STUDIES OF METABOLIC CONTROL AND COMPLICATIONS IN INSULIN DEPENDENT DIABETES MELLITUS.

2 VOLUMES

Volume I

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VOLUME II

CANDIDATES' DECLARATION

I, Peter Howard Winocour certify that I am the author of this work. All books and papers cited in this thesis were consulted by me personally. Some of this work has been performed in collaboration with others; where this is the case it is stated in the text.

The glycosylated albumin assay was initially evaluated by me although I received advice from Professor David Anderson in the early stages. This assay and the fructosamine , filter paper blood glucose and urinary albumin assays were initially performed by me and latterly by technical staff working in the laboratory of Professor David Anderson. I personally carried out all clinical evaluations.

The subject matter of this thesis has not been used in whole or in part for any previous thesis.

January, 1989.

STRUCTURE OF THE THESIS

In Chapter 1 I have discussed the assessment of control of carbohydrate, lipid and protein metabolism in insulin treated diabetes mellitus, those factors which can affect metabolic control, and epidemiological and pathophysiological aspects of diabetic complications. In Chapter 2 the design of the Salford Home Blood Glucose Monitoring Study is reported and the subjects, materials and methods described along with results from the initial laboratory evaluations of the assays for glycosylated serum albumin, urinary albumin and filter paper blood glucose measurement. The effects of continued blood glucose monitoring in the home blood glucose monitoring study when compared to urine glucose monitoring are discussed in Chapter 3. In Chapter 4 I have reported the results of the various analyses on aspects of lipid metabolism that were carried out and in Chapter 5 on the clinical utility of the various measures of glycaemic control used in the home blood glucose monitoring study. The effects of residual C-peptide secretion on metabolic control and complications during the blood glucose monitoring study are presented in Chapter 6 along with an assessment of the association between autonomic neuropathy and renal function in insulin-dependent diabetes mellitus. In Chapter 7 I have presented the results of the psychological evaluations that were carried out at inception to the blood glucose monitoring study and after 1 year. Finally in Chapter 8 I have discussed the results in relation to previously published work and the implications for future research into metabolic control and

complications in insulin-treated diabetes mellitus.

RESULTS

A filter card blood glucose assay was modified for large-scale use and blood glucose levels were found to be stable without the need for preservatives if filter cards were refrigerated. A commercial kit for measurement of glycosylated albumin was evaluated and found to be insensitive at low levels necessitating establishment of a two stage affinity chromatography and immunoturbidimetric method for accurate retrieval and measurement of glycosylated and non-glycosylated serum albumin. The Friedewald formula for the measurement of LDL cholesterol in insulin dependent diabetes mellitus (IDDM) was compared with LDL cholesterol measured by preparative ultracentrifugation and found to correlate closely in subjects without advanced nephropathy.

The Salford Home blood glucose monitoring study initially involved 153 diabetic patients and showed that continued access to a blood glucose meter following an intensified period of management and education was associated with marginal improvements in various measures of glycaemic control over 1 year, and somewhat better retention of diabetic self-care skills. Regardless of the means of glucose monitoring, stable glycaemic control and improved educational skills were maintained for a period of 1 year following the intensive management period. The Reflolux blood glucose meter was found consistently to overestimate low normal and hypoglycaemic blood glucose values.

Conformational changes in low density lipoprotein(LDL) and high density lipoprotein(HDL) are described in stable IDDM in the

absence of nephropathy which were modified by genetically determined structural changes in apolipoprotein E, early diabetic nephropathy, and inadequate diabetic care. 40% of 205 patients with IDDM were found to have hypercholesterolaemia and/or hypertriglyceridaemia at the initial assessment.

The use of several glycosylated blood protein measures provided complimentary information about glycaemic instability in IDDM in addition to direct measures of glycaemia. Glycosylated albumin (GSA) was found to be a more sensitive indicator of short term improvements or fluctuations in glycaemia than HbA_1 or fructosamine. GSA and fructosamine measurements in IDDM provided discrepant information on occasions and fructosamine levels were apparently lowered in hypertriglyceridaemic sera.

Residual C-peptide secretion was associated with minor improvements in glycaemic control and lower insulin requirements, although it was not associated with any differences in measures of lipid metabolism. Subjects with long standing IDDM and residual C-peptide secretion had more peripheral vascular disease but less proliferative retinopathy at entry to the Salford Home Blood Glucose Monitoring Study but after 1 year significant differences were no longer apparent. Autonomic neuropathy was associated with enhanced natriuresis and albumin excretion, particularly in overnight urine collections.

Higher anxiety and depression ratings were reported more frequently in patients with neuropathy, impotence, coronary and peripheral vascular disease and proliferative retinopathy, but bore no relation to glycaemic control apart from a higher anxiety score in patients who allegedly experienced daily hypoglycaemic

episodes.

The importance of these findings to patient management, the natural history of diabetic complications, and the link between metabolic control and complications are discussed.

ACKNOWLEDGEMENTS

I am indebted to many friends and colleagues for their support over the last five years. I am particularly grateful to those individuals who were invaluable in helping me to develop and perform the laboratory assays, and to store and analyse the large amounts of data. I felt it best to express my gratitude for their support in the text of the thesis.

I would especially like to extend my gratitude to Professor David Anderson and Dr. Harold Cohen for continued moral support and guidance, to Sister Carole Shingles and the staff of the Medical Investigation Unit for their help, to Mrs. Chris. Brown in the Department of Medical Illustration, and to Mrs. E. Timperley and Miss C. Cleminson for their assistance in the typing of this thesis.

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Finally and most importantly I gratefully acknowledge J. for her extraordinary patience and ego-boosting during times of despair.

LIST OF FIGURES AND TABLES

CHAPTER 1

INTRODUCTION

INTRODUCTION

The introduction of insulin into clinical use in 1922 for the management of insulin-dependent diabetes mellitus (IDDM) transformed the prognosis from one of inevitable early death to a situation where symptoms could be relieved and life prolonged. At that time controversy existed over the degree of metabolic control that should be achieved owing to the development of hypoglycaemic reactions which were sometimes severe or even, on occasions, fatal (1). This controversy still exists not only because of the acute consequences and possible sequelae of hypoglycaemia (2-5), but with the realisation that the long term vascular complications of diabetes might be the result of inadequate metabolic control (6). Diabetic microangiopathy and the aggressive atherosclerotic phenomena that accompany IDDM have been found primarily to be associated with the duration of the diabetes and the age of the individual (7, 8, 9). Glycaemic control has been suggested from both prospective and retrospective epidemiological studies to be a major determinant of microangiopathic complications (10-15), although the evidence for atheromatous cardiovascular disease is less compelling; here additional environmental, clinical (14-18) and genetic (19) components are likely to be more important.

At present the question of whether effective metabolic control prevents or leads to regression of established vascular disease is unresolved for several reasons:

(i) the means by which effective metabolic control could be achieved has only recently become available.

(ii) objective assessment of long term glycaemic control

has only become possible with the introduction of the measurement of glycosylated (glycated) proteins into clinical practice.

(iii) the intimate association between the development of complications and the duration of diabetes means that the question of prevention of vascular complications will not be answered until prospective studies have run for perhaps ten years or more.

Over the last 5 years several small prospective clinical trials have evaluated the effects of improved glycaemic control on established diabetic microvascular complications. No prospective studies as yet have attempted to investigate the role of glycaemic control in the development or progression of atheromatous (macrovascular) disease in IDDM.

Critical scrutiny of studies of the effects of near-normoglycaemia on established microvascular complications reveals conflicting results (20), due to the paucity of randomised trials with well characterised insulin-dependent diabetics and adequate quantification of end points. Diabetic retinopathy probably improves or its progression is arrested after two years of improved glycaemic control (21, 22), assuming that normoglycaemic re-entry is gradually achieved because of the frequently noted transient deterioration after 1 year (23-25), due possibly to the effects of relative hypoglycaemia on the impaired autoregulation of retinal blood flow. Other studies (26, 27) conducted over 1-2 years, however, did not support such reports. Although established diabetic nephropathy (persistent proteinuria) is unlikely to resolve with improved glycaemic control (26, 28, 29),

the situation for incipient nephropathy (albuminuria excretion rates > 15µg/minute without overt proteinuria) remains inconclusive, and the progression of overt nephropathy may be delayed (20, 24, 30-35). Peripheral neuropathy, likewise, may improve with near normoglycaemia when assessed by neurophysiological measures (21, 36-40), but autonomic dysfunction is probably not amenable to correction (22, 40). Most of these prospective studies (21-35, 37, 39) instituted effective glycaemic control by continuous subcutaneous insulin infusion (CSII) pumps, which however are applicable and effective only in selected highly-motivated patients (41, 42).

In practical terms, there is probably enough evidence for the modern diabetic clinic to employ the general principle that, the better glycaemic control, the better the long term outlook for patients with IDDM. However, despite these recent studies, major questions remain unresolved. Perhaps most important, are how best to manage all individuals with IDDM in the outpatient clinic setting, how to evaluate and improve their metabolic control, and whether effective metabolic control can prevent macrovascular as well as microvascular disease.

Although IDDM is clearly a disorder of lipid and protein as well as carbohydrate metabolism, the assumption has been that control of the latter will normalise protein and lipoprotein metabolism. This is supported by findings in several short-term studies (43-49), but only a few long term studies of small numbers have reported the effects of intensified insulin therapy either by CSII or multiple insulin injections on protein (50) or lipid (50-54) metabolism, where qualitative as well as

quantitative disturbances may be present (54-56). Furthermore, abnormal postprandial patterns of intermediary metabolites of lipid and protein metabolism persist with all modes of subcutaneous insulin delivery (42,43). Macrovascular disease is the major cause of premature death in IDDM, and epidemiological studies have suggested that hypertension and disturbed lipid metabolism are more important than glycaemic control in determining the risk of cardiovascular death and morbidity (17, 18, 57, 58).

The work reported in this thesis extends back to 1983 when over 150 insulin-dependent diabetics were consecutively recruited to a large prospective study to try to answer the following broad questions:-

1. Do different measures of carbohydrate, lipid and protein metabolism give comparable or complementary information in assessing metabolic control in IDDM?
2. What factors determine metabolic control in IDDM, and does home blood glucose monitoring help, independently of other factors?
3. What is the effect of improved metabolic control on the health of patients with IDDM?

1. METABOLIC CONTROL

The concept of 'control' of diabetes relates to pharmacological and dietary efforts to regulate those metabolic disturbances in diabetics which have immediate or long term adverse consequences. The relationship between metabolic control and the development and progression of complications has still to

be fully resolved, perhaps because most studies have relied solely on assessment of glycaemic control to investigate the issue.

If one does take into account other aspects of metabolism it has been questioned whether conventional insulin therapy could ever fully normalize all the biochemical disturbances of IDDM (59).

Within the limitations of measures of glycaemia that are generally used to reflect metabolic control, there are still no accepted criteria as to what constitutes perfect, adequate or poor control. The suggestion that poor metabolic control is reflected by a fasting blood glucose of greater than 8.2mmol/l or a post prandial value greater than 11.2mmol/l (60, 61), would condemn the vast majority of people with IDDM to this category. If one accepts a variation from normal mean blood glucose as assessed by an M value (an index of glycaemic fluctuation) of less than 10 (62) as perfect metabolic control, then even CSII does not normalize such fluctuations (63). Data suggesting that 'impaired glucose tolerance' is a risk factor for vascular disease (18) suggests that even near normoglycaemia may not reduce the risk of angiopathy to that of the general population. The assessment of diabetic control is further complicated by the inherent variability of results on retesting under identical conditions, in individuals who are said to have 'labile' diabetes (64).

The other purpose of the assessment of diabetic control, in addition to investigation of a pathogenetic role, is to allow optimization of therapy which has to take into account all

factors which may determine metabolic control (vide infra). At present, clinical evaluation and quantitative measures of carbohydrate metabolism remain the standards whereby the metabolic status of diabetic patients is judged.

1:1 CARBOHYDRATE METABOLISM

URINARY GLUCOSE MEASUREMENT

Perhaps the first 'qualitative' assessment of glycosuria was made by the Hindu physician Sushrutu 2000 years ago when he described the sweet flavour of the urine of individuals afflicted with diabetes (65). After the introduction of insulin, qualitative assessments of glycosuria by the Clinitabs methods was superceded by semiquantitative and quantitative methods which remain in clinical practice. Most semi-quantitative methods employ glucose as a reducing agent, for example in the glucose oxidase/peroxidase reaction when glucose is oxidised to S-(D)-gluconolactone, with the production of hydrogen peroxide which oxidises the colour sensitive indicator. In the absence of large quantities of ascorbic and salicylic acid, the test is reproducible and accurate (66). Currently Clinistix, Diastix, Clinitest and Diabur-test 5000 are commonly used in clinical practice with reasonable accuracy (67, 68). The use of well timed urine collections has been reported to provide a reasonable indicator of simultaneously recorded continuously analysed blood glucose (69) in insulin-dependent diabetics without advanced nephropathy. Timing of urine analysis is important and post-prandial measurements may reflect more closely the tendency for hyperglycaemia (70), although double voiding of urine does not necessarily improve the precision (71). The question of altered renal threshold and collection problems has focussed disfavour on urinary glucose measurement as an assessment of carbohydrate metabolism particularly in patients with high renal thresholds (70). A diurnal variation of the renal threshold for glucose may

further confound matters (69-70). The less direct information may prejudice against the patient with IDDM achieving better glycaemic control than with blood glucose testing, although this question remains unresolved. A poor quantitative correlation between semi-quantitative measures of urine glucose concentration and simultaneous blood glucose values has been noted (72), although fractional quantitative glucose analysis (gm/hour) is more reproducable (69) but impractical for routine use. Even fractional quantitative urine collection may be inadequate as a basis for therapeutic decision making (73). In the volatile state of childhood IDDM, studies comparing urine and blood glucose testing as a means of assessing glycaemic control have produced conflicting conclusions (74, 75). In careful studies of adult IDDM the degree of glycosuria was found to be of value as a index of glycaemia when the blood/urine glucose relationship was known for each individual, in the absence of established diabetic nephropathy (76). The most recent study to try to isolate the effects of blood versus urine glucose monitoring failed to demonstrate any advantage of the former (77).

The premature suggestion that urine glucose testing is obsolete in any case neglects the fact that many patients with IDDM are reluctant to carry out blood testing and with appropriate advice might use urine analysis to achieve reasonable glycaemic control without hypoglycaemia.

Perhaps more important is the potential role of 24 hour urinary glucose excretion not only as a marker of carbohydrate metabolism but as a possible direct independent mediator of renal damage. Excessive glycosuria for example has direct effects on

renal tubular handling of cations which may explain the disturbed glomerulo-proximal tubular balance in diabetes (78). This may be of relevance to the pathogenesis of diabetic hypertension, in that sodium reabsorption may be altered leading to the phenomenon of increased total exchangeable sodium seen in diabetes (79). Furthermore, glycosuria may be relevant to the development of early changes in the glomeruli such as basement membrane thickening which may result from glycosylation secondary to the free filtration of excess amounts of glucose (80).

GLYCAEMIA

The measurement of glycaemic control as an aid to management of IDDM is less straightforward than is at first apparent. First, there are methodological differences in the measurement of blood glucose. The capillary tends to be higher than the venous blood glucose value, and whole blood glucose values tend to be lower than those in plasma or serum (81). In addition enzymatic measurements of glucose by glucose dehydrogenase and hexokinase may give different degrees of precision, with the hexokinase method more accurate in the measurement of borderline hypoglycaemic levels (81, 82).

The inherent variation in blood glucose in IDDM makes a random measurement of little use in assessing glycaemia, and fasting blood glucose levels, whilst important in reflecting the efficacy of nocturnal insulin delivery and the frequency of the 'dawn phenomenon' in IDDM (83, 84), have been found to correlate poorly with integrated measures of glycaemia such as glycosylated (glycated) haemoglobin (HbA_1) in IDDM (85-88). Mean blood glucose

determination, based on a series of blood glucose measurements, is more closely related to HbA_1 (83, 85, 87, 89-92). However for obvious reasons this often does not include a measure of nocturnal blood glucose levels. Since intermediate acting insulin is frequently administered in the evening in IDDM, and a high fasting blood glucose raises the question of either relative insulin deficiency or an excessive counter-regulatory response as part of the Somogyi effect in hypoglycaemia (83), the failure to use nocturnal glucose measures in mean blood glucose determination may give incomplete information.

In addition mean blood glucose values do not reflect the variability of glycaemia. The mean amplitude of glucose excursions (MAGE) or the mean of daily difference (MODD) reflect the within day or day to day glycaemic variability from the mean value (83, 93-96). This approach may be more fruitful in assessing the potential for changes in transmembrane glucose flux to initiate or aggravate cellular damage in diabetes. The 'M' value of Schlichtkrull is a quantitative index of the deviations of several blood glucose determinations from the mean over a 24 hour period (97), and has been modified to measure glycaemic variability in SI units. It gives as a single figure an expression of mean glycaemia and the effect of glucose swings and may be the most relevant measure of glycaemic stability in that it gives a weighted average of glucose values with hypoglycaemic fluctuations having relatively more bearing on the derived M value (83). Hypoglycaemia per se or normoglycaemiac 're-entry', as stated previously, has clearly been demonstrated to be relevant to exacerbation of retinopathy (3, 5), and potentially

to other microvascular complications.

Therefore the measurement of glycaemia to assess metabolic control effectively should utilise a robust method, stating what the source of blood was, and should provide an estimate of fasting and mean blood glucose levels as well as some estimation of the diurnal instability.

NON-ENZYMATIC GLYCOSYLATION OF BLOOD PROTEINS AS AN INDICATOR OF SHORT AND LONG-TERM INTEGRATED GLYCAEMIA

1. Glycosylated (Glycated) Haemoglobin

Glycosylated haemoglobin was first isolated by starch gel electrophoresis in 1955 (98), and it was identified as a heterogeneous fast fraction in 1958 (HbA_{1a}, HbA_{1b} and HbA_{1c}) (99). However it was only in 1962 that Huisman and Dozy (100) correlated this haemoglobin variant with diabetes mellitus, and in 1969 Rahbar et al (101) confirmed that it was the HbA_{1c} fraction that was specifically elevated. Improvements in elution conditions revealed that glycosylated haemoglobin (HbA_{1c}) was formed as a consequence of non-enzymatic post-translational modifications of haemoglobin (HbA_o). The prime site for glycosylation is the terminal amino group of the β-chain of HbA_o, but glycosylation also takes place at the alpha chain amino termini and at the ε-amino groups of lysyl residues. In health roughly 5-8% of HbA_o has detectable glycosylation (102).

The chemical process involved is common to all blood proteins. First, glucose forms an aldimine adduct with the amino group. This rapidly formed 'Schiff base' is labile and reflects ambient glycaemia in addition to 'prevailing' glycaemic control.

Thereafter 'Amadori' rearrangement, which is a slower process, takes place, to produce a stable ketoamine. Both the labile and ketoamine bonded fractions of HbA_{1c} may be clinically relevant in the assessment of glycaemic control (102-106). A longer term rearrangement after stable glycosylation to the state of protein 'browning' in tissue glycosylated proteins by the 'Maillard' reaction may be relevant to the pathogenesis of diabetic angiopathy.

Assuming a steady state of synthesis of red blood cells, 'a slightly reversible' equation for the determination of the rate of synthesis and net accumulation of HbA_1 was developed. Based on an assumption of red cell life span of 120 days a computer simulated model (107) reached the following conclusions:-

1. The maximal half time for approach to a new equilibrium if a patient with persistent hyperglycaemia was abruptly returned to normoglycaemia would be eight and a half weeks. Decreased red cell survival would clearly shorten the time.
2. A short term loss of glucose control (2-4 fold increase in mean blood glucose) will cause a more rapid rise in HbA_{1c} than the fall which occurs following improved glycaemic control of an equivalent degree.

This model does not necessarily operate precisely the same in different individuals. For example, the rate of glycosylation of HbA_1 has also been found to be dependent on red cell glucose permeability (108), red cell oxygenation and pH, and erythrocyte concentration of 2, 3 diphosphoglycerate (2, 3 DPG) (109). However the extent of these alterations in vivo is unlikely to have a major bearing on HbA_1 levels in clinical practice (110).

The system is likely to be saturable however, and post translational modification of HBA_{1c} may be influenced by the duration of diabetes (92).

METHODS FOR DETERMINING GLYCOSYLATED HAEMOGLOBIN

For clinical use the ideal method should be quick, economical, sensitive and reproducible. The most widely used methods involve spectrophotometric estimation following isolation by chromatographic or electrophoretic procedures.

1. Ion Exchange Chromatography

Cation exchange chromatography utilises the difference in molecular charge arising from glycosylation of the N-terminus of the β chain, and was the method used initially in the assessment of HbA_{1c} in diabetes. When macrocolumns or HPLC (high performance liquid chromatography) are used, HbA_{1c} can be differentiated from HbA_1 and the other minor fast haemoglobin components (HbA_{1a} and HbA_{1b}), although in practice this is unnecessary and measurement of the total fast fraction (HbA_1) has been found to be more easily measured and as precise for clinical use (92,102). The mini column method reliably isolates the total HbA_1 fraction, assuming there is rigid temperature control and due attention to buffer composition and a relatively consistent sample load (92, 102). The labile aldimine fraction will be incorporated into the measurement so preparatory elimination by dialysis, saline incubation or chemical treatment (e.g. cell lysis with saponic reagent at pH 5.0) is required (111). Other factors which may affect values are the presence of haemoglobin variants such as HbF, HbG and HbH, which by virtue of their negative charge lead

to an artefactual increase in 'HbA_1' measurement, or positively charged HbS and HbC where false decreases result (102).

Additional sources of artefactual elevations are marked jaundice, severe lactescence, heparin monoidoacetate in the collection vial, sample ageing and high dose aspirin therapy leading to acetylation of haemoglobin (102, 112, 113). A more clinically relevant limitation is the measurement of carbamylated haemoglobin in uraemic diabetics which would lead to an apparent increase in glycosylated haemoglobin (102, 114, 115).

2. Boronic Acid Affinity Chromatography

Cross-linked agarose activated with carbonyl dimidazole and subsequently coupled to M-aminophenyl boronic acid provides a highly efficient matrix for separation of all sugar cis-glycols (102). This method has the capacity to detect haemoglobin glycosylated at sites in addition to the N-terminus of the β chain (115). Modification of the ligand by addition of sepharose particles allows separation of proteins containing sugar. After application of haemolysate, only glycosylated haemoglobin therefore remains adherent to the gel until it is eluted with sorbitol or acetic acid (116). The percentage of glycosylated haemoglobin is thereafter determined by measuring spectrophotometric absorbance at 414nm by the formula:-

$$\%HbA_1 = \frac{\text{Bound A414}}{\text{Bound A414} + \text{Non Bound A414}} \times 100$$

Unlike ion exchange chromatography, the affinity method is relatively insensitive to changes in pH and temperature (116-118). The labile aldimine fraction has been suggested to account for up to 6% of the total HbA_1 measured in one study where its

removal was recommended (119), although this was not confirmed in a later report (120).

The presence of haemoglobin variants do not affect measurement of HbA_1 by this method (115, 119) and likewise carbamylated and acetylated Hb probably do not affect the assay (115). Further potential benefits are the lack of effect of anticoagulant, hyperlipidaemia or hyperbilirubinaemia on sample analysis, and the capacity for the commercial gel filled columns to be re-used (119) up to 12 times assuming the gel is stored at 4°C in the dark. Overloading the columns with more than 4mg of haemoglobin does however reduce the yield of HbA_1 (119) and recent reports have suggested difficulties in standardisation between different batches of gel (121-124), although individual laboratory modifications of the amounts of equilibration wash and elution buffers may account for these discrepancies.

3. Agar Gel Electrophoresis

This uses electrochromatographic separation, and is insensitive to changes in buffer pH over the range 5-6.5, as well as being temperature independent (102). In addition, positively charged haemoglobin variants (HbS and HbC) can be detected and excluded from measurement although the same does not hold for HbF and HbG (102, 115). Labile aldimine is measured by the assay so pre-incubation with saline or preliminary dialysis or chemical lysis are required (102, 115). Furthermore carbamylated and acetylated haemoglobin can produce artefactual increases in the 'HbA_1' detected (115).

4. Polyacrylamide Gel Isoelectric Focussing

This method is similar to simple agar gel electrophoresis, but is more reliable in separating HbA_{1c} from HbF, unless 30% or more of the HbF is acetylated (102). Likewise high dose aspirin treatment and renal failure may lead to falsely elevated levels of 'HbA_1' (112, 115), although aspirin ordinarily does not interfere with the method (113). Whilst closely correlating with chromatographic methods, the specialised equipment required make this method unsuitable for clinical practice (102).

5. Colorimetric methods

This approach directly estimates the extent of all ketoamine linked glucose by measuring the conversion to hydroxymethylfurfural (HMF) following limited hydrolysis with a weak acid (oxalic) (102, 125). HMF is then converted to a coloured complex by reacting with thiobarbituric acid (TBA). The method theoretically has the advantage of only measuring the stable glycosylated protein fraction, and does not detect either haemoglobin variants or carbamylated or acetylated proteins (102, 105, 113, 115). However despite the excellent specificity and a potential advantage over column methods with regard to reproducability after long term storage of samples (102), the method is lengthy and difficult to standardise due to varying or poor yields of HMF, (92, 102, 105, 122) or de novo formation of HMF from hydrolysis of glucose at high concentrations (92, 102, 105, 126), and poor precision has been reported (102, 126). In addition the colour development involved in the assay varies with the site of the ketoamine bond and is eight times the amount

derived from alpha linked valine compared to lysine residues (105, 127).

Other chemical methods have measured the glycosylated protein bonds indirectly by formaldehyde generation after periodate oxidation (128). Phenol and sulphuric acid hydroxolysis and hydrolysis may also be reliable (129). Most recently phytic acid was employed to saturate 2, 3 DPG binding sites in haemoglobin (130). As HbA_1 cannot bind phytic acid, the change in spectral absorption after phytic acid binding is thus inversely proportional to the percentage of glycosylated haemoglobin, although HbF will also be estimated by the same principle. None of these latter methods, despite their ingenuity, are sufficiently economical in time and cost to be of clinical relevance.

6. Immunoassays

Sheep immunised with HbA_{1c} purified by large column chromatography can produce antibodies which are extensively absorbed (90%) with HbA_0 linked to agarose, although the remaining 10% of the titre is highly specific for HbA_{1c} by radioimmunoassay (131). Apart from the potential ease of application, this method allows precise measurement of HbA_{1c} in the presence of fetal haemoglobin, but has yet to be developed commercially. Nephelometric quantitation of immune precipitants of HbA_1 and antibody may also be advantageous in its potential for automation and in its sensitivity (132), although preliminary separation of HbA_0 and HbA_1 by column chromatography would be required, and the procedure has not been validated in clinical practice.

Glycosylated haemoglobin methods: Conclusions

At present a choice lies between minicolumn and affinity chromatography, agar gel electrophoresis or colorimetry using the TBA reaction. The clinical workload, ethnic population and existing laboratory facilities of the area providing diabetic services should dictate which method is employed.

In the data to be presented in my thesis, HbA_1 (HbA_{1a} + HbA_{1b} + HbA_{1c}) has been measured by cation exchange chromatographic method. The method has operated since 1982, and is precise and reproducible. The Department of Chemical Pathology at Hope Hospital participated in the external quality control scheme for glycosylated haemoglobin determination which would appear to be essential for validation of results, particularly whilst several methods are operated nationally.

A glycosylation index $\left(\frac{\text{observed } HbA_1}{\text{mean laboratory normal range } HbA_1}\right)$ has been suggested as a means to allow comparison of HbA_1 data from different centres, but has not as yet been put into practice.

CLINICAL UTILITY OF GLYCOSYLATED HAEMOGLOBIN

The elevation of HbA_{1c} in diabetes first prompted suggestions that its presence might act as a genetic marker for insulin-dependent diabetes mellitus, although this was not borne out by twin studies (133). The recognition that HbA_{1c} levels were elevated in secondary diabetes (e.g. due to Cushing's syndrome) (134) led to the concept of glycosylated haemoglobin as a monitor of metabolic control. This was borne out by Trivelli et al (135)

in one of the first clinical studies of HbA_1 when highest levels were found in diabetics with ketoacidosis or infection. The interesting observation of a lack of association between HbA_1 concentrations and duration of diabetes or the extent of angiopathy in this study has subsequently been reinforced (105).

HbA_1 or HbA_{1c} concentrations correlate with various parameters of glycaemic control to a greater or lesser degree in studies that combined patients with insulin-dependent and non-insulin-dependent diabetes mellitus (NIDDM). These have included fasting blood glucose, random blood glucose, mean daily blood glucose during the previous 3 months, the sum of bracketed blood glucose values around meals, maximum glucose concentration in the area under an oral glucose tolerance curve, the highest mean blood glucose occurring during the 12 hours of continuous monitoring, and 24 hour urinary glucose excretion obtained 4-12 weeks previously (85-92, 102, 136). Some studies have also revealed a correlation between HbA_1 and triglycerides and cholesterol (137-139).

Unfortunately very few studies have looked solely at IDDM following removal of the labile fraction of HbA_1. The 24 hour glycaemic variability of NIDDM is much less than IDDM and may be predicted from the fasting blood glucose making serial blood glucose sampling of little use and close correlations with HbA_1 unsurprising. When studied, HbA_1 correlated poorly ($r = 0.38$) with fasting blood glucose in IDDM (85-88). The relationship between HbA_1 and the amplitude of glycaemic excursions assessed by MAGE or M values in IDDM has to my knowledge not been examined in detail. HbA_1 has been reported to bear little relation to the

standard deviation from the mean blood glucose of IDDM (85) although in one report on 9 subjects, the standard deviation (SD) of glucose recorded continuously for 12 hours did correlate with HbA_1 presumably due to stable glycaemia (SD 1-3mmol/L) (89). Bearing in mind the observation that whilst a significant increase in stable HbA_1 follows 1 week after induction of poor glycaemic control in IDDM (104), but a similar fall in HbA_1 lags 3-10 weeks behind achievement of improved glycaemic control (102, 137), the HbA_1 assay seems to reflect disproportionately hyperglycaemic rather than hypoglycaemic fluctuations, although the latter may also have a role in the initiation or aggravation of diabetic microangiopathy. Indeed, it has been suggested that since a particular HbA_1 level can result from varying degrees of glucose control between individuals, and mean blood glucose demonstrates a curvilinear relationship with HbA_1 suggesting a saturable system (maximum HbA_1 23%), stable HbA_1 is an insensitive index of fluctuating glycaemic control for a particular individual with a higher range of mean blood glucose levels (140).

Many earlier studies in IDDM failed to remove the labile Schiff base limiting interpretation of the relationships between different measures of glycaemic control and HbA_1. However longitudinal studies have since demonstrated the capacity of serial HbA_1 measurements to parallel improved glycaemic control assessed by mean blood glucose and M value determination (44). The labile fraction of HbA_1 should now be routinely removed prior to HbA_1 measurement, although it has been suggested that in addition to the stable ketoamine component, the labile fraction

may be of particular value in assessing the relationship between short and longer term integrated measures of glycaemia (106).

The introduction of HbA_1 has had a major impact on clinical practice by offering the potential to modify treatment regimes more effectively and allowing reliable prospective examination of the role of glycaemic control in the development of vascular complications. Acceptable levels of HbA_1 for individual IDDM subjects need further clarification. The suggestion of 3% above the mean normal HbA_1 (141) (i.e. 10% in our laboratory) as an acceptable upper limit for insulin-dependent diabetic subjects is I feel far too liberal.

Measurement of stable HbA_1 though now routine, should not be taken out of clinical context and in isolation. Relevant glycaemic data whether self generated by the patient or produced by a technique that allows quality control of the patient's home blood glucose monitoring result (v.i) should also be analysed in a complementary fashion to confirm the reliability for that individual patient and to assess what degree of glycaemic control (near-normoglycaemia, normal HbA_1, minimal hypoglycaemia) is justifiable for the individual patient.

The previously mentioned clinical limitations of certain of the methods for measurement of HbA_1 must also be borne in mind:-

(i) Haematological Disturbances

Unless colorimetric methods or perhaps affinity chromatography is employed, haemoglobinopathies such as sickle cell disease or thalassaemia may lead to falsely low or high values of HbA_1 (102, 115).

Any condition leading to shortened red cell survival secondary to haemolysis may lead to artificially low values of HbA_1 (102). Likewise conditions of accelerated erythropoesis such as pregnancy (102, 142) or treatment of iron deficiency anaemia lead to lower values of HbA_1 (143). By contrast increased HbA_1 levels accompany increased erythrocyte survival following splenectomy (144).

(ii) Aspirin

High dose aspirin therapy can produce elevations of fast haemoglobin due to acetylation which could be detected by electrophoretic methods for measuring HbA_1 (112, 113).

(iii) Uraemia

Diabetic nephropathy affects 45% of IDDM. Most studies reporting high HbA_1 levels have used electrophoretic methods where carbamylated haemoglobin may be falsely detected in such patients (102, 114, 145-148). In addition depressed erythropoesis resulting in secondary anaemia and the possible use of frequent blood transfusions particularly in haemodialysed patients further complicates interpretation of HbA_1 (102, 114).

(iv) Sex and Racial Differences

The effects of gender on HbA_1 are controversial. Studies have suggested that HbA_1 levels are either higher in non-diabetic men (149) or alternatively lower in diabetic men than women (141, 150). Menstruation and psychosocial factors have been

postulated to account for these differences, but most laboratories do not take gender into account when reporting levels, or quote sex dependent normal ranges. A further important factor is ethnically determined differences in HbA_1 levels in normal populations with identical fasting blood glucose levels. Thus a statistically significant graded increase in levels of HbA_1 was noted by Gomo between Caucasian, Negroid and Arab populations (149). This needs confirmation particularly if similar differences can be demonstrated in the respective diabetic populations for given levels of glycaemia, and if this can be shown to have a bearing on the prevalence of vascular complications between different racial groups. For example, Asian diabetics in Natal have been shown to have a higher retinopathy rate than black Africans (151): is this reflected by a higher HbA_1 range in non-diabetic Asians?

Finally, HbA_1 itself may have a pathogenetic role. A higher oxygen affinity of HbA_1 compared to HbA_o has been reported in vitro (110), and it has been hypothesised that the consequent tissue hypoxia leads to diabetic vascular complications (152). However in vivo studies have shown that at mean HbA_1 levels (labile and stable combined) of 11.6-12.2%, the higher oxygen affinity patterns for diabetics do not affect oxygen dissociation once values are normalised with respect to levels of 2,3, DPG and pH (110). A lack of correlation between HbA_1 and vascular complications in IDDM has been reported (7, 105, 135), which further reduces the likelihood that HbA_1 itself contributes to diabetic angiopathy. Nevertheless, non-enzymatic glycosylation of intra-erythrocytic proteins involved in nucleoside and

nucleotide metabolism has been reported (153) and may be relevant to the role of disturbed red cell function (154) in microvascular disease.

2. GLYCOSYLATED SERUM PROTEINS

(a) Glycosylated (Glycated) Albumin (GSA)

Albumin is the predominant serum protein, circulating in a concentration of 35-50g/L and accounting for roughly 55-70% of the measured total serum protein.

Glycosylated serum albumin was first isolated from human serum in 1979 (155), and elevated levels were subsequently demonstrated in diabetic serum (156, 157) although its existence in horse serum had previously been identified in 1956 (158). Human glycosylated albumin has been found to form in vivo by the same chemical reaction as glycosylated haemoglobin with an initial non-enzymatic reaction to form a labile Schiff base, which thereafter undergoes Amadori rearrangement (155). Consequently other non-enzymatic glycosylated proteins have been confirmed to undergo the same process (159).

Unlike haemoglobin, the site of in vivo glycosylation of albumin has been demonstrated to be predominantly at the 525-lysine residue (160). Using 500mg of albumin applied to a large (2.5x18cm) affinity chromatographic column with a slow flow rate (60ml/hour), 10-12% of freshly prepared radioactive human serum albumin was found to be glycosylated, of which 48% was found by cation exchange chromatography to take place at this site (160). Previous work suggested that like acetylation, some glycosylation of albumin also takes at the 189-lysine and 199-lysine residues

(155, 160). Bearing in mind that the half life of albumin is 19 days, the rate of non enzymatic glycosylation of albumin has been estimated to be approximately 9 times greater than that of haemoglobin (160), whose half life is 120 days, and where 5-8% is normally glycosylated. This suggests that direct measurement of glycosylated albumin, in addition to reflecting shorter term improvements in glycaemia than glycosylated haemoglobin, may also be a more sensitive indicator of acute hyperglycaemic fluctuations than HbA_1 (156, 161). As yet, there have been very few studies on the kinetics of human glycosylated albumin, although glycosylated total plasma protein levels were found to increase by 29% from basal levels after 48 hours of maintained hyperglycaemia (22mmol/L), using the Biostator in well controlled IDDM, in whom a maximum rise was noted by 12 hours, which thereafter plateaued (161). Animal work has also suggested that glycosylated serum albumin is formed more quickly than HbA_1 in response to hyperglycaemia, although the half life of GSA was not affected (162).

Early studies suggested that 6-15% of total serum albumin was glycosylated in normal human serum (155), but levels may be higher with different methods, and reference ranges of 11.6-19.5% have been quoted (120). More recently normal reference ranges of 1.4-5.4% have been quoted using affinity chromatography (163, 164), raising the possibility of variable bonding of GSA by different methodologies.

Methods for determining glycosylated (glycated) albumin (GSA)

1 Combined Chromatographic/Chemical Methods

The original description of human GSA (155) was based on an in vitro experiment where albumin was initially purified from fresh serum by affinity chromatography (165), and thereafter incubated with radiolabelled glucose. The albumin was then resolved into 2 fractions by ion-exchange chromatography using carboxymethylcellulose. Radioactivity in one recovered peak isolated the GSA fraction, and this was confirmed by the colorimetric assay for ketoamine protein adducts. This original method is quite impractical for routine clinical assessment but confirmed that both the chemical and physical properties of GSA allow identification of the molecule. Separate affinity chromatography and colorimetric methods have since been shown to correlate closely with one another (157).

A more elaborate combined approach was to purify albumin by a one step chromatographic procedure before measurement by a modification of the fructosamine assay (v.i.) (166). This time consuming method correlated with total plasma fructosamine ($r=0.85$) but is clearly impractical for routine use.

2. Chemical (colorimetric) method

GSA can be measured after isolation of albumin, by its capacity to release hydroxymethylfurfural after weak acid hydrolysis, which in turn reacts with thiobarbituric acid to yield a coloured product with maximal spectrophotometric absorbance at 443nm.

This method was employed in confirming the initial recognition of GSA (155), and was found reliably to detect increased levels in diabetic subjects (156, 157). Colorimetry has been reported to yield about 16% more GSA than the affinity

chromatography method (157), but has the disadvantage for clinical use in that a preliminary affinity column procedure is required to isolate albumin. In addition despite standardisation of the method by optimisation of temperature and pH, free glucose was found to interfere with the assay leading to spuriously high results (167). This could even result from carbohydrate-containing packing materials in packages of glass tubes, necessitating acid washing of all glassware, in addition to dialysis or protein precipitation with trichloro-acetic acid to remove free glucose (v.i.) (167). The overall effect is that this method is too expensive and laborious for routine monitoring of diabetics.

3. Affinity Chromatography

As with HbA_1, the capacity of boronic-acid gel to bind diols selectively allows separation of GSA from non-glycosylated albumin. One modification of the procedure is that after separation of the two fractions, the albumin content of each is measured by a variety of procedures of varying sensitivity. The recognition that albumin is principally glycosylated at the 525-lysine residue was made by a combined approach of affinity chromatography on boronate agarose gel followed by cation-exchange chromatography (160). It was stated that 10-12% of human serum albumin is glycosylated. More recent reports using boronate affinity chromatography have quoted normal ranges of GSA of 11.6-19.5% (120), 6.8-10.3% (168), 2.0-5.4%, (163), 1.0-2.0% (169), 1.4-5.2% (164), 0.43-1.19% (170), 1.3-2.0% (171), and even 0-4.5% (172). The discrepancy in normal values seems to be due

to an accumulation of methodological factors.

The affinity column method as first described showed that the size of the column and the rate of transfer of the sample through the affinity matrix had a definite impact on the recovery of the glycosylated protein (116). The suggestion that GSA comprised 10-12% of albumin assessed by affinity chromatography utilised 500mg of serum albumin on a 2.5x18 cm column of boronic acid gel, with flow rate of 60ml/hour (160). However other studies (120, 161-165, 168-172), have utilised commercial columns (Glycogel B, Pierce Ltd. or Glyc-Affin, Isolab Ltd.), where no more than 10-20mg of albumin is passed through 1ml of boronate gel at a flow rate of around 30ml/hour. The lowest yield of 0.43-1.19% reported previously (170), highlights a source of error in following the instructions given by the manufacturers of kits. It is suggested that 200µl of undiluted plasma is applied to the column, which means in fact that 8-10mg of albumin is added, but it has been shown that when more than 3mg of albumin is loaded, a lower percentage of glycosylated albumin results, possibly due to saturation at binding sites on the affinity matrix (163). This observation has also been made with regard to measurement of total glycated plasma proteins (173), but has yet to be confirmed by other laboratories with regard to GSA.

Another crucial aspect is the method employed to measure albumin in the two fractions after they have been separated by affinity chromatography. Dye binding, immunochemical and radioimmunoassay procedures are described. The main dye-binding method, which is recommended by the manufacturers of one of the commercial methods (Glycogel B, Pierce Ltd.) is Bromocresol

Green. It has been suggested that BCG overestimates low levels of albumin or leads to precipitation of BCG-albumin precipitates, and is non-specific if the reaction is not timed properly (164, 174-176). This may be partly overcome by the use of Bromocresol purple dye (175), but sensitivity in low concentrations and poor reproducability remain a problem (176), and limit interpretation of those studies which have measured GSA by dye binding methods (120, 168, 171, 172, 177, 178).

Immunochemical methods may be more precise and nephelometric (169, 172), immunoelectrophoretic (163), and immunoturbidimetric (164), methods have been employed. Albumin overloading of columns was a feature of the study comparing nephelometry and dye binding (169), which might invalidate the conclusion reached, and although sensitive, nephelometry is not a facility widely available in centrifugal analysers. Rocket immunoelectrophoresis is sensitive but requires an overnight incubation which could make it unsuitable for measuring large numbers of samples. The use of an immunoturbidimetric assay on a centrifugal analyser would provide the best compromise in terms of accessability, economy of effort and time as well as sensitivity. Initial work using this method suggested this was indeed precise with between-run coefficients of variation reported between 0.7-3.2% (164), and analytical recovery of 97-102%. Assuming the column is not overloaded with albumin a precise check on recovery of albumin applied to the column would confirm that GSA measured by boronate affinity chromatography reliably measured all cis-diol groups, effectively the stable ketoamine bond of GSA. A radioimmunoassay method for GSA

measurement has also been reported (170), and would have similar potential to immunoturbidimetry, although its capacity for a clinical service has still to be established.

4. HPLC

An HPLC method has been developed to measure the glycosylated lysine residue (furosine) after isolation of albumin by affinity chromatography (179). The method has been found to estimate glycosylation at 27% of the 525-lysine residues, and can be modified to measure GSA. Whilst reproducable and specific, it is impractical for routine clinical evaluation of glycaemic control.

CLINICAL ASPECTS OF GLYCOSYLATED ALBUMIN MEASUREMENT

The half life of serum albumin is approximately 17 days. It is possible that glycosylation enhances catabolism of albumin; this would be in keeping with clinical observations of GSA in response to fluctuating glycaemic levels (180), and the observations that glycosylation may enhance catabolism of other serum proteins (e.g. apolipoprotein AI in HDL) (181).

As stated previously, GSA may be a sensitive indicator of intergrated glycaemia over relatively short periods of time, as suggested by clinical studies showing established falls in response to improved glycaemic control within 1-3 weeks but not by 2-3 days (182, 183), whilst a rise to a similar degree consequent upon insulin withdrawal, was established for total glycosylated proteins (60% of which is GSA) after 12 hours (161). A similar pattern is suggested from data showing a rise in GSA

following resection of an insulinoma after 17 hours of hyperglycaemia, maximum at 15mmol/L (182). Thus, as for HbA_1, the glycosylation of the specific protein seems to reflect hyperglycaemia disproportionate to hypoglycaemia.

The suggestion that glycosylation of albumin is enhanced in comparison to that of haemogloblin may give GSA a further advantage in improved sensitivity over HbA_1 with regard to measuring minor degrees of glucose intolerance. This is suggested in studies where GSA and HbA_1 have been measured in diabetics and healthy controls, and where hardly any overlap was noted between the 2 groups with regard to GSA whilst a degree of overlap was apparent for HbA_1 (157, 163).

The disparity between GSA and HbA_1 is further suggested by reports of a poor correlation between the two measures in diabetics (r=0.58-0.62) (177, 178), although other studies (163, 164, 180, 184) have shown a much closer relationship between these indices (r=0.78-0.91), reflecting the selection of healthy controls and predominantly non-insulin-dependent diabetics with stable glycaemic control. GSA certainly relates closely to glycosylated total serum protein levels in diabetes (v.i), and to a lesser extent with mean daily blood glucose (r=0.38-0.72) (178, 180) and post-prandial glucose levels (r=0.66) (184), although there appears to be little relationship with either fasting (r=0.30-0.56) (178, 180) or random blood glucose (r=0.33) levels (163).

One small study of stable insulin-requiring diabetics has suggested that GSA and HbA_1 gave broadly similar interchangeable information (178). GSA was not found to be sensitive enough in

this situation to reflect minor glycaemic fluctuations from week to week or month to month. However this is the only study of its kind to date, and the low normal range of GSA quoted and lack of information on precise methodology raises the possibility of column overload thereby leading to falsely low values of GSA.

However, it is clear that GSA may be more reliable than HbA_1 in reflecting glycaemic control in conditions of altered red cell turnover. This may apply in pregnancy where benefits of effective monitoring of short term glycaemic control are likely to occur, although albumin kinetics are also altered. GSA has indeed been reported to be a reliable indicator of glycaemia in cross-sectional (177, 185) and longitudinal surveys (171) of diabetic pregnancy, and to correlate inversely with the birth weight ratio, although again methodological considerations (171, 177) limit the conclusions to be drawn from such reports. GSA may also be clinically relevant if the drug binding capacity of albumin is significantly affected by glycosylation (186).

Finally the glycosylation of serum albumin may also have an important role in the pathogenesis of complications; it is less soluble than the non-glycosylated forms (187) and has reduced transglomerular permeability and reduced urinary excretion compared to non-glycosylated albumin in diabetics with normal albumin excretion rates (AER) (188), although there was no preferential clearance once the AER was increased. However another study found precisely the opposite, demonstrating preferential excretion of all glycated serum proteins including albumin in diabetics with normal AER (189). The observation

of increased amounts of GSA in the renal substance (190) has led to suggestions that glycosylation of albumin may lead not only to enhanced urinary albumin excretion, but may also contribute to diabetic nephropathy by damaging the glomerular and tubular basement membranes leading ultimately to hyalinosis. The observation of mouse glomerular basement membrane thickening following repeated injections of non-enzymatically glycosylated plasma proteins (predominantly albumin) (191) makes this hypothesis quite compelling and merits further investigation.

(b) Glycosylated (Glycated) total serum proteins (GTSP)

The total serum protein pool is comprised of many different proteins with varying physical properties and functions, and half-lives that vary from several hours to 3-4 weeks. Although albumin has been found to account for 55-70% of the serum protein concentration, other large molecular weight compounds - the globulins- also comprise a large percentage of the pool.

When GSA was first isolated, glycosylated fractions of IgM and IgG were also isolated in significant amounts (155). Since then non-enzymatic glycosylation of fibrinogen (192), apolipoproteins (181, 193), and acute phase reactant proteins (haptoglobin and alpha 2-macroglobulin) (194) has been described. The GSA component has been reckoned to account for 60-70% of the GTSP (187). Earlier studies required intricate procedures to separate pure GSA and therefore assessment of GTSP was also made, initially showing cross-sectional and longitudinal parallelism with GSA (171). The lack of prior separation of albumin

obviously makes the measure of GTSP a more attractive procedure for routine assessment of intermediate glycaemic control. However the varying half lives of the different proteins could in theory affect the utility of GTSP as a measure of glycaemia when control is changing. For example, whilst the half-life of albumin may not alter with glycosylation, glycosylated apolipoprotein B (as the major apoprotein of low density lipoprotein (LDL)) has been found to have a prolonged half-life, whilst animal studies have suggested the opposite for Apo AI, the predominant apoprotein of high density lipoprotein (HDL) (181, 193). Although the conclusions of earlier studies can be questioned in the light of methodological problems, the introduction of the fructosamine assay as a measure of GTSP (v.i.) may clarify any differences between GSA and GTSP measurement in IDDM during conditions of fluctuating control.

Methods for measuring GTSP

1. Chemical (colorimetric) method

The capacity of ketoamine bonds to release HMF after weak acid hydrolysis has also been employed to measure GTSP.

An initial report suggested the method was sensitive enough to separate fully diabetic and healthy populations and to correlate not only with GSA ($r=0.99$), but also HbA_1 ($r=0.79$) and fasting blood glucose ($r=0.71$) (187), but it later became clear that this relationship was artefactual. The failure to remove free glucose was found to interfere with the assay, accounting for an elevation of GTSP of 2-3 times that obtained after overnight dialysis of the samples of diabetic subjects (195).

When this correction was carried out, GTSP levels in predominantly insulin-treated diabetics were less clearly separated from healthy controls, and less precise correlations were observed with HbA_1 (r=0.51-0.74) (195-197), and with mean blood glucose levels (based on single measurements on 8 or more occasions over 2 years) (r=0.58) (196). An additional significant correlation with the clinical variance of these glucose values and the M values suggested that GTSP might also reflect glycaemic instability (196).

The methodology involved in colorimetric estimation of GTSP has been criticised, since careful standardisation of the assay is required, with either dialysis (195) or precipitation with trichloroacetic acid (TCA) (198) being employed initially to remove free glucose. It is still not known however whether these methods are equally effective and whether the colour yield after incubation with TCA is similar to that achieved with dialysis (102). The use of a sodium borohydride blank, whilst fully reducing the ketoamine bond within 15 minutes, and the time and temperature dependent hydrolysis, are further time consuming components, although autoclaving at 121°C for 1 hour has been an effective way of shortening the latter procedure (199). The use of purified 5HMF as a standard allowed for variable batch hydrolysis or evaporation of the sample which led to a precision of no better than 8% within batch and 12% between batch variation. Later use of fructose as an alternative standard optimised the assay to produce intra and inter assay coefficients of variation of 4.3% and 8.6% respectively (199). Although automation of the method has been described, it is generally felt

that the colorimetric measure of GTSP is unsuitable for routine use in a clinical laboratory, particularly as poisonous chemicals (oxalic acid and TBA) are employed in the method (102).

2. Protein Precipitation

This method measures both enzymatically and non enzymatically bound glycoproteins and involves precipitation of protein-bound-hexose using perchloric acid initially, with the precipitate then being measured colorimetrically after the addition of sulphuric acid (194). The levels in diabetics correlated with HbA_1 (r=0.41) but not with fasting blood glucose (r=0.25), and correlated with levels of haptoglobin (r=0.59), which was elevated in diabetics (194). The method is unsuitable to monitor glycaemic control as it is too time-consuming and it measures enzymatically derived glycoproteins in addition to non-enzymatic glycosylprotein.

3. Affinity Chromatography

As with GSA and HbA_1, the m-aminophenyl-boronate gel has the capacity to bind selectively the cis-diol groups of all glycosylated serum proteins. A specific eluant then ensures removal and the non-glycosylated and glycosylated fractions can be measured separately to give an estimate of the percentage of GTSP. The method has been shown to distinguish between non-enzymatically glycosylated proteins and enzymatically linked glycoproteins (200).

Two commercial systems (Glycogel, Pierce and Warriner and

GlycAffin, Isolab) were used in initial assessments. It was found that, as with GSA, the Glycogel method was precise (within batch co-efficient of variation (CV) 4%, between batch CV 5.3-6.2%), assuming that precautions were taken to ensure the column was not overloaded with more than 2mg of total serum protein (201). Overloading eventually led to reduced yields of GTSP in diabetic and healthy samples, but unfortunately the direct method recommended by the manufacturers of Glycogel for protein measurement of the glycosylated and non-glycosylated aliquots without a reagent is insensitive unless large amounts of protein (10-15mg) are applied to the gel (201). The introduction of the Bradford method using the Biorad protein assay reagent is sensitive enough to measure the small amounts of GTSP, but only if 2mg is added to the column (201). However several of the clinical reports which followed have failed to take these modifications into account.

The other commercial method (GlycAffin, Isolab) unfortunately used the Coomassie Brilliant Blue-G-250 dye to measure protein which was also too insensitive to detect all the GTSP (120). This commercial method has been assessed further and it was suggested that free glucose had the capacity to interact with the boronic acid binding sites on the gel, necessitating either dialysis or a preliminary chromatographic phase (G25 Sephadex) to remove free glucose (168). Failure to do so led to a false yield of GTSP (25-30% increase) (168). Thus the Isolab method would appear to be unsuitable for general clinical use for several reasons: (1) the preliminary seperation of glucose by chromatography leads to a marked reduction of

analytical recovery of total protein (57%) in addition to introducing an additional time consuming phase to this procedure. Overnight dialysis of free glucose is also time consuming and is not interchangeable with Sephadex chromatography. (2) the method was temperature dependent with poor precision (between-run CV 18%) (3) the addition of large amounts of protein to the column and the consequent measurement by Coomassie Blue may have contributed further to the poor precision.

These two independent affinity chromatography methods seem therefore to have quite different performance characteristics and the suprising difference in the normal ranges of GTSP with Glycogel (3.0-10.3%) and GlycAffin (11.5-16.2% in one report and 8.8-11.6% in another) would appear to confirm this.

The Glycogel affinity procedure in insulin-treated diabetics correlated with HbA_1 levels (r=0.82) but not with random blood glucose values (r=0.38) (201). However the close relationship with the colorimetric (TBA) assay (r=0.70) (201) indicates that both assays measured similar glycosylated moieties although it was suggested that the affinity method might be more accurate. It was noted that unlike early claims when GTSP was measured by colorimetry (196), the discrimination between diabetic individuals and healthy controls was in fact less precise than for HbA_1 (201). A similar degree of overlap between normal and diabetic GTSP ranges has since been confirmed with Glycogel columns, when a virtually identical correlation between HbA_1 and GTSP was observed (r=0.81), although perhaps suprisingly GTSP also correlated closely with fasting plasma glucose (r=0.75) (202). This probably reflects the predominance of non-insulin

dependent diabetics studied confirming previous observations (197), and thereby reduces the relevance of the data to IDDM.

When the Glycaffin column was used (168), GTSP correlated with GSA (r=0.83) although the relationship with HbA_1 was less clear (r=0.81 in one study (120) and r=0.37 in another (168)). No data was provided from these studies on the relationship between GTSP and random blood glucose levels.

3. HPLC

The production of furosine from non-enzymatically (ε-N-lysine) glycosylated proteins by acid hydrolysis can be measured by HPLC and has been reported by Schleicher et al (203). Furosine (lysine-glycosylated serum protein) levels correlated with HbA_1 (r=0.79) and with mean blood glucose (r=0.88) in a mixed group of insulin-dependent and non-insulin-dependent diabetics. A longitudinal survey of 10 insulin-dependent diabetics suggested that furosine was a more sensitive indicator of glycaemia than HbA_1 (203).

CLINICAL ASPECTS

The essential question about the GTSP assay is whether it has the ability to detect short term fluctuations in glycaemia. Using the colorimetric method, it was reported that one week of improved glycaemic control (mean blood glucose falling from 14.9 to 10.7mmol/L) was accompanied by a 34% fall in GTSP ($p<0.01$) which was more marked after 2 weeks, when mean blood glucose levels fell further (204). In contradistinction, when hyperglycaemia (22mmol/L) was induced and maintained in 4 well

controlled subjects with IDDM, an increase of 29% in GTSP was noted within 12 hours which did not change thereafter (161). In clinical practice, the measure of a conglomerate of proteins to determine short term glycaemic control could be of limited use if glycosylation of acute phase reactants during periods of infection/inflamation or ketoacidosis gave a yield of GTSP disproportionate to the overall ambient glycaemia. This has been demonstrated in one study where marked rises in GTSP (measured by colorimetry) were apparent after 3 days in diabetics with minor intercurrent infection (205).

However the opportunity to reflect short term changes in glycaemia over a period of 12 hours to 1 week is likely to be a useful addition to direct glycaemic measures and the use of HbA_1 in the assessment of carbohydrate metabolism in IDDM. The colorimetric method has even been shown to be effective in measuring GTSP in stored filter paper (v.i.) (206), which has obvious potential for validation of glycaemic control at home, particularly when diabetics live in remote areas. The filter papers could then be posted to the hospital for assessment either prior to patients' visits or with a view to suggested modifications in treatment being made by phone without direct patient contact.

As was the case for GSA, the GTSP assay should have advantages over HbA_1 in reflecting glycaemic control under conditions of disordered red cell turnover. The most obvious example is in diabetic pregnancy where GTSP has been shown to correlate with maternal glycaemic control and fetal birth weight (177). Longitudinal assessment in gestational diabetic pregnancy

showed the capacity of GTSP to differentiate normal pregnancies from those who later developed gestational diabetes by 11-14 weeks, although thereafter GTSP levels decreased to early pregnancy values (207). In diabetic pregnancies, improved glycaemic control has been demonstrated to lead to a 50% reduction in GTSP after 4 weeks, whilst it took 12 weeks for similar changes to occur in HbA_1 (171). Unfortunately these studies followed the manufacturer's (Pierce) instructions, thereby inadvertantly overloading the affinity column with protein, which limits interpretation of these data.

In addition to the use of GTSP measures to reflect glycaemia, interest has focussed on a potential pathophysiological role of GTSP in the development and progression of diabetic nephropathy. This was first suggested when repeated intravenous injections of GTSP were found to produce pseudodiabetic renal glomerular changes in mice (191). Glycosylated proteins and peptides have been detected in renal tissues from humans with diabetic nephropathy (190) and also in their urine (188, 189, 208). The suggestion has been made that GTSP excretion is enhanced in diabetics without incipient or established nephropathy (189). The loss of selectivity of GTSP excretion with the development of microproteinuria suggested that the early changes could be related to the development of nephropathy. These findings have recently been challenged, and in fact when urinary GSA was measured by a highly specific method, exactly the opposite finding was recorded in diabetics with normal albumin excretion rates (AER) (188). However both these reports (188, 189) suggested that early changes in ionic change following glycosylation of either the renal

basement membrane or filtered macromolecules may be implicated in the development of nephropathy.

(C). FRUCTOSAMINE

Serum fructosamine measurement is a further colorimetric method designed to estimate all ketoamine bonds and therefore is also a measure of GTSP. I will discuss it separately as its development has superseded that of GTSP and it has now become widely used, perhaps prematurely, because of it's simplicity rather than its clinical utility.

The assay is based on the capacity of stable ketoamine bonds to act as a reducing agent in an alkaline solution. The method was first described in 1982 (209) and is a colorimetric procedure where the colour development secondary to reduction of the dye NBT (nitroblue tetrazolium) is measured spectrophotometrically. Although the method has now become automated (210, 211) and widely used (212), the actual mechanism of the reducing activity has remained obscure and the nature of the active species involved in dye reduction conjectural. It has been suggested that superoxide free radical autoxidation from ketoamine bound glucose is involved in the reduction of NBT and this has been supported by the observation of a diminution in fructosamine levels if the assay was carried out in the presence of superoxide dismutase (SOD), a potent inhibitor of superoxide activity (213). If this hypothesis is sustained then the fructosamine assay may be subject to modification in the presence of substances which can alter the serum oxidant activity, such as serum caeruloplasmin, which acts as a scavenger for SOD (214), and which may increase during pregnancy or with the oral contraceptive (215). The suggestion that

microangiopathy leads to the production of free radicals (213) would also increase the potential for excessive reduction. Other low molecular mass compounds such as glutathione and ascorbic acid in significant amounts have been reported to elevate fructosamine levels falsely (209), and the preservatives fluoride and EDTA can reduce the yield, thereby making serum rather than whole blood necessary for fructosamine estimation in clinical practice (216).

It has only recently been demonstrated that fructosamine measures all serum glycated proteins (217), although fructosamine activity was greater after in vitro incubation of glucose with albumin when compared to alpha, β or gamma globulin protein fractions (218). This suggests that albumin glycosylates more freely. Albumin has a longer half life than the other protein fractions and GSA may therefore give disproportionate weighting over the other proteins in the fructosamine assay. However it is now quite likely that the fructosamine assay does not measure labile (Schiff base) glycated products, as evidenced by the lack of NBT reduction by glucosylpiperidine, the Schiff base of glucose and piperidine (209), and the failure for fructosamine levels to rise after a test meal in diabetics or following the induction of hyperglycaemia in vitro (219). Earlier work demonstrated that the fructosamine colorimetric reaction was not linear at higher concentrations of the standard (deoxymorpholinofructose) (211), suggesting the system was either saturated or less sensitive in response to severe hyperglycaemia.

The majority of these previous considerations are theoretical. In practical terms the method also has

limitations. It currently relies on the use of the synthetic ketoamine DMF (1-deoxy-morpholino-D-fructose) in an albumin solution for standardisation. Variable batch activity of the standard has been reported leading to vastly different normal ranges (220). These have varied from as low as 0.96 - 1.12mmol/L (211) to as high as 2.37-4.05mmol/L (219) and one laboratory has quoted a wide reference range of 1.23±0.62mmol/L (166). The use of pooled human serum has been suggested as an alternative standard (220), but it would seen prudent that both DMF and individual laboratory generated serum pools are measured in each assay run to assess reproducability, which the commercial method currently available (Roche Products, Switzerland) quotes at around 3% . One further modification is to use a pH of 10.3 rather than 10.8 for NBT reduction as originally suggested (209) since the lower pH has been found to increase the yield of fructosamine (221-224).

There is still controversy as to whether the clinical status of the diabetic patient can affect the fructosamine level, and most debate has centred around the effects of differing levels of serum albumin. Whilst most early studies did report a correlation between serum fructosamine and albumin levels (210, 211, 225), clinically relevant significant reductions in fructosamine levels were only encountered in hypoalbuminaemic subjects, in whom a correction factor was suggested to be appropriate (226). Since then there have been reports of a diurnal variation in serum fructosamine and albumin (227), and a routine correction for the serum protein concentration has been advocated (226-229). An additional component to the diurnal

variation in fructosamine independent of changes in serum proteins has also been demonstrated (227), which might imply the need for accurate timing of repeated estimations, although most recently it has been clearly shown that up to 50% of the NBT reduction is due to an as yet unidentified substrate, independent of glycosylated proteins (230). These criticisms are still refuted by the original proponents of the method who have reaffirmed that clinically relevant alterations in fructosamine are not apparent in diabetics who were normoalbuminaemic or who had proteinuria less than 1g/L (231). However it is recognised that other pathological states leading to disturbed protein turnover such as thyroid disease or parenteral nutrition, do modify fructosamine levels (232, 233). Uraemia, per se, does not contribute to significant alterations in fructosamine in the absence of hypoalbuminaemia (211, 225). Likewise, moderate hyperlipidaemia is said to have little impact on the assay (234), although the estimation of fructosamine in frankly lipaemic sera may benefit from prior delipidation (209, 227).

Clinical Utility

As a measure of all glycated total serum proteins (GTSP), the length of time over which fructosamine gives an integrated measure of glycaemic control cannot be stated with certainty, although the average half life of GTSP is probably around 20-30 days (187), and it has been proposed that fructosamine levels provide an index of glucose control over the prevailing 1-3 weeks (225).

Currently, fructosamine tends to be compared with HBA_1, and

has been found to correlate fairly well (r ranging from 0.70-0.79) in independent studies of healthy controls and mixed groups of diabetics (166, 202, 211, 225, 233), although variable correlations with stable HbA_1 of r=0.37 (219), r=0.61 (210) and r=0.82 (235) have been described when only insulin dependent diabetics were studied. The manufacturers of the commercial fructosamine kit quote a correlation coefficient as low as r=0.60 (216) if HbA_1 is measured by HPLC (high pressure liquid chromatography) and as high as r=0.90 if ion exchange chromatography was used to determine HbA_1. Perhaps surprisingly, the correlations with GTSP measured by the TBA colorimetric method has been reported to be as low as r=0.58 (209), although this was attributed to extreme imprecision of the thiobarbituric acid (TBA) method, as evidenced by a small poorly defined difference between duplicate test and blank specimens. More recently correlation coefficients between fructosamine and GTSP have been reported to range between 0.82 and 0.92 (202, 216, 219, 233), when GTSP was measured by either colorimetric or affinity methods. The relationship between fructosamine and GSA has also been reported to be a close one with correlation coefficients quoted between 0.85 and 0.90 (202, 219, 233, 236), although it should be stressed that normal subjects and predominantly type II diabetic data were used in these analyses.

A close relationship with fasting blood glucose has been reported with correlation coefficients ranging between 0.74-0.82 in mixed groups of diabetics and healthy controls (202, 209, 216, 225), although as low as r=0.38 in a study of 45 insulin-treated diabetics (219). The correlation coefficient of r=0.73 between

fructosamine and the 2-hour post-prandial blood glucose values in a study of type II diabetics and healthy controls (225), effectively reflects the fasting blood glucose values in these individuals, and subsequent reports have confirmed the relative insensitivity of fructosamine (235) to reflect the more marked post-prandial glycaemic excursions seen in IDDM, as gauged by the mean amplitude of glycaemic excursions (MAGE). A closer correlation with mean blood glucose and fructosamine values in insulin-dependent diabetics has however, been recorded (235).

Longitudinal studies designed to assess the temporal response of fructosamine to differing glycaemic levels are few and far between. An initial report on 6 insulin-treated diabetics suggested that despite a 48% fall in mean blood glucose and a 38% fall in fasting blood glucose, fructosamine levels only fell by 8% in the first week and that a 16 day lag was observed in one subject before the normoglycaemic levels attained by 3 days were paralleled by normal levels of serum fructosamine (225). In fact, when glycaemic control was improved over 3 weeks in a group of 7 non-insulin dependent diabetics, fructosamine was found to be insensitive as a marker of this ($p=0.27$) (237), with no significant change developing till at least 4 weeks, suggesting that in practice fructosamine does <u>not</u> have the capacity to reflects accurately improved glycaemic over a period of less than 3 weeks. However as with other measures of glycosylated proteins, a hyperglycaemic episode of abrupt onset is accompanied by a significant ($p=0.001$) rise in fructosamine after 1 week (237). An anecdotal report of the response to effective insulin treatment suggesting that fructosamine falls by 1-3

weeks in response to improved glycaemic control (202) has yet to be confirmed in properly designed studies.

Fructosamine has also been studied as a screening test for gestational diabetes (238) and as an alternative to glucose tolerance tests (225). A reasonable sensitivity for these diagnoses (85-89%) was reported with high specificity (5-9% false positives) but further assessment is needed.

At present therefore fructosamine appears an attractive cheaper quicker alternative to glycosylated haemoglobin with which it compares closely both in cross-sectional and longitudinal evaluations. Despite claims that it measures all the GTSP, variable correlations with GSA and GTSP and the lack of a clear temporal relationship of the time span seen with GSA in response both to hyperglycaemic and hypoglycaemic excursions suggests that it gives complementary rather than equivalent information to GSA as an indicator of prevailing glycaemic control. Longitudinal studies of GSA, HbA_1, and fructosamine in IDDM are needed to clarify these points. In addition methodological considerations, in particular the need to correct for the prevailing serum protein concentration, and identification of all substances contributing to NBT reduction, must be taken into account if the assay is to be used to assist in the clinical management of IDDM.

1:2 LIPID METABOLISM

Disordered lipid metabolism is a constant feature of both insulin-dependent and non-insulin-dependent diabetes mellitus.

The excess of cardiovascular mortality in all forms of diabetes is often attributed to hyperlipidaemia although epidemiological studies do not in fact bear this out. Although it is possible that lipids accelerate the development of atherosclerosis by virtually the same mechanism in IDDM and NIDDM, clear differences in the pattern of lipid and lipoprotein abnormalities are apparent between the two. Unfortunately most epidemiological and clinical studies of lipids in diabetes have pooled all diabetics regardless of their endogenous insulin status. Other factors such as age, gender, tobacco and alcohol consumption, relative amounts of exogenous and endogenous insulin, the degree of insulin resistance, body mass index, diet, ambient glycaemic control, the extent of diabetic renal disease and genetic influences all have a bearing on lipid metabolism and should be taken into account.

Although hyperlipidaemia may accompany IDDM, it is becoming clearer that diabetes may also exert a more subtle qualitative influence on lipids and lipoproteins. Prior to reviewing the published work on the quantitative and structural alterations of lipoproteins and their relevance to atheromatous disease in IDDM it is worth outlining current knowledge about normal lipid metabolism to put these changes in IDDM into perspective.

Lipid Metabolism in health

Lipids are a heterogeneous group of compounds which are grouped together by virtue of their solubility in organic solvents and their insolubility in aqueous media. They can be divided into two broad groups: the small rapidly-turning over molecules

involved in immediate fuel supply and the larger molecules involved in lipid transport and storage.

The small lipid molecules are non-esterified (free) fatty acids (FFA), glycerol and ketone bodies. FFA are produced by hydrolysis of triglyceride in adipose tissue and circulate as an energy source for liver and muscle. Glycerol is also formed by lipolysis in adipose tissue. It cannot be re-esterified and more than 90% is taken up for hepatic metabolism. Ketone bodies are formed as by-products of the Kreb's cycle in the liver and circulate to be available as a minor energy source for muscle after oxidation. Levels of all these molecules are increased in the circulation in conditions of enhanced lipolysis, and their half lives can be measured in minutes.

The larger lipid molecules are cholesterol, phospholipid and triglycerides. Half-lives of triglycerides vary from 6-12 hours whilst cholesterol has a half life varying between three and a half and six and a half days. Free cholesterol is the form of sterol relevant to membrane formation and hormone and bile acid synthesis whereas cholesterol ester predominates in the transport and storage forms of cholesterol. These larger lipid molecules are derived from either a dietary source or arise de novo from synthesis at the intestinal, hepatic or adipose tissue level. Lipoproteins have evolved to facilitate transport of these water-insoluble lipids in an aqueous medium (plasma).

Plasma Lipoproteins

Four major classes of lipoproteins are defined, based on either their electrophoretic mobility or their physical characteristics (flotation density determined by either ultra-

centrifugation or precipitation methods) (Table 1). All lipoproteins originate in the liver and/or intestine and appear to have a pseudomicellar structure. Cholesterol esters and triglycerides are neutral but maintained in a soluble stable form by their interaction with the more polar apolipoproteins and phospholipids. Unesterified cholesterol is also present and is intermediate in polarity. The outer surface of lipoproteins is composed of the more polar apolipoproteins, unesterified cholesterol and phospholipids, thereby protecting the polar constituents from the aqueous environment.

Chylomicrons

These molecules have been characterised as large and small triglyceride - rich particles secreted by the intestine, and are the major transport form of dietary fat. Digested dietary fat is presented to the intestinal mucosa as monoglycerides and fatty acids which serve as precursors for triglyceride synthesis. The chylomicron molecule is formed by the combination of resynthesised triglycerides with phospholipids (synthesised de novo or formed from reacylation of absorbed lysolecithin), specific apoproteins synthesised during fat absorption or acquired in the lymph (apo B, apo A I, apo A IV, apo C and apo E) and cholesterol. The cholesterol is derived from absorption of sterol from the

TABLE 1

Composition and classification of the major human plasma lipoproteins (Percent of Dry Weight)

	Chylomicrons	VLDL	LDL*	HDL
Density (g/ml)	<1.006	<1.006	1.006-1.063	1.063-1.21
Electrophoretic Mobility	Origin	PRE BETA	BETA	ALPHA
Triglyceride (%)	85-90	50-55	6-10	3-6
Cholesterol Ester (%)	3-4	14-16	35-45	12-18
Cholesterol (%)	2-3	6-8	8-12	2-4
Phospholipid (%)	6-8	16-20	20-25	25-30
Protein (%)	1-2	8-10	18-22	47-52

* LDL is often subclassified: IDL (LDL_1) density 1.006-1.019g/ml and LDL_2 1.019-1.063g/ml.

(From: Simmons LA and Gibson JC (239))

intestinal lumen, de novo mucosal synthesis and from circulating lipoproteins. Chylomicron molecules are then released into the mesenteric lymph and thereafter into the general circulation where they acquire apo E and apo C from HDL (high density lipoprotein). This enriches the chylomicron with apo C II, the co-factor for enzymatic removal of triglyceride.

Very low density lipoproteins (VLDL) : These molecules have a transport function for endogenous triglycerides, principally derived from the liver, although an intestinal derived VLDL is apparent in post-absorptive and fasting states. The predominant hepatic VLDL contains apo B and apo E derived from the liver whilst apo C and to a lesser extent apo A I are acquired in the circulation.

Low density lipoproteins (LDL): These molecules are the major lipoproteins for cholesterol transport to tissues and in general contain about 80% lipid and 20% protein. The more lipid rich class, IDL (intermediate density lipoprotein), is generated through progressive metabolism of the core triglycerides of VLDL. Cholesterol esters and triglycerides are the major lipid components, whilst apo B and to a lesser extent apo C and apo E make up the protein content of IDL. The turnover of apo B in VLDL and LDL is identical which suggests that both VLDL and IDL are quantitatively converted to LDL, although there may be a capacity for independent clearance of VLDL and IDL and for de novo hepatic synthesis of IDL and LDL in certain circumstances. LDL_2 comprises less triglycerides, apo C and apoE than IDL and is

therefore a smaller denser molecule.

High density lipoproteins (HDL): High density lipoprotein is likely to be responsible for reverse cholesterol transport from peripheral sites to the liver and to other sites of degradation and excretion. At present this has not yet been conclusively established in man. HDL is synthesised both in the liver and the intestine in the form of disc shaped particles (nascent HDL). The molecule contains about 50% lipid (predominantly cholesterol) and 50% protein. The major apoproteins apo A I and apo A II are synthesised by both liver and intestine, although predominantly for the HDL found in the mesenteric-lymph, whilst nascent hepatic HDL in some species contains primarily apoE. Compared to plasma HDL both intestinal and hepatic nascent HDL are enriched with phospholipid and unesterified cholesterol. The major phospholipid species is phosphatidylcholine (lecithin) and as a reactant in the enzymatic reaction catalysed by lecithin cholesterol acyl transferase (LCAT), is involved in plasma cholesterol esterification. Apo A I in HDL has been found to activate the LCAT reaction. HDL has been separated into 2 subclasses, HDL_2 (1.063-1.120g/ml) and HDL_3 (1.120-1.210g/ml). HDL_2 accounts for much of the interindividual variance in HDL cholesterol levels, and particles are larger and contain more unesterified cholesterol and triglyceride than HDL_3. In addition the Apo AI/A II ratio and apo C content may be greater in HDL_2 than HDL_3. The Apo C is transferred from HDL to triglyceride rich lipoproteins to control their rate of lipolysis

and possibly hepatic uptake.

L(p)a Lipoprotein: This lipoprotein class contains predominately apo B in addition to albumin and apo L(p)a. It's density (1.05-1.10g/ml) means that it floats partly in the LDL_2 and partly in HDL_2 range. Its function is unknown but its presence has been related to the development of coronary heart disease.

Lp-X lipoprotein: This lipoprotein has a flotation density similar to LDL, and is characterised by a high proportion of unesterified cholesterol and phospholipid and a low protein content of apo B, apo C and albumin. It is found in patients with biliary obstruction and may arise due to incomplete modification of nascent HDL by LCAT.

The Apolipoproteins: These are the lipid-free protein components of plasma lipoproteins, obtained after treatment with organic solvents or detergents. Not all proteins contained within lipoproteins necessarily have a role in lipid transport, as evidenced by serum amyloid A proteins which are acute phase reactants, and are detected bound to plasma HDL. The amphipathic structure of the apoproteins with water soluble and insoluble amino acids situated on different regions permits the apoprotein to act as soluble stable structures. Their distribution varies through the lipoprotein classes (Table 2).

TABLE 2

Apolipoprotein composition of the lipoprotein classes

Apolipoprotein*	Chylomicrons	VLDL	LDL	HDL
AI	<5%	<5%	Trace	>15%
AII	<5%	<5%	Trace	>15%
AIV	<5%	Trace	Absent	> 5%
B	>15%	>15%	90%	< 5%
CI	10%	10%	Trace	< 5%
CII	15%	10%	Trace	< 5%
CIII	15%	50%	Trace	< 5%
D	Unknown	Minor if present	Trace	< 5%
E	5%	>10%	< 5%	40%

* Refers to % of <u>total</u> protein

Apolipoprotein AI: This is the major protein component of HDL. It is composed of a single chain of 243 amino acid residues, and exists in several isoforms. Apo AI is a potent activator of LCAT, the enzyme responsible for cholesterol ester formation. The relative contributions of the hepatic and intestinal sources of apo AI are ill-defined. More than 90% of plasma apo AI is associated with HDL, less than 1% with VLDL, and no more than 10% with the lipoprotein free plasma fraction.

Apolipoprotein AII: This is also a major constituent of human HDL, accounting for about one third of the total protein and 15% of HDL mass. It exists as a dimer of two identical chains of 77 amino acid residues. The specific role of apo A II in lipid transport is unclear.

Minor HDL Apolipoproteins: The designation apo A III or apo D has been applied to a protein which comprises less than 5% of the HDL apoprotein. It was originally though to activate LCAT but this has not been confirmed. Apo A IV is present in the HDL of some species, but is found predominately in the lipoprotein free fraction of plasma. It is synthesised by both liver and intestine and may be cyclically reincorporated into chylomicrons. Other proteins provisionally called apo F and D-2 have been identified in human plasma HDL but their function is still uncertain.

Apolipoprotein B: Apo B is a major constituent of chylomicrons, VLDL and LDL, comprising more than 90% of the protein of LDL. Two different molecular weight structures of apo B are identified: apoB 100 is synthesised primarily in the liver whilst the smaller form apo B 48 originates from the small intestine. Immunological differences between these large and small varieties of apo B probably exist. In addition to a role in chylomicron and VLDL synthesis, apo B is critical for receptor-mediated LDL uptake. L(p)a, VLDL and chylomicrons together account for no more than 10% of plasma apo B in health.

Apolipoprotein C I: Apo C I comprises 10% of VLDL and 2% of HDL protein, binds phospholipid and activates LCAT. It is a single chain of 55 amino acid residues.

Apolipoprotein C II: Apo C II activates lipoprotein lipase, and accounts for 10% of VLDL, 1-2% of HDL_2 and less than 1% of HDL_3 protein. It is a single chain protein of 78 amino acid residues, and isoelectric heterogeneity of apo C II has been noted.

Apolipoprotein C III: This accounts for 50% of the protein in VLDL and 2% of that in HDL. It is a single chain 79 amino acid residue structure, occurring in at least three polymorphic forms according to the number of sialic acid residues at the end of the carbohydrate chain. About 25% of the Apo C III is in VLDL and 60% in HDL. Whilst lipoprotein lipase activity is diminished in vitro it is doubtful whether this is a physiological property of

Apo C III in vivo.

Apolipoprotein E: This is a component primarily of HDL, IDL and LDL, and it comprises 10-20% of VLDL protein. It usually arises as a single chain and extensive heterogeneity has been defined by isoelectric focussing. Its structure is coded for by three codominant alleles (ε2, ε3 and ε4), located on chromosome 19. Six potential phenotypes are therefore described (Apo E2/2, E2/3, E3/3, E3/4, E4/4, E2/4) and differences in sialic acid content may affect the isoelectric detection. A specific hepatic receptor for Apo E which preferentially binds the more basic isoforms (expression of ε3 and ε4 alleles) has been identified, and their recognition may provide a link in the normal conversion of VLDL remnants to LDL. Apo E is synthesised by the liver and levels correlate closely with those of serum triglycerides. Variable expression of the apo E phenotype accounts for up to 10% of interindividual variation in levels of cholesterol, predominately LDL cholesterol, with expression of an ε2 allele associated with low levels, and correspondingly higher levels in those who express the ε4 allele.

Lipoprotein Metabolism

Anabolism: Absorption:- Dietary triglyceride leads to a post-prandial plasma peak 3-5 hours after a meal. The chylomicron triglyceride generated is normally rapidly metabolised within 15 minutes of absorption. If dietary fat constitutes 22-40% of the total calorie load, plasma cholesterol levels are unlikely to be affected, although the quality of dietary fat may exert an effect

on both cholesterol and triglyceride levels. Dietary polysaturated fats have been reported to decrease plasma lipids possibly secondary to altered intestinal lipoprotein degradation. Although plasma cholesterol levels are relatively stable and do not reflect the post-prandial chylomicron tide, dietary cholesterol is absorbed in chylomicrons and may exert sustained effects on cholesterol via regulation of endogenous cholesterol synthesis. Dietary cholesterol is comprised of both free and esterified components, the free cholesterol is absorbed as a mixed micelle with dietary triglyceride, and 50% is re-esterified with endogenous and exogenous fatty acids in the intestinal mucosa and packaged into chylomicrons. Although only 30-50% of ingested cholesterol is absorbed, the cholesterol content of the diet will have a clear bearing on the plasma concentration of cholesterol for the population studied.

Hepatic Synthesis: - The major stimulus to hepatic VLDL synthesis is the demand for triglyceride transport determined by the availability of the precursors FFA and glycerol. In the absorptive state glucose and residual chylomicron triglyceride fatty acids are used for FFA synthesis while in the post-absorptive state the source is mainly adipose tissue.

The secondary post-prandial triglyceride rise 4-6 hours after a meal represents hepatic VLDL triglyceride synthesis, and varies depending on the dietary carbohydrate and fat load. For example institution of a high carbohydrate diet can lead to hypertriglyceridaemia by the following mechanism. Glucose hepatocyte flux is in excess both of energy demands and glycogen storage capacity. Acetyl Co A and dihydroxy acetone phosphate

are then respectively shunted into fatty acid and glycerol synthesis which primes the system for triglyceride synthesis.

Cholesterol synthesis likewise is related to the demand for triglyceride transport, and is initiated from intrahepatic acetate, and limited by the enzyme HMG Co A reductase, which in turn is regulated by dietary cholesterol and the enterohepatic circulation of bile salts. Therefore the rate of hepatic cholesterol synthesis appears to be inversely related to hepatic cholesterol uptake from chylomicrons and intestinal VLDL. Nascent VLDL requires Apo B and also Apo C and Apo E for its secretion from the liver. LDL formation is thought to be predominantly due to catabolism of VLDL in health, although a minor independent synthetic pathway may be present. Nascent HDL is synthesised in hepatocytes and intestinal mucosal cells but regulating factors have not been identified. After the disc-like nascent HDL structures form with apoproteins combining with phospholipid and free cholesterol, extensive structural and compositional modification takes place after secretion. The most important is the esterification of free cholesterol to form cholesterol ester after LCAT activation, leading to a change in the HDL molecule, which becomes spherical in structure. Cholesterol ester is then transfered to VLDL during catabolism. In addition, apoprotein content varies such that nascent HDL is composed predominantly of apo E relative to Apo A and Apo C, whilst plasma HDL is characterised by a predominance of Apo A. This might be relevant to the LCAT reaction which is facilitated by Apo AI.

Catabolism: The initial step in chylomicron and VLDL catabolism is the acquisition of Apo C-II from HDL which catalyses lipoprotein triglyceride lipolysis by lipoprotein lipase. This enzyme is present on the luminal aspects of capillary endothelium and is released into the circulation by heparin. It's activation results in progressive triglyceride depletion via the IDL intermediate. The lipoprotein structure is maintained by simultaneous removal of phospholipid, unesterified cholesterol and Apo C peptides from the lipoprotein surface to HDL, whilst reciprocal transfer of cholesterol from HDL takes place. VLDL in man is the main determinant of LDL levels, and normally a constant amount of Apo B is maintained per particle, demonstrating that Apo B is a fundamental structural unit of chylomicrons, VLDL, IDL and LDL. LDL circulates as the source of cholesterol for tissue membrane synthesis, and its catabolism is dependent on the expression by the cell of a specific high affinity LDL receptor. Lysosomal uptake is followed by hydrolysis of the LDL protein and cholesterol ester. Free cholesterol is then available for intracellular metabolism. Intracellular cholesterol is involved in a negative feedback process controlling LDL receptor expression: the molecule regulates cholesterol synthesis via its effects on the enzyme HMG-Co A reductase, and in excess leads to ACAT (acyl-CoA transferase) activation, resulting in intracellular cholesterol ester storage. The LDL receptor is in addition involved in specific uptake of Apo E containing lipoproteins as well as Apo B.

The role of HDL in 'reverse cholesterol transport', whereby

it mediates cholesterol removal to degradative and secretory sites, remains speculative in man. However, the kidney is likely to be an important site of HDL catabolism. Apoproteins AI and AII have been demonstrated in the urine of healthy subjects (240) and rat proximal convoluted tubules have been found to degrade Apo A I entering the urine space (241). In addition, of course, cholesterol is excreted and degraded via the hepatobiliary system as faecal sterols, and bile acids from the same source are also critical in the phase of fat absorption.

LIPID METABOLISM IN INSULIN DEPENDENT DIABETES MELLITUS

Disturbed lipid metabolism in insulin-dependent diabetes was probably first recognised by Frederick Allen in 1916 (242). Before the discovery of insulin, it was he who championed a low fat intake to retard the development of ketoacidosis (242). In the 1930's with the wider use of insulin, high fat, low carbohydrate diets were in vogue, and diabetic hyperlipidaemia was quantified and found to be predominatly due to hypertriglyceridaemia (243) and inadequate metabolic control. The increasing use of the liberal carbohydrate intake with correspondingly less fat intake practiced till the early 1970's has been found to be associated with an increased prevalence of macrovascular complications and serum triglyceride concentrations, whilst there was little change noted in microvascular disease and levels of serum cholesterol (244). Since fibre rich, high polyunsaturated: saturated fat ratio diets were advocated for insulin dependent diabetes, further changes in the patterns of lipids and vascular disease are likely to have developed.

Many other factors are also involved in the disturbances of lipid metabolism in IDDM. It is also now clear that lipid metabolism is appreciably different in NIDDM and IDDM. This is due in part to differences in absolute and relative degrees of peripheral and portal insulinaemia and insulin resistance in the two conditions. By the same principle the extent of insulin repletion in IDDM clearly has an important bearing on the pattern of lipid abnormalities. Unfortunately many earlier studies with potentially relevant findings in IDDM tended to merge all

diabetics irrespective of their mode of treatment. However it is still quite feasable that these data as well as that obtained from studies purely on patients with NIDDM could have some bearing on lipid metabolism in IDDM.

In the next section I will summarise published work primarily on IDDM but in addition where pertinent, refer to studies of NIDDM alone, NIDDM and IDDM together, or studies on animal models of insulin-dependent diabetes.

1. Abnormalities of Lipoproteins in IDDM

(a) Chylomicrons and intestinal VLDL

Virtually no studies have been carried out to examine chylomicron or intestinal VLDL synthesis and turnover in IDDM. However the rare chylomicronaemia syndrome associated with massive hypertriglyceridaemia is frequently associated with diabetes, usually IDDM (245). Severe insulin deficiency alone does not lead to the disorder and additional familial forms of hypertriglycerdaemia are required for expression of the condition (246).

Animal models of insulin-dependent diabetes have demonstrated both elevated intestinal cholesterol synthesis and fasting triglyceride production (247, 248), which may provide part of the explanation for the increased whole body cholesterol synthetic rate observed in human insulin-dependent diabetes mellitus (249). In addition exogenous triglyceride clearance assessed by the intralipid tolerance test has been reported to be reduced in untreated IDDM (250), although this was not comfirmed by a later report which suggested that removal of exogenous fat depended

solely on the pre-existing fasting triglyceride level (251).

(b) Hepatic very low density lipoprotein (VLDL)

Elevated levels of VLDL triglyceride and cholesterol accompany diabetic ketoacidosis (252), although if IDDM patients receive adequate insulin repletion VLDL concentration is often normal (253). VLDL triglyceride in particular correlates closely with the degree of glycaemic control in IDDM (253a, 254). A decreased fractional catabolic rate of endogenous VLDL is found in poorly controlled IDDM (251, 255). This has been attributed to the lipoprotein lipase deficiency which accompanies insulin deficiency (256, 257), although the increased VLDL production which accompanies moderate insulin deficiency due to increased fatty acid mobilisation also contributes to the hypertriglyceridaemia (255, 258). However, in normotriglyceridaemic IDDM patients with stable but suboptimal glycaemic control on conventional insulin therapy, VLDL triglyceride production and catabolism rates were found to be normal (259). Intensified insulin therapy by either CSII (259) or the artifical B cell (Biostator) (260), even led to subnormal VLDL triglyceride production rates and to a lesser extent in the fractional catabolic rate (FCR). A fall in VLDL cholesterol also accompanies improved glycaemic control with CSII in IDDM (259). Conformational changes in VLDL are a feature of NIDDM (256), but it is likely that in IDDM there is also lipid enrichment relative to the protein content of VLDL, in addition to the increased VLDL cholesterol: triglyceride ratio previously reported (259). This latter finding might be due to accumulation of VLDL remnants

(intermediate density lipoproteins) in IDDM. Finally, it has been suggested that the lipid metabolism in IDDM may vary with the route of insulin delivery and one unconfirmed report has suggested that intraperitoneal insulin leads to increased VLDL triglyceride levels despite lowering blood glucose (261).

The apolipoproteins CI, CII and CIII are found predominantly in VLDL and to a lesser extent in HDL. Apo C III and CII inhibit and activate lipoprotein lipase respectively, and marginal elevations of both are described in IDDM, with a close correlation with levels of serum triglycerides (262). It is uncertain whether there are functional consequences of glycosylation of Apo CI, described in hyperglycaemic diabetics (263). Apo E is likewise glycosylated in diabetes (263). Although levels are reported to be higher in non-insulin- dependent diabetes mellitus (264), levels of Apo E relative to Apo B were no different, and ApoE was reduced in proportion to VLDL triglyceride in another report (265). Such studies are not available in IDDM, although there is preliminary evidence of reduced levels of Apo E following CSII therapy (266) and reduced levels are found in insulin-dependent diabetic rats (267). Phenotypic variation in ApoE in IDDM has still to be investigated, although in Japanese non-insulin-dependent diabetics the phenotypic frequency was similar to that of non-diabetics (268). However Eto et al (268) did find that the E2/E3 phenotype was the commonest finding in hyperlipidaemic diabetics and non-diabetics.

(c) Low Density Lipoprotein (LDL)

Low density lipoprotein measurements in IDDM reflect

lipoproteins within the density of both LDL_1 (IDL) (1.006-1.019g/ml) and LDL_2 (1.019-1.063g/ml). As yet it is not clear if there are alterations in both these subclasses, although IDL may be increased in IDDM (269). A methodological problem is that in virtually all large studies, the value of LDL cholesterol is calculated indirectly by the Friedewald formula (270), where LDL cholesterol = Total cholesterol - (Total triglycerides/2.2 + HDL). However to my knowledge this ratio has not yet been validated in IDDM.

LDL cholesterol concentrations have been found to vary with the extent of hyperglycaemia, showing a correlation with HbA_1, ranging from r=0.17 (271) to r=0.35 (253a), with closer matching reported in IDDM subjects with HbA_1 levels greater than 13% (253a, 254). In addition improved glycaemic control has led to significant falls in LDL cholesterol in most studies of poorly controlled IDDM treated by CSII, (48, 50-52) and on occasion to levels less than healthy controls (272). However the relatively poor correlation between HbA_1 and LDL cholesterol and the observation from several studies demonstrating similar LDL cholesterol levels in IDDM compared to healthy age and weight matched controls (253), confirms that quantitative alterations are not inevitable in IDDM.

LDL triglyceride levels have been found to be higher than in healthy controls and to be proportional to the degree of hyperglycaemia in IDDM (253). Apo B is the major apoprotein of LDL and has been demonstrated to be a stronger positive risk factor for coronary heart disease in non-diabetics than LDL cholesterol (273). Levels of apoB in IDDM are also related to

glycaemia with a strong correlation (r=0.57) reported with HbA_1 in one study (54), and significant falls in levels accompanying improved glycaemic control (47, 54). However cross sectional comparison of white males with and without IDDM, matched for age, has suggested a quite clear reduction of apoB levels in the IDDM group (54), although no difference was reported in either males or females in another study (274). Thus it would appear that whilst metabolic control may affect levels of LDL, residual qualitative differences could be a feature of IDDM. Both cholesterol and triglyceride saturation of LDL relative to the protein content have been reported in IDDM (54, 56, 275).

Very little work on LDL turnover has been performed in IDDM, but the fractional catabolic rate has been reported to be similar to non-diabetic controls irrespective of the mode of insulin delivery, although improved glycaemic control did lead to decreased LDL apo B production rates to levels less than healthy controls (272).

Studies of LDL metabolism in IDDM have otherwise been confined to predominantly in vitro experiments of LDL receptor activity and have tried to elucidate the cause of the previously described abnormalities of LDL. Although LDL receptor activity in cultured human skin fibroblasts has been shown to be insulin sensitive and LDL degradation sensitive to insulin administration in vivo (276, 277), intrinsic abnormalities in diabetic LDL receptors in vitro were not apparent in one elegant study (278). The demonstration that LDL could be glycosylated in vitro (279) raised the possibility that post-translational modification of the LDL molecule secondary to hyperglycaemia could affect it's

function, and glycosylated LDL has been reported to be internalised and degraded by the LDL receptor to a lesser degree (275, 279, 280) in addition to being a less effective stimulator of cholesterol ester synthesis by cultured fibroblasts (279). The inhibition of LDL catabolism by glycosylation may lead to increased LDL levels in plasma. As expected, therefore, improved metabolic control leads not only to reduced levels of glycosylated LDL, but also to a fall in the triglyceride content of LDL which may in addition enhance LDL catabolism (275). LDL glycosylation has been shown to be closely correlated with HbA_1 (281), which suggests that it is in a dynamic state whereby poor metabolic control in addition to modifying the LDL structure may also affect LDL function. A further intriguing feature of LDL in diabetes is the observation of specific autoantibodies to glycosylated LDL developing in human diabetic subjects (282). The consequent development of LDL immune complexes may prove to be relevant to the development of diabetic vascular complications.

(d) High Density Lipoproteins (HDL)

As with LDL, methodological considerations have been cited to account in part for the disparity in HDL levels reported in IDDM. When comparisons were made, higher levels of cholesterol were measured in the serum high density lipoprotein (HDL) fraction isolated by heparin-manganese chloride precipitation in comparison to ultra-centrifugation, which in turn measured higher levels of HDL than did sodium phosphotungstate-magnesium chloride precipitation (283). It has been suggested that

ultracentrifugation derived HDL in addition isolates Lp(a), present in the density range 1.055-1.085g/ml, whilst the presence of positively charged groups such as on apolipoprotein E, which has disulphide links with apo A II, may contribute to higher HDL levels using the heparin-manganese chloride precipitation method (283).

HDL levels are higher in women than men and may also be increased by alcohol ingestion (284) and training (285), and reduced by smoking (286). There is controversy as to whether ageing is associated with reduced HDL levels. A direct correlation with age and HDL cholesterol has been reported in females with or without (287) and in men with IDDM (288) but this is not apparent in other reports (289, 290). Age related increases in Apo A I are also reported in non diabetics (291). However, body mass does not appear to exert any influence on HDL cholesterol in insulin treated diabetics (292, 293).

Despite such uncertainty there appears to be a general consensus that HDL cholesterol and triglyceride levels are low in poorly controlled hyperglycaemic insulin dependent- diabetics (253a, 254, 256, 289). Otherwise HDL cholesterol and triglyceride levels increase in parallel with improved glycaemic control (254, 256), and HDL tends to be similar (294, 295), or even higher (287, 288, 292, 293, 296) than in non-diabetics matched for age, sex and weight.

Significant increases in HDL cholesterol in patients with suboptimal control of IDDM treated with CSII are well established observations, but were not apparent till at least 8 weeks in most studies (46-48, 51, 52, 54). In a study reporting significant

increases in HDL cholesterol after 2 weeks of intensified conventional insulin therapy, the patients had acute metabolic decompensation (47) thereby accounting for lower than expected baseline values of HDL cholesterol. The complexities of HDL metabolism are such that to date no studies have set out to determine the exact nature of the abnormalities in IDDM. Although improved glycaemic control in individuals has been associated with rising levels of HDL cholesterol, there is with one exception (294) a poor correlation between levels of HDL cholesterol and glucose and HbA_1 in most studies (262, 283, 290, 292, 293, 295, 296), although multivariate analysis has demonstrated an independent relationship between HbA_1 and HDL cholesterol (288). One explanation is the role of VLDL and insulin in HDL metabolism. An inverse relationship between VLDL and HDL cholesterol has been reported (253, 287, 292) and may reflect transfer of esterified cholesterol and triglyceride between these molecules in IDDM. In addition the catabolic pathway which leads to the formation of HDL is dependent on cholesterol enhancement of nascent HDL, which in turn reflects lipoprotein lipase activity. Tissue lipoprotein lipase is activated by insulin and there inevitably is relative peripheral hyperinsulinaemia in well controlled IDDM which would of course activate lipoprotein lipase. For these reasons a relationship between glycaemia and HDL cholesterol in IDDM is apparent although not causal in well controlled insulin replete IDDM with reduced, often low levels of VLDL triglyceride. One further hypothesis is that the state of insulin resistance in IDDM leads to enhanced hepatic output of nascent HDL ultimately leading to

high levels of cholesterol replete HDL (293).

The apparent increase in HDL cholesterol in stable IDDM is contrary to a naive concept that as a 'cardioprotective lipoprotein', HDL levels should low in a population where atherosclerosis is accelerated. This has led to several studies where conformational changes in HDL have been sought. Triglyceride enrichment of HDL in IDDM has been reported (293) although this was not confirmed in another report (292). HDL phospholipid content appears to parallel the cholesterol saturation of the molecule (287). The apoproteins AI and AII have been measured in IDDM with conflicting results. Levels of 'apolipoprotein A' in HDL are no different between IDDM and healthy controls when antisera were used which recognised both apo AI and apo AII (289). More specific analyses have suggested that apo AI levels are increased in IDDM, and an elevated apo AI/AII ratio is described (274, 295, 297). In one of these reports, it was concluded that this reflected an increase in the HDL_2/HDL_3 cholesterol subfraction ratio (295) although this was based on a close correlation between Apo A I and AII in only 4 individuals. Other reports have shown precisely the opposite finding with reduced levels of apoA I and reduced or normal apo AII levels in IDDM (262, 274) although that glycaemic control was poorer in these latter studies. A strong positive correlation between total daily insulin dose and both apoAI and apo AII has been reported, and may help to explain these disparities (274).

The distribution of cholesterol in the HDL_2 and the smaller HDL_3 subfractions may prove to be the most relevant to a pathophysiological role for HDL in atherosclerosis in IDDM, since

HDL_2 cholesterol in non-diabetics is thought to be the fraction involved in cardioprotection. There is disagreement as to whether the HDL_2 (287, 293, 298) or the HDL_3 (299, 300) cholesterol subfraction contributes most to the high HDL cholesterol of IDDM. As HDL_3 is the precursor of HDL_2, a rise in the former might reflect enhanced catabolism prior to its conversion to HDL_2. Support for this hypothesis comes from observations that glycosylation of apoAI and apo AII has been demonstrated in vivo (263) and that catabolism of glycosylated HDL is increased at least in animal models (181). HDL_3 has also been reported to be enriched in triglyceride with reduced particle size in poorly controlled IDDM (256).

The close correlation between post-heparin lipoprotein lipase activity and HDL cholesterol (primarily HDL_2) and phospholipids, and an inverse relationship between HDL and hepatic lipase activity in well controlled IDDM has suggested that exogenous insulin administration may have a key role in determining HDL levels (298). However this hypothesis has been challenged by a report where despite no difference in exogenous insulin dosage, the highest levels of HDL and HDL_2 cholesterol were observed in IDDM without residual endogenous insulin secretion, suggesting that peripheral hyperinsulinaemia leading to enzyme activation and HDL production was not the cause of elevated HDL in IDDM (293).

(e) Lp (a) Lipoprotein

In non-diabetics, this cholesterol-rich, apoprotein B containing lipoprotein is found within the density distribution

of HDL, and high concentrations have been correlated with coronary heart disease (301). In the only study of Lp(a) in IDDM to date, although mean and median values did not differ significantly from controls, elevated levels higher than 20mg/dL were found in 14% of a group of diabetics without retinopathy or nephropathy compared to 5% of controls. Levels did not appear to be influenced by gender, age or glycaemic control (274).

2 Enzymatic regulation of Lipid Metabolism in IDDM

(a) Lipoprotein Lipase

This enzyme is present in a variety of tissues, plays a major role in peripheral removal of plasma triglyceride in man, and it's activity is said to be regulated by insulin. Tissue activity can be indirectly estimated by measuring the post-heparin lipolytic activity (PHLA), a measure of triglyceride hydrolysis after a small dose of intravenous heparin. Levels of PHLA may be low in some uncontrolled hypertriglyceridaemic insulin-dependent diabetics (302-303) although normal PHLA is frequently a finding in such patients (304). Chronic stable IDDM patients tend to have high levels of PHLA (303). PHLA also reflects lipolytic activity from sources other than adipose tissue, including the liver (305), and this may explain why this supposedly insulin-sensitive enzyme activity is not always abnormal in untreated IDDM. However liproprotein lipase activity in adipose tissue and skeletal muscle biopsies is uniformly low in insulin-deficient IDDM, and levels increase in response to insulin treatment (306, 307). Hepatic lipase is also an insulin-sensitive enzyme. Activity has not been found to be

altered in ketoacidotic or stable longstanding insulin-dependent diabetes, although it is elevated in hyperlipidaemic non-insulin dependent diabetes (303). A preliminary observation of reduced activity in well controlled IDDM and an association with a higher HDL_2/HDL_3 ratio (298) suggests that it may be involved in regulation of HDL as well as VLDL.

(b) Lecithin: cholesterol Acyltransferase (LCAT) and cholesterol ester transfer

LCAT catalyses the transfer of an unsaturated fatty acid residue from lecithin to cholesterol with the formation of cholesterol ester and lysolecithin. HDL lipids are the preferred substrate, and this might reflect the capacity of apolipoprotein AI in HDL to activate LCAT. LCAT may have a role in the catabolism of VLDL in addition to structural modification of HDL. In suboptimally controlled IDDM, although the fractional rate of cholesterol esterification was not different, significantly higher mean free cholesterol concentration and mean LCAT activity has been reported in one study when compared to healthy controls (308), although LCAT activity was found to be unaltered in another report in IDDM patients with virtually identical glycaemic control (274) and reduced in diabetic ketoacidosis (309). LCAT activity positively correlated with plasma free cholesterol but not with gender, age, body mass index or glycaemic control (274, 308). The discrepancy with regard to LCAT activity reflects the different methodology employed, but perhaps the report by Mattock et al (308) is most valid in that it gives a measure of the combined effects of enzyme

concentration and substrate characterised in the assayed plasma and may therefore be a more physiological approach to assessing LCAT activity in vivo. The increased plasma phospholipid levels reported in diabetics may enhance LCAT activity to account for these findings (308).

In non-insulin dependent diabetes an increase in the free cholesterol content of VLDL and LDL relative to cholesterol ester and phospholipid has also been reported and may be due to inhibition of cholesterol ester transfer (310, 311). If the same abnormality is present in IDDM then the increased LCAT activity is presumably a secondary compensatory phenomenon to try to catabolise the free cholesterol rich VLDL.

3. Additional factors which exert an influence on lipid metabolism in IDDM

i) Diet: Frederick Allen published his work 'Total dietary regulation in the treatment of Diabetes' in 1919 (242); he recognised that diabetes affected lipid and protein as well as carbohydrate metabolism and suggested that all 3 components of the diet overburdened the system. His radical solution of near-starvation helped to prolong life, in some cases till insulin therapy was available. Although this ruthless regime is now clearly seen as ill conceived in its approach, quantitative and qualitative dietary modification of carbohydrate and fat intake is now widely practised. Indeed, a reciprocal relationship was noted between plasma cholesterol and carbohydrate intake in diabetes as early as 1930 (312).

Consumption of a diet high in polyunsaturated fats (the 'corn-oil' diet) was reported to reduce serum lipids in young diabetics as early as 1955 (313). However longer term follow-up of patients treated in this way demonstrated the development of 'hyperbetalipoproteinaemia', effectively an increase in serum cholesterol levels. These unexpected findings were attributed to an increase in dietary carbohydrate with respect to protein, despite a continued high intake of polyunsaturated fat (314). The observation that a high refined carbohydrate intake can lead to hypertriglyceridaemia had previously been made in normal subjects (315, 316) and has recently been confirmed (317). Whilst this supports the concept that glucose is utilised by the liver as a substrate for VLDL production, such findings are not apparent in studies of diabetes, possibly due to the substitution of sugar by fibre (318, 319). A reduction in HDL cholesterol is also reported to accompany high carbohydrate, low fat diets (318, 320). Therefore it would appear that dietary balance of carbohydrate and lipid is essential to achieve the optimum lipid profile in IDDM. The British Diabetic Association currently recommends an avoidance of saturated fat and its replacement by fibre-rich carbohydrate and polyunsaturated fat containing foods. The relative proportions of the dietary calorie intake suggested are total protein intake 15%, fat 35% and carbohydrate 50% (321).

When such an approach has been put to the test, the effects on lipids and lipoproteins have been rather variable. High fibre diets in diabetics have not led to increases in triglycerides although a reduction in HDL is reported, but concomitant changes in the cholestrol content and the polyunsaturated:saturated (P:S)

fat ratio has made it difficult to assess the independent role of dietary carbohydrate in IDDM (320, 322). Taskinen et al (319) reported that total, VLDL, LDL and HDL triglyceride levels were unchanged, although the HDL_2 triglyceride content increased, in response to a diet rich in fibre (55g/day), with a P:S ratio of 0.4 composed of 60% carbohydrate and 20% fat. Significant reductions in total, LDL and HDL (solely HDL_3) cholesterol were apparent by 2 weeks whilst hospitalised on a metabolic ward, but these alterations were reversed 4 weeks after returning home, suggesting that compliance to such dietary restrictions is poor. In addition adipose tissue lipoprotein lipase activity did not alter during the study, nor did levels of apolipoprotein A I or II in either the HDL_2 or HDL_3 subfractions, although a presumptive transfer of the apo Cs from HDL_3 to HDL_2 developed, as judged by a rise in total protein content in HDL_2 after the diet.

In a report on 6 female insulin-dependent diabetics treated with a more cholesterol restricted, higher P:S fat ratio diet, significant reductions in total, LDL and HDL cholesterol and in levels of apolipoproteins AI, B and CIII were observed (323). Levels of LDL and HDL triglyceride and of the apolipoproteins AII and E were unchanged. However both total and VLDL triglycerides and VLDL cholesterol levels rose significantly (323).

In addition such restricted diets are most unlikely to be palatable enough for routine use and it has even been suggested that a more modest high carbohydrate/high fibre/low fat diet may lead to a deterioration in glycaemic control (324).

More recent alternative dietary strategies in non-diabetics

have employed the use of diets rich in fish oil containing eicosapentaenoic acid (EPA 20:5W3), or commercially available fish oil supplements (Maxepa) to reduce VLDL triglyceride synthesis and also to modulate haemostatic and platelet function. However in a study of normotriglyceridaemic insulin dependent diabetes, 15gm/day of Maxepa led to a significant increase in levels of total and LDL cholesterol and fibrinogen (325) which would outweigh any additional triglyceride lowering effect in hypertriglyceridaemic IDDM.

(ii) Renal Function Diabetic nephropathy characterised by persistent proteinuria and a consequent decline in renal function, ultimately affects up to 45% of patients with IDDM (326). Renal failure and the nephrotic syndrome complicating IDDM can independently lead to disordered lipid metabolism (327-328), a fact often neglected in discussions of lipid metabolism in IDDM.

Previous reports have demonstrated significant elevations of serum triglycerides, total and LDL cholesterol and apolipoprotein B, and reductions in levels of HDL cholesterol and the ratio of apolipoprotein A/apolipoprotein B in IDDM complicated by albuminuria greater than 150µg/minute (329- 331). Progression to renal failure is associated with a more marked reduction in HDL cholesterol levels (330). Abnormalities of lipoproteins may even be apparent prior to the development of proteinuria, and subjects with IDDM and subclinical (incipient) nephropathy (albuminuria excretion rate 20-150µg/minute) have been found to have higher concentrations of apolipoprotein B and a reduced apoA/apoB ratio compared to those with normal

albuminuria excretion rate (less than 20µg/minute) (331).

iii) Genetic: Both diabetes and hyperlipidaemia are heterogeneous conditions and do not segregate according to simple Mendelian principles, but polygenic inheritance of both conditions is a possibility (332).

The insulin gene is located on chromosome 11, and a highly polymorphic locus flanking it has been identified and associated independently with hypertriglyceridaemia (333) and atherosclerosis (334) in NIDDM. Whether such relationships are also apparent in IDDM is unknown, although it is of interest that the genes for the apolipoproteins AI and CIII are also located on chromosome 11 (332), but an uncommon apoA I polymorphism was not found with increased frequency in one study of hypertriglyceridaemic non-insulin dependent diabetic and non-diabetic individuals (335).

An alternative approach designed to examine whether individuals with inherited hypertriglyceridaemia (type IV and V hyperlipidaemia) and diabetes mellitus developed familial diabetes mellitus as a genetic syndrome, failed to demonstrate a dependent inheritance of the two conditions (336). In a survey of 157 insulin-dependent diabetic children, hyperlipidaemia was designated as genetic in source in only 3 of the 14 subjects in whom it was identified (337). However the lack of correlation between measures of glycaemic control and the elevated total serum cholesterol levels in this study raises the possibility of more subtle genetic influences determining lipid levels in IDDM.

It is of interest that in addition to coding for the C3 complement and apolipoprotein E phenotypes (332), chromosome 19 also contains a gene sequence for the insulin receptor (338). Variants of the C3 complement phenotype are associated with familial hypercholesterolaemia (332) whilst structural modification of apolipoprotein E as a consequence of expression of E2/E2 homozygosity confers susceptability to the development of type III hyperlipoproteinaemia (339), which in turn is often associated with glucose intolerance (340). Whilst linkage disequilibrium of the insulin receptor gene and the apoE and/or C3 complement genes is unlikely owing to the location of these genes on chromosome 19, there is still the exciting possibility of a combined genetic defect leading to expression of IDDM and hyperlipidaemia in a homogeneous subset of individuals.

(iv) Demographic variables: Levels of total and low density lipoprotein plasma cholesterol and triglycerides have been found to increase with age both in diabetic (341) and in healthy subjects (342).

Gender has been confirmed to have a bearing on lipoprotein levels in IDDM, and females have been found to have consistently higher levels of total VLDL and LDL triglycerides and total and LDL cholesterol, with correspondingly lower levels of total HDL and HDL_2 cholesterol, in comparison to males with IDDM of similar age and body mass index (287, 343). Similar differences were not apparent between non-diabetic men and women (343).

Diurnal variations in levels of total, HDL and LDL plasma cholesterol (344) have been reported in both normals and insulin-

dependent diabetes but this finding remains unconfirmed, although a post-prandial triglyceride rise is clearly recognised in health and in diabetes (345), in whom subtle abnormalities may prove to be relevant to the pathogenesis of atherosclerosis.

(v) Pharmacological: The management of hyperlipidaemia in IDDM is not stated to differ from that in non-diabetics, although to my knowledge very few if any studies have demonstrated the efficacy of ion-exchange resins, fibric acid derivatives or drugs such as nicotinic acid or probucol in hyperlipidaemia associated with IDDM.

Perhaps more pertinent in practical terms is the potential for agents commonly used in IDDM for associated problems, such as hypertension,to modify lipid metabolism. For example β blockers have been clearly demonstrated to increase plasma triglycerides and VLDL cholesterol, whilst reducing HDL cholesterol levels in normolipidaemic individuals (346, 347), and alpha-adrenergic blockers such as prazosin and doxazosin lead to reductions in triglyceride and cholesterol concentrations (348, 349) in non-diabetic hypertensive patients. In a randomised double blind study of hypertensive insulin-dependent hypertensive diabetics, oxyprenolol surprisingly did not lead to alterations in triglycerides, cholesterol or HDL, although free fatty acids levels fell (350). In distinction methyldopa treatment led to significant increases in levels of triglycerides and reductions in free fatty acids, without affecting HDL or total cholesterol (350).

Other agents have been employed to see whether they had a

specific role to play in treatment of diabetic hyperlipidaemia. Whilst ascorbic acid was not found to modify hyperlipidaemia in a double-blind cross-over study in IDDM and NIDDM (351), an independent role for chlorpropamide in lowering total, LDL, HDL and HDL_3 cholesterol has been observed in IDDM (352). In contrast, despite lowering triglyceride levels, cholesterol levels rose following treatment of IDDM with MaxEpa (325).

1:3 PROTEIN METABOLISM

IDDM is a disorder of protein metabolism, characterised by protein intolerance and the consequence of reduced synthesis of, and altered sensitivity to insulin, which acts as a major anabolic hormone. Although defects of protein metabolism are often reported in associated with hyperglycaemia, it is likely that they both reflect deficient insulin action and therefore are only indirectly linked with one another.

Amino acids, as the substrates for protein synthesis, are often present in abnormal amounts in IDDM. Insulin deficiency is associated with accelerated amino acid oxidation, decreased uptake of exogenous amino acids and repletion of nitrogen in muscle (353). Hyperglycaemia is associated with decreased serum levels of alanine, attributed to a reduction in pyruvate derived from glucose, which in turn usually leads to alanine synthesis. In contrast, the branched-chain (exogenous) amino acids derived from the diet - valine, leucine and isoleucine - are found in increased amounts, probably the consequence of diminished peripheral metabolism as opposed to reduced hepatic release (353). In addition, amino acids serve as precursors for gluconegenesis in IDDM after high protein intake, which further contributes to the hyperglycaemia. Therefore it is not surprising to find that CSII and improved glycaemic control, whilst not associated with significant alterations in serum alanine levels (42, 50), can lead to significant reductions in both fasting and post-prandial levels of the branched chain amino acids (43, 44).

It has also been established in normal subjects and in diabetics with and without established nephropathy that diets low in protein content have the capacity to alter renal haemodynamics and reduce urinary albumin excretion (354-356). However, it could be argued whether quantitation of urinary albumin excretion provides a useful measure of the efficency of protein metabolism in IDDM, as it is undoubtedly sensitive to other factors. Both systolic and diastolic hypertension (357, 358) as well as exercise (359, 360) and the infusion of dibasic amino acids (361) have been found to influence AER. Whilst genetic factors have not yet been found to be important (362), there are sound theoretical reasons why other factors, such as coexistent autonomic dysfunction may affect glomerular and tubular handling of albumin (363) (v.i.). The recognition that elevated urinary albumin excretion might serve as a functional marker of underlying morphological damage to the diabetic kidney and predict ultimate clinical nephopathy (364-366), strengthens the concept of albumin excretion rate measurement as a marker of underlying disease rather than reflecting protein metabolism.

However urinary albumin loss can be regarded as a marker of enhanced catabolism secondary to insulin deficiency in the early stages of diabetic ketoacidosis, and there is clear evidence that poor glycaemic control independently increases the AER (357, 358). The use of CSII to improve glycaemic control is also reported by some (24, 31, 35), although not all (25, 32-34) groups to lead to a reduction in AER. More recent studies have demonstrated that infusions of ketone bodies at a low concentration led to a more marked increase in total urinary

protein excretion than did elevated blood glucose levels (367).

2. DETERMINANTS OF METABOLIC CONTROL

For practical purposes, the management of IDDM is aimed at achieving near-normoglycaemia without excessive hypoglycaemia. Several variables have a bearing on the ability of each patient with IDDM to approximate to ideal metabolic control. Those factors which continue to affect outcome of intensive therapeutic programmes are: (a) type and dose of insulin and its mode of delivery (b) balancing of diet and daily activity (c) self-monitoring of metabolic control (d) patient education, motivation and behaviourial characteristics (e) specific clinical (e.g. blindness) or biochemical (e.g. extent of endogenous insulin reserve) features and (f) flexible modification of factors (a) and (b) based on the information from factor (c).

2:1 INSULIN THERAPY

(i) Formulation and species: After the initial clinical use of the 'pancreatic extract' in IDDM, modification by acidification and later crystallation (368) of insulin led to a sterile short acting insulin of bovine or porcine origin, similar to modern regular (soluble) insulin. In 1936 the fish protein protamine, was complexed with zinc and insulin as PZI (protamine zinc insulin) to retard its absorption and a to prolong its action (369). Modification of PZI by combining insulin and protamine in equal quantities led to the introduction of 'Isophane' insulin in 1950, and this in turn was followed by a formulation whereby insulin was crystallised in an acetate buffer and an excess of free zinc to yield slow acting ('lente') insulins. By careful adjustment of pH, insoluble crystalline (ultralente) and

amorphous (semilente) forms were made available (370). Since the 1950's, the use of bacteriacidal preservatives and availability of neutral (nonacidic) preparations lessened the incidence of bacterial or chemical irritation at injection sites. In the early 1970's the use of purification techniques to remove peptide contaminants such as gluagon and somatostatin, resulted in availability of the so- called 'monocomponent' insulins (371). These purified insulins contained less than 10 parts per million (ppm) of proinsulin and were of bovine or porcine origin (371). The antigenicity of animal- derived insulins leads to the production of antibodies which affect bioavailability of circulating insulin. Even now there is uncertainty as to whether the resultant insulin-antibody immune complexes have a potential pathogenic role in microvascular complications (372, 373), or whether such complexes act as a reservoir for the release of insulin, and consequently a smoother peripheral insulinaemic profile (374, 375). A role for insulin antigenicity in antibody-mediated local cutaneous responses may also influence insulin absorption (376, 377).

Animal insulins have been superceded by the introduction in 1980 of human insulin derived from two different sources. The advent of recombinant DNA technology led to genetic manipulation of organisms such as E. Coli to code for insulin A and B chain production which could then be combined chemically (378). An alternative less economical approach has been the production of semi-synthetic human insulin by enzymatic conversion of porcine to human insulin (379). Both synthetic human insulins and native human insulin have identical composition, although the physico-

chemical structure may differ slightly, but the significance of this is not known (379, 380).

Human insulin would appear to be less antigenic and consequently may have different pharmacokinetic characteristics, with a faster absorption and consequently faster onset and shorter duration of action (381). It has recently been suggested that hypoglycaemic awareness may differ between insulin species (382), perhaps due to altered counter-regulatory responses (383), but reduced antigenicity may confer a further potential benefit for human insulins in the management of cases of insulin resistance or allergy developing whilst on animal insulin (384, 385). It should be stressed however that cross- reactivity between animal insulin antibodies and human insulin is well described (386), and persistent local or systemic allergies to human insulin are also recognised (376, 387).

(ii) Absorption: Factors other than species and formulation are known to affect insulin absorption after subcutaneous administration. The injection site and concentration and volume of injected insulin have all been demonstrated in radiolabelled pharmacokinetic studies to affect bioavailability of insulin (388): subcutaneous abdominal administration produces more rapid and predictable absorption than does injection into the upper or lower limbs (389), the latter of which is also more susceptable to variation during exercise (389, 390). Likewise limb massage (391) or local heating from saunas (392) leads to accelerated rates of absorption secondary to increased subcutaneous blood flow, whilst smoking has the opposite effect (393). It has also

been demonstrated that subcutaneous blood flow is disturbed in diabetic microangiopathy which could lead to erratic absorption (394).

(iii) Frequency and mode of delivery: Virtually all insulin-dependent diabetics administer subcutaneous insulin for control on a day to day basis, although this inevitably produces peripheral hyperinsulinaemia with a peripheral to portal insulin gradient. Other routes of administration may offer a selective advantage in specific circumstances, but are impractical for widespread use.

Peripheral intravenous insulin delivery is the preferred route for treatment of diabetic ketoacidosis (395) and the rare syndrome of subcutaneous insulin resistance (396). However there are difficulties with venous access and thrombosis which result with long term use. The short half-life of intravenous insulin means that interruption of the infusion rapidly leads to insulin deficiency, unlike subcutaneous intermediate acting insulin where a depot of insulin is present. In addition studies have suggested that mealtime hyperinsulinaemia is necessary to achieve normoglycaemia with intravenous insulin (397)

Insulin is absorbed more promptly from the intramuscular than the subcutaneous route (398). It is an effective means by which to treat diabetic ketoacidosis (399). Whilst intramuscular infusion may achieve a better metabolic response in individuals with subcutaneous insulin resistance, haematoma formation and fibrosis at the site of the catheter (400) limit application of this route.

Intraperitoneal insulin is an effective way to control IDDM subjects with nephropathy on chronic ambulatory peritoneal dialysis (CAPD) (401). Direct absorption from the peritoneal cavity to the hepatic portal vein produces excellent control of meal-induced hyperglycaemic excursions and less peripheral hyperinsulinaemia (402). Implantable insulin pumps with peritoneal access have also been used successfully (403), but peritonitis is unfortunately still a frequent complication, although the recent introduction of a subcutaneous implantable reservoir allows peritoneal absorption with less risk of infection (404).

Rapid degradation of insulin by gastointestinal proteolytic enzymes would prevent orally administered insulin from being effective. However if this problem could be circumvented it would become an attractive delivery route as all absorbed insulin would enter the portal vein. Entrapment of insulin in liposomal particles may reduce degration (405) but the delay in absorption inherent in all orally administered drugs make it doubtful whether oral insulin could control acute episodes of hyperglycaemia.

Rectal insulin, however, would be free of such limitations and rapid insulin absorption and hypoglycaemic activity has been reported (406), although the frequent administration necessary to maintain normoglycaemia would be likely to be socially unacceptable.

Nasal administration of insulin produces a rapid hypoglycaemic effect but variable absorption (407) and lack of a stable aerosol formulation has inhibited widespread acceptance of

this approach.

The intraportal route of insulin administration would nearly duplicate the normal pancreatic source of insulin and normalisation of glucose recycling has been demonstrated in animal studies (408), although difficulty with access and the risk of hepatic vein thrombosis make this method of delivery unacceptable for practical diabetes care.

Modification of the frequency and duration of subcutaneous insulin delivery has been found to influence glycaemic control. Once daily acting (Lente or Ultralente) insulins are ineffective in achieving glycaemic control in genuinely insulin-dependent diabetes, and most conventional regimes employ a mixture of soluble and isophane insulin administered twice daily, or split the evening dose, covering the main meal with soluble and administering isophane insulin at bed-time to reduce levels of fasting hyperglycaemia. Intensified conventional therapy is effectively a modification of this regime using multiple insulin injections as necessary to lower post-prandial hyperglycaemia. Alternative approaches to achieving near-normoglycaemia are the use of pen devices, where after a nocturnal dose of subcutaneous ultralente insulin to achieve background insulinaemia, injections of soluble insulin can be administered subcutaneously to prevent post-prandial hyperglycaemia, titrating the dose to the anticipated carbohydrate load. This approach more closely mimics the pulsatility of physiological insulin delivery, although as with CSII, which also ensures continuous background insulin with meal-time boluses, peripheral hyperinsulinaemia is required to achieve near-normoglycaemia (409). There is little doubt that

such regimes may be extremely suitable for a small minority of highly motivated individuals who wish more flexability with their insulin regime. However, studies of both hospitalized patients (410, 411) and outpatients (412, 413) have failed to demonstrate a significant advantage of CSII over intensified conventional therapy, and the high incidence of ketoacidosis and acute metabolic decompensation reported with CSII (41), and excessive weight gain reported with intermittent insulin administered by pen devices (414) will inhibit more widespread usage of these systems. Insulin absorption from repeated injections may be enhanced by the use of a sprinkler needle (415), but this innovation has yet to be subjected to careful scrutiny in controlled studies.

2:2 DIET AND EXERCISE

The acknowledgement that glycaemic control is determined in IDDM by a balance of dietary intake, physical activity and insulin therapy, makes it vital to individualise each patient's meal plan, and in particular to ensure dietary compliance by making the diet realistic.

The precise details of meal times will be dependent to some extent on the type of insulin regime, but in general terms the daily food intake should be reasonably consistent with regard to the calorific intake and balance of the major nutrients. This basic programme should have the capacity to be flexible both with regard to insulin dosage, and the quantity and timing of meals, taking account of variation over week-ends, during holidays, or during the menses. It is more relevant on a day to day basis to

ensure that all patients are encouraged to modify their diet at times of increased energy expenditure (v.i.). Extra carbohydrate, or alternatively, reduced preprandial soluble insulin prior to exercise, should ensure freedom from immediate hypoglycaemia, and it has been suggested that an extra 10-15g of carbohydrate should be provided for every 30-45min of increased activity (416). In my own experience, requirements of additional carbohydrate varies from patient to patient and should be tailored to individual needs. However one frequent observation which is often not stressed is the capacity for delayed (often nocturnal) hypoglycaemia, developing up to 12 hours after the exercise. It is uncertain whether this reflects altered insulin absorption in addition to delayed supression of hepatic glucose output, secondary to depletion of glycogen stores (416). Even without extra physical activity, conventional insulin therapy often predisposes diabetics to preprandial hypoglycaemia, particularly before lunch, following administration of soluble and isophane insulin with breakfast. The principals of regular snacks between meals and at bedtime, and minimal delay with main meals, should be reinforced to minimise this problem. In addition the widespread practice of carrying rapidly absorbed refined carbohydrates at all times is to be encouraged. On the other hand, ingestion of rapidly absorbed simple sugars (glucose, sucrose and maltose) should be discouraged at regular meal times, particularly in those insulin-treated diabetics with residual C-peptide secretion, since dietary glucose alone does not normally mediate insulin secretion and hyperglycaemia could result (416). In general, a diet high in fibre and in polyunsaturated fat which

fulfills the requirements for total energy intake and contains an appropriate distribution of nutrients is to be encouraged.

Total energy intake should vary according to age, sex, body mass index and the degree of physical activity. The Exchange Group System devised by the American Diabetes Association (416) or the British Diabetic Association 'Lines' system, ensure that both the caloric load and nutrient distribution of foods are known, which affords patients flexibility with what they consume. However such concepts have recently been challenged, and it now appears that there is variation in the glycaemic response to various food stuffs containing equivalent amounts of carbohydrate (417). This is particularly apparent with fibre-rich carbohydrate where absorption is delayed and the glycaemic response (the 'Glycaemic Index') is suppressed (418). In addition the timing of the carbohydrate intake affects the glycaemic response. This is most apparent in the morning where the insulin dose is greater than that required to dispose of a meal of similar carbohydrate content eaten at lunch-time (419, 420). Diurnal variation in insulin requirements or an extended 'dawn phenomenon' may account for such observations (421).

Although dietary advice to avoid the use of rapidly absorbed sugars is often ignored, alternative sweeteners without calorific value, such as saccharin, have been introduced into diabetic diets. Recent concern over a possible carcinogenic effect of saccharin (422) has led to examination of alternative nutritive sweeteners such as fructose, or the sugar alcohols sorbitol and xylitol. Whilst they may help to improve dietary compliance, xylitol causes caries, and sorbitol can cause a dose dependent

diarrohea (416, 423). Furthermore the use of sorbitol in diabetes must now be regarded as of dubious value and potentially hazardous, with the realization that increased aldose reductase activity in tissues leads to a build up of sorbitol, which has been implicated in the pathogenesis of the renal, neurological and opthalmological complications of IDDM (424). All the aforementioned sweeteners of course have a calorific value which needs to be taken into account. On the other hand, non-carbohydrate sweeteners like aspartame are non-nutritive, non-calorific, and therefore will not affect glycaemic control, but long term safety data is not yet available (416).

The use of alcohol as an integral part of the diet in IDDM has not attracted much attention previously despite the high sugar and calorie content of many alcoholic beverages and the capacity to potentiate and promote hypoglycaemia by suppressing hepatic gluconeogenesis (425). This is particularly relevant to the development of nocturnal hypoglycaemia when misleadingly high blood glucose levels may be present before retiring to bed due to the caloric load from the alcohol, which could inhibit the patient from taking his/her usual bed time snack.

Further considerations to the diet may be relevant in specific cases. There is increasing recognition that a diet high in saturated fat can impair glycaemic control as well as encourage hyperlipidaemia (312), and likewise a diet rich in refined carbohydrate can encourage hypertriglyceridaemia (315-317). However it is the fibre content that has been suggested to be the major dietary factor determining glycaemic control and lipid levels in IDDM (426). Supplements of non-absorbable fibre

rich substances such as pectins or guar gum have been used to reduce post-prandial hyperglycaemia and cholesterol in diabetics with variable results (427-430), and the high incidence of gastrointestinal side effects would be likely to lead to poor compliance in the long term (430). An alternative approach to reducing alimetary lipaemia and glycaemia in IDDM has been the use of alpha-glucosidase inhibitors, which delay digestion of starch and inhibit intestinal disaccharidase activity, but as with the fibre supplements, iatrogenic malabsorption leads to unpleasant flatulence and non-compliance with treatment (431, 432).

The role of dietary protein and salt has received relatively little attention till recently. Although a liberal protein intake was previously recommended, there is increasing evidence, at least in diabetics with incipient nephropathy and glomerular hyperfiltration, that a reduced protein intake may benefit renal dysfunction (354-356) and there are certainly proponents advocating low protein diets in established renal failure in IDDM (433). I have previously reported a marked reduction in urinary protein excretion and blood pressure accompanied by a fall in glomerular hyperfiltration as a consequence of reduced protein intake (100g) in a young hypertensive diabetic body-builder who was consuming over 250g protein/day and performing regular weight training prior to this (434). High protein diets might also encourage post prandial hyperglycaemia by acting as a substrate for hepatic gluconeogenesis (435).

There is now sufficient evidence to justify sodium restriction in the management of hypertension associated with

diabetes (436), and in addition support for the hypothesis that low sodium diets may improve glucose tolerance (437), although more recently this view has been challenged (438).

Paramount in all aspects of dietary management is the recognition that the diet should be sufficiently flexible and easily understood to ensure compliance, which has been found in the past to be poor (439, 440). A high fibre intake appears to be of undoubted benefit but centres who enthusiastically recommend such diets have reported variation in the willingness of diabetics to maintain them for long periods of time (441, 442).

Physical activity has been encouraged for many years to help achieve good metabolic control. However recent acute and longer term metabolic, hormonal and circulatory studies of the response to exercise suggest that not all the consequences are beneficial for everyone with IDDM, and that as with diet, a programme of exercise should be defined on an individual basis to be relevant, realistic and safe.

The normal acute metabolic response to exercise is an increased muscle consumption of fuel and hepatic glucose production. Glucose levels in serum are normally maintained by appropriate glucose utilisation although they may increase by up to 20% in response to strenuous exercise (416). In IDDM the ambient glycaemic control determines the response. If IDDM is poorly controlled (random blood glucose greater than 15mmol/L), patients should be cautioned against severe exercise as worsening of the hyperglycaemia and ketonaemia is well documented in these cases due to exaggerated hepatic glucose output despite

inadequate glucose utilisation (443, 444), and an associated exaggerated increment in counter-regulatory hormones (445). The idea that chronic hyperglycaemia is best treated by strenous exertion is a common misconception amongst diabetics which should be clarified.

Exercise induced hypoglycaemia in stable IDDM is much more common and was first recognised by R.D. Lawrence 60 years ago (446). It is due to a combination of the failure to inhibit endogenous insulin release (if still present) in response to exercise (416), suppressed hepatic glucose production (447), and an increase of up to 135% in the absorption rate of subcutaneous insulin from the leg (448). This problem should be eminently preventable by anticipation of exercise and modification of either carbohydrate intake or the preprandial insulin dosage.

With regular training however, beneficial alterations in metabolism ensue. Reduced insulin secretion in obesity (449), an attenuated counter-regulatory hormone response to exercise (445), impaired insulin sensitivity (450) and increased insulin receptor binding (451) are all described. Intravenous (452) and post-prandial glucose tolerance (453) may improve in NIDDM and IDDM respectively with a possible reduction in insulin requirements (454). However, there is controversy as to whether glycaemic control actually improves. Whilst it has perhaps unsurprisingly been demonstrated in one study (454), this was not apparent in others (450, 455) and was attributed to a compensatory increase in the caloric intake over the period of training (455). Other encouraging alterations reported in IDDM as a result of training are reductions in body weight, blood pressure, serum

triglycerides (450, 455) and basement membrane thickness (456), although the latter improvement could not be independently attributed to exercise. An increase in serum HDL cholesterol levels has also been reported in IDDM with exercise (450). These observations could make regular exercise in IDDM an attractive pursuit in the prevention of atherosclerosis. There are however cogent reasons to be cautious in adopting this policy on a widespread basis in IDDM, particularly in cases where microvascular disease is established. It is known that diabetics perform less well during exercise than age, sex matched non-diabetics of similar body mass. The maximum heart rate and cardiac output is less whilst a higher blood pressure response is observed leading to a reduction of maximum oxygen consumption (456, 457). Asymptomatic coronary heart disease, diabetic cardiomyopathy and autonomic dysfunction may be in part responsible, but metabolic inefficiency characterised by excessive fat utilisation and production of lactate and pyruvate, and deficient glucose utilisation may also have a contributory role. Glycosylation of haemoglobin could also theoretically reduce oxygen delivery to the tissues (458). I have previously demonstrated that exercise tolerance in hypertensive IDDM with underlying coronary heart disease and autonomic dysfunction is associated with an attenuation of the normal blood pressure response although calcium antagonist not only improved the systolic blood pressure gradient but also improved exercise tolerance and ischaemic E.C.G. features in response to exercise on a treadmill (459). Other reports have confirmed that diabetics with microangiopathy may display a greater blood

pressure response in addition to lower maximal exercise ability, although the increment may be attenuated (456, 457). Exercise is also recognised to increase transcapillary albumin escape (458) and transglomerular albumin loss leading to increased urinary protein excretion (360) and there are anecdotal reports of worsening renal function and retinopathy after exercise (458). In the case of the hypertensive body builder with diabetic nephropathy I previously reported (434), weight training led to obvious acute increases in systemic hypertension, urinary protein loss and renal blood flow. Exercise such as jogging may also not be advisable in diabetic peripheral neuropathy, where altered sensation and disturbed foot pressure may promote the development of neuropathic arthropathy.

It has even been proposed on the basis of some of these observations that exercise may be used to provoke renal and haemodynamic functional abnormalities as a sensitive marker of early diabetic microvascular disease (458).

On balance therefore, the triad of exercise, diet and insulin need to be modified in the light of established complications, and a case could be made for dynamic evaluation of the response to exercise in ostensibly healthy IDDM of long duration before recommending regular physical activity.

2:3 SELF-MONITORING OF METABOLIC CONTROL

It could be argued that blood glucose self-monitoring is primarily a tool for assessing metabolic control and not a factor determining it. However since its advent in 1979, a plethora of

publications has also testified to the pivotal role of 'home blood glucose monitoring' in producing improved glycaemic control perhaps by acting as an aid to education and behaviour modification. Its overwhelming acceptance may have neglected to separate out the parallel and powerful role of increased attention from medical personnel in achieving metabolic control, and psychological aspects and the quality control evaluation of patient-generated results of home monitoring require serious attention. In the following section I will review the literature on 'home blood glucose monitoring' and try to put its role in routine clinical management of IDDM into perspective.

It is perhaps surprising to younger medical practitioners that a concept as logical as home blood glucose monitoring took so long to be introduced into clinical practice. The publication of two papers back-to-back in the Lancet in 1978 (460, 461) are often acknowledged to be the first clinical reports of the effectiveness of home blood glucose monitoring in patients' hands, although reflectance meters capable of measuring blood glucose and sufficiently compact to be used in patients' homes were available in the early 1970's. Patient generated samples for home blood glucose analysis had in fact first been described for use in unstable diabetics or in those with abnormal renal glucose thresholds as early as 1962 (462). This early innovation of filter paper impregnation with blood is a patient-independent determination of blood glucose, and as such denies the opportunity of immediate feedback to the individual. However this early concept has recently undergone a renaissance with the realisation that by laboratory based validation, quality control

of patient generated glycaemic data is possible.

All methods of self-monitoring of blood glucose involve collection of a drop of capillary blood obtained by fingerprick using single prong lancets onto a reagent strip impregnated with glucose oxidase, peroxidase and a chromogen reagent. It is important to stress to patients and health care professionals to avoid application of skin cleansing agents prior to blood letting (463). Glucose oxidase promotes the production of gluconic acid and hydrogen peroxide from the interraction of glucose, oxygen and water. The peroxidase catalyses the reaction between hydrogen peroxide and the chromogen to give a colour change in proportion to the concentration of blood glucose. After a set time of exposure (usually 60 seconds), the blood is removed from the strip by either wiping dry with cotton wool, or by washing with a stream of water, depending on the reagent strip used. The blood glucose level is estimated, after at least a further 60 seconds, by visual comparison of the strip with a colour chart, or by insertion into a reflectance meter. This straightforward method works best if the tips of warm digits are used, preferably the thumb and index fingers which have the best blood supply. Pain may be reduced by using pressure on the target digit using an opposing digit to produce counter-irritation. With repeated use finger tips coarsen and pain diminishes. The use of elastic bands to encourage vascular congestion of the finger tip may also promote free flow of blood for sampling. The use of automated spring- operated devices (e.g. Autolet and Autoclix) has been of particular help in removing the need for visually apparent self-assault.

There are several test strips and meter systems currently available and all initial reports validating these methods were performed by trained personnel in a hospital setting.

(i) Reagent Strips

Five different systems have been or are presently in use and can be read visually or by a meter. The 'Dextrostix' and 'Visidex' strips (Ames, (UK) Ltd) require application of whole blood to the reagent pad for exactly 60 seconds prior to removal with a jet of water. After blotting dry, the strip is then read immediately. Visual interpretation of 'Dextrostix' is only semiquantitative and is frequently inaccurate in most peoples' hands (464), although it provides excellent results when used with a reflectance meter (v.i).

On the other hand 'Visidex' is specifically designed for visual interpretation, and uses a two colour block system (one for values less than 9.9mmol/L and one for values above 11.0mmol/L) to ease interpretation. 'Visidex' readings over a wide range of blood glucose values have been reported to correlate fairly closely with laboratory reference readings (r= 0.81-0.97) (465) and in one study with 'BM Stix' (Boehringer, UK) (466) (v.i.). However at low ranges of blood glucose (4-8mmol/L), a poor correlation (r=0.49) with laboratory measurements was reported (467), and most studies have reported a correlation with laboratory estimates inferior to simultaneous 'BM Stix' readings (467-469). The overestimation of glucose values in the normoglycaemic-hyperglycaemic range reflects instability with storage (467-470), and the need for rigid

adherence to timing of the reaction, in addition to possible between-batch variability (465).

'Visidex' has therefore been superceded by the 'Visidex II' which has an additional advantage in not requiring a wash step, although instability with storage has been described (471, 472).

The alternative sticks ('Stat Tek Glucose', 'Stat Tek Glucose Low Level' and 'BM Test 1-44') are all produced by Boehringer, Mannheim (West Germany) and can be used with whole blood, serum or plasma. After application of the sample to the strip, 60 seconds is allowed to elapse before removal with cotton wool. An additional minute should expire before the strip is read, although glucose levels factor greater than 13.3mmol/L require the whole procedure to last at least 3 minutes, to allow completion of the colour reaction and accurate measurement. 'Stat Tek Glucose' sticks cover the range 2.8-19.8mmol/L, and 'Stat Tek Glucose Low Level' stick measures glucose levels of 0.6-8.3mmol/L, but both methods are semi- quantitative and best read with glucose meters, although they are no longer routinely used in the United Kingdom. 'BM Test 1-44' sticks cover the range 1-44mmol/L. They are divided into two colour blocks with different colour spectra thereby easing identification. In some individuals, the strips can be cut in half to lower costs. When used by patients and health professionals in controlled settings, close correlations were reported with the laboratory glucose determinations (r=0.85-0.96), irrespective of the level of glycaemia (466). The presence of impaired colour vision and retinopathy may (473) or may not (474) impair interpretation of results although a poorer performance in older patients has been

described (474).

One further method, 'Glucoscan Test Strips ' (Lifescan, USA) uses a system of blotting the blood twice, 40 seconds after application, and reading the level after a further 20 seconds. This semi-quantitative method also requires a meter for reliable interpretation and is not used at present in the United Kingdom.

The suggestion that visual assessment of reagent strips is not as accurate as meter recorded blood glucose (466) is therefore not universally accepted and remains an open question.

(ii) Reflectance Meters

'Eyetone' (Ames Ltd) was the first reflectance meter to be introduced in 1973, and was designed for use with 'Dextrostix'. It requires an electrical outlet and a warm-up time of 30 minutes. Although its performance characteristics are acceptable for patient use it is bulkier than more recent machines which have superseded it.

'Statek' (also known as the 'Reflomat') (Boehringer Mannheim) was introduced in 1977 and is used with 'Stat Tek Glucose' or 'Glucose Low Level' reagent strips. As with the 'Eyetone', it has been validated for patient use, but it also requires to be connected to a mains and is rather cumbersome.

The 'R.A.H.C. Glucose Tester' (Australian Biotransducer), although lighter and modified for either 'Dextrostix' or 'Stat Tek' strips, requires either main line current or batteries for operation, and is only available in Australia.

More recent models include the 'Dextrometer' (Ames), which requires recalibration before each use and has no built in timer,

and 'Glucocheck' (Lifescan) which although it has a built-in timer and is mains-operated, cannot be recalibrated with repeated use by patients. Both these systems use 'Dextrostix'. The 'Glucoscan' has recently been replaced by 'Glucoscan II' (Lifescan) which uses its own 'Glucoscan' test strips but which also has no capacity for recalibration.

'Hypocount I and II' (Betascan) (Orange Medical) are small portable battery charged meters with automatic calibration and the ability to use either 'Dextrostix', 'Stat Tek' or 'BM 1-44' sticks. They have an advantage over meters with liquid crystal display such as the 'Glucometer' and 'Reflolux' systems (v.i), in that they use a light emitting device to produce a bright red digital readout, which may be more helpful for patients with poor vision, although they are more expensive.

The most widely used machines in the United Kingdom are the 'Glucometer' (Ames) and 'Reflolux' (Boehringer) systems which have recently undergone modification to provide additional facilities, although at an extra cost.

The 'Glucometer' uses 'Dextrostix' and reads glucose values between 0.6-22mmol/L. A liquid crystal display (dull black) digital readout is provided after 60 seconds, and either calibration with plastic chips or solutions (0.6 and 16.5mmol/L) ensure reproducability. The calibration is maintained when the equipment is not in use. Laboratory glucose values have been found to correlate closely with 'Glucometer' readings (r=0.97) in the hands of technicians over the range 2.0-22.2mmol/l (466), and it has been suggested that the precision at lower levels (less than 10mmol/L) (r=0.92) may be superior to the 'Reflolux' system

(r=0.84), although they are comparable at higher glucose levels (466). The major problem with the system relates to batch to batch variations in the reagent strips. Using Error Grid analysis, it has been clearly shown that the 'Glucometer' may fail to detect hypoglycaemia in patients' hands (475).

This latter problem may have been overcome by the introduction of batch-orientated calibration for the 'BM 1-44' sticks to be used with the 'Reflolux' machine, with the use of the bar code strips for external automatic calibration, produced 'en masse' with large batches of strips to ensure reproducability. The batch bar code strip is inserted into a slot on the hinged flap of the 'Reflolux', prior to use. After ensuring that the battery system is functioning by the display of '888' on the liquid crystal display digital readout, a blank reagent strip is then inserted to confirm calibration by display of '000'. After this, application of a drop of blood onto the test slip is followed by pressing the timer button. The display window then counts up till 60 seconds have elapsed at which time a bell rings and the strip should be wiped with cotton-wool. A further 60 seconds should pass before a further bell is heard, at which time the hinged flap holding the strip should be closed to allow immediate digital display of the result. The 'Reflolux' measures blood glucose values between 2.2-22.2mmol/L. Values less than 2.2mmol/L are record as 'LLL', whilst values greater than 22.2 are displayed as 'HHH'. Although a high correlation is described in several laboratory based evaluations over the range 2.2-22.2mmol/l (466, 476, 477), a consistant over-reading of up to 1mmol/L in relation to the laboratory standard method is

reported in 2 studies (476, 477), with the opposite finding in another (466). All 3 studies seemed to indicate inaccuracy at lower levels of blood glucose, but unfortunately the range chosen (2.2-10.0mmol/L) by including normoglycaemic and hypoglycaemic values, may have flattered the ability of the Reflolux to reliably report hypoglyaemic levels (less than 3mmol/L), with a tendency to overvaluation. The suggestion that this is 'not of therapeutic significance' (476) is I think unfair, particularly when put into the context of individuals with hypoglycaemic unawareness where a 'Reflolux' reading of 3.2mmol/L may lull the subject into a false sense of security despite the fact that the laboratory could have estimated the gluose level at 2.3mmol/L. It has been suggested that the 'Glucometer' may be more reliable than the 'Reflolux' in this critical range (466), although this remains unconfirmed, as does the suggestion that visual assessment of 'BM 1-44' strips is less reliable than the 'Reflolux' based recordings (466). One major benefit of the system is that BM 1-44 strips have been shown to be robust enough to be stored and rechecked by meters in clinics to validate patient generated data (471, 478). To my knowledge, no one as yet has reported long term experience with the 'Reflolux' in patients' hands.

Other systems with similar performance characteristics include 'Diatron Easytest' (Eastlands Electronics), 'Glucocheck II' (Medistron Limited), and Hypocount MX (Hypoguard Ltd.) which allows 'BM 1-44' sticks to be cut in half thereby reducing the cost of the item with major recurrent expenditure (477). The major improvements in the 'Hypocount MX', 'Diatron Easytest' and

the new 'Glucometer M' systems, are their size and their ability to store and automatically to update glucose values. This provides the basis for patient-generated data recorded by a patient-independent method to assess the reliability of home blood glucose estimations (478). However the 'Reflolux' system costs fifty nine pounds, which is less than other systems (range sixty five to eighty five pounds), and was found in a laboratory based assessment to be the most precise and accurate (478). Premature battery failure seems to be unfortunately frequent with most meters in current use. The main refinement in the 'Reflolux II' and 'Glucometer II' instruments over their predecessors relates to their size. They perform to a similar degree of precision and accuracy (479-481), although the 'Reflolux II' reads over a wider range (0.5-27.7mmol/L) of blood glucose values. 'Glucometer II' uses specifically designed reagent strips - 'Glucostix', which are self calibrated in a similar way to 'BM 1-44' strips.

A multitude of alternative instruments have recently come onto the market: 'Glucoscot GT 4310' (Kyoto-Daiichi), 'Glucocheck SC' (Medistron), both of which have memories to store readings, and 'Glucoscan 2000' (Lifescan), 'Trendsmeter' (Orange Medical Instruments), 'Diascan' (Home Diagnostics) and 'Glucokey' (Ulster Scientific) which do not have this facility; of these the 'Glucoscan 2000' came up top for precision and accuracy in a recent report, and 'Diascan' and 'Glucokey' were found to be the most unreliable (481).

On balance it would appear that the 'Reflolux' systems are closest to the requirements for an ideal home blood glucose meter

for general use at the present time. They are compact, light, mains independent, contain the minimum of buttons and mobile parts and glucose results compare favourably (r=0.96) with reference methods. However problems with short battery life and variable performance in the hypoglycaemic range raised the important question of how the system operates in patients' hands. Such an investigation forms a part of the thesis to be presented.

Sonksen et al in 1978 (460) reported an uncontrolled study conducted over four years on 53 insulin-treated diabetics, who used the 'Eyetone/Dextrostix' (Ames) system at home for periods of up to 18 months. Patients were lent machines for variable periods, and the following scheme for blood glucose readings suggested: fasting, pre meals and bedtime recordings as routine, with spot checks at other times, particularly when hypoglycaemia was suspected. They reported a high degree of acceptability of meters by patients, accompanied by an improvement in metabolic control assessed by blood glucose results, which was maintained for up to 18 months. Patients reported less frequent hypoglycaemic episodes and increased ease with adjustment of insulin dosage, without significant complications developing. 70% preferred blood testing to urine testing and 92% wanted to purchase a meter although the cost of the system at the time (two hundred pounds) precluded this option for most patients. Most individuals in this report came from social classes I and II, and in addition to the use of the meters, 55% of patients changed insulin species or the number of daily injections, which clearly also had an impact on metabolic control. Finally, no validation of patient generated data was reported.

In another uncontrolled study, Walford et al (461) reported on 67 insulin-dependent diabetics who used the 'Reflomat' meter (Boehringer) for varying periods in a study that lasted one year. Using a group with a wide scatter of intelligence and social class, a 10 point profile was collected whilst working, and during a rest day. Samples were taken before, one and two hours after each main meal, and before bed. Measurements were repeated as necessary after changes in treatment. A close correlation ($r=0.96$) was reported between 'Reflomat' recordings of 20 individuals and 20 simultaneous auto-analyser recordings. Random blood glucose values from the clinic correlated poorly with patient generated fasting ($r=0.37$) and mean morning ($r=0.41$) glycaemic values. A high degree of acceptability was reported and over 50% of individuals had produced daily profiles in which not more than one blood glucose value exceeded 10mmol/L although no attempt was made to validate patient data generated at home.

Outside the United Kingdom, 4 other uncontrolled reports without laboratory validation of data were published, using a smaller selected sample of individuals. Danowski and Sunder (482) reported on 5 individuals on multiple daily insulin injections who used the 'Eyetone' (Ames) system for over a year. They measured their blood glucose 23 times a week (11 point profile once a week comprising pre and post prandial and nocturnal measurements, and fasting and random glucose evaluations on the 6 remaining days). These manoeuvres were accompanied by almost all reported glucose values ranging between 3 and 8mmol/L over the year.

Peterson et al (483) used the same meter system on 10 subjects who measured their blood glucose 6 times daily over a 6 month period, leading to a fall in HbA_1 from 10.3 to 5.4%. Ikeda et al (484) studied 8 subjects with IDDM, reported similar results, and confirmed a close correlation between patient-generated and simultaneous laboratory determinations at the start of the study.

Skyler et al (485) studied 32 subjects of unspecified age range and reported similar improvements in glycaemic control without an alteration in insulin dose. As well as concluding that self blood monitoring acted as an educational tool, this report demonstrated an effective role for self blood glucose monitoring in the confirmation of suspected hypoglycaemia.

These reports were received with great enthusiasm although an editorial in Lancet at the time (486) correctly pointed out the data was derived from small selected groups of motivated individuals in whom other innovations such as more efficient insulin delivery and increased attention from medical personnel would quite reasonably also be expected to have a bearing on the outcome. Indeed in one presumably highly motivated group of individuals - pregnant diabetics - whilst blood glucose monitoring was quite acceptable to them, glycaemic control in the last trimeter of gestation was not necessarily better, in comparison to women who used urinanalysis to monitor their metabolic state (487). One report has suggested that glucose self monitoring itself produced little impact on control in unstable diabetic patients following renal transplant if there was inadequate contact with medical personnel, irrespective of

whether individuals were compliant or not (488), and reservations have been voiced about widespread use of meters in IDDM, with studies yielding comparable results between visual measurement and meter measurement of reagent strips (466, 489, 490).

The effect of home blood glucose monitoring on glycaemic control clearly needed to be separated from other variables. In perhaps the only study of it's kind to date, Worth et al (77) studied 38 insulin-treated diabetics and reported no improvement in glycaemic control when blood glucose monitoring was used as an alternative to urinary glucose monitoring. In view of the suprising conclusion, this study requires careful scrutiny.

In objectively evaluating the role of home blood glucose monitoring on a large scale, Worth et al started by inviting 154 insulin-treated diabetics to participate. The response rate of 35% (54 subjects) suggests a degree of self-selection, and pregnancy or established renal disease (plasma creatinine greater than 200μmol/L) were deliberately chosen as exclusion critera. This study therefore appears to have studied a selected population with insulin-treated diabetes. The run-in period of 6 months, during which time all individuals were reviewed fortnightly whilst performing regular urine glucose analysis, was designed to separate out the effect of increased attention and patient motivation from the role of blood glucose monitoring. Thereafter patients were randomised to 3 sequential 3 month cycles of continued urine testing or alternatively blood testing using either 'Glucocheck' meters (Medistron Ltd) or visual assessment with 'BM 1-44R' reagent strips (Boehringer Ltd). During the run-in period, patients were educated about dietary

and insulin dosage modification as well as receiving instruction in home blood glucose monitoring. It is not clear whether self-generated glycaemic data during this period was 'fed-back' to patients to allow for alterations in their diabetic care regime. They also provided urinanalysis data from pre-prandial and pre-bed samples (4 times daily) using 'Diastix', and were asked to provide a 7 point capillary blood glucose profile in fluoride-oxalate containers and a 24 hour urine collection, one to two days before each fortnightly visit.

Fortnightly measurements of HbA_1 were made, and cholesterol measured after 6 months run-in and at the end of each of the optimisation periods. Endogenous insulin reserve was assessed by measurement of post-prandial C-peptide. During the cross-over period, patients were asked to carry out blood testing on 7 occasions 2 days each week, and intermittent recordings on intervening days when the patient suspected hypoglycaemia or was concerned about glycaemic instability. Adjustment of insulin dosage was recommended according to a standard algorithm for all individuals. It should be observed that only 46 of the original 54 individuals who commenced the study completed the 6 month run-in period, and of these 38 completed the cross over period. It could be argued that the final drop-out rate of 33% would subject the data to such bias as to invalidate statistical analysis.

Whilst the authors suggested that glycaemic control improved over the 6 month run-in period, this conclusion was based primarily on the graded reduction in urinary glucose excretion based on 24 hour samples and analysis of 'Diastix'. However mean

blood glucose values determined by laboratory measurement of the 7 point daily profiles did not alter over this period, although it should be stressed that patient compliance in producing these profiles was as low as 50% for the fasting sample. It is debatable whether a maximum fall of 1% in HbA_1 is relevant despite reaching statistical significance, particularly when one considers the persistent elevation of HbA_1 throughout this period and return to baseline values by the end of the 6 month run-in period. Other workers have suggested that a fall in HbA_1 of at least 1.4% is required if the original value was 10%, to take account of the limitations of the assay, before one can claim an improvement in overall glycaemic control (115). The fall in cholesterol over the initial 6 months may be more attributable to alterations in diet, particularly as a fall in dietary carbohydrate intake was recorded. Numbers were inadequate to state whether residual C- peptide secretion had any bearing on the degree of glycaemic control throughout the whole study. There were no significant changes in the frequency of hypoglycaemic episodes, daily insulin dosage, or body weight.

During the cross-over period of the study, glycaemic control remained suboptimal as assessed by various criteria, irrespective of the method of monitoring, and cholesterol concentration, dietary carbohydrate intake, frequency of hypoglycaemia and insulin dosage did not alter. A suprising increase in mean serum creatinine concentration was observed in all 3 monitoring groups during the cross-over phase of the study which may reflect failure to revise the level of significance inthe light of multiple comparisons. Despite these disappointing

results, the vast majority of individuals who completed the cross-over phase felt more confident with blood glucose monitoring, although 40% preferred a combination of blood and urine glucose measurements to monitor their own control. There was a suggestion when data was analysed chronologically irrespective of the method of monitoring, that glycaemic control measured by HbA_1 improved over 6 months in the cross-over period in association with a reduction in dietary carbohydrate, but thereafter returned to baseline values.

The major conclusion of the study was that patient education and motivation were paramount in improving metabolic control, and that whilst self blood glucose monitoring was an attractive option, it did not lead to further improvements in control. The subsequent deterioration in glycaemic control was attributed to a 'waning of initial enthusiasm'.

My own interpretation of this study is that the authors may have inadvertently introduced a touch of nihilism in addition to realism. One of the most striking observations was the inability to lower mean levels of HbA_1 much below 10% throughout the study. Although this could be interpreted as failure of any approach to significantly improve control, this data should have been compared with the performance of the much larger group of insulin-treated diabetics under ordinary clinic supervision, as it is possible, and indeed likely in view of the self-selection for the study, that glycaemic control would have been even poorer in the residual clinic population.

Furthermore the approach taken to blood glucose monitoring may have prejudiced against a more obvious improvement. The

failure to monitor blood glucose daily is an important factor, as glycaemic control is likely to respond better to more frequent blood testing (483, 485). The use of algorithms to modify the insulin regime was used by Worth et al (77), and would appear a sensible approach, but it is not clear whether this system was modified for each individual. It is not apparent how often patients adjusted their insulin dosage during the periods of blood glucose monitoring but the lack of change in insulin dosage might infer that very little changed.

At present the question of any independent role for home blood glucose monitoring in improving glycaemic control in IDDM remains unanswered. The study to produce that information should have the following design and fulfil the following criteria:-

1. A large unselected sample representative of the clinic population with IDDM should be studied, and comparison made with data collected in parallel from the clinic patients who do not participate.
2. The independent effects of education and motivation should be separated out by a run-in period of appropriate length during which time glycaemic control should improve by a combined approach of blood and urine glucose monitoring, with advice on adjustment of the insulin dosage appropriate for each individual.
3. The opportunity for continued hospital access should be encouraged during the phase of the study when blood monitoring is compared with urine monitoring, and this should be of sufficient duration to see whether loss of

interest by patients has a major bearing on outcome.

4. In addition to assessing metabolic control, an attempt should be made to assess how well individuals retain knowledge about aspects of diabetic self-care and perhaps more importantly to evaluate how often this information is put into practice.
5. Quality control assessment to validate patient generated glycaemic data is absolutely essential if patient generated data is to be used in statistical analysis.

Following on from the final statement, it is clear that provision of glycaemic data, collected at home but resistant to falsification or technical error, is often not undertaken, although it is necessary in longer term studies. At present there are several techniques available which allow comparison of patient generated data with a laboratory method, but filter paper blood glucose analysis is probably the most realistic approach.

(iii) Filter Paper Blood Glucose Collection

The principle of collecting capillary blood on filter paper for storage and subsequent laboratory analysis was first suggested by Keen and Knight in 1962 as a method for assessment of glycaemic control at home (462), and later reports have used the technique in the same way. More recently Petranyi et al (491) have reported a high incidence of disparity between patient and laboratory determined glucose levels simultaneously collected on filter paper, and have suggested a role for filter paper blood glucose measurement in quality control.

Therefore filter paper blood glucose measurement by diabetics at home provides the potential not only for documentation and consequent modification of glycaemic control, but also in verification of results recorded by patients. Before discussing clinical experience with these methods it is necessary to discuss certain methological considerations. Several approaches to the measurement of blood glucose spotted on filter paper have been described (462, 492-499). The type of filter paper and blood used, method of glucose determination, storage conditions and use of preservatives have led to different conclusions regarding precision and reproducability of the method and have raised the important practical point of stability of blood glucose after application to the filter paper.

The original description of filter paper sampling for blood glucose used Whatman GF1B paper and an insensitive manual colorimetric method which calculated glucose concentration in relation to the prevailing haemoglobin concentration (462). It was suggested that glucose recovery from filter paper was satisfactory for up to one week when stored at room temperature, but that the use of preservatives would be necessary for stabilisation of glucose for longer periods. Seiter et al (492) introduced an automated fluorometric method and used S+S filter paper No. 903. Wakelin et al (493), used plain Whatman No. 4619 filter paper and a manual method for glucose measurement, which was later modified by West et al (494) in the same laboratory for use with an auto-analyser. Abyholm (496) used S and S No. 2992 filter paper and a manual method, whilst Taylor et al (498) used Whatman No. 4619 paper and automation, and Burrin et al (495,

499) in their work, used automated methods with Whatman No. 3 filter paper impregnated with boric acid.

Despite the heterogeneity in methods, there is relative uniformity in the performance characteristics of the assay between those different laboratories, although blood glucose recovery and stability could be subject to variation with different types of filter paper.

Assessment of filter paper glucose measurements has usually been based on either venous or capillary blood spots. However although capillary blood glucose and blood volume may be higher than venous samples (81), the one report to measure duplicate capillary and venous samples taken simultaneously found no significant difference in blood glucose measurements from the two sources (493). The disc of blood-impregnated paper which is subsequently eluted is obtained by the use of a hole puncher. The sample size used in practice has been either 6mm (493, 494, 496, 498, 499) or 10 mm (492, 495) in diameter. It is uncertain whether this difference could affect comparison of these studies, although sample sizes of 15-20mm have been found to yield significantly greater glucose concentrations than smaller blood spots (7-9mm) (496). It is agreed however that blood spots of at least 1cm diameter containing approximately 10µl of blood should be applied to saturate the paper completely with blood. The blood glucose is subsequently eluted from the paper using different protein precipitants and elution times. Seiter et al (492) used 1ml of saturated aqueous benzoic acid for 30 minutes, but 0.3% perchloric acid (PCA) (495, 496), 2.5% sulphosalicylic acid (SSA) (493, 494, 498), or 2% trichloroacetic acid (TCA)

(498, 499) for 60 minutes are more commonly used. Burrin and Price (499) have suggested that TCA has the advantage over SSA and PCA since elution is complete after only 20 minutes, but there is a suggestion that mechanical agitation may accelerate the process regardless of the protein precipitant. Abyholm (496) has suggested the use of a correction factor to take account of the different distribution of glucose in the standard and control serum in comparison to that of filter paper blood, but this has not been confirmed by other reports (498, 499).

Recovery of glucose from the filter papers has been reported to be as low as 79.6% (492), but usually ranges from 80-104% using manual methods of glucose determination (493, 496, 498), whilst continuous flow automated analysers usually yield 97-110% of added glucose (494, 495, 498, 499), in comparison to 94.7% with a Beckman glucose analyser (498).

The accuracy of the procedure is usually calculated at different glucose levels, in comparison to a standard laboratory measurement of glucose in blood without application to filter paper. Surprisingly only one report has examined this important aspect, and a close correlation (r=0.90) was found when the laboratory glucose oxidase method values were compared with filter paper glucose levels eluted by TCA and measured by glucose dehydrogenase (GDH) (499).

The sensitivity of the assay is defined as the lowest reproducable detection limit for that method. It would appear that the GDH method is more sensitive for filter paper blood glucose measurement (0.26mmol/L) than either hexokinase (HK) or glucose oxidase (GOD) methods, and that TCA and PCA appear to be

more efficient eluants than SSA in contributing to this sensitivity (499). These data however, remain unconfirmed.

Within batch precision with glucose levels ranging from 3.2-32.4mmol/L has ranged from 1.8-5.0% in most studies (493-498), with higher precision at higher glucose levels. It has been suggested that at normal blood glucose concentrations (4-5mmol/L), within run reproducability is poorest with the hexokinase method, and that GDH and GOD derived values are comparable, although the combination of GOD with TCA as an eluant led to more variation. At hyperglycaemic levels (15-20mmol/L) the GDH method has the worst coefficient of variation, particularly if SSA was used as the eluant (499). Between batch precision also takes into account stability of filter paper blood glucose, which may be important, particularly if samples are left at room temperature without preservatives. Despite this Seiter et al (492) reported a within batch coefficient of variation (CV) of 4.6% for a normoglycaemic sample stored at room temperature over 3 days, and CVs of 4.2-7.6% have been reported in other studies (493, 496, 498), one of which examined samples at room temperature for up to 6 weeks (498). Between batch coefficient of variation did not vary according to whether manual or automated methods were employed (498) and ranged from 3.2-6.3% for glucose values in the range 8.0-43.9mmol/L (496-498). The observation of between batch coefficients of variation ranging from 2.3-3.7% over a 3 month period when glucose values ranged from 6.4-18.8mmol/L, using the GDH method with TCA as an eluant, is more encouraging, but this information is difficult to interpret since filter cards were stored at -20°C and were

impregnated with boric acid (499).

Of evident relevance is the effect of storage conditions and preservatives to the stability of blood glucose in filter paper. This remains an area of controversy and is addressed in the series of experiments on filter paper blood glucose measurement later in the thesis.

In 1962 Keen and Knight suggested that filter paper glucose levels were stable at room temperature for up to one week (462), and negligible falls were reported by Seiter et al (492) after 72 hours storage at room temperature with or without a dessicator, although a reduction of 10% in glucose values was seen by 13 days. Samples frozen at -20°C under dessication did not lose appreciable amounts of glucose (492). Wakelin et al (493) reported no significant change after 4 days storage at room temperature, although by 8 days the glucose concentrations were significantly reduced ($p<0.05$), irrespective of whether or not preservatives were applied to the paper.

Abyholm (496) demonstrated stability of glucose without preservatives for up to 8 days at room temperature, and suggested no change in initially low glucose values for at least 3 weeks, but an 18% reduction in samples with the highest glucose content by 5 weeks. In addition extremes of humidity (10% or 100%) led to instability and variability in filter paper glucose values, although haematocrits ranging from 0.20-0.80 had little effect on the measured glucose concentration.

Burrin et al (495) suggested that glucose levels on untreated filter paper fell by 20-30% at 5 days, irrespective of the storage temperature (room temperature 4°C or -20°C), but that

prior impregnation of the filter paper with 0.8mmol/L boric acid prevented any degradation for up to 10 days regardless of the temperature. Hochella and Hill (497) have also claimed that borate impregnation prevents glycolysis, although this was not confirmed by Seiter et al (492) or Wakelin et al (493), who likewise found that impregnation with sodium fluoride or benzoic acid did not have a glucose protective effect.

Despite these discrepancies, all the studies to date have suggested that filter paper blood glucose spots for patient self monitoring is a sufficiently robust procedure to allow assessment of glycaemic control at home, and the technique has been applied to the management of adult and paediatric IDDM (495, 500), as well as NIDDM (501). Self blood glucose monitoring using reagent strips or reflectance meters may have an advantage over filter paper glucose recording, in delivering immediate feedback of data to the individual, but the complementary role of the two methods became quite apparent in the report by Petranyi et al (491) who found that in 80% of patients with IDDM who were allegedly experienced in home blood glucose monitoring, visual readings correlated poorly with the laboratory determined data from filter card blood spots. Whilst only 3 of 14 subjects using reflectance meters produced data which correlated poorly with filter card glucose values, this survey highlighted how inaccurate patient recorded data may be, and highlighted the need for quality control of glucose concentrations recorded at home. One further interesting observation from this study which was not expanded on was the recording of biochemical hypoglycaemia at least once over the 48 hours of the study in over 60% of cases. It is not

apparent whether symptomatic hypoglycaemia was observed, but these findings could concur with those who have suggested that hypoglycaemic unawareness is frequently observed in IDDM (502).

Quality control of patients recordings is of course possible using alternative media such as sodium fluoride tubes or Unistep bottles (Owen Mumford, U.K.) for the delivery of samples to hospital for validation. However practical problems such as clotting of samples (493) has inhibited their introduction into general use.

A more sophisticated and inevitably more expensive approach to quality control is to use reflectance meters modified to store recorded glucose values. Whilst these meters have been found to be reliable (503, 504), it was demonstrated that many individuals who used them were not (505) and even pregnant diabetics have been found to under-report high glucose values (506). Whilst this technique suggests that there are psychological barriers to recording hyperglycaemic data, perhaps to receive the doctors' favour, there are a variety of alternative reasons why errors in recorded data may arise, such as unsatisfactory blood monitoring technique, use of poorly calibrated meters or reagent strips after their expiry date, and inappropriate handling or chemical contamination of the strips. The role of memory 'Glucometers' in improving the self-monitoring performance of diabetics needs to be examined in more detail (507), but the more recent studies demonstrate that the introduction of self monitoring equipment leads to altered behaviour patterns which may not always be desirable.

Over the last decade the use of self-monitoring of blood

glucose (SMBG) has become widespread as an aid to diabetic care. Major questions remain as how best to implement recommendations in a recent consensus statement on self-monitoring of blood glucose (508). Whilst there are now well defined subgroups of IDDM where self-monitoring of blood glucose (SMBG) should be of particular benefit, it is useless in isolation. Appropriate instruction in blood testing must be accompanied by general education in aspects of diabetic self care and an insulin regime which is compatable with reasonable glycaemic control. More information is required on reliability, accuracy and precision of meters and reagent strips in unselected patients' hands, which can only be achieved by quality control of user-generated data. It is also important to see how SMBG influences day to day metabolic control and in particular how patients modify their diets, insulin or behaviour in the light of the information they receive.

2:4. EDUCATIONAL AND BEHAVIOURIAL ASPECTS

It has been suggested that the Joslin clinic medal awarded to diabetics of 50 years standing, depicting a charioteer directing the three 'wild horses' of insulin, diet and physical activity, is reflecting the major controlling influence of education in the management of IDDM.

There is certainly little doubt with the introduction of home blood glucose monitoring, diabetic liaison sisters, and improvements in insulin delivery, that patients should also receive adequate education to facilitate self-care.

In addition to education leading to improved self management

and the potential for lessening complications, the diabetic may also be able to enjoy an improved quality of life, to decrease the cost of medical care by decreasing hospitalisation costs, and to improve the general economy by minimising absenteeism from work.

At present national and international diabetic associations have expanded to take account of this innovation with the introduction of education sections. These organisations have in turn highlighted certain deficiencies in the educational process.

Perhaps the most important question is to decide exactly who should be taught and who should teach. It is logical and practical to inform diabetics and relatives, and there is increasing recognition of important roles for diabetic liaison sisters, dieticians, chiropodists and pharmacists. The role of the physician should be pivotal, although there are concerns as to how well they educate patients, and suggestions that whilst knowledge and the commitment to provision of adequate diabetic care may be sufficient in full time diabetologists,it may not be in medical practitioners who manage diabetics in a more sporadic fashion (509). Whilst a programme of education of non-specialist doctors would appear logical to ensure that they are not left behind patients and other health care professionals in the quest for knowledge, implementation of this process may be difficult in practice.

There are two main aspects of the educational process which I will discuss: the content of the education programme, and how best to implement it. In adopting any educational package it would appear fundamental to ensure that it is appropriate and

realistic for the individual. The one, as yet unanswered question, is how to increase the motivation for self-care amongst less compliant diabetics. Even with these provisos, diabetic educators should implement a programme with objectives defined in advance. Whether diabetics respond better to group discussion or a one-to-one approach is difficult to be dogmatic about, although the management of IDDM tends to be idiosyncratic and individualising care may be more appropriate. Involvement of family, friends and colleagues at work would also be advantageous where possible, particularly in the management of hypoglycaemia and illness. The education programme should be designed to impart basic knowledge, and ensure understanding of the principles by encouraging feedback from the patient with the opportunity to demonstrate techniques in self management. The recommended objectives are summarised in Table 3.

It is important to recognise that establishing attitudes and adoption of self-care skills in newly diagnosed IDDM is likely to require a different approach to that which will modify the practice of neglected long-standing diabetics. It is also fair to state that these objectives may need to be modified in selected cases, e.g. mountaineering is not compatable with hypoglycaemic unawareness.

Whilst I do not concur with specific recommendations of those who advise altering the insulin dosage according to algorithms by no more than 1-2 units at a time, regardless of the total daily dosage (510), it is vital to impart information, if necessary in algorithm form, about the onset and duration of action of insulins being administered, to help the patient decide

which type of insulin and which dose (i.e. morning or evening) should be altered to stablise glycaemic control.

TABLE 3 OBJECTIVES OF PATIENT EDUCATION

ATTITUDES

The patient should:

(a) appreciate that IDDM is a permanent condition which can be controlled but not cured at present.

(b) recognise that self care is (now) essential to diabetes management and should be routine, although professional support is available when necessary.

(c) understand that whilst diabetes will impose a few restrictions in lifestyle, he or she can do almost anything a non -diabetic person can do.

KNOWLEDGE

The patient should be able to:

(a) discuss the normal relationship between blood glucose and circulating insulin, and the absence of circulating insulin response in IDDM.

(b) describe the relationship between the primary factors controlling blood glucose (i.e. food intake, energy expenditure and insulin).

(c) discuss the rationale - reducing symptoms and lessening the risk of complications - for careful control of blood glucose.

(d) state the goals of therapy (target blood glucose levels) and discuss the importance of routine self monitoring of blood glucose in attaining and maintaining these goals.

(e) discuss the use of glycosylated haemglobin as an indicator of glycaemic control.

(f) discuss several possible multi-component insulin-regimes,

including the relative advantages and disadvantages of each.

(g) discuss the indications for alterations in insulin dosage

(h) describe the factors that may predispose a patient to hypoglycaemia, how to prevent it, and how to recognise and treat it.

(i) describe the factors that may lead to hyperglycaemia and impending ketoacidosis, and how to intervene to correct these.

(j) describe the effects of physical illness and emotional stress on blood glucose levels.

SELF CARE

The patient should be able to demonstrate the following skills and techniques:

(a) insulin dose measurement and injection technique.

(b) dietary selection and estimation of the calorie, carbohydrate, protein and fat content of meals, and awareness of the importance of high fibre intake and high polyunsaturated: saturated fat ratios.

(c) blood glucose monitoring.

(d) urine glucose and ketone monitoring.

(e) recognition and treatment of hypoglycaemia, including accessability to glucagon.

(f) adjustment of insulin dosage.

(g) use of insulin supplements.

(h) alteration of insulin regime to accommodate exercise.

(i) foor care including cutting of the nails and using appropriate footwear.

(Modified from Krall (509)).

It should be recognised that the educational programme outlined is comprehensive and ideal, and therefore not always practical. Over the last 20 years, various attempts have been made to evaluate diabetic patients' knowledge, to estimate their educational requirements and thereafter to audit the results of implementing educational packages.

Diabetic education programmes gained early acceptance in the United States following reports by Williams et al in 1967, demonstrating widespread ignorance amongst insulin-dependent diabetics regarding diet, insulin administration, foot care and urine testing (511, 512). The indices of knowledge correlated with aspects of self-care although not with the relatively crude measures of metabolic control available at that time. These early findings encouraged the more widespread acceptance of the role of diabetic education in the United Kingdom, although it is sad to relate after almost 20 years, that a recent survey of knowledge amongst 182 Scottish insulin-dependent diabetics revealed a degree of ignorance comparable to those early studies, particularly with regard to insulin requirements during illness and confusion over food constituents and carbohydrate exchanges (513). These disappointing findings have been confirmed by other reports (514), including one from my own unit (515). Indeed, educational defecits have been identified as the cause of up to 47% of urgent hospital admissions in diabetics in one report (516).

These frightening statistics raise the need for more preparative planning of educational programmes as well as highlighting certain limitations in the educational process. In

designing educational material, certain patient characteristics should be taken into consideration. Demographic factors such as learning disabilities and ethnic background are relevant, as are psychosocial factors such as health beliefs, attitudes towards health providers, and compliance, as well as the level of the individual's diabetic knowledge. Whilst this might be self-apparent, medical, nursing and dietetic educators attach different priorities to these variables (517). Educational strategies should be designed not only to instill knowledge, but by improving patient motivation and compliance, to benefit self-care and hopefully metabolic control. Non-compliance remains the major obstacle to improved performance. It is suggested that normal aspects of human behaviour such as self-concept may be disturbed in diabetes leading to the development of psychological defence mechanisms such as denial or projection (518). Failure by the diabetic educator to recognise this response often compounds the problem if the education process becomes a 'teacher-centred' as opposed to 'learner-centred' process. Rather than the educator unloading a mass of information onto the patient, the diabetic subject should be encouraged to reveal his own expectations and fears. If these aspects are incorporated into further education programmes, the behaviour of the individual may be beneficially influenced.

Patients' attitudes to diabetes are the consequence of several interacting factors: the premorbid personality and previous experience of ill health, the individual's views on the cause of the diabetes and the perception of it's severity, the presence of physical symptoms and the immediate and remote

impact of the condition on his/her lifestyle, and the attitude of family, friends, workmates and medical attendants (519). These attitudes will almost certainly have an impact on compliance, although attempts to investigate this relationship have reached distinctly different conclusions (520-522). The health belief and social learning models are usually employed to assess diabetics' attitudes to their illness. The health belief model suggests that the four factors which dictate compliance with a treatment regimen are beliefs about (i) the benefits of treatment, (ii) the barriers to treatment, (iii) the severity of the condition and (iv) vulnerability to the condition. The social learning model or the concept of 'locus of control' attributes perceptions of general health and illness to either internal or external influences. External influences in turn may arise by chance or be the consequence of medical intervention (classified as 'powerful others'). When the health 'locus of control' scale was applied to obese non-insulin-dependent diabetics, compliance was found to be associated with older individuals who saw their condition as significantly more severe and who exhibited more of an internal locus of control than their non-compliant counterparts (520). However compliance in that study was assumed, and based on outcome, determined by weight loss and improved glycaemic control.

In a study by Schlenk and Hart of 30 insulin-dependent diabetic outpatients (521), compliance was assessed as a theoretical concept based upon self-reports detailing the frequency of good diabetic practice. This was assessed by the Multidimensional Health Locus of Control (MHLC) questionaire, a

'social support' scale recording patients beliefs regarding the frequency of appropriate diabetic management by their family, and a 'health value' scale where the importance of health is put into overall perspective. A high degree of compliance was reported with the exception of appropriate foot care and exercise. Compliance was judged to be closely correlated with social support, and the 'internal' (IHLC) and 'powerful others' (PHLC) health loci of control. Multiple regression analysis demonstrated that social support and PHLC accounted for greater than 50% of the variance in compliance scores. In simple terms, appropriate diabetic practice by patients is most likely where there is a high level of family support, and a recognition of the importance of medical personnel in control of their diabetes. Whilst the conclusions from this study are likely to be valid, the high degree of compliance reported suggests a degree of selection in the patients studied, which is confirmed by the 52% response rate to invitations to participate in this study.

Bradley et al examined 286 insulin-dependent diabetic men and women using scales to assess perceived control and health beliefs (523). These were specifically designed for IDDM and were internally validated. The perceived control scales used descriptions of 6 hypothetical events with either negative or positive outcomes (e.g. 'Imagine that you have recently experienced a hypo' as a negative outcome) with responses graded to assess the relative roles of Internality, Treatment, Externality, Chance, Personal Control, Medical Control and Forseeability.

Inevitably self-reporting may bias the assessment of

compliance although patients' opinions of practical issues may be more relevant than questionaires which unintentionally anticipate the appropriate response. The results from the study of Bradley et al (523) also suggested that patients made primarily internal attributions and saw outcomes as forseeable and under their control. In addition situations with positive outcomes were also reported to be due to recommended treatment and medical control, whilst negative outcomes were foreseen to occur by chance. The 'health belief scale' measured patients' perceptions of the benefits of and barriers to treatment, and their impressions of vunerability to and severity of problems associated with diabetes treatment or complications of diabetes. The benefits of treatment were found to outweigh any potential barrier. The diabetics not surprisingly perceived themselves as particularly vunerable to all diabetes-related complications with the exception of cardiac disease. They were reassuringly accurate in reporting insulin reactions as more likely but less severe than hyperglycaemic coma. Of diabetic-related complications, deterioration in eyesight generated most concern.

The consensus of opinion from both these reports (521, 523) is that effective control of diabetes is the consequence of responsable self-management, with adequate support from the medical care team and to a lesser degree, the family. When things go wrong this is attributed to chance.

The dominant concept of 'self-efficacy' in determining compliance has been suggested by Rosenstock (522), and may be achieved by a gradual incremental approach to education with 'proximal rather than distal goals' agreed by the patient and the

health provider. Maintenance of compliance remains a major problem, as suggested by the outcome of longer term longitudinal studies of diabetic education (v.i.). The 'relapse prevention model' is suggested as a solution, whereby patients are encouraged to pre-empt situations which could lead to loss of compliance. If the individual can cope in such situations, 'self-efficacy' is suggested to increase for the next challenge (522).

Such concepts are mainly in the province of educational psychologists but clearly need to be extended to diabetic education programmes in a form that the educators can comprehend. This is apparent when reviewing those studies where deterioration in patient educational skills is almost universal with time, although the success of educational programmes could also be judged by improvements in metabolic control or by a reduction in hospital admissions or contacts with medical personnel. Laurence and Cheely (523a) reported errors in critical areas of self-care developing in one third of cases six months after the initial programme, and suggested reassessment of patients' knowledge every six months.

Korhonen et al (524) found that diabetic knowledge in IDDM could be retained for up to 12 months, regardless of the intensity of the education programme. Performance was not related to age or duration of diabetes, but the level of schooling had a bearing on the outcome. No change in dietary adherance was reported although patients increased their frequency of self-monitoring for urine glucose, irrespective of whether they received standard or intensive education. Perhaps

most important was the observation that a transient improvement in metabolic control after one month in both these groups regressed to base line levels after six months. Those factors which did relate to poor metabolic control were increased levels of anxiety and depression, and poor self-confidence (v.i), whereas the length of schooling was positively correlated with good control. This could suggest that intellect may have a bearing on attaining educational goals in diabetes.

Rettig et al (525) noted that a diabetes home education programme made no impact on the number of preventable diabetes-related hospitalisations, nor in the length of hospital stay, extent of foot problems, number of days off work, or the number of 'emergency room visits' (usually with hypoglycaemia), despite marked improvements in measures of diabetic knowledge and practical skills, when compared to the control group who only received cursory education. This study suggested that the environment for education is crucial and is supported by the report of Beggan et al (526), who despite providing access to a hospital based teaching centre, found a dearth of knowledge amongst the majority of 75 young patients with IDDM of less than 5 years standing. The extent of knowledge was confirmed to correlate poorly with glycaemic control, but an association was found between the duration of formal school education and the level of diabetic knowledge.

A recent report has suggested that the best determinants of compliance and a positive attitude to diabetes are the carriage by patients of a source of sugar and a diabetic identification card (527).

On balance, diabetic educators appear to be crucial in advancing knowledge and practical skills in IDDM, but it is unfair to claim that this is solely responsible for any reported reductions in health service costs, since education programmes are often combined with home blood glucose monitoring and improved methods of insulin delivery, each with their own independent effects (528).

An unfavourable educational background or attitude to diabetes, or a poor self image may contrive to modify compliance, thereby limiting the impact of education on metabolic control and hospital admissions. Even in apparently motivated patients, regular reinforcement of information appears to be fundamental. The introduction of computer-assisted learning programmes may prove to be more cost effective than diabetic educators (529), but this remains to be proven.

Attention was focussed on the psychological and psychiatric aspects of IDDM, even before the biochemical basis for the condition was understood. The hypothesis that diabetes was the consequence of mental illness was proposed by Thomas Willis in 1679, who suggested that 'an ill manner of living ..... sometimes sadness, long grief, also convulsive affections, and other inordinations and depressions of the animal spirits are want to beget and cherish this morbid disposition' (530). In support of this, Willis reported on 'two women obnoxious to convulsive and hypochondriack affections, to whom accrued from thence a great flood of urine and langour, and wasting away of the flesh' (530). The concept of a diabetes-prone personality is now discredited, although there is still considerable support for a role for

psychological processes in the development of diabetes mellitus (531). There is little doubt however, that the patient psyche may have an important bearing on the outcome of all chronic diseases.

There are several important considerations in discussing psychological aspects of IDDM. Firstly, it is important to discuss psychiatric illness in a separate context from the impact of personality traits on the diabetic. Secondly it is important to recognise that the age of the patient and the particular stage of the diabetic condition can have a bearing on how each individual's premorbid personality responds to the stresses accompanying IDDM. Thirdly it is necessary to consider the role of the family and additional social factors.

With the recognition that development of personality is at its most vunerable during childhood and adolescence, a predominance of psychological assessments in this particular age group of diabetics is apparent. The development of diabetes in early adolescence can be viewed as one additional cruel hurdle to overcome in addition to the physical and emotional changes that accompany puberty. Diabetic adolescents have been found to have lower levels of ego development than their peers, and this is particularly apparent in boys (531). Despite this, levels of self-esteem were not found to differ between diabetics and healthy controls, although the duration of diabetes was inversely related to the level of self-esteem (531). Drexler (532) stated that such deficiencies in self-image could lead to disordered development.

Baker et al (533) used a 'psychosomatic model of diabetes'

to demonstrate that emotional arousal led to rises in free fatty acid levels, secondary to increases in catecholamine, cortisol and growth hormone secretion in young diabetics with recurrent ketoacidosis. Furthermore the metabolic changes were blunted by administration of beta blockers, which were claimed to be prophylactic against stress-induced ketoacidosis. It was suggested that such patients had difficulty in handling stress and tended to internalize anger. The biochemical abnormalities were most apparent in response to parental conflict. The suggestion was made that so called 'psychosomatic diabetics' with recurrent ketoacidosis would be best managed in a family setting with their parents rather than being referred for implantation of an intraperitoneal insulin pump. Such cases form a tiny minority of the young diabetic population, although lesser degrees of psychopathology are commonly recognised.

The suggestion by Swift et al (534) that 60% of diabetic children have psychiatric illness has not been substantiated by Simonds (535) in a study designed to eliminate selection bias, where psychiatric diagnoses were made with the same frequency in healthy controls and diabetics (10-15%), irrespective of metabolic control. Inter-personal and non-interpersonal conflicts were reported in significantly fewer diabetics with good control in comparison to either the poor control diabetic group or the healthy controls, which could suggest that by rising to the challenge of diabetes, the former group achieved a state of higher mental well-being. Simonds (535) also found that those diabetics with poor control were perceived by their parents to have more anxiety and depression than the other groups. Bearing

in mind the conclusions of Baker et al (533) and Hauser and Pollets (531) that family interaction was a major factor in the mental health equation, it might be argued that in effect Simonds (535) had demonstrated the concern of parents projected on to their children.

Recently, eating disorders have been recognised as a more subtle manifestation of psychiatric illness in IDDM. Wing et al (536), used validated questionnaires, and found that young diabetics did not differ significantly from non-diabetic control subjects on measures of control of food intake or bulimia. An increased score on the dieting subscale of the questionnaire in diabetics was said to reflect appropriate adherance to the diabetic dietary regime rather than indicating underlying anorexia. It was apparent that features of sub-clinical eating disorders were commoner in both diabetic and non-diabetic females and that self-reported bulimic behaviour was related to poor glycaemic control in IDDM. The consequences of disordered eating patterns are of course much more dangerous in IDDM than in non-diabetics, irrespective of the prevalence. Steel et al (537) have suggested that the 7% incidence of young female diabetics with clinically apparent anorexia nervosa or bulimia in their study was higher than expected from surveys of non-diabetics. As in the report by Wing et al (536) almost all their patients had poor metabolic control, although hyperglycaemia was present prior to the eating disorder, suggesting that poorly controlled young diabetic women are primarily at risk of developing an eating disorder, although it does not exclude a role for dietary indiscretion in further aggravating glycaemic control.

Perhaps the most important finding by Steel et al (537), was the close association between eating disorders and complications, and in particular the development of acute painful polyneuropathy in subjects with anorexia nervosa coinciding with the peak of weight reduction. On balance it would appear prudent to treat young female insulin-dependent diabetics as a high risk group for the development of psychological disturbances, but what of the bulk of adult diabetics outwith this category?

Murawski and colleagues (538) studied the personality patterns of 112 diabetics of long duration, of whom two thirds were said to be free of vascular complications. Those with vascular complications, and males in particular, had significantly higher hypochrondriasis scores, and the highest incidence of abnormal depression scores. The authors acknowledged the difficulty in attributing cause and effect in retrospective studies, particularly in the absence of a non-diabetic control group.

Sanders et al (539) assessed emotional attitudes in an uncontrolled study, by a personality assessment questionnaire and direct interview with 60 insulin-dependent diabetics and their close relatives. Mean values for personality factors were within the normal range, although the patients studied were characterised by a high incidence of anxiety about hypoglycaemia and vascular complications. Those perceived to have problems in day-to-day control of diabetes were found to be significantly more emotionally unstable. It was suggested that anxiety generated by the consequences of hypoglycaemia was often transferred to patients' relatives although data to support this

conclusion was rather sparse.

In the study by Cassileth et al (540), the assumption that certain psychological traits were specifically exaggerated in diabetes was challenged. In a comprehensive evaluation of 758 patients suffering from one of six different chronic illness (including 199 diabetics aged 18-84 years), the Mental Health Index Questionnaire was used to assess anxiety, depression, general positive effect, and emotional ties and control. The diabetic group, irrespective of whether they were insulin-treated or not, did not differ significantly from the general public or from the other groups of physically ill patients for any of the measures of psychosocial status. All groups, including diabetics, had higher psychological scores (i.e. less psychopathology) than a group under treatment for depression. A significant direct relationship between higher mental health scores and ageing was noted, and newly diagnosed diabetic patients had greater anxiety, depression and loss of control. It was concluded that there was effective psychological adaptation amongst patients with chronic illness including diabetes, which was strengthened with increasing duration of disease. Diabetes in particular was not associated with any unique emotional traits.

In a study of 84 insulin-dependent diabetics without advanced complications, Mazze et al (541) found no significant difference in personality, anxiety, depression or quality of life in comparison to non-diabetics, although all psychosocial variables with the exception of personality correlated significantly and positively with glycaemic control, independent

of the type of insulin regime. It was concluded that whilst intensive management had no adverse psychosocial effects, improved glycaemic control was accompanied by reduced anxiety and depression and an improved quality of life, although this was not necessarily a one-way relationship. It would appear, therefore, that well-controlled healthy diabetics are no more depressed or anxious than the general population, in contrast to those diabetics with poor metabolic control. However psychological disturbances in the latter group seem to respond to improved glycaemic control, a feature noted previously in another study of depressed diabetics during a period of home blood glucose monitoring (542). It was unclear however, whether it was the improved glycaemia or other factors which determined mood.

An alternative approach has been to examine the effect of stress reduction on glycaemic control, and one uncontrolled report suggested that biofeedback reduced the range of glycaemic excursions in previously stabilised IDDM as well as alleviating stress (543).

Whilst acknowledgeing that the majority of healthy adult insulin-dependent diabetics appear to be remarkably well adjusted psychologically, it would be unfair to extrapolate this level of well being to those diabetics with long term complications. Although diabetes is the commonest cause of blindness under the age of 65, very little is written about psychological impairment following visual loss in diabetics, although the pattern is likely to parallel blindness from other causes, with denial initially giving way to depression, anger, guilt and latterly acceptance (544-546).

Impotence has a multifactional aetiology (v.i), and may affect 25-50% of male diabetics. Although psychological overlay is recognised in all cases, the effect of established impotence on the psyche of diabetics is uncertain (544, 545), although a higher depression rating has recently been reported (547).

The response to amputation may vary according to the premorbid personality, but has been likened to the grief reaction following bereavement (544).

The uraemia associated with renal failure may itself produce neuropyschiatric features, and it has been suggested that the behaviour of patients with renal disease may regress to resemble the dependency apparent when diabetes was first diagnosed (544). Although myocardial infarction is common in diabetics, there is little written on the pyschological impact of coronary heart disease in diabetes, despite the fact that up to 20% of infarct survivors develop a treatable depression within 5 months of the event (544). Finally, the development of hypoglycaemic unawareness in longstanding diabetes, by imposing certain restrictions on the individual might be expected to lead to anxiety, if not in the patient then at least in a relative (544).

Therefore it is inappropriate to assign psychological stereotypes to all diabetics and the balance of opinion suggests that psychological evaluation should not be a priority for the vast majority of metabolically stable patients with IDDM. A low index of suspicion for psychological disturbance may be helpful in the management of unstable diabetics (particularly young females) and/or those with debilitating complications, in whom

the levels of glycaemic control and mood may have a bearing on one another.

2:5. SPECIFIC CLINICAL AND BIOCHEMICAL FACTORS

It is not stated in standard textbooks with any certainty, but there is a strong clinical impression that metabolic control is poorer in insulin-dependent diabetics with vascular complications when compared to those without. An accumulation of different factors may combine to create this situation. For example, individuals with retinopathy may be less reliable when it comes to monitoring reagent strips visually (473, 474) and diabetics with neuropathy may be more prone to infections and subsequent hyperglycaemia (sensory neuropathy and foot infections, autonomic neuropathy and urinary tract infections). The development of nephropathy-associated hypertension or coronary heart disease may require treatment with β-blockers which could affect metabolic control (548), whilst microvascular changes in the skin might affect the bioavailability of injected insulin leading to erratic glycaemic control (394). Autonomic neuropathy may be accompanied by hypoglycaemic unawareness (549), although the two problems may be associated rather than the former being causal. Whatever the cause, rebound hyperglycaemia (the 'Somogygi' effect) may therefore be more frequent leading to metabolic instability. In addition cross-sectional surveys have testified to the role of metabolic control in the pathogenesis of vascular complications (7-9, 15, 17, 57) when in fact they have provided only circumstantial evidence of this by demonstrating poorer metabolic control in those with established vascular complications who were matched for demographic variables with a group free of complications.

Another controversial issue is the role of residual

endogenous insulin secretion in metabolic control. Whilst it used to be thought that true type I (insulin-dependent) diabetes was characterised by complete loss of β cell function (550), there is now conclusive evidence that this is not so (551). Because insulin and C-peptide are secreted in equimolar amounts after cleavage from proinsulin, measurement of C-peptide gives an estimate of residual endogenous insulin secretion. C-peptide can be measured in serum or urine, following fasting, or in response to physiological or pharmacological stimuli (552, 553). Equally reliable ways to measure all residual β cell function are to measure the C-peptide increment 6 minutes after 1mg of intravenous glucagon, or C-peptide levels 1 hour after a mixed meal (553). Detectable levels of C-peptide have been found in almost 100% of patients with IDDM 2 years after diagnosis, but thereafter there is a gradual fall off in the response so that only 15% of patients have measurable C-peptide activity after 15 years of diabetes, although the incidence is then said to remain constant (551). Those factors which have been found to be important in predicting the quantitative C-peptide response are the duration of diabetes, the insulin antibody titre and the degree of hyperglycaemia, all of which are inversely related to C-peptide, and the age at diagnosis, which is directly related to C-peptide levels (554).

One outstanding question is whether residual β cell function itself has any impact on long term metabolic control and the prevention of complications. There are suggestions that a persistent albeit reduced portal insulin supply may improve glycaemic control (551, 555), but this remains

controversial (551, 554, 556), although more recently C-peptide secretion in middle-aged insulin-treated diabetics was found to have a bearing on lipoprotein levels, in particular on the HDL_2 and HDL_3 cholesterol subfractions (293). At the time of writing of this thesis a report has been published suggesting that residual insulin production assessed by urinary C- peptide excretion, leads to improved metabolic control, and that these patients have a lower prevalance of microvascular complications (557).

3 EPIDEMIOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS OF VASCULAR AND NON-VASCULAR COMPLICATIONS

I have suggested that the excessive morbidity and mortality associated with IDDM is determined by a combination of metabolic, haemodynamic, environmental and genetic factors. The evidence to support this is based predominantly on large cross-sectional and retrospective epidemiological surveys, but also on prospective longitudinal studies. Unfortunately many such studies have examined non-insulin-dependent diabetics along with small numbers of insulin-dependent diabetics.

In addition, the criteria for classification of macrovascular disease is often rather crude and excludes patients with clinically evident but asymptomatic atherosclerosis.

In broad terms, insulin-dependent diabetics appear more susceptable to atherosclerosis than the non-diabetic population, and develop a specific angiopathy of the microvasculature which most significantly affects the eyes and kidneys. The pathogenesis of the somatic and autonomic neuropathy of IDDM appears to have both vascular and metabolic components.

3:1. ATHEROSCLEROSIS (MACROVASCULAR DISEASE)

In assessing the impact of atherosclerotic disease on IDDM , it is necessary to acknowledge limitations in published literature on the subject. Firstly, although less relevant to non-insulin dependent diabetes, temporal factors such as the age and duration of diabetes at the time of the study are of overwhelming importance in both cross- sectional and longitudinal surveys of IDDM. The second point is to take into consideration the ethnic background of the population studied, as this has a bearing on the development of atherosclerosis. In this regard, epidemiological data on non-diabetics from the same ethnic background makes useful comparison. Thirdly, it is important to appreciate that epidemiological surveys may provide different information according to the design of the study. For example morbidity and mortality data are not always comparable, and mortality can be expressed as an acute event, or by using cumulative mortality rates, assess the long term prognosis. The term prevalence refers to the frequency at a particular point in time (i.e. in cross sectional studies) whilst incidence reflects the development of the particular trait over a period of time (i.e. in longitudinal surveys). It is also important to recognise that risk factors are not necessarily causal factors but may simply be acting as a marker for more important unidentified variables.

Notwithstanding these considerations, there is evidence of a changing pattern in the incidence of atheromatous vascular disease in diabetes. Albrink et al (244) found that whilst 10% of diabetics treated with or without insulin during the decade

1930-1939 had clinical evidence of large vessel disease, the frequency in a group of similar age and duration of diabetes was 56% over the period 1951-1961, despite no change in the incidence of microvascular disease. The observation could not be explained by improved diagnostic techniques and was supported by mortality data, suggesting a true increase in diabetic atherosclerosis. More recent evidence has confirmed that atherosclerosis, primarily conorary heart disease, is the leading cause of premature death in diabetics under fifty years of age (558). In a study by Deckert et al (559), renal failure was said to be a more common cause of death than coronary heart disease in Danish insulin-dependent diabetics diagnosed under the age of 31, although the contribution of vascular disease to mortality in those with nephropathy was not disclosed, and an intimate relationship between diabetic renal disease and coronary heart disease has since been described (560).

The prevalence of coronary heart disease in IDDM is difficult to ascertain with certainty because of differing diagnostic criteria. In a study by Reckless et al (561) of 154 middle-aged diabetics of greater than 20 years duration, of whom 70% were insulin dependent, roughly 38% had features of macrovascular disease at varying sites. The WHO Multinational Study of vascular disease in diabetes (8) revealed considerable variation in the apparent prevalence of vascular disease between centres. Over 6,500 diabetics aged 35-54 years were studied, of whom 40% were likely to have been insulin-dependent. The prevalence of large vessel disease ranged from 29-36%, with unequivocal ECG evidence of previous myocardial infarction in 4-

7%, and general features suggestive of coronary heart disease in 25-34% of cases. In a report by Knuimann et al on 179 insulin-dependent diabetics (7), whose mean age was 33.8 years and mean duration of diabetes 12.6 years, the prevalence of macrovascular disease (coronary heart disease, peripheral vascular disease or cerebrovascular disease) was 14.9% in men and 16.7% in women. Deckert et al found objective signs of myocardial infarction in 21% of patients whose duration of IDDM exceeded 40 years (559).

Post-mortem examinations have demonstrated more extensive atheroma and myocardial damage in diabetic coronary arteries, particularly in those who were insulin-treated (562-564). Diabetic atherosclerotic lesions have been reported to have an increase in the extent of fatty streaks, and an excess of raised lesions complicated by ulceration, thrombosis or haemorrhage (562). These adverse features explain in part the worse short-term prognosis following myocardial infarction in diabetics (565). In addition the cumulative 18 year incidence of coronary events in diabetic patients (myocardial infarction, angina or sudden death) in the Framingham study (566) was 1.6 times that of non-diabetic men, and 2.1 times that of non-diabetic women. The cumulative 16 year cardiovascular mortality rates from the Framingham study were even more striking when one looks specifically at insulin-treated patients, where the rate was increased 2-fold in males and over 7-fold in women (567). The Finnish Social Insurance Institutions' Health Survey (564) has also reported a 9-fold excess of coronary heart disease mortality in insulin-treated diabetic women in relation to the general population, whereas in insulin-treated diabetic males it was

increased 3 fold over a 9 year period of observation. In the prospective Whitehall study, Jarrett and Shipley (568) reported that the 10 year coronary heart disease mortality in insulin-dependent diabetics was 1.6 times higher than non-diabetics, although only 46 insulin- dependent diabetics were recruited at the beginning of the study.

Peripheral vascular disease is usually diagnosed if typical symptoms of intermittent claudication and/or absent foot pulses or amputation are recorded. Although involvement of the lower extremities is most common, branches of the thoracic and abdominal aorta may also be involved. The pattern of peripheral vascular disease in diabetes reflects more widespread deposition of atheroma and has a particular propensity for the distal popliteal arteries below the knees. The prevalence of peripheral vascular disease in IDDM is difficult to determine because it is often not categorised separately, and is often recorded as a component of macrovascular disease. In the WHO multinational study, intermittent claudication was reported in roughly 1.5% of cases, whilst amputation was recorded in 1.2% of female and 2.0% of all male diabetics (8).

In the Framingham study (567), intermittent claudication was found to be roughly 5 times more common in diabetic men and 4 times more common in diabetic women than non-diabetics in the 16 year follow up report. The study by Beach et al (569) reported on insulin-dependent diabetics aged 33-35 years whose duration of diabetes averaged 17 years, and found that 26% of 77 men and 22% of 67 women had evidence of peripheral vascular disease. Deckert et al reported the prevalence of amputation and gangrene as 12%

in insulin-dependent diabetics who had survived for more than 40 years (559).

Relatively little attention has been paid to the association between cerebrovascular disease and IDDM, although 7% of premature deaths in young diabetics have been attributed to this cause (558). Cerebrovascular disease is usually diagnosed by the rather crude end point of an established stroke. Cerebral infarction has been reported with increased frequency in post mortem studies of diabetics (570) and in the prospective Framingham study where the rate was roughly two and a half times greater than in non-diabetics (567), but cerebral haemmorhage may not be more common in diabetes (570). In a study of non-insulin dependent diabetics, the degree of hyperglycaemia correlated directly with the severity of the ischaemic stroke (571). In the WHO multinational study completed strokes were recorded in roughly 1-2% of cases (8). Kuebler et al (572) assessed subclinical carotid artery occlusive disease in 482 diabetics whose average age was 58 and duration of diabetes was 8.8 years, of whom 5% were insulin-dependent. They found 20% of all cases and 14% of those with IDDM had detectable carotid artery disease. Deckert et al found a 10% prevalence of stroke in IDDM of more than 40 years duration (559).

There is therefore overwhelming evidence for a more widespread aggressive atherosclerosis in all types of diabetes in comparison to the rest of the population, regardless of the ethnic background. What remains uncertain is whether this is due to an excess of risk factors which predispose to atheroma in general or whether additional specific factors operate in

diabetes.

An abundance of epidemiological studies testify to independent roles for cigarette smoking, hypertension and both hypercholesterolaemia and hypertriglyceridaemia as risk factors for the development of atherosclerosis and premature cardiovascular mortality in the general population (573-578). Additional factors with less predictive value include obesity (577), stress (16) and relative hyperinsulinaemia (579). A role for impaired glucose tolerance, short of overt diabetes mellitus, has been suggested as a mediator of large vessel disease (580), although this relationship has not been found in other studies (581, 582).

There is more disagreement as to the relative importance of these risk factors in the development of macrovascular disease in diabetes, and suggestions that there are marked differences in the associations of risk factors and vascular disease at different sites. One major reason for the dilemna is the failure to separate insulin-dependent from non-insulin-dependent diabetics in the analysis. As a result, differences between these two broad categories in age, body mass index, lipid metabolism and blood pressure, which may be relevant to the pathogenesis of atherosclerosis, have been neglected. In addition, the phenomenon of 'clustering' of risk factors, whereby the presence of one cardiovascular risk factor is associated with another, is more common in diabetes (583), and this will further hinder attempts to demonstrate an independent predictive role for risk factors in diabetics by multivariate analysis. Finally, the recent awareness of the specific susceptability of proteinuric

IDDM for developing macrovascular disease (560) was not appreciated in the earlier population studies.

The WHO multinational study on over 6000 diabetics found that the risk and prevalence of macrovascular disease was the same for diabetic women and men in contrast to non-diabetics (8, 17). Although age and body mass index were often powerful predictive variables for all types of cardiovascular disease, these might have reflected the preponderance of older non-insulin-dependent diabetics. This possibility was supported by the lack of correlation with coronary heart disease and duration of diabetes, which has since been recognised to be important in determining coronary heart disease in IDDM (559, 584). Using multiple regression analysis, coronary heart disease was most strongly related in insulin-treated diabetics to serum triglyceride levels, and to a lesser extent cholesterol, whilst systolic blood pressure was a weak independent risk factor (17). Neither fasting blood glucose levels nor the duration of diabetes were related to coronary heart disease, although both were important determinants of peripheral vascular or cerebrovascular disease, as was systolic blood pressure. Serum triglycerides were not found to determine non-cardiac vascular disease, although cholesterol levels were weakly predictive of strokes. The fact that many individuals were receiving anti-hypertensive medication may have minimised the importance of blood pressure as an independent risk factor, particularly since one major longitudinal study by Christlieb et al (58) has suggested that hypertension is the major risk factor for vascular disease in insulin-dependent diabetes. Perhaps even more suprising was the

failure to find an effect of cigarette smoking on vascular disease, although this may reflect the weakness of cross-sectional prevalence studies.

In contrast, the Framingham study was able to prospectively evaluate risk factors for diabetic vascular disease (566, 585). 293 diabetics were incorporated into the original cohort. The major finding from multivariate analysis was the overriding predictive value of HDL cholesterol for coronary heart disease, particularly in insulin-treated diabetic women (585). Low density lipoprotein cholesterol and measures of hypertension were also found to be significantly associated with macrovascular disease in both males and females, but triglyceride levels were not independently correlated with coronary heart disease. Body mass was related but reflected the large number of obese non-insulin-dependent diabetics in the study. The cluster effect may again have 'blurred' any independent role for cigarette consumption in diabetic vascular disease .

The Whitehall study comprised 168 diabetics (of whom only 46 had IDDM) out of a total of over 18,000 civil servants (568, 580). Subsequent death from macrovascular disease, primarily related to systolic blood pressure in the diabetic group, was predominantly due to myocardial infarction, but the IDDM group was too small for independent analysis. The most striking feature was the lack of relationship between macrovascular disease and smoking, cholesterol or post-prandial glucose values.

Beach and colleagues examined risk factors for peripheral vascular disease in both insulin-dependent and non-insulin-dependent diabetics in cross-sectional and retrospective studies

(569, 586). Neither fasting blood glucose nor glycosylated haemoglobin values correlated with the prevalence of peripheral vascular disease, but smoking was found to be the major determinant, and independent associations were also found with duration of diabetes, and VLDL triglyceride and LDL cholesterol levels.

Zimmerman and colleagues found that high levels of triglycerides were the only distinguishing feature of lipid metabolism in diabetics with peripheral arterial disease (587)).

The report by Tunbridge (558) on premature death in young diabetics commented that deaths from myocardial infarction were associated with hypertension, smoking and obesity, but no attempt was made to assess their role as independent risk factors.

Knuiman et al (7) pointed out in a cross-sectional survey of 1084 diabetics (179 with IDDM) that there were few differences in the risk factor profiles for large vessel disease in IDDM and NIDDM, after correcting for time-related risk variables. The major association for macrovascular disease was with age, but interestingly stepwise regression analysis also found direct associations with renal dysfunction (reflected by serum creatinine), autonomic dysfunction (implied by the postural blood pressure gradient), glycaemic control (assessed by glycosylated haemoglobin), plasma cholesterol, and an inverse correlation with HDL cholesterol.

In a cross-sectional analysis of risk factors for carotid artery occlusive disease (CAOD) in 482 diabetics (of whom only 5% had IDDM), those subjects with CAOD were older and had significantly higher levels of cholesterol and systolic blood

pressure, but no correction for age or regression analysis was carried out (572). Likewise, in the cross-sectional study by Reckless et al (561), those insulin-dependent diabetics with vascular disease had significantly higher levels of total and LDL cholesterol and total and VLDL triglyceride, and were older, more hypertensive and obese, but the specific contribution of each of these risk factors was not stated, although as in the Framingham study (585) diabetic women had low HDL cholesterol levels in the presence of vascular disease, regardless of their type of treatment.

Whilst blood pressure appears on balance to be an important determinant of macrovascular disease in diabetes (including IDDM), the suggestion of an independent role for cigarette smoking or cholesterol is not overwhelming, although as stated earlier (576), this may reflect limitations in study design. The important inverse relationship between HDL and vascular disease in women with IDDM needs to be restated although confirmation is required in prospective studies. However even if all associated risk factors are taken into consideration and one accepts the principle that small increments in combined risk factors could make a sizeable impact on large vessel disease (588), this would fail to account for the excess of cardiovascular mortality which is particularly apparent in insulin-dependent diabetic women. Therefore currently unrecognised risk factors need to be incorporated into the equation. In this respect, it is of interest to find in the study by Knuimann et al (7), that serum creatinine levels figured prominently in the risk equation for macrovascular disease. The development of renal dysfunction in

IDDM characterised by proteinuria has been found to predict mortality not only from renal disease but also from cardiac disease (560). In fact the life expectancy of non-proteinuric patients was not much different from non-diabetics of similar age (560). It may be more than coincidental that the rises in blood pressure which predict vascular disease accompany the development of nephropathy (357, 358). However the additional potential for disturbances of lipoprotein metabolism (331), vascular permeability (589), and coagulation (590, 591) in early diabetic renal disease to enhance the risk of macrovascular disease should not be underestimated.

Platelet dysfunction and disturbed coagulability and haemorheology are features of poorly controlled IDDM which may also accompany diabetic nephropathy (592-594). Fibrinogen in particular may accelerate diabetic macrovascular disease, and elevated levels have been reported in association with diabetic nephropathy (591, 595). Therefore, whilst previous epidemiological studies have failed conclusively to demonstrate independent roles for all the traditional risk factors in the development of cardiovascular disease in IDDM, the recognition that it is the proteinuric sub-group of IDDM who are most at risk of macrovascular disease should make clear the need for a prospective examination of the role of risk factors in IDDM with or without nephropathy to define their pathogenic role more clearly. In particular, the combination of hypertension, disturbed lipoprotein metabolism and altered haemorheology in those insulin-dependent diabetics with established nephropathy may act synergistically to propagate atherosclerosis. Whilst

such speculation will remain hypothetical for some time, it is worth considering how these additional factors may influence current concepts on the mechanism of atherogenesis.

The prime mover in the process of atherogenesis is thought to be endothelial injury, whereby the integrity of the arterial intimal endothelia is breached. It is thought that prior to this, circulating monocytes adhere to endothelial walls and then migrate across intact endothelial barriers, accumulating lipid and thereby transforming into foam cells in the sub-endothelial space (16, 596, 597). Haemodynamic factors such as arterial stiffening and hypertension (598), immune complex deposition associated with microangiopathy (599), and peripheral hyperinsulinaemia (16) and the modified lipoproteins of IDDM (597) might all be expected to affect endothelial function. An increased transcapillary escape rate of albumin is described in diabetics with incipient nephropathy (589), and a similar process might operate in larger vessels to facilitate the entry of atherogenic blood components into the arterial wall. Further evidence for endothelial damage in diabetes is provided by the observation of reduced levels of endothelial-derived prostacyclin, and elevated circulating levels of factor VIII related antigen (597).

The accumulation of foam cells in the arterial intima may be enhanced in diabetes by the effects of glycosylation and oxidation of lipids (600). There is evidence to support a cytotoxic effect of lipid peroxides on the arterial intima, and glycosylation of low density lipoprotein (LDL) in particular may promote lipid deposition in the intima (597). In addition there

is evidence for preferential uptake of very low density lipoprotein (VLDL) remnants by both foam cells and smooth muscle cells, and levels of these remnants (the so called intermediate density lipoproteins (IDL)) may be particularly high in diabetes (597).

The next stage in the atherogenic process is the migration of smooth muscle cells from the media to the intima, possibly in response to mitogens produced by the foam cells. The smooth muscle cells then proliferate with the accumulation of connective tissue and the deposited lipid, leading in turn to the formation of a fibrous plaque. The adherance of platelets to the exposed sub-endothelial connective tissue promotes extension of the atheroma, by secreting platelet-derived growth factor (PDGF), which further stimulates smooth muscle cell proliferation and uptake of LDL. Most studies have demonstrated an increased capacity of diabetic platelets to adhere and aggregate both in vitro and in vivo (592-594), and an excess of PDGF has also been recorded in diabetes (601). These latter abnormalities are of course also relevant to the final thrombo-embolic sequelae of atheroma.

The effects of diabetes on the metabolism of the connective tissue which accumulates in the atherosclerotic plaque may also encourage progression of the lesion. Increased amounts of glycosylated collagen are apparent in diabetic skin and muscle (602), and may also be present in arterial wall collagen. This cross-linked glycosylated collagen is less susceptable to enzymatic degradation, and appears to act as a stimulus for further influx into and platelet adherence to the atheromatous

plaque (597, 600).

The final compounding variables in promoting both atheroma and thrombosis in diabetes are disturbed coagulability, characterised by increased level of clotting factors and platelet dysfunction (591-594), hyperviscosity as a consequence of reduced erythrocyte deformability, and elevated levels and enhanced aggregability of fibrinogen (590, 591, 595, 598). These abnormalities are often reported in those diabetics with microangiopathy (591, 595), which strengthens the hypothesis that the development of microvascular disease (exemplified by diabetic nephropathy) is a vital link in explaining the excess of atheroma in IDDM.

3:2 MICROVASCULAR DISEASE-RETINOPATHY AND NEPHROPATHY

Both retinopathy and nephropathy make a substantial contribution to morbidity, and nephropathy is also implicated in the excess mortality of IDDM. Whilst metabolic and temporal factors appear to play a dominant role in epidemiological and pathophysiological studies of diabetic microangiopathy, there is continued resistance to the idea that these complications are specifically caused by metabolic derangements (603). As with many aspects of diabetic complications, there are likely to be additional undetermined immunological, genetic and vascular components to the problem. In discussing clinical studies which have attempted to shed light on the natural history of these complications, it is important to state that apparent contradictions when comparing cross-sectional analyses with either prospective or retrospective studies may reflect a role for both hyperglycaemic and hypoglycaemic fluctuations in the pathogenesis of microangiopathy.

Diabetic retinopathy is the commonest cause of blindness in the under-sixties in the western world, and is equally common amongst males and females (11). Deckert et al (559) found that 16% of those who had survived over 50 years of IDDM were blind, whilst a further 14% had severe impairment of vision. Virtually all surveys (7-13, 40, 604, 605) have demonstrated that the risk of retinopathy increases with increasing duration of the diabetes. Consequently prevalence data is dependent upon the mean duration of diabetes in the population studied.

The WHO multinational study (8) had the benefit of

studying over 6,000 diabetics aged 35-54 years, of whom over 1,000 were insulin-treated diabetics of at least 7 years duration. Retinopathic blindness was present in 1% and 6% of all diabetics with a duration of disease less than 7 years and greater than 14 years respectively, whilst new vessel formation was a feature of 0.8% and 8% of the two respective groups. Minimal microvascular disease of the eye (microaneurysms or no discernable retinopathy) was found in roughly 6.5% of diabetics of more than 14 years duration.

Using a slightly different classification, Knuimann et al (7) found vision-threatening retinopathy (proliferative, obstructive, ischaemic or macular retinopathy) in 10.3-10.9% of insulin-dependent diabetics whose average age was 34 and whose duration of diabetes averaged 12.6 years. Interestingly over 60% of cases had no opthalmoscopic or fundus photographic evidence of retinopathy.

Telmer et al (606) reported a prevalence of proliferative retinopathy of 16.7-20.4% in 668 insulin-dependent diabetics whose mean age was 36.5 and duration of diabetes 21 years. These figures are very much in keeping with those of Borch-Johnsen et al (584) who found proliferative retinopathy in 24% of the 187 insulin-dependent diabetics of more than 40 years duration. Background changes were recorded in 38% of cases, but at least 26% of the patients had no opthalmoscopic evidence of retinopathy.

In a prospective evaluation, Young et al (40) recorded that 7% of the 75 young insulin-dependent diabetics whose duration of diabetes ranged from 7-17 years had early background retinopathy,

whilst after a further average follow up of 2.5 years, 33% had detectable retinopathy, and 12% had either pre-proliferative or proliferative changes.

In general, whilst these studies reaffirm the development of a high incidence of significant retinopathy with time, it appears that as many as one quarter of long-standing insulin-dependent diabetics will never develop clinically detectable retinopathy, although angiographic evidence is said to be present in up to 90% of diabetics of over 20 years duration (607).

Several cross-sectional evaluations of risk factors for retinopathy have demonstrated that the duration of diabetes is the strongest predictor (7-9, 604, 605), but thereafter all markers of glycaemic control are independently associated with retinopathy. Blood pressure and systolic levels in particular have also been reported to be independent risk factors for retinopathy (7-9, 604), above all for proliferative changes (584, 605).

Various markers of renal dysfunction have been evaluated as predictors of retinopathy. Borch-Johnsen et al (584) and Knuimann et al (7) found that increased urinary albumin excretion was independently associated with retinopathy whilst levels of serum urea (608) and the inverse of the creatinine clearance (604) have also been recorded as associated factors.

There is less certainty as to whether or not lipid levels are independently associated with retinopathy. Cholesterol levels correlated with severe retinopathy in the WHO study in women in particular, using univariate analysis (8), but this probably reflected the stronger relationship with body mass index

in obese non-insulin-dependent female diabetics. Testa et al (604) also found a relationship between cholesterol and retinopathy, but this was not apparent once duration of diabetes had been taken into account, and Knuimann et al (7) also failed to find a relationship. There are however several smaller cross-sectional studies where a relationship between retinopathy and lipids was apparent. Groop et al (605) studied 44 insulin-dependent diabetics with proliferative retinopathy, who were matched with a similar number without retinopathy. Cholesterol and triglyceride levels were increased and HDL cholesterol levels reduced in the retinopathy group, but only if proteinuria was also present. In addition, glycaemic control was appreciably poorer in the group with retinopathy, which could account in part for the altered lipid levels. In a similar study, Dornan et al (609) also found higher total and LDL cholesterol levels and lower HDL/LDL ratios in insulin-dependent diabetics with proliferative retinopathy after correction to a standard creatinine clearance. However glycaemic control again remained poorer and was not corrected for in the group with proliferative retinopathy. Kissebah et al (608) found higher levels of triglycerides and cholesterol in a rather atypical group of non-insulin-dependent diabetic retinopaths with insulin deficiency but apparently similar glycaemic control to a group without retinopathy. However the renal factor may yet again have explained the lipid abnormalities, since serum urea levels were also higher in the group with retinopathy, and Wardle et al (595) reached similar conclusions.

Eckel et al (329) looked at relationships between lipids,

apoproteins and microangiopathy in insulin-dependent diabetics using multiple linear regression analysis. They found that the number of microaneurysms correlated with the ratio of apolipoprotein AI/AII in HDL, and the HDL/LDL cholesterol ratio, but only in women. Retinopathy was also found to be associated independently with triglyceride, glucose and total and LDL cholesterol levels, but again the relationship was predominantly in women. The high degree of concordance between retinopathy and nephropathy, and the small numbers of patients limit the conclusions that can be drawn from this study.

The association between smoking and retinopathy remains contentious. Several cross-sectional studies have not defined a role for smoking (8, 9, 605), and Telmer et al (606) and Christiansen et al (610) failed to demonstrate a difference in the prevalence of proliferative retinopathy in smoking and non-smoking diabetics. Mulhauser et al (611) used a rather different approach comparing insulin-dependent diabetic smokers and non - smokers matched for sex, age and duration of diabetes. Roughly 59% of the subjects in each group had no retinopathy after a mean duration of diabetes of 14 years, but a higher prevalence of proliferative retinopathy was recorded in the smoking group. The authors suggested that smoking represented a risk factor for the progression of retinopathy to the proliferative stage.

Such associations do not of course necessarily confirm that each risk factor has a pathogenetic role in retinopathy, but they do provide the rationale for those prospective studies where improved glycaemic control was associated with an improvement in the degree of established retinopathy (21, 22).

Testa et al (604) and Young et al (40) have attempted to evaluate prospectively whether changes in risk factors relate to changing patterns of retinopathy. Testa et al (604) used CSII and conventional insulin therapy, and reported that the progression of retinopathy correlated inversely with the mean treatment levels of plasma glucose, M values, HbA_1 and serum cholesterol, and with the changes during treatment in plasma levels of glucose and triglycerides. In fact the single most important predictor for the progression or retinopathy was lower plasma glucose levels. Decreased creatinine clearance was also able to discriminate between mild and moderate progress of the retinopathy.

In contrast, Young et al (40) studied conventionally treated insulin-dependent diabetics and found that the deterioration in retinopathy was most strongly associated with increased blood glucose levels and the duration of the diabetes. There were also parallel changes in diastolic blood pressure, urinary albumin excretion and neurophysiological abnormalities. Smoking did not appear to influence the outcome. It was concluded that hyperglycaemia had an essential permissive role for the progression of microangiopathy but that other influences were also important.

Whilst these two reports might appear contradictory, what is apparent and has been confirmed in clinical practice is that both hyperglycaemic and marked hypoglycaemic fluctuations can aggravate established diabetic retinopathy (23-25).

In trying to elaborate the pathogenesis of retinopathy, other potential risk factors have been identified. Abnormalities

of platelet function (612-614), increased whole blood and plasma viscosity (615), elevated levels of fibrinogen (595, 615) and alpha$_2$ macroglobulin (59, 595), reductions in IgG and complement (605, 616), and abnormal glycoprotein metabolism (59) have all been reported in diabetic retinopathy, although their relative importance remains open to speculation. There are discrepant data as to whether or not there is a real excess of insulin-binding antibodies and immune complexes in diabetic retinopathy (599, 605, 617).

The pathogenic role of lipids in retinopathy remains a matter for dispute. Hard exudates, which are lipid rich particles, have clearly been shown to regress in response to diets rich in corn oil or other polyunsaturated fats, clofibrate or p-amino-salicylate, all of which lead to improvements in hyperlipidaemia (618-620), but it remains uncertain whether the long term prognosis for vision is affected by such manoeuvres.

Diabetic retinopathy is characterised pathologically by early structural abnormalities with the loss of pericytes and development of retinal capillary microaneurysms in association with endothelial cell proliferation and deposition of glycoprotein in the vasculature leading to thickened excessively permeable membranes. The subsequent development of haemorrhages and exudates may be a response to lipid thrombi (621), hyperviscosity, or haemodynamic alterations. The proliferation of retinal blood vessels and inappropriate vasodilation that follows may be a response to hypoxia and consequent ischaemia (622). At this point, vitreous haemorrhage, retinal detachment, and rarely rubeosis iridis, lead to serious visual impairment.

At the present time a prudent policy for the prevention and management of retinopathy would appear to be a graded reduction in hyperglycaemia without excessive hypoglyaemia, effective treatment of hyperlipidaemia and hypertension, and encouragement to stop smoking.

A variety of abnormalities of renal function, structure and biochemistry are recognised at different stages of insulin-dependent diabetes (Table 4), which have been incorporated into the hyperfiltration hypothesis for the pathogenesis of diabetic nephropathy (623), but this attractive unified concept remains to be proven in human diabetes. Furthermore, a clear disparity between structural abnormalities and the extent of functional disturbances has been found (624). Features of diabetic glomerulosclerosis are universal in renal biopsies from long-standing insulin-dependent diabetics, many of whom have no apparent functional disturbances (625, 626).

There are however, unequivocal findings regarding the extent of the problem based upon studies of diabetic nephropathy in which there is established proteinuria or chronic renal failure. Uraemia, secondary to diabetic nephropathy, has been reported to be either the leading (559) or the second commonest (558) cause of death in insulin-dependent diabetes. The mortality risk in IDDM is characteristically increased once persistent proteinuria has developed (560).

A clear relationship between the prevalence of overt nephropathy and increasing duration of diabetes is recorded. In the WHO study (8), 'severe' proteinuria was found in 5% of those whose duration of diabetes was less than 7 years, and in 16%

after 14 years. Lesser degrees of proteinuria were apparent in 13% of diabetics of less than 7 years duration, and in 16% after 14 years. Oakley et al reported proteinuria in 9% of individuals with long-standing IDDM (627). Knuimann et al (7) found that serum creatinine levels were elevated (greater than 120μmol/L) in 6.4-6.9% of insulin-dependent diabetics whose mean age was 33.8 and average duration of diabetes 12.6 years.

In perhaps the most comprehensive study, Andersen et al found that the cumulative incidence of proteinuria in insulin-dependent diabetics was 45% after 40 years or more of diabetes, although persistant proteinuria did not develop in over 50% of cases (326). The prevalence of nephropathy was 21% in diabetics of 20-25 years standing which thereafter was followed by a decline to 10% (secondary to mortality) after 40 years.

TABLE 4

Functional and morphological changes at different stages of the neuropathic process in insulin-dependent diabetes mellitus

Stage	Duration	Anatomy and Morphology	FUNCTIONAL CHANGES GFR	RPF	ALBUMIN EXCRETION RATE (µg/min) Basal	Exercise Induced	Blood Pressure
I Nephromegaly and Hyper-function	At diagnosis. Continues if control remains poor	Increased kidney size. Increase in size of glomerulus and nephron	↑ By 20-40%	Normal or slightly increased	↑	↑	Normal
II Early Glomerular Lesions	Detectable after 18 months of diabetes. Progressive over several years	Increased glomerular basement membrane thickness and mesangial expansion	↑ By 20-30%	Normal or slightly increased	Normal	May increase especially during poor metabolic control	Normal
III Incipient Diabetic Nephropathy EARLY	After 10-15yrs in 30-40% of patients.	Focal but diffuse glomerulo-sclerosis mesangial thickening	↑ By 20-30% or Normal	Incipient slow Decline	15-70	Abnormal aggravation of baseline elevations.	Normal or slightly increased, especially during exercise.
LATE	After further 2-5 years						
IV Overt Diabetic Nephropathy (Clinical Nephropathy)	After 15-20 years in up to 40% of cases	Diffuse nodular sclerosis, capsular drops. Fibrinoid caps. Arteriolar hyalinosis. Capillary narrowing.	↓ By 0.75-1 ml/min/ 1/73m^2/ month	↓without treatment by 5ml/min/1.73^2 month	Progressive increase Loss of selectivity for albumin	Not studied	Abnormally high (Diastolic pressure >95 mmHg in 51%)
V End Stage Renal Failure	Final outcome after 25-30 years	Glomerular closure. Hyalinosis. PAS-positive material deposition	<10ml/min/ 1.73m^2	Low	Persistent but may decline due to capillary closure.	Not studied	High

A male preponderance of diabetic nephropathy (1.79:1) was demonstrated in this study, although since then Borch-Johnsen et al have revealed equivalent absolute mortality rates for male and female insulin-dependent diabetics with proteinuria (560). In addition they found that the relative mortality (i.e. in relation to the non-diabetic population) was higher in proteinuric diabetic women than men. Cardiovascular disease was recorded in both these studies as a common cause of death, particularly in cases where the duration of diabetes exceeded 20 years at the time of death.

Most cross-sectional studies have reported systolic, diastolic, or longitudinal increments in systolic blood pressure as being independently associated with proteinuria in IDDM (8, 9, 584, 628), although Knuimann et al (7) did not find an association between blood pressure and increased serum creatinine levels, but this could reflect the absence of abnormal creatinine levels in the study group.

Serum lipid levels (cholesterol and the inverse of HDL cholesterol) were independently associated with proteinuria in the WHO study (8) and with serum creatinine levels in the study of Knuimann et al (7), although no such correlations were reported in other studies (9, 560). There is however a well recognised link between hyperlipidaemia and the nephrotic syndrome, and it is therefore likely that the association between lipids and proteinuria may depend on the degree of proteinuria. Vannini et al (331) found increased levels of triglycerides, total and LDL cholesterol, and apolipoprotein B, with reduced HDL cholesterol levels, in proteinuric insulin-dependent diabetics

compared to a group with normal albumin excretion rates who were similar with regard to age, duration of diabetes, and glycaemic control. Eckel et al (329) reported significant associations between proteinuria and increased levels of triglycerides and total LDL cholesterol, and with reductions in the HDL/LDL cholesterol ratio, particularly in women.

Whilst the role of smoking in retinopathy is uncertain, smoking in most circumstances is independently associated with proteinuria (8, 584, 606, 611), but interestingly predominantly in men, who of course have more proteinuria and smoke more often than women.

Additional independent associations have been reported with proteinuria, namely a low age at diagnosis, daily insulin requirements and the age and insulin-antibody titre at the time of assessment (584). Rather suprisingly, glycaemic control has not been found to be an important independent correlate with proteinuria in several studies (8, 9, 584, 606). This may reflect the relative unimportance of glycaemic control in established diabetic nephropathy. Notwithstanding this, established proteinuria has been reported to be associated with fewer previous ketoacidotic episodes (629), and higher levels of HbA_1 (15, 326) and plasma glucose (9) at the time of assessment.

The pathophysiological relevance of these epidemiological observations can be determined from those clinical studies which have investigated the impact of risk factors on the natural history of established diabetic nephropathy.

It has been suggested that death supervenes on average 6 years after the establishment of proteinuria in IDDM, but a

highly variable course (2-32 years) has been observed between individuals (630). Likewise, although Jones et al (631) reported that once serum creatinine levels exceeded 200μmol/l, a predictable linear progression in renal failure was observed with time, there appeared to be remarkable intra-individual variation in the rate of decline. Mogensen et al (632) reported a decline in glomerular filtration rate (GFR) ranging from 0.2-2.1ml/min per month in insulin-dependent diabetics with established nephropathy, which correlated positively with diastolic blood pressure measured after a mean observation period of 34 months. Parving et al (633) studied insulin-dependent proteinuric diabetics with normal serum creatinine levels in all but two cases. Over a mean observation period of 26 months, they recorded a mean fall in GFR from 107 to 87ml/min/1.73m^2, in effect a fall of 0.75ml/min/1.73m^2 per month, although again the variation (0.1-1.5ml/min/1.73m^2) was considerable. Rises in blood pressure and proteinuria excretion over this period were more uniform, but the decrease in GFR did not correlate with either, nor with the duration of diabetes, age of onset, insulin requirements, blood glucose, or initial GFR.

In contrast, Hasslacher et al (15) found that hypertensive proteinuric diabetics developed elevated serum creatinine concentrations more often, and the delay till serum creatinine levels rose was negatively correlated with blood glucose levels. Thereafter the decay in renal function occurred faster in patients with persistent as opposed to intermittent hypertension.

Takazakura and colleagues (634) performed serial renal

biopsies in a prospective study and demonstrated that progressive glomerulosclerosis accompanied poor glycaemic control. However, Thomsen et al (626) found that the severity of glomerulosclerosis and arteriolar hyalinosis bore no relation to the presence of clinically overt diabetic nephropathy, although increases in interstitial connective tissue and glomerular mesangial tissue, and a reduction in the number of open glomerular capillaries were specific histological findings in those diabetics with proteinuria.

The recognition of the importance of hypertension in the progression of diabetic renal disease was reinforced by Parving et al (626), who demonstrated that diastolic hypertension in particular was common in insulin-dependent diabetics with nephropathy but normal serum creatinine levels. Since then the consensus from long-term interventional studies is that aggressive anti-hypertensive therapy can at least delay progression, and may possibly reverse the decline of renal function, in addition to reducing the transcapillary escape rate of albumin (635-638). The selection of specific anti-hypertensive agents such as ACE inhibitors may have an additional role in reducing proteinuria in diabetics, independent of the blood pressure lowering action (639).

The impact of improved metabolic control on the progression of established nephropathy is likely to be of less importance and remains controversial. Viberti et al (28) suggested that greatly improved metabolic control made no impact on the decline in renal function in the six insulin-dependent diabetic nephropaths studied, although in one case it led to a dramatic

reduction in the rate of decline of GFR, and overall a non-significant reduction in the rate of decline of GFR from 1.35 to 0.69ml/min/month was observed. Nyberg et al (640) suggested that the decline in GFR was directly related to the degree of glycaemic control, and that poor glycaemic control independently contributed to one third of the progressive reduction in GFR.

In addition to these factors, more marked proteinuria and renal histological changes may act as poor prognostic markers (641), whilst a reduction in dietary protein intake may reduce proteinuria excretion and keep renal failure at bay (642). Residual endogenous insulin secretion has also been associated with less nephropathy, possibly due to improved glycaemic control (643).

The recognition that sub-clinical increases in urinary albumin excretion ('microalbuminuria') during the stage of incipient nephropathy can predict later clinical nephropathy (364-366, 644), has led to intense interest in factors controlling albumin excretion. However, a note of caution should be introduced. Firstly, what occurred in the intervening period from the stage of microalbuminuria to proteinuria in these retrospective studies remains obscure. Secondly it could be argued that the detection of albumin excretion rates (AER) of 30µg/min or more merely identifies individuals in whom the development of proteinuria is inevitable, particularly if one regards an AER of 70µg/min as the predictive cut off point (644). In addition there is still no agreement about the level of microalbuminuria and the collection conditions with which to categorise individuals at risk of nephropathy.

Several studies have attempted to define those factors that could promote the transition from incipient nephropathy to clinical nephropathy. Unlike established clinical nephropathy, increases in AER do not appear to be more apparent with increasing duration of diabetes (584, 645), and intra-individual day to day variations in AER of up to 50% have been reported (646, 647). Consequently the prevalence of microalbuminuria could vary according to the duration of diabetes and the frequency of urine collections in the various studies.

Borch-Johnsen et al (584) reported that elevated AERs (greater than 30mg/24hours) were present in 45% of 184 insulin-dependent diabetics who had survived at least 40 years. Hommel et al (647) reported the prevalence of microalbuminuria (20-200µg/min) in 1254 insulin-dependent diabetics as 14%. 5% of 265 diabetics (type not defined) had microalbuminuria (greater than 30µg/min) in the study of Gatling et al (648). Mogensen et al (645) reported a 12% prevalence of microalbuminuria in a clinic survey of 1082 insulin-dependent and non-insulin-dependent diabetics with a wide range of ages and duration of diabetes.

Cross-sectional studies designed to examine correlates of microalbuminuria have reached different conclusions. Borch-Johnsen et al (584) found the most important in long term survivors was the age at diagnosis, but the systolic blood pressure increment, insulin-binding antibody capacity, and regular smoking were also significantly correlated, although glycaemic control (assessed by HbA_1), was not.

Wiseman et al (357) matched normoalbuminuric with microalbuminuric insulin-dependent diabetics for age, sex, and

duration of diabetes, and found significant independent associations between albumin excretion, HbA_1 and arterial pressure. Mathiesen et al (644) found from their longitudinal data that AER was only predictive of nephropathy at levels greater or equal to 70µg/min, and that cross-sectional analysis demonstrated that such patients had significantly higher systolic and diastolic blood pressures, without significant differences in levels of serum creatinine, HbA_1, or GFR, when compared to normoalbuminuric patients.

Christensen and Mogensen reported a significant correlation in incipient nephropathy (AER greater than 15µg/min) between AER and systolic but not diastolic blood pressure (649). Correlations between renal haemodynamics and AER were not reported separately for patients with incipient nephropathy, but when data from diabetics with normal AER and those with incipient and clinical nephropathy were combined, significant negative correlations were reported between AER and both GFR and renal plasma flow. A later study from the same authors reported that GFR as well as diastolic blood pressure was higher in insulin-dependent diabetics with incipient nephropathy compared to the group with normal AER (650).

Together these papers suggest that the true relationship between AER and GFR is probably more like a bell shaped curve than a negative linear regression line. Therefore whilst renal haemodynamics and glycaemic control may be relevant, cross-sectional studies would suggest that blood pressure is the most important pathogenic determinant of the elevated AER of incipient nephropathy.

In attempting to clarify how all these variables influence the progression from incipient to overt nephropathy, retrospective and prospective observational studies and prospective interventional studies have been carried out. Hasslacher et al (15), in a retrospective analysis of 52 insulin-dependent diabetics, found that systolic blood pressure and the median post-prandial glucose levels were significant indicators of future nephropathy. This confirmed the earlier observation of Pirart (14) in a prospective study based on clinic blood glucose recordings over 40 years, where poor glycaemic control was clearly associated with the later development of nephropathy. In prospective studies using CSII to improve glycaemic control, Viberti et al (651) reported an accompanying fall in AER in an uncontrolled study of 7 patients with microalbuminuria, although a more recent report from the same group found no beneficial effect of CSII on AER once intermittent clinical proteinuria was established (29). The Kroc group found that CSII significantly reduced elevated AER (greater than 12µg/min) during an eight month controlled study (24). Longer term controlled studies have reported that at best, persistent near-normoglycaemia stabilises AER rather than leading to continued reductions (20, 25, 26, 29, 30, 33-35).

In prospective non-interventional studies, Mathiesen et al (644) reported that nephropathy developed in seven cases where the AER was initially greater than 70µg/min, whilst only three of the remaining 64 patients with less marked elevations of AER developed clinical nephropathy. In the studies of Christensen and Mogensen (649, 650), the mean AER increased from 58.8 to

118.1µg/min over an average period of 4.9 years in 10 individuals. There appeared to be great intra- individual variation in the rate of yearly increase in AER, ranging from -7.3 to +58.5%, but a significant correlation was observed between yearly increases in AER and parallel changes in diastolic blood pressure and renal vascular resistance. The lack of change in glycaemia in the study suggested that haemodynamic factors might operate independently of metabolic factors in the pathogenesis of diabetic nephropathy.

Young et al (40) suggested that rises in both HbA_1 and diastolic blood pressure significantly paralleled rises in AER over a two and a half year period, but multiple regression analysis showed that blood pressure was the more important determinant of progression.

It would therefore appear that as in established nephropathy, haemodynamic factors exert a dominant influence over metabolic factors in the early stages of renal dysfunction. This is not to say that improved glycaemic control has no role to play. If one acknowledges that the higher GFR in incipient nephropathy may be relevant to progression, then the observation of reductions in glomerular hyperfiltration with CSII is encouraging (652, 653). More recent work has suggested that CSII may achieve this by reducing not only hyperglycaemia but also intermediary metabolites, since ketone bodies have been reported independently to increase glomerular filtration, and perhaps more significantly, tubular protein excretion (367). Reduced dietary protein intake has also been found to normalise glomerular hyperfiltration and reduce fractional albumin clearance (355,

356). Glomerular hyperfiltration probably only improves after insulin administration by virtue of its effects on lowering blood glucose and ketone body levels, rather than by a direct effect of insulin on the renal vasculature (654, 655).

Whilst metabolic and dietary factors appear to exert an influence on renal haemodynamics, hormonal disturbances such as elevated levels of growth hormone and glucagon do not characterise diabetic patients with high glomerular filtration rates (656).

Alternatively the intrarenal prostaglandin system could promote renal vasodilatation. Administration of non-steroidal anti-inflammatory drugs did not affect glomerular hyperfiltration in insulin-dependent diabetics of less than 5 years duration (657), although reductions in GFR and AER in established nephropathy have been reported with indomethacin (658), and one report has suggested that specific thromboxane synthetase inhibitors can reduce microalbuminuria (659).

Autonomic neuropathy is common in insulin-dependent diabetes (v.i.) and has been found to modulate renal haemodynamics (660), but its role in albumin excretion and nephropathy remains to be elucidated.

Non-enzymatic glycosylation of serum albumin and of the glomerular basement membrane may lead to changes in ionic change which might lead to increases in AER (188, 189).

The use of antihypertensive agents in incipient nephropathy holds much promise and there are some early reports of reductions in AER following administration of ACE inhibitors to insulin-dependent diabetics with microalbuminuria (661). It

remains to be demonstrated whether such therapy can also improve morphological abnormalities.

Nephromegaly associated with hyperfiltration is an early feature of IDDM (662) which persists in early nephropathy and is closely related to glomerular filtration function, but which does not appear to relate to either urinary albumin excretion or the extent of mesangial expansion (663). Histological features of diabetic nephropathy developed in donor kidneys following transplantation into insulin-dependent diabetics (664, 665), but the most dramatic representation of the relative importance of a diabetic environment for the development of nephropathy is the report demonstrating improvements in glomerulosclerosis following transplantation of diseased diabetic kidneys into healthy recipents (666).

As yet there are no controlled studies of the effects of improved glycaemic control on renal morphology, but the data from renal biopsies following transplants supports the concept that the risk for nephropathy is acquired with the diabetic state for whatever reason, rather than inherited.

In addition to haemodynamic and metabolic factors, there is continued support for immunological and haemostatic mechanisms of nephropathy. High levels of immune complexes (559) and insulin antibody titres have previously been associated with nephropathy (584). A role for platelet dysfunction (667) in the establishment and progression is also suggested, and antiplatelet agents such as dipyridamole (668) and thromboxane synthetase inhibitors (659) have been successfully used at different stages of diabetic nephropathy.

Early intervention with aggressive antihypertensive therapy, effective glycaemic control, and advice to stop smoking, are likely to become cornerstones in the management of incipient diabetic nephropathy but properly designed controlled studies have yet to be carried out. Roles for antiplatelet, lipid lowering agents, and aldose reductase inhibitors (v.i.) in the treatment and prevention of diabetic nephropathy also remain to be defined.

3:3. POLYNEUROPATHY

Whilst peripheral sensori-motor and autonomic neuropathy are recognised complications of diabetes, it has only recently been appreciated that both subclinical and symptomatic disturbances of both nerve conduction and cardiovascular autonomic tone are commonly found, and are often present together (669, 670). Although the somatic and autonomic neuropathies may have a common pathogenesis, the clinical manifestations and implications of the two conditions are quite different, and will be discussed separately.

(a) SENSORY AND MOTOR NEUROPATHY

The diabetic peripheral nerve appears to be acutely sensitive to metabolic peturbations but is also subject to progressive long-term damage. Diagnostic criteria for peripheral neuropathy abound, and sensory symptoms from concomitant peripheral vascular disease may be difficult to distinguish from a neuropathic source. Consequently the prevalence of diabetic peripheral neuropathy has been reported to vary between 0 and 93% (671). The lowest prevalence rates were obtained from history taking, which emphasises the observation that despite abnormal clinical and neurophysiological findings, diabetic neuropaths are often symptom-free.

In a study by Boulton et al (672), which was carefully designed to exclude 'false positives', symptomatic diabetic neuropathy was present in 10.7% of 382 insulin-treated diabetics aged 15 to 59 years, whose duration of diabetes ranged from 12.3 to 21.5 years. Diabetics whose symptoms could have been related

to associated peripheral vascular disease or alcohol abuse were excluded, and the prevalence would have been nearer 20% had they also been included in the survey.

Knuimann et al (7) examined lower limb sensation and reflexes in 179 insulin-dependent diabetics, mean age 33.8 and average duration of diabetes 12.6 years, and found objective clinical evidence of sensory neuropathy in 8-9% of cases. In the huge prospective study by Pirart of 4,400 diabetics (14), neuropathy was clinically detectable in 8% of cases at the time of diagnosis, and in 50% after 25 years of diabetes.

It is acknowledged that abnormal electrophysiological findings are often a feature of asymptomatic diabetic patients. One such study confirming this is that by Young et al (673), who found abnormalities of peripheral somatic neurophysiology in almost three-quarters of 79 cases of IDDM without symptoms of neuropathy.

Temporal and biological factors which could contribute to the development of neuropathy have been examined. Both age and duration of diabetes are positively associated with clinical sensory neuropathy and abnormal electrophysiology (7, 14, 36, 40, 672).

There is strong circumstantial evidence for a pathogenic role for hyperglycaemia in symptomatic neuropathy, operating either directly or indirectly. Boulton et al (672) and Young et al (673) found that HbA_1 correlated with either clinical or subclinical peripheral neuropathy in cross-sectional studies, and in a prospective evaluation of the same group of patients, Young et al (40) confirmed that deterioriation in nerve condition

velocities paralled rises in HbA_1. Knuimann et al (7) could not confirm an association between HbA_1 and neuropathy but found that plasma creatinine, insulin dose and diastolic blood pressure all correlated independently with sensory neuropathy. Young et al (40) suggested that subclinical neuropathy was also independently associated with diastolic blood pressure, renal dysfunction (assessed by AER), and the presence of retinopathy.

Prospective intervention studies have produced conflicting results as to the role of improved glycaemic control on neuropathy. The well recognised associations between neuropathic symptoms and acute hyperglycaemia (671) were supported by the report from Pirart (14) who demonstrated less severe neuropathy in diabetics whose control had been 'good'. Pietri et al (37) found an improvement in symptoms and some, but not all, measures of nerve conduction velocity in response to improved metabolic control, using CSII in a short term uncontrolled study. Holman et al (38), and Service et al (39) found in longer term studies that improved metabolic control by either intensified conventional therapy or CSII led to sustained improvements in vibration sensation and nerve conduction velocities, but vibration sensation was not appreciably different from baseline after 2 years of improved metabolic control in the study by Lauritzen et al (22). In addition, increased residual insulin production and the associated relative improvement in glycaemic control was not associated with less abnormalities of sensory and motor nerve conduction velocity in one study (557). Young et al (40) suggested that although a certain level of hyperglycaemia was essential to initiate neuropathy, there was inherent or

acquired variation in the susceptability to its development. In addition it was suggested that the strong association between renal and microvascular complications independent of hyperglycaemia meant additional factors were implicated in their pathogenesis.

Diabetic neuropathy is characterised by distal axonal degeneration associated with segmental demyelination (671). The clinical course is quite variable, and the onset of symptoms may be abrupt or insidious, whilst progression of symptoms can be rapid or slow, and spontaneous remission is well described. The suggested role of hyperglycaemia in the pathogenesis of neuropathy is likely to be indirect since nerves are relatively insensitive to insulin. However the accumulation of sorbitol in nerves secondary to hyperglycaemia and saturation of the capacity of sorbitol dehydrogenase to convert sorbitol to fructose despite the continued ability of aldose reductase to convert glucose to sorbitol, as a low affinity system, may be important. Both sorbitol accumulation and myo-inositol deficiency are thought to reflect abnormalities of the polyol pathyway operating in human diabetic neuropathy as well as in animal experimental models. Clinical studies of aldose reductase inhibitors have produced inconclusive findings (674, 675). A high incidence of drug toxicity was observed in earlier studies, and the conclusions of one early open study demonstrating both subjective and objective improvements in neuropathy (676) has been the subject of criticism (677). Whilst neurophysiological measures do seem to improve in asymptomatic patients (678), aldose reductase inhibitors may (676) or may not (679) provide symptomatic

benefit, with variable changes in nerve condition over an 8 week period, although it should be appreciated that short term treatment may be an unrealistic approach to a condition that usually develops over several years. An alternative suggestion is that longstanding neuropaths have extensive nerve damage which is not amenable to correction and therefore aldose reductase inhibitors might be more logically used as prophylactic agents for subclinical neuropathy.

The use of dietary myo-inositol supplements has likewise led to inconclusive results (671).

Alternative proposed mechanisms for neuropathy are 'in vogue' at present. A microvascular aetiology was first suggested by observations of small vessel occlusion in human diabetic nerves (680, 681), and more recently abnormal platelet function has been reported in insulin-dependent diabetics with neuropathy but without evidence of microvascular renal or opthalmological disease (682). Haemodynamic alterations could induce microvascular damage to nerves and the close association between neuropathy and diastolic blood pressure (7, 40) may support such a hypothesis. As with retinal and renal complications, the pathogenesis is likely to be multifactorial and metabolic and haemodynamic abnormalities are likely to act in concert with one another in those who are genetically predisposed, and effective treatment might therefore require a combination of different modalities.

(b) AUTONOMIC NEUROPATHY

The diverse clinical features of advanced diabetic autonomic

neuropathy were first comprehensively described in 1945 (683). Since then, awareness of the poor prognosis associated with symptomatic autonomic neuropathy (684) has led to widespread interest in the pathophysiology of the earlier subclinical stage of autonomic dysfunction (685).

There are several problems inherent in studying diabetic autonomic neuropathy, quite apart from the tendency to mix groups of patients with IDDM and NIDDM (686). The first is that subclinical autonomic dysfunction often affects many organ systems without a predictable sequence of denervation (685), and secondly there is still no clear agreement as to what are the most appropriate sensitive tests of autonomic tone (685). Thirdly, the demonstration of functional changes in autonomic tone may not always represent structural damage, as suggested by the observation of disturbed cardiovascular autonomic function tests in newly-diagnosed insulin-dependent patients (687), in whom established nerve damage would be unlikely. Finally, in applying tests of autonomic tone to diabetic patients, the importance of age-related normal ranges has recently been emphasised (688-693). Failure to recognise that apparent functional changes in autonomic tone are a normal feature of the ageing process can lead to detection of 'false positives' amongst elderly diabetic patients, and exclusion of 'false negatives' amongst younger diabetic patients. In addition, whilst autonomic tests were previously categorised as measuring either parasympathetic or sympathetic tone, it is apparent that this classification is physiologically imprecise and that both systems act in combination to produce the standard cardiovascular

reflexes (685).

A suggested sequence of autonomic nerve damage (685) remains to be confirmed in prospective studies. Impaired sweating of the feet, impotence (v.i), and bladder dysfunction have been suggested to be the earliest features, followed by abnormal thermoregulation of the hand, cardiovascular reflex abnormalities, and finally the late severe symptomatic manifestations of excessive body sweating, hypoglycaemic unawareness, postural hypotension and gastroparesis. It is still uncertain whether there is inevitable progression through these various stages.

Most studies of the prevalence of autonomic neuropathy employ measures of cardiovascular autonomic tone. It has been suggested that such disturbances correlate closely with abnormal autonomic function in the gastro-intestinal, optic and neuroendocrine systems (685). Although there are many tests the commonest in use assess heart rate or blood pressure changes at rest, or during deep breathing, the Valsalva manoeuvre, adopting a lying from standing position or vice-versa, or the response to sustained handgrip. Ewing and Clarke have pioneered the use of a battery of tests, suggesting that one single test is misleading, as autonomic dysfunction is often not an all or none phenomenon (694). This is supported by reports of poor correlations between different measures of cardiovascular autonomic tone in healthy individuals (689). Whilst Ewing has suggested that the maximal beat-to-beat variation in heart rate detected during the course of 6 deep breaths in and out over one minute is the most reproducable test (685, 694), others have

suggested that a single deep breath is a more potent stimulus (693, 695). It has been suggested that even with the use of four standardised tests with age corrected ranges, up to 2% of the normal population will have more than one abnormal test result recorded (693).

The heart rate response to lying down may prove to be the most sensitive discriminator of autonomic dysfunction (696), although this requires confirmation. Despite methodological limitations, it is clear that asymptomatic autonomic dysfunction is detectable in a sizeable minority of insulin-dependent diabetic patients.

Hilsted and Jansen (697) measured beat-to-beat variation during maximal respiration in 119 asymptomatic insulin-treated diabetic patients whose average age was 36 years and duration of diabetes ranged from 3 months to 41 years. They recorded that 37.8% had abnormal values, although no age related normal range was reported.

Dyrberg et al (688) reported on 75 insulin-dependent diabetic patients whose mean age was 40 years and whose duration of diabetes ranged between zero and 40 years. In comparison to a group of healthy age matched controls, 27% of the diabetic patients had reduced beat-to-beat variation during forced respiration, whilst 17% had an abnormal heart rate response to the Valsalva manoeuvre, but none had an altered heart rate response to standing. When three sub-groups with increasing duration of diabetes but equivalent glycaemic control and age were compared, the prevalence of autonomic dysfunction significantly increased with the longer duration of diabetes. In

addition an association between autonomic neuropathy and nephropathy and proliferative retinopathy was recorded.

Young et al (673) used a series of five cardiovascular autonomic reflex tests and found that 31% of 79 asymptomatic teenage insulin-dependent patients had at least one abnormal heart rate response (Valsalva ratio, maximum-minimum heart rate, or the 30:15 lying to standing ratio), although the blood pressure response to handgrip dynamometry was normal in all cases. Those individuals with autonomic dysfunction tended to have poorer glycaemic control and an increased duration of diabetes.

Masaoka et al (691) confirmed that both the age and the duration of diabetes independently affected autonomic tone in diabetic patients compared to age related healthy controls. They reported that 36% of 102 asymptomatic patients with IDDM had reduced heart rate variability during deep breathing. Pronounced inter-individual variability was observed, which could explain why three of the patients with asymptomatic autonomic neuropathy (18%) had normal heart rate variability during deep breathing. This last finding again confirms the ability of autonomic neuropathy to damage selectively some reflexes whilst sparing others.

There are no adequate studies of the prevalence of symptomatic diabetic autonomic neuropathy, but it is uncommon and related to the age of the patient and increasing duration of the diabetes (685).

The pathophysiology of diabetic autonomic neuropathy is still far from clear. As with somatic neuropathy, axonal loss

and segmental demyelination take place (671), although whether this is patchy or progressive and generalised is unknown (685). As sympathetic fibres are larger, it is often suggested that parasympathetic nerve fibre damage takes place before changes in sympathetic nerves (685), although it is now clear from microneurographic findings that sympathetic nerve activity is disturbed relatively early in diabetic neuropathy compared to other types of polyneuropathy (698). Once established, progression seems to be the rule (684, 685). As with peripheral neuropathy, metabolic and vascular aetiological mechanisms are proposed for diabetic autonomic neuropathy (671). The close association between both symptomatic and subclinical autonomic and peripheral neuropathy in IDDM and NIDDM suggests a common pathogenesis (669, 670, 687).

The observation of lymphocytic infiltration of autonomic ganglia (699) has led to suggestions of an immunological basis for autonomic neuropathy, and the recent demonstration of increased levels of immune complexes and reduced complement (C_3) levels in diabetic autonomic neuropathy strengthens this hypothesis (700).

Relatively few studies have examined the impact of potential risk factors on established autonomic dysfunction. Young et al (40) found an increased prevalence of abnormal heart rate variation during deep breathing in insulin-dependent diabetics after a two and half year period of poor metabolic control, although the mean value was not different. The change in heart rate independently paralleled changes in albumin excretion rate and HbA_1, suggesting a role for glycaemic control. A weaker

assocation was reported with the development of retinopathy although the heart rate variability during the Valsalva manoeuvre and the blood pressure response to sustained handgrip did not alter during this period.

The effect of improved glycaemic control on established autonomic dysfunction has also been examined in prospective studies with variable results. The Steno study group (22) found that beat-to-beat variation during maximum respiration did not decline and could improve when CSII was used to improve glycaemic control in diabetics without symptomatic autonomic dysfunction.

The St. Thomas's Diabetic Study Group also found that the beat-to-beat variation in heart rate declined least where glycaemic control improved most with intensified conventional therapy over a 2 year period (701). However the Valsalva ratio, postural blood presure response, and tests of pupillary autonomic tone all worsened over this period. Other groups have reported that autonomic cardiovascular tone improved with improved glycaemic control (702, 703).

The possible role of sorbitol as the pathogenic agent for both autonomic and peripheral neuropathy (671) led to a report of a beneficial effect of Sorbinil, an aldose reductase inhibitor, on autonomic function in one uncontrolled study (676). However two later double-blind cross-over studies in insulin-dependent diabetic patients with symptomatic neuropathy failed to demonstrate any change in autonomic tone after 8 weeks (675, 679), although it could be argued that longer periods of treatment would be required to demonstrate a beneficial effect.

Gangliosides and dietary myo-inositol supplements have been used without effect in diabetic autonomic neuropathy (685).

The effects of autonomic denervation on haemodynamics has been reported to be implicated in diabetic foot ulceration (670, 704), although this suggestion has been contested (705), but altered renal and cardiac haemodynamics have been reported to accompany diabetic autonomic neuropathy (660), and might be expected to modify the natural history of diabetic cardiac and renal disease.

The diagnosis of early autonomic neuropathy has implications beyond the potential for associated morbidity. In a five year prospective study, Ewing et al (684), reported a 50% mortality in patients with advanced diabetic autonomic neuropathy, usually due to renal failure. Cardio-respiratory arrest has been documented in severe diabetic autonomic neuropathy (706), and could account for the association with sudden death (684, 685), whilst Zitomer et al (707) reported in a retrospective study that 35% of diabetics with gastro-intestinal autonomic neuropathy had died within three years.

At present the recognition of more sensitive markers of autonomic dysfunction should allow the opportunity for further understanding of the natural history of diabetic autonomic neuropathy in its early stages, but those factors which lead to the advanced stage remain to be ascertained.

3:4 IMPOTENCE

Impotence (the ability to achieve and maintain an erection when desired) has been recognised as a diabetic complication for almost 90 years (708). Whilst therapeutic nihilism was previously the rule, this could change with recent innovations such as penile implants and vacuum-assisted erection devices (709, 710).

The prevalence of diabetic impotence is often quoted as between 18 and 71% (711). This wide range reflects the difficulty in diagnosis and the multifactorial aetiology. Whilst autonomic dysfunction is often associated with impotence (685), most impotent diabetic men appear to have normal cardiovascular autonomic tone (684). Psychological and vascular factors are also likely to be important in some instances, but hormonal causes of diabetic impotence are rare (712).

In recognising the increased prevalence of impotence amongst diabetic men, the crucial impact of ageing was first recognised in a study by Rubin and Babbott of 198 cases (713). The prevalence of impotence was reported as 25% in the 30-34 age group and 54% in the 50-54 year old age group.

Kolodny et al (714) reported that 49% of 175 diabetic men whose average age was 49 were impotent. These patients were older and had more frequent evidence of neuropathy than the rest, but interestingly had no excess of cases of hypertension, retinopathy, or nephropathy. The onset of impotence was gradual and libido appeared to be intact in all cases.

A more recent comprehensive report by McCulloch et al (711) on 541 diabetic men aged 20 to 59 years revealed that 35% had

erectile impotence. In this study libido was reduced in 28% of those who were impotent. Linear regression analysis showed that impotence was most strongly independently associated with age, retinopathy, and symptomatic peripheral and autonomic neuropathy. Impotence was less clearly associated with poor glycaemic control, coronary heart disease, and nephropathy. In a 5 year prospective study of the same cohort, McCulloch et al (715) found that 28% of patients who were initially potent subsequently lost their ability to achieve erections. The independent predictors of impotence were age, alcohol consumption, poor glycaemic control, and intermittent claudication and retinopathy at the outset of the study. It was also found that established impotence was only reversible in younger patients with a short duration of the disease, when impotence was of acute onset and likely to have been psychogenic in origin.

In assessing the impotent diabetic it is clearly important to screen for autonomic neuropathy, which from present knowledge would suggest that the position is irreversable (684, 685). Peripheral vascular disease, alcohol abuse, androgen deficiency and poor glycaemic control should be detected and corrected. There may be a small residue of cases where psychological factors predominate who may benefit from behavioural modification. Whilst psychogenic factors have a bearing on impotence whatever its cause, the effect of impotence on the psyche may be more marked, although this aspect has not previously received much attention (547).

3:5 HYPOGLYCAEMIA

Sporadic episodes of hypoglycaemia have complicated the management of IDDM from the outset (1). The ability to achieve near-normoglycaemia with intensified conventional insulin regimes has caused understandable concern as to whether meticulous metabolic control would be accompanied by an unaccetpable increase in the frequency of hypoglycaemia (4). Besides the obvious acute symptoms and signs of hypoglycaemia, there may be additional psychological and biological sequelae which in themselves may be to the detriment of the patient (2).

Assessment of the magnitude of the problem is hampered by inevitable difficulties in the diagnosis of lesser degrees of hypoglycaemia and this may account for the paucity of information on the subject. Severe hypoglycaemia may be defined as loss of consciousness or an insulin reaction that required assistance from a relative, friend, or hospital practitioner, who would have administered parenteral glucose or glucagon. Milder hypoglycaemic episodes, which although clinically significant, are self-managed, may also provoke clinical, biological and psychosocial disturbances. Inevitably their reliable documentation proves difficult in practice, and of course asymptomatic hypoglycaemia goes undisclosed.

The assessment of biochemical hypoglycaemia would in theory detect all three categories of clinical hypoglycaemia although the pick up rate would obviously reflect the frequency of blood glucose testing. Detection of biochemical hypoglycaemia might however allow estimation of duration which may itself have important pathophysiological and pathopsychological consequences.

In one of the few studies of its kind, Gale and Tattersall documented previously unrecognised prolonged nocturnal biochemical hypoglycaemia in up to 50% of a selected group of unstable insulin-dependent diabetic patients, in whom symptoms were often mild or absent (502).

Reports of an increased number of severe hypoglycaemic episodes during intensified therapy (716) led to an investigation of the incidence of biochemical hypoglycaemia during a 24h period of continuous blood glucose monitoring following either intensified conventional therapy or CSII (717). Biochemical hypoglycaemia was defined as less than 2.8mmol/L and was recorded on 31 occasions in the 19 patients studied. Post-prandial hyperinsulinaemia was frequently recorded in association with hypoglycaemia and the incidence of problems was similar with either method of insulin delivery. Although only 25% of cases studied had evidence of angiopathy, biochemical hypoglycaemia was only accompanied by symptoms in 40% of the episodes.

In a large survey based on almost 10,000 capillary blood glucose samples collected at home by 99 insulin-dependent diabetic patients over an unspecified period, the frequency of biochemical hypoglycaemia (less than 3mmol/L) was found to relate inversely to median blood glucose concentrations (718). The prevalence was not reported, but was apparently more frequent preprandially than postprandially, particularly before lunch during treatment with conventional insulin therapy.

An alternative approach to assessing the extent of hypoglycaemia has been to record the incidence of severe hypoglycaemia in large groups of insulin-treated diabetic

patients. Basdevant et al (719) reported that 30% of insulin-dependent diabetic patients experienced a severe reaction at least once a year, and the same workers later reported an incidence of 26% per year for severe hypoglycaemia, whilst mild hypoglycaemia was recorded at least once a month in 58% of the 172 insulin-dependent diabetic patients interviewed (720).

Other groups have reported much lower figures. Potter et al (721) found that 9% of known insulin-treated diabetic patients attended Casualty with 'severe hypoglycaemia' at least once a year, which was readily acknowledged to underestimate the true figure by excluding those incidents which were not managed in hospital. Goldstein et al (722) reported a 4% incidence of hypoglycaemia per year in 147 insulin-dependent diabetic children. Casparie and Elving reported an 8% patient/year incidence of severe hypoglycaemia in 400 insulin-treated diabetics (723), which is comparable to the 9-10% reported by Mulhauser et al (724) during a one year assessment of 434 adults with IDDM. Mulhauser et al (725) reported in a later study that intensified conventional therapy did not lead to an increased frequency of hypoglycaemia when compared to traditional insulin regimes.

The causes of hypoglycaemia are multifactorial but Unger has suggested that unrealistic therapeutic goals, and inappropriate patient selection (advanced age $\pm$ diabetic complications) for meticulous glycaemic control are the main contributory factors (4). The latter problem is implicated in the report by Barbosa and Johnson of grave hypoglycaemia complicating the management of diabetic patients treated with CSII following renal

transplantation (716).

Defective counter-regulatory responses (726), hypoglycaemic unawareness associated with autonomic neuropathy (685), or a central (727), hypothalamic or metabolic defect (4) (e.g. absence of ketone bodies as an alternative cerebral fuel) may also predispose certain patients to 'severe hypoglycaemia'. In addition the response to hypoglycaemia may be attenuated following a period of optimised glycaemic control (728, 729).

Patients themselves often attribute hypoglycaemia to imbalance of their insulin dosage, calorie intake and energy output, but emotional disturbances are often reported as a precipitant of hypoglycaemia (720). In a survey of hypoglycaemia during a teaching and treatment programme, individuals with an extraordinarily high incidence were identified as those with excessively high insulin doses, inappropriate alcohol consumption, pregnancy, or hepatic/renal disease (725, 730). However evaluation by patients and their medical practitioners still failed to reveal an obvious cause for hypoglycaemia in up to 11% of cases in one study (720).

The consequences of acute hypoglycaemia may go beyond the initial neuroglycopaenic and adrenergic response. Whilst hypoglycaemic encephalopathy is an unacceptable and hopefully rare complication, accelerated diabetic retinopathy is now recognised to be a consequence of too-rapid tightening of glycaemic control (4, 5). Angina has also been reported anecdotally to complicate hypoglycaemia (2), and the myriad of haemodynamic and haemostatic changes that accompany hypoglycaemia may also aggravate pre-existing complications (2).

Finally, whilst many surveys have testified to the overwhelming fear of hypoglycaemia amongst insulin-treated diabetic patients, there is little data on the psychological correlates of recurrent hypoglycaemia and the impact on metabolic control thereafter.

CHAPTER 2

THE SALFORD HOME BLOOD GLUCOSE MONITORING PROJECT

PATIENTS, MATERIALS AND METHODS

1.THE SALFORD HOME BLOOD GLUCOSE MONITORING PROJECT

1:1 SELECTION OF PATIENTS AND STUDY PROTOCOL

During the period from September 1983 to December 1984, insulin-treated diabetics were identified for changeover to U100 insulin by a combined approach of writing to all general practitioners in the Salford Area, and by identifying all insulin-treated diabetic patients at the two diabetic clinics within Salford. At the time the study began no diabetic register existed and there was no computerisation of patient details.

It was originally planned to recruit 200 insulin-treated diabetics for the study during the period of U100 changeover. In the event over 500 insulin-treated diabetic individuals were identified, of whom 153 individuals participated, whilst another 50 individuals were followed up in parallel at the diabetic clinic to serve as a control group.

Eligibility for the study was based on the following criteria:

i) Aged 16 - 70 years.

ii) Receiving insulin treatment continuously since diagnosis or for a minimum period of 1 year following therapeutic failure with oral hypoglycaemic agents.

iii) Absence of advanced diabetic nephropathy (serum creatinine greater than 200μmol/L), severe proliferative retinopathy, blindness or foot complications, mental retardation, psychiatric illness, previous lower limb amputation or pregnancy.

Women who became pregnant during the study continued to participate to allow data collection although they received more regular follow-up than the rest of the study population.

iv) Duration of diabetes of at least 3 months.

v) Written consent to participate. All were informed about the nature of the study which involved intensive efforts to improve glycaemic control and regular visits over a 2 year period.

vi) Suboptimal glycaemic control (random blood glucose greater than 10 mmol/L at the clinic) or inappropriate insulin regime (eg once daily insulin in young patients with IDDM).

vii) Not currently performing home blood glucose monitoring.

viii) Absence of advanced cardiovascular or hepatic disease or cancer of any type.

Suitable patients were identified from the sources outlined above and invited to participate. The vast majority who were approached consented to take part; the numbers of insulin-treated diabetics suitable and those participating at different phases of the study are shown in Table 19. Although the study was designed to last 2 years, I only present data in this thesis from the first year of the study.

PROTOCOL (Tables 5-7)

Following verbal agreement to take part all participants came to the Medical Investigation Unit at Hope Hospital after an overnight fast of 10 - 12 hours. Blood (roughly 50ml) was taken for measurement of the following parameters:

fasting blood glucose, glycosylated haemoglobin (HbA_1), glycosylated albumin (GSA), fructosamine, serum urea, creatinine, albumin, electrolytes, cholesterol,triglycerides, the cholesterol content of low density lipoprotein (LDL), high density lipoprotein (HDL), and its subfractions HDL_2 and HDL_3, and apolipoprotein B and

insulin antibodies (V.I.).

The usual morning insulin dose was administered, followed after 30 minutes by a breakfast composed of their prescribed carbohydrate content. Blood and double voided urine glucose measures were thereafter made before meals, 1 and 2 hours post prandially and prior to retiring to bed at night (V.I.). Serum C-peptide was measured following a standard lunch (V.I.).

Collection of 24 hour urine was started from admission as timed daytime (erect) and nocturnal (supine) samples. Estimates of creatinine clearance, albumin excretion and glycosuria were made from the samples (V.I.).

During the day patients rotated between myself, the diabetic liaison sister, the dietician, and when necessary the chiropodist.

Structured interviews and clinical assessment were carried out and recorded in an initial trial entry document (Appendix 1).

Following breakfast the patient was interviewed by either the diabetic liaison sister or the dietician. The dietician assessed the calorie and carbohydrate intake (Section A of Appendix 1) and the dietary history of each patient (Section B question 6 in Appendix 1). Recommendations were made about fibre and fat intake according to the British Diabetic Association dietary guidelines and patients were encouraged to ask questions regarding their diet. No specific instructions were given to modify the diet other than to have a prescribed number of carbohydrate exchanges for each individual which could be modified in line with altered activity or insulin dosage.

Most of that day was spent with the diabetic liaison sister

and myself. The former completed an assessment of the patients' attitudes to diabetes and aspects of self management (Sections B and E of Appendix 1). The educational assessment of diabetic knowledge and practical skills was scored after interview from 1 (excellent) to 5 (terrible) (v.i.). Thereafter a programme of education and self management was instituted by myself and the diabetic sister which continued over the next 6 to 10 weeks according to individual capability. The components of the teaching and treatment programme were as follows:-

i) INSULIN TREATMENT Proper subcutaneous insulin delivery was demonstrated and the importance of weekly rotation of injection sites emphasised. The background intermediate or long acting insulins were continued, with soluble insulin prescribed in all cases, and circumstances for its use were stressed. Patients were instructed on how to mix the different insulin preparations and were told to take their insulin 20 - 30 minutes before meals. All patients but two received Plastipak syringes (Becton and Dickinson, U.K.) which they were encouraged to use for up to 3 days each during the study.

ii) HOME BLOOD GLUCOSE MONITORING All patients received a Reflolux blood glucose meter (BCL, UK) which was loaned to them during the 6 - 10 week run-in period of the study and which used BM Glycaemie 20-800R reagent strips (Boehringer, Mannheim, FRG). Autolets (Owen Mumford, UK) were prescribed to ease blood letting.

Patients were given instructions on how to perform both visual and meter-assisted evaluations of blood glucose, and were

asked to make at least three routine recordings daily with a full 10 point profile (pre-meals,1 and 2 hours post-prandial and pre-bed) once every fortnight. Additional measurements were requested if the patient felt unwell, suspected hypoglycaemia, or produced unexpected glycaemic results, or following manipulation of the insulin dosage or diet. Blood glucose results along with urinanalysis, documentation of hypoglycaemia and other comments were to be recorded in a diabetic log book. In addition the use of filter paper blood glucose cards was demonstrated for later in the study.

iii) URINANALYSIS Patients were instructed in the use of either Ketodiastix (Ames, UK) or Ketodiabur (Boehringer, Mannheim, FRG) and asked to carry out double voided urine tests at home following each blood glucose recording. The importance of monitoring for ketones during illness or hypoglycaemia was stressed.

iv) GLUCAGON All participants received glucagon sets (Glucagon, Novo, Copenhagen, Denmark) for subcutaneous or intramuscular administration in cases of severe hypoglycaemia, and whenever possible the diabetic liaison sister personally instructed relatives or friends of the patients in its use; otherwise patients were asked to instruct relatives.

v) SELF MANAGEMENT All patients received practical verbal and written advice about the causes, symptoms and treatment of hypoglycaemia, hyperglycaemia and ketoacidosis, and information on the implications of illness, exercise, alcohol intake, foot

care and altered dietary practice.The emphasis was to liberalise management allowing adjustment of their insulin and diet requirements according to their blood and urine glucose results, whilst at the same time encouraging them to attain glycaemic and glycosuric target levels.

The acceptable blood glucose target range was set at 3-10 mmol/L although on occasion this was 'shifted to the right' if patients complained of hypoglycaemic symptoms with blood sugar values recorded at less then 5 mmol/L. Preprandial target values were usually 4-6 mmol/L and post-prandial values 6-8 mmol/L.

All recommendations were reinforced during the 6 - 10 week run in period whilst efforts to achieve target glycaemic levels were in practice. The diabetic liaison sister visited patients at least once a fortnight at home during this period and additional hospital contact either direct or by 'phone was encouraged with free access to either myself or the diabetic liaison sister for advice.

Following lunch and C-peptide estimation on the initial assessment day, the patient had an ECG and chest x-ray carried out, and underwent a series of 5 tests of cardiovascular autonomic tone as previously described by Ewing and Clarke (694). Resting heart rate was assessed from the ECG. Thereafter with the assistance of the staff of the MRC Trauma Unit and the ECG Department at Hope Hospital, I carried out measurements of heart rate variation during the Valsalva manoeuvre, during deep respiration and after adopting an erect from supine posture. I later personally manually analysed all data. In addition I assessed the blood pressure response to standing and the

diastolic response to sustained handgrip using the dynamometer normally used to assess handgrip strength (maximum grip strength 300 mmHg). Using the standard criteria (694), each of the 5 tests was scored as 0 (normal), 1 (borderline) or 2 (abnormal) to give a total score ranging from 0 to 10. The prevalence of impotence was assessed by direct enquiry.

After collecting basic demographic data and assessing social circumstances (Section D, Appendix 1), I then made an assessment of patients' glycaemic control over the preceding year based upon the number of hospital admissions and blood glucose recordings at previous clinic visits, and undertook a full history and examination with particular reference to the cardiovascular, respiratory and peripheral nervous systems. The presence of lower limb pulses was assessed clinically and graded (0 = absent, 1 = reduced, 2 = palpable), and the femoral pulses were auscultated for bruits. Definite peripheral vascular disease was diagnosed by a history of intermittent claudication associated with one or more absent foot pulses. Pulse and heart rate were assessed with the patient supine at 45 degrees after resting on a couch for 15 minutes. Blood pressure was measured 3 times following resting for 15 minutes with the patient lying flat and at an angle of 45 or 90 degrees to horizontal and the mean figure taken for analysis. Korotkoff phase V was taken as the diastolic pressure. Hypertension was classified as systolic blood pressure greater then 150 mmHg and/or diastolic blood pressure greater or equal to 95 mmHg, and/or current treatment for hypertension. Visual acuity was assessed using a Snellen's eye chart and retinopathy assessed by direct opthalmoscopy after

pupillary dilation with tropicamide 0.5%. Acuity was graded from 0(6/6) to 6(6/60). The presence of cataracts, microaneurysms, haemmorhages, exudates, new vessels, retinitis proliferans,or laser scars was recorded and graded as 0 to 4 according to severity. Referral to the opthalmologist was made if necessary and recorded. Feet were examined for ulceration and their state of hygiene. Where necessary the chiropodist was involved. The presence or absence of ankle jerks, vibration sense, fine touch and pinprick sensation was examined for by routine clinical methods. Absence of parameters was scored at 0, normality as 2 and equivocal results as 1. A maximum total score of 16 was therefore possible.

Ischaemic heart disease was classified as a history of myocardial infarction (relevant clinical history with positive electrocardiographic and cardiac enzyme evidence), typical angina pectoris (exertional chest pain relieved by glyceryl trinitrate), pronounced ECG Q wave abnormalities, or T wave inversion and ST segment depression, or both (Minnesota codes 1-1,1-2,8-1,8-3).

Renal glucose threshold was calculated as either high, low, or normal or the basis or paired blood and urine glucose readings carried out during the initial hospital evaluation and from subsequent recordings at home. Persistant glycosuria with blood glucose values less than 8 mmol/L was categorised as a low renal threshold, whilst the absence of glycosuria despite simultaneous blood glucose levels greater than 11 mmol/L constituted a high renal threshold. Glycosuria in response to glycaemic values of 8 - 11 mmol/L was taken as a normal renal threshold. Clinical nephropathy was categorised as urinary protein concentration

(assessed by Albustix) >0.3 g/L and subsequently confirmed as greater than 500 mg/24 hours.

The following day their insulin regime was switched to twice daily bovine or biosynthetic human insulin if glycaemic control was unsatisfactory on the 10 point blood glucose profile (mean blood glucose greater then 10 mmol/L). The type of insulin was determined from a randomised schedule with equal numbers receiving bovine or biosynthetic human insulin according to a 10 x 20 randomised schedule matrix. Patients with acceptable control remained on their pre-existing type of insulin.

Finally, questionaires were administered which gave ratings for intelligence, anxiety, depression, obsessionality and assessed patients' attitude to illness (V.I.). Intelligence quotient was assessed by the Mill Hill scale, and standard progressive matrices and vocabulary sections were scored separately to assess integrative and communicative aspects of intelligence. An assessment of anxiety was made using the Epstein-Fenz questionnaire whereby 3 facets of anxiety were assessed: autonomic symptoms, muscle tension and insecurity.

The Zung Depression Scale was used both as originally devised and as modified by Dr. Chris Main of the Dept. of Psychology at Hope Hospital to give a depression rating. The Leyton Observation Inventory was used to assess symptoms and traits of obsessionality.

The Multi Health Locus of Control (MHLC) questionnaire assessed the attitude to illness and patients' perception of who controlled their health when applied to diabetes. As well as the total score, subscales for the role of chance, self-help,

powerful others and internal-external factors were assessed.

Patients were then discharged home and were formally reviewed thereafter fortnightly and data collected regarding glycaemic control, the incidence of hypoglycaemia, general health and hospital contacts (Appendix 1). Insulin regimes were adjusted at these visits and if necessary at home to achieve blood glucose levels of 3 - 10 mmol/L on the blood glucose meter. Blood sticks from the ten point glucose profile were saved and made available to validate their accuracy. Simultaneous urine glucose estimates were encouraged to assist in the classification of each individual's renal threshold.

After a minimum period of 6 weeks, patients were considered for randomisation to the second phase of the study if their glycaemic control had reached the target levels, and they were judged to be adept in all aspects of self management. If patients had not achieved target levels by 10 weeks or were not adept at self-management they were withdrawn from the study.

At the point of randomisation all patients were performing both blood and urine testing and had received instruction on insulin dose adjustment using either glycaemic or urinary glucose data. Measurements of lipid and carbohydrate metabolism were made at this time. They were then randomised to spend the ensuing year continuing with blood testing using the Reflolux meter or alternatively to relinquish it and continue to perform urine analysis as a means of monitoring their control. The randomisation schedule was organised in blocks of 20, anticipating that 200 would ultimately be randomised. Both groups of patients were reviewed 3 monthly when estimates of

metabolic control were made and after 1 year all were readmitted to the Investigation unit for clinical, biochemical, educational, and psychometric assessment. In the second year of study all those who had been performing urine analysis in the first year received a blood glucose meter. Those who had been carrying out blood testing for the first year were asked about their satisfaction with the method, and spent either the first or second 6 months of the ensuing year carrying out direct visual assessment of blood glucose sticks, retaining the meter for the other 6 month period.

As with the first year of the study, all patients were reviewed by myself and the diabetic liaison nurse at 3 monthly intervals (Appendix 1) and a comprehensive assessment was made at the end of the second year.

Following randomisation to either blood or urine glucose monitoring after the 6 - 10 week run in period, all participants received 10 point filter paper cards to perform glucose profiles once a month at home (V.I.). Those who had a blood glucose machine were asked to carry out simultaneous recordings to allow validation of their self-generated data. The urine testing group also carried out 10 point monthly filter paper blood glucose profiles. At the 3, 9, 15 and 21 month follow-up visits blood was taken for measurement of HbA_1, GSA, albumin and fructosamine, and at 6, 12, 18 and 24 months additional measurements of fasting glucose, lipids and lipoproteins were also taken. A 24 hour urine collection and assessment of renal function was made at the 1 and 2 year visits. (Table 7). Insulin antibody titres were measured initially and at 3, 6 and 12 month time points.

Throughout the 2 year period any changes in health, hospital contacts, hypoglycaemic episodes and days off work (where relevant) were recorded.

Psychometric tests were re-evaluated after one year in the study in a subgroup of patients.

To serve as a control group 52 other insulin dependent diabetic patients were subsequently studied. They attended the clinics on a regular basis without individualised attention or access to a blood glucose meter. None were carrying out regular blood glucose monitoring. Blood was taken for assessment of glycaemic control and lipid and lipoproteins on 2 occasions 6 months apart.

Table 5 STUDY DESIGN

1. OPTIMISATION PERIOD 6 - 10 WEEKS

- BLOOD GLUCOSE MONITORING WITH REFLOLUX METER
- URINANALYSIS WITH EITHER KETODIASTIX (AMES) OR KETODIABUR (BOEHRINGER)
- INTENSIVE CONVENTIONAL THERAPY AS NECESSARY TO IMPROVE GLYCAEMIC CONTROL
- INTENSIVE EDUCATION - HOME VISITING BY DIABETIC LIAISON SISTER
- FORTNIGHTLY BIOCHEMICAL ASSESSMENT

2. RANDOMISATION TO EITHER URINANALYSIS OR BLOOD GLUCOSE TESTING WITH REFLOLUX METER FOR 1 YEAR

BLOOD GLUCOSE TESTING	URINE GLUCOSE TESTING
	KETODIABUR OR KETODIASTIX

BOTH GROUPS :.
- MONTHLY 10 POINT FILTER PAPER BLOOD GLUCOSE PROFILE
- 3 MONTHLY FOLLOW UP FOR BIOCHEMICAL ASSESSMENT AND ENQUIRY ABOUT CONTROL AND COMPLICATIONS; AND DAYS OFF WORK WHERE RELEVANT
- ANNUAL CLINICAL REVIEW
- PSYCHOLOGICAL SCREENING

3. .URINANALYSIS YEAR 1 —> BLOOD GLUCOSE TESTING WITH REFLOLUX YEAR 2

. BLOOD GLUCOSE TESTING YEAR 1 —> 1ST OR 2ND 6 MONTHS OF YEAR 2 CONTINUING WITH METER OR VISUAL BLOOD GLUCOSE TESTING WITH BM STICKS

BOTH GROUPS:.MONTHLY 10 POINT FILTER PAPER BLOOD GLUCOSE PROFILE

.3 MONTHLY FOLLOW UP FOR BIOCHEMICAL ASSESSMENT AND ENQUIRY ABOUT CONTROL, COMPLICATIONS AND ABSENTEEISM FROM WORK

.ANNUAL CLINICAL REVIEW AT 2 YEARS

TABLE 6 MEASUREMENTS AT VARIOUS VISITS ACCORDING TO STUDY PROTOCOL

	RUN IN PERIOD						YEAR 1 (METER vs URINE)				YEAR 2 (METER vs VISUAL)				
WEEK	0	2	4	6	(8	10)									
VISIT	1	2	3	4	5	6	1	2	3	4	1	2	3	4	
MONTH							3	6	9	12	15	18	21	24	
DIETARY ASSESSMENT	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
EDUCATIONAL ASSESSMENT	+	–	–	+	(+	+)	–	–	–	+	–	–	–	+	
CLINICAL EXAMINATION	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
CHEST X-RAY	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
ECG	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
I.Q. TEST	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
PSYCHOLOGICAL SCORES	+	–	–	–	–	–	–	–	–	+	–	–	–	–	
FASTING BLOOD GLUCOSE	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
MEAN BLOOD GLUCOSE	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
M VALUE	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
FILTER CARD PROFILE (X3)	–	–	–	–	–	–	+	+	+	+	+	+	+	+	
INSULIN ANTIBODIES	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
HBA_1	+	–	–	+	(+	+)	+	+	+	+	+	+	+	+	
GSA	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
FRUCTOSAMINE	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
SERUM ALBUMIN	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
CHOLESTEROL	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
TRIGLYCERIDES	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
LDL CHOLESTEROL	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
HDL CHOLESTEROL	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
HDL_2 CHOLESTEROL	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
HDL_3 CHOLESTEROL	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
APOLIPOPROTEIN B	+	–	–	+	(+	+)	–	+	–	+	–	+	–	+	
APOE PHENOTYPING	–	–	–	–	–	–	–	–	–	–	–	–	–	+	
POST PRANDIAL CPEPTIDE	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
SERUM UREA	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
SERUM CREATININE	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
24 HOUR URINE GLUCOSE	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
24 HOUR URINE PROTEIN	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
DAY AER	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
NIGHT AER	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
TOTAL AER	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
CREATININE CLEARANCE	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
URINE ALBUSTIX	+	–	–	–	–	–	–	–	–	+	–	–	–	+	
HYPOGLYCAEMIA ENQUIRY	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
DAYS OFF WORK	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	
HOSPITAL CONTACTS	+	+	+	+	(+	+)	+	+	+	+	+	+	+	+	

TABLE 7

Time table for day 1 of home blood glucose monitoring study

A.M.	FASTING	VENUPUNCTURE.
		COMMENCEMENT OF URINE AND BLOOD GLUCOSE PROFILE.
		START OF A 24 HOUR URINE COLLECTION.
		INSULIN ADMINISTRATION AND BREAKFAST.
	10am - 12 noon	EDUCATION SESSION WITH DIABETIC
		SISTER AND DIETICIAN.
	12noon	LUNCH.
P.M.	1.15 - 1.30	POST PRANDIAL C-PEPTIDE ESTIMATION.
	1.30	AUTONOMIC NERVE TESTS.
	2.30	CLINICAL EVALUATION.
	4.00	PSYCHOLOGICAL QUESTIONNAIRES.

MATERIALS AND METHODS

Table 6 reveals the many measurements that were carried out throughout the study. I am very happy to acknowledge the assistance of colleagues in the Chemical Pathology Department at Hope Hospital who supervised the measurements of HbA_1, and measures of renal function other than albumin excretion rate and urinary glucose excretion. In particular I would like to thank Dr. Ian Holbrook for assistance in ensuring reproducability and accuracy of the HbA_1 assay.

I would especially like to thank Miss Monica Ishola of the Department of Medicine at the Manchester Royal Infirmary for providing me with extensive lipid and lipoprotein data; Dr. Chris Gordon of the Department of Medicine of the Manchester Royal Infirmary for the C-peptide measurements; Dr. Terry Wilkin of the Department of Medicine at the University of Southampton for providing the insulin antibody results; and Dr. Lesley Tetlow of the Department of Chemical Pathology at the Christie Hospital, Manchester for performing apolipoprotein E phenotyping.

I was personally directly involved in the development and use of the following assays during the study: - lipids and lipoproteins, filter card blood glucose and the urine glucose measurements, albumin excretion rate (AER), glycosylated albumin and fructosamine. I will discuss these assays in detail and those experiments leading to their effective use. During the 3 years of the study I spent an average of 2 days per week in the laboratory performing these assays.

MATERIALS AND METHODS

1:2 URINARY ALBUMIN EXCRETION RATE (AER)

The method used in the study was effectively the enzyme-linked-immuno-sorbent-assay (ELISA) for urine albumin described by Barbara Fielding and colleagues in the Department of Child Health at the Royal Manchester Children's Hospital (731). I would like to extend my appreciation to Dr. Fielding and also to Dr. Denise Bu'lock of the Department of Chemical Pathology at Hope Hospital who together enabled me to establish the ELISA assay for AER for use in the home blood glucose monitoring study. I was personally involved in the initial development and the early runs but thereafter Dr. P. Kalsi and Mrs H. Dhar, who worked with me as research technicians, performed the majority of assays.

THE ELISA ASSAY: ELISA is a technique where an enzyme labelled antibody or antigen is used to amplify an antibody-antigen reaction. In the assay the concentration of urinary albumin is below that which would form visible immunoprecipitates using gel techniques, therefore one of the reactants is adsorbed to a solid support. After adsorption of antigen or antibody to a solid support (in this case rabbit anti-human albumin antibody to the polystyrene wall of the microtitre plate), the complimentary antibody or antigen (urinary albumin) is bound. An enzyme labelled antibody (or further antibody and later enzyme-linked antibody in the AER assay - the modified double antibody sandwich ELISA) is subsequently attached to the bound molecules, and a chromogenic substrate for the enzyme is added. The intensity of the coloured reaction products generated is

proportional to the amount of conjugated enzyme bound, and thus indirectly reflects the amount of sample molecules. The major components of the system are therefore the solid phase support, the first, second and third antibodies, the substrate, buffers and standards, and a photometer to read the absorbance of the reaction.

Reagents: Tween 20 and bovine serum albumin were obtained from Sigma Chemical Co., London, UK. The following antisera and peroxidase conjugate were obtained from Miles Laboratories Limited, Slough, UK.: Rabbit anti-human albumin (lyophilised) cat. no. 65-051, goat anti-human albumin (liquid) cat. no. 61-015 and horseradish peroxidase conjugated to anti-goat Ig G (heavy and light chain) cat. no. 61-201. Inactivated rabbit serum was obtained from the Wellcome Foundation, London, UK.

Buffers: A solution of phosphate buffered saline and Tween 20 (PBS-Tween) was made up by dissolving 7.88g Na_2 HPO_4, 2.04g of NaH_2PO_4-$2H_2O$, 9g of NaCl, and 0.5ml of Tween 20 in 1 litre of distilled water to a pH of 7.3.

Diluent was prepared by dissolving 2.5g of bovine serum albumin (BSA) in 1 litre of PBS-Tween. This concentration of BSA did not cross react with antisera in the assay system but was sufficient to inhibit non-specific binding.

The coating buffer which acted as the first antibody diluent was made by dissolving 1.59g of Na_2 CO_3, 2.93g of $NaHCO_3$ and 0.20g and NaN_3 in 1 litre of distilled water at a pH of 9.6.

The substrate solution comprised 20mg of O-phenylene-diamine (Sigma Labs.) in 100ml of 0.2 mmol/L phosphate solution at pH 6.0, to which 160 µl of 300g/L hydrogen peroxide solution was

added. The substrate solution was stored at 4°C in a dark bottle, and was found to be stable for 1 week.

Standards: Human serum albumin (Sigma cat. no. A-8763) was dissolved in the diluent solution (PBS-Tween), to give a stock standard concentration of 200mg/L, which was stored at -20°C. From a working standard of 400µg/L, doubling dilutions were made to give concentrations of 3.13, 6.25, 12.5, 25, 50, 100 and 200 µg/L.

Antisera: The first antibody (Rabbit anti-human albumin) was diluted 10,000 fold in the coating buffer whilst the second antibody (goat anti-human albumin) was diluted 10000 fold in the PBS-Tween buffer to which normal rabbit sera diluted 1/400 had been added. The peroxidase conjugate (Rabbit anti-goat Ig - Horseradish Peroxidase) was likewise diluted 1/10000 in PBS/Tween.

Equipment: Nunc-Immuno plates (Gibco Europe Ltd., Middlesex, UK, cat. no. 439 454) were used as microtitre plates and the reaction was read using the Microelisa minireader (Dynatech Laboratories Ltd., West Sussex, UK) which was lent to us by the Immunology Department at Hope Hospital.

Samples: Timed day time (erect), night time (supine) and 24 hour (pooled day and night) urine samples were taken from all participants in the study and from 52 healthy control volunteers. 2ml aliquots of the samples were stored at -20°C with 20µl of inactivated rabbit serum which was added to minimise adsorption of albumin to the storage tubes.

Assay Procedure: The assay was carried out at room temperature:-

(1) Initially 200µl of the rabbit anti-human albumin is added to each well on the plate, and incubated for one and a half hours. The wells are then decanted and washed three times with the PBS Tween buffer.

(2) 200 µl of the standard or diluted (1/250) samples were then added in duplicate to the coated wells and incubated for 1 hour. The wells were again decanted and washed three times with the PBS Tween solution.

(3) 200 µl of the goat anti-human albumin was then added to each well and incubated for 1 hour, and thereafter decanted with the same washing procedure as previously.

(4) 200 µl of the peroxidase-conjugate solution was added, incubated for one further hour and washed with PBS/Tween buffer as before.

(5) 200 µl of the substrate solution was then added to each well at timed intervals.

(6) the plate was then incubated in the dark for 30 minutes, and then 50 µl of 1M HCl solution was added to each well at timed intervals.

(7) The absorbance of the contents of the wells was then read at 490 nM using the microtitre plate reader.

Results: STANDARD CURVES A typical standard curve is shown in figure 1.

SENSITIVITY The concentration of the lower standard (3.13 µg/L) was reliably detected, which constituted 625 pg of albumin per well or 0.78 mg of albumin/litre of urine after 1/250 dilution.

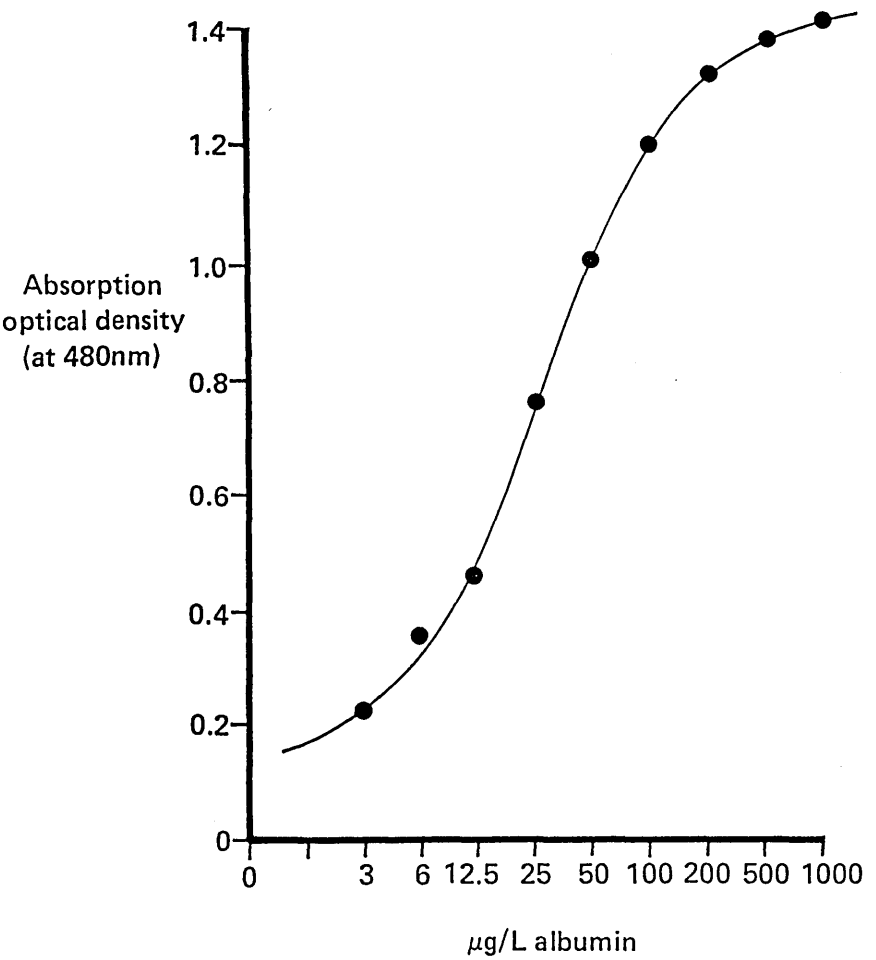


FIG. 1 Standard curve for Elisa Assay of Urinary Albumin.

The range of the assay was initially set at 3-1000 µg/litre, although further dilutions allowed albumin concentrations of up to 3500 µg/litre to be recorded but with less precision (greater than 10% variation) at concentrations greater than 1000 µg/L.

PRECISION 'Within batch' precision was estimated by assaying 3 urine samples with differing albumin concentration 20 times in one assay. The coefficients of variation (CV) for the 3 concentrations was as follows: 12 µg/L CV 5.9%, 40 µg/L CV 3.8%; and 210 µg/L CV 4.9%.

'Between-batch' precision was estimated by assaying 3 samples with differing albumin concentrations on 6 separate occasions with the following results: 12 µg/L CV 10.4%; 82 µg/L CV 8.5%; and 96 µg/L CV 15.1%.

ANALYTICAL RECOVERY The recovery of albumin from diluted urine samples was between 92 - 108%.

PARALLELISM Adequate parallelism was demonstrated when samples were analysed neat, or in dilutions of up to 1/16.

NORMAL RANGES Based on timed 24 hour collections (split into day and night intervals) from 52 healthy volunteers (27 men and 25 women) aged 36.8±11.3 (mean ±SD) years, normal ranges were established. Repeated (2-4) collections were made on 10 healthy volunteers and the mean between collection variability of the 24 hour, daytime and nightime samples was estimated at 45%, 60% and 30% respectively. The observed range of AER for the daytime (erect) samples was 0.1 to 15 µg/minute, for daytime (supine) samples 0.1 to 7.9 µg/minute, and for pooled 24 hour collections 0.1 to 9.1 µg/minute. Therefore the upper limit of normal for daytime and 24 hour collections was set at 15 µg/minute and for

nightime samples at 10 µg/minute.

1:3 FILTER PAPER BLOOD GLUCOSE MEASUREMENT

The establishment and assessment of an automated method for glucose determinations in filter paper blood spots was carried out between August and September 1983. I am most grateful to Dr. Graham MacKinnon of the Department of Chemical Pathology who was principal in establishing the method and with whom I collaborated in assessing its potential for use in the home blood glucose monitoring study. The majority of measurements of filter card glucose during the study were carried out by Dr. P. Kalsi and Mrs. H. Dhar, Research Technicians in the Department of Medicine at Hope Hospital, although I continued to assist with these measurements on a regular basis.

The technique for measurement of filter paper blood glucose involves an enzymatic reaction and the use of a continuous flow auto-analyser to increase throughput.

Reagents: Glucose concentrations were determined enzymatically using the GOD-PAP reagent (BCL glucose kit) obtained from Boehringer Corp., England (kit no. 166391). Bottle 1 containing glucose oxidase is made up to 200 ml with deionised water and stored at 4°C, whilst the phenol in bottle 2 is added to 1 litre of 0.9% NaCl in distilled water. 1ml of 30% Brij is latterly added to this NaCl/Phenol reagent and stored at room temperature. In addition we made up 5 litres of 1.5% sulphosalicylic acid solution and 500 ml of 1% boric acid, which were both stored at room temperature.

Standards: (1) Aqueous Standards - a stock standard of 50mmol/L glucose solution in saturated benzoic acid was obtained

from BDH Chemicals Ltd., UK. Working standards equivalent to 1.0-25.0 mmol/L concentrations are prepared by dilution of the stock solution with saturated benzoic acid, and stored at 4°C. Before use standards are diluted 1 in 50 (100µL + 5ml) in 1.5% sulphosalicylic acid.

(2) Blood spot secondary standards - specimens which had been analysed on a Yellow Springs Glucose Analyser (Clandon Scientific Ltd,. England) were obtained with values which corresponded roughly to the aqueous standards. Samples were spotted onto filter paper, allowed to dry, and frozen to allow 4 spots on each glucose value to be measured in each assay run.

Filter papers: We used pre-printed cards designed by Professor DC Anderson, with a superimposed strip of Whatman 31 PKU filter paper (see Appendix III). A quantity of the filter papers were saturated with 5% boric acid for 1 hour and allowed to dry overnight at room temperature. Blood spots were cut out using a hand held 6mm diameter paper punch (Velos Ltd, England).

Apparatus: A single channel continuous-flow autoanalyser system was set up comprising a Newton Sampler and Turntable, Technicon AAII proportioning pump, Technicon AAI heating bath and coil, Fisons Vitatron UPS Colorimeter with a 510 mm filter, and a Fison's Vitatron flat bed lin-log UR 400 recorder with a curve regenerator fitted and a chart speed of 2cm/minute (Figure 2).

Assay Procedure: Blood is initially spotted onto the filter paper to produce a spot at least 10mm diameter, and allowed to dry. A 6mm diameter blood impregnated disc or the standard is added to a LP3 test tube, to which 500 µL of 1.5% sulphosalicylic

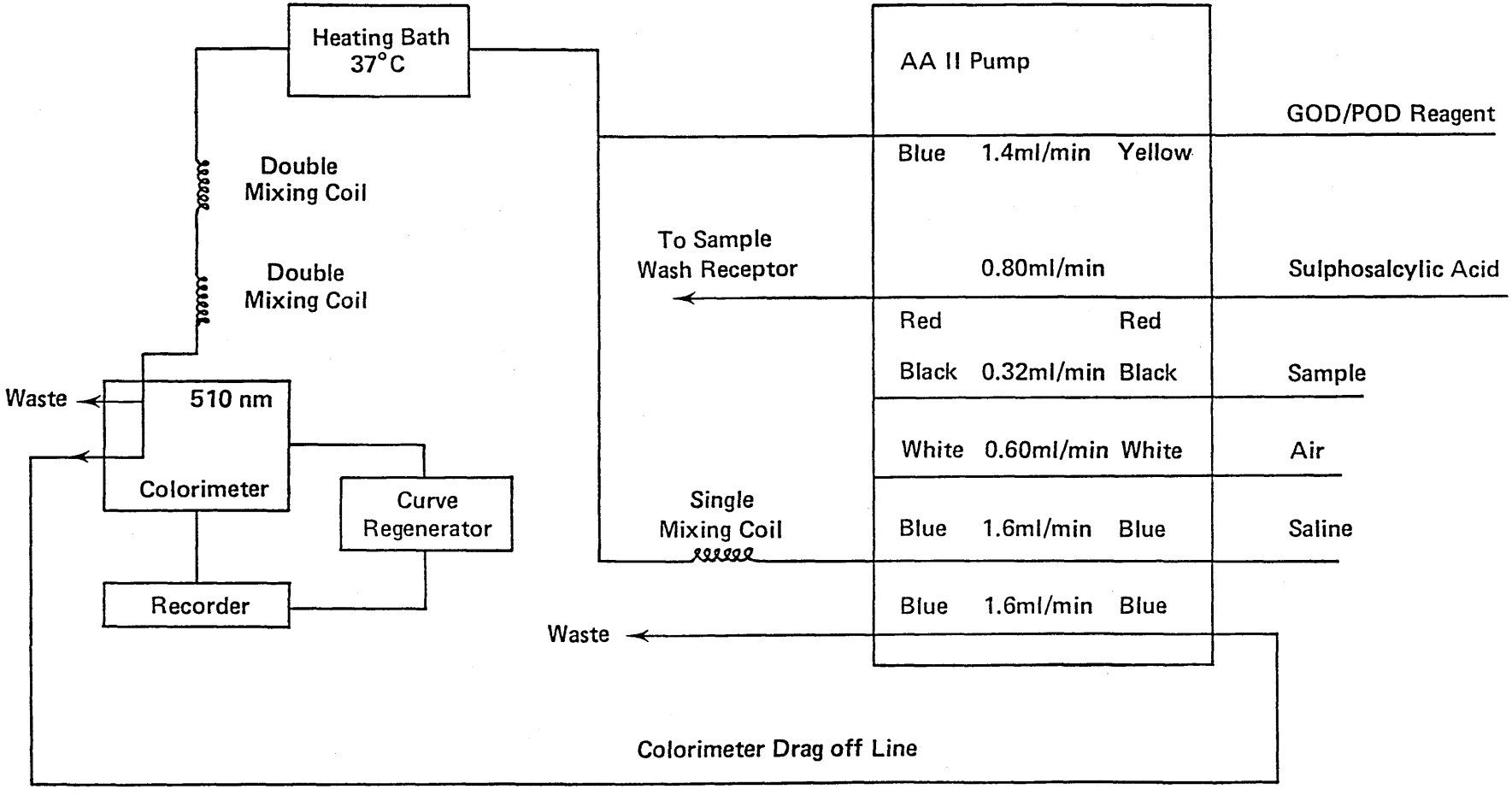


FIG. 2 **Diagram of continuous flow autoanalyser system for filter paper blood glucose measurement.**

acid is added. The sample is then put in a mechanical shaker for 30 minutes - 1 hour, and precipitated protein is removed by centrifugation of the tubes for 10 minutes at 3000 revs/minute. The supernatant is then put into autoanalyser cups, and dialysis was not required after such treatment. Water is pumped out of the sampler wash of the flow system and 1.5% sulphosalicylic acid is run through to ensure that no water is sampled during the run which would give a negative baseline. Diluted aqueous standards are put through first, followed by the blood spot standards and blood spot samples in pairs. A diluted aqueous control is also placed at every tenth sample position to act as a control. Samples with values greater than the top standard are diluted in 1.5% sulphosalicylic acid and repeated. The linearity of the assay is checked using the aqueous standards, and samples are reported to the nearest 0.5 mmol/l. Glucose is estimated spectrophotometrically by the colour development following the reaction:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + 4 \text{ aminophenazone} \xrightarrow{\text{POD}} \text{phenol } 4H_2O +$$

4 (p.benzoquinone-mono-amino) phenazone.

Results: a) Initial experience with the system - We found that complete extraction of glucose was obtained within 30 minutes of the initial elution with 1.5% sulphosalicylic acid and the subsequent mixing, which halved the time suggested by previous workers (493, 496, 498). Continuous sampling of the 30 mmol/l glucose standard produced a pink-red colour. The extinction of this was set at 100 chart units by the use of the 'Span' control. Single sampling of this standard on a 25/25

second wash/sample cycle gave a peak readout of 93 units on the chart. The method therefore achieved in the order of 93% of the steady state, adequate for the analysis. Because baseline drift was apparent in the early stages of development, the sensitivity of the recorder was reduced by backing off the 'Span' control so that a 30 mmol/L glucose sample would read about 75 chart units. In addition two double mixing coils were added to the line between the heating bath and the flow cell. This allowed for colour development to maximise before readout and reduced baseline drift. It was also found that using a smaller pump tube reduced reagent consumption and flow rate without impairing the sensitivity of the method. Using a total sample volume of 100 µl, a sampling rate of 102 per hour was achieved.

b) Collection of filter paper blood spot samples - The dry weight of 100 µL of blood was calculated by weighing 6 filter papers dry and again after spotting them with 100 µl samples in the form of 5 spots on each card. A disc was punched from each spot and weighed individually. The mean volume per disc for the six samples was 10.7 µL (SD 1.5 µL; coefficient of variation 14%). The coefficient of variation for reproducibility of the punching procedure was 2.1%.

c) Glucose Measurement - The analytical precision of the method was calculated from 50 direct measurements of three levels of diluted aqueous controls (mean glucose levels 3.8, 8.3 and 17.2 mmol/L), performed over a period of 12 weeks. The between batch precision was 4.2%, 3.8% and 3.3% respectively.

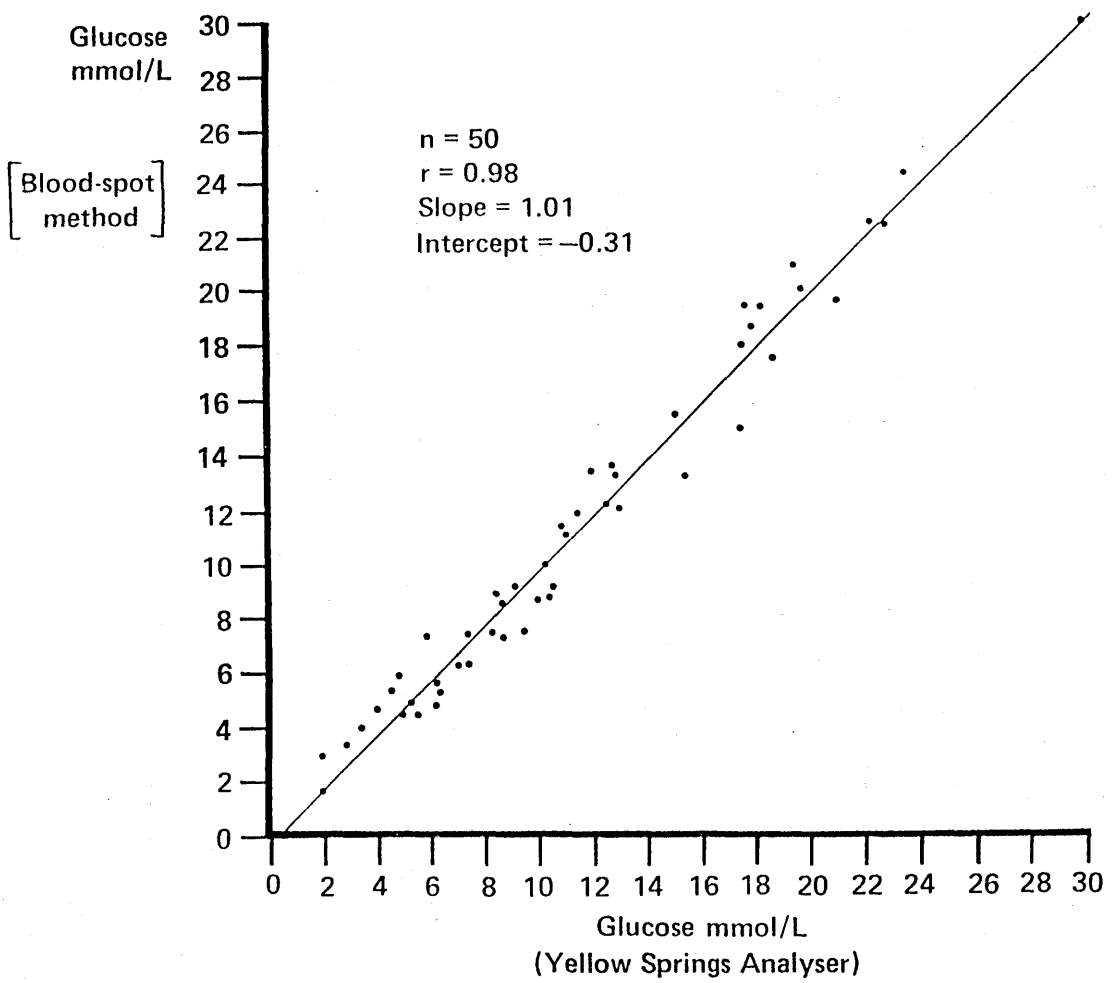


FIG. 3 **Correlation between Yellow Springs analyser and filter paper blood glucose levels in 50 diabetic samples.**

The within-batch precision of the filter paper derived venous blood glucose measurements were also assessed. Twenty four specimens were each assayed ten times, with 8 specimens from one of three possible ranges for glycaemia:

Range 1 3.0 - 7.0 mmol/l. Mean blood glucose 4.7 mmol/l, mean within batch coefficient of variation 4.6%

Range 2 7.0 - 13.0 mmol/l. Mean blood glucose 10.5 mmol/l, mean within batch coefficient of variation 3.6%

Range 3 13.0 - 22.0 mmol/l. Mean blood glucose 17.8 mmol/l, mean within batch coefficient of variation 3.7%.

Between batch precision was evaluated from eight successive duplicate measurements of seven diabetic patients' venous blood samples collected on filter paper, and stored at -20°C over a 4 week period of analysis. The mean and range of glucose concentrations was 11.4 and 3.7 - 26.4 mmol/L, respectively. The mean between batch coefficient of variation was 3.6% (SD 0.4%). The accuracy of the assay was investigated using venous samples from 50 diabetic patients measured on filter paper and compared with results using the Yellow Springs Glucose Analyser (Clandon Scientific Ltd., England). An excellent correlation was found ($r = 0.98$) (Figure 3). Glucose levels in quality control serum measured by both methods also showed a close correlation ($r = 0.99$).

d) Stability Studies - i) Untreated filter paper - The effect of storage of filter paper at varying temperature was investigated. Sixty blood spots were collected from each of eleven diabetic patients, of which 20 were kept at room temperature, and a further 20 were stored at 4°C and -20°C in

sealed plastic envelopes. Duplicate analyses of each specimen were made on the day of collection (day 0) and on days 1, 3, 7, 14, 21, and 28. The range of initial glucose concentrations was 4.2 - 26.1 mmol/L. The mean of duplicate results was calculated as a percentage of the initial value. (Figure 4). After an initial fall in blood glucose concentration over 24 hours irrespective of the storage temperature, samples stored at room temperature fell on average to 81% of the baseline value at one week ($p < 0.001$). Samples stored at 4°C showed less degradation, although this was still significant after 7 days. At -20°C, no significant change in glucose concentration was found over 28 days ($p > 0.5$). To examine the change in glucose concentrations during the first 48 hours after collection, samples from a further 4 diabetic patients were analysed at room temperature after 1,3,5,10, 24 and 48 hours (Figure 5). No change was apparent by 10 hrs, although a significant difference was seen by 24 hours ($p < 0.05$), when a mean fall of 4% was noted, and this had risen to 7% by 48 hours.

ii) The effect of Boric Acid Impregnation of filter paper

Boric Acid has been suggested to prevent glucose degradation in stored filter paper (495). Samples of 6 diabetic patients were collected onto untreated filter paper and filter paper impregnated with 5% boric acid, stored at room temperature and analysed on the day of collection (day 0) and subsequently on days 1,2,4,7,21 and 28. Results for the first seven days are seen in figure 6. Initial levels of filter paper blood glucose for all samples showed that borate impregnation resulted in an

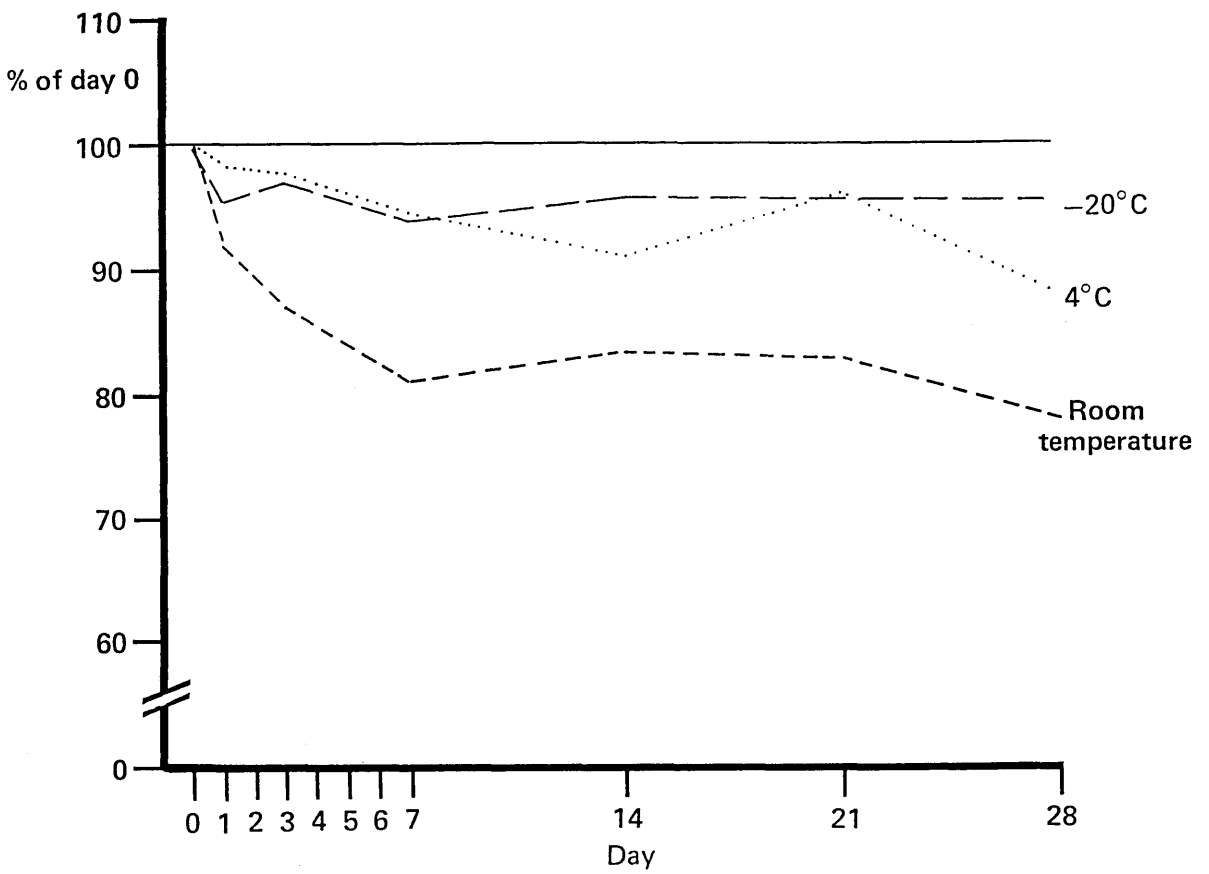


FIG. 4 Long term stability study of filter card blood glucose levels.

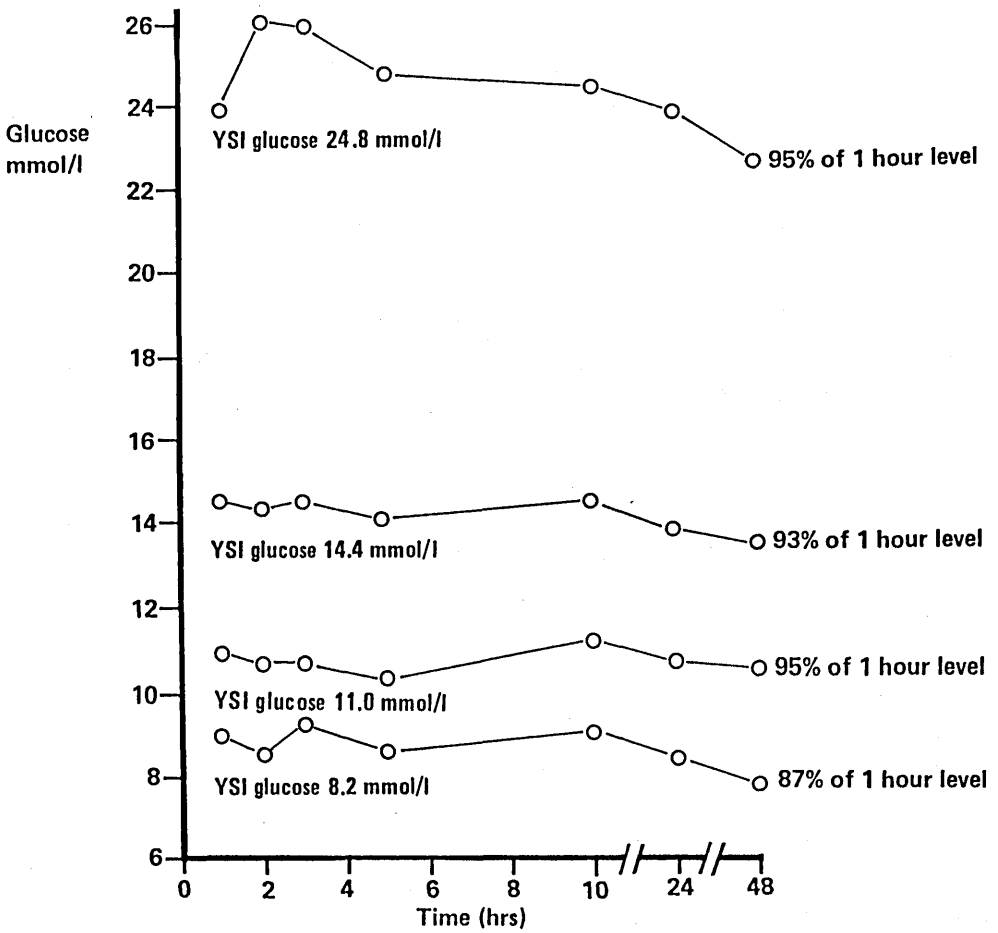
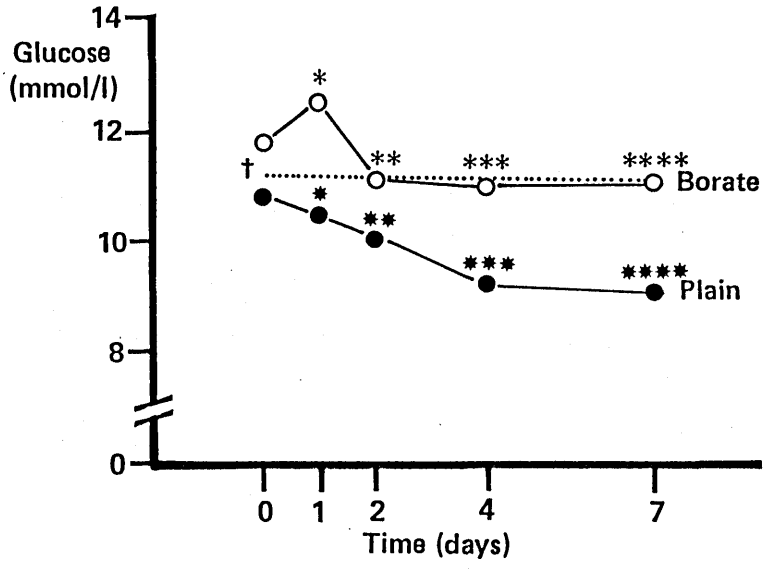


FIG. 5 Short term stability study of filter card blood glucose levels.

apparent rise of 1 mmol/L when compared with untreated filter paper values (mean blood glucose on borate treated paper 11.8 mmol/L, compared to 10.8 mmol/L on untreated paper; $p<0.02$).

Comparison of the borate treated samples with the standard laboratory Yellow Springs Analyser method showed a similar anomaly (mean Yellow Springs Analyser result 11.1 mmol/L; $p<0.025$), whilst there was no apparent difference between the initial untreated filter paper glucose and Yellow Springs blood glucose levels ($p>0.4$). Paired borate-treated and untreated samples were subsequently different on all days of analysis ($p<0.05$). In terms of degradation, glucose levels in borate treated specimens had fallen by 5% by day 2 ($p<0.05$) (in comparison to initial value), which was comparable to the 6% fall ($p<0.01$) seen in the untreated specimens. From day 4 onwards both sets of samples showed significant degradation, but borate impregnation was shown to have a conserving effect by day 7 which was still apparent after 28 days.

e) Comparison with patient derived Reflolux blood glucose recordings - Prior to commencing the home blood glucose monitoring study, I compared filter paper blood glucose results with glucose concentrations determined simultaneously by 14 diabetic patients who were experienced at blood glucose monitoring. 140 (10 x 14) filter paper spots and Reflolux recordings were available for comparison, of which 131 where suitable for analysis. A close correlation ($r = 0.89$) was found, (Figure 7), which reassured us of the feasibility of such a comparison during the home blood glucose monitoring study as well



* Comparison with Day 0 $p < 0.01$ * $p < 0.5$

** " " " " $p < 0.05$ ** $p < 0.1$

*** " " " " $p < 0.01$ *** $p < 0.05$

**** " " " " $p < 0.05$ **** $p < 0.001$

Dotted line represents whole blood glucose value

FIG. 6 Long term study of blood glucose degradation at room temperature in borate-treated and plain filter paper.

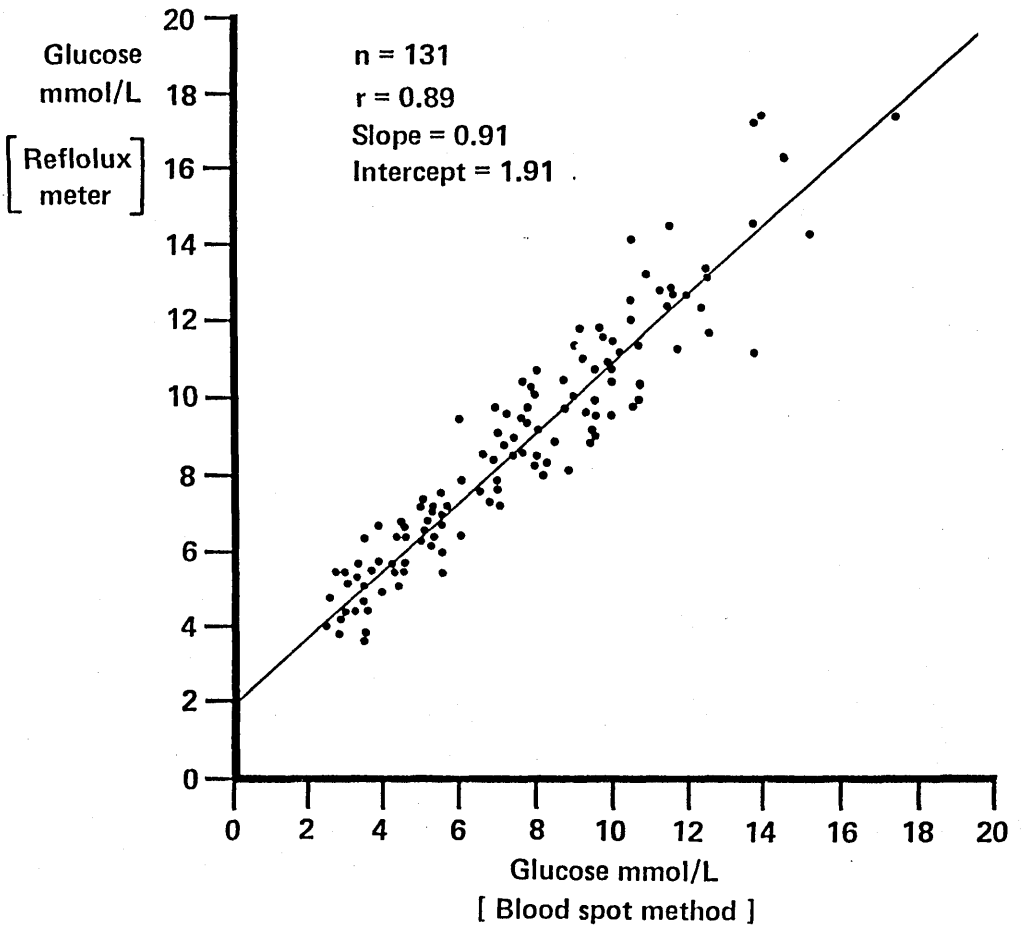


FIG. 7 Correlation between Reflolux meter and filter paper blood glucose levels.

as confirming reliability of data collection, at least in these patients.

Comment: Filter paper blood glucose measurement was investigated and validated for use in the home blood glucose monitoring study. The method developed was relatively inexpensive, and up to 20 profiles (200 estimations) could be estimated in a single working day. Both refrigeration and borate impregnation of the filter cards retarded degradation of blood glucose although the former was more efficient in this respect. The reason for the artefactual rise in glucose following impregnation with borate was unclear, but a small significant loss of glucose thereafter still occurred if filter papers were kept at room temperature. For practical purposes we did not routinely impregnate filter paper with borate but instead used sealed envelopes and instructed patients to store filter cards in the refrigerator prior to bringing them up to hospital the day after completion; thereafter they were kept at -20°C till analysis.

1:4 GLYCOSYLATED (GLYCATED) SERUM ALBUMIN (GSA)

This assay was established in two stages. Initially I performed a series of experiments to validate the performance of a commercial assay. I was assisted in this work by Mrs. Maureen Newman, research technician in the University Department of Medicine, to whom I am most grateful. The realisation of the need for an albumin assay with extremely low sensitivity led to collaboration with the University of Manchester Department of Chemical Pathology in developing an immunoturbidimetric assay. I carried out these later experiments in conjunction with Drs. Paul Reed and Deepak Bhatnagar from Chemical Pathology and Mrs. Hankah Dahr, my research technician. After validation of the assay, the majority of evaluations for the home blood glucose monitoring study were carried out by Mrs H. Dahr and later Dr. P. Kalsi, although I assisted regularly. I also acknowledge the help of Mr. G. Warriner of Pierce and Warriner, Chester, UK, who kindly supplied me with Glycogel B kits for the duration of the study.

i) EVALUATION OF GLYCOGEL B AS AN AFFINITY CHROMATOGRAPHIC METHOD FOR DETERMINATION OF GSA

The Glycogel system (Pierce and Warriner,Chester, UK) is an affinity chromatographic support which incorporates an immobilised ligand (m - aminophenyl-boronic acid) capable of specifically interacting with non-enzymatically glycosylated proteins. My own evaluation of this system for the measurement of GSA was carried at the same time as John and Jones (163)and was later published after modification of the original method (V.I.).

Material and Methods

Sample Collection Clotted blood was collected by venepuncture from diabetic and non diabetic individuals in plastic containers free of preservative. Serum was separated within four hours of sampling and initially stored at -20°C. Samples that could not be analysed within 4 weeks of venepuncture were stored at -40°C.

Materials - Glycogel Analytical Mini Columns containing 1.0ml of immobilized m-aminophenyl-boronic acid on cross linked 6% beaded agarose gel in an aqueous medium containing 0.02% sodium azide were used as the affinity matrix. The Equilibration/Wash Buffer consisted of 250 mmol/L ammonium acetate, 50 mmol/l magnesium chloride, and 3 mmol/l sodium azide, pH 8.3. The Elution Buffer comprised sodium citrate and 0.02% sodium azide, pH 4.5. Colorimetric determination of albumin was made using bromocresol Green reagent (containing bromocresol green, citric acid and trisodium citrate), pH 4.8. In addition bromocresol Purple reagent (containing bromocresol purple, sodium acetate, acetic acid, Brij and sodium citrate (BDH Chemicals, Poole, UK)), pH 5.2, was also assessed as a sensitive indicator of albumin. 1 litre volumes of 0.1N and 0.001N HCl were made up in deairated deionised water to serve as column regeneration and storage solutions respectively. All materials were stored at 4°C between use, and Glycogel B columns were kept out of direct light during storage and during assay runs.

Standard Assay Procedure - The columns and buffers are removed from storage at 4°C and allowed to return to room temperature (20-25°C). The 0.001N HCl storage solution is

withdrawn after removal of the top column stopper only. The bottom column stopper is then removed and the column is placed in a rack. The columns are then equilibrated by adding 5ml of the Equilibration/Wash Buffer (EWB) solution, and the effluent is discarded. The column is then ready for use. Duplicate runs are carried out for all samples. A 200µl sample (either neat or diluted serum) or saline blank is added to the top disc and allowed to pass through. This is followed by 0.5ml of EWB to ensure complete transmission of the sample through the top disc and into the gel. 4.5ml of EWB is then applied to the column followed by 3 consecutive 5ml fractions of the same buffer and the total 20.2ml (0.2ml sample, 20ml EWB) effluent containing the non-glycosylated albumin fraction is collected and mixed well. Thereafter the remaining bound glycosylated albumin fraction which is adherent to the affinity gel is released by applying 3ml of the elution buffer to the column, and the total fraction is collected. 1ml of the non-bound albumin fraction is added to 3ml of the BCG albumin reagent whilst 2ml of the bound albumin fraction is mixed with 1ml of the BCG reagent, and both samples are mixed well. The spectrophotometer is set at 630nM and the absorbance to zero using the column blank non-bound tube. The absorbance of the two fractions is recorded and the % of GSA is calculated from the following equation:-

$$\%GSA = \frac{1.5\ (A_{630}\ B)}{20.2\ (A_{630}NB)+1.5(A_{630}B),} \times 100$$

where A_{630} B is the absorbance at 630 nM of the bound (glycosylated) fraction and A_{630} NB is the absorbance of the non-bound (non-glycosylated) fraction. After use the columns are

regenerated with 5ml of 0.1N HCl solution, discarding the effluent, 5ml of 0.001N HCl column storage solution is then run through the column of which 2ml is kept in the column, with the rest discarded. The columns were then stored in the dark at 4°C for re-use.

The method outlined above is that recommended by the manufacturers and was used in the initial assessment although modifications were made of volumes of sample and albumin reagent when assessing BCP (bromocresol purple) and BCG reagents of differing pH.

Furthermore the method finally found to maximise yield of GSA without overloading the columns with albumin used 100µl of serum diluted 1/16 with 1.5ml of deionised water, of which 100 µl is applied to the column and eluted with only 9.9ml of the EWB. In addition the glycosylated albumin was subsequently eluted by 4ml of a Sorbitol buffer comprising 200 mmol/L Sorbitol, 50 mmol/L EDTA, 100 mmol/L tris (hydroaymethyl) aminomethane and 3 mmol/L of sodium azide with a pH of 8.3 - 8.8.

Results: (1) Normal and Diabetic Ranges - Samples were taken from 25 healthy non-diabetic individuals without clinical or biochemical evidence of hepatic or renal dysfunction, and compared with 53 samples from insulin treated diabetic patients with varying degrees of glycaemic control. The observed range in the healthy controls was 0.46 - 3.42%, mean value 1.7%; 95% confidence intervals 0.3 - 3.1%. This compared favourably with the 0.80 - 3.20% range, mean value 2.0%, quoted by the manufacturers of the kit. The range of values in the diabetic samples was 0.70 - 20.06% with a mean of 7.61% which was

significantly higher ($p<0.0001$) using Student's unpaired t-test. The highest value in the control population belonged to an obese individual who was later found to have impaired glucose tolerance in response to a 75g glucose tolerance test. The lowest value from the diabetic samples belonged to a well controlled insulin-treated diabetic with chronic pancreatitis secondary to alcoholism who had experienced recurrent hypoglycaemic episodes. The maximum figure of 20.06% was recorded in a young insulin dependent diabetic female who was in diabetic ketoacidosis at the time of sampling, having stopped her insulin therapy for a period of 2-3 weeks prior to hospital admission.

(2) Effect of Albumin Concentration (Experiments 1-4) - The effect of varying the volume of sample and thereby the concentration of albumin was investigated to examine whether applying increasing albumin concentrations exceeded the capacity of the columns to remove all glycosylated albumin. 5ml of a healthy control serum (GSA value estimated from 200 µl sample as 1.44%) was applied to the column, and the serum collected after passage through the column gel. When this was subsequently reapplied to a fresh column, a GSA value of 1.16% was estimated which demonstrated that the column was inefficient at trapping all GSA when such high albumin loads (roughly 200 mg) were applied. In fact it took a further 4 passages of the original 5cc volume before no further GSA was detected.

I then went on to examine the effects of loading much smaller volumes of serum (10 µL to 400 µL) with albumin loads roughly varying between 0.4 and 16mg. It became apparent that no reproducable measurement was obtained with serum volumes of less

than 50 µL because of the insensitivity of the BCG dye at such low albumin concentrations (v.i.). However a clear relationship between reducing serum volumes and increasing GSA values was observed in healthy control and controlled and ketotic diabetic sera (Table 8), which confirmed that 50-100 µL was probably a more suitable volume of serum to apply, although this was later found to stretch the capacity of the BCG method to determine albumin levels.

Table 8 EFFECT OF SERUM VOLUME (ALBUMIN LOAD) ON RECOVERY OF GSA (%)

	VOLUME OF SERUM APPLIED			
Sample	50 µL	100 µL	200 µL	400 µL
HEALTHY CONTROL	3.58%	2.81%	2.33%	2.20%
CONTROLLED DIABETIC	7.51%	7.33%	6.43%	6.10%
KETOACIDOTIC DIABETIC	23.63%	18.11%	18.55%	17.60%

It can be seen that applying 50 µL of serum (roughly 2mg of albumin) increased the yield of GSA by around 54% in healthy controls and by 17-27% in the diabetic samples. A similar pattern emerged in several other patients when different amounts of sera were applied to columns, which tended to confirm that overloading the column with albumin reduced the yield of GSA.

I then attempted to demonstrate whether GSA was definitely lost with the elution buffer during overloading or whether it remained adherent to the column matrix. I applied 200 µL of diabetic serum to 2 columns, directly measuring the % GSA by the standard method in one case, and eluting the second column with 5

5 ml of wash buffer, which would in theory have only removed non-glycosylated albumin. The eluted 5.2ml volume was then reapplied to a fresh affinity column to which 3 ml of albumin elution buffer was applied, to remove any GSA that had not adhered to the original column. 8.77% of albumin was found to be glycosylated after direct measurement by the standard method, but 1.44% of albumin was found to be glycosylated in the eluted fraction after the second passage. This confirmed that adding 200 µL of serum (8mg of albumin) did overload the column, leading to loss of GSA from the matrix during elution with the wash buffer. In effect some 14.1% of the glycosylated albumin $\left(\frac{1.44}{8.77 + 1.44} \times 100\ \%\right)$ passed through the column without adhering thereby underestimating the true % GSA.

These observations do not of course preclude the possibility that the failure of the columns to adhere all the glycosylated albumin was secondary to too high a concentration of albumin rather than the absolute quantity of albumin applied to the column. Accordingly I studied the effect of prediluting the 200 µL serum samples of 2 diabetic patients with 2ml of wash buffer prior to passage through the column, and examined whether reapplying the 200 µL of serum once it had been washed through the column with 5ml of wash buffer would alter the recovery of GSA. In both cases the routine procedure was followed after these initial modifications (Table 9).

Table 9. EFFECT OF PREDILUTION AND POSTDILUTION OF 200 µL SERUM SAMPLE ON RECOVERY OF GSA FROM DIABETIC SERA

	STANDARD METHOD	PREDILUTION WITH 2ml of EWB	REPASSAGE (POSTDILUTION) of 5.2ml (SAMPLE PLUS 5ml EWB)
% GSA			
DIABETIC (1)	11.28	11.16	10.15
DIABETIC (2)	10.85	10.02	9.15

Both diabetic samples show that predilution does not increase the yield of GSA, and that rather then encouraging adherance of GSA which was erroneously eluted during the initial passage by reapplication, repassage of the diluted sample leads to a further reduction of GSA which is outwith the standard deviation for the assay performance at this level of GSA (v.i.) and suggests if anything that repassage of the sample with wash buffer might lead to elution of GSA which is less strongly bound to the boronate matrix.

3. Precision - (Experiments 5-6) - The within and between batch variation was analysed for 10 pairs of duplicate samples at 3 different levels of glycosylation (non-diabetic, and well controlled and poorly controlled diabtic serum) and the results are shown in Table 10.

Table 10 PRECISION FOR GSA USING AFFINITY CHROMATOGRAPHY

GSA (%)	WITHIN BATCH (no. = 10)	BETWEEN BATCH (no. = 10)
NON DIABETIC MEAN (SD)	2.78 (0.20)	1.85 (0.39)
COEFFICIENT OF VARIATION (%)	11	23
WELL CONTROLLED DIABETIC MEAN (SD)	7.00 (0.45)	7.40 (0.55)
COEFFICIENT OF VARIATION (%)	7	8
POORLY CONTROLLED DIABETIC MEAN (SD)	16.80 (0.60)	16.80 (0.96)
COEFFICIENT OF VARIATION (%)	3	8

As expected the method was much less accurate within the normal range although it appeared reproducable in diabetic samples.

4. Effect of Freezing and Thawing Samples (Experiment 7) - I studied the effect on recovery of GSA of storage and thawing separated aliquots of the same diabetic sample on 5 occasions over a 2 week period. The initial level was 3.79% and over the period of observation after repeated rethawing the mean value was 3.43%, which is not signifcantly different, as the between run mean and standard deviation for the sample was 3.21 $\pm$0.41%. In addition all other samples measured during the two week period after thawing were within the standard deviations of the between run mean value (Figure 8).

This suggests that rethawing of samples does not alter the recovery of GSA. The fact that the % GSA after 2 weeks freezing was 3.43% in comparison to 3.79% in the initial run also confirmed that freezing itself did not alter the level of GSA.

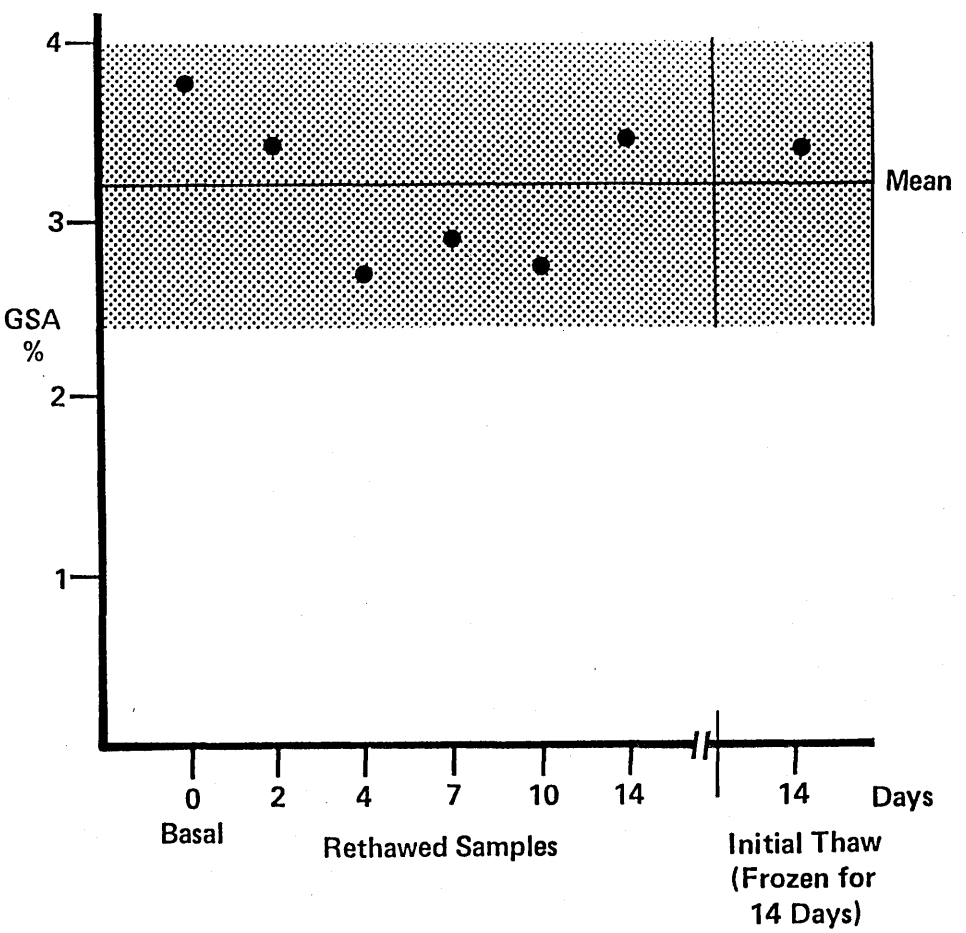


FIG. 8 Effect on recovery of GSA of freezing and rethawing samples.

5. Regeneration of the Gel (Experiments 8 - 10) - The efficiency of the boronate gel to bind GSA after being used several times was assessed by measuring a sample from a diabetic subject on a fresh column and on columns that been used 2,6 and 10 times. On these columns the initial GSA was 6.77% and was thereafter 6.70%, 6.68% and 6.60%. Other experiments using columns where colour changes or air bubbles had developed in the gel showed that either of these alterations led to significant reductions in recovery of GSA (greater than 20% of the original values).

6. Effect of Prior Dialysis of Samples (Experiments 11 -12) In order to investigate the possibility that the Glycogel Affinity Chromatography might bind labile Schiff base GSA as well as the stable fraction, which would have necessitated a time consuming preliminary pretreatment of samples by hydrolysis or dialysis, I examined the change in GSA in duplicate samples run before and after dialysis to remove any free glucose bound to albumin. 5 diabetic samples with high levels of ambient blood glucose at the time of sampling were studied before and after a 24 hour period of dialysis of the samples against normal saline at 4°C. The results are shown in table 11.

Table 11. EFFECT OF DIALYSIS WITH SALINE ON GSA RECOVERY, AND LEVELS OF BLOOD GLUCOSE AND SERUM ALBUMIN

	BLOOD GLUCOSE mmol/l		SERUM ALBUMIN g/L		GSA (%)	
SAMPLE NO.	PRE	POST	PRE	POST	PRE	POST
1	17.0	0.1	36	35	3.98	3.98
2	19.0	0.5	43	38	5.57	5.78
3	15.8	0.2	37	32	5.76	6.06
4	19.0	0.8	37	35	14.86	15.79
5	17.1	0.8	45	42	7.54	7.56

This experiment confirmed that the affinity chromatography method satisfactorily measured stable GSA and was unaffected by removal of free glucose which could artifically have raised GSA levels if labile GSA was also isolated by affinity chromatography. Even with dilution of samples with saline as evidenced by falls in albumin concentration, no change in GSA has resulted. This suggests that separation of the labile (Schiff base) and stable forms of GSA took place during the initial passage of serum through the affinity matrix.

I considered whether the mechanism of this separation was due to glucose separating from albumin and itself adhering to the boronate ligand in the gel. However after passing a sample of serum down the column, followed by a glucose oxidase reagent (Glucose Oxidase/ Peroxidase, Phenol and 4-amino-phenozene) which would have produced a specific colour change in the presence of significant amounts of glucose, no such reaction took place after 24 hours incubation at room temperature, suggesting that the

boronate adhered only stable GSA without affinity for free glucose or labile GSA.

7. Linearity (Experiment 13) - In order to assess linearity of the GSA assay, the % of GSA was determined in samples from a healthy control and a poorly controlled diabetic, and was found to be 2.07% and 15.04% respectively. 200 µL serum loads of a pooled sample in varying amounts (0 - 100 % of diabetic serum mixed with control serum) were then applied to the column, in varying dilutions (up to 1/4) and left on the columns for varying periods of time (0 - 90 minutes). The most reproducable and linear results were obtained when samples were diluted 1/4 with saline and left on the column for 1 hour, confirming that linearity of GSA with varying amounts of diabetic Vs control sera was a feature of the assay (Figure 9).

8. Effect of Temperature Variation (Experiment 14) - I investigated the effects of the ambient temperature on the affinity system in both the normal and diabetic range by cooling the chamber where experiments were carried from 25°C to 20°C. A decrease in GSA% of less than 5% was found when the temperature fell from 25°C to 20°C for both diabetic and control samples with negligible differences observed for the former.

9. Effect of Variation in pH and Type of Dye (Experiments 15 -16) - Suggestions of improved sensitivity using more specific dyes to measure albumin led to assessment of bromocresol green (BCG) dyes of varying pH and bromocresol purple. The standard BCG dye which was supplied with the kit had a pH of 4.8. Increasing or

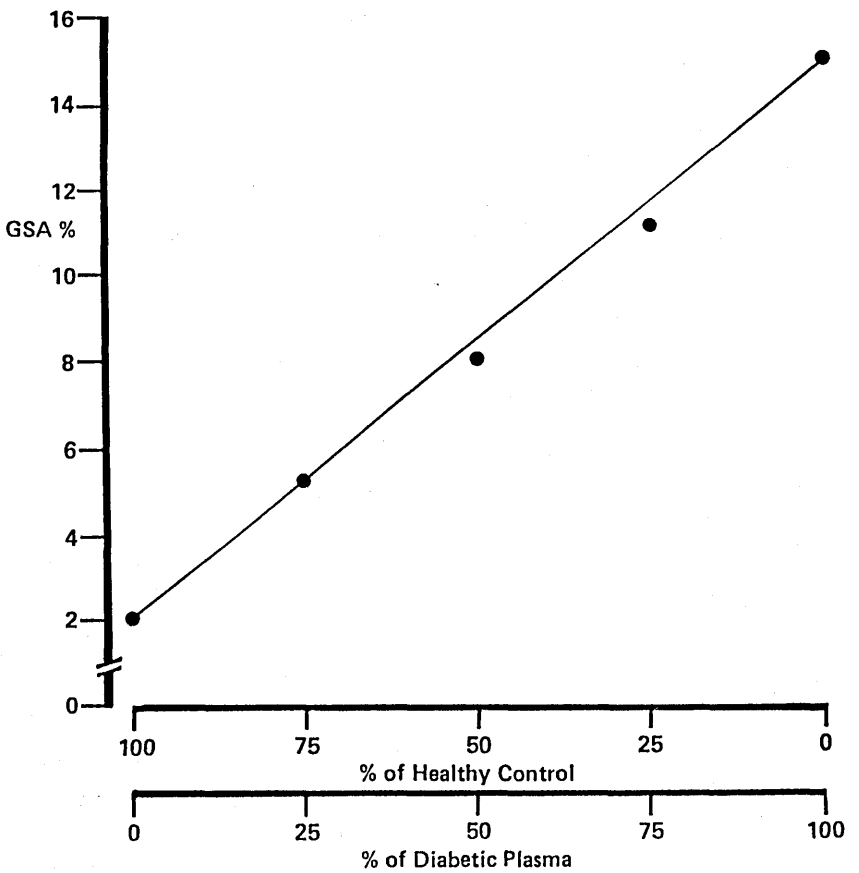


FIG. 9 Linearity of GSA Assay.

reducing the pH of BCG within the range 4.2 -7.0 produced reactions but standardisation was difficult and did not lead to improved reproducibility over the suggested method. Because bromocresol purple (BCP) is supposed to be more sensitive than BCG in measuring smaller quantities of albumin, I investigated whether BCP would prove more useful in measuring GSA in the DIABETIC range. Paired duplicate runs using the BCP and BCG dyes were carried out on 5 diabetic samples. The pH of the commercial BCP was 5.2 and the consequent yellow colouration after reaction with buffers was different to the green colouration with the BCG dye. It was found by trial and error that whilst the maximum absorption with BCG was with the spectrophotometer setting at 630 nm, it was 590 nm for BCP. The measured GSA comparing BCP or BCG reagents is seen in table 12.

Table 12. COMPARISON OF BROMOCRESOL PURPLE (BCP) WITH BROMOCRESOL GREEN (BCG) IN THE DETERMINATION OF GSA

DIABETIC SAMPLE	STANDARD BCG METHOD	BCP METHOD
1	5.70%	0.94%
2	8.11%	3.45%
3	7.75%	4.07%
4	8.07%	3.22%
5	8.10%	4.52%

A consistent reduction in measured GSA was observed using the BCP. Despite increasing the absorption by setting the spectrophotometer to 590 nm, the pH of albumin elution and wash buffers were found to be outside the range where BCP produced a reproducable measureable colour reaction, so that the BCP solution of pH 5.2 was unsuitable for GSA measurements.

10. Accuracy - John and Jones (163) claimed that despite apparent reproducibility of the assay the lack of a standard and insensitivity of BCG to detect low levels of albumin precluded the use of the kit for clinical practice in its existing form. Consequently I investigated the accuracy of both BCG and BCP to detect low concentrations of albumin to definitely state whether or not dye binding techniques could be applied to the measurement of albumin in both glycosylated and non-glycosylated fractions.

A standard human albumin solution (Hoechst Pharmaceuticals, Hounslow, UK) was made up in doubling dilutions in saline in the range 5-1280 µg/ml. The absorption on the spectrophotometer was measured at 630 nm for BCG (added in a ratio of 1/2 with the albumin) and at 600 nm for BCP (also added in a ratio of 1/2 with the albumin solution). The capacity of the two dyes to measure varying concentrations of albumin is seen in figure 10.

Whilst BCP is undoubtedly more accurate at measuring albumin that BCG, there is a definite lack of correlation below albumin concentrations of 80 µg/ml. In trying to exploit the assay to its full it was previously stated that the affinity column gave the highest yield of GSA when 50 µL of undiluted serum was applied thereby taking care not to overload the capacity of the boronate gel to efficiently trap all glycosylated albumin (163). Assuming an average albumin concentration of 40g/L of serum, roughly 2mg of albumin would be present in the 50 µL serum sample, effectively a concentration of 40 mg/ml of albumin, which would be the maximum load which could safely be applied to the column. Previous experiments suggested that the ideal volume

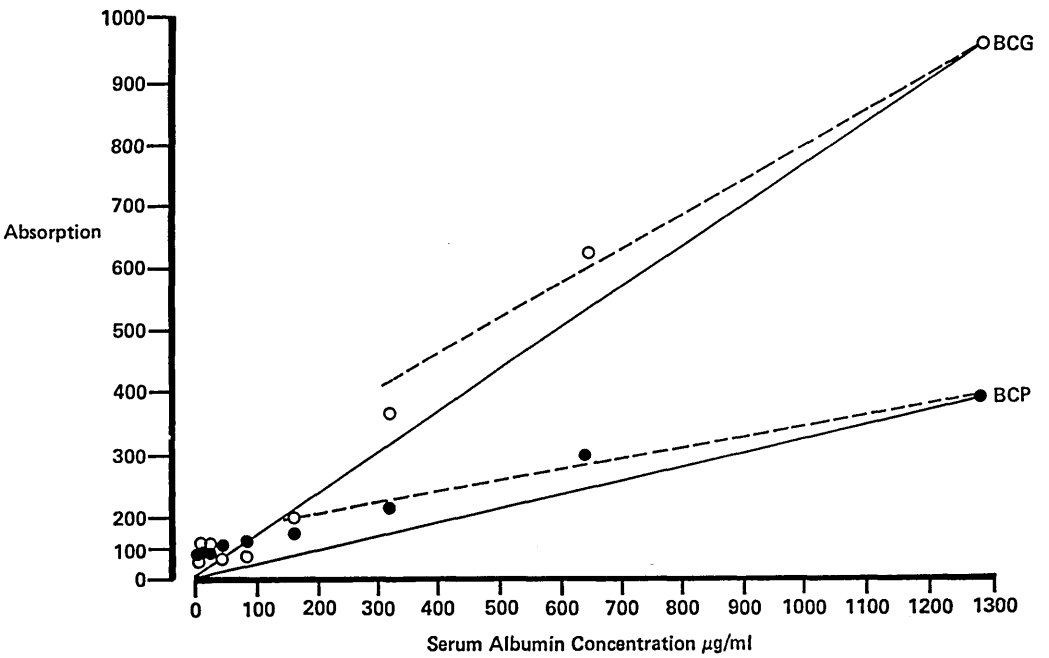


FIG. 10 Accuracy of BCG and BCP in measurement of low albumin concentration.

of serum to apply for maximum affinity for GSA was between 25 and 50 µL which is roughly equivalent to albumin concentrations of 1000 µg/ml and 2000 µg/ml. Whilst both BCP and BCG would be expected to measure these concentrations of albumin reliably in undiluted serum from the accuracy experiments, these dyes also have to be capable of measuring the glycosylated fraction. In a diabetic sample where 10% of albumin may be glycosylated only the BCP could reliably estimate the 100-200 µg/ml of glycosylated albumin. In normal samples where perhaps only 2.5% of albumin is glycosylated, even BCP would be unreliable in measuring the 25 - 50 µg/L concentrations.

My conclusion from the above experiments is that the affinity column component of the method is robust and efficient in separating glycosylated from non-glycosylated albumin if small volumes (less than 100 µl) of plasma are applied to the column, but that dye binding methods of measuring albumin are too insensitive to measure the small amounts of albumin present in the glycosylated fractions.

If a 50 µL non-diabetic serum sample is applied to the column and GSA diluted with 3ml of albumin elution buffer, a concentration of roughly 13 µg/ml of albumin might be present if 2% of the original albumin load was glycosylated. With this is mind I then went on to explore other more accurate methods capable of determining such low levels of albumin concentration.

ii. VALIDATION OF GSA MEASUREMENT BY AFFINITY CHROMATOGRAPHY AND IMMUNOTURBIDIMETRY

I carried out an assessment of a combined affinity

chromatography and immunoturbidimetric assay for the precise measurement of GSA with the assistance of Mr. Paul Reed and Dr. Deepak Bhatnagar of the Dept. of Chemical Pathology at Hope Hospital and Mrs Hankah Dahr who worked as a research technician with me during the study.

a) Materials and Methods

Samples - Fasting venous blood samples were collected and sera separated by ultracentrifugation and stored at -20°C as previously described.

Affinity Chromatography - The method employed the Glycogel B kit which was used as previously described except that 100µL of serum diluted 1/16 in water was applied to the column and only 9.9ml of wash buffer was then applied to the column. It was also found that glycosylated albumin was best eluted using 4ml of sorbitol buffer (200 mmol/L sorbitol, 50 mmol/L EDTA, 100 mmol/L Tris and 3 mmol/L sodium oxide; pH 8.8), as opposed to the sodium citrate solution in the original experiments.

Immunoturbidimetric measurement of albumin

Reagents: Antiserum - Goat antihuman albumin (Atlantic Antibodies Antiserum ALB2) of nephelometric grade was obtained from American Hospital Supplies (UK) Limited, Didcot, UK. This was diluted 1 in 170 with 4% PEG (polyethylene glycol 6000) in phosphate buffered saline (0.3g/L NaH_2PO_4, $2H_2O$, 1.15g/L Na_2HPO_4, 8.6g/L NaCl; pH 7.4) (Mercia Brocades M99A, UK), left for 10 minutes and centrifuged for 5 minutes at 1500g at room temperature before use.

Standards: A stock standard was prepared from BR99 (reference normal human serum, Unipath Limited, Bedford, UK) by 1/1000 dilution in the affinity chromatography wash buffer (pH 8.8) with 0.05% sodium azide as a preservative. This solution was stable for at least one month at 4°C. Before each assay appropriate dilutions were made in wash buffer to cover a standard curve from 1mg/L to 20 mg/L. A pooled serum sample from healthy controls and from poorly controlled diabetics (20ml samples) served as glycosylated albumin standards.

Controls: Working controls were prepared at concentrations corresponding to 2.6 mg/L (QC1), 8.7 mg/L (QC2) and 15.4 mg/L (QC3) from a stock solution prepared by a 1/1000 dilution of Hoechst 041015 (material of human origin, Behring Diagnostics, Hoechst House, Hounslow, UK) in affinity chromatography wash buffer (pH 8.8) with 0.05% sodium azide added.

Instrumentation for Albumin Measurement: The glycosylated and non-glycosylated albumin fractions were separated using affinity chromatography as previously described with diluted serum, and sorbitol was used as an albumin elution buffer. The eluates containing glycosylated albumin were assayed neat whilst those containing non-glycosylated albumin were diluted 1/2 in the wash buffer. Albumin in the two eluates was measured by immunoturbidimetry on a centrifugal analyser, the Multistat III plus F/LS (Instrumentation Laboratories (UK) Limited, Warrington, Cheshire, UK), which provided initial data. The method was later adapted to run on the Cobas Fara analyser (Roche Products Ltd., Welwyn Garden City, Herts., UK). Instrument settings for both

centrifugal analysers use shown in Tables 13 and 14. The % glycosylated serum albumin was determined from the following equation:

$$\% \text{ GSA} = \frac{\text{GSA concentration} \times 100}{\text{GSA concentration} + 4.88\ (\text{non-glycosylated albumin concentration})}$$

b. Results

1. Normal and Diabetic ranges of GSA - GSA was measured in 25 healthy control subjects and in 37 type 1 insulin-dependent diabetic patients. The range of % GSA obtained in the healthy controls was 2.3 to 6.3% with a mean value and standard deviation of 3.42±0.97%. The predicted normal range was therefore 1.5-5.4% (95% confidence intervals) which is similar to although greater than the 0.3-3.1% range described using the Glycogel B method with subsequent albumin measurement by dye binding techniques. In the 37 insulin-dependent diabetics GSA varied from 2.1 to 15.1% with a mean value and standard deviation of 7.9 ±3.3%, so that considerable overlap between the control and diabetic ranges was observed (figure 11).

2. Optimisation of Albumin Measurement - Increasing the percentage of polyethylene glycol from 4% to 6% which is added to the antisera had no apparent effect on the sensitivity of albumin detection. However when the sample size for measurement was increased from 20 µL to 36 µL with an equivalent alteration in the dilution of antisera (originally 1/300; with alteration in sample dye 1/200 x 36/20 = 1/170 as described in method section), the Δ Albumin across the standard curve doubled and precision was improved. Altering the pH of the antisera solution altered the yield, more alkaline pH increasing and acidic pH reducing the

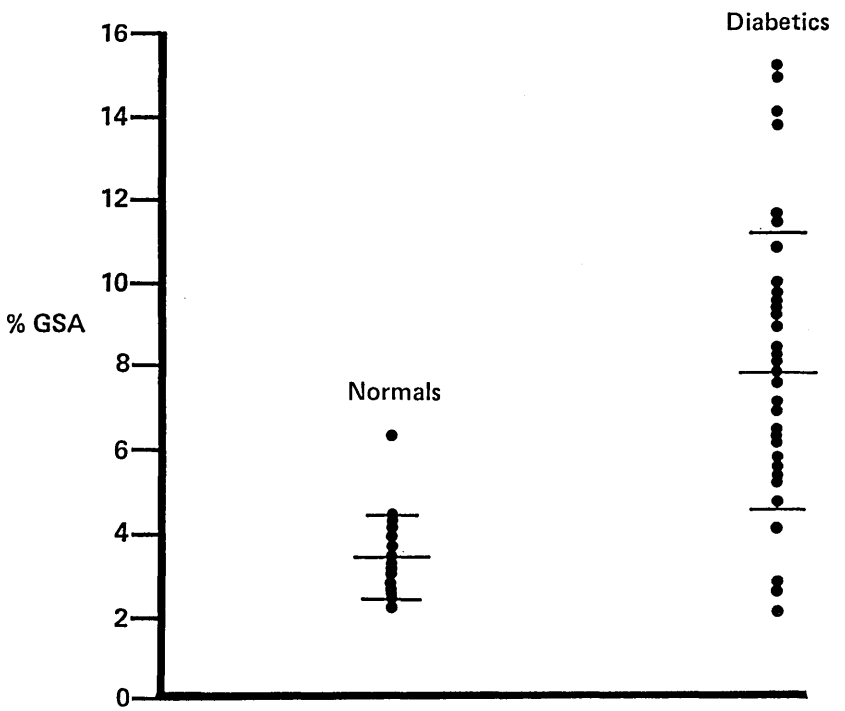


FIG. 11 Range of GSA in normals and diabetics.

Lines are Mean ± 2 SD

results, so both eluents had to have the same pH, which is why sorbitol rather than sodium citrate was used to elute GSA from the affinity column.

3. Standardisation and Parallelism - Human serum albumin standard prepared in the wash buffer or the sorbitol buffer gave almost superimposed standard curves (figure 12) thus enabling one set of standards prepared in wash buffer at pH 8.8 to be used in the assay of both glycosylated and non-glycosylated fractions. In addition when serial dilutions of human serum albumin were made in wash buffer (pH 8.8) and put through the albumin assay, parallelism was observed with the standard curve showing linearity of the assay to 18mg/L.

4. Accuracy - The lower limit of detection was assessed by assaying the albumin eluted in column wash buffer 10 times within batch. The resultant mean value was 0.39 mg/L with a standard deviation of 0.21 giving a detection limit of 0.81 mg/L (upper 95% confidence limit).

5. Carry Over - Wash buffer was placed after the highest standard (20 mg/L) in the assay run on 6 occasions, and carry over of albumin was found to be negligible compared with the detection limit (range -0.3 to 0.9 mg/L, mean 0.32 mg/L).

6. Stability Studies - Both glycosylated and non-glycosylated albumin fractions in 10 samples of varying concentrations were found to be stable when stored at 4°C for 22 hours (Table 15).

Table 13. INSTRUMENT SETTINGS ON THE MULTISTAT III PLUS F/LS FOR ALBUMIN MEASUREMENT

The DILUTOR:

SAMPLE VOLUME	36 µL
VOLUME DILUENT	60 µL
VOLUME 2nd REAGENT	0
POSITION 2nd REAGENT	0
VOLUME DILUENT	10 µL
VOLUME 1st REAGENT	200 µL
POSTION 1st REAGENT	1
1st SAMPLE	2
LAST SAMPLE	20
REFERENCE CUVETTE	1w

ROTOR FORMAT:

CUP 1	REFERENCE, EMPTY
CUP 2	BLANK, WASH, BUFFER
CUPS 3 - 7	STANDARDS
CUP 8	QC 2
CUP 9	QC 1
CUPS 10 - 17	SAMPLE ELUATES
CUP 18	HIGH POOLED GSA STANDARD ELUATES
CUP 19	LOW POOLED GSA STANDARD ELUATES
CUP 20	QC 3

INSTRUMENT PARAMETERS:

STANDARD 1	1mg/L
STANDARD 2	5mg/L
STANDARD 3	10mg/L
STANDARD 4	15mg/L
STANDARD 5	20mg/L
TEMPERATURE	30 °C
CALIBRATION MODE	2
REAGENT BLANK	2
UNITS	mg/L
DATAPOINTS	12
FILTER CODE 1	340nM
FILTER CODE 2	340nM
STARTMODE	2
DELAY INTERVAL	3 secs
DATA INTERVAL	90 secs
DATA MODE	4
ANTIGEN EXCESS CHECKPOINT	5
ANTIGEN EXCESS DETECTION	10
PRE INCUBATION TIME	0 secs

Table 14. INSTRUMENT SETTINGS ON COBAS FARA FOR ALBUMIN MEASUREMENT

REACTION MODE	P-T-AO
CALIBRATION MODE	NON-LINEAR
WAVELENGTH	340 nM
TEMPERATURE	30 °C
SAMPLE	34 µL
REAGENT	200 µL
DILUENT	56 µL
FIRST READING	0.5 secs
INTERVAL	19 secs
NUMBER OF READING	25
STANDARDS	1. WASH BUFFER
	2. 1.0 mg/L
	3. 5.0 mg/L
	4. 10.0 mg/L
	5. 15.0 mg/L
	6. 20.0 mg/L
CALCULATION MODEL	POLYNOMIAL 5

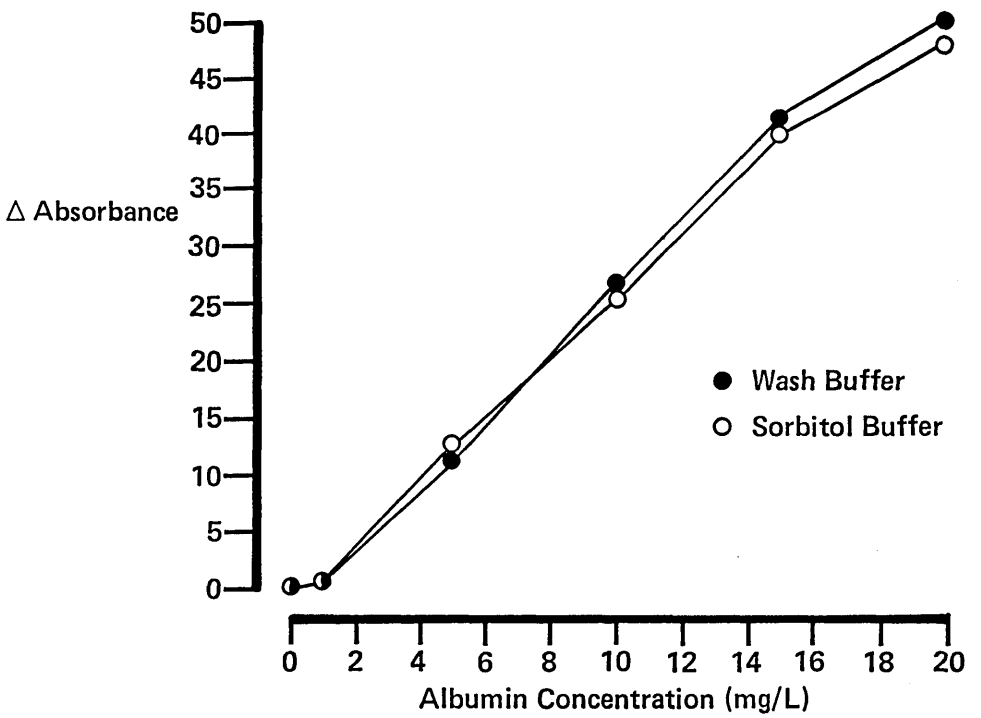


FIG. 12 Standard curves for albumin measurement with standards prepared in wash buffer and sorbitol buffer.

7. Recovery - i) FROM AFFINITY CHROMATOGRAPHY - The total albumin concentration was measured in 10 cases in the original sample prior to application to the column and related to the albumin content measured in the bound and non-bound albumin fractions after passage through the columns. The mean recovery was 101.5%. Recoveries of less than 85% were subsequently encountered in up to 10% of cases which necessitated routine assessment of recovery and repeating measurements when necessary.

Table 15 STABILITY OF GLYCOSYLATED AND NON-GLYCOSYLATED ALBUMIN FRACTIONS STORED FOR 22 HOURS AT 4°C.

	GLYCOSYLATED FRACTION mg/L		NON-GLYCOSYLATED FRACTION mg/L		% GSA (ABSORPTION)	
	4 hours	22 hours	4 hours	22 hours	4 hours	22 hours
1	3.2	3.7	16.4	17.4	3.8	4.1
2	3.8	4.3	13.1	15.4	5.6	5.4
3	4.1	4.5	13.6	15.6	5.8	5.6
4	4.8	5.5	13.3	13.8	6.9	7.6
5	5.5	6.2	14.1	14.4	7.4	8.1
6	2.1	2.4	16.6	17.1	2.5	2.8
7	6.7	6.7	15.2	16.3	8.3	7.8
8	7.8	8.0	13.8	15.3	10.4	9.8
9	10.3	11.8	12.8	14.9	14.2	14.0
10	1.4	1.7	14.1	16.1	2.0	2.1

Recovery - ii) FROM IMMUNOTURBIDIMETRIC ASSAY OF ALBUMIN - Known amounts of human serum albumin in wash buffer were added to fractions containing 3mg glycosylated albumin and put through the immunoturbidimetric assay. The percentage recovered when 5mg/l was added to give a concentration of 8.0 mg/l was 96%, 99% when 10mg was added to give an expected concentration of 13mg/l and 98% when 15mg was added to give an expected yield of 18mg.

8. Precision - 10 duplicate samples of working controls of varying concentrations were measured simultaneously to calculate the within-batch coefficient of variation and 3 other duplicate samples of varying concentrations were measured on 20 separate occasions to produce between-batch coefficient of variations to assess precision of the immunoturbidimetric component of the assay. The data are shown in table 16. I also assessed the precision of the combined affinity chromatography and immunoturbidimetric procedure for the determination of GSA of varying levels in diabetic samples. The results are shown in Table 17, and confirm that the method was reproducable.

Table 16. WITHIN AND BETWEEN BATCH PRECISION ON ALBUMIN CONCENTRATION IN WORKING CONTROLS MEASURES BY IMMUNOTURBIDIMETRY

WITHIN BATCH COEFFICIENT OF VARIATION (CV)

Sample Concentration (mg/l)	CV(%)	Standard Deviation
1.59	20.4	0.3
4.55	6.5	0.3
8.05	1.5	0.1
13.50	4.9	0.7
18.57	2.4	0.4

BETWEEN BATCH COEFFICIENT OF VARIATION (CV)

Sample Concentration (mg/l)	CV(%)	Standard Deviation
2.65	13.8	0.4
8.89	4.7	0.4
15.51	4.6	0.7

Table 17. WITHIN AND BETWEEN BATCH PRECISION (CV) OF GSA MEASUREMENT IN DIABETIC SAMPLES.

% GSA	No. of Measurements	CV(%)	Std. Deviation
i. WITHIN BATCH			
3.2	9	9.9	0.3
7.7	9	4.4	0.3
13.2	9	3.5	0.5
ii.BETWEEN BATCH			
3.0	10	11.0	0.3
7.6	9	6.2	0.5
17.7	8	3.7	0.7

Comment: The immunoturbidimetric measurement of albumin was found to be sensitive and reproducable enough to allow reliable measurement of the small concentrations of albumin that could safely be applied to the affinity chromatographic columns without overloading them. The major advantage of this method was that by measuring the total albumin concentration in serum prior to application to the column, and the albumin concentration in the glycosylated and non-glycosylated fractions, I could confidently state the concentrations of glycosylated albumin and confirm whether or not recovery of albumin from the column was complete. In the event mean recovery was 101.5% in the initial series of experiments although subsequent experience found less than 85% albumin recovery from the columns in up to 10% of cases. This was usually in columns where the boronate gel was discoloured or where air bubbles were evident within the gel. Discarding such columns minimised the frequency of reduced recovery of albumin from columns. Although no glycosylated albumin standard was initially available we confirmed reliabilty between-batches by the use of pooled high and low GSA sera.

1:5 FRUCTOSAMINE

Serum fructosamine was measured using a commercial kit which measured NBT reduction on the Cobas Fara centrifugal analyser. The kit was validated by Dr. Deepak Bhatnagar of the Dept of Chemical Pathology at Hope Hospital and both he and I in collaboration with Dr. Paramjeet Kalsi, Research Technician in the Department of Medicine at Hope Hospital, carried out the initial assays, although the samples were subsequently analysed by Dr. Kalsi.

Reagents and Hardware

I employed the Fructosamine kit manufactured by Roche Products Ltd., Welwyn Garden City, Herts. UK, and adapted the method of Baker et al (209) for use on the Cobas Fara centrifugal analyser. The commercial reagent consisted of 0.25 mmol/l nitroblue tetrazolium chloride (NBT) in a 0.1 mmol/l carbonate buffer ($NaHCO_3/Na_2CO_3$) with a pH of 10.35. The settings of the Cobas Fara analyser are seen in Table 18.

The standards used were a commercial secondary standard solution of 'glycosylated albumin' calibrated against a primary series standard curve of synthetic deoxy-morpholino-fructose (DMF) at a concentration 3.22 mmol/L, and pooled patients samples with good or poor glycaemic control were used in each batch to ensure quality control.

Table 18. PARAMETER LISTINGS FOR THE SERUM FRUCTOSAMINE ASSAY ON THE COBAS FARA (ROCHE) CENTRIFUGAL ANALYSER.

General Parameters

Measurement Mode	Absorb
Reaction Mode	P-T-AO
Calibration Mode	Std nonlin
Reagent Blank	Reag
Wavelength	530 nM
Temperature	37.0°C
Unit	mmol/l

Analysis

Sample 20 µl	Diluent, 40 µL
Reagent 200 µl	
+ Delay Time 1 sec.	
AO Readings	
First 500 seconds	Number 2
Interval 300 seconds	

Calculation

Conversion Factor, 1.00	Off 0.000
Reaction Direction Increase	Check On
Test Range low 0.00	High 5.00
Norm Range low 0.60	High 1.85
Calcs steps I	
Calcs steps A Endpoint	
Readings First 1	Last 2
Limit Calibration 3.50	Point 2

Calbration interval. On request
Standard non linear

1.	0.00	2.	0.50	7.	5.00	8.	0
3.	1.00	4.	1.50				
5.	2.00	6.	2.50				

Calc model	Lin. Regress
Std. Replicate	Single
Calib. correction	Off
Reag. Range low	- 0.10 High 1.00
Blank Range low	- 0.10 High 1.00

Procedure

The colorimetric fructosamine assay is based on the fact that ketoamine bonds in alkaline media form corresponding eneaminols which then reduce NBT to the corresponding formazan. The reagent is warmed to 37°C prior to use and the optical density absorbance change between 10 and 15 minutes is recorded at a wavelength of 530 nM. Serum albumin was also measured on a separate occasion on the Cobas Fara analyser and fructosamine levels corrected to a standard albumin concentration of 40g/l.

Results

Normal Range - Serum from 50 non-diabetic ostensibly healthy bus drivers was analysed to produce a normal range of 1.87 - 2.97 mmol/l.

Precision and Quality Control - Using pooled patients' sera and samples from healthy and diabetic individuals we attained within batch precision of 1-2% and between batch precision averaging 3% throughout the range of samples measured. Thereafter internal quality control was ensured despite the lack of a non-synthetic standard by using pooled sera.

I did not routinely correct for albumin concentration in initial assessment of the assay although in the light of recent publication suggesting this is necessary (227,229) where proteinuria or hypoalbuminaemia is present, I also standardised the fructosamine values to an albumin concentration of 40g/l, to examine whether or not it affected interpretation of results.

1:6. LIPID AND LIPOPROTEIN MEASUREMENTS

I fully acquainted myself with the techniques for isolation and measurement of lipids and apoproteins within the lipoprotein fractions during the course of the study. The assays were mainly carried out by Ms. Monica Ishola, Research Technician in the Laboratory of Dr. Paul N. Durrington, Senior Lecturer in the University Department of Medicine at the Manchester Royal Infirmary.

Lipid concentrations were measured enzymatically, cholesterol with reagent supplied by Diamed AG, (Murten, Switzerland) and triglycerides by the GPO-PAP method by Boehringer (Mannheim, W.Germany). To isolate HDL and HDL_3 by ultracentrifugation, the background density of plasma was adjusted to 1.063 g/ml and 1.125 g/ml respectively, by addition of a solution of sodium chloride and potassium bromide (731): after ultracentrifugation at 10^5g for 48 hours ('Superspeed 65' ultracentrifuge with 18 x 6.5 ml rotor, MSE Ltd., Crawley, Sussex, UK), the infranatants were separated by tube-slicing ('Spinco' Tube Slicer, Beckman Instruments, Palo Alto, California). The concentration of cholesterol in HDL_2 was determined by subtraction of HDL_3 cholesterol from total HDL cholesterol (299) and LDL cholesterol was calculated from total serum cholesterol, triglycerides and the HDL concentration by the Friedewald formula (270). In addition LDL cholesterol was determined directly in 48 insulin-dependent diabetic patients after preparative ultracentrifugation (v.i.) by subtraction of HDL (density greater than 1.063 g/ml) and VLDL (density less than 1.006 g/ml) cholesterol from total cholesterol. HDL cholesterol

was also isolated following the precipitation of other lipoproteins with sodium phosphotungstate and magnesium chloride as previously described (732) and LDL cholesterol indirectly estimated using this HDL. Serum apolipoprotein B concentrations were determined by immunoelectrophoresis (733) using goat anti-human serum obtained from Immuno, Dunton Green, Kent, UK.

Apolipoprotein E phenotypes were measured by Dr. Leslie Tetlow of the Department of Chemical Pathology at the University Hospital of South Manchester. The method was a refinement of a previously described method (734) and was carried by flat bed agarose gel isoelectric focussing. Prior to delipidation of the isolated VLDL fraction containing the bulk of apolipoprotein E, serum was mixed with neuraminidase to remove sialic acid and non-enzymatically bound glucose residues attached to apo E that might have affect interpretation of the different isoforms. Apo E bands were identified by nitrocellulose blotting of the agarose gel followed by incubation of the nitrocellulose with mouse monoclonal anti-apo E (Biogenesis, Bournemouth, UK) and thereafter with immunoperoxidase linked rabbit antimouse antibody (Dako, High Wycombe, Bucks., UK).

Validation of the FRIEDEWALD FORMULA for the measurement of low density lipoprotein cholesterol in insulin-dependent diabetes mellitus

The Friedewald formula was devised in order to obviate the need for preparative ultracentrifugation in the measurement of LDL cholesterol, and allows LDL cholesterol levels to be calculated with a knowledge of the concentrations of total serum

cholesterol and triglycerides and of the HDL cholesterol (270).

It relies on the assumption that the mass ratio of serum triglycerides to very low density lipoprotein (VLDL) cholesterol is 5:1 (equivalent to a molar ratio of 2.2:1), when LDL cholesterol is equal to :

$$\text{Total serum cholesterol} - \text{HDL cholesterol} + \frac{\text{total serum triglycerides}}{2.2}$$

The formula is based on the assumption that the relative quantities of VLDL and LDL triglycerides remain constant, as does the ratio of cholesterol to triglyceride within VLDL.

However compositional abnormalities of both VLDL and LDL in IDDM might lead to an underestimation of LDL cholesterol by the Friedewald method. I therefore compared the LDL cholesterol levels derived from the Friedewald formula and preparative ultracentrifugation in 48 subjects with IDDM.

42 of the subjects were normotriglyceridaemic (fasting triglycerides less than 2.50 mmol/l), and 6 had hypertriglyceridaemia (fasting triglycerides 2.98-5.30 mmol/l). Renal function was well preserved in all cases. Serum urea was 5.8 (1.8) mmol/l (mean (SD)) and serum creatinine 87 (20) µmol/l. Urinary protein excretion was 0.1 (0.05-6.6) g/24hours (median (range)), and greater than 0.5g/24 hours in 6 patients.

Results

Glycaemic control for the group as a whole was moderately good (fasting blood glucose 8.8±3.5 mmol/l (mean±SD), HbA_1 8.8± 1.7%).

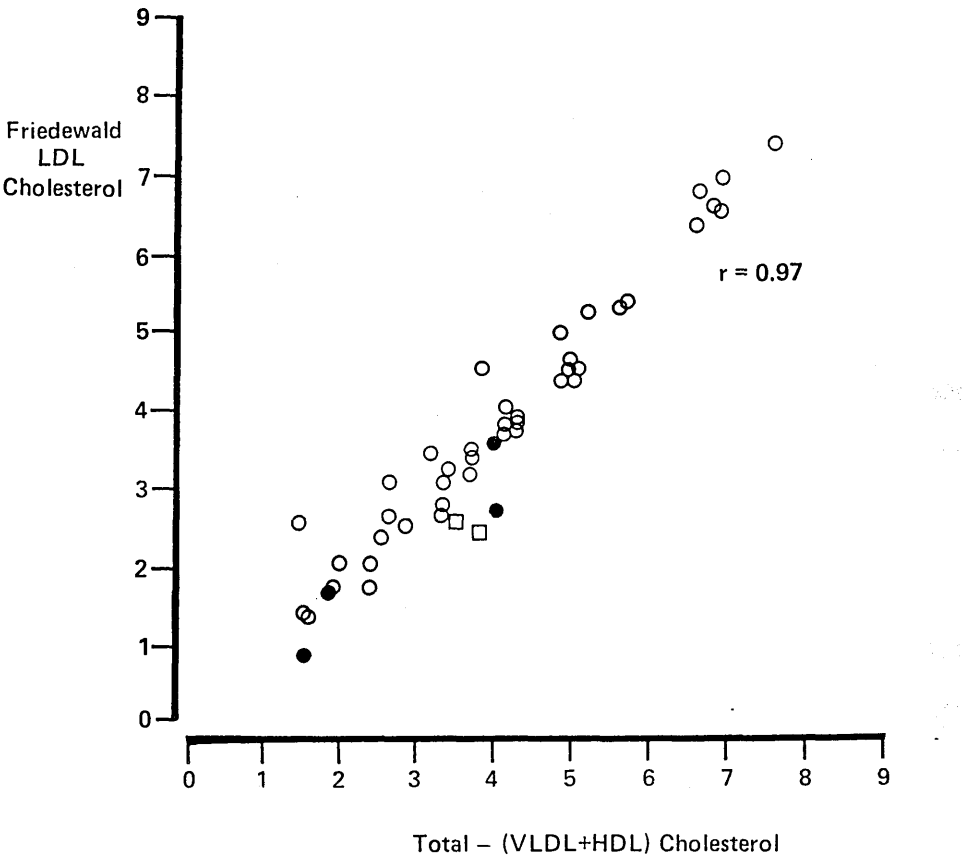


FIG. 13 Correlation between LDL Cholesterol calculated by the Friedewald formula and determined by preparative ultracentrifugation in insulin-dependent diabetes mellitus.

Lipid and lipoprotein values for the group as a whole were as follows: total cholesterol 5.87±1.65 mmol/l; total triglycerides 1.67±1.10 mmol/l; HDL cholesterol 1.36±0.34 mmol/l. The mean value for LDL cholesterol when LDL was calculated directly was 3.89±1.27 whereas the mean LDL cholesterol using the Friedewald formula was slightly less at 3.68±1.13 mmol/l.

In the 42 normotriglyceridaemic (<2.5 mmol/l) individuals a close correlation between LDL calculated by the Friedewald formula and determined by preparative ultracentrifugation was noted ($r = 0.98$, $p<0.0001$; slope 0.98), and this close relationship persisted when data from the 6 patients whose serum triglycerides exceeded 2.5 mmol/l (figure 13) were incorporated.

Discussion

The Friedewald formula was found to be an appropriate method to derive LDL cholesterol values in insulin-dependent diabetics with relatively well preserved renal function when they were normotriglyceridaemic or moderately hypertriglyceridaemic, irrespective of compositional abnormalities that might take place in VLDL and LDL in IDDM. The close correlation and the slope of unity when the linear regression line was calculated suggests that 2.2 is an appropriate value for the molar ratio of triglyceride to cholesterol in IDDM in the absence of advanced nephropathy.

1:7 C-peptide and other Measurements

C-peptide secretion was assessed 1 hour following a standard meal which has previously been found to yield results comparable to that obtained following stimulation with 1mg glucagon administered intravenously (553). The assays were carried out by Dr. Chris Gordon of the Department of Endocrinology of the Manchester Royal Infirmary. A radioimmunoassay method (IRE, Belgium) was used. The lower limit of detection of the assay was 0.015 pmol/ml, the between batch variation of the assay averaged 10% for pooled plasma and 5.5% to 10% for quality control sera; the intra-batch coefficient of variation ranged between 1.3 and 3.1%.

HBA_1

Glycosylated (glycated) haemoglobin measurements were made in the Dept. of Chemical Pathology at Hope Hospital by ion exchange chromatography using a commercial kit (Boehringer, Mannheim, W.Germany). The normal range in our laboratory for 50 healthy individuals was 5.0-8.0%.

Insulin Antibodies

Antibodies to bovine and human insulin were measured by the laboratory of Dr. T. Wilkin at the University Dept of Medicine at Southampton University, using an enzyme-linked immunosorbent assay (ELISA) (735).

Other Measurements

Serum biochemical profiles (including serum albumin) and urinary creatinine clearance were measured in the Dept. of Chemical Pathology at Hope Hospital with a multichannel

autoanalyser. Total urinary protein was measured by turbidimetry using benzethonium chloride routinely used in the same laboratory and the lower limit of detection was found to be 0.05g/l. Creatinine clearance and urinary protein excretion were calculated from timed urine collections and expressed per 24 hours corrected for body surface area (to 1.73 m^2). Urinary sodium excretion was determined by flame photometric methods.

Data Collection and Analysis

All data was initially collected on trial document forms (see Appendix). Data was then put on computer file either by myself or by Mrs Glynis Medlicott, Diabetic Clerk to whom I am indebted. Latterly I was also assisted by Mrs. Hazel Downs, Diabetic Clerk, in transposing data onto the microcomputer for analysis.

We used a Sirius Microcomputer with a Winchester disk drive to extend the capacity for storage of information. Initial plans to transfer all data directly to the mainframe (AMDAHL) computer at the Regional Computer Centre in the University of Manchester via a link proved a time consuming and ultimately inappropriate approach and subsequently floppy discs containing data were taken to the Computing Centre for analysis. Analysis of data was carried out with the assistance of Dr. Valerie Hillier of the Dept. of Computation at Manchester University for whose help I am most grateful. Data was analysed using the Statistical package for Social Sciences (SPSS). Data are usually presented as mean (SD) or (SEM) unless stated otherwise. The distribution of characteristics was assessed initially to see whether parametric

or non-parametric statistics were appropriate using the Kolmogorov Smirnoff goodness of fit test. Log transformation was carried out for analysis of triglyceride and M Value data. Analysis for paired or more usually unpaired data utilised Student's t-test, one-way analysis of variance, Wilcoxon's Rank Test, the Mann-Whitney U-test or the Kruskal-Wallis ANOVA for quantitative outcome measures. Groups were compared at each time point by the Wilcoxon Rank sum test and the Kruskal-Wallis one-way test ANOVA. To assess the changes over time within the groups the paired samples t-test, analysis of variance for repeated measurements, the Wilcoxon's matched pairs signed rank test and the Friedman two-way ANOVA were applied. The chi-squared test or the Fischer's exact test were used to analyse variables with categorical classification. The McNamar test was used to compare the frequencies of complications initially and after the first year. Correlations were made using Wilcoxon's signed rank sum test or Kendall's correlation coefficient. In all cases statistical significance was assumed at a two tailed $p<0.05$. A glossary of the statistical tests used in the study and their roles is in the appendix section of the thesis.