve expensive reagents, and most automated HPLC methods require sophisticated dedicated instrumentation (*1*). A method that involves simple chemicals and that can be performed with standard laboratory equipment would have significant advantages for a busy clinical laboratory.

Recently, Somani et al. (*2*) outlined a manual method for measuring hemoglobin $A_1$ in which, after acetone extraction of heme, glycated globin reduces nitroblue tetrazolium (NBT) to its formazan derivative.[1] To overcome the solubility problems of extracted globin in the assay reagent, to investigate the reaction kinetics, and to design a colorimetric method suitable for automated analysis, I added *p*-chloromercuribenzoic acid (CMB) to the reagent to suppress the reducing activity of the thiol groups on the globin molecule. I investigated the reaction between glycated globin and NBT and describe a reformulated reagent optimized for the Cobas Mira S analyzer (Roche Instruments, Welwyn Garden City, U.K.).

## Materials and Methods

CMB, Triton X-100, sodium carbonate, copper sulfate, potassium sodium tartrate, potassium iodide, sodium hydroxide, and acetone (GPR grade) were obtained from BDH, Poole, Dorset, U.K. NBT was purchased from Sigma Chemicals Co., Poole, Dorset, U.K. All studies were carried out with the Cobas Mira S analyzer.

For the initial studies I used a diluent reagent containing, per liter, 75 mmol of sodium bicarbonate, and 225 mmol of sodium carbonate (pH 10.35). The dye reagent was prepared by mixing equal volumes of solution A (NBT, 3 mmol/L) and solution B (6 mmol of CMB, 150 mmol of sodium carbonate, and 450 mmol of sodium bicarbonate per liter).

The optimized reagents (adjusted to pH 10.35) consisted of the diluent reagent, containing 75 mmol of sodium bicarbonate, 225 mmol of sodium carbonate, and 24.2 g of Triton X-100, per liter; and the dye reagent, prepared by mixing equal volumes of solution A (as above) and solution B (as above, but containing 12 mmol of CMB per liter).

The analyzer protocol involved pipetting into the reaction cuvette 85 μL of globin solution with 10 μL of water, followed by 85 μL of diluent reagent and 95 μL of dye reagent. The change in absorbance between 10 and 15 min after mixing was recorded. All incubations were conducted at 37 °C.

To perform the assay, collect samples of whole blood into EDTA tubes by venipuncture, and store specimens at 4 °C. Add 0.1 mL of whole blood to 10 mL of 0.9 g/L sodium chloride reagent and mix the cell suspension well. After centrifugation, discard the supernate and add 1 mL of distilled water to the cell pellet. Vortex-mix, then let the hemolysate stand for 15 min. After further centrifugation, slowly add 1 mL of supernate to 10 mL of ice-cold acetone containing 50 μL of 1 mol/L hydrochloric acid reagent. Mix the solution, let it stand for 30 min, then centrifuge the tubes for 10 min at 1000 × *g*. Discard the supernate and immediately redissolve the precipitated globin in 0.5 mL of 0.9 g/L sodium chloride solution.

Throughout the study, I used pooled human serum diluted 10-fold and fivefold with 0.9 g/L NaCl solution as the glycated globin and total globin calibrators, respectively. Glycated globin values were assigned to the calibrators by assaying against deoxymorpholinofructose, and were expressed as millimoles of glycated globin per gram of hemoglobin. Globin concentration was measured by a biuret method on the Mira S simultaneously with glycated globin, as follows. Globin extract (40 μL) was added to 250 μL of biuret reagent, which contained, per liter, 14.3 mmol of copper sulfate, 38 mmol of potassium sodium tartrate, 36 mmol/L of potassium iodide, and 700 mmol of sodium hydroxide.

Hemoglobin ($A_1$ fraction; $HbA_1$) was measured by boronate affinity chromatography (Pierce and Warriner, Chester, Cheshire, U.K.). The leukocyte count was determined with a Symex K1000 counter (Toa Medical Electronics, Kobe, Japan).

## Results

*Solubility of globin in assay reagents.* The changes in absorbance that occurred during the 15-min period after adding extracted globin to the initial reagent system are shown in Figure 1. A rapid increase in absorbance was followed by a plateau, during which absorbance changed very little. After 15 min, a white precipitate, presumed to

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[1] Nonstandard abbreviations: NBT, nitroblue tetrazolium; CMB, *p*-chloromercuribenzoic acid; RER, residual error of regression; and $HbA_1$, hemoglobin, $A_1$ fraction.

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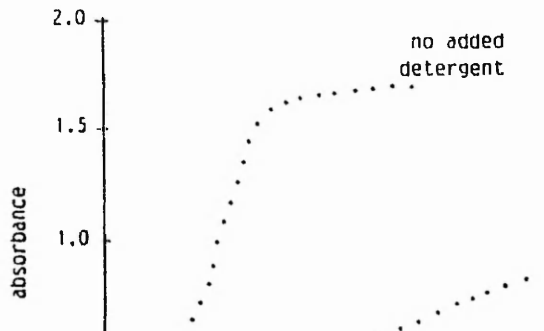


Fig. 1. Time course of reaction with and without Triton X-100 detergent

precipitated globin, appeared in the reaction mixture. To avoid this problem, I added Triton X-100 to the reaction mixture; a final concentration of 7.5 g/L in the assay reagent prevented globin precipitation (Figure 1).

*Effect of increasing concentration of surfactant.* Reagents containing 7.5 g and 15 g of Triton X-100 per liter were compared to determine whether changing the concentration of detergent affected the assay. Although changes in absorbance were similar, these methods differed in their agreement with the affinity chromatography method. With Triton X-100 at 7.5 g/L, the correlation was good [$y = 1.92x + 6.0$, $r = 0.94$, residual error of regression (RER) around $y = 2.5$]; at a detergent concentration of 15 g/L, the correlation was poor ($y = 1.89x + 5.9$, $r = 0.81$, RER around $y = 3.8$). Therefore, there appeared to be no advantage in increasing the concentration of detergent beyond 7.5 g/L. All changes in absorbance were read after precisely 15 min, with no delay before reading.

*Effect of CMB concentration on reaction kinetics.* Despite addition of surfactant, the reaction kinetics did not show a continuous monotonic increase in absorbance (Figure 2). Increasing the concentration of CMB in solution B to 3 mmol/L produced a smooth increase in absorbance, without the point of inflection that appears in Figure 2. This effect

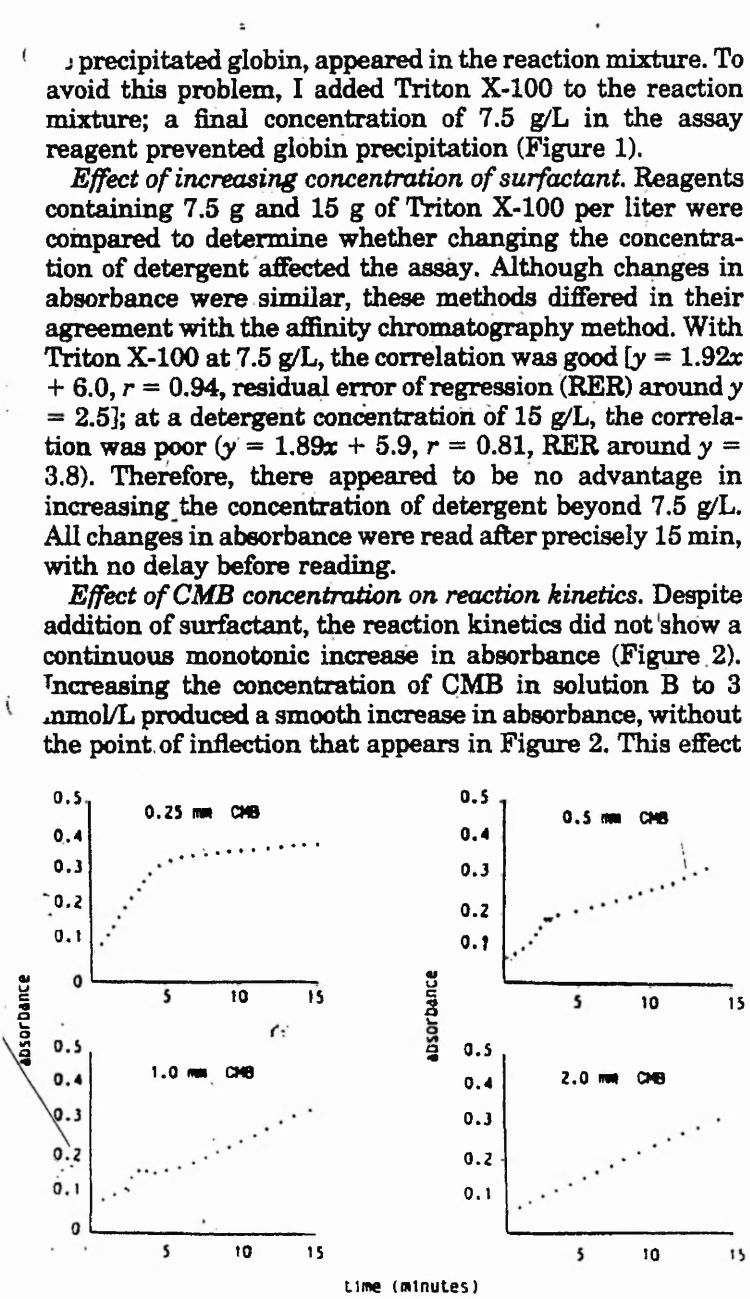


Fig. 2. Time course of reaction with various concentrations (mmol/L) of CMB

is probably attributable to incomplete suppression of thio group-mediated reduction of NBT at lower concentration of CMB.

*Pre-incubation period and read time.* The specificity wit which glycated proteins in serum can be measured with us of NBT reduction presumably is affected by the length c pre-incubation time (*3*). (Pre-incubation time is the tim that elapses between mixing the reactants and taking th first absorbance reading.) Various lengths of pre-incuba tion time and read time (the period during which abso bance measurements are taken) were investigated by ca culating the regression equation of results by this metho vs those by affinity chromatography. When no pre-incuba tion period was used and absorbance changes were rea over the first 5 min of reaction, the correlation was poo ($r = 0.78$; RER = 3.00; $y = 1.2x + 6.3$, where $y = HbA_1$ an $x$ = glycated globin). A 5-min pre-incubation followed by 5-min read time gave considerably better correlation ($r$ = 0.96), with a lower RER (1.88) and the following regressio equation: $y = 2.1x + 7.4$.

Increasing pre-incubation time to 10 min followed by 5-min read time produced similar good correlation ($r$ = 0.97), with RER = 1.58 and the regression equation bein $y = 2.0x + 5.9$.

*Precision.* The precision of the method was assessed b taking fresh blood samples from individual diabetic pa tients through the entire extraction and measuremen procedure. Table 1 shows the within-run and between-ru precision.

*Linearity.* With a globin solution diluted as described i *Materials and Methods,* the results of the glycated globi method showed a linear response over the range of 4–20 g/ (Figure 3), roughly equivalent to a total hemoglobin con centration of 50–200 g/L, with 5%–20% $HbA_1$. The pres ence of an intercept despite the use of a reagent blan suggests the presence of a nonspecific component in th reaction.

*Method comparison.* Whole-blood samples from 69 dia betic patients were analyzed by this method ($y$) and boronate affinity chromatography method ($x$). The regres sion equation, calculated by using the Deming method, wa $y = 2.0x + 5.9$ ($r = 0.97$, RER = 1.58).

*Interferences.* Traces of heme/acetone persisted to th final stage of the globin preparation procedure. To tes whether heme/acetone would interfere with the assay, added increasing volumes of supernate from the extractio stage of globin preparation; no substantial effect was seer with ≤100 μL of supernate.

Leukocytes, especially phagocytes, produce superoxid radicals (*4*), which under alkaline conditions reduce NBT Leukocytes also contain the enzyme superoxide dismutase (EC 1.15.1.1), which inhibits NBT reduction. To test th effect of including leukocytes in the sample, I analyzed samples from nondiabetic patients with leukocyte counts >11 000/μL for glycated globin before and after removal o

**Table 1. Precision Data for Proposed Assay**

| | CV, %, for determinations of glycated globin | |
|---|---|---|
| $HbA_1$, % | Within-batch | Between-batch |
| 5.0 | 7.6 | 8.5 |
| 10.2 | 3.1 | 4.2 |
| 12.8 | 2.0 | 3.5 |

n = 30 each.

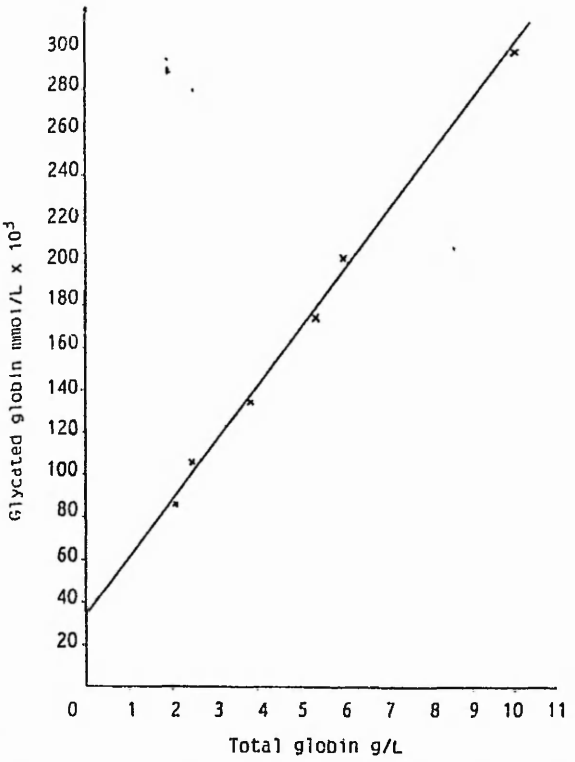


Fig. 3. Linearity of results for glycated globin at various dilutions

the buffy layer. Removal of the buffy cell layer reduced the leukocyte count to <4000/μL in all cases. A difference of <5% glycated globin was observed for each patient.

Reducing compounds in serum, e.g., ascorbate and urate, which might interfere with NBT reduction, were removed during the saline wash step, which dilutes plasma 100-fold.

To test the effect of high concentrations of ascorbate, I added 100 μL of a 50 mg/L solution of ascorbic acid to 100 μL of blood and compared the result for this with that for a control containing no ascorbic acid. The recovery of glycated globin in the supplemented sample was <3% more than that of the control. I conclude that extracellular low-molecular-mass compounds are unlikely to interfere with this method. To test the effect of urate, I added 50 μL of serum from a patient with renal failure (urate concentration 717 μmol/L) to triplicate samples of blood, but found no detectable interference.

*Stability and storage.* Extracted globin was stable in saline solution at room temperature for at least 12 h. $HbA_1$ in whole blood is stable for at least one week. Hemolysates of erythrocytes may be stored up to one month at −20 °C.

## Discussion

Although this method requires preparation of globin before automated analysis, the preparation stage is simple and contains no critical steps that require accurate measurements. A sample processor capable of fast liquid handling can reduce technician time to <1 h, comparable with that of other methods for $HbA_1$ analysis, e.g., affinity chromatography.

Results by this method correlate well with those by affinity chromatography and show a magnitude of intercept similar to that seen in comparisons between affinity chromatography and another chemical method (*5*) (thiobarbituric acid). However, Somani et al. (*2*) reported a negligible intercept in a similar comparison of affinity chromatography and a manual NBT method (*2*). The presence of an intercept on the dilution curve requires further investigation.

Adding detergent to alkaline reduction methods for glycated protein has previously been shown to be beneficial for the assay system (*6*). In this case, adding detergent ensures solubility of extracted globin.

Dye-reduction methods for glycated proteins have until recently been calibrated against deoxymorpholinofructose. The shortcomings of this material as a calibrant have been discussed elsewhere (*7*). Glycated polylysine has been suggested as a suitable alternative (*8*). Another approach would be to calibrate the glycated globin assay via a well-validated method for $HbA_1$ (e.g., affinity chromatography) or $HbA_{1c}$ (e.g., HPLC). The disadvantage is that methods based on different principles measure different proportions of the glycohemoglobin subclasses.

This method appears to be worthy of further development; however, larger clinical studies are required before the assay can be recommended as a routine test.

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Ann Clin Biochem 1990; 27: 413–424

# Recommendations for adopting standard conditions and analytical procedures in the measurement of serum fructosamine concentration

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*Additional key phrases: diabetes mellitis; glycated proteins; calibration*

Improvements in the treatment of diabetes mellitus, particularly since the availability of insulin therapy, have led to many fewer deaths from the acute medical problems of severe insulin deficiency. Increasing attention is now being focused on the long-term complications of the disease. Maintenance of blood glucose as near as possible to non-diabetic levels is widely accepted as a treatment goal, which if achieved, may significantly reduce the common sequelae of diabetes.[1] Multiple daily glucose measurements are often not a practical way of assessing diabetic control[2] although these measurements remain the 'gold standard' by which other indices should be judged. During the 1970s techniques for measuring glycated haemoglobin, which were suitable for routine clinical use, became available providing an objective retrospective indication of blood glucose levels over several weeks. Measurement of $HbA_1$, has been widely adopted as a useful measurement of diabetic control. Most techniques attempt to measure $HbA_{1c}$ which was originally described as the largest of the minor fractions of haemoglobin to be elevated in diabetic subjects. These early methods were not technically robust and varied in their specificity. (For a review of glycated haemoglobin methods and their clinical use see reference 2.) Over the last decade methods have been developed which, although tedious and labour intensive, are more reliable. Most of the common methods in the UK are not specific for $HbA_{1c}$, measuring varying proportions of other glycated haemoglobins. Specific assays for $HbA_{1c}$ have to date been mainly used for research.

The discovery that other serum proteins become glycated in an analogous way to haemoglobin[3] led to interest in their clinical significance. Glycated albumin has been suggested as a useful index of glycaemic control over a 2–3 week period,[4] a considerably shorter period than glycated haemoglobin which reflects diabetic control over 4–6 weeks. Methods for measuring glycated proteins include affinity chromatography,[5,6] spectrophotometric methods based on the thiobarbituric acid reaction[7] and high performance liquid chromatography of glycated lysine residues after hydrolysis of the glycated proteins.[8] Each of these methods is capable of giving good results in experienced hands. However, with the exception of the affinity chromatography method, they are generally too expensive or too cumbersome for routine use in a clinical chemistry laboratory.

In 1983 Johnson, Metcalf and Baker[9] described a fructosamine assay based on the ability of ketoamine-linked glucose residues on glycated serum proteins to reduce the dye nitroblue tetrazolium (NBT). The term 'fructosamine' refers to the structure of the ketoamine rearrangement product (1-deoxy-1(ε-lysyl-albumin) fructose) formed as a result of the non-enzymatic reaction between glucose and the ε amino group on the lysine residues of albumin (Fig. 1). The contribution of other reducing substances in serum which can interfere with the test is kept to a minimum by performing the reaction at pH 10·35 and by delaying absorbance readings until non-specific reactants such as glucose and urate have completed their reaction course. Fructosamine concentration largely reflects glycated albumin concentration although approximately 20% of the reducing activity is derived from other serum proteins.[10]

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Correspondence: Dr R P Hill.

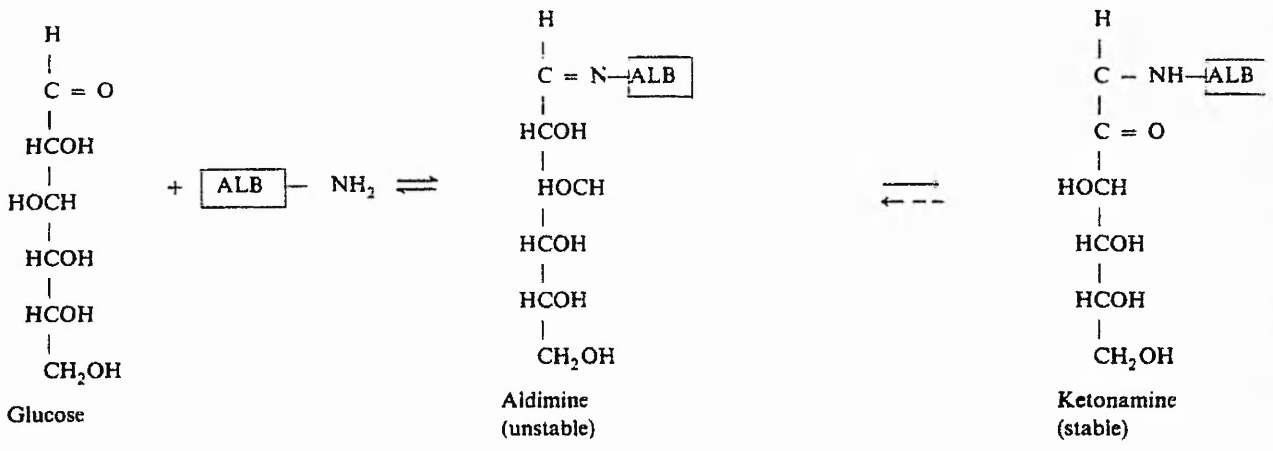


FIGURE 1. *Reactions leading to the formation of glycated albumin.*

The ease with which serum fructosamine measurement can be automated and the excellent between-batch analytical imprecision (typically most modern instruments show coefficients of variation of less than 5%) make the assay a potentially attractive test for monitoring diabetic patients.[11] Despite these advantages, problems of calibration and optimization of reaction conditions have limited its acceptance as an index of diabetic control. More recently, the effects of protein concentration, other diseases, biological variability and analytical specificity have led to confusion over the interpretation and clinical value of the assay, especially since much of the data are conflicting. The lack of consensus on important characteristics of the assay reduces confidence in published clinical evaluations and produces difficulties when comparing fructosamine concentrations from different centres. An agreed method for the assay, and recognition of the situations where fructosamine measurement may be inappropriate, will allow a more accurate assessment of the clinical utility of the assay.

It is the aim of this report to recommend guidelines which will produce more agreement on analytical procedures for the estimation of serum fructosamine. It is not our intention to recommend the use of the serum fructosamine assay in preference to any other measurement of glycated proteins. Rather it is hoped that a consensus method will allow the assay to be clinically evaluated in more centres in the UK.

In making recommendations we have considered in detail (i) assay reaction conditions, (ii) calibration and (iii) the influence of protein concentration on the measurement of serum fructosamine.

## ANALYSIS

### Reaction mechanism

The precise reaction mechanism by which glycated proteins reduce nitro-blue tetrazolium to its blue formazan form is unknown. Jones *et al.*[12] observed that 47% of the reducing activity of fructosamine could be inhibited by the addition of superoxide dismutase, a superoxide-radical scavenging enzyme; this led them to propose a reaction mechanism implicating free radical intermediaries. The ketoamine which results from the glycation of protein exists in equilibrium with its eneaminol tautomer (Figs 1 and 2). Either the ketoamine or the eneaminol is capable of reducing molecular oxygen thus generating superoxide radical which in turn reduces NBT. Formation of the superoxide radical implies single electron auto-oxidation of the ketoamine producing a free radical intermediate of glycated protein (Fig. 2).

Sakuri and Tsuchiya[13] recently proposed an alternative mechanism (Fig. 3) in which the enediol form of the ketoamine is oxidized to an alkoxy radical by metal ions present as trace contaminants in the assay buffer. The alkoxy radical is then capable of producing superoxide radicals.

### Reaction conditions

Where instrument design imposes restrictions upon the choice of assay conditions, proper consideration should be given to possible effects of altered reaction conditions on assay bias and specificity. The most important variables in assay conditions are considered in this section.

*Temperature*

The reaction between glycated protein and NBT

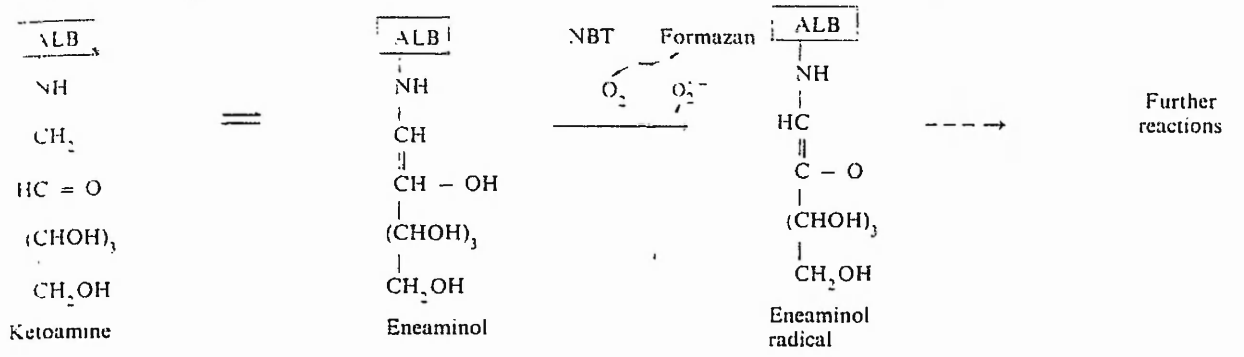


FIGURE 2. *Proposed reaction mechanisms for the reduction of NBT by glycated albumin. (Mechanism proposed by Jones* et al.[12])

dye proceeds more quickly at elevated temperatures, the highest temperature investigated and reported in the literature is 40°C. The temperature used for the assay needs to be high enough to provide adequate absorption changes and also to be readily available on the variety of instruments which are used in clinical chemistry laboratories; in practice this means 25, 30 or 37°C. The temperature should be held constant during the entire reaction period.

Changes in temperature have a proportionally greater effect on the reaction between DMF and NBT than on that between glycated protein and NBT. Therefore assays using DMF as calibrant may be subject to substantial bias if the temperatures used are significantly different from 37°C,[14] the temperature originally used. Convenience and precedent suggest that 37°C is a suitable temperature for analysis.

RECOMMENDATION. The reaction should take place at 37°C ± 0·1°C.

*Wavelength*

Glycated protein reduces NBT to yield a blue formazan product which binds mainly to albumin. The absorption spectrum of the glycated protein/reduced dye complex appears to differ according to the source of the glycated protein. Evidence for this comes from observations that varying the wavelength at which the assay is performed produces significant variations in patient results when bovine glycated albumin is used as the calibrator.[15] Howey *et al.*[16] observed the detailed absorbance spectrum of reduced NBT in the presence of human serum and the absence of detergent (Fig. 4) and found the wavelength of maximum absorbance to be 540 nm.

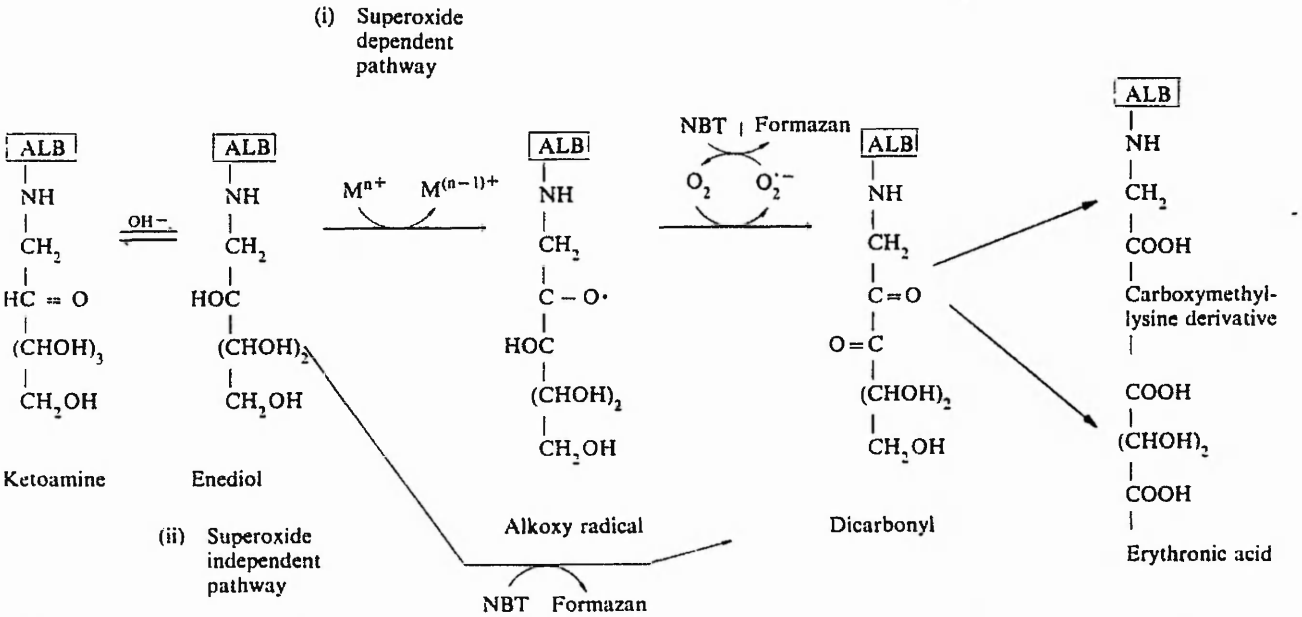


FIGURE 3. *Proposed reaction mechanisms for the reduction of NBT by glycated albumin. (Mechanism proposed by Sakurai and Tsuchiya.*[13]*) $M^{n+}$ and $M^{(n-1)+}$ indicate metal ions postulated to be present in trace quantities.*

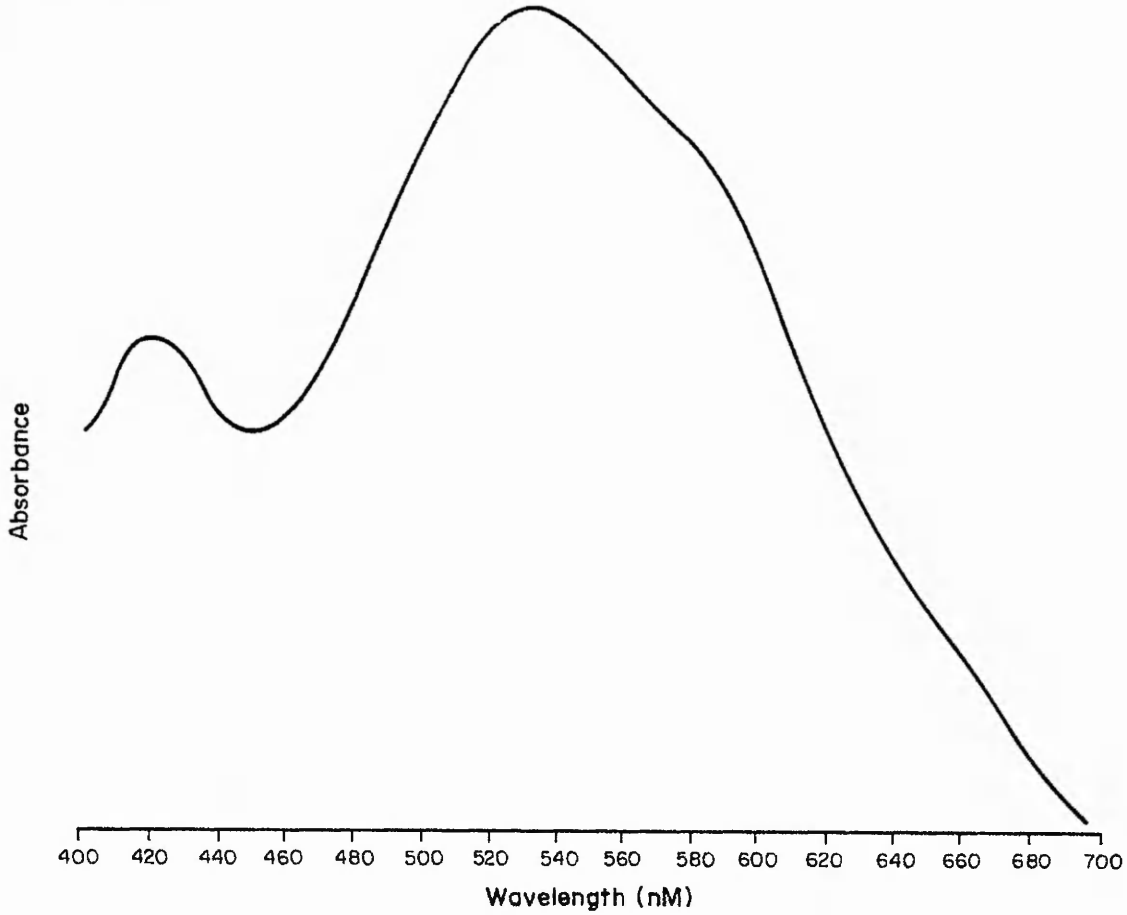


FIGURE 4. *Absorbance scan of glycated human serum protein/NBT reagent (after Howey* et al.[16]).

Addition of detergent (Triton X-100[17] or a mixture of ionic and non-ionic detergent[18]) changes the spectral characteristics of both the glycated human serum/formazan reaction product and the DMF/formazan reaction product, making them almost identical. Other calibrants such as dihydroxyacetone[17] and glycated polylysine[19] also form formazan adducts with very similar absorbance spectra to human serum/formazan.

RECOMMENDATION. Detergent should be present in the reaction mixture to reduce matrix effects between calibrants and human serum. Absorbance readings should be taken between 520–530 nm for detergent-containing reagents although wavelengths outside this range may be used if instrumentation constraints require this.

*Reagent buffer and pH*

Baker and Johnson described a buffered reagent comprising 75 mmol/L $Na_2CO_3$ plus 25 mmol/L $NaHCO_3$,[20] producing a pH of 10·35 at 25°C. Assays using DMF dissolved in a solution of albumin as calibrant may show significant bias if the assay pH is outside the range 10·35 ± 0·05. The pH of the assay buffer should be checked by calibrating a suitable pH meter at pH 10·00 and pH 11·00. Commercial buffer preparations are available for this purpose. (Hydrion dry buffer pH 10·0, Cat. No. 23 912–7, Hydrion dry buffer pH 11·0, Cat. No. 23 913–5. Aldrich Chemical Company Ltd, Gillingham, UK.) Assays using glycated protein standards and/or detergent in the reagent may not require such stringent pH control.[14]

RECOMMENDATION. The pH of the buffered NBT reagent should be 10·35 ± 0·05.

**NBT concentration**

The composition of the reagent specified by Johnson and Baker[20] was 0·25 mmol/L. Siedel *et al.*[18] described a modified reagent containing detergent, uricase and 0·6 mmol/L NBT. Using the method of Siedel *et al.* at an NBT concentration of 0·48 mmol/L, the fructosamine assay was linear up to 3·5 times the upper limit of the reference range for non-diabetic subjects.[21]

RECOMMENDATION. NBT concentration should

be sufficiently high to ensure assay linearity up to at least three times the upper limit of the reference range for non-diabetics. Linearity should be checked by diluting glycated human serum albumin.

*Pre-incubation period*

The original fructosamine assay of Johnson *et al.*[9] involved a pre-incubation of specimens and reagent for 10 min before making the first absorbance measurement followed by measurement of the absorbance change over the next 5 min. Since the reaction is non-linear, it is only necessary to measure absorbance at 10 and 15 min after initiation. Continuous monitoring of absorbance change, when available, may be useful as a qualitative check on assay performance. Although reducing the pre-incubation time has been reported to introduce assay bias[22] and to increase analytical interference,[23] later work[15,24,25] has suggested that shorter incubation times are possible. In view of these conflicting data, the effect of using shorter pre-incubation times (i.e. between 5 and 10 min) should be carefully assessed before the assay is used clinically.

RECOMMENDATION. The assay reaction mixture (including serum) should be pre-incubated for 10 min. Absorbance measurements should be taken at 10 and 15 min after initiation of reaction.

*Sample/reagent ratio*

Recovery of DMF from serum has been reported to be highly dependent upon protein concentration.[26] As the sample/reagent ratio is increased, recovery is decreased, leading to higher measured serum fructosamine concentration in patient specimens. Insufficient work has been completed to determine the optimum sample/reagent ratio. Precedent and convenience have led to sample/reagent ratios of between 1:10 and 1:17·5 being used.[14]

RECOMMENDATION. Sample dilution should exceed sample/reagent ratio of 1:10.

*Modifying agents*

RECOMMENDATION. Add Uricase (4 U/L) and detergent (20 g/L) (see p 8).

**Calibration**

*Calibration using 1-deoxy-1-morpholino-D-fructose (DMF)*

1-Deoxy-1-morpholino-D-fructose (DMF), a synthetic ketoamine compound, was suggested as a suitable calibrant for the fructosamine assay by Johnson *et al.*[9] The units in which the test results are universally reported are derived from DMF standards. Use of chemically well defined standards which can be prepared simply provides a stable reference point for the assay and ensures transferability of results. As originally described, DMF was dissolved in a solution of human albumin and used as a working calibrant. The presence of albumin prevents the formazan reaction product from precipitating and also changes the spectrum of reduced tetrazolium blue to more closely resemble that obtained when the dye is reduced by serum glycated proteins. Unfortunately albumin itself contributes to the fructosamine concentration of such standards and must be taken into account when constructing calibration curves.

*Problems with the use of non-serum calibrants*

The chemical nature of the ketoamine linkage in calibrants may not be the same as those in patient specimens, e.g. synthetic ketoamines clearly differ from the glycated proteins found in patient specimens. Such disparities may lead to a different relationship between the reducing activity of patient specimens and calibrants. Variations in assay temperature,[14] wavelength,[15] pH[11] and pre-incubation time[22] between laboratories can lead to systematic bias and to significantly different results being obtained for the same patient material even when using identical calibrants. This problem is most acute when DMF-containing calibrants are used in reagent formulations which contain no detergent[14] since serum specimens and DMF/protein standards give proportionately different absorbance changes as reaction conditions are altered. In an attempt to overcome this problem and the reported differences in recovery between different batches of albumin, DMF calibrators have been prepared using DMF in pooled human serum[27] or in sera from individual patients.[16]

Baker and Johnson now recommend glycated protein standards as suitable secondary calibrants for the assay.[14] Use of secondary glycated protein standards has been shown to decrease, but not eliminate, the effect of altering reaction conditions.[28] Differences in the distribution of glycated proteins between the major serum protein classes, or differences in spectral characteristics of the protein-formazan reaction product in individual sera probably account for these observations. Recent evidence suggests that a secondary calibrant of pooled human serum containing no DMF produces the least inter-laboratory variation.[29]

Use of detergent in the reaction mixture[17,18] largely overcomes matrix related problems and should reduce inter-laboratory variation (see p 4).

*Other calibrants and reference materials*

Several approaches have been suggested in an attempt to find a suitable reference material for the fructosamine assay and a suitable reference method for measuring glycated proteins. Dihydroxyacetone[17] has been suggested as a potential candidate reference material which can be used directly in the fructosamine assay. This material has the advantage that, like DMF it is a simple compound of known molecular weight requiring no reference method to assign fructosamine values. Further work is required to confirm that this calibrant is robust under routine analytical conditions and that the resulting fructosamine concentration measured in human serum represents the true concentration of glycated amino acid residues. Lever *et al.*[30] reported some preliminary work on the use of ε-glycated lysine and valine as standards. Farr *et al.*[31] have suggested the use of purified globin as a reference material. The molar concentration of glycated amino acid residues was calculated from fluorometric measurements of the release of formaldehyde from the glycated globin after periodate oxidation.[32]

Schleicher and Wieland[8] have developed a specific method for glycated protein using high performance liquid chromatography to measure furosine [ε-N(2-furoylmethyl)-L-lysine], the derivative of glycated lysine released from glycated protein after acid hydrolysis. This method has potential use as a reference method but includes a precipitation step and a hydrolysis step which may lead to lack of reproducibility. The method has been used to estimate the true fructosamine concentration of serum and as such is a useful research tool.

Recently Schleicher and Vogt[19] suggested glycated polylysine as a suitable primary standard for the fructosamine assay, the fructosamine content was determined by $^{14}C/N$ elemental analysis. Use of glycated polylysine as a calibrant in the fructosamine assay was shown by these authors to produce strikingly lower results in patient sera when compared with a DMF calibrated assay. However the results were in good agreement with the analytically more rigorous HPLC method of Schleicher and Wieland.[8] Glycated polylysine may be used directly in the fructosamine assay or to assign values to secondary calibrants.

RECOMMENDATION. DMF/albumin calibrants should no longer be used. There is currently too little available information to make a clear recommendation on the most suitable reference material for calibration. Both dihydroxyacetone and glycated polylysine are candidate materials which may provide acceptable alternatives to DMF/albumin. It is clearly highly desirable to achieve a consensus concerning the source of calibration data for the fructosamine assay.

*Secondary calibrants*

It is likely that secondary calibrants will continue to be used to calibrate fructosamine assays from day to day, the criteria shown in Table 1 should be met when choosing a suitable secondary calibrant.

**Compounds interfering with the analysis**

*Effect of protein concentration*

Measurement of glycated haemoglobin and total serum glycated proteins have traditionally been expressed as % glycated protein or mmol glycated protein/mmol non-glycated protein. The assumption behind this calculation is that the proportion of total protein which has been glycated reflects diabetic control most accurately. The fructosamine assay was first described as a simple concentration measurement of glycated serum protein without reference to total protein.

Johnson *et al.*[9] and later Staley[33] have argued that within the physiological range of albumin

TABLE 1. *Criteria to be met by secondary calibrants for fructosamine assay*

| | |
|---|---|
| 1. | The matrix should consist of pooled human serum which has been tested and found negative for HB$_s$Ag and HIV. If HIV testing is unavailable the serum should be heat treated at 56°C for 1 h |
| 2. | The concentration of fructosamine in serum should not exceed the limit of assay linearity |
| 3. | The fructosamine content should be stable during long-term storage (up to 2 years) |
| 4. | The secondary calibrant should have albumin and globulin concentrations within the locally accepted reference intervals |
| 5. | The spectral characteristics of the reduced NBT reaction product should be identical to those of patient specimens |
| 6. | The material should not have been excessively glycated during any manufacturing step. Most commercial sera, whether lyophilized or liquid, have fructosamine concentrations elevated well into the diabetic range |

concentration the rate of protein glycation is independent of albumin concentration because serum protein is always far in excess of the reacting carbonyl form of glucose. However, a number of studies have reported statistically significant correlations between serum albumin and serum fructosamine within the reference range[16,34] during pregnancy,[35] during treatment of ketoacidosis[36] and in subjects with serum albumin varying from 20–55 g/L.[11]

Other workers have found less significant correlations between serum fructosamine and serum albumin. Baker *et al.*[37] could only demonstrate a relationship when serum albumin falls below 30 g/L. Furrer *et al.*[38] found that application of albumin correction to fructosamine results from 148 diabetics with serum albumin levels between 35·5–42·5 g/L resulted in a difference of less than 6%, a deviation which they regarded as being clinically insignificant. Johnson *et al.*[10] found that fructosamine measurements in diabetic patients were not correlated with serum albumin concentrations in individuals with urinary albumin excretion < 1 g/L and concluded that routine correction for serum albumin is unnecessary. Allgrove and Cockrill[39] studied 61 diabetic children and found a correlation of $r = 0{\cdot}69$ between Hb $A_{Ic}$ and fructosamine. Correcting fructosamine for total protein gave an almost identical correlation, $r = 0{\cdot}68$. They observed little individual variation in total protein in these patients and concluded that applying an albumin correction factor to serum fructosamine values did not offer any advantages.

Subjects with serum albumin less than 30 g/L or with urinary protein concentrations of greater than 1 g/L may have abnormal rates of albumin turnover. In such cases, both measured and corrected fructosamine concentrations are likely to be inaccurate indications of glycaemic control when compared to the reference population. However, it is possible that serial fructosamine measurements in such individuals may provide useful information regarding deteriorating or improving diabetic control providing their protein status remains unaltered.

Glycaemic changes apart, fluctuations in serum fructosamine concentration are likely to arise from abnormal serum protein turnover regardless of actual protein concentration[38] and/or redistribution of body water.[37] Since the relationship between fructosamine and protein in these two states will differ, the validity of any single correction formula derived from a random population in which both types of effect may be operating will be suspect. Thus, while in a given population a relationship between serum fructosamine and protein may be apparent, the clinical utility of routine fructosamine correction has not been clearly established and further studies are needed.

The progressive decrease in serum albumin concentration during the second and third trimester of pregnancy, along with an observed drop in fructosamine concentration during the same period, has led Van Dieijen-Visser and coworkers to propose correction of fructosamine for albumin or total protein concentration in this situation.[35] Other groups have found poor correlations between protein concentration and fructosamine and have not applied corrections.[40,41] The importance of good diabetic control during pregnancy requires that any expected changes in fructosamine during normal pregnancy are taken into account.

Lack of agreement in the literature concerning correction for albumin or total protein during pregnancy suggests that, if such corrections are made, each laboratory should establish its own correction formula. In practice, trimester-related reference ranges are a more suitable way of presenting data and should prove widely acceptable.

TABLE 2. *Effect of interfering substances on fructosamine determination (adapted from Blair* et al.[34])

| Instrument | COBAS BIO | RA 1000 |
|---|---|---|
| Reagent source | Baker[9] | Roche |
| Preincubation time | 10 min | 7 min |
| Added analyte | Observed difference (mmol/L fructosamine) | |
| Heparin (IU/L) | | |
| 90 909 | −0·16 | |
| 80 000 | | −0·28 |
| Bilirubin (μmol/L) | | |
| 439 | +0·31 | |
| 428 | | −0·51 |
| Haemoglobin (g/L) | | |
| 2·3 | | −0·58 |
| 5·0 | −0·01 | |
| Cysteine (mmol/L) | | |
| 0·20 | | −0·05 |
| 0·50 | −0·12 | |
| Glutathione (mmol/L) | +0·06 | +7·69 |

*Effect of other substances*

The effects of other interfering reducing substances have been studied in detail by Baker *et al.*[14] using reaction conditions as originally described

by them. Heparin (80 000 IU/L), EDTA (5·5 g/L), bilirubin (428 umol/L), triglyceride (7·45 mmol/L), urate (0·93 mmol/L), ascorbate (0·57 mmol/L) and cysteine (0·5 mmol/L) produced analytical interference greater than 0·1 mmol/L fructosamine although none of these substances interfered by more than 0·3 mmol/L fructosamine at the stated concentrations. Blair *et al.*[23] used the Technicon RA-1000 analyser to test the effect of reducing pre-incubation time to 7 min (the maximum allowed by the instrument). Table 2 compares the data from this study with those of Baker *et al.*[14] from a pre-incubation time of 10 min on a Cobas-Bio centrifugal analyser. Each potential interfering substance was dissolved in diluent and added to pooled human serum (0·1 ml added to 1·0 ml serum). From the apparent fructosamine concentration of the spiked serum a blank was subtracted which contained, in addition to reagent, serum diluent only. The data shown here indicate that shortening the incubation period to 7 min did produce large interference problems, provided that haemolysis and very high levels of bilirubin are avoided. However, it also shows that there is a need to reassess the effect of interfering substances whenever the reaction conditions are changed from the recommended conditions. The effect of different pre-incubation times on assay bias has already been considered. Raised serum concentrations of superoxide dismutase and caeruloplasmin have been reported to inhibit the reducing activity of glycated protein towards NBT.[12] Although there are no published data on the fructosamine activity of subjects with conditions known to be associated with elevated serum antioxidant activity (e.g. rheumatoid arthritis) it is possible that falsely low fructosamine values could arise in such conditions.

The modified fructosamine assay described by Siedel *et al.* contains uricase (4 U/L), which reduces interference from urate, and a mixture of non-ionic and ionic detergents (22 g/L), which eliminates interference from triglyceride.[18] Severe hyperuricaemia (> 0·93 mmol/L) and severe hyper triglyceridaemia (> 7·45 mmol/L) are possible metabolic consequences of diabetes and diabetic nephropathy, although the prevalence of either may not be high in many diabetic populations.

### Specificity

The specificity of the fructosamine assay has been challenged by Schleicher *et al.*[42] who observed that a variable fraction of serum NBT reducing activity persists after reduction of serum with sodium borohydride. Borohydride treatment is thought to reduce the keto group on the ketoamine thereby preventing glycated protein-derived ketoamine from reducing NBT. The remaining NBT reducing activity (up to 50% of the fructosamine concentration of non-diabetic serum) was concluded to be independent of the glycation of albumin, since glycated human albumin when reduced by sodium borohydride showed no significant reaction in the fructosamine assay.

The precise nature of the reducing agents responsible for borohydride-resistant NBT reduction is unknown. The reaction mechanisms by which such substances reduce NBT is superoxide independent, unlike that described for ketoamines derived from glycated protein (see 'Reaction Mechanism' above). Sakurai and Tsuchiya[13] have postulated an alternative superoxide independent mechanism which described NBT reduction occurring as a result of direct oxidation of the enediol form of the ketoamine (Fig. 3). This reaction is suggested to proceed simultaneously with a modified superoxide dependent pathway. (For further details of the reaction mechanism see reference 13.) However, Sakurai and Tsuchiya's mechanism suggests that the superoxide independent reaction involves ketoamine-derived intermediates which are still bound to serum albumin and other serum proteins, a conclusion which is at variance with the observations of Schleicher *et al.*[42]

Modified methods, which take steps to reduce interference in the assay by adding detergent and uricase may lower borohydride resistant reducing activity. However, there are currently no published data to establish whether such modifications entirely remove this non-specific interference.

### Storage of reagents and patient specimens

*Storage of reagents*

NBT reagent should be stored protected from the light in dark glass bottles at 4°C.

*Storage of samples*

*Anticoagulants.* Serum specimens are widely used for fructosamine assay. Heparin (80 000 IU/L) and EDTA (5·5 g/L) decrease fructosamine activity in the original method of Baker and Johnson by a small but statistically significant amount (0·16 mmol/L and 0·15 mmol/L, respectively).[14] The modified method of Siedel *et al.*[43] eliminates heparin interference and reduced EDTA interference to less than 3%. If plasma specimens are

being used the effect of anticoagulant upon fructosamine activity should be taken into account.

RECOMMENDATION. Either plasma or serum may be used.

*Storage temperature*

Fructosamine activity, even in specimens with high glucose concentrations, is unaffected by storage at ambient temperatures for up to 24 h even if the serum remains in contact with the clot.[44] Patient samples may be stored for several months at −20°C[9] and for 2 weeks at +4°C (M. Lemon, unpublished observation). However, Koskinen and Irjala[45] have reported considerable changes (−20% to +26·7%) in specimens stored at −20°.

RECOMMENDATION. Separate samples within 24 h and store at +4°C. Analyse within 2 weeks.

## PERFORMANCE CRITERIA

**Imprecision and internal quality control**

Data on biological variation can be used to define an analytical goal for the imprecision of an assay based on the view that the tolerable analytical variation should not exceed one half of the intra-individual variation. Howey *et al.*[16] have investigated the biological variation of serum fructosamine, deriving an analytical goal for imprecision of CV <2·6%. This goal is widely attainable with current instrumentation which is capable of producing an analytical CV of <2% at non-diabetic levels of serum fructosamine. Internal quality control materials which conform to the recommendations for suitable calibrants (see Calibration Recommendation) should be used.

**Accuracy and external quality assessment**

Problems with calibration of the assay make accuracy difficult to assess. Low between-laboratory bias is desirable. Currently only local external quality control schemes are available. A list of UK schemes which will accept new participants is included in Appendix 1. External quality assessment is particularly important due to the calibration problems discussed earlier. Such schemes allow laboratories to identify sources of bias and if necessary to modify their technique. Insufficient information is available to define maximum acceptable bias. Imprecision should be less than CV 2·6% over the range 1·0–4·0 mmol/L.

**Linearity and working range**

Methods should show linearity of response to three times the upper limit of the reference range for non-diabetics, making dilution of specimens unnecessary.

**Reference intervals**

Calibration difficulties and variations in assay conditions imposed by instrument design make construction of locally-derived reference intervals essential. Guidelines for interpretation of fructosamine values should ideally be derived by reference to mean blood glucose levels observed over a period of 2–3 weeks at various times during the day in subjects with normal protein turnover. In practice these data are difficult to collect and most guidelines contain arbitrary reference intervals.

## SUMMARY OF RECOMMENDATIONS

**Clinical use**

The main application for serum fructosamine measurement is in monitoring the degree of glycaemic control achieved by diabetic patients over a period of 1 to 3 weeks. Fructosamine measurements should not be used for the diagnosis of diabetes mellitus: this can only be made by blood glucose measurements and/or a glucose tolerance test according to the WHO criteria for diabetes.[46]

**Assay conditions**

Where insufficient technical details are available from the literature, or where variations in assay conditions are unlikely to cause major problems, the assay conditions are not fully defined:

| Reagent concentrations: | |
|---|---|
| Buffer pH | 10·35 ± 0·05 |
| Sodium carbonate (anhydrous) | 75 mmol/L |
| Sodium bicarbonate | 25 mmol/L |
| Nitro-blue tetrazolium | 0·48 mmol/L |
| Detergent | 20 g/L |
| Uricase | 4 U/L |

Reagents should be stored in the dark at 4°C. (Stable for 2 weeks at 4°C):

| | |
|---|---|
| (1) Sample:reagent ratio | Sample dilution ⩾ 1:10 |
| (2) Temperature | 37 ± 0·1°C |
| (3) Wavelength | 520 nm–530 nm |
| (4) Pre-incubation time (defined as the period during which serum is incubated with assay reagents prior to taking | 10 min |

| | | |
|---|---|---|
| | the first absorbance reading) | |
| (5) | Reaction time | 5 min |

**Calibration**

Secondary calibrants consisting of human serum and containing no DMF should be used. Values should be assigned to secondary calibrants by primary reference materials (see p 6).

**Interferences**

Analytical interference is minimized by avoiding haemolysed and icteric specimens, by adding uricase and detergent and by using a 10 min pre-incubation period. Shorter pre-incubation periods may be feasible but require careful evaluation.

**Storage of samples**

Plasma or serum may be stored at 4°C and should be analysed within 2 weeks.

**Performance targets**

A between batch impression of CV $< 2{\cdot}6\%$ is desirable throughout the assay working range.

**Protein correction**

Protein correction is not recommended for routine use. Patients with serum albumin concentration less than 30 g/L (as measured by an immunochemical method) or with urinary protein concentration greater than 1 g/L are unsuitable for fructosamine estimation. If serum fructosamine results are not accompanied by serum albumin or urinary protein measurements, clinicians should be made aware that suspected hypoproteinaemia may lead to misinterpretation of serum fructosamine concentrations.

## CONCLUDING REMARKS

The recommendations in this document were made taking into account the published work on fructosamine assay. The authors suggest that further development of the assay should be encouraged. It is likely that, as with the introduction of any new method, modifications to the recommended procedure may be proposed which improve the clinical value or technical performance of the assay. In particular efforts should be made to investigate further the specificity of the assay and to continue research into methods of calibration.

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## APPENDIX 1

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# The effect of calibration on the between-laboratory variation of serum fructosamine

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SUMMARY. Human pooled serum from diabetic and non-diabetic subjects, and calibrators containing glycated protein or 1-deoxy-1-morpholinofructose (DMF) in a variety of matrices, were distributed to 10 laboratories. When they used their own assay conditions and calibrators, the inter-laboratory variation was unacceptably high. However, when the pool from diabetic patients was reassayed using a calibrator with an assigned value prepared from freeze-dried human serum, and containing no DMF, inter-laboratory variation was reduced significantly. Inter-laboratory agreement for the pool from non-diabetic subjects remained poor despite recalibration. Recalibration using either serum or albumin based solutions of DMF as calibrator failed to effect any significant reductions in inter-laboratory variation. Secondary calibrators based on a protein matrix with no added DMF are recommended for routine use.

Since Johnson *et al.*[1] first described a method for monitoring diabetic patients by measuring serum fructosamine, there has been considerable debate concerning the most appropriate calibrant for the assay.[2–6] Originally, DMF was added to human albumin and used as a 'primary' working calibrator. It was later suggested[7] that DMF should be added to human serum, as this would minimise the matrix effects, especially when an internal standardisation technique was used. Baker *et al.*[2] now recommend a secondary working calibrator containing glycated protein, and Roche Products Ltd (Welwyn Garden City, Herts, UK) produce a kit using glycated bovine albumin as calibrator. Although, secondary glycated protein calibrators are believed to reduce matrix effects, such materials themselves require calibration. No satisfactory primary standard is currently available making the values assigned to secondary standards unreliable.

In view of these different approaches to standardisation, three calibration techniques have been assessed to determine which minimised inter-laboratory variation.

The heads of the clinical chemistry laboratories in the Trent Regional Health Authority, which were carrying out assays for fructosamine, agreed to participate in an inter-laboratory trial. This was organised through the Trent Regional Chemical Pathology Panel and Quality Control Group.

## Materials and methods

Each laboratory received two liquid human serum pools, one collected from poorly controlled diabetic outpatients (Pool 1), the other from non-diabetic volunteer blood donors contributing to the Blood Transfusion Service (Pool 2).

TABLE 1. Source of material distributed

| Pool no. | Identification |
|---|---|
| 1 | Human serum pool collected from poorly controlled diabetic out-patients |
| 2 | Human serum pool collected from non-diabetic volunteer blood donors |
| 3 | Freeze-dried human serum (Assigned value 5·2 mmol/L). Obtained from Wolfson Research laboratories |
| 4 | Pool 2 containing added DMF (final concentration 4·3 mmol/L) |
| 5 | Human albumin (40) g/L) in saline containing DMF (final concentration 4·5 mmol/L) |

TABLE 2. Summary of methodology questionnaire returns

| Lab no. | Kit | Instrument | λ nm | Buffer (pH) | Lag time (min) | Measurement interval (min) | Calibrant |
|---|---|---|---|---|---|---|---|
| 1 | In-house | Centrifichem | 550 | 10·80 | 10 | 5 | Human serum |
| 2 | Roche | Cobas Bio | 530 | 10·35 | 10 | 5 | Bovine albumin |
| 3 | In-house | Cobas Bio | 530 | 10·35 | 10 | 6 | Human serum |
| 4 | In-house | Cobas Bio | 530 | 10·35 | 10 | 5 | DMF/albumin |
| 5 | Roche | Cobas Bio | 530 | 10·35 | 10 | 5 | Bovine albumin |
| 6 | In-house | Cobas Bio | 530 | 10·35 | 10 | 5 | Bovine albumin |
| 7 | Roche | IL Multistat II | 520 | 10·35 | 10 | 5 | Bovine albumin |
| 8 | Roche | Baker Encore | 520 | 10·35 | 10 | 5 | Bovine albumin |
| 9 | In-house | Gilford 203·5 | 546 | 10·35 | 15 | 1 | Human serum |
| 10 | In-house | Kem-O-Mat 1 | 550 | 10·35 | 10 | 5 | Human serum |

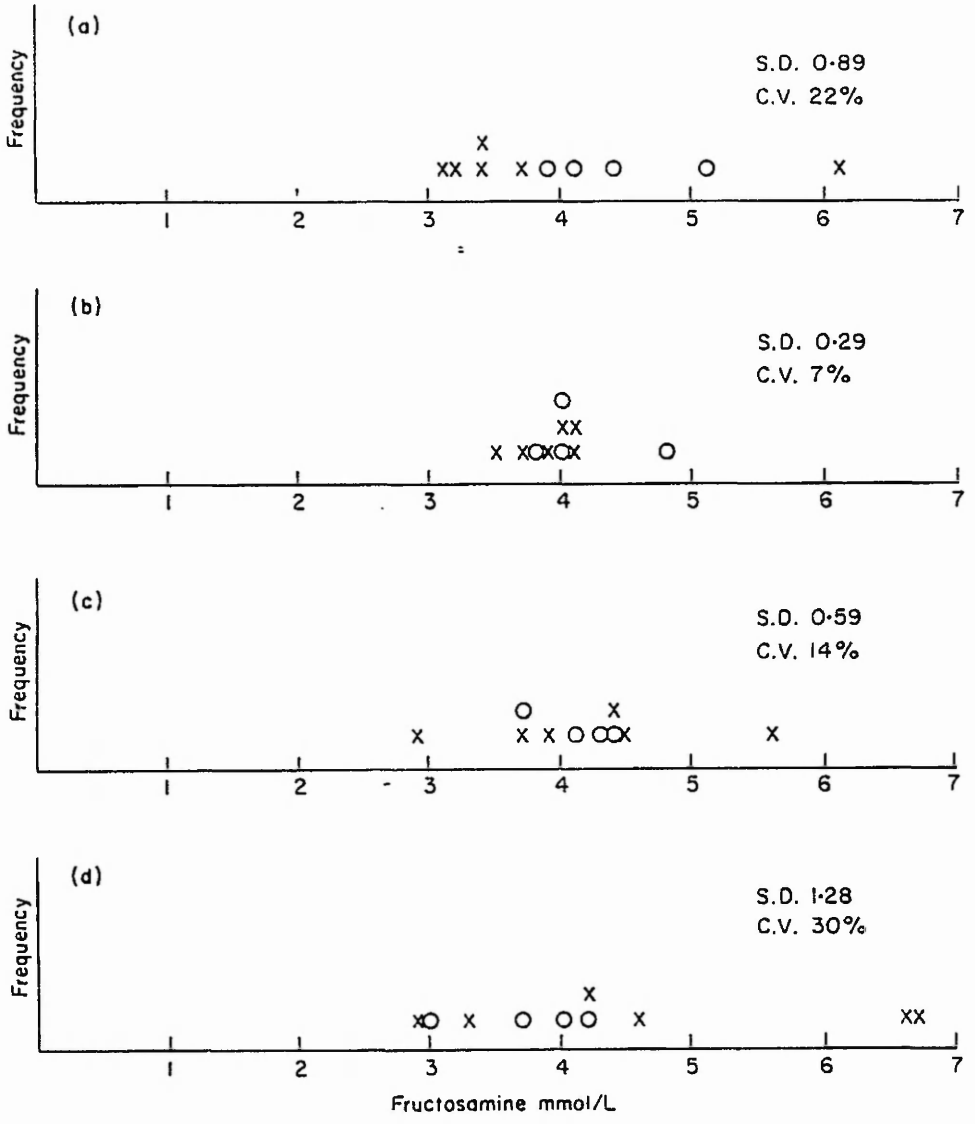


FIG. 1. Distribution of results for diabetic pool (Pool 1). ○ Roche kit users; × Others. (a) uncorrected data. (b) data recalculated using human serum as calibrator. (c) data recalculated using DMF weighed into human serum as calibrator. (d) data recalculated using DMF weighed into human albumin as calibrator.

In addition, three sets of calibrator materials (Pools 3–5) were circulated and analysed, simultaneously, with the serum pools (see Table 1). All analyses were performed singly within the same batch. Each laboratory was asked to fill in a questionnaire about its method of analysis. Each calibrator pool was assigned a value equal to the mean of all results returned for that pool.

## Results

Table 2 summarises returns from the methodology questionnaire. None of the participating laboratories corrected their fructosamine analysis for protein concentration. Figures 1a and 2a show the distribution of results obtained for the diabetic and non-diabetic pools, respectively. The large spread of results returned for both diabetic and non-diabetic pools indicate poor inter-laboratory agreement. Each laboratory used its own calibration system, details of which appear in Table 2.

## Human serum as calibrator

Figures 1b and 2b show the same data recalculated using Pool 3 (human serum, assigned value 5·2 mmol/L) as calibrator. Comparison of variances using the *F*-test shows that there is a

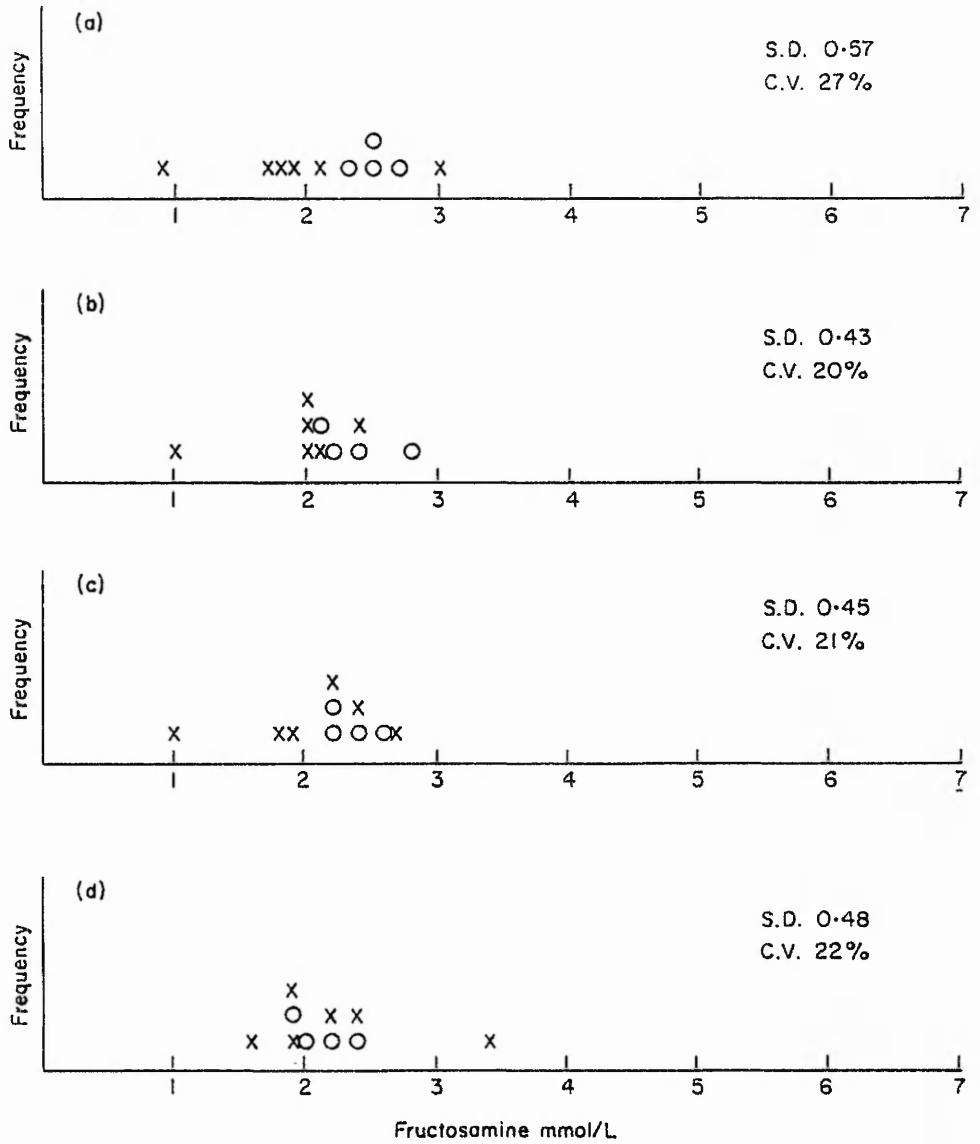


FIG 2. Distribution of results for non-diabetic pool (Pool 2). ○ Roche kit users; × Others. (a) uncorrected data. (b) data recalculated using human serum as calibrator. (c) data recalculated using DMF weighed into human serum as calibrator. (d) data recalculated using DMF weighed into human albumin as calibrator.

highly significant reduction in variance ($P<0{\cdot}01$) when the diabetic pool data is recalculated. The coefficient of variation (C.V.) is reduced from 22% to 7%. Figure 2b indicates that although a decrease in variation occurs after recalibration of the non-diabetic pool, it is not significant ($P>0{\cdot}05$) and the spread of results remains unacceptably high.

**Human serum spiked with DMF as calibrator**

Figures 1c and 2c show the data recalibrated using Pool 4 (DMF added to human serum; assigned value 4·3 mmol/L) as calibrator. Although, the use of Pool 4 as calibrator reduced the spread of results in both the diabetic and non-diabetic pools, the reduction in variance is not statistically significant ($P>0{\cdot}05$) in either case. Recalibration using a DMF based serum calibrator has less effect in reducing inter-laboratory variation of the pool from diabetic patients, (C.V. 14%) than recalibration using human serum with no added DMF (C.V. 7%).

**Human albumin spiked with DMF as calibrator**

Figures 1d and 2d show the data recalibrated using Pool 5 (DMF added to human albumin assigned value 4·5 mmol/L). The spread of results for the diabetic pool increased after recalibration (from C.V. 22% to 30%) while results for the non-diabetic pool showed a small decrease (from C.V. 27% to 22%), but remained unacceptably high. Neither of these changes was statistically significant. Recalibration using a DMF/albumin calibrator is least effective in reducing inter-laboratory variation.

**Discussion**

The observed improvement in inter-laboratory agreement has been mainly achieved as a result of adopting an assigned value for the calibrator. Nine out of the 10 laboratories which participated in this study used either glycated bovine albumin or glycated human serum as secondary calibrators for their assays indicating that the main contribution to variance is the unreliability of the values which have been assigned to such calibrators. Once agreement has been reached, inter-laboratory variation can be substantially reduced. The disappointing inter-laboratory variation which persists when the non-diabetic pool is analysed, even after recalibration, could be explained by increased analytical imprecision at lower fructosamine concentrations, although between batch estimates of imprecision at this level (typically <7%) do not support this explanation. Alternatively, non-linearity in some participants' assays may be contributing to the observed variation, since the mean fructosamine value for this pool before recalculation (2·1 mmol/L) is considerably lower than the calibrator (5·2 mmol/L).

If calibrators containing DMF dissolved in a protein matrix are used, small changes in reaction conditions can markedly affect apparent fructosamine concentrations.[2] This may explain why inter-laboratory variation was not significantly improved when results were recalculated after recalibration with either DMF/serum or DMF/albumin calibrators. These results suggest that secondary standards comprising glycated protein, without added DMF are more appropriate than DMF based calibrators for routine use. Baker *et al.*[8] in a larger study, found much closer agreement between laboratories, but this may reflect a higher proportion of laboratories in their group using the Roche kit which would provide a greater degree of calibration uniformity. Modifications to the originally described method may reduce the problems associated with using DMF as a working calibrator.[7] However, this has not been demonstrated on a multi-laboratory trial.

Assigning appropriate values to secondary standards remains a difficult problem. The use of DMF weighed into human albumin or serum, as a primary calibrator for assigning values to secondary calibrators, is open to challenge on the same grounds that preclude its use as a working calibrator, namely poor reproducibility between laboratories using slightly different reaction conditions. Agreement should be reached on how values are assigned to secondary calibrators. Discussion of the precise reaction conditions and instrumentation used in the process is important. Meanwhile, other approaches aimed at providing primary reference material for glycated protein measurements should be encouraged.

This preliminary study suggests there is merit in the common calibrant approach and a larger study to test these conclusions would seem appropriate. Further work is required to establish techniques for assigning reliable values to secondary calibrators. Adoption of secondary calibrators with accepted assigned values will reduce inter-laboratory variation allowing greater transferability of data.

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