ASPECTS OF GLUCOSE METABOLISM IN URAEMIA

A Thesis submitted

by

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ABBREVIATIONS

CAPD	continuous ambulatory peritoneal dialysis
CHb	carbamylated haemoglobin
CI	confidence interval
CRF	chronic renal failure
CV	coefficient of variation
2,3-DPG	2,3-diphosphoglycerate
EDTA	ethylenediaminetetraacetic acid
EDTA	European Dialysis and Transplant Association
GFR	glomerular filtration rate
GH	growth hormone
GHb	glycosylated haemoglobin (colorimetric)
GLC	gas liquid chromatography
Hb	haemoglobin
HbA_1	chromatographic fraction of haemoglobin A_1
HbA_{1a+b}	chromatographic component of haemoglobin A_{1a+b}
HbA_{1c}	chromatographic component of haemoglobin A_{1c}
HD	haemodialysis
HDL	high density lipoprotein
5HMF	5 hydroxymethylfurfural
IPD	intermittent peritoneal dialysis
IPH	isopropyl hydantoin
LDL	low density lipoprotein
MRC	metabolic clearance rate
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
PCA	perchloric acid
PCV	packed cell volume

PTH	parathyroid hormone
SD	standard deviation
SEM	standard error of the mean
TBA	thiobarbituric acid
UFV	ultrafiltration volume
VLDL	very low density lipoprotein

NB: Other abbreviations used are fully explained in the appropriate part of the text.

SUMMARY OF THESIS

Aspects of Glucose Metabolism in Uraemia

This thesis takes the form of a tripartite study on aspects of glucose metabolism in uraemia. The work examines certain biochemical aspects of glucose metabolism in a clinical context.

Continuous ambulatory peritoneal dialysis (CAPD) is a relatively new but now established mode of renal replacement therapy, that presents an unique clinical situation in that glucose is continuously self-administered intraperitoneally to control fluid balance. The metabolic consequences are thus clinically important.

The first 4 chapters review the background to the work and include the topics of glucose homeostasis, glucose metabolism in uraemia, CAPD and glycosylated haemoglobin. Chapter 5 describes the biochemical methods and chapters 6, 7 and 8 detail the three projects performed.

1. The first project "Pancreatic Beta Cell Function in Uraemia" evaluated beta cell function in renal failure. The specific objective was to determine if CAPD adversely affected the beta cell secretory capacity. Beta cell integrity was also investigated in diabetics to assess the effect of uraemia and dialysis.

2. Residual beta cell function was assessed, in 70 patients, by using intravenous glucagon stimulation and measuring in the peripheral blood the beta cell peptide response.

3. Fasting glucose and insulin concentrations were normal in uraemic patients but c-peptide was grossly elevated due to impaired renal catabolism. Following glucagon stimulation, an exaggerated blood glucose response with delayed glucose peak was observed, while the peak insulin response was normal, but the return to basal concentrations was delayed. The c-peptide response was exaggerated and peak concentrations were greatly increased. Responses were similar in all non-diabetic uraemic patients. The above features reflect glucose intolerance in uraemia which persisted despite dialysis therapy.

4. The glucagon challenge showed significant increments in the beta cell peptides reflecting adequate residual pancreatic function. The response was similar in both new and established CAPD patients compared to non-dialysed uraemics and haemodialysis subjects. Thus, it can be concluded, that despite continuous glucose administration, CAPD does not adversely affect pancreatic beta cell function.

5. Type I insulin-dependent diabetics had no beta cell response to glucagon, but type II uraemic diabetics had raised fasting c-peptide concentrations which might suggest hypersecretion of the beta cell. However, absolute concentrations can be misleading as the increase was largely due to renal impairment. This was confirmed by the lack of response to glucagon challenge, indicating no significant residual beta cell function. The effect of long-standing diabetes overshadowed any discernible effect of uraemia or dialysis.

6. The second project "Glucoregulatory Hormones and Intermediary Metabolites in CAPD" assessed the acute metabolic consequences of intraperitoneal glucose administration. Fasting hormones and metabolites were evaluated in non-dialysed uraemic and haemodialysis patients for comparison with CAPD subjects. Metabolic profiles were monitored during a CAPD cycle comparing high and low dextrose dialysate solutions. Specific groups of CAPD patients were studied including diabetics, the elderly and patients with peritonitis.

7. In the fasting state blood glucose and insulin were normal in most uraemic patients, but c-peptide was grossly elevated and glucagon moderately increased. Growth hormone was normal and cortisol, although normal in CAPD patients was increased in other uraemic subjects. The gluconeogenic precursors lactate, pyruvate and alanine were normal, but ketone bodies and glycerol tended to be elevated particularly in the elderly CAPD patient.

8. The hormone and metabolite profiles during a CAPD cycle showed greater changes with high dextrose solutions, particularly during the first two hours of dialysate dwell. Peritoneal glucose absorption induced hyperglycaemia, hyperinsulinaemia and a transient fall in glucagon, while growth hormone and cortisol were unchanged. Lactate increased, largely due to absorption from the dialysate, rather than via endogenous glycolysis, as the blood lactate increment was independent of dialysate glucose concentration. Ketone bodies and glycerol were suppressed in the early part of the cycle during the period of maximum glucose absorption. In general, the metabolic variables returned to

their basal state by the end of the 6 hour cycle. Thus peritoneal glucose absorption causes hyperglycaemia which induces hyperinsulinaemia, which in turn stimulates glycolysis, inhibits gluconeogenesis and suppresses ketogenesis and lipolysis.

9. Peritonitis increased the rate of glucose and lactate absorption from the dialysate due to increased permeability of the peritoneum. Elderly patients had similar profiles to younger CAPD subjects.

10. The effect of peritoneal glucose absorption in a state of insulin deficiency was evaluated in diabetics deprived of insulin. Dialysate glucose absorption induced hyperglycaemia but no beta cell response and glucagon, growth hormone and cortisol were unchanged. Lactate increased but pyruvate and alanine were essentially unaltered. Ketone bodies and glycerol remained elevated and were not suppressed despite the ambient hyperglycaemia. Thus in a state of insulin deficiency peritoneal glucose absorption failed to stimulate glycolysis or inhibit gluconeogenesis and ketogenesis and lipolysis were maintained.

11. The third project evaluated "Glycosylated and Carbamylated Haemoglobin in Uraemia". Glycosylated haemoglobin in renal failure is a controversial subject and was reappraised in a uraemic population of over 200 patients, including non-dialysis, dialysis, transplants, diabetics with and without renal failure.

12. The study confirmed that glycosylated haemoglobin was increased in non-diabetics with uraemia, when measured by mini-column

ion-exchange chromatography, but was normal when total ketoamine glycosylation of the red cell was measured by colorimetry.

13. In diabetics glycosylated haemoglobin was increased independent of methodology and both techniques showed excellent correlation. Although colorimetry is unaffected by uraemia and may be the method of choice, the chromatographic method was still useful for assessing glycaemic control in diabetics with renal failure.

14. All chromatographic HbA_1 fractions increased in uraemia, but the HbA_{1c} component was more influenced by diabetes and the HbA_{1a+b} component by uraemia.

15. Carbamylated haemoglobin was detected in all subjects but was grossly elevated in renal failure. The increase paralleled the rise in the HbA_1 fractions in non-diabetic uraemics and correlated with the severity of uraemia.

16. The concept that carbamylated haemoglobin in uraemia is analogous to glycosylated haemoglobin in diabetes warrants further investigation. From the work of this thesis further research is being done to assess the clinical usefulness and possible pathophysiological significance of carbamylated haemoglobin.

PREFACE

In the early 1980's, while a registrar in renal medicine in Glasgow, I developed an interest in continuous ambulatory peritoneal dialysis (CAPD). At that time, CAPD was a new mode of treatment and clinical experience was limited, but from this my interest grew. An increasing number of young diabetic patients with end stage renal disease were also being treated, particularly with CAPD. This nurtured my interest in diabetics with renal failure and initiated my plans to study glucose metabolism in uraemia. While thinking about a research project in the field of glucose metabolism I was attracted to a research post in Sheffield.

On arrival in Sheffield in 1984, I was full of enthusiasm to get started only to realise that no formal research programme or expertise existed in this busy department of nephrology. However, with an abundance of renal failure patients, particularly a large CAPD population, and encouragement and cooperation from many departments in the Royal Hallamshire Hospital, Sheffield and the metabolic departments in Newcastle, I set about designing and exploring certain aspects of glucose metabolism in uraemia.

The first project, "Pancreatic beta cell function in uraemia" was designed to determine if CAPD treatment, which involves continuous glucose administration, had an adverse effect on the beta cell secretory function. This seemed to me an obvious, yet unanswered question of great clinical significance.

The second project followed, that continuous intraperitoneal glucose administration was a unique clinical situation, with possible metabolic consequences and yet, the physiological or pathophysiological effects had not been investigated. This stimulated

me to study glucoregulatory hormones and intermediary metabolites during CAPD treatment in a variety of patients including diabetics and the elderly.

The third project, "Glycosylated and carbamylated haemoglobin in uraemia" originated from both, my endeavours to achieve good blood glucose control in uraemic diabetics, and the confusing and conflicting data on glycosylated haemoglobin in the literature. The suggestion that uraemia interfered with the analysis of glycosylated haemoglobin directed me to measure carbamylated haemoglobin. From the work of this thesis, I believe that carbamylated haemoglobin may have a clinical usefulness as well as a pathophysiological significance and in 1987 this continues to be an ongoing research interest.

CHAPTER 1

GLUCOSE METABOLISM AND HOMEOSTASIS

1. INTRODUCTION

Glucose is the major energy source for the human body and is the only metabolic fuel utilized by the brain under most conditions encountered in normal life. The brain cannot synthesize or store glucose and thus the maintenance of blood glucose concentration is critical. The concentration of glucose in the blood is kept within a narrow range although there is some variation as circumstances change, such as feeding or fasting. The blood glucose concentration depends on the balance between glucose entering or leaving the extracellular compartment.

Glucose entry into the circulation results from absorption of glucose derived from digested dietary carbohydrates and release of endogenous glucose from the liver. Endogenous glucose is derived from hepatic glycogen (glycogenolysis) and glucose synthesized in the liver (gluconeogenesis). Gluconeogenic precursors include lactate and pyruvate transported to the liver from a variety of extrahepatic tissues and carbon skeleton intermediates which are derived from amino acids, principally alanine from muscle and to a smaller extent glycerol from adipose tissue.

Glucose exits from the circulation into a variety of tissues where it is rapidly metabolized. Glucose has several metabolic fates. It can be stored in the liver and muscle as glycogen, undergo glycolysis to pyruvate which can be reduced to lactate, transaminated to alanine or converted to acetyl-CoA. Acetyl-CoA can be oxidized to carbon dioxide and water via the tricarboxylic acid cycle (Krebs or citric acid cycle), converted to fatty acids (lipogenesis) and stored as triglycerides, or utilized for ketone body synthesis (ketogenesis). A schematic presentation of glucose metabolism is summarized in Figures
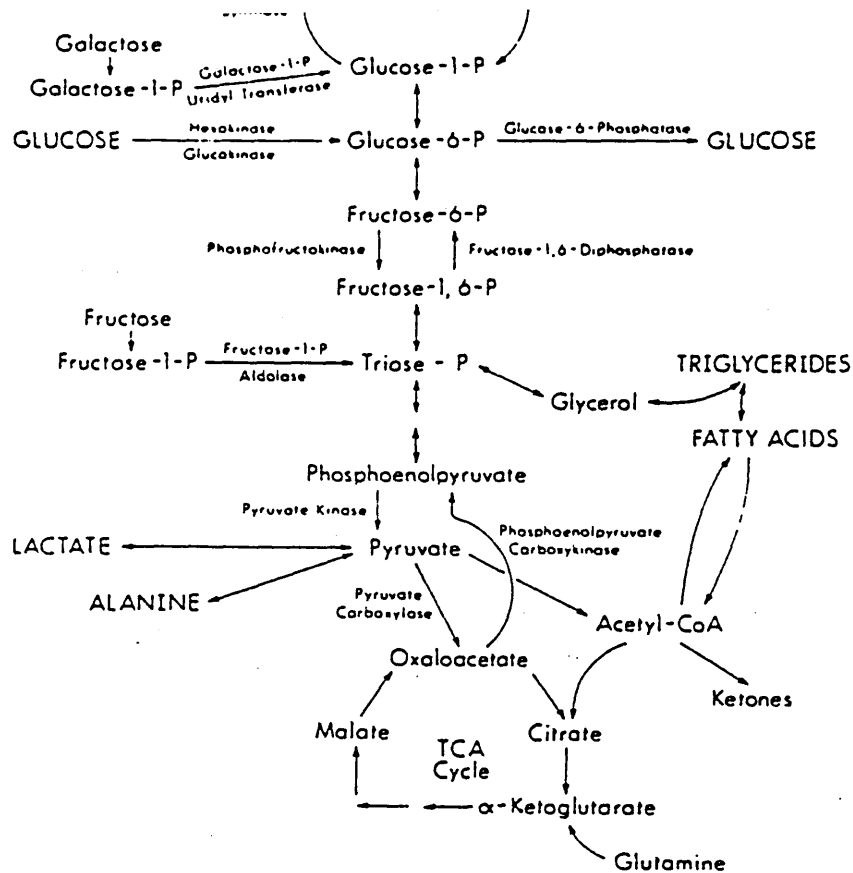
Figure 1. 1 Schematic representation of glucose metabolism

Figure 1. 2 Inter relationships between glucose and substrates
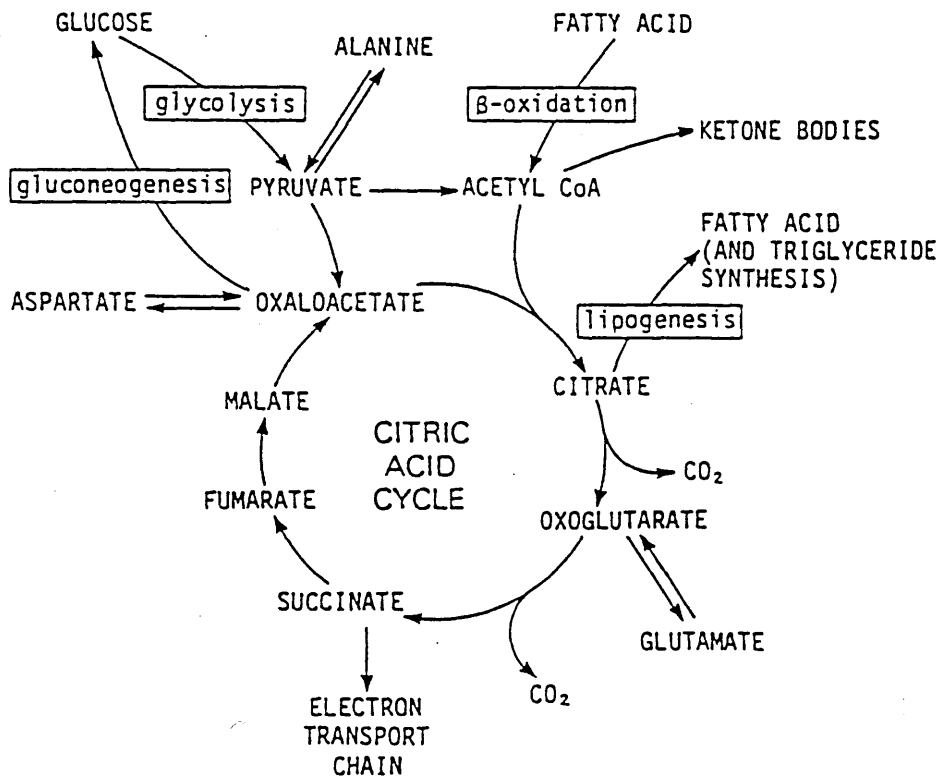
1.1 and 1.2. The inter-relationship between glucose and the various substrates are shown.

2. THE LIVER

The liver is central in the role of glucose metabolism and flexible depending on the metabolic demands at any given time, such as fasting or feeding. It is the single most important organ ensuring a constant energy supply to other tissues (see Figures 1.3 and 1.4). The liver can store excess glucose as glycogen (glycogenesis). The rate of glycogen synthesis from glucose 6-phosphate is modulated by insulin, which is secreted from the pancreatic beta cells in response to systemic hyperglycaemia. The excess of glucose, following a meal, results in the hepatic conversion to fatty acids which are ultimately stored as triglyceride in adipose tissue. The entry of glucose into hepatic cells (as well as cerebral cells) is not affected directly by insulin but depends on the extracellular glucose concentration. The conversion of glucose 6-phosphate, the first step in glucose metabolism in all cells, is catalysed in the liver by the enzyme glucokinase, which has a low affinity for glucose compared to that of hexokinase found in most tissues. Glucokinase activity is induced by insulin secreted in response to systemic hyperglycaemia. Thus proportionately less glucose is extracted by hepatic cells during fasting compared to the post prandial state. This helps maintain a fasting glucose supply to vulnerable tissues such as the brain.

Under aerobic conditions the liver can synthesize glucose by gluconeogenesis using lactate, glycerol or carbon chains resulting from deamination of most amino acids (mainly alanine) which are products of metabolism of other tissues. The liver contains the enzyme glucose 6-phophatase, which hydrolyses glucose 6-phosphate

yielded from glycogen breakdown or by gluconeogenesis to produce
glucose and maintain extracellular glucose homeostasis. Hepatic
glycogenolysis is stimulated by glucagon from the alpha cells of the
pancreas.

During fasting the liver can convert fatty acids released from
adipose tissue to ketones which can be used by other tissues,
including the brain, as an energy source when glucose is in short
supply (Figure 1.4). The renal cortex is also capable of
gluconeogenesis and of converting glucose 6-phosphate to glucose, but
this is generally only significant under certain conditions such as
prolonged fasting. Although other tissues, such as skeletal muscle,
store glycogen it can only be utilized locally in that tissue as
muscle does not have the necessary enzyme glucose 6-phosphatase. Thus
muscle glycogen cannot directly maintain blood glucose concentration.

The effects of an oral glucose load are schematically presented in
Figure 1.3. The liver modifies the potential hyperglycaemic effect of
a high carbohydrate meal by extracting relatively more glucose from
the portal blood than in the fasting state. Some glucose passes
through the liver unchanged and a rise in the systemic concentration
stimulates the pancreas to secrete insulin which further stimulates
hepatic and muscle glycogenesis. The entry of glucose into adipose
tissue and muscle cells, unlike that of liver and brain, is stimulated
by insulin, and the blood glucose falls rapidly to near fasting
levels. This does not happen if there is a relative or absolute
insulin deficiency (as in diabetes mellitus). Conversion of
intracellular glucose into glucose 6-phosphate in adipose and muscle
cells is catalysed by hexokinase which, because its affinity for
glucose is greater than that of hepatic glucokinase, ensures that
glucose enters the metabolic pathways in these tissues at lower
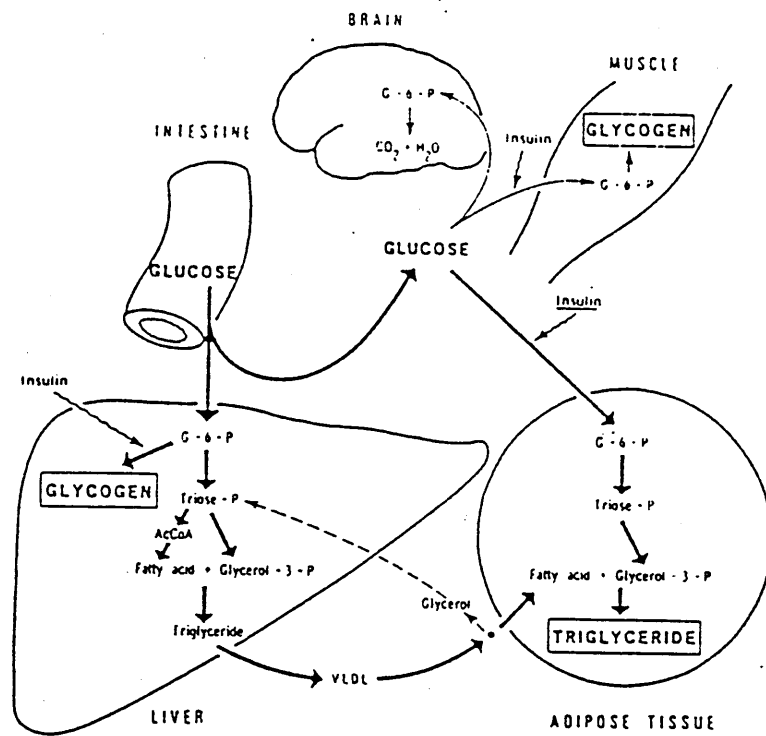
Figure 1. 3   Post-prandial state - feeding
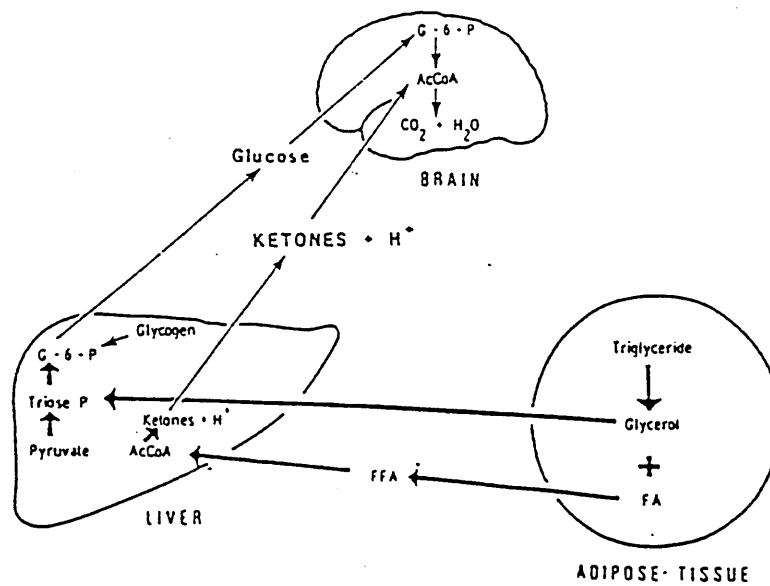
Figure 1. 4   Post-absorptive state - fasting

concentrations than in liver. Muscle and adipose tissue store the
excess post-prandial glucose although the mode and function of each is
very different.

3. ADIPOSE TISSUE AND THE LIVER

Adipose tissue triglyceride is the most important long term energy
store in the body and increased utilization of fat stores can be
associated with ketosis.

Adipose tissue, in conjunction with the liver, converts excess
glucose to triglyceride and stores it in this form rather than
conversion to glycogen. The component fatty acids are derived from
glucose entering the liver and the component glycerol from glucose
entering adipose tissue cells. In the liver triglycerides are formed
from glycerol 3-phosphate (from triose phosphate) and fatty acids
(from acetyl-CoA). This triglyeride is transported to adipose tissue
in VLDL (very low density lipoprotein), where it is hydrolysed by
lipoprotein lipase. The released fatty acids (of hepatic origin)
condense with glycerol 3-phosphate derived from glucose entering
adipose tissue under the influence of insulin and the resultant
triglyceride is stored. More energy can be stored as triglyceride
than glycogen.

During fasting, when exogenous glucose is unavailable, endogenous
adipose tissue triglyceride is reconverted to free fatty acids (FFA)
and glycerol by lipolysis (Figure 1.4). These are transported to the
liver where glycerol enters the gluconeogenic pathway at the triose
phosphate stage. The glucose synthesized can be released into the
circulation to maintain the blood glucose concentration. Most tissues
other than the brain use the FFA as a metabolic fuel after conversion
to acetyl-CoA. The liver can also form acetoacetate by enzymatic

conversion of acetyl-CoA, acetoacetate in turn can be reduced to 3-hydroxybutyrate and decarboxylated to acetone. The ketone bodies can be used as an energy source by brain and other tissue when glucose supply is reduced.

Ketosis occurs when fat stores are the main energy source and can be seen in conditions such as fasting and reduced nutrient absorption due to vomiting. Mild ketosis may occur after 12 hours of fasting, but is not usually associated with acidosis unless the fasting is prolonged. Diabetic ketoacidosis is accompanied by hyperglycaemia unlike the ketotic hypoglycaemia seen in prolonged fasting such as in anorexia nervosa. However the mechanism of ketosis is similar. In starvation ketosis the supply of glucose to cells of adipose tissue is insufficient for normal glycolysis and lipogenesis. In diabetes the insulin deficiency induces intracellular glucose deficiency due to impaired entry of high extracellular concentrations of glucose into the fat cells. The high extracellular concentrations of glucose is thus a misleading index of intracellular events.

4. <u>MUSCLE AND THE LIVER</u>

Glucose enters skeletal muscle post-prandially under the influence of insulin and is stored as glycogen (Figure 1.3). The glycogen cannot be reconverted to glucose due to the absence of glucose 6-phosphatase and therefore muscle glycogen can only supply local needs.

During muscle activity (Figure 1.5) glycogenolysis is stimulated by adrenaline and the resultant glucose 6-phosphate undergoes glycolysis to pyruvate and lactate. Under aerobic conditions pyruvate is oxidized via the tricarboxylic acid cycle. However, the oxygen availability during exercise is readily depleted and anaerobic

$CO_2 + H_2O$

BRAIN

MUSCLE

Glycogen

Adrenaline

Glycogen

$G - 6 - P$

$G - 6 - P$

Glucose

(Cori Cycle)

Pyruvate

Pyruvate

ALANINE

Lactate + H⁺

LACTATE + H⁺

Lactate + H⁺

Pyruvate

$CO_2 + H_2O$

ALANINE

ALANINE

ALANINE

LIVER

AMINO ACIDS
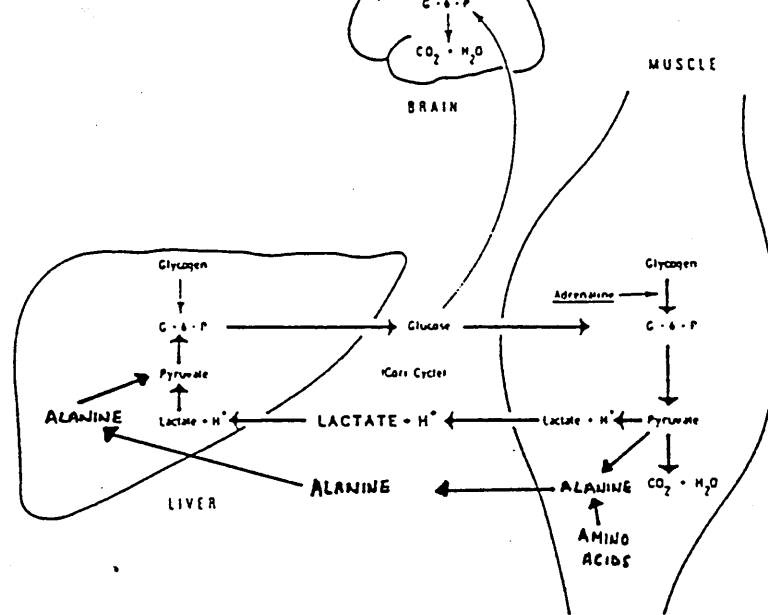
Figure 1. 5   Muscle activity

Glycogen

Glycogen

$G - 6 - P$

$G - 6 - P$

Pyruvate

Pyruvate

Lactate + H⁺

LACTATE + H⁺

Lactate + H⁺
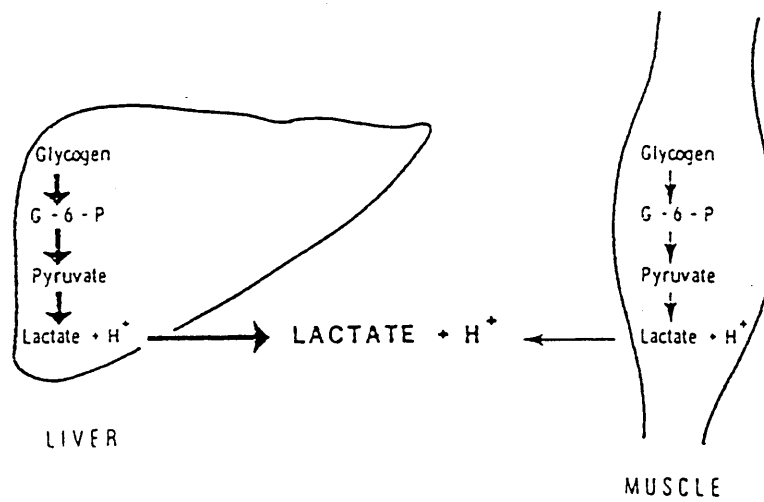
LIVER

MUSCLE

Figure 1. 6   Lactate production

glycolysis with production of lactate becomes important. Lactate is
transported from muscle to liver in the circulation where it can be
used for gluconeogenesis (Cori cycle) and provides further glucose for
muscle activity. Alanine can also be transported from muscle to liver
where it serves as a gluconeogenic precursor (glucose-alanine cycle).
During gluconeogenesis hydrogen ion is also neutralized. Under
aerobic conditions the liver consumes more lactate than it produces.
The physiological accumulation of lactate during increased muscle
activity is temporary and disappears at rest when slowing of anaerobic
glycolysis allows the aerobic processes to equilibrate.

5. LACTIC ACIDOSIS

Lactic acid produced by anaerobic glycolysis may be oxidized to
carbon dioxide and water in the tricarboxylic acid cycle or be
reconverted to glucose by gluconeogenesis in the liver. Pathological
accumulation of lactate can occur due to increased production or
decreased utilization. This may be due to increased production by
anaerobic glycolysis and decreased utilization by impaired
gluconeogenesis or impairment of the tricarboxylic acid cycle. This
is seen in tissue hypoxia due to poor tissue perfusion. The
combination of impaired gluconeogenesis and increased anaerobic
glycolysis converts the liver from a lactate consuming to a lactate
producing organ (Figure 1.6).

6. GLUCOSE HOMEOSTASIS AND INTERMEDIARY METABOLISM

In the post-absorptive state (after an overnight fast) blood
glucose concentrations are stable indicating that production and
utilization are equivalent. The brain accounts for 60% of glucose
utilization (Sherwin, 1980) and the remainder is used for other

glycolysing tissues such as red blood cells, renal medulla, muscle and fat. Hepatic glucose production after an overnight fast is largely from glycogenolysis (75%) and the remainder from gluconeogenesis (25%). Gluconeogenesis from lactate, pyruvate, alanine and glycerol is estimated to represent 13, 1, 4 and 4% of endogenous glucose production respectively and, therefore, 52, 4, 16 and 16% of gluconeogenesis respectively (Cryer, 1985).

Gluconeogenesis is important in providing new glucose and replenishing hepatic glycogen storage in the post-absorptive state due to the limited availability of preformed glucose. The glucose pool consists of free glucose in the extracellular fluid and in the cells of some tissues especially the liver, but also small amounts in the kidney, blood cells, pancreatic islets, brain and intestinal mucosa. The glucose pool amounts to 15 to 20 g in the normal adult (Searle, 1976) and glycogen can be mobilized to provide 70 g of glucose (Nilsson, 1973). In more prolonged fasting plasma glucose falls and then stabilizes, hepatic glycogen falls and gluconeogenesis becomes the only source of glucose production. Muscle protein is degraded providing amino acid substrate and glucose utilization by muscle and fat is inhibited. Lipolysis and ketogenesis accelerate and circulating ketone concentrations increase with ketone bodies becoming the major fuel for the brain.

After a meal, in the post-prandial state, glucose absorption results in a large increase in exogenous glucose delivery to the circulation, often doubling the rate of post-absorptive glucose production. As glucose is absorbed, endogenous glucose production is suppressed and glucose utilization by the liver, muscle and adipose tissue increases. Thus exogenous glucose is assimilated and blood glucose returns towards the post-absorptive state.

In this way glucose homeostatic mechanisms result in keeping blood glucose within a relatively narrow range. The regulation of systemic blood glucose involves hormonal, neural, and autoregulatory factors.

7. HORMONAL GLUCOREGULATORY FACTORS

Glucoregulatory hormones include insulin, glucagon, adrenaline, growth hormone and cortisol (Figure 1.7). Insulin is the dominant glucose lowering hormone. It suppresses endogenous glucose production and stimulates glucose utilization. Insulin inhibits hepatic glycogenolysis and gluconeogenesis and in association with other factors, including hyperglycaemia and hypoglucagonaemia converts the liver into an organ of glucose consumption and fuel storage in the form of glycogen and triglycerides. Insulin stimulates glucose uptake, storage and utilization by other tissues such as muscle and fat. Insulin also increases fatty acid and triglyceride synthesis and triglyceride transport via VLDL, increases fatty acid uptake and incorporation into triglyceride in adipose tissue and suppresses tissue lipolysis. The latter results in decreased fatty acid flux to the liver which coupled with direct hepatic effects of insulin results in decreased ketogenesis. Insulin also stimulates amino acid uptake and net protein synthesis in muscle. Thus insulin promotes the storage of carbohydrate, fat and protein.

Glucose-raising or counterregulatory hormones include glucagon, adrenaline, growth hormone and cortisol. Glucagon is secreted by the alpha cells of the pancreas into the portal circulation and acts predominantly on the liver under physiological conditions. Glucagon is a potent stimulator of glycogenolysis and gluconeogenesis and rapidly increases hepatic glucose production. Glucagon also stimulates hepatic ketogenesis particularly when insulin

Somatostatin

(−)   (−)

D cells

A cells   B cells

Glucagon ←   → Insulin

ISLET

(+)   (+)

Glycogenolysis	(+) ←— adrenaline —→ (−)	Glucose uptake
Gluconeogenesis	(+) ←— Cortisol	
LIVER AND KIDNEY	Growth hormone —→ (−)	LIVER, ADIPOSE TISSUE AND MUSCLE

DIET →   INFLUX   GLUCOSE   EFFLUX → BRAIN

EXTRACELLULAR FLUID

Hormonal influences on glucose homeostasis   (+) Stimulation. (−) Inhibition.
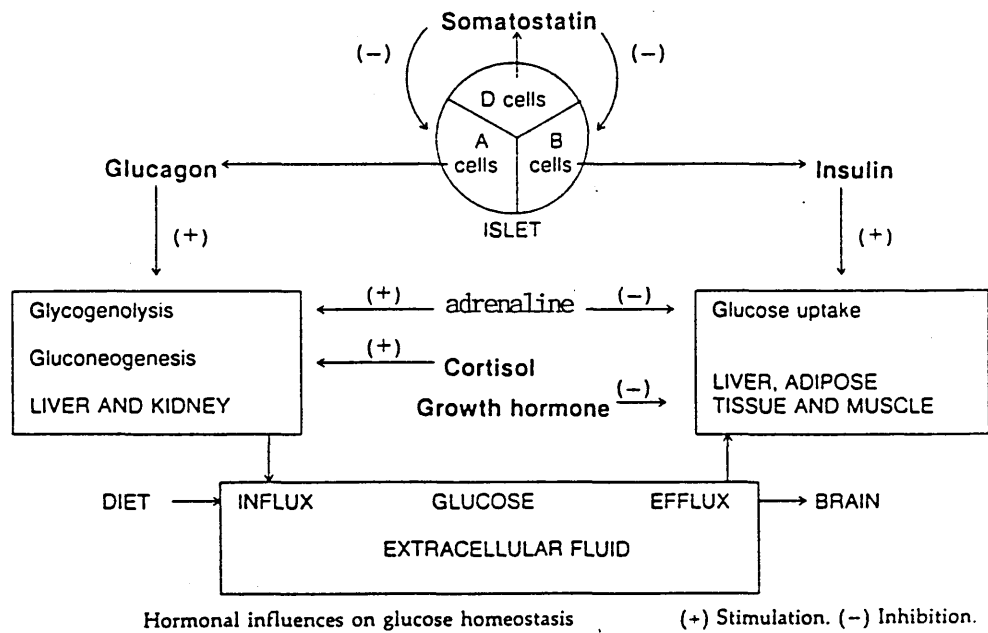
Figure 1. 7
Hormonal influences on glucose homeostasis
(+) Stimulation (−) Inhibition

concentrations are low. However, hepatic glucose production induced by hyperglucagonaemia is only transient and other factors such as glucose induced insulin release and autoregulation due to hyperglycaemia may also be involved (Cryer, 1985).

The hyperglycaemic effect of adrenaline is complex. Adrenaline stimulates hepatic glucose production and restricts utilization and these effects are mediated via alpha and beta adrenergic mechanisms (Rizza et al., 1980). Adrenaline can also increase hepatic glycogenolysis and gluconeogenesis independently of other hormones. Adrenaline, like glucagon, acts rapidly and produces a transient increase in glucose production and continues to support glucose production even in the fasting state. In contrast to glucagon, adrenaline also limits glucose utilization. Sustained increases in adrenaline causes persistent hyperglycaemia due to reduced glucose utilization.

Long term elevation of growth hormone limits glucose transport into cells and can produce an insulin resistant state. Growth hormone may have a glucose lowering effect but it is not obvious for hours (MacGorman et al., 1981). Thus growth hormone is unlikely to be important for rapid glucose counter-regulation. Infusion of cortisol does not increase glucose production by limiting glucose utilization (Shamoon et al., 1981) and thus, like growth hormone, cortisol does not appear to have a major role in the short term glucose control. In the long term growth hormone and cortisol may have a permissive role to play in glucose homeostasis in that their presence is necessary even though they do not have a direct effect (Cryer, 1984). Furthermore, the hyperglycaemic effects of glucagon, adrenaline and cortisol may be synergistic and thus have a role in glucose counter-regulation (Shamoon et al., 1981).

Neural and autoregulatory factors also have a role in glucose homeostasis. Vagal stimulation increases hepatic glycogen synthesis and direct hepatic sympathetic nerve stimulation decreases glycogen and increases blood glucose (Lautt, 1980). The concept of hepatic glucose autoregulation, that is, the role of hepatic glucose production is altered in inverse relation to the blood glucose concentration independent of the effects of circulating hormones, has been advocated (Sacca et al., 1979).

a) Glucose Counter-Regulation

Glucagon plays a primary role in promoting glucose recovery from hypoglycaemia. Glucose recovery from insulin induced hypoglycaemia is essentially normal when glucagon secretion is intact and partially impaired when glucagon secretion is inhibited. The latter is associated with an increased adrenomedullary adrenaline response (Rizza et al., 1979). Glucose recovery is impaired little, if at all, during adrenergic blockade. However, when glucagon secretion is inhibited glucose recovery from hypoglycaemia is markedly impaired by adrenergic blockade. Thus glucagon plays a primary role in recovery from insulin induced hypoglycaemia and glucagon deficiency is largely compensated by increased adrenomedullary adrenaline secretion. Glucose recovery is severely impaired in the absence of both glucagon and adrenaline. The acute release of cortisol and growth hormone has little role in recovery from insulin induced hypoglycaemia, although chronic deficiency of both cortisol and growth hormone increase the sensitivity to insulin (Rizza et al., 1979).

b) Post-Absorptive State

Diminished insulin secretion is fundamental to the maintenance of

the post-absorptive blood glucose concentration in that it permits hepatic glucose production to proceed via hepatic glycogenolysis and gluconeogenesis and limits glucose utilization by the liver, muscle and adipose tissue. Thus obligatory glucose utilization (brain, renal medulla, red blood cells) does not result in hypoglycaemia. However, insulin is not the sole determinant of the post-absorptive blood glucose concentration. Glucagon and possibly adrenaline may also have a role in maintaining the fasting glucose concentration (Rosen et al., 1984).

c) Post-Prandial State

After glucose ingestion blood glucose concentration rises as a result of glucose absorption and endogenous glucose production is markedly suppressed. The circulating concentration of glucose declines rapidly due to accelerated glucose utilization coupled with diminishing glucose absorption towards fasting levels. When glucose absorption is complete glucose production resumes. The transition from endogenous glucose declining to endogenous glucose production is regulated by the coordinated actions of insulin and glucagon. In the state of glucagon deficiency (induced experimentally by somatostatin and partial insulin replacement) adrenaline has a counter-regulatory effect but this is not observed in the presence of glucagon (Cryer, 1984).

8. GLUCOSE METABOLISM IN DIABETES

A decrease in insulin production and release and/or decreased insulin activity in target tissue is fundamental to the development of diabetes. Consequent to insulin deficiency glucagon concentrations may rise and a fall in the insulin:glucagon ratio causes increased

production of glucose by the liver, while an absolute decrease in insulin concentration or insulin action reduces glucose utilization in peripheral tissues resulting in hyperglycaemia. A further decline in the insulin:glucagon ratio can lead to syndromes of decompensation such as diabetic ketoacidosis and hyperosmolar non-ketotic coma. The release of catecholamines and other stress hormones can act in many ways and affect both insulin and glucagon secretion from the islets. Catecholamines can reduce endogenous insulin and increase glucagon stimulated lipolysis, decrease muscle glucose utilization and increase hepatic glucose production. Growth hormone can increase insulin and glucagon secretion, stimulate lipolysis from adipocytes, promote hepatic glucose production and inhibit muscle glucose utilization. Cortisol can also have similar actions to growth hormone (Unger and Foster, 1985).

Insulin deficiency blocks glucose utilization by insulin requiring tissues, activates lipolysis in adipose tissue, increases proteolysis in muscle, causes hyperglucagonaemia and enhances glucagon effects on the liver. Glucagon when opposed by a normal insulin response is primarily responsible for the hepatic components of diabetic decompensation, i.e. increase glycogenolysis, gluconeogenesis and ketogenesis (Unger and Foster, 1985).

The metabolic pathways in liver, muscle and adipose tissue during insulin deficiency are depicted in Figure 1.8. In the liver (Figure 1.8a) glucose release is mediated via glucagon although adrenaline, cortisol and growth hormone have a complementary role. Amino acid catabolism is stimulated by glucagon although cortisol does have an auxillary role. Ketone body production is modulated by glucagon and adrenaline also has a 'permissive' role. In muscle (Figure 1.8b), when the action of insulin is insufficient there is increased fatty

LIVER

Glycogen

Glucose ⇌ Glucose ⇌ Glucose-6-P → Phosphogluconate Pentose Pathway

α-Glycerol-P

Triglycerides

Amino Acids ⇌ Amino Acids ⇌ Pyruvate

Fatty Acids ⇌ Fatty Acids

Acetoacetyl CoA

Protein

TCA Cycle ← Acetyl CoA

$CO_2 + H_2O$

Ketone Bodies → Ketone Bodies

a

MUSCLE

Glycogen

Glucose ⇌ Glucose → Glucose-6-P

Amino Acids ⇌ Amino Acids ⇌ Pyruvate

Acetyl CoA ← Fatty Acids

TCA Cycle

Ketone Bodies

Protein

$CO_2 + H_2O$

b

ADIPOCYTE

Glycogen

Glucose ⇌ Glucose → Glucose-6-P → Phosphogluconate Pentose Pathway

α-Glycerol-P

Triglycerides ← Triglycerides

Pyruvate

Fatty Acids ⇌ Fatty Acids

With Insulin ——

TCA Cycle

Acetyl CoA

Insulin Deficiency ----

$CO_2 + H_2O$

c

Thick solid arrows (———) show the pathways that are favored in the presence of insulin, while thick broken arrows (------) depict those that predominate when the action of the hormone is insufficient.

Figure 1. 8   Major metabolic effects of insulin deficiency

acid and ketone body uptake. Adrenaline and growth hormone both contribute to fatty acid utilization in muscle. In the adipocyte (Figure 1.8c), triglycerides are broken down during insulin deficiency and the fatty acid release is modulated by adrenaline, cortisol and growth hormone.

This introductory chapter outlines glucose metabolism and its complex inter-relationships. The metabolic pathways and the role of the liver, muscle and adipose tissue were briefly discussed. Glucose and intermediary metabolism are regulated by hormonal and other factors to produce accurate control and reliable homeostasis. Aspects of glucose metabolism in diabetes were briefly summarized, including hormone physiology and intermediary metabolism. This chapter gives the biochemical and endocrine background to part of the research work of this thesis, in particular the study of glucoregulatory hormones and intermediary metabolites in uraemia and continuous ambulatory peritoneal dialysis (CAPD).

CHAPTER 2

GLUCOSE METABOLISM IN URAEMIA

1. INTRODUCTION

Abnormalities of carbohydrate metabolism in uraemia have been recognised for many years. Neubauer in 1910 first described hyperglycaemia in renal disease and this was soon confirmed by Hopkins in 1915. The prevalence of hyperglycaemia was probably overestimated prior to the mid 1960's as the methods for measuring glucose did not discriminate between glucose and non-glucose reducing substances (Reaven, 1974). However, these observations of 'uraemic pseudodiabetes' (Westervelt and Schriener, 1962) did not become clinically significant until the advent of dialysis. The development of dialysis and transplantation in the 1960's created a new significance for uraemic carbohydrate intolerance. The limited facilities for dialysis and restricted availability of donor kidneys resulted in exclusion of diabetics for renal replacement therapy. In many centres diabetics had a significantly higher morbidity and mortality than non-diabetics (Comty and Shapiro, 1975; Kjellstrand et al., 1972). Thus, it became important to distinguish between true diabetes and uraemia induced glucose intolerance.

In the past 20 years there has been a multitude of reports on carbohydrate metabolism which have widened our understanding of the complicated pathophysiology of renal failure.

2. THE ROLE OF THE KIDNEY IN PANCREATIC PEPTIDE METABOLISM

The kidney plays an important role in the metabolism of low molecular weight proteins including the alpha and beta cell peptides. This is characterised by a high extraction from the circulation and negligible urinary excretion of the intact molecule suggesting that hormone removed by the kidney is degraded locally (Katz and Emmanouel,

1978). Renal disposition involves both glomerular filtration and
tubular uptake and degradation. The kidney plays an important
function in the lowering and stabilizing of peptide hormone
concentrations but does not regulate them, this being a function of
the secreting endocrine gland. The increased circulating levels of
certain peptide hormones in uraemia suggests that the secretory rate
remains inappropriately high for the degree of reduction in their
metabolic clearance. This reflects either impaired feedback control
mechanisms, possibly in some instances due to end-organ resistance, or
to the inability of the endocrine gland to reduce secretion rates
appropriately (Emmanouel et al., 1981).

a) Insulin

Insulin is normally rapidly removed from the blood by the liver,
which clears 40-60% in a single passage (Ferrannini et al., 1983).
This proportion of insulin removed is dependent, in part, on the
plasma insulin concentration. The renal arterio-venous concentration
difference is about 30-40% (Rabkin et al., 1970; Katz and Rubenstein,
1973) and this is unaffected by changes in the plasma insulin
concentration within the physiological range (Maude et al., 1981).
The renal clearance of insulin is about 200 ml/min in man and from
this it is estimated that 6-8 units/day are degraded by the kidney
(Rubenstein et al., 1975). This accounts for a quarter of the daily
pancreatic secretion in man. However, only 0.1% of the endogenous
pancreatic insulin is excreted in the urine (Rabkin et al., 1984).
Since the normal glomerular filtration rate (GFR) (120 ml/min) is
significantly less than the renal clearance of insulin (200 ml/min)
peritubular uptake must be responsible for a significant portion of
the renal removal. The physiology of the renal handling of insulin

has been recently reviewed (Rabkin et al., 1984).

The clinical importance of these observations is that there is little change in the metabolic clearance rate of insulin until the GFR is decreased to 40 ml/min. Marked prolongation of the half life does not occur until the GFR is below 20 ml/min (Rabkin et al., 1970; Rubenstein et al., 1975). However, with progressively declining GFR there is a compensatory increase in peritubular insulin uptake such that insulin clearance changes only minimally. However, when renal functional mass is reduced to a critical level and the GFR is less than 20 ml/min the metabolic clearance rate falls precipitously (Rubenstein et al., 1975).

Impaired hepatic degradation of insulin may contribute to the prolonged half life observed in uraemia (DeFronzo et al., 1978a). Improved insulin clearance was observed after haemodialysis and was attributed to increased degradation by non-renal tissues, liver and muscle (Mondon et al., 1978). The accumulation of uraemic toxins that might inhibit insulin degrading systems may be removed by haemodialysis and account for the improved clearance of insulin (DeFronzo et al., 1978a). Metabolic clearance rates and secretory rates of insulin have been reported to improve after haemodialysis (Navalesi et al., 1975; Ferrannini et al., 1979). However, several direct studies have not detected any hepatic abnormality of insulin clearance even in hepatic cirrhosis (Proietta et al., 1984; Taylor et al., 1985).

b) C-peptide

Insulin and c-peptide are secreted into the portal circulation in equimolar concentrations, but in the peripheral blood c-peptide is 5-7 fold higher due to differences in the metabolic clearance rate (MCR).

The MCR for insulin (11 ml/min/kg) is higher than for c-peptide (4.4 ml/min/kg). Unlike insulin, c-peptide passes through the liver without significant extraction and is almost exclusively metabolized and degraded by the kidney. The immunological half life for insulin (5 mins) is considerably shorter than c-peptide (11-33 mins depending on experimental conditions). Normally 5-20% of pancreatic secretion of c-peptide is excreted in the urine. Renal dysfunction causes an increase in serum c-peptide level and the relative concentrations may be dependent upon the quantitative role which the kidneys play in their removal (Jaspan et al., 1977).

c) Proinsulin

Small amounts of proinsulin are released into the circulation with insulin and c-peptide in normal subjects, but this accounts for less than 15% of the total circulating insulin immunoreactivity. There is no evidence to suggest that proinsulin is converted to insulin in the circulation (Rubenstein et al., 1977). The metabolic clearance independent of plasma levels, is slower for proinsulin (3.1 ml/min/kg) compared to insulin (11 ml/min/kg) and the half life for proinsulin is about 25 mins compared to 5 min for insulin. In contrast to the difference in their MCR, the renal disposition of both peptides is similar with high extraction and very low urinary clearance (Katz and Rubenstein, 1973). The extraction rate for proinsulin is approximately 36% while the fractional urinary clearance is about 0.6% indicating more than 99% of the amount filtered is sequestered in the kidney. Thus the kidney is the major organ for proinsulin degradation and thus high concentrations of proinsulin may be expected in renal failure (Katz and Rubenstein, 1973; Mako et al., 1973).

d) Glucagon

Circulating glucagon is heterogeneous, consisting of proglucagon (molecular weight 9000) and the biologically active glucagon (molecular weight 3500). Glucagon is freely filtered by the glomerulus, reabsorbed and catabolized in the proximal tubule. There is active uptake and degradation by the peritubular membrane (Emmanouel et al., 1976). The metabolic clearance rate is approximately 10 ml/min/kg and the half life is 5.5 mins (Alford et al., 1976). The fractional extraction by the kidney is about 40% and less than 5% is excreted in the urine. Compared to insulin the liver plays a less important role in glucagon degradation (Felig et al., 1976) and renal excretion accounts for about half of glucagon degradation. In chronic renal failure the marked increase in circulating glucagon levels is largely due to proglucagon, but there is a significant rise in the biologically active glucagon (Emmanouel et al., 1976). The hyperglucagonaemia in uraemia is due to reduced renal clearance (Kuku et al., 1976) as the pancreatic secretion of glucagon is normal (Lefebvre and Luyckx, 1975).

3. GLUCOSE INTOLERANCE

Elevated fasting plasma insulin concentration with normal fasting glucose has been well documented in uraemia (Hutchings et al., 1966; Briggs et al., 1967; Horton et al., 1968). The fall in blood glucose following exogenous insulin administration is delayed and decreased (Westervelt and Schriener., 1962; Hampers et al., 1966; Horton et al., 1968; Spitz et al., 1970). The fall in blood glucose following IV tolbutamide was also diminished and delayed (Cerletty and Engbring, 1967; Spitz et al., 1970) and the concomitant insulin levels were elevated. All these studies suggest insulin antagonism. However,

there are conflicting reports on the plasma insulin response to both oral and IV glucose tolerance tests.

The early (2-10 mins) insulin response following IV glucose has been reported as normal (Horton et al., 1968), increased (Hutchings et al., 1966) or decreased (Hampers et al., 1966). Insulin levels during the latter part of the test (25-60 mins) have been uniformly increased (Horton et al., 1968; Hutchings et al., 1966; Hampers et al., 1966). Following an oral glucose stimulus the early release of insulin was found to be normal (Briggs et al., 1967; Cerletty and Engbring, 1967) or increased (Spitz et al., 1970) while late responses were elevated in all the studies.

These effects could be explained in part by prolongation of the half life of insulin, true hypersecretion of insulin, relative hypersecretion secondary to the higher glucose concentrations found later in the tests or a combination of these. Furthermore, the conflicting results could be explained by differences in the uraemic populations studied, such as the severity of renal failure (i.e. residual renal function) and the use and the 'adequacy' of dialysis. The decreased insulin response to IV glucose, in contrast, to the increased response to oral glucose (Hampers et al., 1966; Hampers et al., 1968) has been suggested to be due to insulin-releasing gut hormones which may have a role for maintenance of insulin secretion in uraemia (Creutzfeldt et al., 1970).

4. TISSUE SENSITIVITY TO INSULIN AND BETA CELL SENSITIVITY TO GLUCOSE

DeFronzo (1978b) suggests that the seemingly conflicting results in the literature concerning the plasma insulin response in glucose tolerance tests may represent two distinct groups of uraemic patients. He postulates that one group have 'normal' glucose tolerance and

increased plasma insulin levels, while the other group have impaired
glucose tolerance and diminished or normal plasma insulin responses to
infused glucose.

DeFronzo proposed that since peripheral antagonism (tissue
insensitivity) to insulin is uniform in uraemia, glucose tolerance
would only remain normal if the pancreatic beta cells were able to
increase their insulin secretion to overcome the insulin resistance.
This would conform to the first group of patients with 'normal'
glucose tolerance and increased plasma insulin responses. However, if
significant inhibition of insulin secretion was superimposed on a
state of insulin antagonism glucose intolerance would become overtly
impaired. This would conform to the second group, with impaired
glucose tolerance and normal or decreased insulin responses. Thus an
interaction between tissue sensitivity to insulin and beta cell
sensitivity to glucose might explain the seemingly discrepant results
concerning the insulin response to glucose. DeFronzo (1978a)
quantitated the relative contributions of impaired insulin secretion
and insulin resistance using clamp techniques. Using the
hyperglycaemic clamp technique the plasma insulin response was
biphasic, the early (0-10 mins) response was normal but the later
(10-120 mins) was higher in uraemics. After 10 weeks haemodialysis
the late plasma insulin response decreased and was not significantly
different from the controls. The decline in the insulin response was
mainly due to an increase in the metabolic clearance rate of insulin.
The change in insulin secretion was quite variable. In the majority
of patients insulin secretion increased negligibly (suggesting that
uraemia had an inhibitory effect on the beta cell). In some insulin
secretion fell post-dialysis (suggesting that the primary disturbance
was one of impaired insulin action). During the hyperglycaemic clamp

performed post dialysis the decreased insulin response resulted from a
small increase in insulin secretion and a larger increase in the
metabolic clearance rate of insulin. The amount of glucose
metabolized during the clamp studies was significantly less in
uraemics than controls. Although haemodialysis resulted in a marked
improvement in glucose metabolism it did not restore it to normal. To
provide an independent measure of tissue sensitivity to insulin,
DeFronzo's group (1978a) employed the euglycaemic insulin clamp
technique and found that tissue sensitivity to insulin was
significantly reduced in uraemics and following haemodialysis insulin
mediated glucose metabolism improved to a level only slightly less
than controls. The observations for the above clamp techniques has
promoted the concept than insulin resistance is present in most
patients with chronic renal failure and plays a dominant role in
glucose intolerance in uraemia. The normal beta cell response to this
insulin antagonism would be to augment its insulin secretion in an
attempt to override the insulin resistance. In some patients,
however, uraemia also impairs insulin secretion. In those subjects in
whom both tissue insensitivity to insulin and impaired beta cell
response to glucose occur together, the greater decline in glucose
tolerance is observed (DeFronzo, 1978a;1978b).

However, it should be noted that the increased circulating insulin
in uraemia may not necessarily be due to resistance to the action of
insulin, but delayed insulin degradation due to loss of renal
parenchyma may also be important (Reaven and Olefsky, 1978). The
insulin response to a constant glucose infusion was studied in an
animal model of acute uraemia induced without loss of renal mass.
There was no difference between pre- and post-insulin responses
(Swenson et al., 1973). The authors concluded that there was no

increase in insulin response when there was no loss of renal

parenchymal tissue. These results suggest that the elevation of the

insulin response in uraemia may be due to a defect in insulin removal

rather than insulin hypersecretion. Thus the rise in insulin

concentration does not necessarily mean an increased pancreatic

insulin response.

5. <u>THE SITE OF INSULIN RESISTANCE</u>

Impairment of insulin-mediated glucose metabolism in uraemia could

result from increased hepatic glucose production that does not

suppress normally following insulin, diminished glucose uptake by the

liver or impaired glucose uptake by peripheral (muscle and adipose)

tissues. Using the euglycaemic insulin clamp technique in combination

with radioisotope (H_3-glucose) turnover methodology and

hepatic/femoral vein catheterisation (DeFronzo <u>et al</u>. 1978a; 1980;

1981) showed that both suppression of hepatic glucose production and

splanchnic (hepatic) glucose uptake are normal in uraemia. In

contrast, the ability of insulin to increase glucose uptake in the leg

was markedly impaired in uraemic subjects. These results suggest that

the major site of insulin resistance resides in the periphery. This

is consistent with the forearm perfusion studies with constant

intra-arterial insulin infusion which showed reduced glucose uptake in

uraemia (Westervelt, 1969). Following haemodialysis tissue

insensitivity to insulin was found to return towards normal and

overall glucose intolerance improved (DeFronzo <u>et al</u>., 1978a).

6. <u>INSULIN RECEPTOR AND POST RECEPTOR DEFECTS</u>

Insulin, like other peptide hormones, is known to initiate its

effects on target tissues by binding to specific surface receptors

(Pastan et al., 1966). The resultant hormone receptor interaction triggers a sequence of membrane and intracellular events that produce the biological response. Thus insulin resistance could result from either a receptor or post receptor (intracellular) defect.

Decreased insulin binding to red cells has been reported in non-dialysed uraemics, however, after 1 year of haemodialysis erythrocyte binding decreased relative to controls (Gambhir et al., 1981). In contrast, insulin binding to rat adipocytes was normal (Maloff and Lockwood, 1981) and insulin binding to circulating monocytes in uraemic subjects was also normal (Smith and DeFronzo, 1982). Thus, by inference if receptor binding is normal in uraemia then it may be predicted that intracellular defects are responsible for the insulin resistance.

The site of insulin resistance has been studied by constructing an in vivo dose-response curve using the euglycaemic clamp technique. Insulin resistance can be classified as being due to either a decreased sensitivity or a decreased responsiveness to insulin. These two abnormalities are manifest by a shift to the right in the dose-response curve or a reduced maximum response respectively. The spare receptor concept indicates that reduced insulin receptor binding usually results in a decreased sensitivity while a post-receptor defect results in insulin unresponsiveness. The maximal responsiveness to insulin was decreased in uraemics and partially corrected by dialysis (Schmitz et al., 1983). A similar study showed both a decreased sensitivity and reduced responsiveness but the monocyte insulin binding was normal in keeping with a post-binding defect (Smith and DeFronzo, 1982). Thus, there is evidence that the site of insulin resistance is due to impaired intracellular glucose metabolism or abnormal glucose transport within the cell.

7. THE ROLE OF THE LIVER

The role of the liver in the development of glucose intolerance has been examined by measuring the rate of glucose turnover and by quantifying the hepatic responses to glucose and insulin infusion.

Data on hepatic glucose production in uraemia are conflicting. Rubenfeld and Garber, 1978, observed a 50% increase in glucose turnover, a 2-fold rise in alanine and that gluconeogenesis (mainly from alanine) increased in non-dialysed uraemic patients. Following haemodialysis there was a reduction, but not a complete reversal of the accelerated gluconeogenesis (Rubenfeld and Garber, 1979). They postulated an increase in glucose utilization following dialysis treatment. The increased gluconeogenesis could also be due to increased hepatic sensitivity to glucagon (Sherwin et al., 1976).

The importance of gluconeogenesis for glucose homeostasis has been underlined by the observation of hypoalaninaemia and decreased rates of gluconeogenesis in debilitated uraemic patients presenting with hypoglycaemia (Garber et al., 1974). The kidney also has a role in gluconeogenesis. During prolonged starvation the normal kidney has been reported to contribute about half of the glucose production through recruitment of the gluconeogenic pathway (Owen et al., 1969).

However, in contrast, normal rates of glucose production have been reported (DeFronzo et al., 1981; Ricanati et al., 1983). Both suppression of hepatic glucose production and splanchnic (hepatic) glucose uptake were normal in uraemia. Kalhan et al. (1983) showed that in chronic renal failure glucose production did not change, but glucose carbon recycling increased and glucose oxidation decreased. Haemodialysis had no effect on any of these variables.

8. GLUCAGON AND GLUCOSE METABOLISM

Increased circulating levels of glucagon have been implicated in the development of carbohydrate intolerance and insulin resistance in uraemia. Although proglucagon is primarily responsible for the elevated levels of increased circulating glucagon, there is up to 3-fold elevation of the biologically active glucagon (MW 3500) (Emmanouel et al., 1976). High circulating plasma glucagon concentrations result from decreased renal clearance (Kuku et al., 1976). The increase in glucagon (MW 3500) is due to the decrease in metabolic clearance rate as glucagon secretion is normal (Lefebvre and Luyckx, 1975). Compared to insulin the liver plays a much less important role in glucagon degradation (Felig et al., 1974). The primary effect of glucagon is to stimulate hepatic glucose production. Basal hepatic glucose production is not increased and is normally suppressed by insulin in uraemia and glucagon has no clear effect on the peripheral utilization of glucose (DeFronzo et al., 1978a). Thus it is unlikely that hyperglucagonaemia per se plays a role in glucose intolerance under conditions where the plasma insulin is elevated.

However, the plasma glucose response to glucagon is enhanced in uraemia indicating increased hepatic sensitivity to glucagon. Following glucagon infusion the increase in plasma glucose concentration in uraemia was 3-4 fold greater than controls. Haemodialysis corrected the excessive glycaemic response to glucagon (Sherwin et al., 1976). These findings suggest that hepatic sensitivity to glucagon is increased while tissue sensitivity to insulin is decreased in uraemia. In the situation, for example after a protein meal, when the plasma glucagon response is high and the insulin response is relatively low excessive stimulation of hepatic glucose output may ensue and result in glucose intolerance. Elevated

glucagon levels may also contribute to increased gluconeogenesis from alanine in skeletal muscle (Rubenfeld and Garber, 1978). Furthermore, DeFronzo and Smith (1985) have found increased alanine uptake by splanchnic tissue (liver) in uraemia suggesting gluconeogenesis is increased in chronic renal failure. This has important clinical implications and may explain the muscle wasting and negative nitrogen balance commonly observed in uraemia.

9. PARATHYROID HORMONE AND GLUCOSE METABOLISM

Abnormalities in the metabolism of parathyroid hormone (PTH) have also been implicated in the pathogenesis of carbohydrate intolerance associated with uraemia.

Increased insulin secretion was observed in uraemic patients with severe secondary hyperparathyroidism which improved following parathyroidectomy (Lindall et al., 1971). This observation was attributed to the parathyroid hormone effect on pancreatic islet function. However, others have failed to show any relationship between secondary hyperparathyroidism and glucose tolerance, insulin secretion and insulin sensitivity (Amend et al., 1975).

More recently studies in animals (Akmal et al., 1984) and humans (Mak et al., 1983) suggest that excess PTH inhibits the compensatory increase in insulin secretion in a insulin resistant state, thus contributing to the glucose intolerance of chronic renal failure. Graf et al. (1985) using the hyperglycaemic clamp technique evaluated the role of PTH on glucose metabolism in chronic renal failure and dialysis. They found that patients on haemodialysis had normal peripheral glucose uptake. The beta cell response to hyperglycaemia (during the early phase as well as during the steady state) was the same in controls and non-parathyroidectomised uraemics, whereas, those

who had parathyroidectomy, i.e. previous severe and long standing
secondary hyperparathyroidism, had markedly increased insulin
secretion. Tissue sensitivity to insulin was normal in the
non-parathyroidectomised haemodialysis group but was decreased in the
parathyroidectomy group. Thus PTH may have a suppressive effect on
insulin secretion.

10. GROWTH HORMONE AND GLUCOSE METABOLISM

Growth hormone (GH) concentrations are frequently elevated in
renal failure but do not correlate with glucose intolerance in
individual patients (Horton et al., 1968; Saaman and Freeman, 1970;
Orskov and Christensen, 1971). Further, despite an apparent
improvement in carbohydrate intolerance and insulin sensitivity with
haemodialysis levels of growth hormone remain unaffected (Saaman and
Freeman, 1970). Abnormal growth hormone concentration in chronic
renal failure results from both impaired degradation and abnormalities
in secretion. Peripheral disposal of this peptide depends largely on
renal mechanisms (Cameron et al., 1972; Gottheiner et al., 1979). The
normal response of reduction in growth hormone following glucose
administration does not occur in renal failure suggesting that the
secretory dynamics are altered. In fact, levels of growth hormone
often show a paradoxical rise during glucose tolerance testing (Wright
et al., 1968; Saaman and Freeman, 1970). The latter change has been
attributed to protein and caloric malnutrition since this paradoxical
response is observed in conditions such as kwashiorkor and marasmus
(Pimstone et al., 1966).

DeFronzo (1978b) found no difference in fasting GH levels pre- and
post-dialysis. The majority of patients showed no change in GH
response with sustained hyperglycaemia. Thus the deranged growth

hormone physiology in uraemia is unlikely to be related to
carbohydrate metabolism.

11. LIPID METABOLISM AND CARBOHYDRATE METABOLISM

There is an important inter-relationship between carbohydrate and
lipid metabolism in uraemia. Type IV - (carbohydrate induced and
accompanied by glucose intolerance) hyperlipidaemia is common in renal
failure.

Hypertriglyceridaemia with normal or increased cholesterol is
common in uraemia. The lipid abnormalities in chronic renal failure
include an increase in very low density lipoproteins (VLDL), normal or
increased low density lipoproteins (LDL) and decreased high density
lipoproteins (HDL). There is also an increase in saturated fatty acid
esters and a reduction in linoleic acid. Low concentrations of
HDL-cholesterol, high total serum cholesterol (Kannel et al., 1979),
high VLDL-triglyceride (Norbeck et al., 1980) and relatively low
linoleic acid and high fatty esters (Norbeck et al., 1982) are thought
to promote atherosclerosis.

The aetiology and pathogenesis of lipid abnormalities in uraemia
are still unclear. Abnormalities from deranged carbohydrate
metabolism have been long implicated (Bagdade et al., 1968; Olefsky et
al., 1974). Increased hepatic synthesis of triglycerides or impaired
removal of triglycerides from the circulation have been suggested as
the cause of hypertriglyceridaemia.

Peripheral insulin resistance combined with hyperinsulinaemia,
resulting from insulin antagonism and/or impaired degradation, have
been reported to increase the hepatic synthesis of VLDL triglyceride
(Bagdade et al., 1968). However, later studies have failed to show
any relationship between hyperinsulinaemia and hypertriglyceridaemia

in chronic renal failure (Cattran et al., 1976). Indeed, reduced

rates of triglyceride synthesis has been reported (Cattran et al.,

1976; Sanfelippo et al., 1977).

The main abnormality appears to be diminished catabolism of

lipoproteins. Impaired removal of circulating triglycerides has been

well documented (Cattran et al., 1976; Ibels et al., 1976). This may

be due to low enzyme levels of both serum and hepatic lipoprotein

lipase (Chan et al., 1982).

Serum lipoprotein abnormalities occur early in chronic renal

failure and are only marginally, if at all, affected by haemodialysis

(Hass et al., 1983) or low protein diets (Attman et al., 1984). In

uraemia increased dietary intake of carbohydrate markedly increases

triglyceride concentrations which may reverse with dietary restriction

(Sanfelippo et al., 1978). In CAPD, patients are exposed to large

quantities of glucose and subsequently develop an increase in both

triglyceride and cholesterol (Gokal et al., 1981).

12. URAEMIA AND DIABETES

In type I (insulin dependent) diabetes mellitus, destruction of

the beta cells leads to loss of insulin secretory capacity and the

insensitivity to insulin appears to be secondary to the metabolic

consequences of insulin deficiency (Nankervis et al., 1984).

In type II (non-insulin dependent) diabetes mellitus, the

mechanism of the disorder is still not clear. Abnormalities of both

insulin secretion and insulin action are found in patients with

established type II diabetes (Weir, 1982) and in glucose intolerance

(Reaven and Miller, 1979).

Insulin insensitivity was found to be a more constant feature of

type II diabetes than defective insulin secretion which might result

from secondary decompensation of the beta cells in response to hyperglycaemia (Reaven, 1984). In contrast, others have found impaired beta cell secretory activity as the dominant feature (Kadowski et al., 1984). Recently, using the technique of continuous infusion of glucose with model assessment (CIGMA), O'Rahilly et al. (1986) found beta cell function the primary defect in type II diabetes.

In uraemia, insulin insensitivity appears to be the dominant feature whereas the beta cell response is variable and probably plays a minor role. Basal hepatic production of glucose is normal or only minimally elevated in both uraemia and type II diabetes (DeFronzo, 1978b). Futhermore, glucose production decreases in both groups following physiological hyperinsulinaemia. Thus uraemia and type II diabetes share some similarities, with insulin resistance being more prominent in uraemia and beta cell dysfunction being more characteristic of type II diabetes (Figure 2.1). However, despite insulin insensitivity it is uncommon for uraemics to develop overt diabetes unless there is an underlying genetic diabetic predisposition. Both type I and II diabetes often have marked deterioration in their glucose control with progression of renal failure. Insulin requirements may need to be increased and type II diabetes may need insulin supplementation to control their glycaemia. However, when the GFR falls below 20 ml/min a paradoxical situation arises. The clearance of insulin becomes markedly reduced and less insulin is degraded resulting often in better control or reduced requirements and improved glucose tolerance. Following the institution of dialysis a complex situation situation develops. Dialysis may increase the tissue sensitivity to insulin thereby decreasing insulin requirements. However, dialysis may also improve
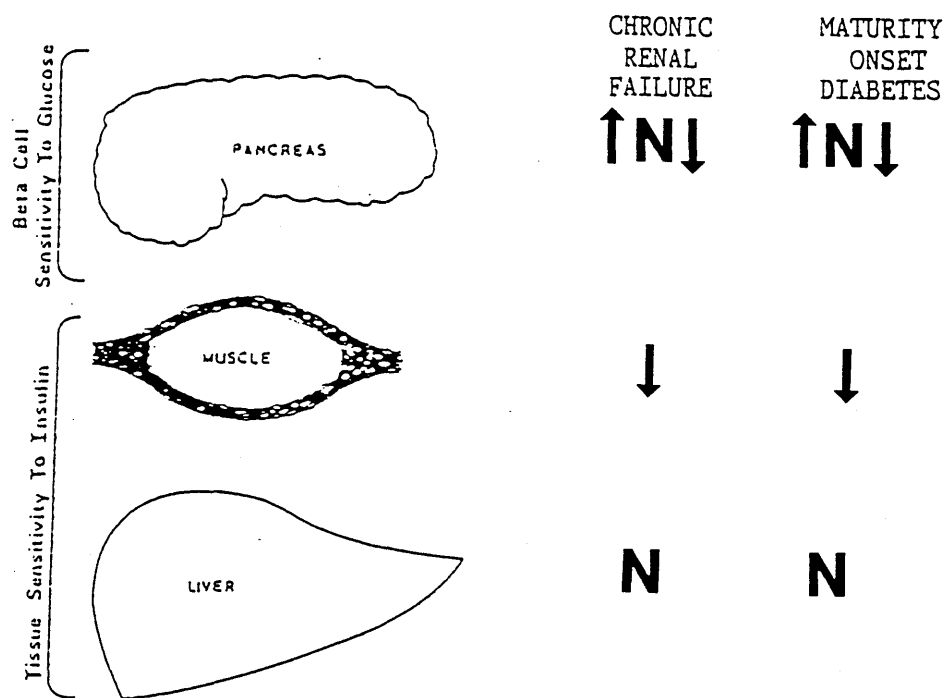
Figure 2. 1   Comparison of mechanisms of glucose intolerance in
uraemia and diabetes
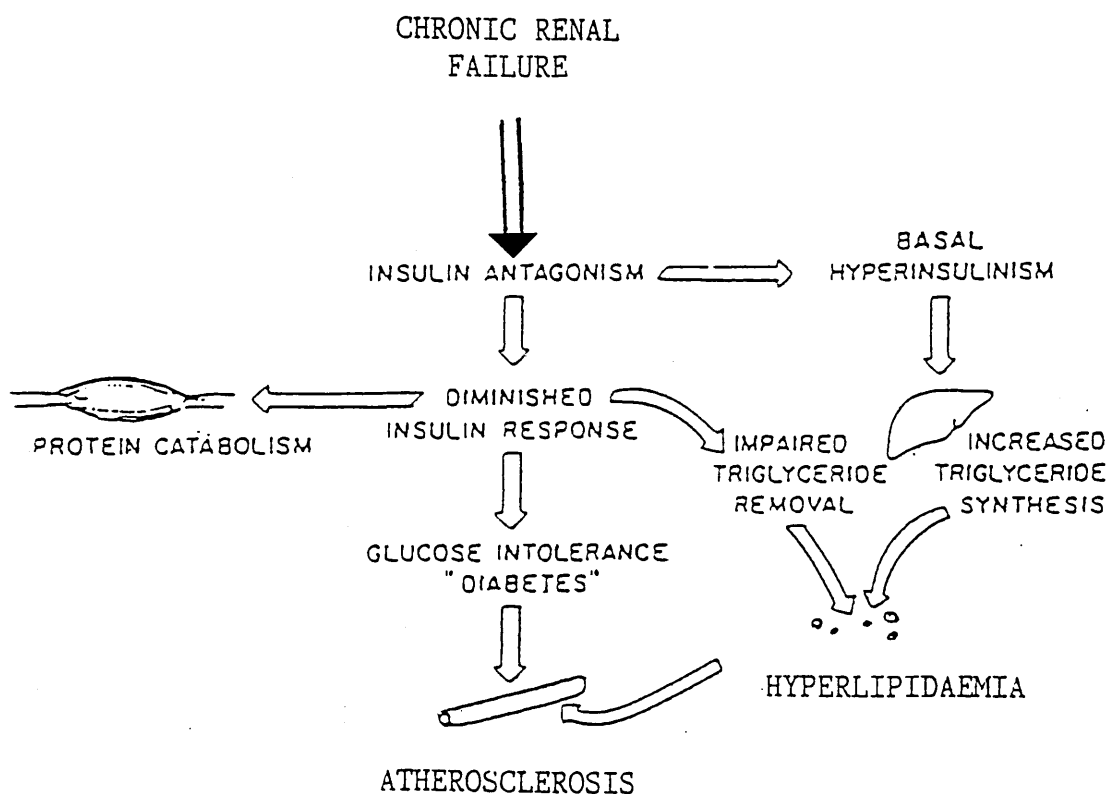
Figure 2. 2   Interrelationship between insulin antagonism and
disturbed carbohydrate, lipid and protein metabolism
in uraemia

insulin degradation towards normal thereby increasing the need for insulin. In view of this paradox it is difficult to predict for any individual what will happen to their insulin requirements.

13. CLINICAL IMPLICATIONS OF GLUCOSE INTOLERANCE IN URAEMIA

As previously discussed, it is unusual for uraemic patients to develop overt diabetes despite their underlying glucose intolerance. However, impaired glucose metabolism in uraemia may have potential adverse effects.

Patients with chronic renal failure have a higher incidence of cardiovascular disease (Linder et al., 1974). Figure 2.2 shows the possible inter-relationship of disturbed carbohydrate, lipid and protein metabolism. However, there is no direct evidence that glucose intolerance per se contributes to atherosclerosis and its subsequent complications. Nevertheless, some insight can be gained from data available on patients with diabetes mellitus.

Accelerated atherosclerosis is common in diabetes. Hyperinsulinaemia in diabetes has been implicated as a causative factor in the pathogenesis of atherosclerosis (Stout, 1979). Insulin has been shown to increase the transport of cholesterol into arteriolar smooth muscle cells and to stimulate the proliferation of endothelial cells lining the arterial vascular wall. Thus the uraemic hyperinsulinaemic state may play a role in hyperlipidaemia.

Diabetes has been associated with an increase in cardiovascular mortality in clinical (Kessler, 1971), life insurance (Goodkin, 1975) and population studies (Garcia et al., 1974). Although other risk factors such as hypertension, obesity and hyperlipidaemia are well known, the presence of fasting hyperglycaemia and a diabetic glucose tolerance curve is an independent risk factor in cardiovascular

disease (National Diabetes Data Group, 1979).

However, most uraemic patients do not fulfil the criteria for the diagnosis of diabetes but do have impaired glucose tolerance. Some cross sectional population studies have shown that impaired glucose tolerance carries an increase in prevalence of coronary artery disease and is independent of hypertension and hyperlipidaemia (Keen et al., 1965; Yano et al., 1982).

Although it is difficult to separate the other risk factors in uraemia, in particular hypertension and hyperlipidaemia, there is suggestive evidence that impaired glucose tolerance carries an increase risk of cardiovascular complications. Hyperglycaemia per se can result in production of abnormal circulating proteins such as glycosylated haemoglobin and albumin and this may contribute to abnormalities in structural proteins of the capillary basement membrane in muscle and kidney in diabetics (Skyler, 1979; Jovanovic and Petersen, 1981). Glycosylated haemoglobin has also been found to be elevated in uraemia in the absence of diabetes and is discussed in detail in Chapters 4 and 8. If uraemia per se increases glycosylated haemoglobin or some similar substance then possibly structural changes may ensue which might have a role in atherosclerosis (DeFronzo and Smith, 1985).

14. SUMMARY

In summary, abnormal glucose metabolism in uraemia is characterised by fasting normoglycaemia, abnormal glucose tolerance, hyperinsulinaemia and hyperglucagonaemia. Impaired renal degradation plays a significant role in the circulating concentrations of both insulin and glucagon. Glucose intolerance results from impaired glucose utilization manifested by reduced glucose uptake in peripheral

sites such as skeletal muscle due to the resistance to the action of insulin. In addition and possibly inter-related to tissue insensitivity to insulin impaired beta cell function to glucose may play a role. This concept could explain some of the conflicting evidence in the literature. Increased glucose production by the liver is controversial. Recent studies have shown that hepatic glucose production is normal but increased gluconeogenesis from amino acid pathways in skeletal muscle may be important. Hepatic sensitivity to glucagon is increased while peripheral insulin sensitivity is reduced. The site of peripheral antagonism appears to be post-receptor, that is, an intracellular rather than a receptor defect. The suppressive effect of parathyroid hormone on insulin secretion remains controversial but recent work suggests that it may have a role in carbohydrate metabolism in uraemia.

The pathophysiology of carbohydrate metabolism in uraemia remains complex and probably multifactorial. The abnormalities may partly be improved with dialysis treatment but complete reversal is uncommon. The similarities between glucose intolerance in uraemia and diabetes mellitus, and the inter-relationship between disturbed carbohydrate and lipid metabolism and the resultant hyperlipidaemia, provide suggestive evidence that impaired glucose metabolism predisposes to cardiovascular disease.

CHAPTER 3

GLUCOSE METABOLISM AND CAPD

1. CONTINUOUS AMBULATORY PERITONEAL DIALYSIS

The term dialysis was introduced by the Scottish chemist Thomas Graham who demonstrated the process of separating substances using a semipermeable membrane made of parchment and albumin (Graham, 1854). As well as designing a 'dialyser' he suggested that tissue could be used as a functioning semipermeable membrane. However, it was not until the 1920's that both haemodialysis and peritoneal dialysis were used in man to treat acute renal failure. In the 1960's significant advances were made in the treatment of chronic renal failure. Vessel access in the form of arteriovenous shunts and the subcutaneous fistula were developed for haemodialysis, while Tenckhoff and Schechter (1968) designed a permanent peritoneal catheter for chronic intermittent peritoneal dialysis. Further development in machine and artificial dialyser technology made haemodialysis a more efficient and acceptable treatment for general use. However, intermittent peritoneal dialysis remained less popular because of inferior solute clearances, the high incidence of peritonitis and the relatively long periods patients were attached to fluid cycling machines. Continuous ambulatory peritoneal dialysis (CAPD) was developed in 1975 when Popovich and Moncrief described a 'portable equilibrium dialysis technique' (Popovich et al., 1976). Modifications (Oreopoulos et al., 1978) and clinical experience have now made CAPD an acceptable mode of renal replacement therapy.

a) Principle

CAPD is based on solute diffusion across a semipermeable membrane and fluid movement by osmosis. The fluid compartments, blood and dialysis fluid in the peritoneal cavity are separated by a natural semipermeable membrane, the peritoneum. The process of

transperitoneal equilibration between dialysate and plasma is determined by time of exposure of the dialysate to the peritoneum and the molecular size of the solute.

Small molecules, such as urea (60 Daltons) achieve equilibration within 2 hours of dialysate dwell whereas larger solutes (500-5000 Daltons) take proportionately longer to approach equilibrium. Although it may be expected that after small solutes achieve rapid equilibration clearances would be reduced with dwell times longer than 3-4 hours the continuous nature of the system compensates such that weekly clearances are satisfactory to control uraemia. Small solute clearances in CAPD are superior to intermittent peritoneal dialysis though inferior to haemodialysis. The process of equilibration for large solutes continues for long periods and favours long dwell times. Clearance of large molecules falls less rapidly with long exchanges than small solutes. Large solutes are cleared more efficiently in CAPD than either intermittent peritoneal dialysis or haemodialysis. Thus CAPD has a low solute flow rate but this is compensated by the continuous process of the technique.

b) Technique

The procedure consists of instilling by gravity dialysis fluid, contained in plastic (PVC) bags, through a plastic tube (transfer set) which is connected to a permanent peritoneal catheter. The fluid equilibrates within the peritoneal cavity for 4 to 6 hours and is then drained out by gravity. Generally, three 2 litre exchanges are performed during the day and a longer (8 hours) exchange is done overnight. Following instillation of the fluid the empty plastic bag is rolled up and attached to the body, allowing the patient freedom to carry out normal daily activities, till the end of the cycle. At the

end of the exchange the bag is disconnected and replaced by fresh

dialysis fluid. The instilling and drainage procedure takes about 25

minutes. Several modifications of the system and innovations in

'connector' technology have improved the technique and reduced the

incidence of peritonitis.

Variations in the technique can be applied to meet the needs of

the individual patient. Continuous cyclic peritoneal dialysis (CCPD)

is a variation of CAPD which involves using a machine to instil and

drain fluid automatically. Generally several cycles are performed

during the night when the patient is asleep and one exchange (or none

- empty peritoneum) is done during the day. Currently experience with

this technique is limited.

2. THE ROLE OF GLUCOSE IN CAPD

a) Dialysis solutions

The chemical composition of peritoneal dialysis solutions is

fundamental in the process of CAPD. Commercially available solutions

are now relatively standardised and provide satisfactory electrolyte,

mineral and acid-base balance. Ultrafiltration is achieved by an

osmotic agent which to date has been glucose. Dialysis solutions and

systems in CAPD have been recently reviewed by Winchester (1986).

There are minor differences in the non-osmotic composition of

fluids which vary between manufacturers. In general, the constituents

are sodium (130-134 mmol/l), chloride (100-103 mmol/l), calcium

(1.35-1.75 mmol/l), magnesium (0.5-1.5 mmol/l) and lactate (35

mmol/l). Fluids are usually potassium free. The osmotic component of

the fluid is glucose (dextrose), which varies between 1.36 g/dl (%)

and 4.25 g/dl. This allows manipulation of the osmotic strength of

the fluid to achieve the desired degree of ultrafiltration.

A sodium content of about 132 mmol/l is adequate in most clinical situations in CAPD and any extracellular fluid contraction and postural hypotension due to excessive ultrafiltration can be corrected by adjustment to the osmotic strength of the fluid or to the sodium and water oral intake. This is in contrast to intermittent peritoneal dialysis when a lower sodium concentration is often recommended, particularly when the dwell times are short and hypertonic solutions result in disproportionately greater removal of extracellular water than sodium. Potassium is generally not required to maintain normal serum concentrations and any change in serum potassium can be corrected by oral or dietary manipulation. A positive calcium balance is maintained in most patients, but underlying renal osteodystrophy may require alterations in either the calcium content of the fluid or more usually oral supplementation with calcium and vitamin D analogues. Magnesium concentrations are usually well maintained and rarely require alteration, but the increased use of magnesium containing phosphate binders may in the future warrant changes in the dialysate magnesium concentration.

Acid-base balance is achieved by lactate. Bicarbonate would be ideal but there are two drawbacks related to the manufacturing process that prevents its use. In the presence of bicarbonate insoluble calcium and magnesium salts precipitate on storage and the alkali pH of bicarbonate produces caramelisation of glucose during autoclaving. Acetate and lactate have been used as alternative buffers without the above problems. However, recently the use of acetate has been associated with loss of ultrafiltration (International Study Group, 1984) and a possible association with the rare, but lethal, condition of sclerosing peritonitis (Slingeneyer et al., 1983). Thus lactate is now exclusively used as the buffer in CAPD fluids.

b) <u>Osmotic agents</u>

Fluid removal during peritoneal dialysis depends on osmotic

forces. Glucose has been used as an osmotic agent since the early

experimental days and has proven to be safe, effective and

inexpensive. Indeed dextrose is the only commercially available

osmotic agent at present. However, the high rate of peritoneal

glucose absorption has lead to obesity, hypertriglyceridaemia and the

potential hazardous effects on carbohydrate metabolism and accelerated

atherosclerosis has generated interest in alternative osmotic agents.

Glucose in dialysis fluids can undergo spontaneous breakdown to

aldehyde, 5-hydroxymethylfurfural, laevulinic and formic acids. This

process may be accentuated by extreme heat during the sterilization of

fluids during manufacture. The effect of storage and heating of the

dialysis solution by the patient prior to instillation has been shown

to increase glucose metabolites (Henderson <u>et al</u>., 1984). Henderson

<u>et al</u>. (1984) propose that metabolites (5-hydroxymethylfurfural) may

combine with lactate to form Schiff bases which can alter the

properties of tissue components. Thus glucose metabolites may have an

adverse effect on the peritoneal membrane. To prevent the

caramelisation of glucose during autoclaving, hydrochloric acid is

added to the dialysis solution to keep the pH below 5.5. This low pH

may have disadvantages, as it has been attributed to the pain some

patients have on initial instillation. It can also inhibit

phagocytosis and affect intracellular killing of bacteria, but this is

probably only transient as the dialysate pH rises to 7 within 1 hr

after instillation (Vas <u>et al</u>., 1981).

Alternative agents have been investigated and reviewed by

Winchester (1986). Currently, no agent has shown any definite

advantage over dextrose. Some agents have shown good ultrafiltration characteristics but adverse effects have prevented their use. Sorbitol, fructose, xylitol and polyanions have revealed toxic effects. Amino acid solutions are potentially useful and have both good ultrafiltration properties and nutritional value but at present are too expensive for general use. Glucose polymers which are not significantly absorbed, with sustained ultrafiltration characteristics and reduced caloric load look promising but some have shown prolonged retention and impaired metabolism. Future research may soon find a suitable alternative to dextrose.

3. CLINICAL EXPERIENCE

Since its conception in 1975 there has been a vast global expansion in CAPD. The reasons are multiple and include medical, social and economic factors.

In recent years many reports on the clinical experience with CAPD have revealed variable results (Chan et al., 1981; Kurtz et al., 1983; Ramos et al., 1983; Heaton et al., 1986; Morgan et al., 1986, Tsakiris et al., 1986). Although patient selection can have a strong bias on clinical data (Coward et al., 1982), with experience clinical outcome has significantly improved. Peritonitis is undoubtedly the major complication (Williams et al., 1981; Gokal et al., 1982; Smith et al., 1986), but catheter related problems are responsible for many hospital admissions and technical failures. CAPD is particularly suitable for diabetics (Flynn, 1983), the elderly (Nicholls et al., 1984) and young children (Balfe and Watson, 1986). These groups were often denied treatment in the recent past as they were considered 'poor candidates' for haemodialysis or transplantation.

The merits of CAPD are generally compared to those of

haemodialysis as it is accepted that for most patients a successful transplant is the optimal treatment. In brief, CAPD provides adequate control of uraemic symptoms and signs. Salt and water removal is achieved by hypertonic glucose solutions and helps maintain fluid balance and controls hypertension. Electrolyte, mineral and acid-base balance are regulated by the chemical constituents of the dialysis fluid. The advantages of CAPD are; a feeling of well being (perhaps due to several factors including better clearance of 'uraemic toxins'), less restricted fluid and diet control, steady state biochemistry, improved control of anaemia and hypertension and is generally more appropriate for diabetics, young children and the elderly. Social factors such as relative independence, freedom to travel and lower financial cost are also important. The disadvantages include peritonitis and catheter-related problems, particularly if they require regular hospital admissions, obesity and other forms of malnutrition, hyperlipidaemia and the uncertainty of long term viability of the peritoneum.

It is estimated that about 30,000 patients are on CAPD worldwide (Gokal, 1986). In the UK the 'CAPD explosion' has resulted in more patients being treated with CAPD than home haemodialysis (EDTA report, 1985). Patient and technique survival have improved and are now similar to haemodialysis. Patient survival is about 80% at 2 years and 50% at 4 years, and the main reason for 'drop out' from CAPD is now transplantation (Gokal, 1986).

a) Diabetes and CAPD

Prior to 1970 most diabetics with end stage renal disease were denied treatment largely due to the high morbidity and mortality (Drukker et al., 1971; White et al., 1973). Progress on the

understanding of the pathophysiology of diabetes, improved control of glycaemia and management of complications as well as advances and improved facilities in dialysis and transplantation have dramatically changed the outlook. It is now estimated that 25% of new dialysis patients and 20% of transplant recipients in the USA are diabetic (Friedman, 1985). In the UK the proportion of diabetics on renal replacement therapy has increased from 1.4% in 1975 to 11.1% in 1984 (Cameron and Challah, 1986).

The advantages of CAPD over haemodialysis for diabetics is partly factual, partly anecdotal and still debatable (Legrain and Keen, 1983; Whitley and Kjellstrand, 1984; Friedman and Peterson, 1986). In brief, the advantages of CAPD include steady state control of uraemia, good control of hypertension and a stable cardiovascular state without rapid fluid shifts and tighter blood glucose control by the use of intraperitoneal insulin. Heparinisation and vessel access are avoided. However, the peritoneal glucose load may promote obesity, hyperglycaemia, hyperlipidaemia and aggravate accelerated atherosclerosis. Despite the theoretical advantage of removal of middle molecules and uraemic toxins in CAPD peripheral neuropathy is generally unchanged.

b) Blood glucose control and intraperitoneal insulin

Improved blood glucose control is seen in most diabetics on CAPD and is largely due to the use of intraperitoneal insulin administration (Flynn et al., 1979; Khanna et al., 1983; Rottembourg et al., 1983). Normally insulin is secreted into the portal vein in response to various stimuli and about 50% is removed by passage through the liver. Intraperitoneal insulin acts like an 'artificial pancreas' and mimics this physiological route. Clinical experience in

CAPD suggests that intraperitoneal insulin is superior to the subcutaneous route. However, the dose of insulin requires to be increased (often 3-4 fold) to achieve adequate glycaemic control. In addition to the peritoneal glucose load, insulin binding and retention to the dialysate bags, slow transperitoneal absorption because of its large molecular size, losses to the dialysate and possibly other metabolic and kinetic factors contribute to the higher insulin requirements (Khanna et al., 1986).

The use of intraperitoneal insulin to reduce hypertriglyceridaemia is unclear. A fall in triglyceride concentrations has been reported by some (Moncrief et al., 1981) but not by others (Beardsworth et al., 1983). Despite the use of intraperitoneal insulin, which usually involves injecting directly into the dialysate bags, the incidence of peritonitis in diabetics is no different from non-diabetics on CAPD (Rottembourg et al., 1983).

The use of glycosylated haemoglobin in monitoring glycaemic control in diabetics is well established but the value of this indicator in uraemic diabetics is controversial and is discussed and investigated in Chapters 4 and 8.

The survival of diabetics on dialysis and transplantation has significantly improved in recent years (Friedman and Peterson, 1986). Recent data from two large centres show that CAPD (Toronto) and haemodialysis (Minnesota) have similar morbidity and mortality and this was largely due to accelerated atherosclerosis (Khanna and Oreopoulos, 1986).

4. GLUCOSE ABSORPTION AND CAPD

CAPD provides the patient with a continuous glucose infusion and quantification of this carbohydrate load is important due to its nutritional consequences.

The average daily absorption of glucose from dialysis solutions varies between 100 to 200g (Grodstein et al., 1981; Von Baeyer et al., 1981). On a typical regimen of 3 isotonic (1.36%) and 1 hypertonic (3.86%) 2 litres exchanges about 120g of glucose will be absorbed per day (Nolph et al., 1979). During a 6 hour cycle 60-80% of glucose instilled into the peritoneal cavity is absorbed. This amounts to 45-60g from a 3.86% solution and 15-22g from a 1.36% solution (Grodstein et al., 1981). These workers calculated that on average 182g of glucose was absorbed per day, which represented 8.4 Kcal/Kg/day or between 12% and 34% of the total energy intake. Similar studies have reported that glucose absorption from peritoneal dialysis fluid accounts for about 20% of the total energy intake (Lindholm and Bergstrom, 1986).

The rate of glucose absorption varies between patients, indicating individual differences in peritoneal permeability. However, within an individual patient the glucose absorption is fairly constant for a given fluid regimen (Grodstein et al., 1981; Lindholm and Bergstrom, 1986).

The rate of glucose absorption may increase during episodes of peritonitis due to increased peritoneal permeability and increased diffusive transperitoneal transport of glucose (Rubin et al., 1981a; Verger et al., 1984). The accelerated glucose absorption results in a rapid decrease in dialysate glucose concentration and a reduction in the osmotic driving force for water removal (Henderson, 1985). This may result in the need for more hypertonic and/or more frequent

exchanges to maintain adequate ultrafiltration.

The long term effect of glucose absorption through the peritoneum is unknown. Loss of ultrafiltration has been reported with time on CAPD and several aetiological factors have been proposed, including the use of hypertonic dextrose, acetate, impurities such as particulate matter, endotoxins and glucose metabolites. Morphological and functional changes in the peritoneum have also been observed and associated with some of these factors. This subject has been reviewd by Diaz Buxo (1984) and more recently by Henderson and Gokal (1986).

5. METABOLIC AND NUTRITIONAL ASPECTS OF CAPD

The continuous absorption of glucose in CAPD may contribute to: hyperglycaemia, hyperinsulinaemia, excess weight gain, aggravation of lipid and lipoprotein abnormalities, alteration in protein and amino acid metabolism which may induce malnutrition, and possibly promote premature atherosclerosis.

a) Glucose intolerance in CAPD

Glucose intolerance is common in uraemia and is largely due to impaired peripheral insensitivity to the action of insulin (DeFronzo et al., 1978a). The effect of CAPD on glucose intolerance is unclear. Further deterioration in glucose tolerance and a decreased rate of insulin secretion was found with oral glucose testing (Armstrong et al., 1980) and a suggestion of beta cell depletion after intravenous glucose loading has also been reported (Panzetta et al., 1982). In contrast, others have found no change in the degree of glucose intolerance up to 1 year after commencing CAPD (Lindholm et al., 1981; Von Baeyer et al., 1983). The continuous glucose administration could potentially alter pancreatic beta cell function possibly by depleting

the beta cells. This is discussed in Chapters 2 and 6.

During CAPD a hypertonic exchange induces hyperglycaemia and hyperinsulinaemia in a similar manner to an oral or intravenous glucose load. Isotonic solutions, however, appear to have only a marginal effect on blood glucose and insulin (Heaton et al., 1983; Armstrong et al., 1985). Although the occasional patient on CAPD may develop diabetes mellitus de novo, there is no convincing data currently to suggest that CAPD induces diabetes. However, it is not uncommon to find type II diabetics on CAPD to require insulin supplementation to control their blood glucose (DeFremont et al., 1981). It is possible, therefore, that patients with a genetic predisposition to diabetes may have precipitation of type II disease induced by the continuous hyperglycaemic stress of CAPD.

b) Nutritional state

Weight gain, particularly during the first year on CAPD, is well documented (Kutz et al., 1983; Young et al., 1983; Tsakiris et al., 1986). This may be due to increased body fat (Young et al., 1983; Bouma et al., 1984) and has been attributed to excessive glucose absorption and hyperinsulinaemia. However, most patients return to their premorbid non-uraemic weight after 1 year on CAPD (Rubin et al., 1983). Weight gain has also been attributed to an increase in lean body mass indicating an improved nutritional state (Rubin et al., 1983; Lindholm et al., 1981). Accumulation of body water is usually easily recognisable and readily correctable provided there is no loss of ultrafiltration. In the early years of CAPD a liberal oral intake of fluid was prescribed which often resulted in the frequent use of hypertonic solutions to control fluid balance and lead to excessive caloric intake.

On the contrary, malnutrition and loss of appetite may also be related to glucose absorption. The abdominal distension due to the volume of fluid in the peritoneal cavity may suppress satiety and the continuous peritoneal absorption of glucose has been reported to contribute to anorexia (Von Baeyer et al., 1983; Young et al., 1983).

c) Lipid metabolism

Lipid metabolism is deranged in uraemia and further changes occur in CAPD. Excessive carbohydrate and abnormalities in glucose metabolism (hyperinsulinaemia and insulin insensitivity) may lead to hyperlipidaemia (Olefsky et al., 1974; Coulston et al., 1983). Serum VLDL and total triglyceride concentrations in renal failure strongly correlate with the quantity of carbohydrate consumed (Cattran et al., 1976; Sanfelippo et al., 1977). Thus in CAPD with continuous glucose absorption one might anticipate deterioration in lipid status. Indeed, within the first year of CAPD hyperlipidaemia is very common (Gokal et al., 1981; Nolph et al., 1984) and about one third of patients may develop hypertriglyceridaemia. The latter change has been associated with protein loss in the dialysate (Gokal et al., 1981) similar to that seen in the nephrotic syndrome. However, many studies have shown that after about 1 year on CAPD there is a tendency for both cholesterol and triglycerides to fall (Khanna et al., 1983; Lindholm et al., 1983). This may indicate a metabolic adaption to the glucose load and also correlates with weight reduction in many patients. This may be due to changes in energy intake over time. It has been shown that the total carbohydrate intake in some CAPD patients is regulated by a spontaneous reduction of the oral carbohydrate intake (Von Baeyer et al., 1981).

d)   Protein and amino acid metabolism

Protein and amino acid abnormalities are well recognised in uraemia and CAPD.   In CAPD protein is lost (5-15 g/day) in the dialysate and this is membrane permeability dependent (Rubin et al., 1981b; Blumenkrantz et al., 1981).   This explains the large interindividual difference in dialysate loss.   Variations in ultrafiltration volume and the use of hypertonic exchanges appear to have little effect on protein losses (Rubin et al., 1981b; Blumenkrantz et al., 1981).   Small proteins may be depleted rapidly, particularly after peritonitis, that synthesis or oral replacement is insufficient.   Thus protein depletion can contribute to malnutrition in CAPD.   A high protein diet is generally recommended but compliance can be a problem.   Patients may have a reduced appetite for reasons previously discussed or may genuinely have difficulty consuming protein, especially if they have been accustomed to a low protein diet for many years as part of their chronic renal failure management. CAPD patients often require to be re-educated to reduce their carbohydrate and fat intake and increase the protein content of their diet.

Deranged amino acid metabolism is observed in chronic renal failure, haemodialysis and CAPD (Bergstom et al., 1978).   In CAPD the loss of amino acids depends largely on the plasma concentration and ultrafiltration volume (Dombros et al., 1982).   The decreased essential amino acids and reduced valine-glycine ratio seen in CAPD is also observed in non-dialysed uraemics.   This tends to suggest that the amino acid abnormalities may largely reflect the underlying uraemia.   Sustained hyperinsulinaemia in CAPD has been reported to contribute to reduced amino acid concentrations (Dombros et al., 1982; Martin et al., 1982).   Glucose administration to non-uraemic patients

has been shown to lower plasma amino acid concentrations (Martin et al., 1982). Thus peritoneal glucose may affect amino acid metabolism. Dialysate solutions containing amino acid have been studied and may have a further role in selected patients (Winchester, 1986).

6. SUMMARY

CAPD, at least in the short term, has proven to be an effective and acceptable treatment for end stage renal failure. Glucose plays a major role in this therapy being responsible for the osmotic and ultrafiltration effect. However, the long term effect of glucose on the integrity of the peritoneum is unknown. Glucose exerts metabolic and nutritional changes during CAPD. The hyperglycaemia and hyperinsulinaemia can, at times, be an advantage in promoting anabolism and improving the nutritional state. However, CAPD may also be detrimental by promoting nutritional imbalances in the form of protein deficiency and net catabolism. Furthermore, the increased body fat in some patients, in addition to hyperlipidaemia may aggravate atherosclerosis. The continuous effect of peritoneal glucose absorption on pancreatic beta cell function is still not known. Nevertheless, despite these possible adverse effects of glucose, the use of CAPD has resulted in good control of uraemia, hypertension and blood glucose.

The future of CAPD ultimately depends on the preservation of the peritoneum to retain its solute clearances and ultrafiltration. New dialysis solutions, substituting non-glucose osmotic agents, may prove advantageous in reducing the metabolic and nutritional consequences of glucose.

CHAPTER 4

GLYCOSYLATED HAEMOGLOBIN

Glycosylated haemoglobin has become universally accepted in the past 10 years as an independent and objective indicator of glycaemic control in diabetes. Its clinical value is clear, but it also provides an important tool for research as a model of non-enzymatic glycosylation and has opened up new avenues into the pathogenesis of the complications of diabetes as well as advancing the concept of non-enzymatic carbamylation.

1. INTRODUCTION

The non-enzymatic 'browning' reaction was recognised in 1916 by Maillard who pioneered work on sugar and amino acid condensation in food. In the past 20 years there has been a great surge of interest in non-enzymatic glycosylation, that is, the attachment of glucose to certain amino acid residues of proteins, particularly in the field of diabetes. Current interest in glycosylated haemoglobin began with the potential clinical application of the measurement of modified haemoglobins. Blood from diabetic patients was observed to contain increased amounts of unusual haemoglobin fractions that migrated faster on electrophoresis or chromatography (Rahbar et al., 1968; Trivelli et al., 1971). In addition to identifying the haemoglobin species it was also demonstrated that non-enzymatic glycosylation occurred slowly and cumulatively, and the measured percentage of glycosylated haemoglobin in an individual could reflect ambient glucose concentrations over an integrated period of time (Bunn et al., 1976; Koenig et al., 1976).

In the past 10 years, there has been an explosion of data, on glycosylated haemoglobin as a tool in assessing diabetic status, on the development of new and improved methodology, on the understanding of the nature and use of haemoglobin glycosylation and the widespread

use of glycosylated haemoglobin as an indicator of blood glucose control in diabetics.

Non-enzymatic glycosylation of haemoglobin has stimulated research into the pathogenesis of the complications of diabetes. Increased glycosylation of other proteins, in particular proteins derived from specific tissues typically involved in diabetic complications suggest that the glycosylated proteins may have altered structure and function (Cohen, 1986).

It has been suggested that non-enzymatic carbamylation of haemoglobin, that is, the attachment of urea-derived cyanate to haemoglobin, occurs in uraemia (Fluckiger et al., 1981) and this is analogous to non-enzymatic glycosylation in diabetes. However, little is known about carbamylated haemoglobin and the possible clinical relevance and pathophysiological significance in uraemia remains to be seen.

2. CHEMISTRY

a) Structure and Biosynthesis of Glycosylated Haemoglobin

Enzymatic glycosylation is a normal post-translational process which greatly expands the structure and function of proteins that are synthesized from the 20 amino acids. Collagen, basement membrane protein, HLA antigens, some cell surface receptors and certain hormones are but a few glycoproteins synthesized enzymatically. When a protein is exposed to a high glucose concentration for a relatively long duration non-enzymatic incorporation of glucose can occur.

Structural heterogeneity of a protein is generally due to either multiple gene coding for that protein or to post-translational modifications. Normal human red cells contain one major haemoglobin component, HbA [α_2, β_2] and two minor components, HbA$_2$ [α_2, δ_2] and

HbF $[\alpha_2, \gamma_2]$ each with 4 polypeptide chains. These proteins are coded by 4 different globin genes. Haemoglobin consists of HbA which accounts for 97% of the total and HbA_2 and HbF, 2.5% and 0.5% respectively. Additional minor components arise because of non-enzymatic glycosylation. Chromatographic analysis of HbA has identified HbA_{1a}, HbA_{1a2}, HbA_{1b}, HbA_{1c}, HbA_{1d}, HbA_{1e}. The latter two are not clearly elucidated and will not be discussed further.

The components of HbA_1 result from post-translational non-enzymatic modification of HbA_1 by a variety of small molecular weight substances, such as glucose, phosphorylated sugars, cyanate and aspirin, and comprise about 7% of the total haemoglobin in normal subjects. Each of the chromatographically determined components is not homogenous and may be composed of many different entities. The major component of the HbA_{1c} peak is glycosylated haemoglobin (about 70% in normal subjects).

Glycosylated haemoglobin is formed when a glucose molecule attaches to the N-terminal amino group (valine) of the β chain of haemoglobin to form a Schiff base (Bunn et al., 1975). The labile Schiff base, often called 'pre HbA_{1c}' or the 'labile fraction', is an intermediate which is reversible and increases rapidly in amount within a few hours after incubation of HbA with glucose (Higgins et al., 1981). The Schiff base subsequently undergoes an Amadori rearrangement to a stable ketoamine linkage (Bunn et al., 1975). This is present in increased amounts in diabetics as a consequence of increased blood glucose concentrations. The post-translational non-enzymatic glycosylation of HbA occurs slowly but continuously within the red cell throughout its 120 day life span in the circulation.

The attachment of glucose to haemoglobin occurs non-enzymatically

via a 2 step mechanism (Figure 4.1). In the initial rapid and reversible condensation the Schiff base aldimine is formed and slowly undergoes the Amadori rearrangement to the more stable ketoamine form. The Schiff base formation is dependent on the glucose concentration and is readily reversible with dialysis of the reaction mixture or lowering of the blood glucose concentration. The Schiff base accounts for the labile fraction as it elutes from the cation exchange resin with the same mobility as the more stable ketoamine form. Although there is equilibrium between the 2 configurations the balance is in favour of the ketoamine.

The formation of 5-hydroxymethylfurfural (5HMF) by mild acid hydrolysis of the Amadori rearrangement of HbA_{1c} is shown in Figure 4.2. The characteristic spectrum of the adduct formed with thiobarbituric acid is specific for ketoamine linked hexoses and forms the basis of the specific chemical method to detect total ketoamine glycosylation of proteins (Gabbay et al., 1979).

HbA_{1a1} and HbA_{1a2} (Table 4.I) are predominantly the β chain N-terminal adducts of fructose-1,6,-diphosphate and glucose-6-phosphate respectively (McDonald et al., 1978), while HbA_{1b} is not yet fully clarified but may be a deamidisation product of HbA (Krisnamoorthy et al., 1977). In addition to the NH_2 termini of the β chain, glucose adducts can form with the amino terminal of the α chain as well as free amino groups in the haemoglobin molecule. Various lysine residues (Table 4.II) in the α and β chains become glycosylated on exposure to glucose (Bunn et al., 1979) and glucose can also condense with ϵ-amino groups of lysine residues along the polypeptide chains of many proteins. Thus haemoglobin glycosylation is a general and non-specific process. The amount of HbA in red cells, like HbA_{1c}, is increased in diabetics, but unlike HbA_{1c}, the modification of

HbA   'pre HbA$_{1c}$'   HbA$_{1c}$

Glucose   aldimine
(Schiff base)   ketoamine
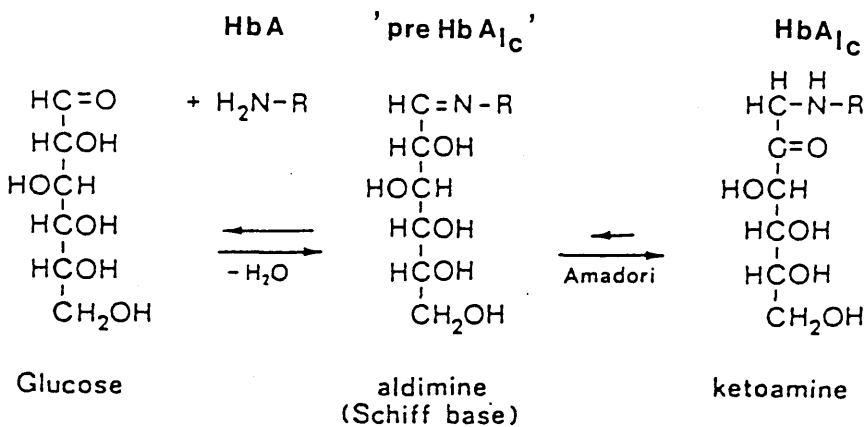
Figure 4. 1 Non-enzymatic interaction of glucose with amino groups. The labile and easily reversible aldimine (Schiff base) is readily formed. The aldimine slowly enolizes to form the more stable ketoamine configuration via an Amadori rearrangement

Schiff
Base

Ketoamine
(Hb A$_{1c}$)

Schiff Base
of 5-HMF

5-HMF

$$5\text{-HMF} + 2\text{-Thiobarbituric Acid} \longrightarrow \text{Adduct}$$
$$(\text{Abs}_{max}\ 443\,nm)$$
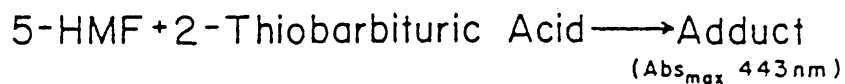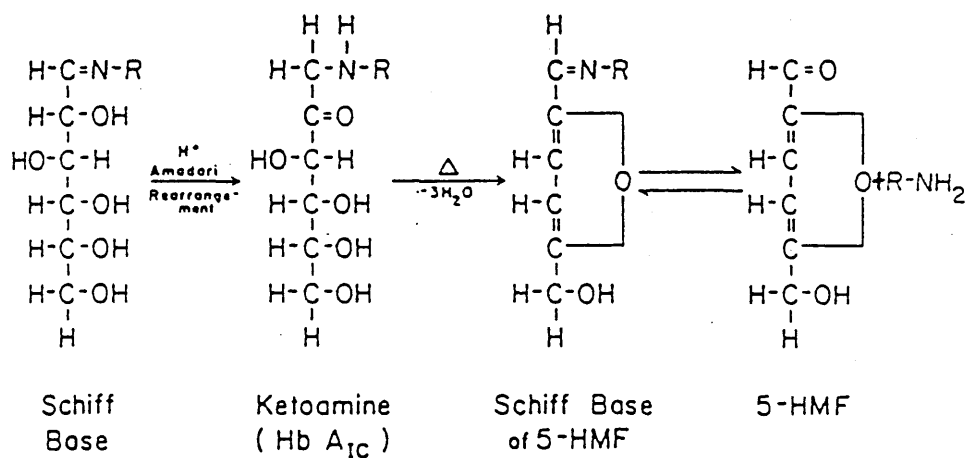
Figure 4. 2 Chemistry of the TBA colorimetric test for detection of glycosylation. Only the ketoamine rearranged form reacts to yield 5-hydroxymethylfurfural (5-HMF). The latter is detected by forming an adduct with thiobarbituric acid

Table 4. I   Minor Components of Haemoglobin A

Haemoglobin	Modification	Abundance (%)
A $(\alpha_2\beta_2)$	...	95*
A_{Ia_1}	$\alpha_2(\beta\text{-}N\text{-fructose-1,6-diphosphate})_2(?)$	0.2
A_{Ia_2}	$\alpha_2(\beta\text{-}N\text{-glucose-6-phosphate})_2$	0.2
A_{Ib}	$\alpha_2(\beta\text{-}N\text{-carbohydrate})_2(?)$	0.5
A_{Ic}	$\alpha_2(\beta\text{-}N\text{-glucose})_2$	4

*An estimated 8% to 10% is glycosylated at sites other than N termini.

Table 4. II   Sites on Human Haemoglobin which
Undergo Nonenzymatic Glycosylation

In Vitro	In Vivo
β Val 1	β Val 1
α Lys 16	β Lys 66
β Lys 66	α Lys 61
β Lys 17	β Lys 17
α Val 1	α Val 1
α Lys 7	α Lys 40
	β Lys 8

glucose at these other sites do not result in changes in the electrophoretic or ion exchange chromatographic properties (Gabbay et al., 1979).

b) Modifying Factors

Non-enzymatic glycosylation takes place under physiological conditions in normal individuals. The reaction follows second order kinetics (McDonald et al., 1979) and the amount of glycosylated product is proportional to the concentration of reactants. The major determinant is the glucose concentration which according to the law of mass action will cause a proportionate increase in the amount of Amadori product formed. The second main determinant is the time of exposure of the protein to the increased glucose concentration. Thus, in vivo, the degree and duration of hyperglycaemia are the prime factors in determining the amount of glycosylated haemoglobin.

Temperature and pH are important in vitro factors and are critical, particularly in column chromatography (see later). The half-life of the protein in the circulation or tissue is also significant. Reduced half-life of the protein such as in haemolytic anaemia and shortened red cell survival can cause a decrease in HbA_{1c} even in diabetics (Bunn et al., 1976) and old erythrocytes contain more HbA_{1c} than young ones (Fitzgibbons et al., 1976).

The permeability and availability of glucose in different tissues will influence the extent of glycosylation. The erythrocyte is freely permeable to glucose and hence the ease of haemoglobin glycosylation. It is interesting that several characteristic complications of diabetes occur in tissue that do not require insulin for glucose transport and can dispose of glucose via insulin-independent pathways. This adds to the concept that non-enzymatic glycosylation contributes

to the pathogenesis of diabetic complications in these tissues.

Biochemical factors such as the number of free amino groups, accessibility and pK of the amino group within the structure of the protein also influence the formation of glycosylated proteins (Cohen, 1986).

c) The Maillard Reaction

The ketoamine adduct formed from the reaction between glucose and protein amino groups can undergo a series of dehydration, rearrangement and cleavage reactions collectively known as the Maillard or browning reaction (Figure 4.3). The products of such reactions are highly cross-linked, insoluble pigmented and fluorescent polymers. The formation of these substances has been studied extensively in vitro, particularly in the food industry but recent evidence indicates that these products can form in vivo, particularly in long-lived proteins and when non-enzymatic glycosylation is increased. These advanced glycosylation products have recently been implicated in the pathogenesis of diabetic complications (recently reviewed by Cohen, 1986) as well as in the aging process. Thus the Maillard browning reaction involves three stages, first the formation of a ketoamine (as seen in Figure 4.1), i.e. non-enzymatic glycosylation, then deamination followed by dehydration, cyclization and fusion to generate secondary products such as hydroxymethylfurfural (Figure 4.2) and the final stage involves polymerization (Figure 4.3). The biological significance of advanced glycosylation end-product formation is at present unclear but has stimulated a new area of research in the field of glucose metabolism.

Reducing Sugar + Amino Compound ⇌ Schiff's Base

Ketoamine ⟵ Aldosylamine

1,2 enolization   2,3 enolization

Derivatives   Derivatives
(HMF)   (reductones,
                          fission products)

+RNH₂

Aldols   Aldimines, ketimines

Polymerization   Polymerization
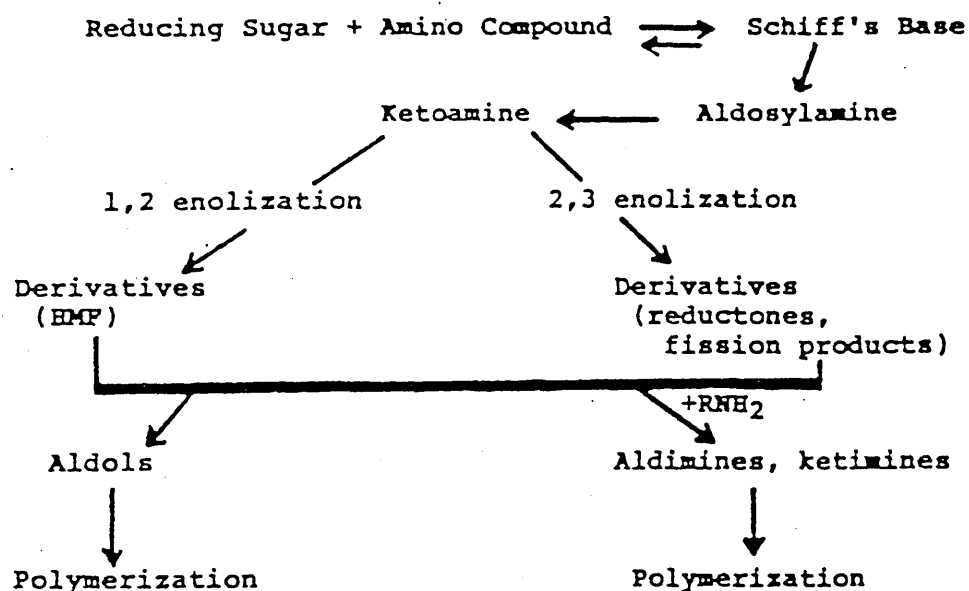
Figure 4. 3 Simplified scheme of the Maillard reaction. HMF, 5-hydroxy-
methylfurfuraldehyde. .

<u>Other Designations</u>

N-Terminal Modification

A_{1a1}
A_{1a2}
A_{1b} Fast Haemoglobin   Total
A_{1c} HbA_1 Glycosylated
                                       Haemoglobin

Modifications at sites other
than N-terminal
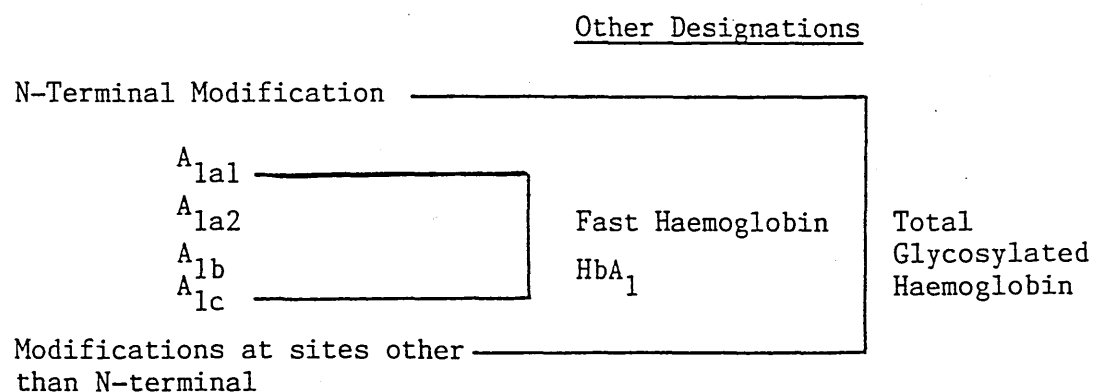
Figure 4. 4   Nomenclature for glycosylated haemoglobins

3. MEASUREMENT OF GLYCOSYLATED HAEMOGLOBIN

a) Nomenclature

There has been some confusion about the nomenclature of glycosylated haemoglobin and this is mainly due to the fact that our knowledge of the subject has been changing rapidly. Glycosylated haemoglobin (is synonomous with glycohaemoglobin and glycated haemoglobin) refers to a series of stable adducts that are formed between haemoglobin and sugars and whose concentrations are increased within the red cell of patients with diabetes. Some assay methods quantify all glycosylated haemoglobin species regardless of the haemoglobin tetramer and this is called total glycosylated haemoglobin. Other assays measure the products formed by glycosylation of the amino termini of HbA (α_2, β_2). Some are specific for one product, e.g. HbA_{1c}, while others measure the sum of HbA_{1a}, HbA_{1b} and HbA_{1c}, which is called HbA_1 or 'fast' haemoglobin (Figure 4.4). It is generally accepted that regardless of the particular glycosylated haemoglobin, values obtained from the same blood sample, but assayed by different methods are clinically comparable. However, there are exceptions such as in renal failure which will be discussed later.

b) Methods

Glycosylated haemoglobin is currently estimated by 2 basic methods which operate on different principles and yield independent results which are nonetheless related. The chromatographic methods depend on a net change in the charge of the haemoglobin molecule when the N-terminal position of the β chain is modified. Thus the components of HbA_{1a-c} are eluted readily from a cation exchange resin and can be quantified and expressed as a percentage of total haemoglobin.

Haemoglobin molecules glycosylated at sites other than the β N-terminal do not have sufficiently altered net charge to increase their mobilities and are thus not measured by these techniques. These chromatographic operations are very sensitive to minor changes in buffer pH and temperature which can significantly affect the values obtained.

Separation based on a charge difference is the principle for the macro-column (Biorex-70 column chromatography - Trivelli et al., 1971), isoelectric focus ing (Spicer et al., 1978), agar gel electrophoresis (Nathan, 1981), high performance liquid chromatography (HPLC) (Dunn et al., 1979a) and the convenient commercial mini-column kits (Bio-Rad, Richmond, Calif.). The latter columns can measure the sum of HbA_{1a-c} (HbA_1) or specific fractions (HbA_{1c} and HbA_{1a+b}). These are detailed in Chapter 5.

The other general type of method involves direct chemical measurement of total glycosylation in the red cell. In the thiobarbituruic acid (TBA)-colorimetric method (Fluckiger and Winterhalter, 1978), furfural compounds are generated from the ketoamine-linked carbohydrate moieties upon heating under acidic conditions and are quantified colorimetrically with TBA (Figure 4.2). This method is detailed in Chapter 5.

Several new methods have been developed in recent years and one of the most promising is affinity chromatography (Klenk et al., 1982), which uses affinity gel columns to separate glycosylated haemoglobin from the non-glycosylated fraction. Results from this method suggest that glycosylation accounts for only about 70% of the HbA_{1c} peak isolated by cation-exchange chromatography. This is consistent with the finding that a variety of non-glucose substances can modify the N-terminal amino acid of the β chain such as carbamylation and

acetylation (see later).

c) Limitations of Assay Methods

Despite the variety of methods all have some limitations although
all are adequate for assessing clinical status and glycaemic control.
Ion exchange chromatography is greatly influenced by buffer pH, the
temperature of reagents, interference by non-glucose adducts, aldimine
intermediates (pre-HbA$_{1c}$) and haemoglobinopathies. It also requires a
high degree of operator skill for precision, but has the advantage of
being rapid to perform, relatively inexpensive to run and suitable for
the small laboratory. HPLC has similar limitations and is expensive
to install but is useful particularly for research purposes.
Electrophoresis is subject to interference by several reactants
including non-glucose adducts, can be time consuming and relatively
expensive. The TBA-colorimetry method is unaffected by non-glucose
adducts, aldimine intermediates and haemoglobinopathies and is
relatively inexpensive to run. However, standardization can be a
problem (with no universally accepted standard) and hydrolysis
conditions must be carefully controlled for precision. Generally it
is a time consuming manual process but can be semiautomated for use in
large laboratories.

d) Interference

The chromatographic-type procedures can under certain conditions
produce falsely abnormal results due to interference. These are
listed in Table 4.III (adapted from Gabbay, 1983). Conditions such as
uraemia, chronic aspirin intake, antibiotic therapy and recent alcohol
intake can lead to false elevation of glycosylated haemoglobin. These
effects are due to changes in charge of the haemoglobin molecule

I – Conditions leading to false elevation of HbA$_1$

    A. Chromatographic abnormalities:
       1.   Hyperlipidaemia – due to lactescence
       2.   Elevated temperature and/or buffer pH
       3.   Negatively charged Hb variants – e.g. HbF
       4.   Acute hyperglycaemia – 'fast glycosylation'

    B. Other post-translational Hb modifications:
       1.   Aspirin – acetylation
       2.   Antibiotics – penicilloylation
       3.   Alcohol – 5-deoxy-xylulose-1-phosphate
       4.   Uraemia – carbamylation

II – Conditions leading to falsely low Hb A$_1$ values

    A. Chromatographic abnormalities:
       1.   Low temperature and/or buffer pH
       2.   Positively charged Hb variants – e.g. HbS,C

    B. Altered red blood cell dynamics:
       1.   Increased destruction – e.g. haemolytic anaemia
       2.   Active erythropoeisis – e.g. pregnancy
       3.   Recent transfusions

Table 4. III   Factors interfering with HbA$_1$

$$NH_2 - \overset{\overset{\displaystyle O}{\|}}{C} - NH_2 \; \rightleftharpoons \; NH_4^+ + NCO^- \; \longrightarrow \; \overset{\overset{\displaystyle H}{|}}{N} = C = O$$

(Urea)   (Isocyanate)

$$R - NH_2 + \overset{\overset{\displaystyle H}{|}}{N} = C = O \; \longrightarrow \; R - \overset{\overset{\displaystyle H}{|}}{N} - \overset{\overset{\displaystyle O}{\|}}{C} - NH_2$$

'Carbamylated protein'
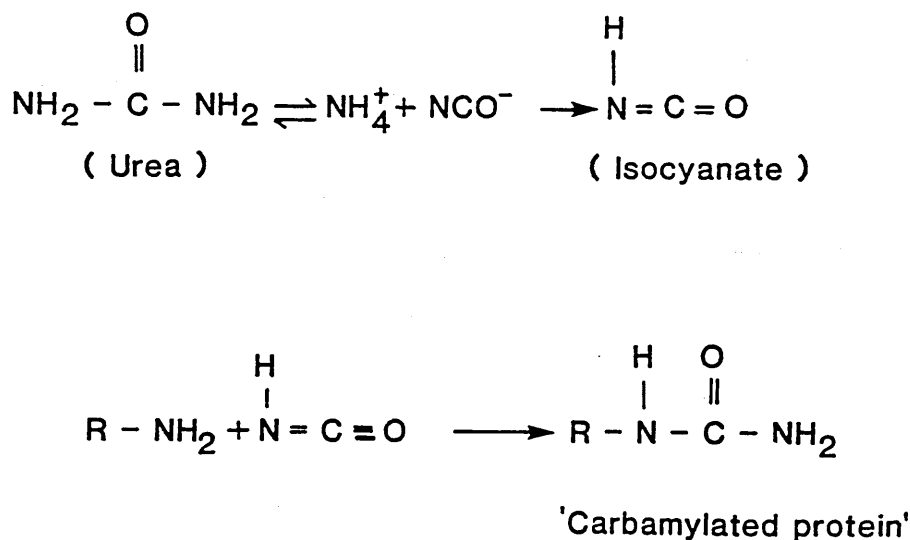
Figure 4. 5 Formation of isocyanate from urea and its reaction
with an amino group

brought about by post-translational modification occurring via mechanisms other than glycosylation. Elevation of HbA_1 in uraemia has been reported to be due to a carbamylation species (Fluckiger et al., 1981) which is detected by the chromatographic technique but not by the TBA-colorimetric method. Glycosylated haemoglobin measured by any method will be significantly reduced in patients with decreased red cell life span (Elseweidy et al., 1983) such as in haemolytic anaemia, uraemia or in patients with active erythropoiesis such as pregnancy. The use of whole blood haemolysates in chromatography may give rise to artefacts which can falsely elevate HbA_1 or HbA_{1c}. The presence of the labile fraction or pre-HbA_{1c} is influenced by the ambient blood glucose concentration and in a poorly controlled diabetic acute hyperglycaemia can cause false elevation of HbA_{1c} unless the labile fraction is removed (Nathan et al., 1981). Hyperlipaemic blood, common in both diabetes and uraemia, can also increase the 'fast' (HbA_{1a-c}) fraction due to interference from lactescence, which elutes with the HbA_1 fraction and is absorbed at the same wavelength (Dix et al., 1979a). However, these complications are circumvented by the TBA-colorimetric method with the exception of shortened red cell survival.

4. CLINICAL SIGNIFICANCE

The clinical usefulness of glycosylated haemoglobin in diabetes has been firmly established.

a) Blood Glucose Control

Periodic monitoring of glycosylated haemoglobin is useful in documenting the degree of glucose control that has prevailed during an interval of weeks to months before the sample is taken since

glycosylated haemoglobin concentration reflects the time averaged concentration of glucose within the erythrocyte during that period. The evidence supporting the correlation between glycosylated haemoglobin and glucose control is well established (Gabbay et al., 1977; Gonnen et al., 1977; Koenig et al., 1976; Jovanic et al; 1981). Periodic measurement of glycosylated haemoglobin concentrations provides an objective assessment of glycaemic control that complements and extends information obtained from traditional methods (such as regular home urine and blood glucose testing). This assessment is independent of the daily variations due to meals, insulin dosage and physical activity and improves the clinical management of diabetes. The technique employed to measure glycosylated haemoglobin is less critical than the consistent use of the same technique with reliable performance, since normal ranges will differ according to what is being measured by different methods.

b) Diagnosis

Attempts to use glycosylated haemoglobin as a more sensitive or specific test and to replace the oral glucose tolerance test have not been corroborated in clinical studies (Santiago et al., 1978; Dunn et al., 1979b; Flock et al., 1979). The combination of an elevated fasting blood glucose and elevated glycosylated haemoglobin usually obviates the need for further testing and indicates clinically significant hyperglycaemia. However, the value of glycosylated haemoglobin in detecting degrees of glucose intolerance is limited. Screening studies have shown that there is considerable overlap in glycosylated haemoglobin values in patients with normal and abnormal glucose tolerance (Dix et al., 1979b; Cederholm et al., 1984; Hall et al., 1984).

c) Complications of Diabetes

The complications of diabetes include microvascular and macrovascular disease, retinopathy, neuropathy and nephropathy, but the biochemical basis for the development of such sequelae is not known. Moreover, considerable controversy exists about the relationship between the degree of control of blood glucose and the eventual development of such sequelae. Strict control of carbohydrate metabolism should reduce the risk of secondary complications, but it has been difficult to document this because of the lack of means of measuring both the degree of control and the pathological changes that occur with chronic disease. Long term prospective clinical studies are necessary to assess the contribution of the severity and duration of hyperglycaemia to the development and progression of diabetic complications.

Several short term studies show a correlation between HbA_{1c} and cholesterol possibly indicative of hyperglycaemia as a causative factor in macroangiopathy (Gabbay et al., 1977; Sosenko et al., 1980). Muscle basement capillary membrane thickness has been reported to decrease after optimal glucose control assessed by glycosylated haemoglobin (Petersen, 1980; Raskin, 1983). Increased glomerular filtration rate (GFR), an early marker of diabetic nephropathy, has been shown to fall in conjunction with normalisation of HbA_1 (Wiseman et al., 1985). Thus microangiopathy may be influenced by persistent hyperglycaemia. However, although glycosylated haemoglobin can be used as an index of metabolic control much more investigation is needed before a definite link can be established between hyperglycaemia and the complications of diabetes.

5. PATHOPHYSIOLOGICAL SIGNIFICANCE

a) Haemoglobin

The amino terminal of the B chain of haemoglobin, to which glucose attaches, is also a site where 2,3-diphosphoglycerate (2,3-DPG) binds. 2,3-DPG is an important red cell glycolytic intermediate which influences the affinity of haemoglobin for oxygen through its binding to B chain residues of deoxyhaemoglobin. Addition of organic phosphate decreases the oxygen affinity of haemoglobin, whereas removal increases the oxygen affinity of HbA and HbA_{1c}. The availability of this site for interaction with 2,3-DPG is compromised when it is covalently linked to glucose (Bunn et al., 1970). Hence HbA_{1c} exhibits greater oxygen affinity than HbA in the presence of 2,3-DPG (McDonald et al., 1979). The increase in HbA_{1c} in red cells of diabetics shows a slight increase in oxygen affinity of these cells in the presence of 2,3-DPG compared to non-diabetics (Arturson et al., 1974). This difference could be due to interference by the NH_2-terminal glucose of HbA_{1c} to the binding of 2,3-DPG.

The impact of the minor shift in the haemoglobin-oxygen dissociation curve arising from increased HbA_{1c} in diabetics is unclear. It has been suggested increased HbA_{1c} coupled with low red cell 2,3-DPG could compromise oxygen delivery to the tissues and promote hypoxia and diabetic complications (Ditzel et al., 1979). However, this has been challenged by the fact that certain haemoglobinopathies produce greater shifts in the oxygen dissociation curve without significant effects on tissue oxygen (Bunn et al., 1981).

b) Other Proteins

Glycosylation of other proteins may also have pathological significance. Like HbA_1, measurement of glycosylated albumin provides an index of blood glucose control during the preceding 1-2 weeks (Dulhofer et al., 1981). Furthermore, glycosylated albumin has been implicated in the pathophysiology of diabetic microangiopathy as it is filtered through the glomerulus faster than normal albumin (Ghiggeri et al., 1984) and may contribute to the increased capillary membrane permeability and proteinuria seen in diabetic nephropathy. Glycosylated lipoproteins are increased in poorly controlled diabetics (Curtiss et al., 1985) suggesting a possible role in atherosclerosis. Glycosylation of lens protein has also been implicated in the pathogenesis of cataract formation (Kasai et al., 1983) and glycosylation of myelin has been observed in the central nervous system and peripheral nerves in diabetics (Vogt et al., 1982; Vlassara et al., 1983).

Increased glycosylation of collagen is found in many tissues of diabetics including aorta, skin, tendon, glomerular and lens capsular basement membrane collagen (Vogt et al., 1982). Non-enzymatic glycosylation may induce resistance to collagenase digestion and cause a premature aging process which correlates clinically with complications, such as the premature atherosclerosis seen in Type I diabetes.

Covalent interactions or cross-linking between glycosylated proteins and other soluble proteins such as albumin and IgG have been reported (Brownlee et al., 1983). This may explain the increased concentration of albumin in glomerular basement membrane of patients with diabetic nephropathy (Michael et al., 1981). Glycosylation can also alter the immunogenic properties of proteins (Bassiouny et al.,

1983) which may initiate an immune response against tissue components and could also explain the deposition of albumin and IgG in the microvascular matrix of diabetic patients.

c) Advanced Glycosylation

Glucoadducts produced as a result of non-enzymatic glycosylation can give rise to advanced glycosylation end-products, which can induce molecular modifications and subsequent trapping or binding of unrelated proteins. Thus advanced glycosylation has the potential for disturbing structural and biological characteristics of proteins or for exerting toxic effects on cell processes. It has been postulated that a period of hyperglycaemia could initiate excess non-enzymatic glycosylation, but deleterious effects could ensue as a result of protein interactions and abnormalities brought about by the subsequent formation of advanced end-products even after hyperglycaemia is corrected (Cohen, 1986). However, further studies are needed to confirm this possible link between diabetic control and the chronic complications of diabetes.

6. GLYCOSYLATED HAEMOGLOBIN AND HAEMOGLOBIN CARBAMYLATION IN URAEMIA

Uraemia influences the chromatographic measurement of HbA_1 (DeBoer et al., 1980; Lunetta et al., 1981; Fluckiger et al., 1981). Elevated concentrations of both HbA_1 and HbA_{1c}-like haemoglobins have been reported in renal failure. Glucose intolerance is common in uraemia and some dialysis patients are dialysed against fluid with a high glucose content. This has lead to the assumption that the increase, at least in part, may be due to increased formation of glycosylated haemoglobin resulting from abnormalities in carbohydrate metabolism.

However, the colorimetric estimate of glycosylated haemoglobin

does not show an increase in uraemia (Fluckiger et al., 1981). The

increase in fast (HbA_{1a-c}) haemoglobin has been suggested to result

from haemoglobin carbamylation occurring via the condensation of

urea-derived cyanate with the N-terminal amino groups of the

haemoglobin chains (Figure 4.5). This form of non-glycosylated

haemoglobin is detected by column chromatography, but not by the

TBA-colorimetric method which is specific for the detection of

glycosylation. Fluckiger et al. (1981) identified valine hydantoin,

which is released from the carbamylated N-termini of the haemoglobin

chains upon acid hydrolysis and showed that valine hydantoin

correlated with time averaged blood urea of the previous 2-3 months in

uraemic patients. These authors also suggest that the increase in

HbA_1 in uraemia was entirely due to an increase in the HbA_{1a+b}

component.

Thus carbamylated haemoglobin may reflect the urea concentration

over a period of time, a situation analogous to that of glycosylated

haemoglobin in diabetes. It should also be noted that the

interpretation of glycosylated haemoglobin in uraemia is complicated

by the presence of shortened red cell survival which would tend to

reduce glycosylated haemoglobin independently of the method of

measurement. Glycosylated haemoglobin status in uraemia is complex

and caution is required in interpreting results in both diabetic and

non-diabetic uraemic patients. The relevance of carbamylated

haemoglobin is unclear, but the implication that it may be analogous

to glycosylated haemoglobin suggests that future research is needed to

explore possible clinical applications and pathophysiological

consequences.

7. GLYCOSYLATED AND CARBAMYLATED HAEMOGLOBIN IN URAEMIA —

   REVIEW OF THE LITERATURE

The use of glycosylated haemoglobin as an indicator of long-term

blood glucose control in diabetics with normal renal function is well

established (Koenig et al., 1976; Gabbay et al., 1977). However, the

role of glycosylated haemoglobin in uraemia and diabetics with renal

failure is unclear. Confusion is partly related to methodology and

nomenclature and partly to conflicting data in clinical studies.

Generally chromatographic techniques measuring HbA_1 or HbA_{1c} have

shown elevated concentrations of glycosylated haemoglobin in

non-diabetic uraemia patients (Casparie et al., 1977; DeBoer et al.,

1980; Kovarik et al., 1981), but other have found low concentrations

(Dandona et al., 1979; Freedman et al., 1982). Chemical methods such

as the thiobarbituric acid-colorimetric technique, which measures the

total glycosylation of the red cell have revealed normal values of

glycosylated haemoglobin (Fluckiger et al., 1981; Oimomi et al., 1981;

Nath et al., 1982) in non-diabetic uraemic patients. Thus differences

in methodology reveal different results in the presence of uraemia.

Clinical studies showing increased HbA_1 or HbA_{1c} in non-diabetic

uraemic patients initially suggested that the elevation was due to

glucose intolerance which is common in uraemia (DeFronzo and

Alvestrand, 1980) or coexisting hyperglycaemia in renal patients

(Casparie et al., 1977; Stanton et al., 1978). However, later studies

did not find any correlation between glucose intolerance, fasting

blood glucose or 24 hour glycosuria and HbA_1 (Kovarik et al., 1981;

Panzetta et al., 1983). The association between uraemia and elevated

HbA_1 was strengthened by the common finding of a linear relationship

between urea or creatinine and HbA_1 (Graf et al., 1980; Fluckiger et

al., 1981; Kovarik et al., 1981). However, others did not find a

correlation between HbA_1 and urea in haemodialysis patients (De Marchi et al., 1983a). The latter group found HbA_1 strongly correlated with arterial pH and plasma bicarbonate. De Marchi et al. (1983b) reported, in a larger study in non-dialysis chronic renal failure patients, a weak correlation between HbA_1 and urea, but again emphasised the role of acidosis. The effect of acidosis, however, was not confirmed by others (Panzetta et al., 1983).

The increase in HbA_1 and sub-fractions has also been related to the presence of a non-glucose adduct of haemoglobin that is chromatographically indistinguishable from glycosylated haemoglobin. Fluckiger et al. (1981) demonstrated that HbA_1 was elevated largely due to haemoglobin carbamylation resulting from a condensation of urea-derived cyanate with the N-terminal amino groups of haemoglobin. These authors showed that this non-glycosylated haemoglobin was detected by column chromatography but not by a chemical method specific for the detection of glycosylation. In addition to elevation of HbA_1 they found an increase in HbA_{1a+b} fraction. Carbamylation of haemoglobin was detected in vitro from the components of the chromatographic peaks. Although frequently suggested as a possible cause for the elevation of glycosylated haemoglobin in uraemia no other studies have directly measured carbamylated haemoglobin in uraemia. Oimomi et al. (1984) studied the in vitro addition of urea to erythrocytes and found the amount of cyanate produced was proportional to the amount of HbA_1.

Reduced concentrations of HbA_1 have been reported by one group (Dandona et al., 1979; Freedman et al., 1982) in a wide range of uraemic patients. They suggested that low concentrations were due to shortened red cell life span although no measurement of erythrocyte survival was done. It is well documented that red cell survival is

reduced in uraemia (Shaw, 1967; Hocken, 1982; Hefti et al., 1983). Furthermore, glycosylated haemoglobin is reduced in conditions with shortened red cell life span but not in non-haemolytic anaemias (Fitzgibbons et al., 1976).

The effect of uraemia on diabetes is clinically important if glycosylated haemoglobin is to be used as an integrated index of glycaemic control. The data on glycosylated haemoglobin in diabetics with uraemia measured by direct chemical means is apparently unaltered by uraemia and should therefore reflect glycaemic control in diabetics with renal failure. This would then appear to be the method of choice but the effect of shortened red cell life span must also be considered. To date this has not been directly studied.

HbA_1 has been reported to be higher in uraemic diabetics than non-diabetic uraemics (Kumar et al., 1983), while others found little difference between similar groups of patients (Panzetta et al., 1983). Saloranta et al. (1986) assessed HbA_1, HbA_{1c}, HbA_{1a+b} by column chromatography in diabetics and non-diabetic uraemic patients. They found the expected increase in HbA_1 which weakly correlated with urea and creatinine in non-diabetics, while diabetics had even higher HbA_1 which was less influenced by urea and creatinine concentrations. Saloranta and colleagues also found that the HbA_{1c}/HbA_{1a+b} ratio was higher in diabetics than non-diabetics confirming the findings of others (Lantz et al., 1981; Oimomi et al., 1981). This suggests that the diabetics state influences HbA_{1c} more than HbA_{1a+b}. However, Saloranta et al. (1986) did not find any change in the HbA_{1c}/HbA_{1a+b} ratio in non-diabetic uraemic patients. This is contrary to the results of others (Fluckiger et al., 1981; Lantz et al., 1981) who found that uraemia influenced the HbA_{1a+b} components more than the HbA_{1c} fraction. In a small number of diabetics with renal failure

repeated measurements of HbA_1 and blood glucose showed correlation and the authors (Saloranta et al., 1986) concluded that HbA_1 or HbA_{1c} measured chromatographically was still a useful index of glycaemic control.

In summary, current data suggests that HbA_1 is elevated in uraemia when measured chromatographically. The cause of this elevation could be due to glucose intolerance, acidosis or carbamylation of haemoglobin. The latter is an attractive possibility, bearing in mind the similarities with non-enzymatic glycosylation. Shortened red cell survival in uraemia would be expected to reduce or have an inhibitory effect on glycosylated haemoglobin independent of the assay method used and theoretically, could also influence the concentration of carbamylated haemoglobin. Assessment of diabetics with uraemia requires further clarification and although the direct chemical colorimetric methods appear to be unaffected by uraemia the usefulness of the common chromatographic procedures are still unclear.

8. THE ROLE OF NON-ENZYMATIC CARBAMYLATION IN URAEMIA

Non-enzymatic carbamylation of haemoglobin in uraemia has been suggested as being analogous to non-enzymatic glycosylation in diabetics (Fluckiger et al., 1981). To date, no studies have been published to implicate a clinical or pathophysiological role for non-enzymatic protein carbamylation in uraemia. However, recently an in vitro study demonstrated reduced biological activity of insulin after incubation with cyanic acid (Oimomi et al., 1987).

Carbamylated adducts of haemoglobin have been known for many years and were used experimentally both in vitro and in vivo in the treatment of sickle cell disease. Sodium cyanate was used as a covalent agent to inhibit sickling of red cells. Cyanate binds firmly

to both alpha and beta chains of haemoglobin and increases the oxygen affinity and reduces the capability of the red cell to sickle (Nigen et al., 1974). Red cell survival was increased and the increase in red cell life span reflected the degree of carbamylation (Cerami et al., 1971; Gillette et al., 1974). Unfortunately, carbamylation in vivo was not confined to the NH_2 groups of haemoglobin, but affected the other enzyme systems (De Furia et al., 1972). Numerous symptoms including weight loss, nausea, vomiting, epigastric pain and drowsiness were reported (Gillette et al., 1974; Petersen et al., 1974). Peripheral nerve conduction was also abnormal but resolved on cessation of cyanate therapy (Petersen et al., 1974). Thus toxicity limited the potential clinical benefit of prolonging red cell survival and inhibition of sickling.

Theoretically, many of the characteristics of non-enzymatic glycosylation may also be applicable to non-enzymatic carbamylation. Fluckiger and coworkers suggested that carbamylated haemoglobin may reflect time averaged blood urea. Thus carbamylated haemoglobin may be used as an integrated index of uraemic control. This could conceivably have a clinical value in several situations. Carbamylated haemoglobin may have a role in distinguishing acute from chronic renal failure, detecting the timing of deterioration in chronic renal failure, assessing compliance in protein diet restriction therapy and evaluating the efficiency of dialysis therapy. Carbamylated haemoglobin may give a mean estimate of the uraemic state prevailing during the previous 4-8 weeks.

Non-enzymatic carbamylation may also have a wider application if it can modify other proteins analogous to non-enzymatic glycosylation. Carbamylated plasma proteins and carbamylated insulin have recently been demonstrated in vitro (Oimomi et al., 1985; Oimomi et al., 1987).

It is interesting that vascular disease, lipoprotein abnormalities and neuropathy are common to both diabetes and uraemia and it is tempting to speculate that non-enzymatic modification of proteins are implicated. The effects of cyanate used in sickle cell disease are similar to common uraemic symptoms such as the nausea, vomiting and tiredness and peripheral neuropathy with prolonged nerve conduction time.

Thus non-enzymatic carbamylation of proteins would appear to warrant further investigation in uraemia to assess its potential clinical usefulness and pathophysiological relevance.

9. SUMMARY

The structure, biosynthesis and measurement of glycosylated haemoglobin has been outlined, with particular emphasis on the difference between methods, modifying and interfering factors. The clinical usefulness of glycosylated haemoglobin as a measure of integrated long-term glucose control is well established in diabetics without uraemia. The effect of interference due to uraemia is unclear, but may be circumvented by using colorimetry as opposed to ion-exchange chromatography. The process of non-enzymatic glycosylation has wide implications from the evidence that it can alter the structure and function of proteins. The association between non-enzymatic glycosylation and the complications of diabetes provides a possible link between glucose control and the sequelae of diabetes. This has long been suspected but has generally lacked direct evidence. Thus, glycosylation of proteins has an important pathophysiological significance.

The controversial data on the role of glycosylated haemoglobin in non-diabetic patients with renal failure is reviewed. The concept of

a non-glucose adduct of haemoglobin interfering with the chromatographic, but not the colorimetric measurement of glycosylated haemoglobin is presented. The formation of carbamylated haemoglobin from urea-derived cyanate is discussed and the analogy between glycosylated haemoglobin and diabetes, and carbamylated haemoglobin and uraemia is developed. The potential role of carbamylated haemoglobin in uraemia including the possible clinical usefulness and pathophysiological significance is postulated. This Chapter provides the background to the project "Glycosylated and Carbamylated Haemoglobin in Uraemia" (Chapter 8).

CHAPTER 5

METHODS

This Chapter describes the biochemical procedures performed during the work of this thesis. Brief details are given of the methods used to assay hormones which were undertaken by others. A more in depth description of the methods carried out by myself include the measurement of intermediary metabolites, glycosylated haemoglobin and carbamylated haemoglobin.

PREPARATION OF SAMPLES

1. Containers

   a) Plain tubes without anticoagulant were used for serum separation and lithium heparin tubes were used for plasma separation.

   b) Standard tubes coated with potassium oxalate and sodium fluoride were used for blood glucose estimation by the colorimetric oxidase method.

   c) Aprotinin and EDTA were added to plain tubes for pancreatic glucagon analysis.

   d) Whole blood was added to perchloric acid for the assay of intermediary metabolites.

   e) Lithium-heparin tubes were used for analyses of glycosylated haemoglobin, haemoglobin A_1 and carbamylated haemoglobin.

   f) Full blood count and platelets were measured in EDTA-K_3 vacutainers.

2. Separation, Storage and Transportation

   All hormone and metabolite specimens were kept on ice until centrifuged at $0^{o}C$ (3000 rpm for 5 minutes) and the separated serum or plasma was stored at $-20^{o}C$ until assayed. Specific sample preparation is detailed where appropriate later in the Chapter. Frozen specimens were transported between laboratories in card ice ($-80^{o}C$).

BIOCHEMICAL ASSAYS

1. **GENERAL**

   a) Biochemical profile: Urea, creatinine, electrolytes, liver
   function tests and proteins were analysed by the SMAC (Sequential
   Multiple Analyser plus Computer), a Technicon instrument.
   Haematological profile: Full blood count was analysed by the
   Technicon H6000 autoanalyser.

   b) Blood glucose was measured by two methods: a) Automated
   colorimetry using the oxidase method with 4-aminophenazine in the
   colour reagent (Barham and Trinder, 1972) and b) Enzymic
   fluorometric continuous flow analysis (Lloyd et al., 1978) which
   is detailed in below.

   c) Serum lipoproteins were measured by Cobas biocentrifugal analysis
   using standard Boehringer reagents. Cholesterol was assayed by
   automated enzymatic determination (Deeg and Ziegenhorn, 1982) and
   total triglycerides by enzymic hydrolysis (Wahlefeld, 1974).

2. **HORMONES**

   a) INSULIN: Serum insulin was measured by double antibody
   radioimmunoassay (Soeldner and Slone, 1965) [Courtesy of the
   Department of Clinical Biochemistry, University of Newcastle].
   The standard was human insulin (RD13, Wellcome Diagnostics,
   Dartford), the first antibody was antisera to insulin raised in
   guinea pig (RD10, Wellcome) and the second antibody was rabbit
   anti-guinea pig serum (RD18, Wellcome). In diabetics, free
   insulin was measured by the removal of endogenous insulin
   antibodies with polyethylene glycol (PEG) precipitation
   (Desbuquois and Auerbach, 1971). This was done immediately after
   serum separation and prior to storage. Details of the PEG

solution are given in Appendix 2. All insulin samples were assayed in duplicate. The intra- and inter-assay coefficients of variation were between 5% and 8%.

b) C-PEPTIDE: Plasma c-peptide was measured by the Department of Clinical Biochemistry, University of Newcastle. The modified method (Heding, 1975), used the Novo radioimmunoassay kit for human c-peptide (Novo Biolab, Denmark). The standard was synthetic human c-peptide and the antibody antisynthetic human c-peptide guinea pig serum (M1230). This antiserum had about 10% cross reactivity with proinsulin and no prior separation of c-peptide was needed with non-diabetics subjects. In insulin treated diabetics with insulin antibodies a significant amount of proinsulin might be bound to the antibodies, and to prevent co-determination of the proinsulin, c-peptide was determined by PEG precipitation to remove antibody bound proinsulin. The sensitivity of the assay showed a lower limit of detection of 0.02 nmol/l. Precision over the linear range of the standard curve had a within-assay C.V. of 3% and between-assay C.V. of 5%. Uraemic patients with high serum c-peptide concentrations had dilution of serum samples so that the working samples were within the linear part of the standard curve.

c) GLUCAGON: Glucagon was measured by c-terminal specific radioimmunoassay using wick chromatography (Orskov et al., 1968) [Specimens were sent to Professor Hans Orskov, Aarhus, Denmark for assay]. Due to the instability of pancreatic glucagon, blood was added immediately after venesection to a mixture of EDTA and the proteinase inhibitor aprotinin (Trasylol, Bayer). Details of this solution are in Appendix 2.

d) GROWTH HORMONE: Serum growth hormone (GH) was measured by the

Supraregional Assay Service (SAS), Royal Victoria Hospital,

Newcastle upon Tyne. The method was a double antibody

radioimmunoassay using human GH, UK6 standard (EQAS, Edinburgh),

the first antibody was anti-human GH raised in rabbit (RD16,

Wellcome) and the second antibody was goat anti-rabbit gamma

globulin (Calbiochem-Behring, Cambridge). The assay precision

was;

GH level mu/l	Between Batch C.V.%	Within Batch C.V.%
1.7	11.1	7.1
4.2	8.6	7.2
1.8	6.0	5.0

e) CORTISOL: Plasma cortisol was measured by the SAS laboratory in
Newcastle using a double antibody radioimmunoassay technique. A
commercial cortisol standard was supplied by Seron o Biodata, the
first antibody was antisera to cortisol raised in rabbits (NZ06,
Cambridge Medical Diagnostics) and the second antibody was goat
anti-rabbit gamma globulin. The precision of the assay was;

Cortisol level nmol/l	Within Batch C.V.%	Between Batch C.V.%
130	8.9	10.7
435	7.3	10.5
996	8.8	11.5

3. INTERMEDIARY METABOLITES

Blood glucose, lactate, alanine, 3-hydroxybutyrate and glycerol were measured by enzyme fluorometric continuous flow assays by the method of Lloyd et al., 1978. Dialysate glucose and lactate were also measured by the same technique. Blood acetoacetate and pyruvate were measured by manual spectrofluorometry. A detailed description of both techniques are described.

Preparation of samples

Two ml of whole blood was deproteinised in 5 ml of 5% ice cold perchloric acid (0.8 mol/l). The sample was mixed and the bottle reweighed, centrifuged (3000 rpm for 5 minutes at $0^{\circ}C$) and the acid supernatant was removed for analysis. The dilution factor was calculated from the weight change observed. Glass bottles were weighed before and after the addition of perchloric acid (PCA) and then again after the addition of blood.

$$\text{Dilution factor (DF)} = \frac{\text{Wt. of PCA} + \text{Wt. of blood}}{\text{Wt. of blood}}$$

The supernatant was stored at $-20^{\circ}C$ until specimens were assayed in batches. All metabolites were analysed within 2 weeks of collection except for acetoacetate and pyruvate which were measured within 24-48 hours.

a) AUTOMATED ENZYME FLUOROMETRIC ANALYSIS

The method used was adapted from that of Lloyd et al., 1978.

Principle: The principle of the assay is the detection of changes in fluorescence due to altered concentrations of NADH. Reduced NAD has a characteristic fluorescence, whereas the corresponding oxidised form does not exhibit the same property. Thus with the use of a fluorometer and specific hydrogenase enzymes one can measure

metabolites that require NAD in the reduced or oxidised form as
coenzymes.

Reagents: En zymes and coenzymes were from Boehringer Corporation
Ltd., Sussex. The following enzymes were used; glucose-6-phosphate
dehydrogenase (G-6-P-DH) 700 kU/1, hexokinase 1400 kU/1, lactate
dehydrogenase (LDH) 4000 kU/1, L-alanine dehydrogenase (ADH) 150 kU/1,
glycerokinase 600 kU/1, glycerol-3-phosphate dehydrogenase (G-3-P-DH)
1700 kU/1, 3-hydroxybutyrate dehydrogenase (3-OHB-DH) 15 kU/1, NAD,
NADH (Grade II Analar).

Standards: Glycerol, L-alanine, glucose (50 mol/1): Analar grade
(British Drug Houses, Dorset). Lithium lactate (Grade L, Sigma
Chemical Co. Ltd., Surrey). DL-3-hydroxybutyrate (Boehringer Corp.
Ltd.). All other reagents were Analar grade.

Method:

GLUCOSE

$$\text{GLUCOSE} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{GLUCOSE-6-PHOSPHATE} + \text{ATP}$$

$$\text{GLUCOSE-6-PHOSPHATE} + \text{NADP}^+ \xrightarrow{\text{G-6-P-DH}} \text{6-PHOSPHOGLUCONATE} + \text{NADPH} + \text{H}^+$$
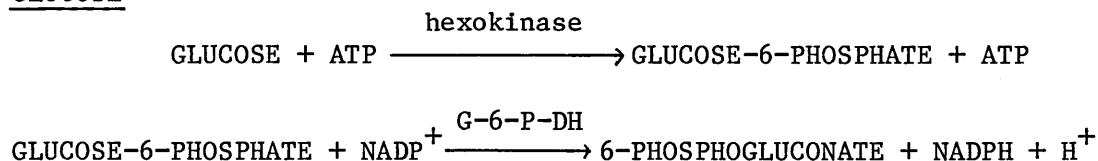
Buffer: prepare 0.1 mol/1 triethanolamine buffer, pH 8.0
containing 2 mmol of magnesium chloride per litre. Enzyme-coenzyme
reagent : Dissolve 37 mg ATP, 32 mg NADP, 140 U of glucose-6-phosphate
dehydrogenase and 160 U hexokinase in 10 ml of 0.4 mol/1
triethanolamine buffer, pH 7.4.

LACTATE

$$\text{LACTATE} + \text{NAD}^+ \underset{\text{LDH}}{\rightleftharpoons} \text{PYRUVATE} + \text{NADH} + \text{H}^+$$
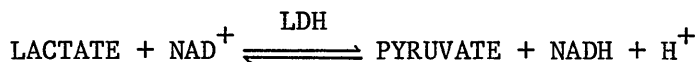
Buffer: prepare 0.5 mol/1 glycine buffer, pH 9.6, containing 0.2
mol of hydrazine and 2 g of disodium ethylene-diaminetetracetate per
litre. Enzyme-coenzyme reagent : Dissolve 20 mg NADH and 60 U of
lactate dehydrogenase in 10 ml of 0.1 mol/1 phosphate buffer, pH 7.4.

ALANINE

$$\text{L-ALANINE} + \text{NAD}^+ + \text{H}_2\text{O} \xrightleftharpoons{\text{ADH}} \text{PYRUVATE} + \text{NADH} + \text{NH}_4^+$$
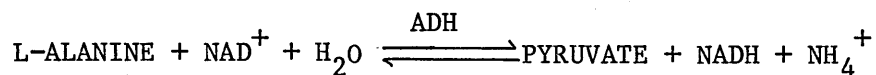
Buffer: Prepare 40 mol/l tris (hydroxymethyl) methylamine buffer, pH 10.0, containing 1 mol of hydrazine and 500 mg of disodium ethylenediaminetetracetate per litre. Enzyme-coenzyme reagent : Dissolve 20 mg of NAD and 15 U of L-alanine dehydrogenase in 10 ml 0.1 mol/l phosphate buffer, pH 7.4.

GLYCEROL

$$\text{GLYCEROL} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{L-GLYCEROL-1-PHOSPHATE} + \text{ATP}$$

$$\text{L-GLYCEROL-1-PHOSPHATE} + \text{NAD}^+ \xrightleftharpoons{\text{G-3-P-DH}} \text{DIHYDROXYACETONE PHOSPHATE} + \text{NADH} + \text{H}^+$$
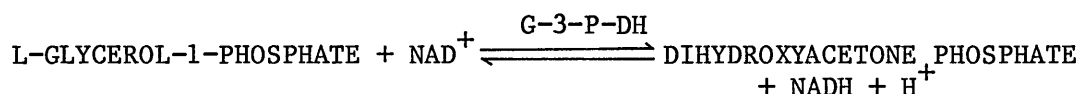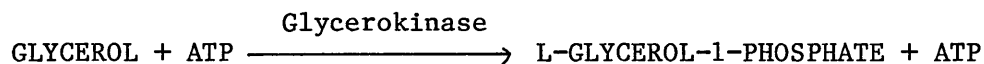
Buffer: Prepare 0.2 mol/l glycine buffer containing 1 mol of hydrazine and 0.01 mol of magnesium chloride per litre. Enzyme-coenzyme reagent : Dissolve 20 mg NAD, 20 mg of ATP, 20 U of glycerokinase and 30 U of glycerol-3-phosphate dehydrogenase in 10 ml of 0.4 mol/l triethanolamine buffer, pH 7.4.

3-HYDROXYBUTYRATE

$$\text{D(-) 3-HYDROXYBUTYRATE} + \text{NAD}^+ \xrightleftharpoons{\text{3-OHB-DH}} \text{ACETOACETATE} + \text{NADH} + \text{H}^+$$
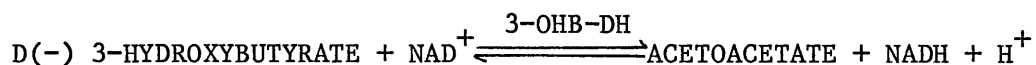
Buffer: Prepare 0.1 mol/l of tris (hydroxymethyl) methylamine buffer, pH 9.0, containing 500 mg/l disodium ethylenediaminetetracetate. Enzyme-coenzyme reagent : Dissolve 20 mg NAD and 1.5 U of 3-hydroxybutyrate dehydrogenase in 10 ml of 0.1 mol/l phosphate buffer, pH 7.4.

Dialysate glucose and lactate were analysed in an similar manner.
The dialysate solutions were diluted 1 in a 100 with 0.5 mol/l
perchloric acid.

Standard solutions:

Stock solutions were prepared in deionised water and all dilutions
of stock were made with perchloric acid 0.5 mol/l. The range of
working standards were (mmol/l)

Glucose	1.0 - 5.0
Lactate	0.5 - 2.0
Alanine	0.04 - 0.30
3-Hydroxybutyrate	0.01 - 0.10
Glycerol	0.02 - 0.12

Instrumentation:

Modules consisted of an Autoanalyser II sampler and peristaltic
pump (Technicon, Hants), fluorescent spectrophotometer (Model 1000
with a 355 nm excitation filter and a 485 nm emission filter) and a
twin channel recorder (Perkin-Elmer, Bucks).

Procedure:

Details of the manifold are shown in Figure 5.1. Perchloric acid
(0.5 mol/l) was pumped through the sample line from the wash reservoir
and the appropriate buffer through the other line until a steady
baseline was reached on the recorder. Enzyme-coenzyme reagent was
then pumped into the system and the baseline readjusted to give a 5%
deflection for NADH. Standards were run at the beginning of each
batch and at intervals of 5 samples to compensate for drift changes
during analysis. The native fluorescence of each sample was measured
by omitting the enzyme-coenzyme reagent on a blank run for all
samples. All assays were done at room temperature (20-25°C).

Manifold for Intermediary Metabolites
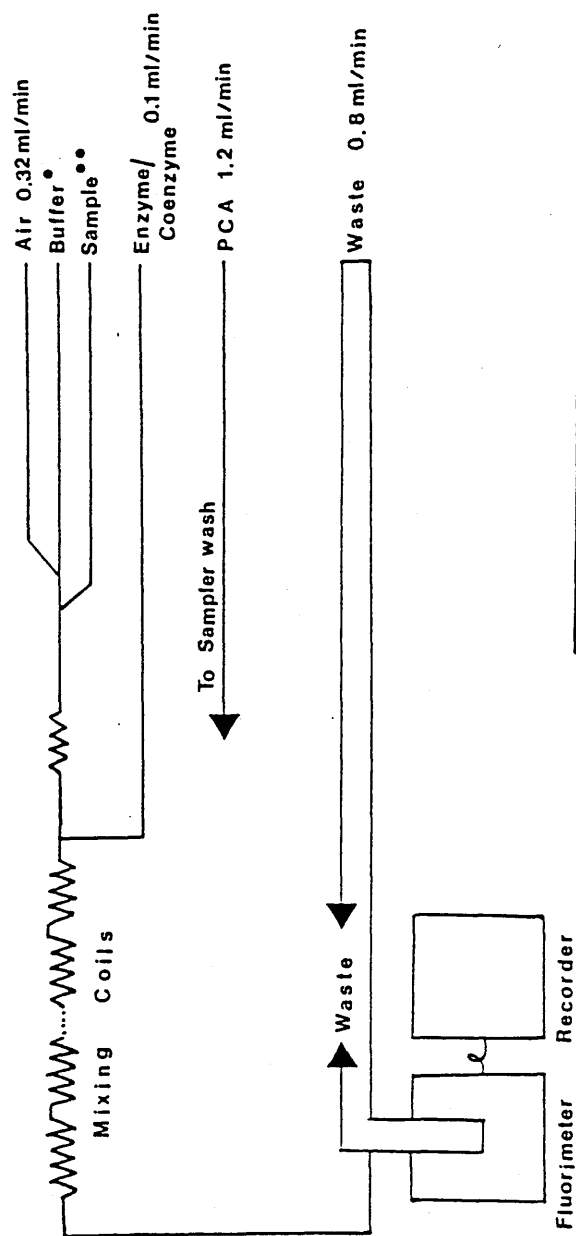
METABOLITE	BUFFER LINE• ml/min	SAMPLE LINE•• ml/min	No. of Mixing Coils
GLUCOSE	2.0	0.05	2
LACTATE	1.6	0.1	2
ALANINE	1.6	0.1	4
3-OH BUTYRATE	1.6	0.1	4
GLYCEROL	1.6	0.1	4

Figure 5. 1 Continuous-flow enzyme assays for intermediary metabolites

Native fluorescence:

This assay system depends on changes in fluorescence resulting from alterations in NADH concentrations produced by enzyme action. Any fluorescence that is non-enzymatic in origin must be taken into account to increase the accuracy of the analysis. Serum from uraemic patients has a native fluorescence, the intensity correlating with the level of serum creatinine and the presence of drugs can also affect the fluorometric analysis (Hadjivassiliou et al., 1984). Thus all samples were run with blanks (i.e. omitting the enzyme-coenzyme stage in the assay) to exclude all non-enzyme (native) fluorescence.

Precision:

Analytical recovery was not performed during the assays but previous estimates from the laboratory by adding a known quantity of stock solution showed a recovery range of between 90-95%. Within-batch and between-batch precision was under 5% for each metabolite, except 3-hydroxybutyrate which had a within assay C.V. of 8% and between assay C.V. of 12%.

Calculation of metabolites:

The metabolite concentration was determined by subtracting the blank from the standard activity and calculating the concentration relative to the appropriate working standard. Metabolite concentrations were expressed in mmol/1 by multiplying the whole blood dilution factor for each sample.

b) MANUAL SPECTROPHOTOMETRIC ANALYSIS OF ACETOACETATE AND PYRUVATE

Acetoacetate (AcAc) and pyruvate (Pyr) are unstable relative to the other metabolites even at $-20^{\circ}C$ storage. Thus a manual spectophotometric method was used for the analysis, which was performed usually within 24 hours, but always within 48 hours of

collection. Samples were stored at -20°C during this short storage period. This method is based on the following reactions:

$$\text{PYRUVATE} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{LACTATE} + \text{NAD}^+$$

$$\text{ACETOACETATE} + \text{NADH}^+ + \text{H}^+ \xrightleftharpoons{\text{3-OHB-DH}} \text{D(-)3-HYDROXYBUTYRATE} + \text{NAD}^+$$
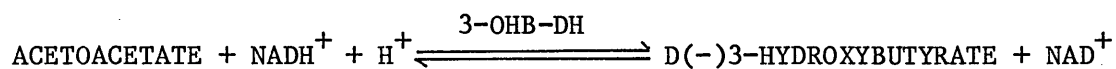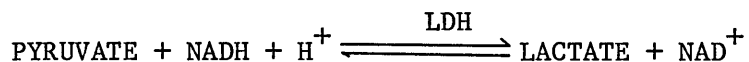
Reagents:

Enzymes: lactate dehydrogenase (LDH) 4000 kU/l, 3-hydroxybutyrate dehydrogenase (3-OHB-DH) 15 kU/l.

NADH 0.3%: NADH (13.33 mg) was dissolved in 4 ml of deionised water.

Acetoacetate standard: 10.8 mg was dissolved in 10 ml water.

Pyruvate standard: 11.0 mg was dissolved in 10 ml water.

Standards were frozen in aliquots and working standards were prepared on the day of assay by dilution (1:100) to give a final concentration of 0.1 mmol/l. Phosphate buffer, pH 7.4, contained 61 ml Na_2HPO_4, 0.1 M (1.42 g/100 ml) and 39 ml NaH_2PO_4, 0.1 M (1.56 g/100 ml).

Procedure:

All manipulations were carried out at room temperature although all reagents were kept on ice during procedure.

Sample neutralization:

To 1.4 ml of sample (volume 1) was added 4 drops of universal indicator solution (BDH) and a few drops of 20% potassium hydroxide until the solution was alkaline (pH 10). This was back titrated with 5% perchloric acid till neutral (pH 7.0). The total volume of solution was noted (volume 2). The sample dilution factor was volume 2/volume 1.

Cuvettes: (volumes-ml)

	Spec	Reag	AcAc STD	Pyr STD	Sample
Phosphate buffer	-	1.0	1.0	1.0	1.0
Distill H_2O	3.0	2.0	1.0	1.0	1.0
NADH	0.04	0.1	0.1	0.1	0.1
STD	-	-	1.0	1.0	-
Sample	-	-	-	-	1.0

STD = standard : Spec = Spectrometer blank : Reag = Reagent blank.

Cuvette solutions were mixed well and the optical densities read on a SPS 400 UV/VIS spectrophotometer (Pye, Unicam, Cambridge) at 340 nm wavelength using a tungsten lamp.

Then, 10 µl of diluted lactate dehydrogenase (5 mg/1) [1:1 in 3.2 M $(NH_4)_2SO_4$] was added, mixed and read after 10 minutes. The change in optical density estimates the pyruvate concentration. Then, 10 µl 3-hydroxybutyrate dehydrogenase was added, mixed and read after 20 minutes. The change in optical density estimates the acetoacetate concentration.

Calculation of metabolite concentration

The concentration of metabolite (mmol/1) was determined from:-

$$\Delta OD = \frac{1}{E} \times TVc \times \frac{1}{V_2}/V_1 \times B.D.F.$$

ΔOD = change in optical density

E = Extinction coefficient of NADH at 340 nm = 6.22

TVc = total volume in cuvette = 3.10 ml

V_2/V_1 = sample dilution factor

B.D.F. = whole blood dilution factor

Precision: The within-assay C.V. was 5% and 8% for pyruvate and acetoacetate respectively (n=10), and between-assay C.V. was 8% and 12% for pyruvate and acetoacetate respectively (n=6).

4. GLYCOSYLATED HAEMOGLOBIN

a) SEMIAUTOMATED COLORIMETRIC METHOD

This method of determining the total glycosylation in the red cell was based on the colorimetric assay described by Fluckiger and Winterhalter, 1976. The automated aspect being loosely based on the methods of Ross and Gibson (1979) and of Burrin et al., 1980. The modifications were made by M. Benton (unpublished) and now form the routine method of determining glycosylated haemoglobin in the Department of Clinical Chemistry, Royal Hallamshire Hospital, Sheffield.

Principle: Blood was collected in lithium heparin tubes to prevent coagulation, the red cells were spun, separated and then washed with saline. The cells were then lysed, diluted and heated with oxalic acid to hydrolyse off the glycosyl (frucrosyl) moiety which converts to 5-hydroxymethylfurfural (HMF). The HMF was then measured colorimetrically by forming an adduct with thiobarbituric acid (TBA). The reaction is shown in Figure 5.2.

Sample preparation: Blood (5-10 ml) was collected in tubes containing an anticoagulant (lithium heparin), centrifuged at 3000 rpm for 5 minutes, the plasma was removed and the red cells washed twice with isotonic saline to eliminate interference from glucose and glycosylated plasma proteins. The red cells were then hydrolysed by the addition of an equal volume of 0.1% Brij. The haemolysate was stored at $4^{\circ}C$ prior to analysis. All assays were determined within 5 days of haemolysate storage.

Preparation of working haemolysate:

The original haemolysate was diluted 1:30 in 0.1% Brij. A small amount of haemolysate (0.25-0.5 ml) was left undiluted and stored in case adjustment of the total haemoglobin content was required (see

HbA   'pre HbA$_c$'   HbA$_c$

$\begin{array}{l} HC=O \\ HCOH \\ HOCH \\ HCOH \\ HCOH \\ CH_2OH \end{array}$ $+ H_2N-R$ $\begin{array}{l} HC=N-R \\ HCOH \\ HOCH \\ HCOH \\ HCOH \\ CH_2OH \end{array}$ $\begin{array}{l} H\ H \\ HC-N-R \\ C=O \\ HOCH \\ HCOH \\ HCOH \\ CH_2OH \end{array}$

$\xrightarrow{-H_2O}$ $\xleftarrow{\text{Amadori}}$

Glucose   aldimine   ketoamine
                     (Schiff base)
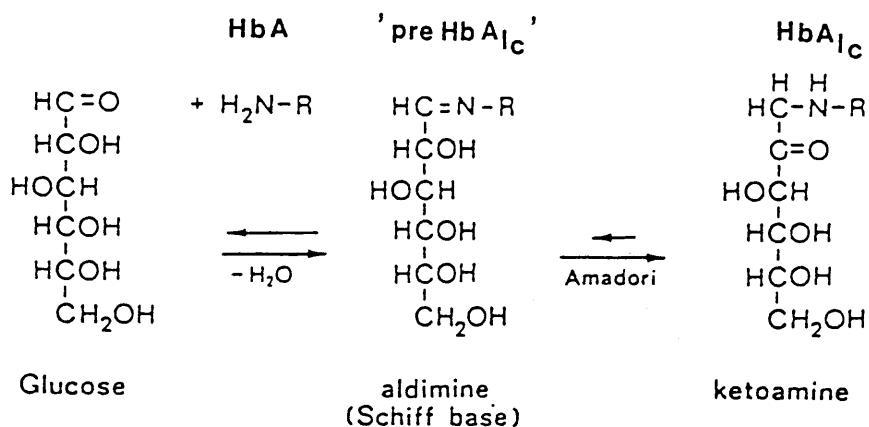
Figure 5. 2a   Non-enzymatic interaction of glucose with amino groups. The labile and easily reversible aldimine (Schiff base) is readily formed. The aldimine slowly enolizes to form the more stable ketoamine configuration via an Amadori rearrangement

$\begin{array}{l} H-C=N-R \\ H-C-OH \\ HO-C-H \\ H-C-OH \\ H-C-OH \\ H-C-OH \\ H \end{array}$ $\xrightarrow[\text{Rearrangement}]{\substack{H^\bullet \\ \text{Amadori}}}$ $\begin{array}{l} H\ H \\ H-C-N-R \\ C=O \\ HO-C-H \\ H-C-OH \\ H-C-OH \\ H-C-OH \\ H \end{array}$ $\xrightarrow[-3H_2O]{\Delta}$ $\begin{array}{l} H \\ C=N-R \\ C \\ H-C \\ H-C \\ C \\ H-C-OH \\ H \end{array}$ O $\rightleftharpoons$ $\begin{array}{l} H-C=O \\ C \\ H-C \\ H-C \\ C \\ H-C-OH \\ H \end{array}$ O $+R-NH_2$

Schiff   Ketoamine   Schiff Base   5-HMF
Base   (Hb A$_{IC}$)   of 5-HMF

┌──┐
│ 5-HMF + 2-Thiobarbituric Acid ⟶ Adduct   │
│   (Abs$_{max}$ 443nm) │
└──┘
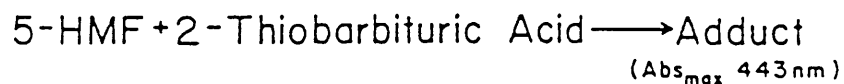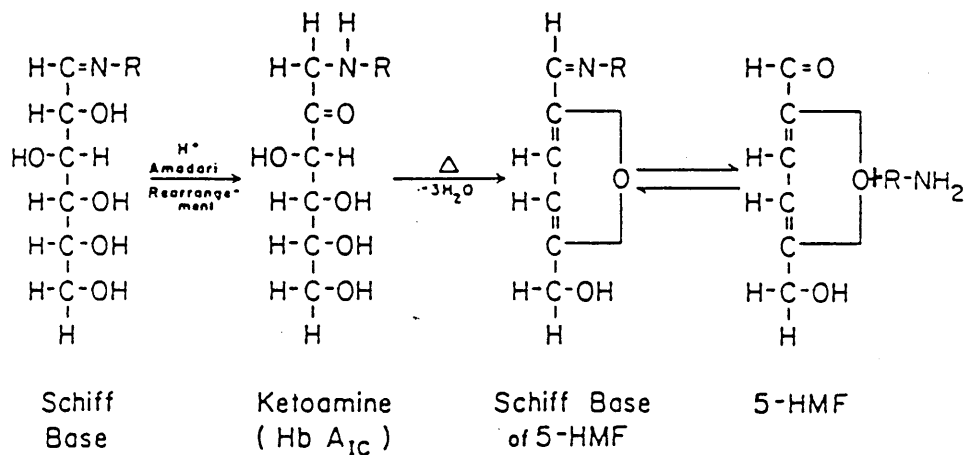
Figure 5. 2b Chemistry of the TBA colorimetric test for detection of glycosylation. Only the ketoamine rearranged form reacts to yield 5-hydroxymethylfurfural (5-HMF). The latter is detected by forming an adduct with thiobarbituric acid

below).

Determination of Total Haemoglobin

Haemoglobin reagent: (Van Kampen and Zijlstra, 1961)

Potassium Ferricyanide	400 mg (Sigma Ltd.)
Potassium Cyanide	100 mg (Sigma Ltd.)
Potassium Dihydrogen Phosphate	280 mg (Sigma Ltd.)
Sterox SE	1 ml (Monsanto Chemical Co.)

These were dissolved in 2 litres of water. This reagent was stable for at least 2 weeks at room temperature. Working haemolysate (1 ml) was added to 5 ml of haemoglobin reagent and the optical density (OD) was determined at 540 nm using a digital spectrophotometer (Cecil Instruments). The haemoglobin concentration of the working reagent was calculated from the formula:-

$$\frac{\text{Dilution factor X OD}}{\text{m molar extinction coefficient}} = \frac{6 \text{ X OD}}{11} \text{ mmol/l}$$

There was an inverse and non-linear relationship between the concentration of haemoglobin in the reaction mixture and the final yield of 5 HMF per mol of haemoglobin. Concentrations of haemoglobin were expressed in terms of the monomer (M Wt = 16,520). The haemoglobin concentration was kept within 10% of 0.275 mmol/l (range 0.250 - 0.300). This range adjustment of the total haemoglobin of the haemolysate was made by further dilution with 0.1% Brij, if greater than 0.300 or by taking the remainder of the original haemolysate and making a weaker dilution if the concentration was less than 0.250.

Determination of Hydroxymethylfurfural (HMF)

Reagents:

Brij 0.1% solution in water.

Oxalic acid (BDH, Dorset) 0.5 M : Oxalic acid (126 g) was dissolved in water and made up to 2 litres.

Thiobarbituric acid (BDH, Dorset).

TBA reagent 0.2% in 0.25 M oxalic acid : TBA reagent (1 g) was dissolved in 0.25 M oxalic acid and made to 500 ml with 0.25 oxalic acid, 0.5 ml of Brij was also added. This solution was stable for at least 1 week at 4°C.

Standard Hydroxymethylfurfural (HMF)

Stock HMF (Sigma Chemical Co., Dorset) 10 mmol/l : HMF (126 g) was dissolved and made to 100 ml with water. A 1:100 dilution was made with water and the optical density was determined at 283 nm on a Cecil spectrophotometer. This was normally between 1.68 and 1.72. The volume of stock was adjusted if necessary and then stored in 10 ml aliquots at -20°C. Substock solutions were prepared in 0.1% Brij at 150, 300, 450, 600 and 750 umol/l. This method was used to prepare standard HMF because solid HMF is deliquescent and accurate weighing of the pure substance is difficult.

Working standards

Since HMF standards degrade on heating, a set of standards were diluted 1:30 as in the tests, then diluted 1:1 oxalic acid and hydrolysed before being assayed colorimetrically.

Hydrolysis

Hydrolysis liberates fructose and converts it to 5 HMF. Haemolysate (4 ml) was placed in a 10 ml vial and 4 ml of 0.5 M oxalic acid containing 0.1% Brij was added. This mixture was capped and placed on a heating block at 100°C for 6 hours and then cooled in

water immediately.

Colorimetry

The flow diagram shows the manifold for the glycosylated haemoglobin method (Figure 5.3). The sampler, peristalic pump, dialyser, colorimeter and dual channel recorder were all standard autoanalyser II equipment (Technicon, Hants.).

Samples of hydrolysate were aspirated, air segmented and dialysed with a 'C' type membrane into the TBA reagent. The sample stream was dialysed either directly into the TBA reagent (to increase sensitivity) or into 0.25M oxalic acid for the blank measuremnt. The recipient stream was passed through a double length mixing coil (DMC) to develop the colour, this reaction as well as the dialysis, being carried out at $47^{\circ}C$ to increase the sensitivity.

Calculation

The values are read from a conventional chart reader, the difference between test and blank being the concentration of HMF in the working haemolysate in μmol/l. The final result is obtained by dividing the level of glycosylated haemoglobin (i.e. HMF) in μmol, by the concentration of haemoglobin in the sample in mmol. This is expressed as mmol HMF/mol Hb (i.e. multiplying each by 1000).

Reference range for normal adults in the laboratory was 29-39 mmol HMF/mol Hb, established by testing 80 healthy subjects.

Precision

Within batch and between-batch coefficients of variation were 3.5% and 6.0% respectively at a mean glycosylated haemoglobin level of 35 mmol HMF/mol Hb. A very strong correlation was found between this method and the micro-column method of determining HbA_1 (Bio-Rad Lab. Ltd., Herts.) [r=0.92, n=130].

MANIFOLD FOR GLYCOSYLAYED HAEMOGLOBIN

Flow Rate

0.32 ml/min
1.2 "   "
1.2 "   "
0.32 "   "
1.0 "   "
1.6 "   "

Air

Sample (G Hb)

Recipient (TBA/Acid)

Air

From F/C

Reservoir

Waste

Dialyser 47°C

D.M.C.

Waste   Waste

Pump

COLORIMETER

RECORDER

T.B.A. for the test or 0.25mol Oxalic Acid for the blank

Figure 5. 3   Semi-automated colorimetric method

b) HAEMOGLOBIN Al, Alc, Ala+b BY MINICOLUMN CHROMATOGRAPHY

Haemoglobin A_1, A_{1c}, A_{1a+b} were measured using commercial kits. Haemoglobin A_1 and haemoglobin A_{1c} column tests (Bio-Rad, Herts.).

Principle

In the Bio-Rad haemoglobin A_1 column test whole blood was mixed in a haemolysis reagent which lysed the red cells and liberated haemoglobin. An aliquot of haemolysate was then applied to a weak acidic cation exchange resin in a disposable column. An elution/developing reagent was then added to the column, which separated the 'fast' moving glycosylated haemoglobin component (HbA_1a,b,c) from the remaining 'slow' haemoglobin fraction.

The total haemoglobin was measured by mixing an aliquot of haemolysate with the elution/developing reagent. After the column eluate containing HbA_1 (A_1a+b+c) had been collected the relative concentrations of the glycosylated and the total haemoglobin were determined spectrophotometrically (415 nm) and the percentage glycosylated haemoglobin (HbA_1) was calculated.

In the Bio-Rad HbA_{1c} column assay separate determination of HbA_{1c} and HbA_{1a+b} was achieved by using 2 elution/developing reagents. Haemolysate was prepared as in the HbA_1 method which lysed the cells and initiated removal of the Schiff base (aldimine). A low ionic strength borate/phosphate buffer was added to the column after application of the haemolysate. This first buffer eluted the HbA_{1a} and HbA_{1b} and further dissociated the labile Schiff base fraction. The HbA_{1c} and HbA_o fractions remain on the column. HbA_{1c} was then eluted from the remaining haemoglobin fraction by the addition of the second elution/developing reagent.

The total haemoglobin was prepared as in the HbA_1 method and the relative percentage concentrations of HbA_{1a+b} and HbA_{1c} were

determined by spectrophotometric analysis of the first and second elutes respectively.

The assays were performed according to the manufacturers instructions, but several modifications were made to improve the sensitivity and precision. The exact method for each assay is detailed below. All assays were carried out at a constant $26^{o}C$ in a water bath. Positive displacement micropipettes (50 µl and 500 µl) were used to obtain adequate reproducibility and accuracy. Assays were performed in batches of 10 samples and analysed within 24-48 hours after collection.

Haemoglobin A1 Assay

Reagents

Haemolysis reagent : polyoxyethylene ether (0.33% v/v). Elution/developing reagent : phosphate buffer pH 6.7. Resin columns : disposable columns containing a measured amount of weakly acidic cation exchange resin. All stored at room temperature.

Procedure

1. Whole blood in a lithium heparin tube was mixed well and 100 µl (2x50 µl) was added to 500 µl of haemolysis reagent, vortexed and allowed to stand for a least 5 minutes.

2. Resin columns were shaken, resuspended and the waste fluid allowed to drain. The columns were placed in a bath at $26^{o}C$ (controlled by a Grants Thermostatic heater) throughout the assay.

3. Within 15 minutes of preparing the columns 100 µl (2x50 µl) of haemolysate or control was carefully pipetted dropwise onto the centre of the resin bed.

4. After 5 to 7 minutes 10 ml of elution/developing reagent was

added to the column without disturbing the resin bed. The column

eluate was collected within 45 minutes (Solution A).

5. Total haemoglobin of the sample was determined by adding 50 μl of

haemolysate or control to 10 ml of elution/developing reagent

(solution B).

6. Samples were then read on a spectrophotometer (Digital

spectrophotometer CE 393, Cecil Instruments, Cambridge) at 415 nm

after 'zeroing' with a elution/developing reagent blank.

Calculation of HbA1

The purpose of the test was to determine what percentage of total

haemoglobin was the A_1 fraction. This was calculated from the

formula:-

$$\%HbA_1 = \frac{\text{Absorbance of Solution A}}{\text{Absorbance of Solution B}} \times \left[\frac{1}{2}\right]^* \times 100$$

*correction factor for differences in sample concentration

Precision

Within-batch precision, in the normal range (10 replicate samples)

gave a mean and standard deviation of 7.73±0.26%, i.e. coefficient of

variation of 3.36%. In the upper or 'diabetic' range (10 samples)

mean and SD was 14.42±0.62%, C.V. was 4.3%.

Between-batch precision (5 replicate samples), in the 'normal'

range was 7.60±0.47% with a C.V. of 6.2%. In the 'diabetic' range

mean and SD was 13.63±0.95, C.V. was 7.0%.

Haemoglobin A1c and A1a+b assay

Reagents

Haemolysis reagent : polyethylene ether (0.33% v/v).

Elution/developing reagent. First elution buffer : borate/phosphate

buffer, pH 6.7. Second elution buffer : phosphate buffer, pH 6.7.
Resin columns : disposable columns with weakly acidic cation exchange
resin. All stored at room temperature.

Procedure

1. Whole blood in a lithium heparin tube was mixed well and 100 μl
    (2x50) was added to 500μl of haemolysis reagent, vortexed and
    allowed to stand for 5 minutes.

2. Resin columns are shaken, resuspended and waste fluid allowed to
    drain. The columns were placed in a constant temperature bath
    (26°C) throughout the assay.

3. Within 20 minutes of preparing the columns 100 μl (2x50) of the
    haemolysate or control was carefully pipetted on to the column
    resin bed.

4. After 5 minutes 4.0 ml of the first elution/developing reagent
    was added to the column without disturbing the resin bed. Within
    30 minutes the first elution buffer was drained and collected for
    HbA_{1a+b} estimation (Solution A).

5. Then 10 ml of the second elution/developing reagent was added and
    after 45 minutes the second eluant was collected for HbA_{1c}
    determination (Solution AA).

6. The total haemoglobin of the sample was prepared by adding 50 μl
    of haemolysed sample or control to 10 ml of the second elution
    buffer (Solution B).

7. Samples were then read on a spectrophotometer at 415 nm.

Calculation of HbA1a+b and HbA1c

HbA$_{1a+b}$ was calculated using the formula:-

$$HbA_{1a+b}\% = \frac{\text{Absorbance of Solution A}}{\text{Absorbance of Solution B}} \times \left[\frac{1}{2}\right]^{*} \times \left[\frac{10}{4}\right]^{+} \times 100$$

HbA$_{1c}$ was calculated using the formula:-

$$HbA_{1c}\% = \frac{\text{Absorbance of Solution AA}}{\text{Absorbance of Solution B}} \times \left[\frac{1}{2}\right]^{*} \times 100$$

Correction factors:

* to correct for difference in sample concentration

+ to correct for difference in sample volume

Precision

HbA$_{1a+b}$ within-batch precision was (on 10 replicate samples);

a) in the normal range : C.V. 4.1% (mean 1.46%, SD 0.06)

b) in the diabetic range : C.V. 4.4% (mean 2.04%, SD 0.09)

Between-batch precision was (on 5 replicate samples);

a) in the normal range : C.V. 4.8% (mean 1.65%, SD 0.08)

b) in the diabetic range : C.V. 6.6% (mean 2.12%, SD 0.14)

HbA$_{1c}$ within-batch precision was (on 10 replicate samples);

a) in the normal range : C.V. 2.8% (mean 5.96%, SD 0.17)

b) in the diabetic range : C.V. 2.4% (mean 9.71%, SD 0.23)

Between-batch precision was (on 5 replicate samples);

a) in the normal range : C.V. 3.4% (mean 6.21%, SD 0.21)

b) in the diabetic range : C.V. 4.9% (mean 9.2%, SD 0.45)

Quality control

Periodicaly during the HbA_1 and HbA_{1c} assays precision and accuracy was checked using lyphochek controls (Bio-Rad, Hants). Lyphochek was prepared from human whole blood and exhibits column elution profile and temperature restrictions comparable to those of patient whole blood haemolysate.

HbA_1 lyphochek for normal levels gave a mean value of 7.4% (manufacturer's mean value 7.1%, range 6.4-7.7) and for the higher (diabetic) levels a mean value of 13.8%, (manufacturer's assayed value, mean 13.3%, range 12.2-14.3).

HbA_{1c} lyphochek for normal levels gave a mean value of 6.1%, (manufacturers assayed value, mean 5.8%, range 5.3-6.3) and for the higher (diabetic) levels a mean of 9.8%, (manufacturer's assayed value, mean 9.4%, range 8.6-10.1).

5. CARBAMYLATED HAEMOGLOBIN

Carbamylated haemoglobin was measured by the methods of Fluckiger et al. (1981) and Manning et al. (1973) with several modifications which are detailed.

Principle

Red blood cells were washed in normal saline and the haem was removed by washing in acid-acetone. The globin was then hydrolysed with acetic acid and hydrochloric acid to split off isopropyl hydantoin (IPH). IPH was extracted into ethyl acetate and estimated by gas liquid chromatography (GLC). The reaction is shown in Figure 5.4.

Reagents

Sodium hydroxide 10 M

Concentrated hydrochloric acid

Sodium chloride; saturated solution

Sodium bicarbonate; 5% solution

Acid-acetone; 2% HCl in acetone

Acetic acid

Diethyl ether

Analar grade reagents used when available

Standards

Isopropyl Hydantoin (MW 142) (Aldrich Chemical Co., Dorset)

Stock solution; 172.5 mg in 25 ml methanol

Working solution; Dilute 2 ml to 100 ml with de-ionised water; 100 μl of this solution was used for the standard, i.e. working standard contained 13.8 μg of isopropyl hydantoin.

CARBAMYLATED HAEMOGLOBIN
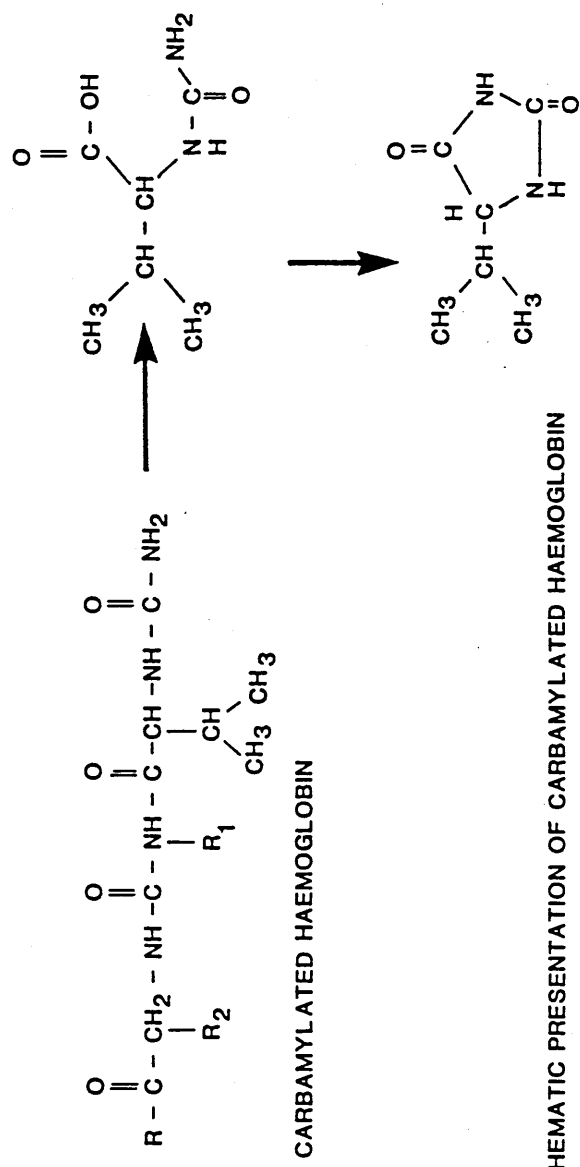
5 – isopropyl hydantoin

(2,4 – imadazolidine – dione)

Figure 5. 4   SCHEMATIC PRESENTATION OF CARBAMYLATED HAEMOGLOBIN

Internal Standard

Hexobarbitone (MW 236) (May and Baker, Dagenham).

Stock solution; 70.8 mg in 25 ml methanol.

Working solution; dilute 2 ml to 100 ml with de-ionised water; 100 μl of this solution was used for the working internal standard, i.e. working internal standard contained 5.7 μg of hexobarbitone.

Preparation of sample

Blood was taken into lithium heparin tubes and the red cells separated within 24 hours. The red cells were washed in normal saline. The red cells were then resuspended in an equal volume of physiological saline. To 1 ml of this suspension, 10 ml of ice cold acid-acetone was slowly added on a vortex mixer. The globin was washed several times (on average 5) in 10 ml volumes of ice cold acetone to removed the haem and finally washed with diethyl ether and dried overnight at room temperature. The globin (white powder) was then stored at $4^{\circ}C$ until hydrolysis was carried out (usually within 1-2 weeks).

Hydrolysis of Globin

Globin (50 mg) was resuspended in 0.5 ml of 50% acetic acid in a B 14 tube and 0.5 ml of concentrated hydrochloric acid was added. The tube was then capped with a glass marble and heated for 1 hour at $100^{\circ}C$ and then cooled on ice.

Extraction

Sodium hydroxide 10 M (0.65 ml) was added to the hydrolysate. The pH should be between 3.0 and 5.0. Saturated sodium chloride (0.5 ml)

was added, then 100 µl of the internal standard, hexobarbitone, 100 µl of de-ionised water and finally 5 ml of ethyl acetate. The tube was stoppered and mixed gently for 3 minutes. The ethyl acetate was transferred to another B14 tube and washed with 1 ml of 5% sodium bicarbonate. After centrifuging 4 ml of ethyl acetate was taken off into a conical glass centrifuge tube and evaporated to dryness at 60°C under a stream of air. The residue was redissolved in 100 µl of ethyl acetate and 5 µl prepared for injection on to the GLC column.

Standard

'Working' internal standard, hexobarbitone (100 µl) and isopropyl hydantoin (100 µl) 'working' standard were added to a mixture of 0.5 ml of 50% acetic acid and 0.5 ml hydrochloric acid and then treated as in the sample extraction.

Gas liquid chromatography

GLC analysis was performed using a Sigma I gas chromatograph (Perkin Elmer, Bucks). Aliquots (5 µl) were chromatographed on a glass column, 2 mm x 1 m, packed with GP 2% SP 2110/1% SP-2510 DA on 100/200 supelcoport (Supelco Inc., Bellfonte, PA, USA). The carrier gas was Argon, flow rate 40 ml/min, using a nitrogen detector and an oven temperature of 170°C.

The retention times (Figure 5.5) were 2 minutes for isopropyl hydantoin and approximately 5 minutes for hexobarbitone. Periodically the column was repacked when accumulation of 'contaminants' produced extra or erratic peaks.
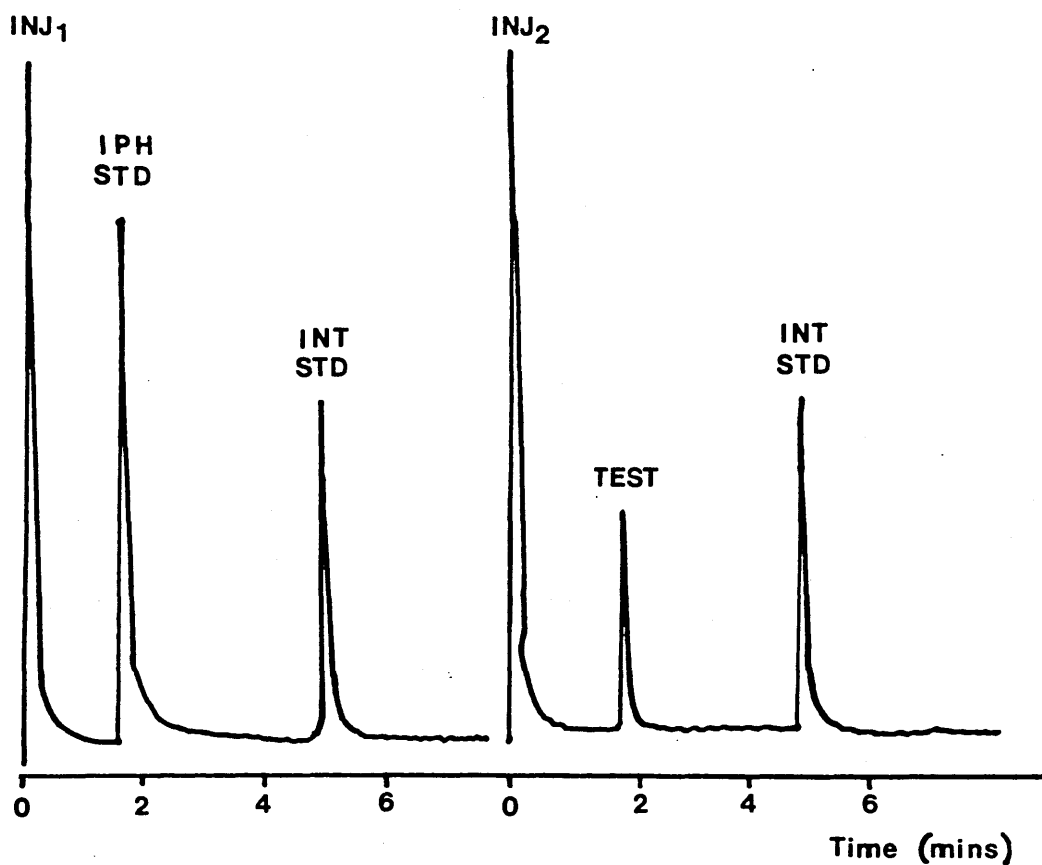
GLC peaks and retention times

Carbamylated Haemoglobin is determined by the release
of isopropyl hydantion, calculated from:

$$\frac{\text{Ht. of Test/Ht. of INT STD}}{\text{Ht. of IPH STD/Ht. of INT STD}} \times \frac{13.8^*}{50} = \text{ngIPH/mg globin}$$

INT STD - internal standard (hexobarbitone)
IPH STD - Isopropyl hydantoin standard
HT - peak height

* 13.8 ng is weight of IPH injected onto column
   50 mg is weight of globin hydrolysed

Figure 5.5   GLC peaks and retention times
              Calculation of carbamylated haemoglobin

Calculation

Carbamylated haemoglobin was calculated using the formula:-

$$\frac{\text{Ht. of Test sample/Ht. of Internal Std.}}{\text{Ht. of Std. IPH/Ht of Internal Std.}} \quad X \quad \frac{13.8}{50} \quad = \text{ng IPH/mg}$$

where 13.8 ng is the weight of IPH injected into the column and 50 mg is the weight of globin hydrolysed.

Precision

A reference range has not been previously recorded, but in 40 subjects (20 healthy controls and 20 patients with normal renal function) concentrations were less than 40 ng IPH/mg globin. A mean value with 95% C.I. was 27 (22-32) ng IPH/mg globin.

All samples were assayed in duplicate and a coefficient of variation of 12% was found between test samples.

STATISTICS

Multics is the operating system on the Joint Cardiff Computing Service mainframe computer (Honeywell DPS - 8/70 M). This facility was used at the University of Wales College of Medicine.

Much of the data in this thesis were analysed using the Minitab Statistical Package on the Multics system. Some data were also analysed using the HP 67/97 and 41Cx Hewlett Packard statistical package on a micro computer.

Specific statistical methods are described in the appropriate chapters under the method section.

CHAPTER 6

PANCREATIC BETA CELL FUNCTION IN URAEMIA

INTRODUCTION

The abnormalities of carbohydrate metabolism in uraemia have been discussed in Chapter 2 and include the effect of tissue sensitivity to insulin and the beta cell response. It has been suggested that the beta cell response may be altered in uraemia. To compensate for the increased peripheral antagonism to the action of insulin the beta cell may hypersecrete in an attempt to overcome the insulin resistance. However, despite the tissue insensitivity to insulin a normal or decreased insulin response to oral glucose has also been observed. Thus beta cell function may be normal, hyperactive or relatively inhibited in uraemia.

1. HAEMODIALYSIS

The glucose intolerance of uraemia has been reported to improve markedly with haemodialysis (Hampers et al., 1966; Alfrey et al., 1967). In contrast, more recent studies, using glucose specific methods, have shown that haemodialysis produces only marginal improvement or no significant improvement in glucose tolerance (Swenson et al., 1974; Ferannini et al., 1979; Marumo et al., 1979). However, studies on the metabolic clearance rates have shown that haemodialysis significantly improves but does not fully correct the abnormalities of carbohydrate metabolism in uraemia (DeFronzo et al., 1978a; Graf et al., 1985).

2. INTERMITTENT PERITONEAL DIALYSIS

During intermittent peritoneal dialysis large quantities of glucose are absorbed from the dialysis fluid and can induce hyperglycaemia (Nolph et al., 1970). The serum insulin response to an oral glucose load has been shown to decrease within a few days after

intermittent peritoneal dialysis suggesting possible exhaustion of the pancreatic beta cells (Spitz et al., 1970).

3. CONTINUOUS AMBULATORY PERITONEAL DIALYSIS (CAPD)

CAPD is a unique clinical situation that involves constant glucose administration. The effect of this persistent glycaemic stress is a potential hazard and its effect on beta cell function is of paramount importance. The continuous glucose absorption from the dialysate could affect insulin release with profound effects on carbohydrate, lipid and amino acid metabolism (see Chapter 3).

There are several similarities between uraemia and diabetes, particularly type II (maturity onset) as discussed in Chapter 2. The tissue insensitivity to insulin and impaired beta cell reponse to glucose are seen in both conditions although to different degrees. Continuous glucose administration in maturity onset diabetes has been shown to exhaust insulinogenic reserves (Seltzer and Harris, 1964). Thus CAPD with its continuous peritoneal glucose administration may possibly exhaust beta cell function. Alternatively, continuous stimulation of the beta cell may further augment serum insulin concentrations that are already increased in response to the insulin resistance in uraemia. Thus CAPD may adversely affect beta cell secretory function, either by depletion of the beta cell or even to stimulate further secretion, both of which would affect glucose homeostasis. The inability to adapt to the extra glycaemic stress in CAPD might even lead to overt diabetes mellitus.

Present data on glucose metabolism in CAPD is limited because the duration of experience of this mode of dialysis is short. Few centres have more than 7 years experience and relatively few patients have been on CAPD for longer than 3 years due to the initial high failure

rate of this technique (see Chapter 3).

Deterioration in the insulin response to oral glucose has been reported (Armstrong et al., 1980). In contrast, others have shown that although glucose intolerance was present prior to CAPD, no further deterioration was observed in the short term (Lindholm et al., 1981; Von Baeyer et al., 1983). A similar study in children showed no change in glucose intolerance after 1 year on CAPD (Broyer et al., 1983).

4. DIABETES AND DIALYSIS

CAPD is not only a viable alternative to haemodialysis for the diabetic with end stage renal disease but is, in the United Kingdom, the preferred mode of dialysis treatment (Cameron and Challah, 1986). In other parts of Europe haemodialysis is the main modality of treatment (Jacobs et al., 1983) for diabetics. At present most diabetics on CAPD are type I but increasing numbers of older type II diabetics are being treated (Cameron and Challah, 1986). It initially may seem paradoxical that a treatment consisting of continuous glucose administration is actually beneficial. The advantages of this treatment are discussed in Chaper 3. Although insulin requirements increase in type I and many type II diabetics require supplemental insulin blood glucose control with intraperitoneal insulin is much improved (Flynn, 1979; Khanna et al., 1983; Rottembourg et al., 1983).

Type I diabetics have negligible beta cell function, especially after 20 to 30 years, when they reach end stage renal disease, but type II diabetics often have some residual beta cell function which could be depleted by continuous glucose administration.

5. BETA CELL SECRETORY FUNCTION

Stimulation of the beta cell normally results in extrusion of the beta cell granule where proteolytic cleavage of proinsulin to insulin and c-peptide takes place. The secretory products of the beta cell consists of equimolar amounts of insulin and c-peptide, which account for 94% of the secreted products. The remaining 6% is due to proinsulin intermediates (Rubenstein et al., 1977). In the peripheral blood the circulating c-peptide concentration is approximately 5-fold greater than insulin in healthy fasting subjects and this falls to about 2-3 fold after pancreatic stimulation (Faber et al., 1978). This reflects the slower metabolic clearance of c-peptide compared to insulin (see Chapter 2).

Beta cell secretory activity has been assessed using intravenous glucagon stimulation and measuring the c-peptide response in peripheral blood (Faber and Binder, 1977; Binder and Faber, 1978). This dynamic test is simple, specific, sensitive, rapid to perform, causes no discomfort to the patient and applicable to both diabetic and non-diabetic subjects. Diabetic patients with insulin antibodies and those taking exogenous insulin present a problem when measuring plasma insulin concentrations. The plasma of these patients contain both free (unbound) and antibody bound insulin which makes interpretation of plasma insulin difficult unless specific extraction procedures are carried out (Heding, 1969; Desbuquois and Auerbach, 1971; Hanning et al., 1985). Hence the value of c-peptide measurement. In non-diabetics both insulin and c-peptide measurements are complementary.

6. OBJECTIVES

The primary objective of this study was to evaluate beta cell function in a mixed uraemic population and to determine if CAPD treatment per se affects the beta cell secretory capacity. However, as beta cell peptides can be elevated in uraemia it was necessary to compare non-dialysed uraemic and haemodialysis patients with those on CAPD to distinguish the effects of uraemia from dialysis therapy.

The secondary aim of this study was to determine beta cell integrity in diabetics with renal failure including those on haemodialysis and CAPD.

1. PATIENTS

Beta cell function was investigated in 67 subjects. Forty non-diabetic uraemic patients on different modes of dialysis were studied. They were classified into four groups (with 10 patients in each): chronic renal failure (CRF) on conservative treatment, i.e. non-dialysis; haemodialysis (HD); NEW CAPD (on CAPD for less than 6 months); and CHRONIC CAPD (established on CAPD for more than 1 year). Eight healthy subjects were used as controls.

In the diabetic groups all 19 patients had proven diabetes of varying duration and most, but not all, had diabetic nephropathy as the cause of their renal failure. The selection of diabetic subjects with renal failure was limited due to the relatively small number available for study. Diabetic patients were classified in two ways i) by mode of renal therapy, i.e. CRF, HD or CAPD and ii) by type of diabetes, i.e. type I insulin dependent or type II non-insulin dependent. Diabetics with normal renal function (NRF) were used as controls. Normal renal function was judged by normal serum creatinine concentrations, but lesser degrees of diabetic nephropathy such as impaired glomerular filtration rate and microalbuminuria were not excluded.

All subjects had normal liver function assessed by history and absence of clinical manifestations of hepatic disease and normal biochemical indices of liver function. The non-diabetic subjects had no clinical or biochemical features of diabetes. No subjects were taking steroids, beta blockers or thiazide diuretics at the time of the study. All subjects continued with their usual diet prior to the study. Haemodialysis patients were studied on their interdialysis day (i.e. 24 hours after their last dialysis) and all CAPD patients

omitted their overnight dialysis exchange prior to the study.
Diabetics on insulin omitted their morning dose and oral hypoglycaemic
drugs were delayed until after the study.

2. PROTOCOL

All subjects had a 12 hour oral fast and CAPD patients also had an
overnight 'peritoneal' fast by omitting their overnight exchange. The
study was done between 9.00 and 11.00 a.m. All subjects remained
semi-supine throughout the 1 hour test. An intravenous cannula was
inserted into a forearm vein for serial blood sampling and kept patent
by heparinised saline flushes after each blood sample was taken.
Patients were constantly under medical supervision throught the study.
The Sheffield hospitals medical ethics committee approved the study.

3. PILOT STUDY

Prior to the main study 5 patients were given 0.5 mg or 1.0 mg of
glucagon intravenously and samples were taken at frequent intervals to
determine the optimal dose and timing of samples. From this study, 1
mg of glucagon and eight time intervals were chosen for the glucagon
stimulation test.

4. MAIN STUDY

After a fasting blood sample (time zero), 1 mg glucagon (Novo
Labs) was given by I.V. bolus and blood specimens were taken at 5, 10,
15, 20, 30, 45 and 60 minutes post injection for glucose, insulin and
c-peptide determination. Fasting serum lipoproteins, glycosylated
haemoglobin, full blood count and a 'routine' biochemical screen
including liver function tests were measured. Specimens were kept on
ice until transported to the laboratory. Blood for peptide

estimations were separated within 3 hours of collection and stored at $-20^{\circ}C$ until assayed.

5. ASSAYS

Blood glucose was measured by the glucose oxidase method (Barham and Trinder, 1972). Serum insulin was determined by modification of the double antibody radioimmunoassay technique of Soeldner and Slone (1965). Serum free or unbound insulin was measured in diabetic subjects by using PEG precipitation after serum separation but prior to storage. Plasma c-peptide was determined by a modification of the Heding (1975) technique using a radioimmunoassay kit (Novo Biolab). Serum insulin had a 5% and 8% intra- and inter-assay precision respectively. The intra- and inter-assay coefficients of variation for c-peptide were 3% and 5% respectively. C-peptide antibody had less than 10% cross-reactivity with proinsulin. Glycosylated haemoglobin was measured by colorimetry (a modification of the Fluckiger and Winterhalter (1976) technique) and serum lipoproteins by Cobas biocentrifugal analysis (Deeg and Ziegenhorn, 1982; Wahlefeld, 1974). A more detailed description of each assay is given in Chapter 5.

6. STATISTICS

Data within each group was analysed by the paired 't' test and data between groups by analysis of variance. Analyses of the glucagon response with time curves was done by the analysis of variance technique using repeated measures analysis to investigate differences between the groups and their response patterns with time.

RESULTS

Intravenous glucagon was given without any adverse effects although approximately 30% of subjects experienced transient nausea lasting about 1 minute which occurred usually between 3 and 4 minutes post-injection. Results of the study are divided into two sections:-1. Non-diabetics, 2. Diabetics.

1. NON-DIABETICS

Clinical data (values are mean and S.D.) are given in Table 6.I. The mean age and weight of each group were comparable. The difference in duration of dialysis was clearly seen particularly distinguishing NEW CAPD from CHRONIC CAPD. Serum creatinine although higher in the CHRONIC CAPD group was not statistically different from the other uraemic groups. Fasting total serum triglycerides were increased in all uraemic groups but this was only statistically significant in the HD group ($p < 0.05$). Glycosylated haemoglobin was normal in all groups.

Fasting lipoprotein profiles are shown in Figure 6.1 (values are mean and SEM). Total triglycerides and cholesterol were increased in all uraemic groups and HDL-cholesterol was decreased in all uraemics except the CRF group. However, there was marked variation within each group and the only statistically significant change was the increase in total triglycerides in HD group compared to controls.

a) Basal and peak responses to glucagon

Fasting and maximum responses (values are mean and SEM) of glucose, insulin and c-peptide to I.V. glucagon are shown in Table 6.II and Figure 6.2. Basal glucose concentrations (3.6 to 4.4 mmol/l) were similar in all groups and there was also no difference between peak concentrations (6.4 to 7.4 mmol/l). The increment (59-80%)

	CONTROL	CRF	HD	NEW CAPD	CHRONIC CAPD
Subjects M/F	3:5	5:5	7:3	5:5	4:6
Age	42±12	61±6	50±12	54±12	59±5
Weight (Kg)	69.0±12.0	65.5±13.1	63.2±11.3	67.1±16.5	71.8±10.5
Duration of Dialysis (months)	-	-	24±32	3.6±2	35.4±13.5
Serum Creatinine (μmol/1)	89±16	779±257	889±145	951±238	1118±189
Serum total triglycerides (mmol/1)	1.22±0.70	1.93±0.81	2.56±1.70 *	2.48±1.24	2.43±1.27
Glycosylated haemoglobin (mmol HMF/mol Hb)	37.1±2.7	37.8±2.8	34.9±3.6	37.1±2.5	37.0±3.3

Table 6. I

Clinical Data
Values are mean and SD
* p < 0.05 compared to control
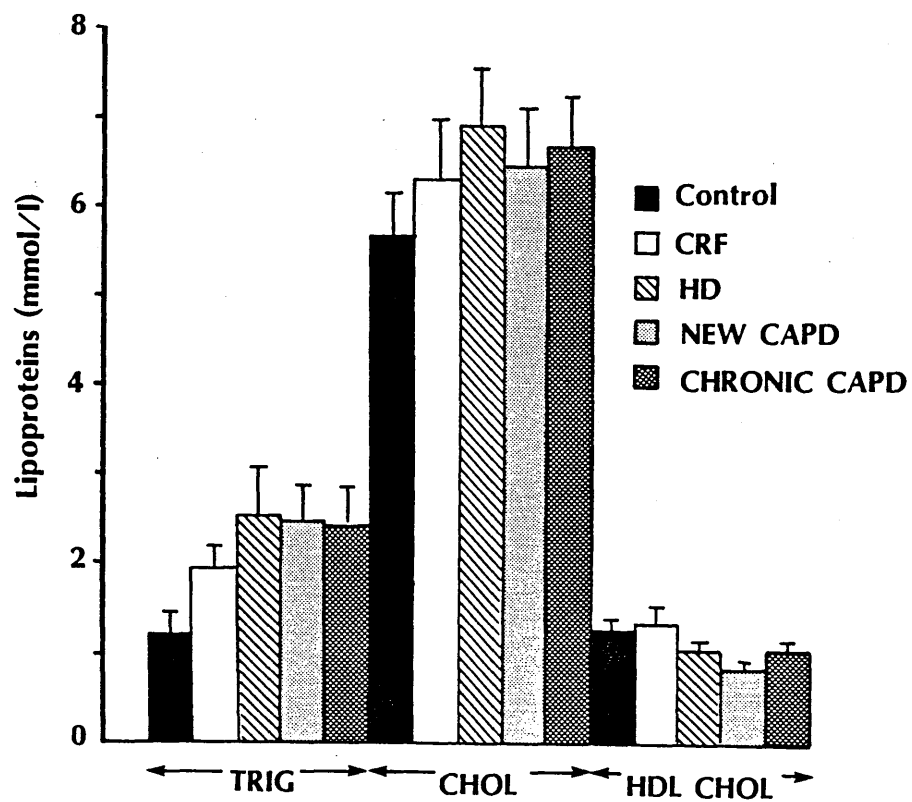
Figure 6. 1 Fasting serum lipoproteins
Values are mean and SEM
(n = 10 in each group, except controls n=8)

		CONTROL	CRF	HD	NEW CAPD	CHRONIC CAPD
GLUCOSE (mmol/l)	B	3.6 ± 0.2	4.4 ± 0.4	4.2 ± 0.3	4.1 ± 0.2	4.2 ± 0.2
	M	6.4 ± 0.5	7.0 ± 0.3	7.3 ± 0.4	7.4 ± 0.4	7.1 ± 0.5
	p	<0.001	<0.001	<0.001	<0.001	<0.001
INSULIN (mu/l)	B	7.5 ± 1.6	11.5 ± 1.7	11.7 ± 1.6	9.5 ± 1.0	11.7 ± 1.4
	M	53.4 ± 9.3	49.9 ± 5.3	59.2 ± 6.5	57.9 ± 6.8	55.0 ± 10.2
	p	<0.001	<0.001	<0.001	<0.001	<0.01
C-PEPTIDE (nmol/l)	B	0.48 ± 0.06	1.84 ± 0.38	2.25 ± 0.39	1.94 ± 0.20	2.38 ± 0.30
	M	1.51 ± 0.13	3.00 ± 0.45	4.30 ± 0.55	4.04 ± 0.34	3.92 ± 0.40
	p	<0.001	<0.001	<0.001	<0.001	<0.001

Table 6. II   Basal (B) and maximum (M) response to glucagon
Values are mean and SEM
p values are differences between B and M
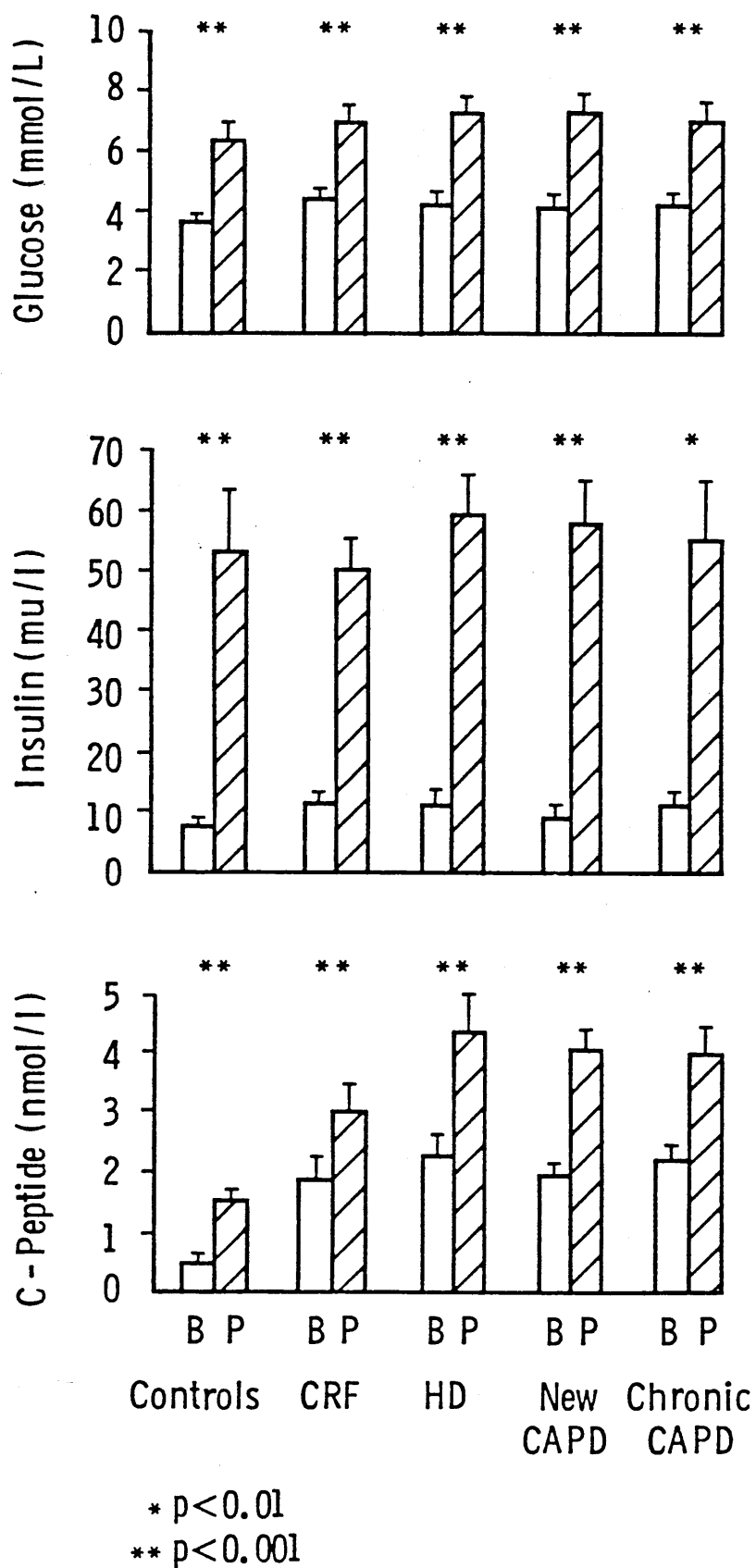
Figure 6. 2 Basal (B) and Peak (P) responses to glucagon
Values are mean and SEM
p values are differences between B and P

between basal and peak was significant (p<0.001) in all groups.

Fasting serum insulin was higher (9.5 to 11.7 mu/l) in the uraemic

groups but this was not statistically significant. There was also no

difference between the uraemic groups maximum response (49.9 to 59.2

mu/l) and the controls (53.4 mu/l). The increment between fasting and

maximum responses was highly significant in all groups. Fasting

plasma c-peptide concentrations were significantly increased in all

the uraemic groups (1.84 to 2.38 nmol/l) compared to controls (0.48

nmol/l) (p<0.001). There was a 4-5 fold rise in the uraemic groups.

Similarly peak c-peptide concentrations were significantly increased

(p<0.001). Peak c-peptide concentrations in the uraemic groups were

2-3 times higher than controls. There was no difference between any

of the uraemic groups in either basal or peak concentrations. The

increment from basal to peak stimulation by glucagon was highly

significant in all groups (p<0.001). This increment was between 163%

and 208% in the uraemic groups compared to 314% in the controls.

b) Glucagon-response curves

The glucose response to glucagon over 60 minutes, Figure 6.3

(values are mean and SEM), showed that the four uraemic groups were

different from the controls ($F_{7,301}$=14.74, p<0.001). The rate of rise

in the early phase (0-15 minutes) was similar in all groups, but the

uraemic groups continued to rise for 30 minutes and had a prolonged

elevation and delayed fall in glucose concentration. Repeated

measures analysis of variance showed no difference between any of the

uraemic groups.

The insulin response to glucagon, Figure 6.4, revealed significant

differences between the 5 groups ($F_{28,301}$=3.17, p<0.001). However,

this was due to the difference between controls and the 4 uraemic
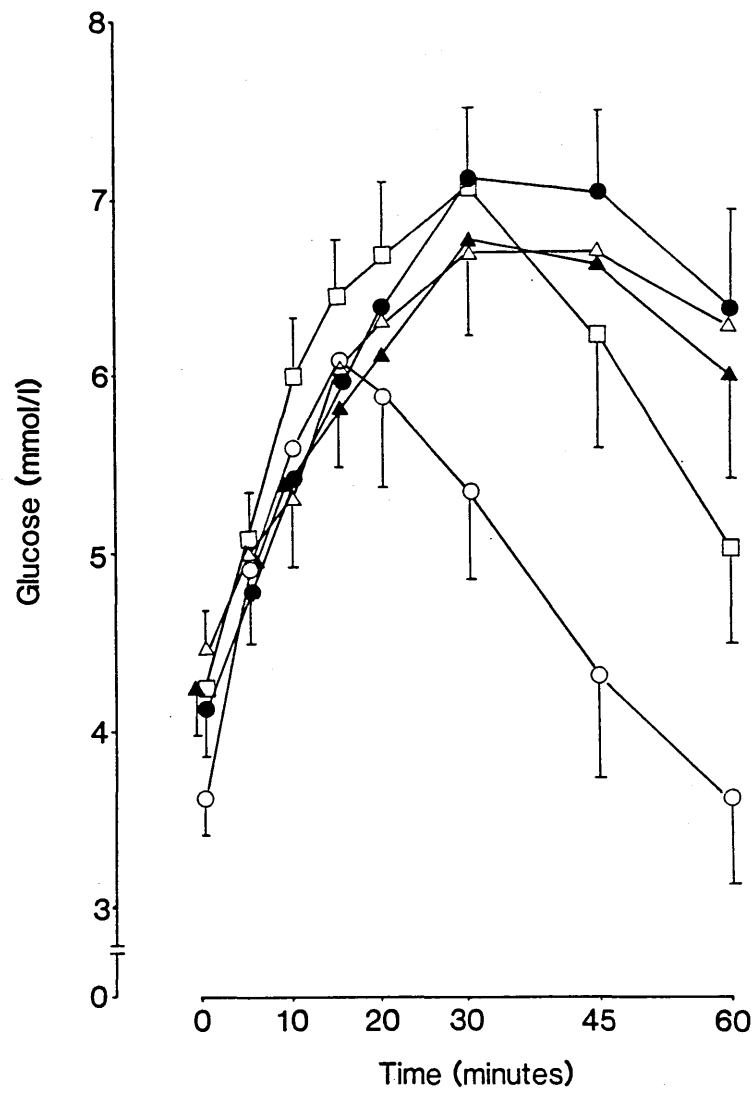
Figure 6. 3   GLUCOSE - TIME RESPONSE TO GLUCAGON

(○) CONTROL, (△) CRF, (□) HD, (●) NEW CAPD, (▲) CHRONIC CAPD

Points are mean and SEM
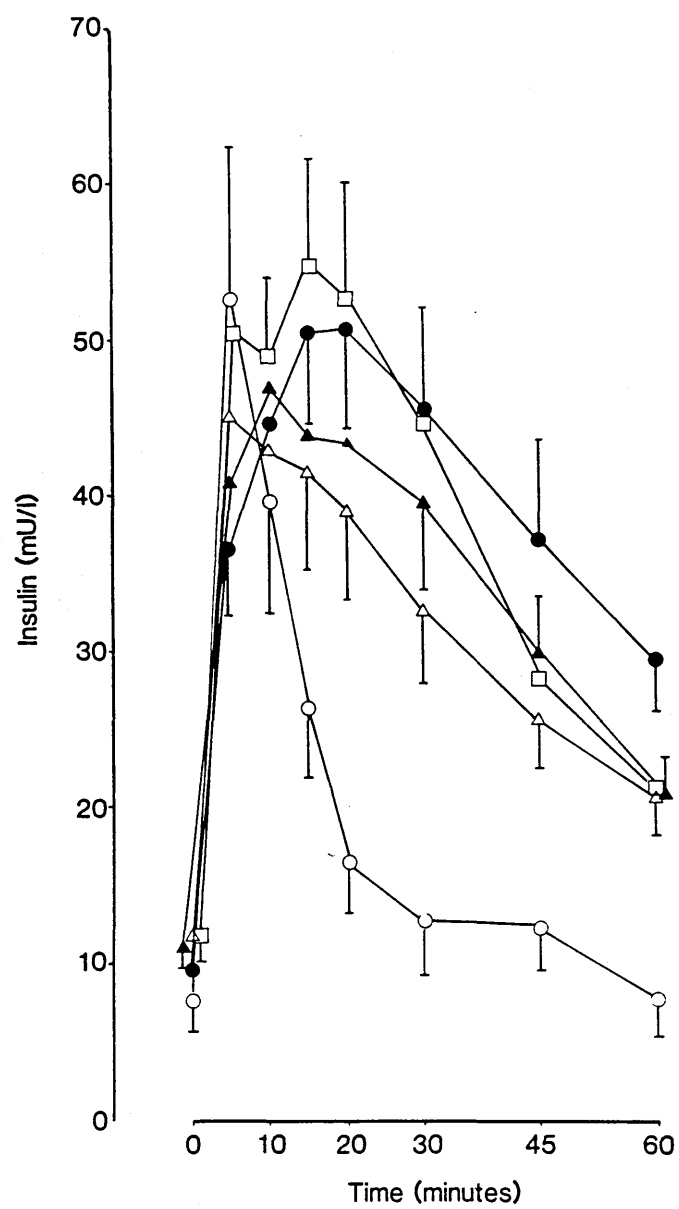
Figure 6. 4   INSULIN - TIME RESPONSE TO GLUCAGON

(○) CONTROLS, (△) CRF, (□) HD, (●) NEW CAPD, (▲) CHRONIC CAPD

Points are mean and SEM
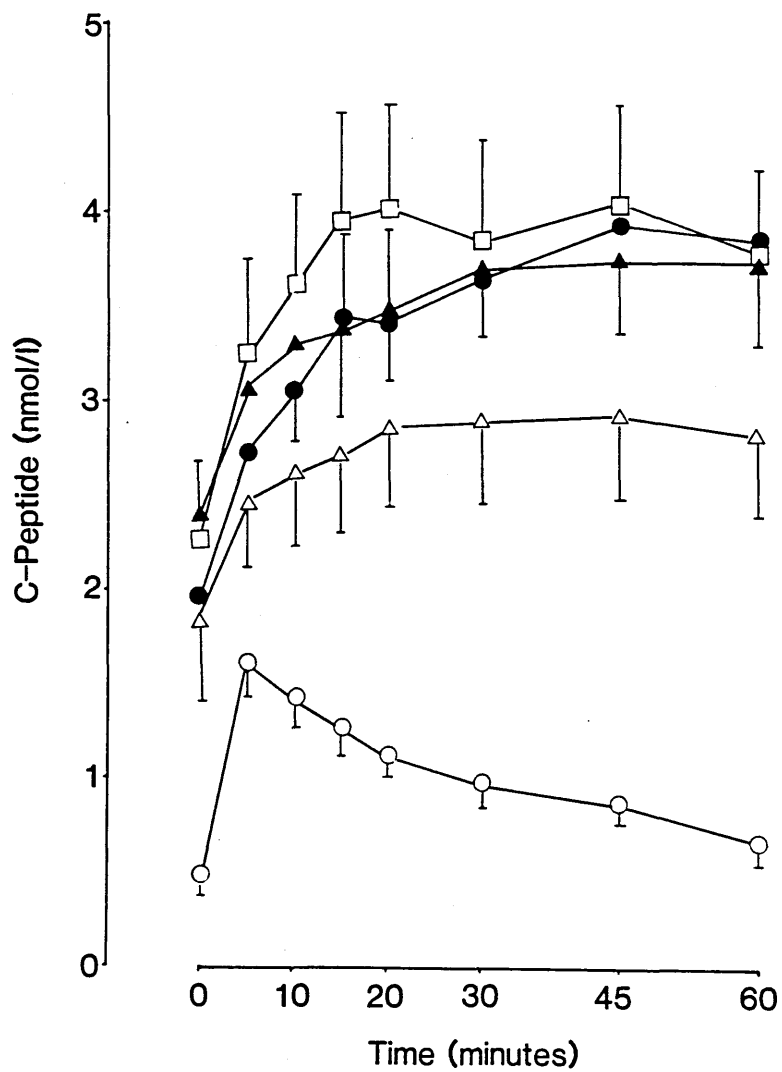
Figure 6. 5   C-PEPTIDE - TIME RESPONSE TO GLUCAGON

(○) CONTROL, (△) CRF, (□) HD, (●) NEW CAPD, (▲) CHRONIC CAPD

Points are mean and SEM

groups ($F_{7,301}$=8.78, p<0.001). There was no difference between the uraemic groups, all of whom showed a similar rise and delayed fall in serum insulin concentration.

The c-peptide response to glucagon, Figure 6.5, showed significant differences between the groups ($F_{28,301}$=4.90, p<0.001). This was mainly due to differences between the controls and the four uraemic groups ($F_{7,301}$=13.6, p<0.001), but there was a small difference between the CRF group and the 3 dialysis groups ($F_{7,301}$=2.04, p<0.05). The control group had a prompt rise reaching a peak at 5 minutes followed by a progressive fall over the remaining hour. The uraemic groups had a supranormal basal concentration, which increased within 20 minutes of stimulation and then had a plateau effect over the remainder of the test period.

c) <u>Time to maximum response</u>

The time course of glucose, insulin and c-peptide from basal to maximum response was prolonged in all uraemic groups. The mean time in minutes, for individuals to reach their maximum response is shown in Table 6.III (values are mean and SD). Glucose peaked significantly earlier in controls (p<0.001). The uraemic groups had similar time courses. The insulin response peaked at 6 minutes in controls which was significantly shorter (p<0.001) than the uraemic groups. There was no difference between any of the uraemic groups. C-peptide also peaked at 6 minutes in controls which was markedly shorter than the uraemic groups (p<0.001). Thus, all uraemic groups had a protracted time course in response to glucagon. It should be noted that there was marked variation in the time to reach maximum response within each uraemic group. The timing of the absolute maximum response in individual patients was occasionally difficult to assess as many had

small increments over a period of time before their absolute maximum. This is clearly seen in Figures 6.3, 6.4 and 6.5. This probably accounts for the small difference ($p<0.05$) in time course between the HD group and the other uraemic groups with respect to the glucose and c-peptide responses.

d) Molar ratio of c-peptide/insulin

The molar ratios of c-peptide to insulin, calculated from 1 mu/l insulin equals 0.00689 nmol/l, are shown in Table 6.IV (values are mean and SD). The elevated basal ratios in the uraemic groups reflected the high c-peptide concentration. The maximum response after glucagon stimulation resulted in a decrease in the ratio due to a relatively greater increase in insulin. All uraemic groups had higher basal ($p<0.001$) and maximum response ($0.1<p<0.05$) ratios compared to controls. The fasting molar ratio was increased 2-3 fold in uraemics and the stimulated ratio was increased by about 2-fold compared to controls. However, all groups showed a significant fall in the c-peptide to insulin ratio with beta cell stimulation. There was no difference between any of the uraemic groups.

Fasting serum triglycerides showed a strong positive correlation with fasting serum insulin in controls ($r=0.923$, $p<0.01$) but no significant correlation was found in uraemic subjects. Fasting plasma c-peptide showed a positive correlation with serum creatinine in the CRF group ($r=0.800$, $p<0.01$) but no correlation was found in the control or any of the dialysis groups. Fasting c-peptide showed a positive correlation with fasting serum insulin in the control ($r=0.795$, $p<0.01$), CRF ($r=0.861$, $p<0.01$) and HD ($r=0.710$, $p<0.05$) groups but no significant association was found in either of the CAPD groups.

Table 6. III Time to maximum response after glucagon stimulation
Values (minutes) are mean and SD

	CONTROL	CRF	HD	NEW CAPD	CHRONIC CAPD
GLUCOSE	16 ± 4	37 ± 13	26 ± 9	37 ± 9	36 ± 8
INSULIN	6 ± 2	14 ± 14	12 ± 6	13 ± 9	16 ± 11
C-PEPTIDE	6 ± 2	39 ± 13	26 ± 19	44 ± 11	41 ± 16

Table 6. IV MOLAR RATIO OF C-PEPTIDE/INSULIN IN RESPONSE TO GLUCAGON

	CONTROL	CRF	HD	NEW CAPD	CHRONIC CAPD
BASAL	11.0 ± 4.7	22.6 ± 8.3	28.2 ± 9.8	32.5 ± 14.1	32.1 ± 16.2
MAXIMUM	5.2 ± 2.9	9.1 ± 3.3	10.7 ± 3.4	12.4 ± 5.8	12.6 ± 6.6
p	$\approx$0.01	<0.001	<0.001	<0.001	<0.001

p values represent differences between basal and maximum molar ratios. The molar ratio c-peptide/insulin was calculated considering 1 mU/1 insulin equal to 0.00689 nmol/1. Values are mean and SD.

2. DIABETICS

Diabetics were classified by renal status and diabetic type. When classified by renal status the diabetic patients were grouped as normal renal function (NRF), CRF, HD and CAPD (Table 6.V). When classified by diabetic type the diabetic groups consisted of NRF type I (insulin dependent), uraemic type I and uraemic type II (non-insulin dependent) (Table 6.VI).

a) Clinical data

When patients were classified by renal status (Table 6.V, values are mean and SD), subjects were of comparable age and weight. Serum creatinine in the three uraemic groups was also similar although there was more variation in the CRF group. The mean time on dialysis was 2.5 times longer in the haemodialysis group than the CAPD group but there was marked variation between patients in both groups. Fasting triglycerides were increased in all diabetic subjects compared to healthy controls. There was no difference in triglyceride concentrations between the diabetic groups. Glycosylated haemoglobin was raised in all groups compared to healthy controls (37.1 ± 2.7 mmol HMF/molHb), but there was no difference between diabetic groups.

When the same patients were reclassified by diabetic type (Table 6.VI, values are mean and SD) it was seen that there were more type I uraemic diabetics than type II. It was also observed that all CRF diabetics were type I as were most of the CAPD group. Type II diabetics were older but there was marked variation in age in type I diabetics. Serum creatinine did not differ between the uraemic groups. Serum triglycerides were higher and glycosylated haemoglobin (although increased compared to controls) lower in the type II group.

	NRF	CRF	HD	CAPD
Subjects	4	5	5	5
Age	49 ± 17	44 ± 11	49 ± 13	46 ± 11
Weight (Kg)	75.5 ± 17.5	65.8 ± 6.0	70.6 ± 8.5	66.6 ± 12.1
Duration of Dialysis (months)	–	–	53 ± 40	21 ± 20
Creatinine (µmol/1)	116 ± 12	618 ± 216	846 ± 168	728 ± 143
Triglycerides (mmol/1)	2.0 ± 0.7	2.5 ± 1.1	3.0 ± 0.8	2.3 ± 0.7
Glycosylated Haemoglobin (mmol HMF/mol Hb)	56 ± 8	55.0 ± 7	46 ± 7	51 ± 10

Table 6. V

Clinical Data: Diabetics classified by renal status
Values are mean and SD

DIABETICS: DIABETIC TYPE

	NRF Type I	Uraemic Type I	Uraemic Type II
Subjects	4	11	4
CRF	–	5	–
HD	–	2	3
CAPD	–	4	1
Age	49 ± 17	43 ± 1	55 ± 6
Weight (Kg)	75.5 ± 17.5	64.8 ± 8.3	74.0 ± 7.5
Creatinine (µmol/1)	116 ± 12	712 ± 210	782 ± 134
Triglycerides (mmol/1)	2.0 ± 0.7	2.41 ± 1.0	3.1 ± .3
Glycosylated Haemoglobin (mmol HMF/mol Hb)	56 ± 8	54 ± 7.0	41 ± 3

Table 6. VI

Clinical Data: Diabetics classified by diabetic type
Values are mean and SD

b) <u>Basal and peak responses to glucagon</u>

Basal and peak responses to glucagon are shown in Figures 6.6 and 6.7 (values are mean and SEM).

When diabetics were classified by renal status (Figure 6.6) the glucose response to glucagon showed a significant increase in hepatic released glucose in all diabetics ($p<0.05$). There was no difference between the groups both in basal or peak concentrations but the increment (40-61%) from basal to peak (mean 4.0 mmol/1) was statistically significant. Basal serum free insulin (unbound) was lower in the CAPD diabetics than the other groups but this is not statistically significant. Peak free insulin also showed much variability between the groups, but again no significant difference was found. The increment from basal to peak was not significant in any of the groups ($p>0.05$), although the HD group had a greater rise in free insulin. Plasma c-peptide concentrations were almost undetectable in the NRF and CRF groups and did not change with glucagon stimulation. The HD group had a relatively high basal c-peptide concentration and the CAPD group had basal levels within the normal range (0.18-0.52 nmol/1). After glucagon stimulation neither the HD nor CAPD groups showed a change in c-peptide concentration.

When diabetics were reclassified by diabetic type (Figure 6.7) basal glucose was similar in all groups. Peak response was also similar in the three groups but the increment (about 50%) from basal to peak (mean 4.2 mmol/1) was significant. Basal free insulin was similar in all groups but peak free insulin in the type II uraemic group was higher than in both type I groups, although the difference was not significant. The change from basal to peak was not significant in any of the groups. Basal and peak c-peptide were negligible in both type I groups. Type II uraemic diabetics had both
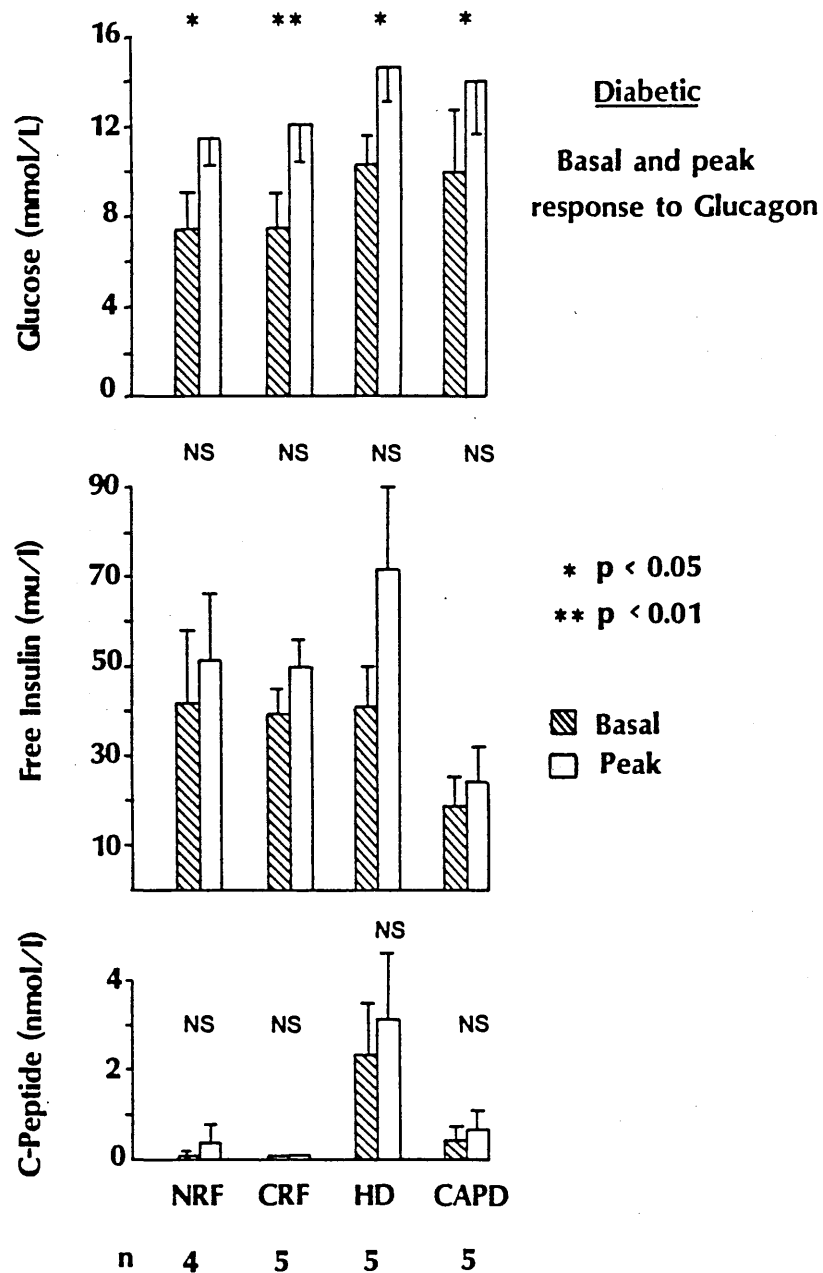
Figure 6. 6   Basal and peak responses to glucagon in diabetics
classified by renal status
Values are mean and SEM

DIABETIC: BASAL AND PEAK RESPONSE TO GLUCAGON

* P < 0.05
** P < 0.01

Basal

Peak

NRF = Normal renal function
UD = Uraemic diabetic
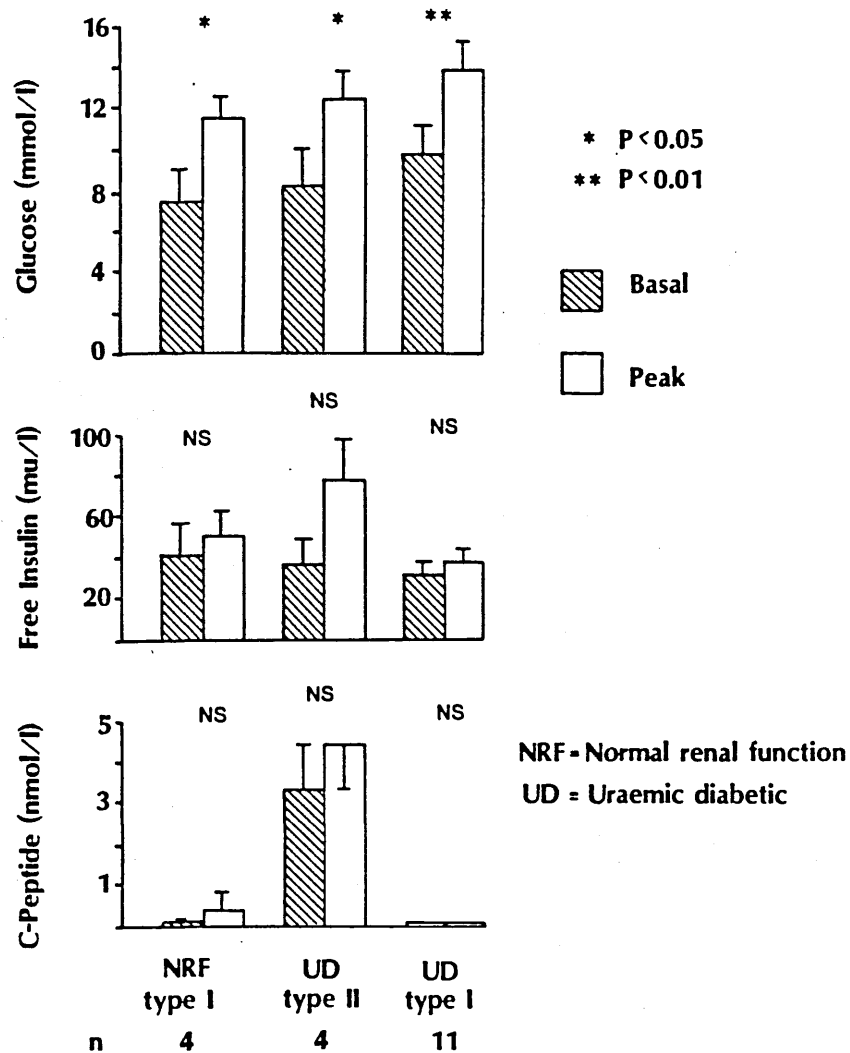
Figure 6. 7   Basal and peak responses to glucagon in diabetics
classified by diabetic type
Values are mean and SEM

increased basal and peak concentrations (p<0.05) compared to the other

diabetic groups, but this did not change with glucagon stimulation.

Thus all diabetics independent of diabetic type and renal status

had a positive glucose response to glucagon. None of the diabetic

groups showed a beta cell response as neither free insulin nor

c-peptide concentrations changed with glucagon stimulation. The high

c-peptide concentrations in the HD group corresponded to the type II

diabetics in this group.

c) Glucagon response curves

Diabetics classified by renal status (Figure 6.8, values are mean

and SEM), showed a small but steady rise in glucose in the first 20

minutes following glucagon and thereafter glucose levels remained

relatively stable. There was much variability within each group (for

clarity some of the error bars have been omitted) and overall there

was no difference between any of the groups.

The free insulin time response showed that the HD group had a

slight but not significant rise in response to glucagon and

concentrations throughout remained higher than in the CAPD group.

Diabetics with NRF, CRF and CAPD showed no response to glucagon and

free insulin levels remained unchanged. Absolute concentrations of

free insulin in the NRF and CRF groups were intermediate between the

HD and CAPD groups. Statistically there was a small difference

between the HD and CAPD groups (p<0.05).

Plasma c-peptide concentrations were low to negligible in the NRF,

CRF and CAPD groups, but were elevated in the HD group (p<0.05). No

change occurred in the curve patterns with time in any group, i.e.

there was no positive beta cell response.

When the same patients were reclassified by diabetic type (Figure

DIABETIC RESPONSE TO GLUCAGON
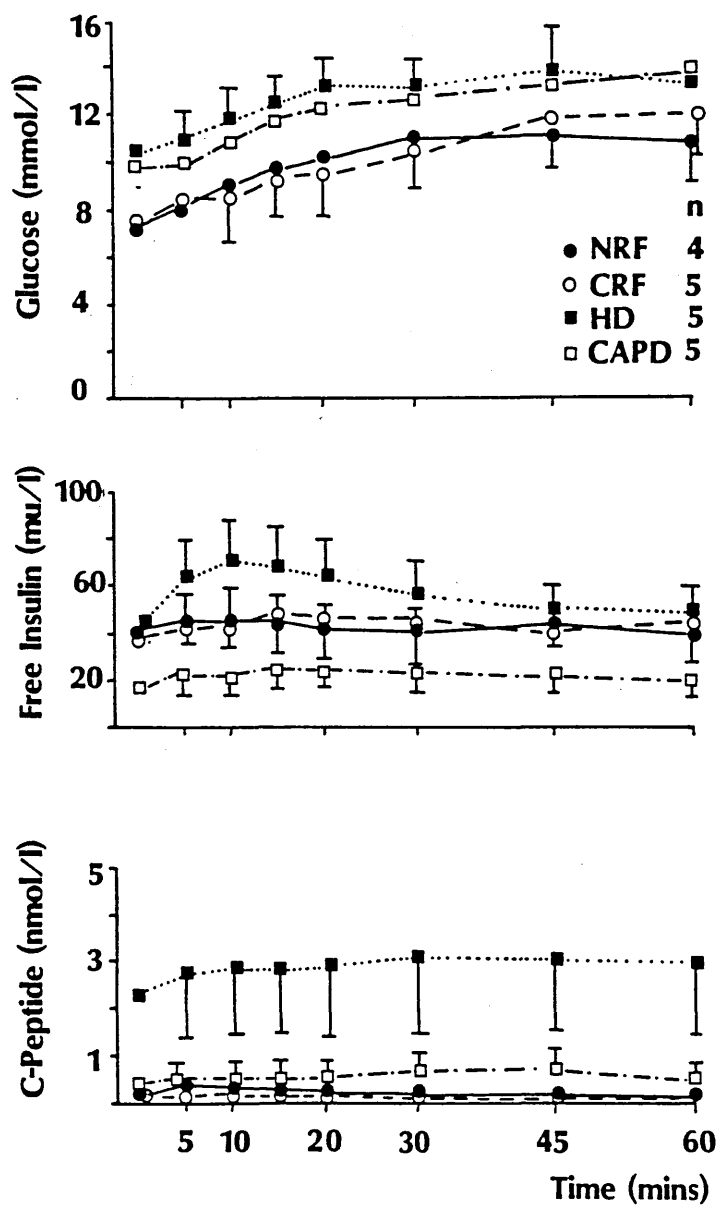
Figure 6. 8   Time – response to glucagon stimulation in
             diabetics classified by renal status
             Values are mean and SEM
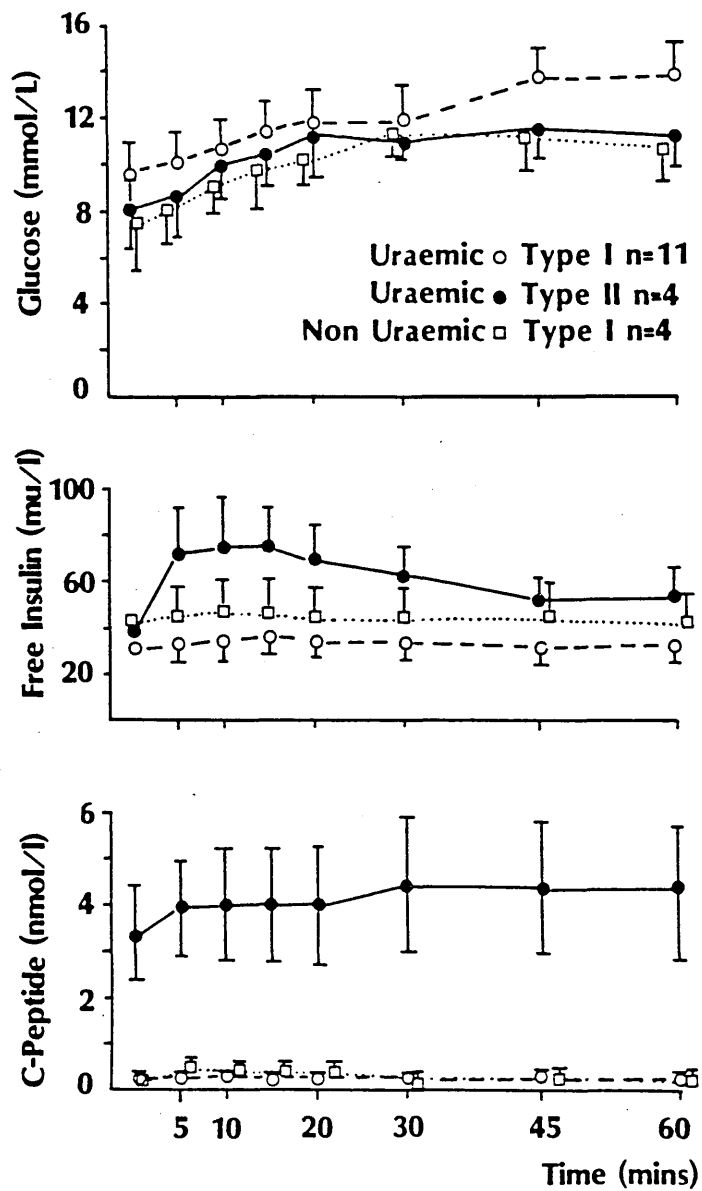
Figure 6. 9   Time – response to glucagon stimulation
in diabetics classified by diabetic type
Values are mean and SEM

6.9, values are mean and SEM), the glucose response showed a slow steady rise with time but there was no difference between any of the groups.

The free insulin time response was similar in both uraemic and non uraemic type I diabetic groups, neither or which responded to glucagon stimulation. Type II uraemic diabetics had a small but not statistically significant rise in free insulin after glucagon. Although the type II group had a higher free insulin level in the first 20 minutes following beta cell stimulation this was not significantly different from the other groups.

C-peptide was negligible in both type I groups and no change occurred with glucagon. C-peptide concentrations were elevated in type II diabetics ($p < 0.05$) but no change occurred with glucagon.

Thus all diabetics independent of renal status or diabetic type had a slow but steady increase in glucose especially within 20 minutes of glucagon administration. Although there was variability in the free insulin concentrations the small rise following glucagon in the HD group could be accounted for by the presence of type II diabetics in that group. Likewise, the elevated c-peptide concentrations found in the HD group largely corresponded with the type II diabetics. However, neither free insulin nor c-peptide increased in any group following glucagon, thus there was no positive beta cell response.

DISCUSSION

1. NON-DIABETICS

In this study CAPD was temporarily discontinued 12 hours before the glucagon challenge to achieve a 'peritoneal' as well as on oral fast, for comparison with the other groups. Haemodialysis patients were studied on their interdialysis day, that is 24 hours after their last dialysis, which represented a state midway between the two extremes of pre and post dialysis. These conditions were applied in an attempt to achieve comparable basal states. Steroids, which can decrease hepatic and peripheral tissue sensitivity to insulin and induce hyperglycaemia (Pagano et al., 1983; Rizza et al., 1982), and beta blockers, which can impair the insulin response in uraemia (Pun et al., 1985), were part of the exclusion criteria of the study. A minority of patients (4 out of 10) in the chronic renal failure group had been taking frusemide, but there is no direct evidence that loop diuretics unlike thiazides can cause deterioration in glucose tolerance (Taylor, 1986).

The glucose, insulin and c-peptide responses to glucagon were abnormal in uraemics compared to controls. These were manifested by a prolonged elevation and a delayed fall in glucose and insulin, as well as an increased fasting and persistent elevation of c-peptide. These features reflect glucose intolerance induced by uraemia.

Normal fasting blood glucose was found in all subjects. This is a common finding in uraemia despite the presence of glucose intolerance (DeFronzo et al., 1973), in contrast, to maturity onset diabetes where fasting hyperglycaemia and often a normal fasting insulin are found (Turner and Holman, 1978).

Although fasting serum insulin was slightly increased in the uraemic groups this was not statistically significant. Data on the

fasting insulin state in uraemia are conflicting. Normal fasting

insulin concentrations have also been reported by many (Spitz et al.,

1970; Roth et al., 1973; DeFronzo, 1978b). Spitz et al. (1970) have

also reported a group of uraemics with increased basal levels.

Several reports, particularly in the late 1960's (Hutchings et al.,

1966; Briggs et al., 1967; Horton et al., 1968) found basal

hyperinsulinaemia in patients with chronic renal failure some of whom

were on haemodialysis. More recently, decreased fasting insulin

concentrations were found in CAPD patients (Von Baeyer et al., 1983).

The reason for the discrepancies is not clear and is probably

multifactorial.

Studies in the late 1960's used assay techniques which by today's

standards were crude and less specific. Both insulin standards and

antibody preparation were less pure and specific and probably much

cross-reactivity with proinsulin occurred. This may have resulted in

over-estimation of serum insulin. The relatively more sensitive and

specific assay used in this study (detailed in Chapter 5) was a double

antibody radioimmunoassay with minimal cross-reactivity with

proinsulin. It has been suggested (DeFronzo, 1978b) that two

categories of uraemic patients may exist, some having normal basal

insulin (possibly reflecting impaired beta cell function) and others

having increased basal insulin (in an attempt to overcome peripheral

insulin resistance). In this study 8 or the 40 uraemic patients had

basal levels above the laboratory normal reference range (2.5-13.5

mu/ml). However, the majority of these had only marginally elevated

concentrations. Even allowing for the heterogeneity of the uraemic

population there was no difference between any of the groups.

Fasting plasma c-peptide concentrations were increased in all

uraemic patients. This confirms the findings of others (Jaspan et

al., 1977) that renal failure impairs the catabolism of c-peptide. The elevated c-peptide basal concentrations reflect impaired renal metabolism rather than basal hypersecretion of the beta cell and this in keeping with the essentially normal basal insulin concentrations in all the uraemic groups. This has important clinical implications as elevated c-peptide in uraemia may mistakenly be interpreted as increased beta cell secretory activity. Similarly, the occurrence of hypoglycaemia in renal failure in association with elevated c-peptide might erroneously suggest the presence of endogenous hyperinsulinaemia due to an insulinoma. Increased c-peptide concentration may also be due to the presence of proinsulin. The antibody to c-peptide used in the radioimmunoassay (Novo Biolab M1230) had a 10% cross-reactivity, on a molar basis, with proinsulin. Proinsulin is increased in uraemia, largely due to impaired renal degradation (Katz et al., 1973) and, therefore, may have contributed in a small way to the increase in c-peptide.

The time dependent glucose response to glucagon showed prolonged elevation with a delayed rate of decline in all uraemic patients although the absolute maximum responses were similar to healthy subjects. This is typical of the glucose intolerance induced by uraemia and was seen in both non-dialysis and dialysis patients. The abnormal time dependent changes may be explained by several mechanisms. Increased hepatic sensitivity to glucagon, which is documented in uraemia (Sherwin et al., 1976), could account for the prolonged hepatic glucose production and the impaired degradation of glucagon in uraemia (Lefebvre et al., 1975) could also explain the prolonged glucose response. However, peripheral resistance to the action of insulin causing impaired peripheral glucose utilization (DeFronzo, 1973; 1978b) may also induce the prolonged hyperglycaemia.

The insulin response to glucagon was similar in all uraemic groups but differed from the controls. Although the absolute maximum response was similar in all subjects a slower rate of rise, prolonged elevation and delayed fall in insulin was found in uraemic patients. Several mechanisms could be responsible for this type of response. Impaired beta cell secretion might account for the slower rate of rise, but the similar maximum responses in all groups suggests that beta cell secretion was adequate. The prolonged hyperinsulinaemia could be due to tissue insensitivity to insulin (peripheral insulin resistance) with relative secondary hypersecretion of the beta cell (DeFronzo, 1978b). The prolonged elevation and delayed decline could also be explained by delayed renal degradation of insulin (Rubenstein et al., 1975; Rabkin et al., 1984).

The c-peptide response to glucagon differed markedly in uraemic patients compared to normal subjects. The increased basal concentrations most likely due to impaired renal catabolism have been discussed. However, all uraemic groups despite their elevated basal concentrations showed a significant increase in response to glucagon. The metabolic clearance rate of c-peptide is much slower than that of insulin, thus differences in hepatic extraction and peripheral kinetics (Faber et al., 1978) explain the difference in rate of decline between insulin and c-peptide in all groups. The slower rate of rise followed by persistent elevation (plateau effect) can be explained by the impaired renal clearance of c-peptide (Jaspan et al., 1977; Faber et al., 1978). The small difference between the non-dialysis (some residual renal function) and the 3 dialysis groups reflects the quantitative role the kidney has in c-peptide removal.

The mean time to reach the maximum response was 6 minutes for both insulin and c-peptide in normal subjects and confirms the findings of

others (Faber and Binder, 1977). The time course for all uraemic subjects was prolonged in keeping with glucose intolerance.

The molar ratio of c-peptide to insulin is an index of beta cell function. The basal ratio in normals was similar to that found by Jaspan et al. (1977) and Heding (1975). Following stimulation the ratio fell, due to a relatively greater increase in insulin because of its lower basal concentration, and was comparable with data of Heding (1975). In uraemia the increased basal ratio was due to the high basal c-peptide concentration. The basal ratios in this study were comparable to that found by Jaspan et al. (1977), who studied a group of haemodialysis patients. Following stimulation, although both insulin and c-peptide increased there was a fall in the ratio as there was a relatively greater increase in insulin. The CRF non-dialysis group had marginally lower basal and maximum molar ratios than the dialysis groups due to lower c-peptide concentrations which reflected a degree of residual renal function.

Fasting plasma c-peptide strongly correlated with fasting serum insulin in controls, CRF and haemodialysis subjects but the two variables did not correlate in either CAPD group. The reason for this is not clear but possibly peritoneal clearance of c-peptide (Wideroe et al., 1984) may play a role, although absolute concentrations of c-peptide and insulin in both CAPD groups were similar to the other uraemic groups. Plasma c-peptide showed a strong positive correlation with serum creatinine in the non-dialysis chronic renal failure group, but no linear relationship was found in the dialysis groups. This may be explained by the differential clearances of both molecules by dialysis.

Serum lipoproteins were abnormal in all uraemic groups. The characteristic pattern of hypertriglyceridaemia, increased total

cholesterol and low HDL-cholesterol was found in agreement with numerous previous studies (Kannel et al., 1979; Norbeck et al., 1980). However, as previously documented, there was much interpatient variability in lipoprotein concentrations.

This study confirms that glucose, insulin and c-peptide responses to glucagon stimulation in renal failure are different from controls and that the abnormalities are due to uraemia. The significant rise in beta cell peptides to glucagon confirms adequate pancreatic reserve. There were no differences between the non-dialysis, haemodialysis or CAPD patients. Thus, there is no evidence that CAPD therapy per se has any adverse effect on beta cell function.

2. DIABETICS

The limited number of diabetics on dialysis restricted the study and this has to be taken into account when interpreting the results. Diabetics were classified by renal status to determine if mode of treatment affected beta cell function and by diabetic type which clearly has a profound effect on the beta cell integrity.

The majority of diabetics studied were type I. All of the CRF group and most of the CAPD group were insulin dependent, of variable duration, but generally had diabetes for at least 15 years. Type II diabetics were in the minority but accounted for three-fifths of the HD group. The duration of type II diabetes was difficult to assess with any accuracy but was at least 10 years in all patients.

Hypertriglyceridaemia was found in all diabetic patients and this was most marked in the older type II patients. This association between hyperlipidaemia and diabetes is well established (Steiner, 1981; Gibbons, 1986).

All diabetic patients independent of diabetic type or renal status

had an increase in blood glucose in response to glucagon. This is in agreement with others (Faber and Binder, 1977), who found an increase in hepatic glucose release to glucagon in diabetics. Diabetics had persistent hyperglycaemia throughout the 1 hour test suggesting no effective counterregulatory insulin response.

Serum free (unbound) insulin was measured by PEG precipitation after separation of serum and prior to storage which improves the accuracy of the assay (see Chapter 5). However, immediate precipitation after venesection has been shown to further improve the sensitivity and precision of the method (Hanning et al., 1985). There was considerable variation in free insulin concentrations, particularly between type I and II diabetics, the latter having much less or negligible antibody bound insulin. However, there appears to be no definite correlation between total insulin and free insulin concentrations in diabetics (Nakagawa et al., 1973). Thus a direct comparison between total insulin concentrations in non-diabetics and free insulin concentrations in diabetics is not possible. All diabetics had similar basal free insulin concentrations which did not significantly change after glucagon stimulation, indicating no significant beta cell reserve. The time dependent changes essentially showed no response, but in the type II group (predominantly HD patients) there was a suggestion of an increase in free insulin although this was not significant. This has to be interpreted with caution in view of the small numbers studied. The absolute concentrations of free insulin were higher than found by Nakagawa and colleagues (1973). The reason for this is not entirely clear. Several explanations may account for the differences. The radioimmunoassay may have been more sensitive, the presence of uraemia in the majority of patients may have contributed to elevated insulin

by inducing peripheral insensitivity to insulin as well as impaired renal degradation. Furthermore, type II diabetics may have relatively 'normal' or even 'high' circulating insulin concentrations due to defects in insulin receptor binding. This has been reviewed by Bailey et al. (1984).

Fasting plasma c-peptide was just detectable in the type I diabetics confirming negligible beta cell function which did not change with glucagon stimulation. However, in the type II diabetics elevated basal c-peptide was observed but again there was little change following glucagon. Thus, it is evident that the circulating c-peptide levels were high largely due to the presence of uraemia rather than significant residual beta cell function. The time dependent changes confirm no effective residual beta cell function. The high c-peptide concentrations in the type II diabetics obviously reflect some degree of beta cell secretion, perhaps even relative hypersecretion as a secondary compensatory mechanism, but as no change occurred with glucagon stimulation the main mechanism behind the elevated levels must be the presence of uraemia. Elevated proinsulin in uraemia, which has some cross-reactivity with c-peptide estimations, may also have contributed to the increase.

Type I, insulin dependent diabetics generally have negligible beta cell function, particularly after 10-20 years, when end stage renal disease is reached and therefore it is not unexpected that no response to glucagon occurred. Type II, non-insulin dependent diabetics present a more complex problem. Defects in both insulin secretion and insulin action have been demonstrated (Reaven, 1984; Truglia et al., 1985; O'Rahilly et al., 1986). This is further complicated by the effect of uraemia (discussed in detail in Chapter 2). From this study the effects of diabetes predominate over the uraemic effects and it is

not possible to conclude what effect if any, dialysis per se has on beta cell function in diabetics. The small though insignificant rise in insulin in the HD group reflects the presence of type II diabetics rather than any specific effect of haemodialysis.

The important points derived from this study are that c-peptide and possibly free insulin concentrations may be altered by the presence of uraemia. The interpretation of beta cell function by random c-peptide measurements may falsely imply adequate reserve. The use of c-peptide determination as a means of assessing deficiency of endogenous insulin secretory capacity and to categorise type II diabetics into those requiring diet, oral drugs or even insulin therapy has been widely studied (Madsbad et al., 1981; Rendell, 1983). However, the presence of renal failure will increase the c-peptide concentration and thus limit the potential of this test.

The use of recent techniques such as the hyperglycaemic and euglycaemic clamp (DeFronzo et al., 1978a) and continuous infusion of glucose with mathematical model assessment (O'Rahilly et al., 1986) may in the future elucidate residual beta cell function in diabetics with uraemia.

SUMMARY

Pancreatic beta cell function was evaluated in uraemic patients by measuring the beta cell peptides in the peripheral blood after glucagon stimulation. Non-diabetic subjects in chronic renal failure, on haemodialysis, new to and established on CAPD were studied. Fasting glucose and insulin concentrations were normal and did not differ between the uraemic groups, but c-peptide concentrations were markedly increased. Following glucagon stimulation an exaggerated blood glucose response with delayed glucose peak was observed, while the peak insulin response was normal but the return to basal concentrations was delayed in uraemia. The c-peptide response was also exaggerated and peak concentrations in uraemic subjects were greatly increased. The possible underlying mechanisms are discussed. Glucose intolerance persisted despite dialysis therapy confirming the abnormalities were due to the underlying uraemia. However, the glucagon test showed significant increments in beta cell peptides reflecting adequate residual beta cell function. The response was similar in both new and established CAPD patients to those on haemodialysis and non-dialysed chronic renal failure patients. Thus, it can be concluded that despite continuous intraperitoneal glucose CAPD per se does not adversely affect beta cell function.

Glucagon stimulation in diabetics was assessed by classifying patients by diabetic type and mode of renal therapy. As expected, type I diabetics had no beta cell response, although glucagon caused a rise in glucose. Type II diabetics showed hepatic glucose release and a very small beta cell response. However, the absolute concentrations of c-peptide in uraemic diabetics can be misleading as concentrations are elevated due to impaired renal degradation. This dynamic test of residual beta cell function confirmed no significant reserve in any of the diabetic subjects. The effect of diabetes predominated over any possible uraemic effect and dialysis per se had no detectable effect on beta cell function.

CHAPTER 7

GLUCOREGULATORY HORMONES AND INTERMEDIARY METABOLITES IN CAPD

INTRODUCTION

The continuous peritoneal glucose absorption that occurs in CAPD could conceivably affect glucoregulatory hormone homeostasis and intermediary metabolism, but to date this has not been adequately investigated. The normal physiological and biochemical changes in the post-absorptive state (after an overnight fast) induce a tendency to stimulate gluconeogenesis, lipolysis and ketogenesis. After a meal or oral glucose load increased glycolysis and inhibition of gluconeogenesis, lipolysis and ketogenesis occurs. This has been discussed in Chapter 1.

The hyperglycaemia and hyperinsulinaemia in CAPD have been documented (Heaton et al., 1983; Armstrong et al., 1985), but only one group has studied the changes in intermediary metabolism (Heaton et al., 1983). In a small group of patients Heaton et al. showed elevation of gluconeogenic precursors and decreased ketone bodies during CAPD. No other reports have been published.

Furthermore, although high and low dextrose dialysis solutions may affect hormone and metabolite homeostasis, other factors such as age, diabetes mellitus and peritonitis may also have additional consequences. CAPD patients are frequently elderly or diabetic and peritonitis is the commonest complication of this mode of treatment.

1. Age

Disposal of an oral or intravenous glucose load is impaired with aging. This is probably multifactorial and factors such as reduced diet, physical inactivity and decreased lean body mass may all play a role. Pancreatic beta cell function appears to be normal but evidence of insulin antagonism is strong though not conclusive (Davidson, 1979). Peripheral insensitivity to insulin has been suggested as the

mechanism of glucose intolerance in the elderly (DeFronzo et al., 1978a) and probably due to a post-receptor or intracellular defect rather than a receptor defect (Jackson et al., 1982). A similar mechanism has been suggested for the glucose intolerance seen in uraemia (Smith et al., 1982; Pedersen et al., 1985) and is discussed in Chapter 2. CAPD has been a particularly suitable treatment for the elderly (Nicholls et al., 1984), and at least one third of CAPD patients are over 60 years of age (Nolph et al., 1985). Hence the importance of assessing the effect of CAPD on glucoregulatory hormones and intermediary metabolites in the elderly. To date no studies have been reported.

2. Diabetes

The effect of diabetes on gluconeogenesis and ketogenesis is well documented and is discussed in Chapter 1. Diabetics have deranged glucoregulatory hormones and under certain conditions, particularly infection and poor blood glucose control, can have disturbances of intermediary metabolism, such as lactic acidosis and increased ketogenesis. Furthermore, diabetics with end stage renal failure are frequently treated by CAPD (Flynn et al., 1983; Khanna et al., 1986). Indeed in the U.K. CAPD is the preferred mode of dialysis for diabetics (Cameron and Challah, 1986). Thus it is relevant to investigate the hormonal and intermediary metabolic effects of intraperitoneal glucose absorption, particularly as no studies have been previously reported.

3. Peritonitis

Peritonitis is by far the commonest complication of CAPD. Single centre studies show an incidence of about 1.7 episode of peritonitis

per year (Smith et al., 1986) and large multicentre registry data shows similar results with a 66% risk of patients developing peritonitis in 1 year (Nolph et al., 1985). Occasionally, peritonitis can be recurrent and is one of the main reasons for failure of CAPD therapy (Heaton et al., 1986; Tsakiris et al., 1986). Peritonitis is associated with increased glucose absorption from the dialysate (Rubin et al., 1981), which can induce loss of ultrafiltration (Verger et al., 1984) resulting in the need for more hypertonic exchanges to achieve adequate fluid balance. Although the effect of increased permeability of the peritoneum to glucose is known the hormonal and intermediary metabolite status during peritonitis have not been studied.

4. Objectives

Thus, the primary objective of the study was to determine the glucoregulatory hormone and intermediary metabolite status in CAPD patients. This was investigated in several ways. Firstly, fasting concentrations were determined in CAPD patients as well as other uraemic patients to distinguish the effects of CAPD from those of uraemia. Secondly, low and high dextrose dialysate solutions were studied to assess the quantitative effect of peritoneal glucose absorption. Thirdly, specific categories of CAPD patients were investigated, namely, diabetics, the elderly and patients with peritonitis to determine if any of these factors had any metabolic consequences on glucoregulatory hormone homeostasis and intermediary metabolism.

METHODS

1. Patients

Glucoregulatory hormones and intermediary metabolites were measured after an overnight fast in a 'mixed uraemic population'. Ten healthy subjects acted as controls, 6 non-dialysis patients with advanced chronic renal failure were the CRF group, 6 patients on haemodialysis were the HD group and 42 patients on CAPD were also studied. After a 12 hour overnight fast CAPD patients had hormone and metabolite profiles measured during a 6 hour peritoneal dialysis cycle.

The CAPD patients were classified into 7 groups, with 6 patients in each; isotonic, hypertonic, new, elderly, peritonitis, diabetic and overnight peritoneal dialysis. The isotonic group was given low dextrose (1.5% or isotonic) dialysis solution during the study. All the other groups were given high dextrose (4.25% or hypertonic) dialysis solution. The group named 'hypertonic' (H) was used as the reference group during the CAPD profiles. The 'new' (N) group were patients recently established on CAPD (for more than 1 month but less than 6 months). Most patient had about 3 months of therapy prior to the study. The 'elderly' (E) group were patients over the age of 65 years. The 'peritonitis' (P) group consisted of patients with proven bacterial peritoneal infection who were studied within 4 days of the onset of peritonitis. The 'diabetic' (D) group were patients who had diabetic nephropathy as the cause of their renal failure. All were taking insulin supplementation in the form of intraperitoneal insulin. Four patients were 'insulin dependent' and 2 were 'insulin treated'. The last group, overnight peritoneal dialysis (OPD), were patients who continued their peritoneal dialysis prior to the study, i.e. they did not omit their overnight exchange and had an isotonic cycle prior to

the metabolic profile.

2. Protocol

All subjects had a 12 hour oral fast and all CAPD patients except the OPD group omitted their overnight dialysis cycle. Haemodialysis patients were studied on their interdialysis day (18-24 hours after their last dialysis). Fasting blood specimens were taken between 0900 and 1000 hours and CAPD profiles were studied between 0900 and 1500 hours.

None of the CAPD patients were taking steroids, B-blockers or thiazide diuretics prior to the study. Diabetics omitted their intraperitoneal insulin during the study and blood glucose was monitored regularly with BM stixs (Boehringer). Patients with peritonitis also omitted their intraperitoneal antibiotics during the study cycle.

An intravenous cannula was inserted into a forearm vein and kept patent by heparinised saline flushes after each blood sample. Any residual peritoneal fluid was drained from the peritoneal cavity into the empty overnight bag which was attached the previous night to keep the CAPD system closed and sterile. Body weight and blood pressure were recorded and dialysis fluid bags were weighed and an aliquot taken for analysis prior to instillation. The exact time of instilling the fluid was recorded and the mid-point was taken as zero time. Subsequent blood specimens were taken at 15, 30, 45, 60, 90, 120, 180, 240 and 360 minutes. At the end of the cycle the dialysate effluent was drained, weighed and an aliquot was taken for analysis.

During the 6 hour exchange no food or fluids were given except water. Most patients consumed between 200 and 400 ml of water. Patients remained semi-supine for most of the study. At the end of

the study cycle an isotonic exchange was performed and patients continued with their usual CAPD regimen. Intraperitoneal insulin was adjusted according to blood glucose estimations and intraperitoneal antibiotics were resumed in the peritonitis group. Patients continued their usual diets which had a caloric value of 35-45 Kcal/Kg/day and protein intake of about 1.2 g/Kg/day.

The study was approved by the Sheffield Hospitals Medical Ethics Committee and all subjects gave informed written consent.

3. Assays

Blood for glucose, lactate, pyruvate, alanine, 3-hydroxybutyrate, acetoacetate and glycerol was taken into ice cold perchloric acid. The automated enzymic fluorometric continuous flow assays (Lloyd et al., 1978) and the manual spectrofluorometric assay for acetoacetate and pyruvate are detailed in Chapter 5. Dialysate glucose and lactate were measured by the same technique. Blanks were run for all metabolites to exclude non-specific (native) fluorescence due to uraemia. Blood for hormone assays was kept ice cold until separated (within 4 hours). Frozen plasma or serum was stored at $-20^{o}C$ until assayed. Serum insulin was measured by double antibody radioimmunoassay, a modification of the method of Soeldner and Slone (1965). Free insulin in diabetics was measured after antibody precipitation with polyethylene glycol. Plasma c-peptide was measured by a radioimmunoassay kit (Novo Biolab) and glucagon by c-terminal specific radioimmunoassay with wick chromatography (Orskov et al., 1968). Growth hormone and cortisol were measured by double antibody radioimmunoassays from 'in house kits'. Details of all assays are described in Chapter 5.

4. Peritoneal dialysis solutions

All CAPD patients used Fresenius standard dialysis solutions (Perito-Flex, Fresenius, The Dylade Co. Ltd., Runcorn, U.K.). The isotonic fluid contained 1.5% glucose (15 g/l or 84 mmol/l) and the hypertonic solution contained 4.25% glucose (42.5 g/l or 237 mmol/l). The other constituents of the peritoneal fluid were:-

Sodium	134 mmol/l	(5.786 g/l NaCl)
Calcium	1.75 mmol/l	(0.257 g/l $CaCl_2$)
Magnesium	0.50 mmol/l	(0.102 g/l $MgCl_2$)
Chloride	103.5 mmol/l	
Lactate	35.0 mmol/l	(3.924 g/l Na lactate)

The isotonic fluid contained glucose in the form of dextrose monohydrate 16.5 g/l, equivalent to anhydrous dextrose 15 g/l. The hypertonic fluid contained dextrose monohydrate 46.5 g/l, equivalent to 42.5 g/l of anhydrous dextrose. The osmolality of the isotonic fluid was 358 mosm/l and the hypertonic solution was 511 mosm/l.

5. Statistics

Statistical analysis was performed using the paired 't' test to compare changes within a group and by analysis of variance to compare changes between groups. Generally parameters had a normal distribution but metabolites had a slightly skewed distribution. Correlations were sought using linear regression analysis.

RESULTS

Glucoregulatory hormones and intermediary metabolites were determined in a mixed uraemic population. In the fasting state healthy control subjects were compared with chronic renal failure, haemodialysis and CAPD patients. This latter group, CAPD (n=30), was an amalgamation of 5 CAPD subgroups (viz: isotonic, hypertonic, new, elderly and peritonitis). The CAPD subgroups were abbreviated by the letter(s):-

I = Isotonic

H = Hypertonic

N = New

E = Elderly

P = Peritonitis

D = Diabetic

OPD = Overnight peritoneal dialysis

It should be noted that during the CAPD cycle all patients were given a hypertonic (high dextrose) solution except those in the isotonic group who were given isotonic (low dextrose) solution.

Results are presented in five sections:-

1. General data including clinical variables

2. Fasting hormone and metabolite status

3. Hormones and metabolites during a CAPD cycle

4. Hormones and metabolites in diabetics on CAPD

5. Dialysate glucose and ultrafiltration

1. GENERAL DATA

Clinical data for the control and three uraemic groups (CRF, HD, CAPD) are shown in Table 7.I (values are mean and SEM). Age and serum creatinine were comparable in the uraemic groups. Fasting serum total

	CONTROL	CRF	HD	CAPD
No of subjects	10	6	6	30
Sex M:F	6:4	6:0	4:2	8:22
Age	34.0 (2.4)	56.2 (6.2)	49.0 (4.0)	58.0 (2.8)
Serum Creatinine (µmol/l)	83 (6)	756 (118)	809 (69)	923 (92)
Cholesterol (mmol/l)	4.92 (0.20)	5.28 (0.59)	5.03 (0.86)	7.05^b (0.03)
Triglycerides (mmol/l)	0.88 (0.10)	1.98^c (0.27)	2.12^c (0.29)	2.50^c (0.18)
HDL – Cholesterol (mmol/l)	1.28 (0.10)	0.72^c (0.08)	0.72^c (0.08)	0.95^a (0.04)

TABLE 7. I Clinical Data of control and uraemic subjects
Values are mean and (SEM)

$a = p < 0.05,$ $b = p < 0.01,$ $c = p < 0.001,$
p values compared to control

	I	H	N	E	P	D	OPD
No of Patients	6	6	6	6	6	6	6
Sex M:F	3:3	3:3	1:5	1:5	0:6	3:3	4:2
Age	61.3 (1.9)	54.2 (2.9)	52.3 (3.7)	71.0 (0.9)	52.3 (4.6)	47.7 (4.2)	63.2 (2.5)
Weight (kg)	65.5 (4.7)	66.8 (5.6)	60.5 (4.3)	65.3 (6.2)	60.8 (4.2)	66.0 (4.2)	74.3 (6.7)
Serum Creatinine (µmol/l)	1053 (109)	1192 (116)	755 (84)	859 (82)	758 (71)	709 (48)	1278 (94)
Cholesterol (mmol/l)	2.15^c(0.22)...						
Cholesterol (mmol/l)	6.15^c(0.72)	9.02^c(0.58)	6.43^a(0.64)	6.78^b(0.54)	6.88^b(0.71)	6.73^c(0.27)	6.83^a(1.1)
Triglycerides (mmol/l)	2.15^c(0.22)	3.87^b(0.98)	2.22^c(0.19)	2.17^c(0.36)	2.12^c(0.22)	2.70^c(0.40)	3.63^c(0.76)
HDL – Cholesterol (mmol/l)	0.82^b(0.06)	0.95^b(0.06)	0.97^a(0.09)	0.97^a(0.13)	1.05 (0.12)	0.85^b(0.12)	0.85^a(0.16)

TABLE 7. II Clinical Data of CAPD subgroups
Values are mean and (SEM)

I – Isotonic,   H, Hypertonic,   N – New,   E – Elderly,
P – Peritonitis,   D – Diabetic,   OPD – Overnight Peritoneal Dialysis

a = $p < 0.05$,   b = $p < 0.01$,   c = $p < 0.001$,
p values refer to comparison with controls

cholesterol was elevated in the CAPD group (p<0.01), whereas the CRF
and HD subjects, although they had a high cholesterol level this was
not significantly different from controls. Fasting serum total
triglycerides were markedly increased in all the uraemic groups
(p<0.001). Fasting HDL-cholesterol was significantly decreased in the
uraemic subjects.

CAPD patients were subgrouped as shown in Table 7.II (values are
mean and SEM). This classification is used throughout the result
section of the chapter. More females participated in the study
reflecting the female predominance in CAPD at the centre. Most
patients were between 50 and 65 years of age except for the elderly
group, all of whom were over 65 years. The mean weight in each group
was comparable and varied between 60 and 75 Kg. Fasting serum total
cholesterol and triglycerides were increased and HDL-cholesterol was
decreased in all subgroups.

2. GLUCOREGULATORY HORMONES AND INTERMEDIARY METABOLITES IN THE

FASTED STATE

a) Glucoregulatory Hormones

Fasting hormone concentrations for each group and CAPD subgroups
are shown in Table III (values are mean and SEM). Glucose
concentrations were not significantly different in any of the groups
or subgroups compared to controls. Fasting serum insulin
concentrations were similar to controls in all but two CAPD subgroups.
The hypertonic (H) subgroup had a higher basal insulin concentration
(p<0.05), although the mean value was in the accepted normal reference
range (2.5-13.5 mU/1) for the assay. The OPD subgroup also had a
higher basal insulin concentration but this set of patients were not
in a true fasted state as they have peritoneal dialysis immediately

GROUPS	NO.	GLUCOSE (mmol/1)	INSULIN (mu/1)	C-PEPTIDE (nmol/1)	GLUCAGON (pg/ml)	GRO.HOR. (mu/1)	CORTISOL (nmol/1)
CONTROL	10	4.8 (0.1)	6.3 (1.6)	0.32 (0.06)	68 (11)	3.6 (1.5)	297 (23)
CRF	6	5.0 (0.1)	8.2 (1.6)	2.34^c (0.37)	200^b (36)	5.5 (2.2)	488^b (59)
HD	6	5.2 (0.2)	10.4 (2.6)	3.17^c (1.00)	303^b (46)	8.3^a (2.1)	468^b (65)
CAPD	30	5.0 (0.1)	8.9 (0.8)	2.25^c (0.18)	107^a (9)	4.4 (0.6)	331 (19)
CAPD SUBGROUPS							
I	6	5.0 (0.1)	6.5 (0.4)	2.48^c (0.35)	135^b (21)	8.2^a (1.5)	301 (26)
H	6	4.9 (0.1)	13.3^a (2.6)	2.21^c (0.25)	94 (15)	4.5 (1.1)	356 (53)
N	6	5.2 (0.2)	8.8 (1.1)	2.01^c (0.29)	105 (16)	3.8 (1.4)	322 (49)
E	6	5.0 (0.1)	9.2 (1.2)	2.49^c (0.66)	83 (15)	3.1 (0.5)	332 (44)
P	6	4.6 (0.1)	7.0 (1.3)	2.06^c (0.43)	108 (23)	2.3 (0.5)	345 (41)
DIABETIC	6	7.3 (1.5)	13.3 (2.8)	0.99 (0.45)	92 (19)	6.4 (2.5)	304 (43)
OPD	6	5.8 (0.4)	12.7^a (1.7)	3.67^c (0.72)	------	1.6 (0.2)	375^a (24)

TABLE 7. III   FASTING HORMONE CONCENTRATIONS

Values are Mean and (SEM)

$a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$
p values refer to comparison with controls

prior to the study. Plasma c-peptide was grossly elevated (p<0.001) in all uraemic patients with the exception of diabetics on CAPD. Basal c-peptide in diabetics was similar to controls (p<0.05). Fasting plasma glucagon was increased in all the uraemic groups particularly the CRF and haemodialysis groups (p<0.01). However, in CAPD patients basal glucagon was variable and was only significantly increased in the I subgroup (p<0.01). Serum growth hormone concentrations showed wide variation in the fasting state and although increased in some uraemic patients overall there was no difference between controls and any of the uraemic groups or CAPD subgroups. Fasting plasma cortisol was increased (p<0.01) in the CRF and HD groups. However, the main CAPD group and all subgroups, (except the OPD subgroup), showed no significant change in basal cortisol concentrations.

Figures 7.1 to 7.6 show the fasting glucose and hormone concentrations for each main group and the subgroups. Values are mean and SEM. The p values are derived from one way analysis of variance and compare controls with the appropriate uraemic group or subgroup. The CAPD subgroup, N (new), OPD (overnight peritoneal dialysis) and D (diabetic) have been omitted. These subgroups were omitted because the N group behaved in an identical manner to the H group, the OPD group did not represent a true fasted state and the D group is presented later in the diabetic section.

b) Intermediary Metabolites

Fasting intermediary metabolites for each main group and CAPD subgroups are shown in Table 7.IV (values are mean and SEM). Glucose was unchanged as previously stated. Fasting blood lactate, pyruvate and alanine were similar to control values and the lactate:pyruvate
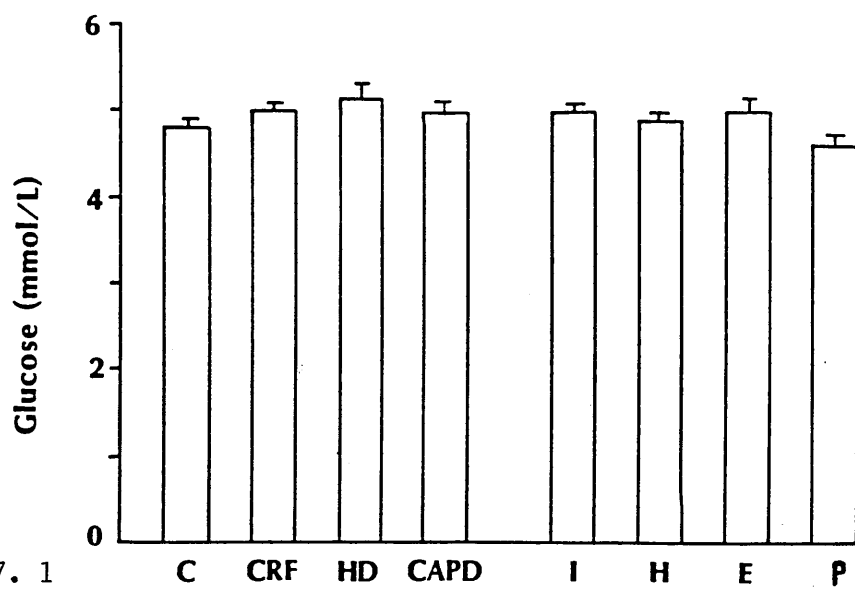
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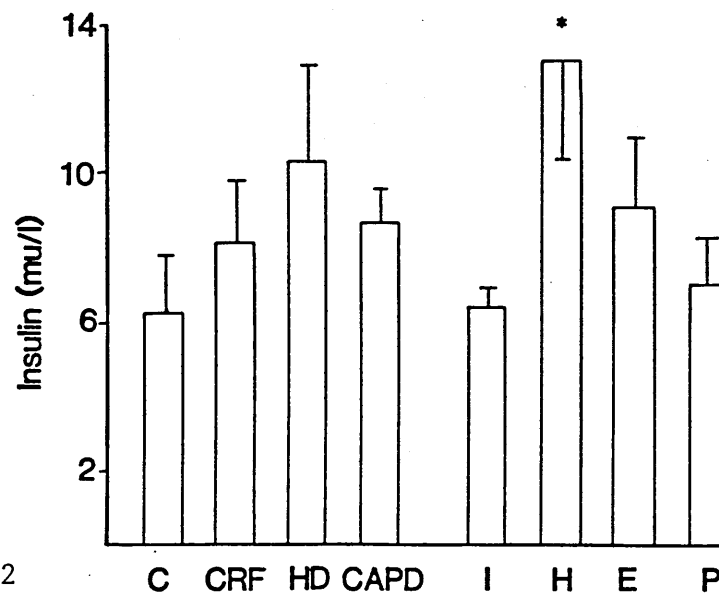
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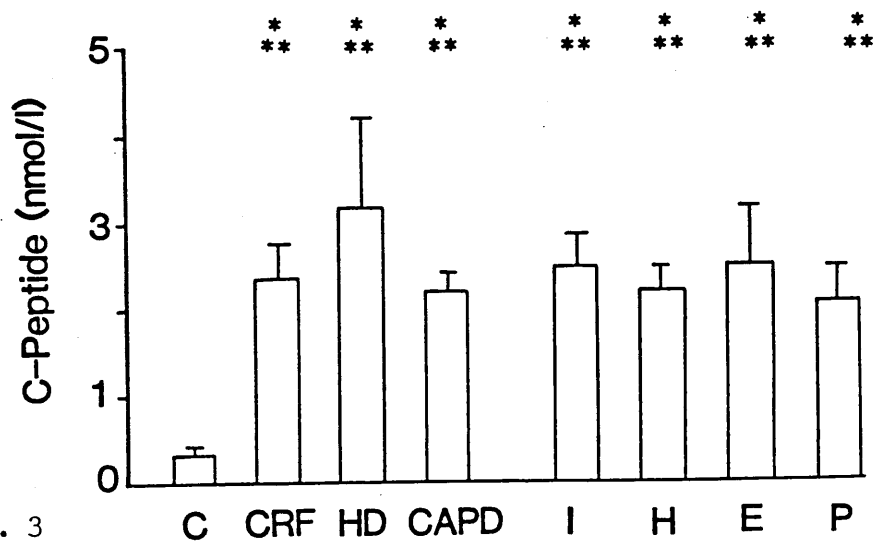
Figure 7. 3

Fasting hormone concentrations for each uraemic group. Values are mean and SEM

* p < 0.05, **p < 0.001 compared to controls
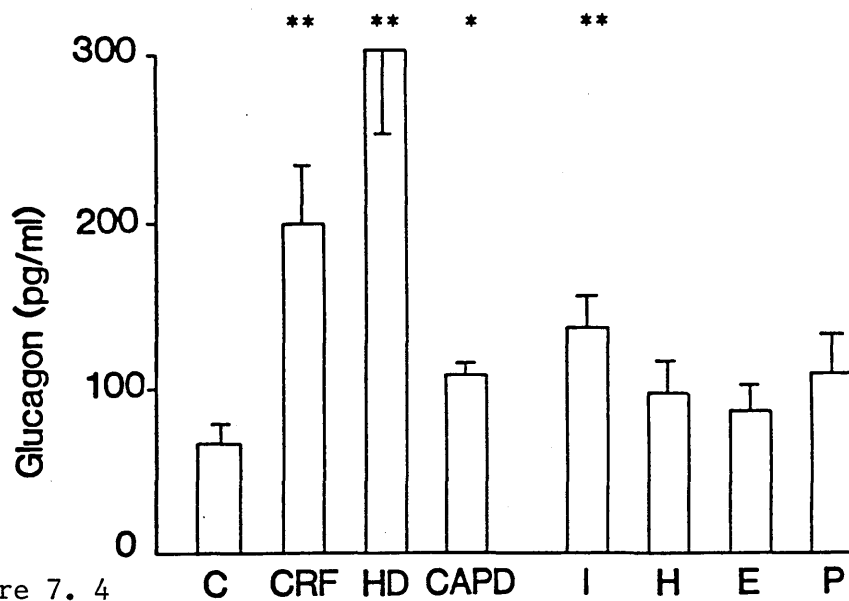
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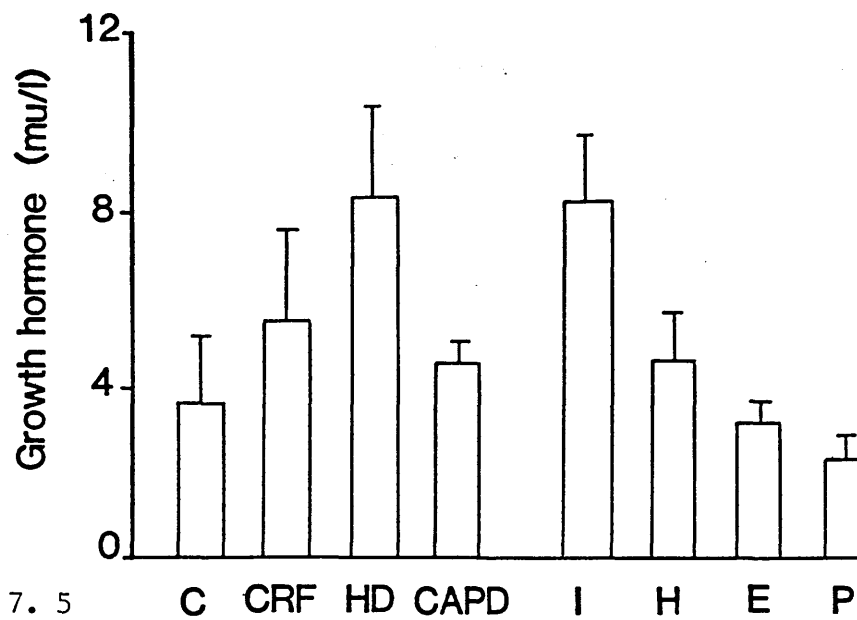
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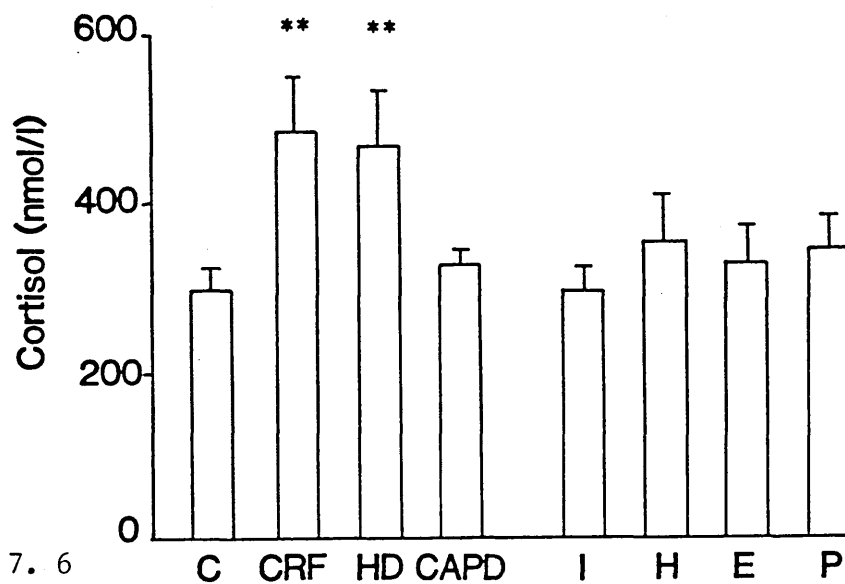
Figure 7. 6

Fasting hormone concentrations for each
uraemic group. Values are mean and SEM

* p < 0.05,   ** p < 0.01 compared to controls

ratio was normal in all groups and subgroups. Fasting
3-hydroxybutyrate was higher in the uraemic patients but this was only
significantly different in the CRF group ($p<0.05$) and the elderly CAPD
subgroup ($p<0.05$). Basal acetoacetate was also higher in uraemia but
was only significantly increased in the CAPD group ($p<0.05$) and
largely due to the elderly subgroup ($p<0.01$). The
3-hydroxybutyrate:acetoacetate ratio was essentially normal but was
moderately increased in the CRF group due to a relative increase in
3-hydroxybutyrate. Fasting glycerol concentrations were generally
higher in uraemia and this was most marked in the CAPD group ($p\leqslant0.05$)
and again predominantly due to the elderly subgroup ($p<0.01$). The
diabetic and OPD subgroups also had significantly raised glycerol
concentrations.

Figures 7.7 to 7.12 show the fasting intermediary metabolite
concentrations for each group and CAPD subgroups. Values are mean and
SEM. Three subgroups have been omitted for reasons explained above.

In summary, in the fasting or post-absorptive state total
triglycerides were increased and HDL-cholesterol decreased in all
uraemic patients. Total cholesterol was significantly increased only
in CAPD patients. Fasting glucose and insulin were normal but
c-peptide was grossly elevated in uraemia. Glucagon was increased in
the CRF and haemodialysis patients and in many of the CAPD subjects.
Lactate, pyruvate and alanine were normal but the ketone bodies and
glycerol were increased in uraemic patients, particularly the elderly
subjects on CAPD.

GROUPS	No	GLUCOSE mmol/1	LACTATE mmol/1	PYRUVATE mmol/1	L/P	ALANINE mmol/1	3OH-BUT mmol/1	ACAC mmol/1	3OH-BUT/ACAC	GLYCEROL mmol/1
CONTROL	10	4.8 (0.1)	0.917 (0.108)	0.070 (0.008)	13.1	0.318 (0.025)	0.042 (0.008)	0.034 (0.008)	1.2	0.059 (0.009)
CRF	6	5.0 (0.1)	0.796 (0.121)	0.076 (0.016)	10.5	0.271 (0.066)	0.097[a] (0.019)	0.037 (0.007)	2.6	0.043 (0.006)
ID	6	5.2 (0.2)	0.653 (0.083)	0.079 (0.016)	8.3	0.283 (0.046)	0.053 (0.026)	0.046 (0.011)	1.2	0.073 (0.023)
CAPD	30	5.0 (0.1)	0.715 (0.051)	0.063 (0.019)	11.3	0.275 (0.014)	0.073 (0.008)	0.059[a] (0.005)	1.2	0.101[a] (0.011)
CAPD SUBGROUPS										
I	6	5.0 (0.1)	0.702 (0.097)	0.064 (0.010)	11.0	0.257 (0.024)	0.088 (0.013)	0.063[a] (0.009)	1.4	0.064 (0.008)
H	6	4.9 (0.1)	0.626 (0.058)	0.063 (0.007)	9.9	0.309 (0.007)	0.068 (0.026)	0.042 (0.006)	1.6	0.107 (0.024)
N	6	5.2 (0.2)	0.697 (0.136)	0.062 (0.009)	11.1	0.323 (0.048)	0.071 (0.014)	0.053 (0.013)	1.3	0.096 (0.020)
E	6	5.0 (0.1)	0.753 (0.077)	0.064 (0.006)	11.8	0.244 (0.034)	0.097[a] (0.024)	0.081[b] (0.015)	1.2	0.155[b] (0.039)
P	6	4.6 (0.1)	0.800 (0.186)	0.061 (0.009)	13.1	0.237 (0.021)	0.043 (0.007)	0.052 (0.013)	0.8	0.090 (0.011)
DIABETIC	6	7.3 (1.5)	0.762 (0.075)	0.079 (0.009)	9.6	0.304 (0.020)	0.082 (0.017)	0.064[a] (0.013)	1.3	0.125[a] (0.020)
CPD	6	5.8 (0.4)	0.732 (0.076)	0.091 (0.030)	8.0	0.324 (0.030)	——	0.054 (0.13)	—	0.145[b] (0.029)

TABLE 7. IV   FASTING INTERMEDIARY METABOLITE CONCENTRATIONS

Values are mean and (SEM)

a = $p < 0.05$,   b = $p < 0.01$

p values refer to comparison with controls
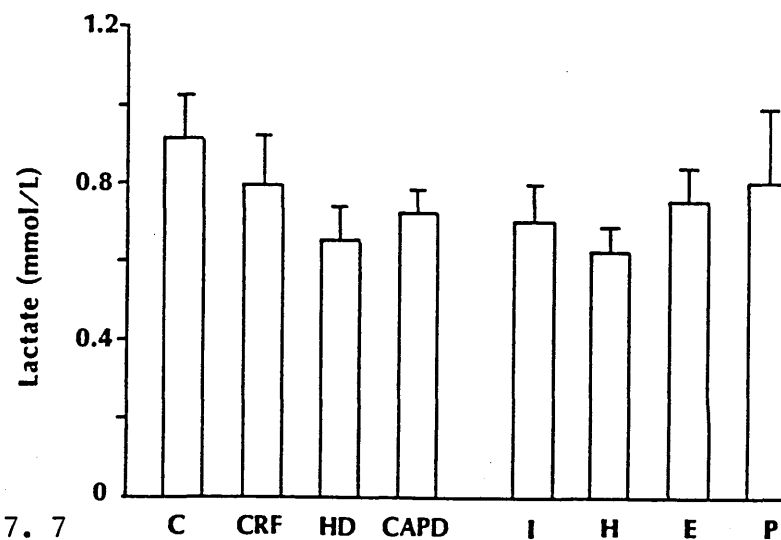
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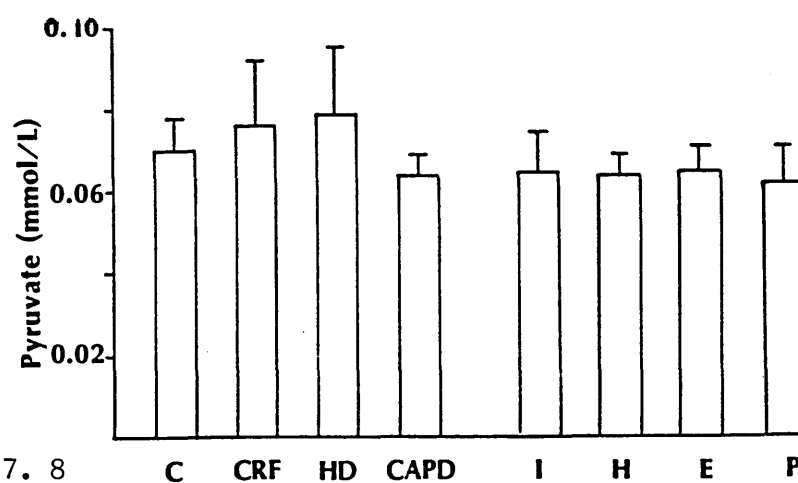
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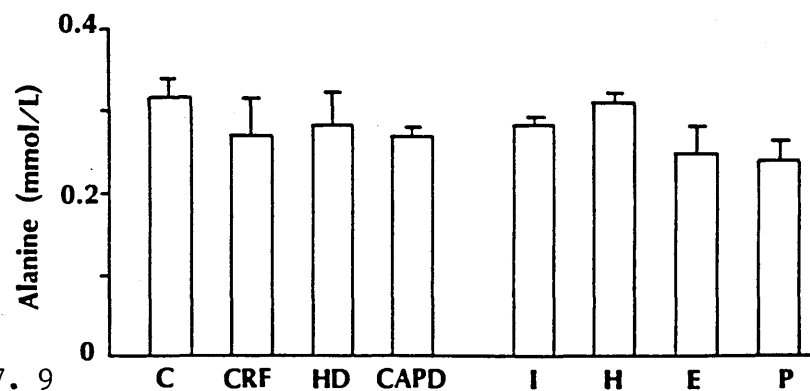
Figure 7. 9

Fasting intermediary metabolite concentrations
for each uraemic group
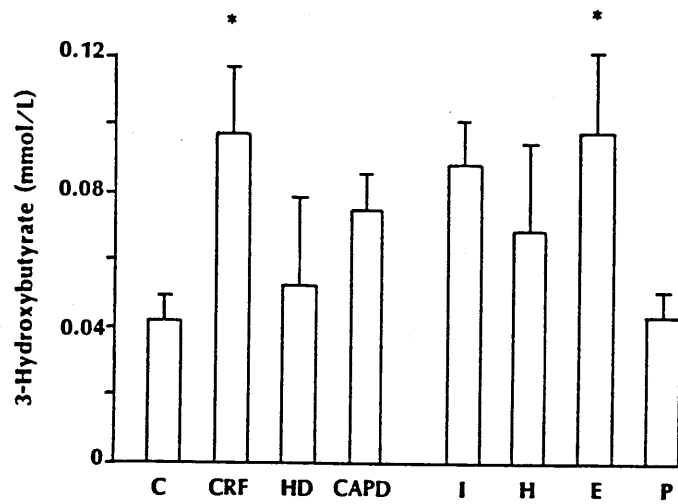
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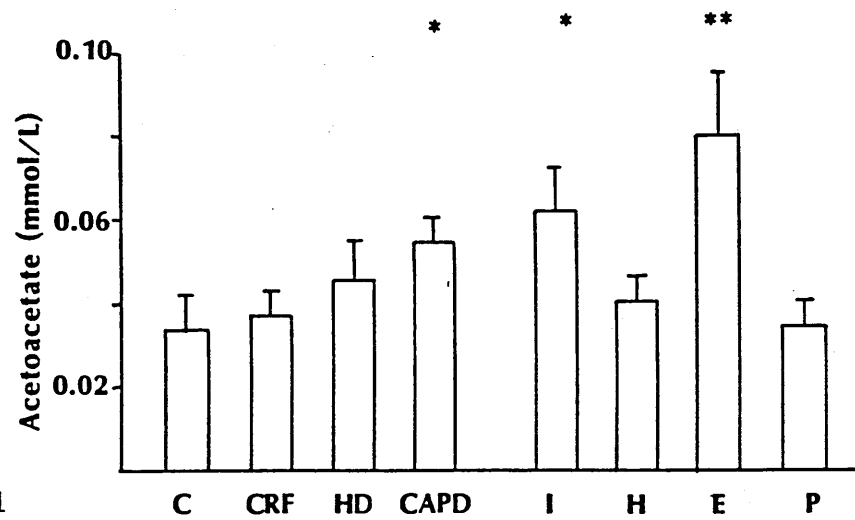
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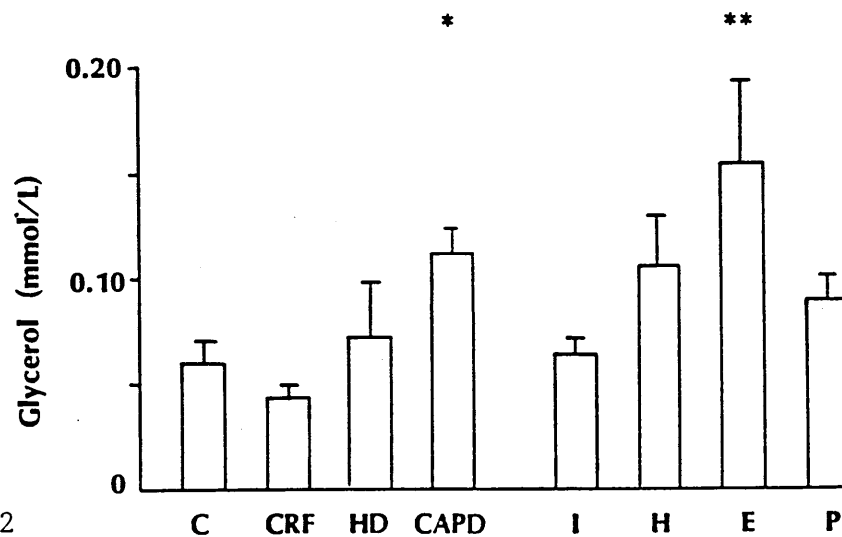
Figure 7.12

Fasting intermediary metabolite concentrations for each uraemic group

$* \ p < 0.05$, $* * p < 0.01$ compared to controls

3. <u>HORMONE AND INTERMEDIARY METABOLITES DURING A PERITONEAL DIALYSIS</u>

<u>CYCLE</u>

The hormone and metabolite changes with peritoneal glucose absorption over a 6 hour dwell period are shown in Figures 7.13 to 7.24. The mean concentrations are plotted at 10 time intervals but the SEM has been omitted from some points for clarity of illustration, where there was no significant difference. The new, diabetic and overnight peritoneal dialysis groups have been omitted for reasons previously stated.

a) <u>Glucoregulatory Hormones</u>

<u>Glucose</u>: Blood glucose (Figure 7.13) significantly increased in all groups ($p < 0.01$) and especially with hypertonic dialysate. Glucose showed a small but significant increase from 5 to 6 mmol/1 with isotonic dialysate reaching a maximum at 30 minutes. Hypertonic dialysate increased blood glucose from 5 to over 9 mmol/1 and peaked at 60 minutes. Patients with peritonitis had a more rapid rise and increased blood glucose concentration which was significantly higher than the H (hypertonic) group ($p < 0.05$). The H group had a markedly increased blood glucose compared to the I (isotonic) group ($p < 0.01$). Blood glucose concentrations returned to their basal levels by 4 hours.

<u>Insulin</u>: The rise in serum total insulin (Figure 7.14) paralleled that of glucose. The rapid rise in the first 15 minutes was followed by smaller increments over the first hour. All groups showed a significant rise in insulin from basal concentrations ($0.001 < p < 0.05$). The isotonic fluid produced much smaller increments compared to hypertonic fluid. There was approximately a 3-fold increase in
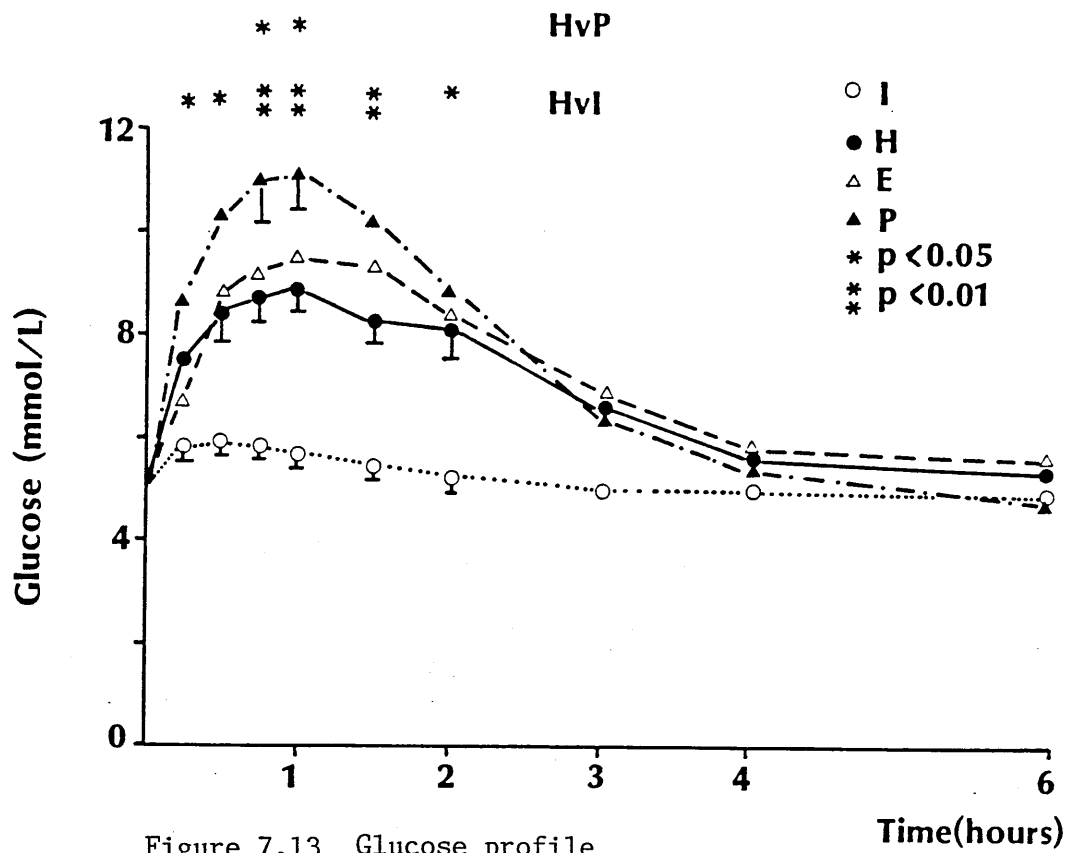
Figure 7.13   Glucose profile
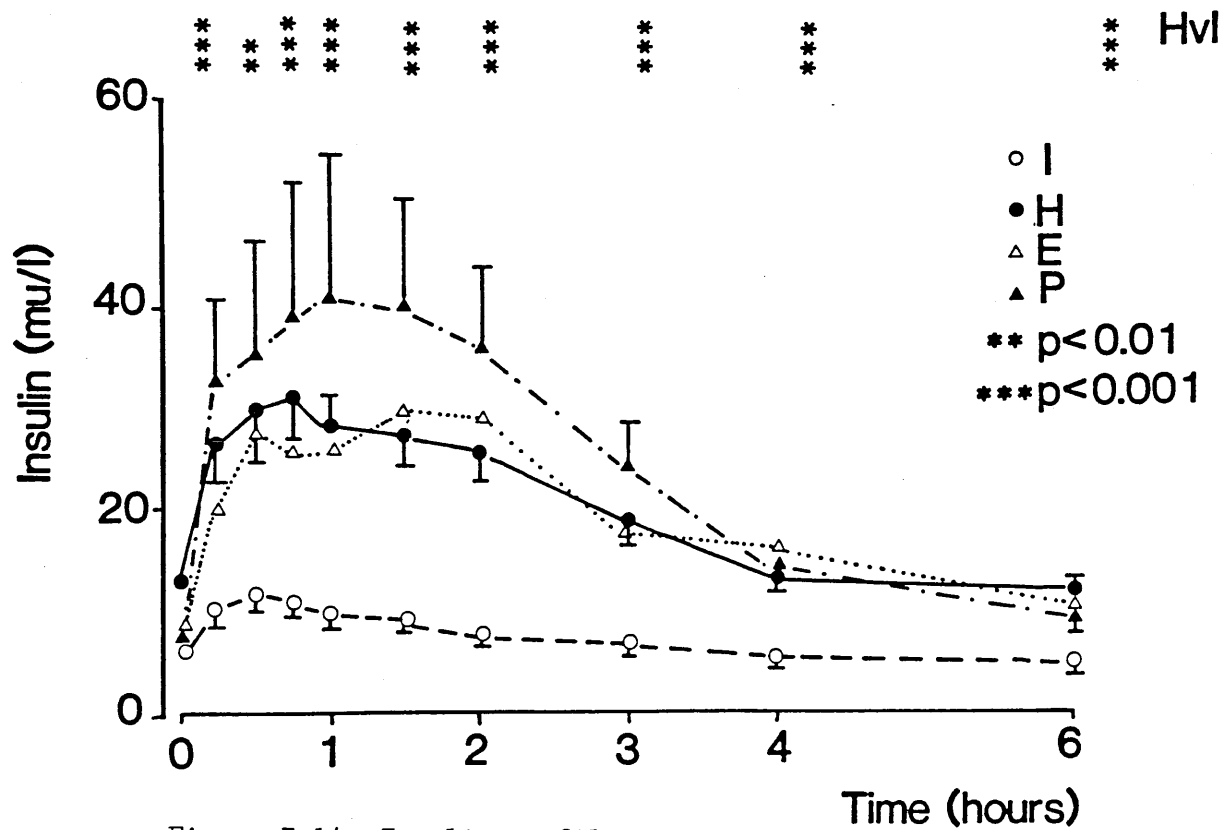
Figure 7.14   Insulin profile

insulin in response to hypertonic solution. The difference in serum insulin between the hypertonic (H) group and the isotonic (I) group was highly significant throughout most of the cycle ($p < 0.001$). The peritonitis (P) group had a higher insulin concentration over the first 3 hours of the cycle but this was not statistically different from the H group. Insulin concentrations returned to basal level by 4 hours.

C-peptide: Plasma c-peptide concentrations (Figure 7.15) were elevated at the start and increased further during peritoneal dialysis. The isotonic solution produced a small but significant increase ($p < 0.05$) but the 3 groups using hypertonic fluid had a marked increase ($p < 0.01$). The rate of rise was slower than that of glucose and insulin, reaching a maximum by 2 hours with hypertonic fluid. There was a very slow decline and c-peptide concentrations did not return to their basal levels by 6 hours in groups using the high dextrose dialysate. The difference between the H and I groups was significant throughout most of the cycle ($p < 0.05$). The peritonitis (P) group had a more rapid rise and reached higher concentrations than the other groups using hypertonic fluid but the difference was not significant.

Glucagon: There was marked variation in plasma glucagon concentrations within each group (Figure 7.16). The general trend showed a fall in glucagon over the first hour of the dialysate dwell. Although basal glucagon was high in the isotonic group no significant change occurred during the dialysis cycle. The hypertonic (H) and elderly (E) groups showed a small but significant fall in glucagon ($p < 0.01$), while patients with peritonitis (P), although they had a
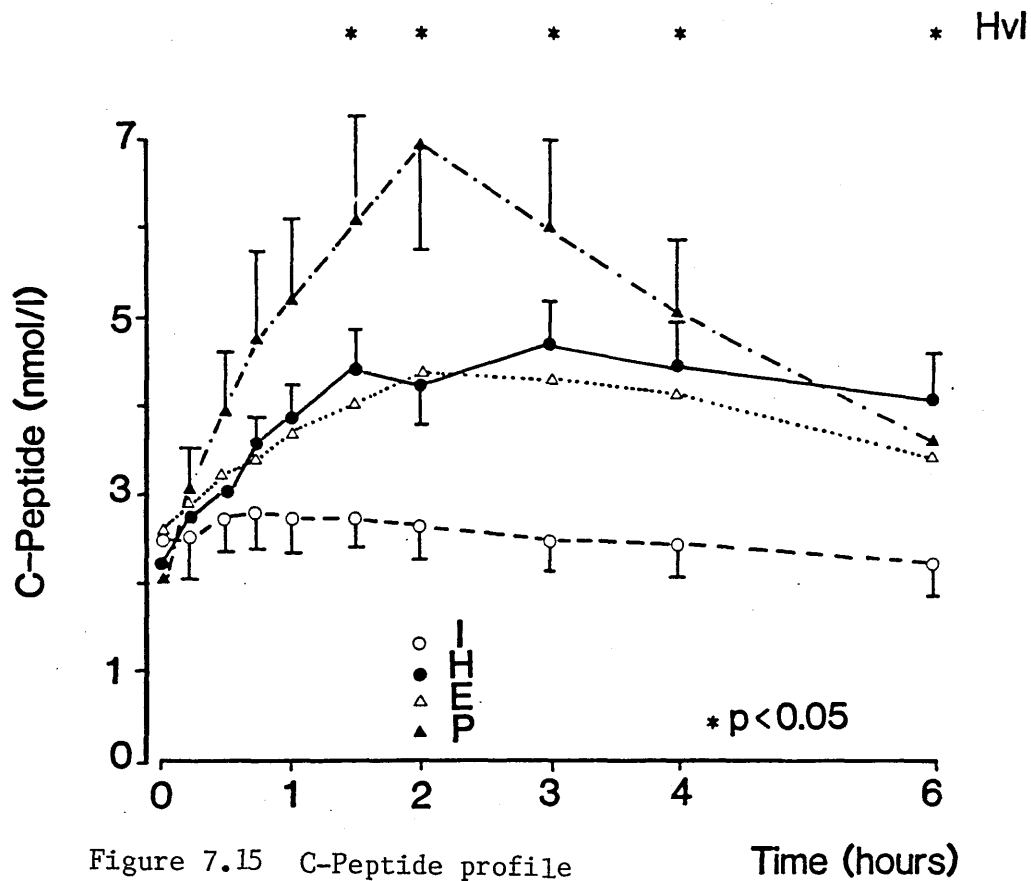
Figure 7.15   C-Peptide profile
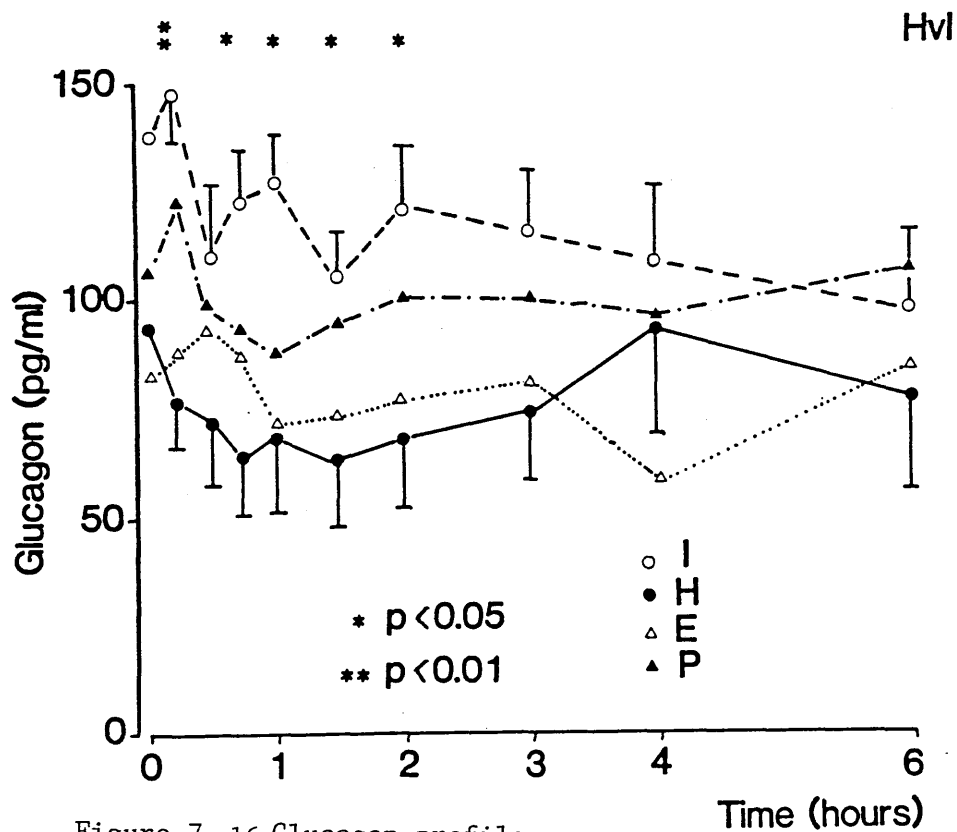
Figure 7.16 Glucagon profile
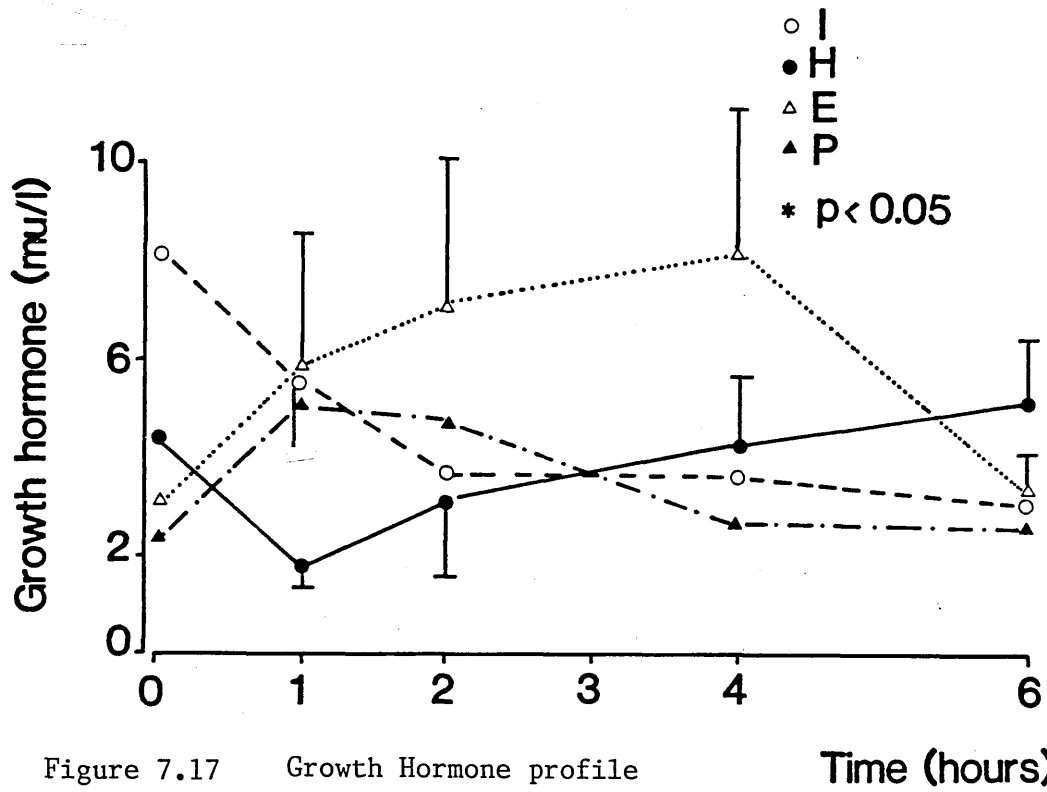
Figure 7.17   Growth Hormone profile
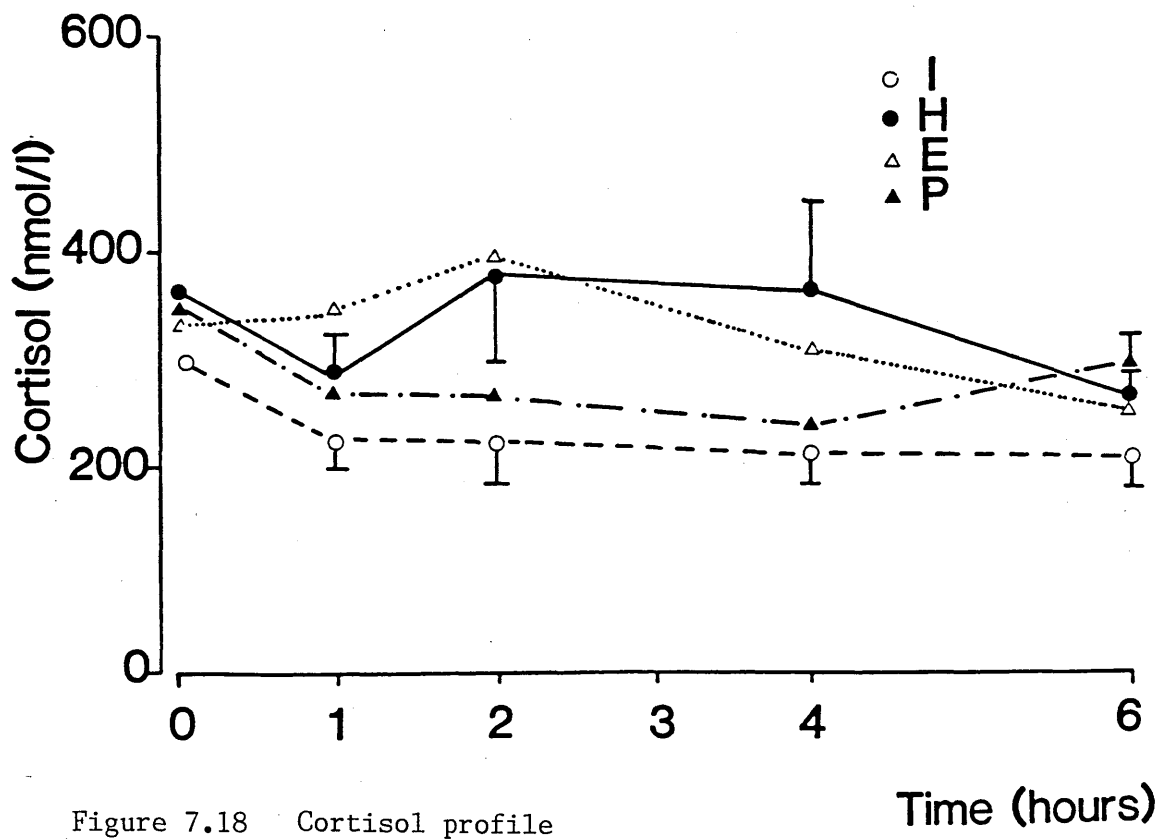
Figure 7.18   Cortisol profile

similar trend did not produce a significant fall in glucagon.
Glucagon was significantly decreased (p<0.05) during the first 2 hours
of dialysate dwell in the H group compared to the I group.

Growth hormone: Serum growth hormone concentrations fluctuated
both within and between groups (Figure 7.17). Overall there was no
clear trend in response to the peritoneal glucose load. The isotonic
group showed a fall in growth hormone over the 6 hours (p<0.05) while
the other groups showed marked variability and no real change. There
was no difference between any of the groups.

Cortisol: Plasma cortisol concentrations (Figure 7.18) also
showed considerable variation both within and between groups. Overall
there was no change during the peritoneal dialysis cycle and no
difference between any of the groups.

b) Intermediary Metabolites

Lactate: Blood lactate (Figure 7.19) increased in all patients
during peritoneal dialysis (p<0.05) reaching a peak by 60 minutes and
returning to basal concentrations by 4 hours. There was no
significant difference in lactate concentrations throughout the cycle
between the isotonic (I) and the hypertonic (H) or elderly (E) groups,
although the elderly group did have higher levels in the first 2
hours. However, lactate concentrations were significantly higher
(p<0.01) in the peritonitis (P) group compared to the others. A rapid
rise within the first hour of dwell and approximately a 3-fold
increase from basal concentrations was observed.

Pyruvate: Pyruvate (Figure 7.20) behaved similarly to lactate but
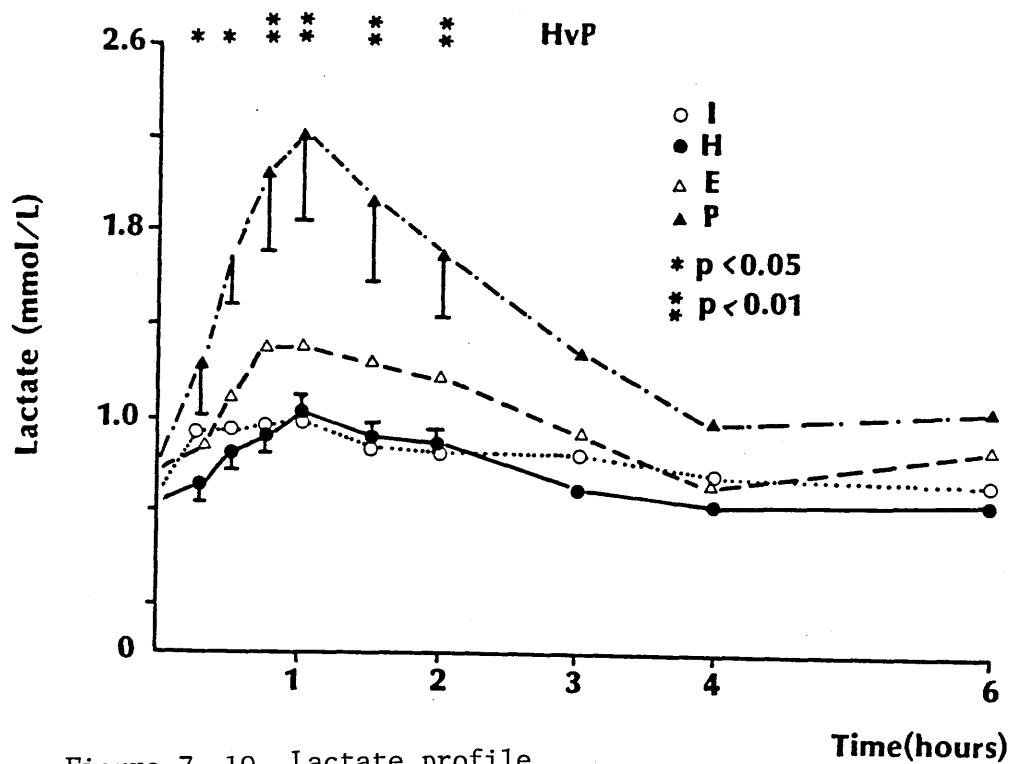
Figure 7. 19   Lactate profile
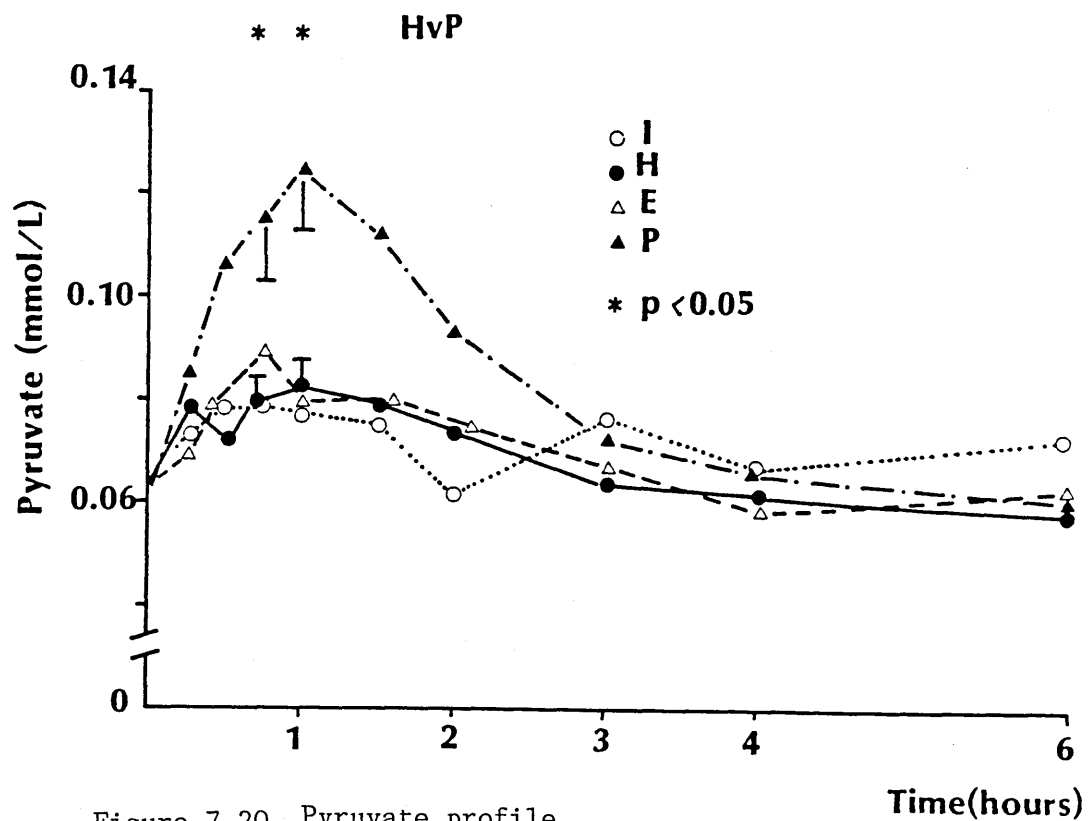
Figure 7.20   Pyruvate profile

the increase from basal concentrations was smaller. The rise in

pyruvate concentration was not significant in the hypertonic (H)

group, but small and significant changes (p<0.05) were seen in both

the isotonic and elderly groups. The peritonitis group showed a

marked increase in pyruvate (p<0.01) which reached a maximum at 60

minutes and declined to basal levels by 4 hours. The difference in

peak pyruvate between the hypertonic (H) and peritonitis (P) groups

was significant (p<0.05).

Lactate:pyruvate ratio: The lactate:pyruvate ratio (Table 7.V)

was essentially normal (10:1) throughout dialysis in most groups. The

exceptions were in the elderly and particularly in the peritonitis

group which showed an increased ratio due to the relatively greater

increase in lactate.

Alanine: Blood alanine concentrations (Figure 7.21) were variable

but remained normal during dialysis. There was a small but

statistically significant rise in alanine concentrations (p<0.05) in

the elderly and isotonic groups during the first 2 hours of dwell. No

change occurred in either the hypertonic (H) or the peritonitis

groups. Furthermore, there was no difference between any of the

groups during the cycle.

3-Hydroxybutyrate: Blood 3-hydroxybutyrate (Figure 7.23)

decreased from high fasting concentrations to trough levels between 2

and 3 hours and then increased over the remainder of the cycle. The

decrement was generally greater with the high glucose dialysis

solutions. However, the decrease in 3-hydroxybutyrate was significant

in the isotonic and elderly groups (p<0.05) but not in the hypertonic

Time (mins)	0	15	30	45	60	90	120	180	240	360
Group										
I	11.0	12.9	12.1	12.3	12.8	11.6	13.8	11.1	11.4	10.1
H	9.9	9.0	11.9	11.6	12.2	11.6	11.5	11.1	10.4	11.5
N	11.1	11.1	11.3	10.0	11.5	13.6	11.9	11.3	12.2	12.8
E	11.8	13.2	13.6	14.8	16.4	15.7	15.5	13.6	12.8	14.1
P	13.1	14.4	16.3	17.8	17.7	17.3	17.9	17.3	14.5	17.3
D	9.6	10.4	11.9	10.9	11.5	11.7	12.9	11.4	10.3	10.6
OPD	8.0	8.5	9.4	10.1	10.7	11.0	9.3	9.3	10.4	10.6

TABLE 7. V   Lactate/Pyruvate ratios during CAPD
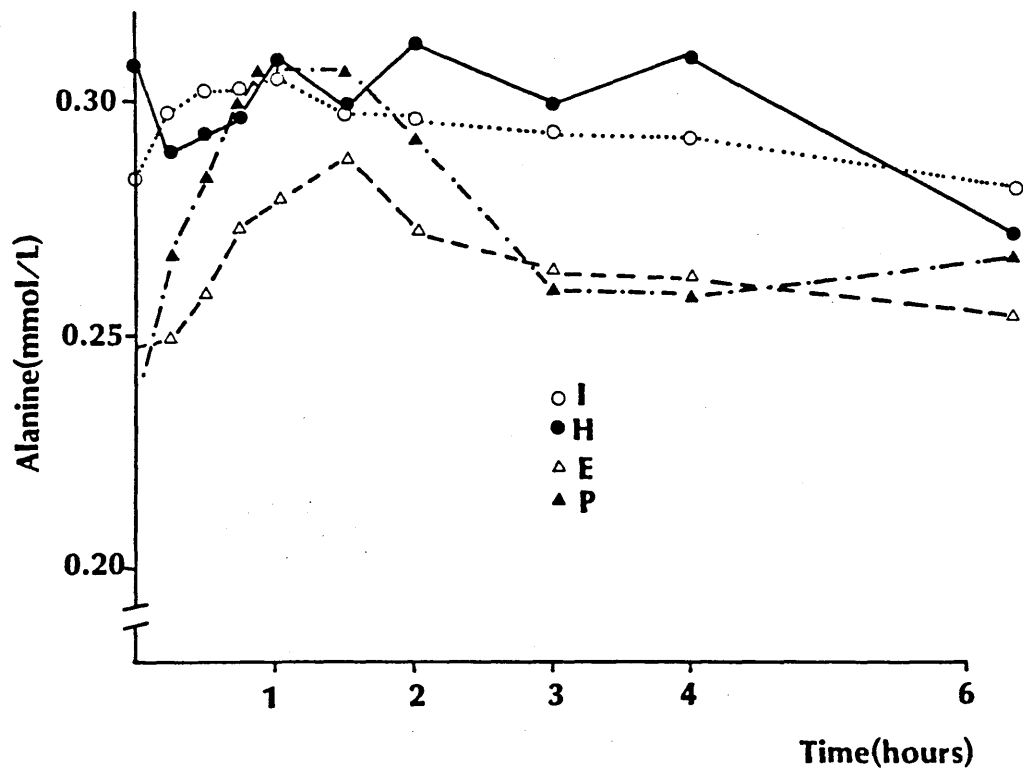
Figure 7.21 Alanine profile
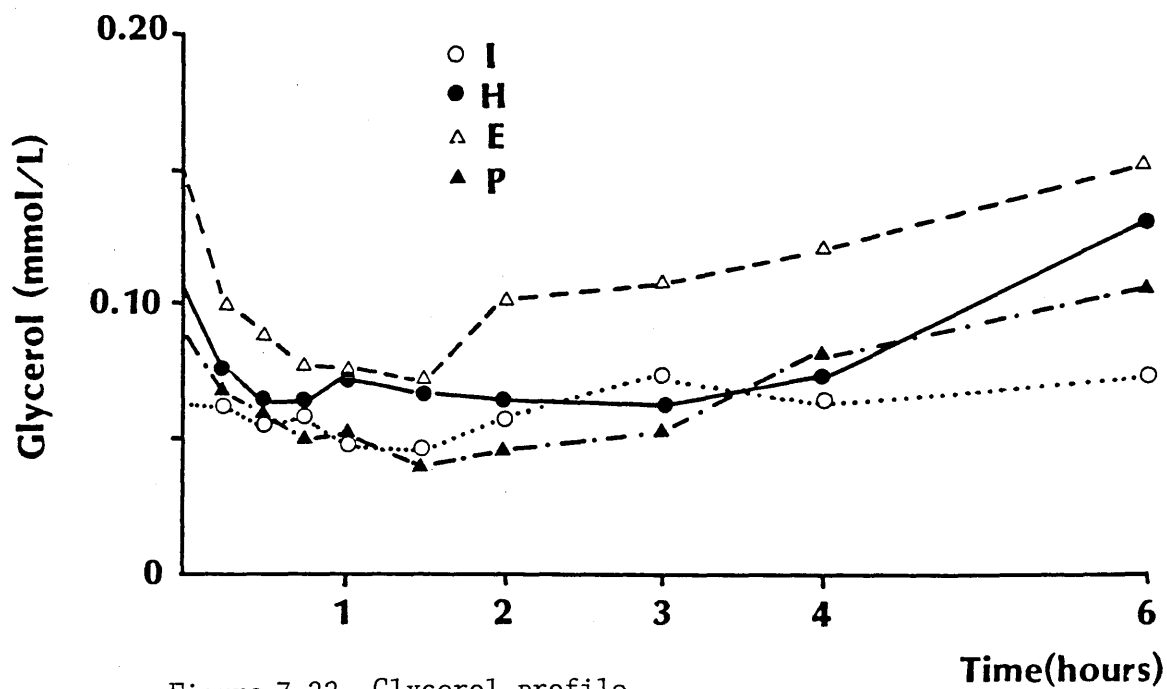
Figure 7.22 Glycerol profile
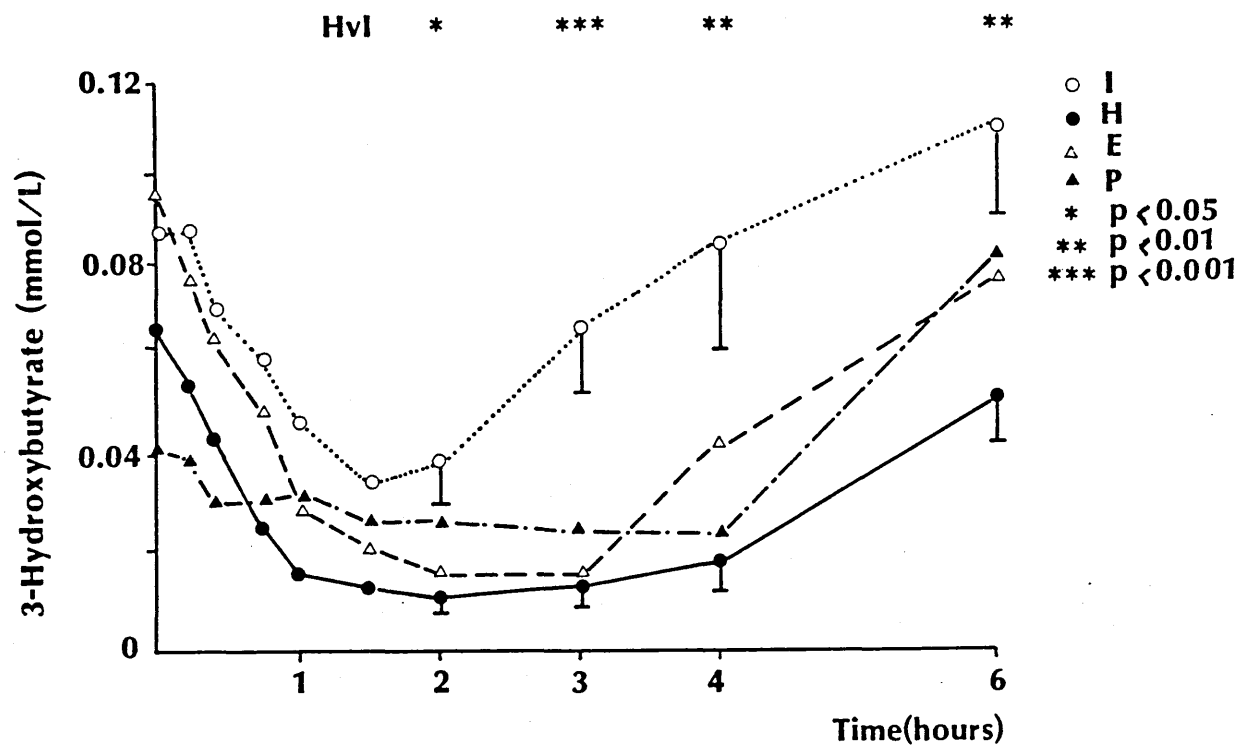
Figure 7.23   3-Hydroxybutyrate profile
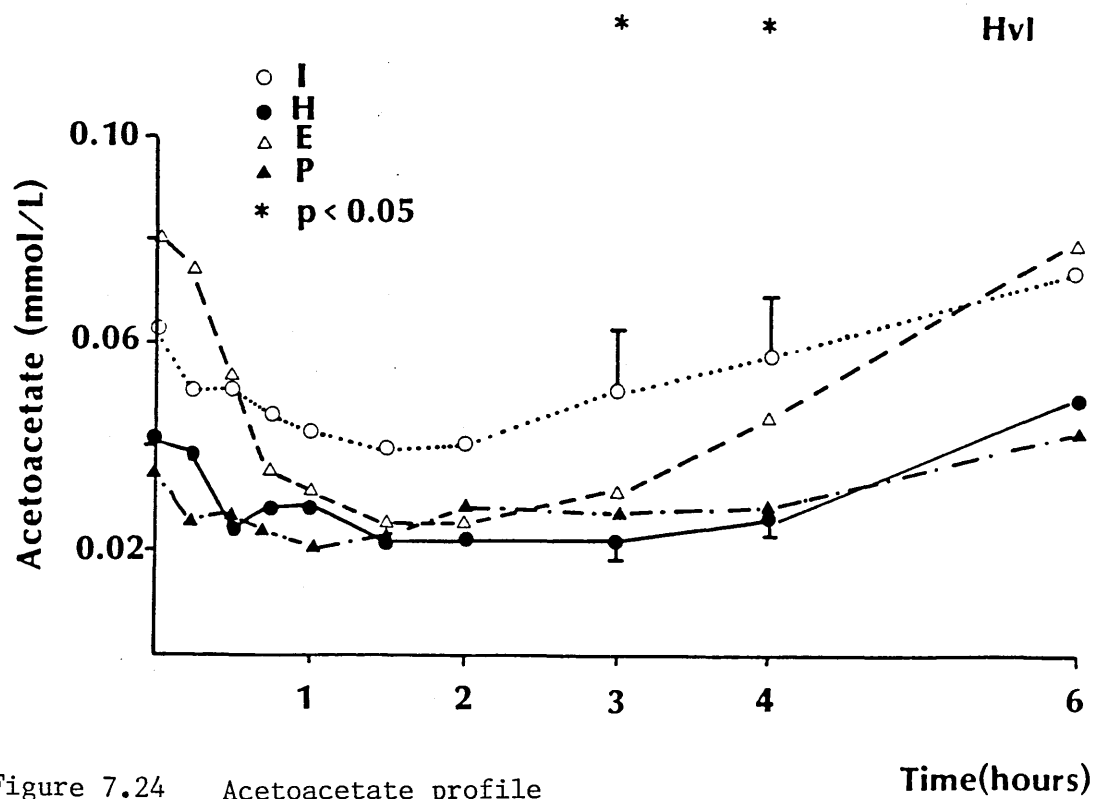
Figure 7.24   Acetoacetate profile

Time (mins)	0	15	30	45	60	90	120	180	240	360
Group										
I	1.4	1.7	1.4	1.3	1.1	0.9	1.0	1.4	1.5	1.5
H	1.6	1.4	1.9	1.5	0.6	0.6	0.5	0.6	0.7	0.9
N	1.3	1.5	1.2	1.2	1.0	0.5	0.5	0.5	1.0	1.4
E	1.2	1.1	1.3	1.4	1.0	0.8	0.6	0.5	0.9	1.0
P	0.8	1.5	1.2	1.3	1.6	1.1	1.0	0.9	1.0	1.1
D	1.3	1.3	1.5	1.3	1.4	1.5	1.4	1.3	1.2	1.1
OPD	-	-	-	-	-	-	-	-	-	-

TABLE 7.VI   3 Hydroxybutyrate/Acetoacetate ratios during CAPD.

(H) and peritonitis groups. The increment from the mid cycle trough to the end of the dwell was more prominent with the low glucose dialysate than with hypertonic fluids. The difference in 3-hydroxybutyrate concentrations between the isotonic (I) and the hypertonic (H) groups was significant ($p < 0.01$) in the latter half of the cycle.

Acetoacetate: Acetoacetate (Figure 7.24) responded similarly to 3-hydroxybutyrate. All groups showed a significant fall from high fasting concentrations to trough levels at about 1 hour ($p < 0.05$). There was no difference between any of the groups, with the exception of higher acetoacetate concentrations in the latter half of the cycle in the isotonic (I) compared to the hypertonic (H) group ($p < 0.05$).

3-hydroxybutyrate:acetoacetate ratio: The 3-hydroxybutyrate: acetoacetate ratio (Table VI) was essentially normal (1:2) throughout most of the dialysis cycle in most groups. However, the hypertonic (H) and elderly (E) groups had low ratios in mid cycle due to the relatively greater decrease in 3-hydroxybutyrate.

Glycerol: Blood glycerol concentrations (Figure 7.22) decreased ($p < 0.05$) in all groups in the first 60 minutes and then gradually returned towards basal levels particularly during the last 2 hours of the dialysis dwell. The hypertonic solutions appeared to have a more suppressive effect on glycerol but there was no significant difference between any of the groups.

A summary of the glucoregulatory hormone and intermediary metabolite changes induced by peritoneal glucose absorption during a

TABLE 7. VII Summary of changes in hormones and intermediary metabolites during CAPD

	ISOTONIC (I)	HYPERTONIC (H)	ELDERLY (E)	PERITONITIS (P)
Glucose	↑	↓	↓	↓↓
Insulin	↑	↓	↓	↓↓
C-Peptide	↑	↓	↓	↓↓
Glucagon	↑	→	→	(→↓)
Growth Hormone	↑↑	↑↑	↑↑	↑↑
Cortisol	↑	↑↓	↑↓	↑↓↓
Lactate	↓	↓	↓	↓↓
Pyruvate	↓	↓	↓	↓↓
Alanine	↑	↑	↑	↑↑
3OH-Butyrate	→	→	→	→→
Acetoacetate	→	→	→	→→
Glycerol	→	→	→	→→

→ little or no change, ↑ increase, ↑↑ marked increase, ↓ decrease

dialysis cycle are shown in Table 7.VII. Generally, greater changes
were observed with hypertonic than isotonic fluid. The presence of
peritonitis showed a greater increase in glucose, insulin, c-peptide,
lactate and pyruvate compared to other patients given similar
hypertonic solutions.

4. HORMONAL AND METABOLITE CHANGES IN DIABETICS ON CAPD

a) Post-Absorptive State

Fasting serum total cholesterol and triglycerides were increased
($p<0.001$) and HDL-cholesterol decreased ($p<0.01$) in diabetics on CAPD
compared to controls (Table 7.II). Fasting blood glucose was higher
(7.3 ± 1.5 mmol/1), but due to the wide individual variation this was
not significantly different from controls. Serum free (unbound)
insulin was measured in diabetics and, therefore, not directly
comparable with the total insulin measured in non-diabetics. Fasting
c-peptide, glucagon, growth hormone and cortisol were not
significantly different from controls. The mean fasting c-peptide
concentration was within the normal range but this was due to 2
patients having high basal levels and 4 having low concentrations.
Lactate, pyruvate and alanine were normal but the ketone bodies
(3-hydroxybutyrate and acetoacetate) and glycerol were increased in
diabetics ($p<0.05$).

b) During a Hypertonic Dialysate Cycle

Changes in hormone and metabolite concentrations in diabetics
during a hypertonic exchange are shown in Figures 7.25 to 7.35 (values
are mean and SEM). It should be noted that no exogenous insulin
supplementation was given prior to or during the dialysis cycle. The
non-diabetic group used for comparison with the diabetics was the

hypertonic (H) group.

Glucose: Blood glucose (Figure 7.25) increased from 7.3 to 14.5
mmol/l in the first 90 minutes (p<0.05) and then reached a plateau
over the remainder of the cycle. There was no decline in glucose
during the 6 hour cycle. The difference in glucose concentrations
were significant between diabetic and non-diabetics from 1 hour
onwards (p<0.05).

Insulin: Diabetics showed no change in free (unbound) insulin
(Figure 7.26) in contrast to the endogenous insulin release in
non-diabetics with hypertonic dextrose solution. Accepting the
limitations of comparing free with total insulin, it can nevertheless
be seen that there was an obvious difference in insulin response
between the groups (p<0.05), particularly during the first 2 hours of
the dialysis exchange.

C-peptide: Plasma c-peptide concentrations (Figure 7.27) remained
unchanged in diabetics throughout the hypertonic exchange. In
contrast, non-diabetics had a progressive increase in c-peptide in the
first half of the cycle and then remained persistently elevated in the
second half of the exchange. Significant differences were found
between the 2 groups throughout (0.001<p<0.05). The diabetics had a
mean c-peptide value of about 1 mmol/l. This was due to 2 patients
having concentrations in excess of 2 mmol/l and 3 patients having
negligible concentrations. However, none of the diabetic patients
showed any tendency to increase their c-peptide with peritoneal
glucose absorption.
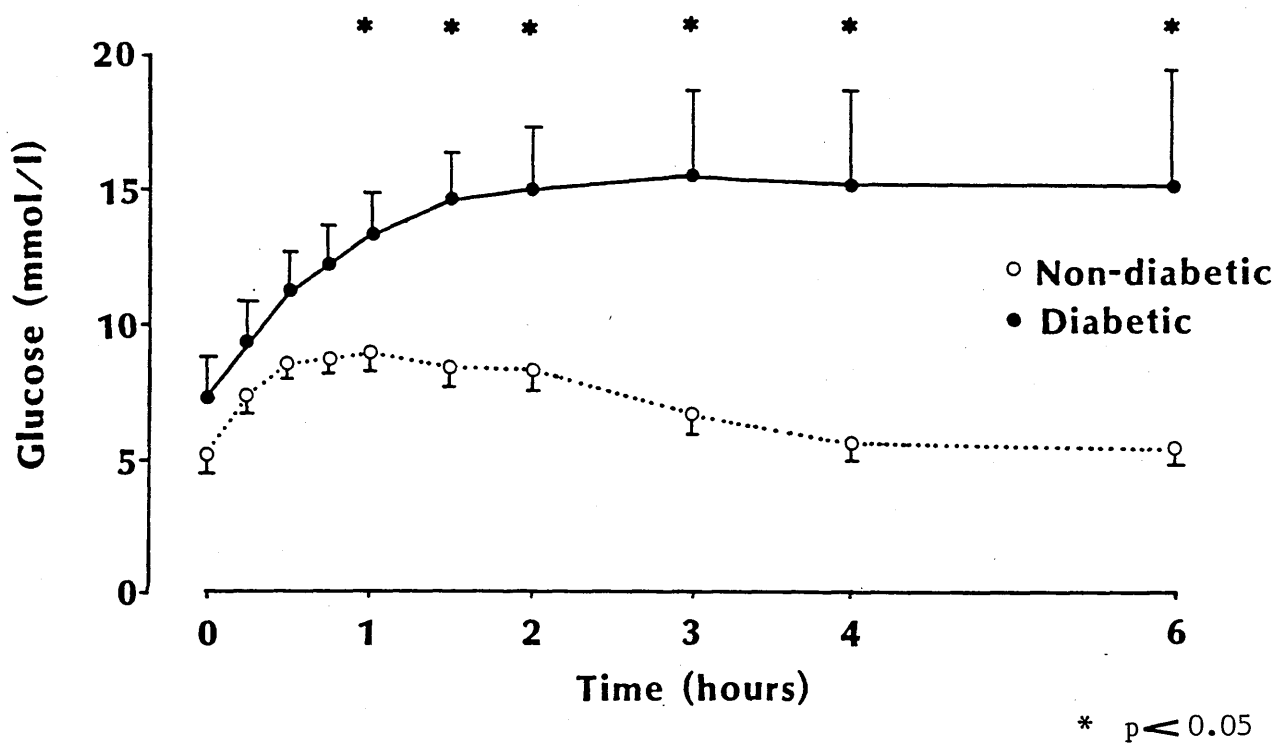
Figure 7.25   Diabetic: Glucose profile

*   p < 0.05

*
*   p < 0.01
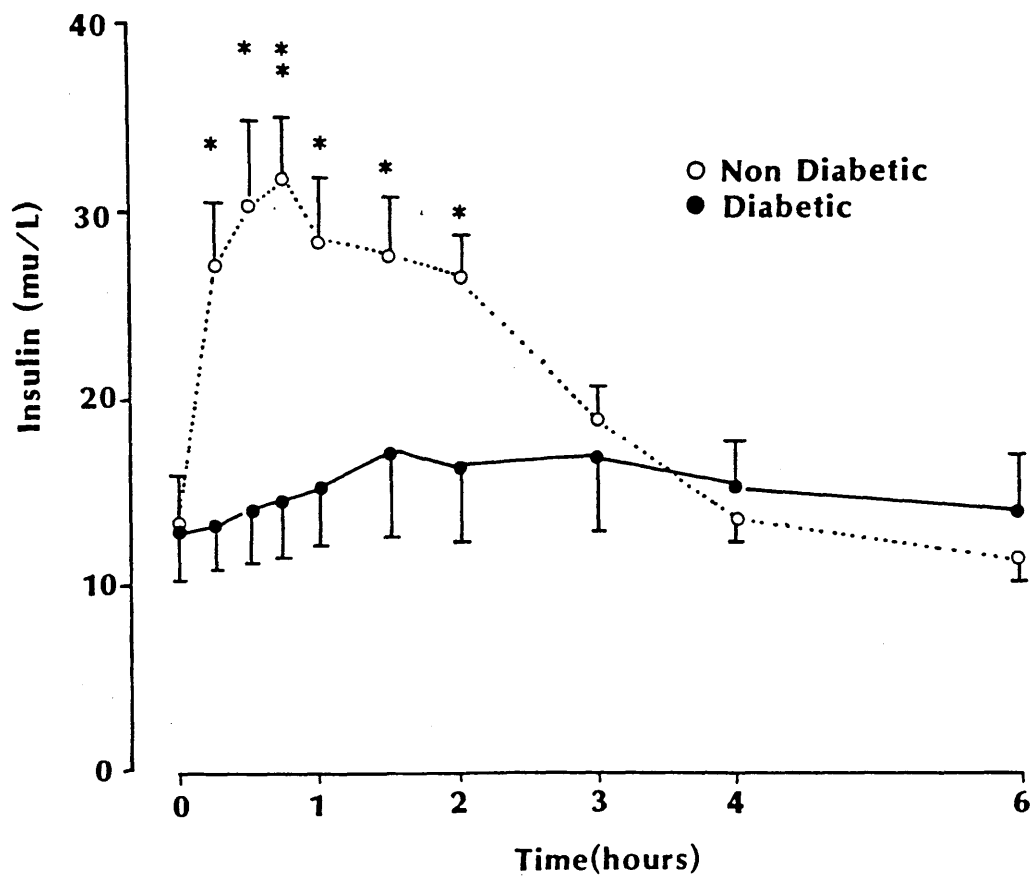
Figure 7.26   Diabetic: Insulin profile
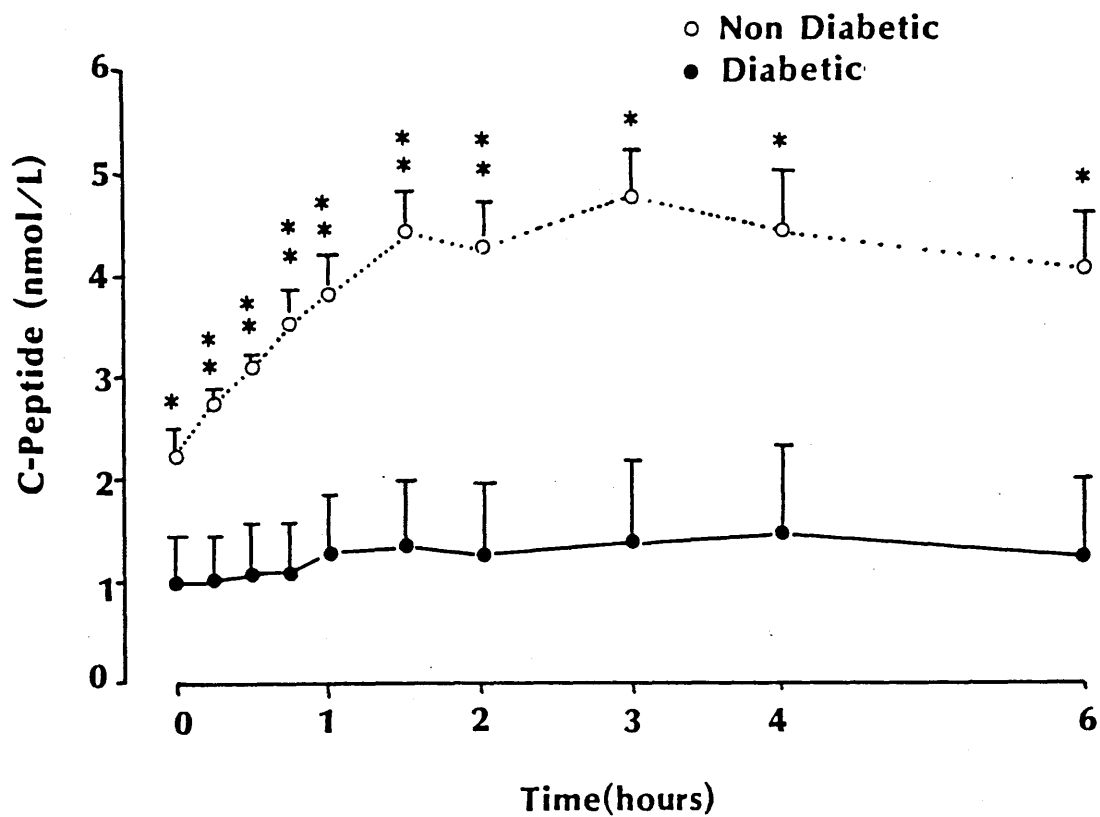
Figure 7.27 Diabetic : C-Peptide profile

* $p < 0.05$
* * $p < 0.01$
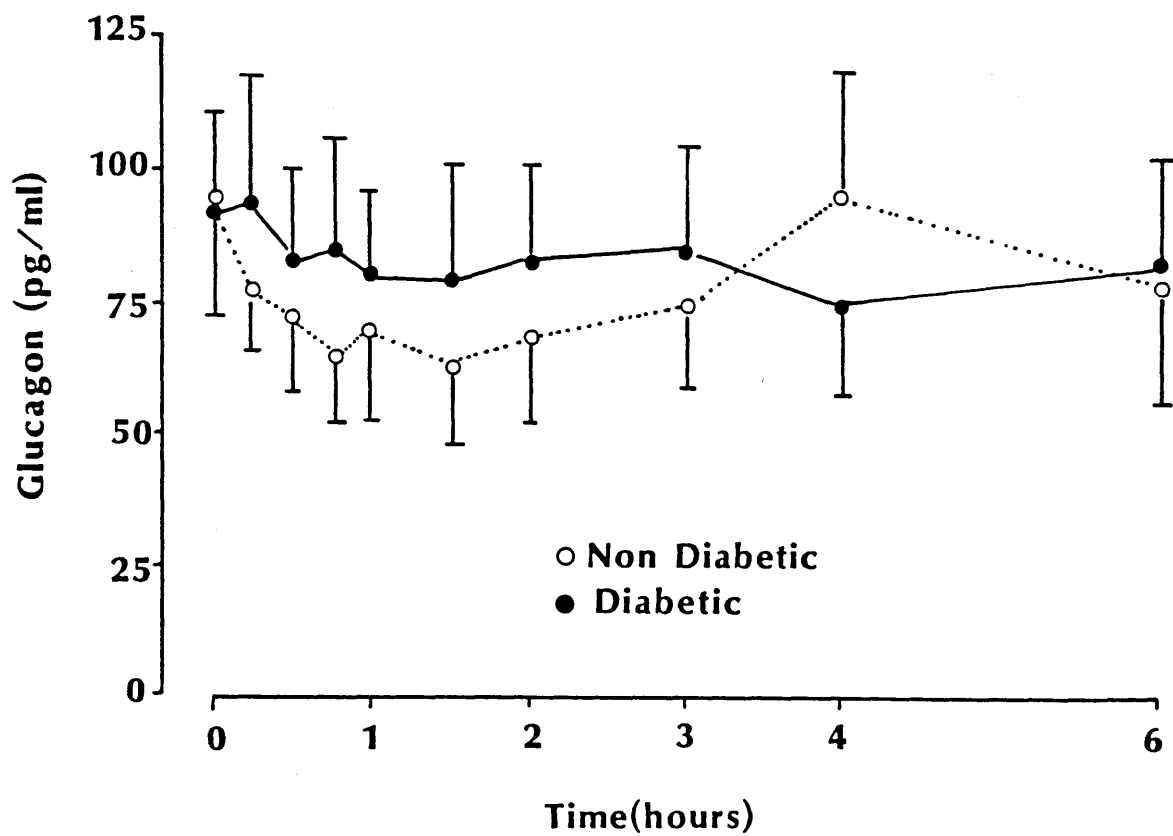
Figure 7.28 Diabetic : Glucagon profile

Glucagon: Plasma glucagon (Figure 7.28) showed no change in diabetics throughout the dialysis cycle. Although the non-diabetic group had a lower concentration of glucagon in the first half of the cycle there was no significant difference between the groups.

Growth hormone and cortisol: Serum growth hormone and plasma cortisol (Figure 7.29) did not show any change during the 6 hour dwell and there was no difference in either hormone between diabetics and non-diabetics.

Lactate: Blood lactate (Figure 7.30) in diabetics increased ($p < 0.05$) over the first hour of dialysis and gradually decreased from 2 to 6 hours. This was similar to non-diabetics and no difference was found between the groups.

Pyruvate: Pyruvate (Figure 7.31) did not show any significant change in diabetics over the 6 hour exchange. There was no difference between diabetics and non-diabetics throughout the cycle. The lactate:pyruvate ratio was essentially normal in diabetics throughout the exchange (Table 7.V).

Alanine: Blood alanine (Figure 7.32) did not show any change during the dialysis cycle and there was no difference between diabetic and non-diabetic patients.

3-hydroxybutyrate: 3-hydroxybutyrate (Figure 7.34) remained elevated and did not change in diabetics during the hypertonic glucose load. In contrast, the non-diabetics showed a decrease from fasting concentrations to a trough level in 1 hour, although the decrement was
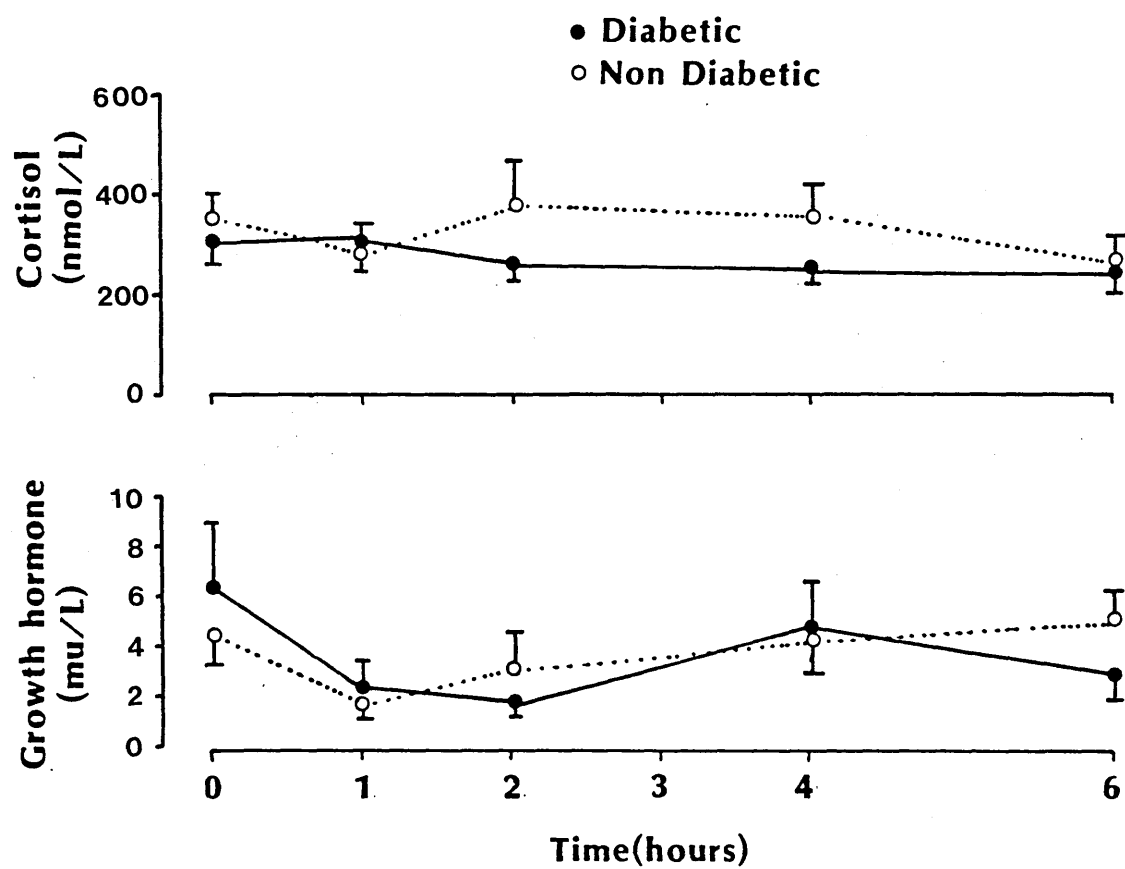
Figure 7.29   Diabetic: Growth hormone and Cortisol profiles
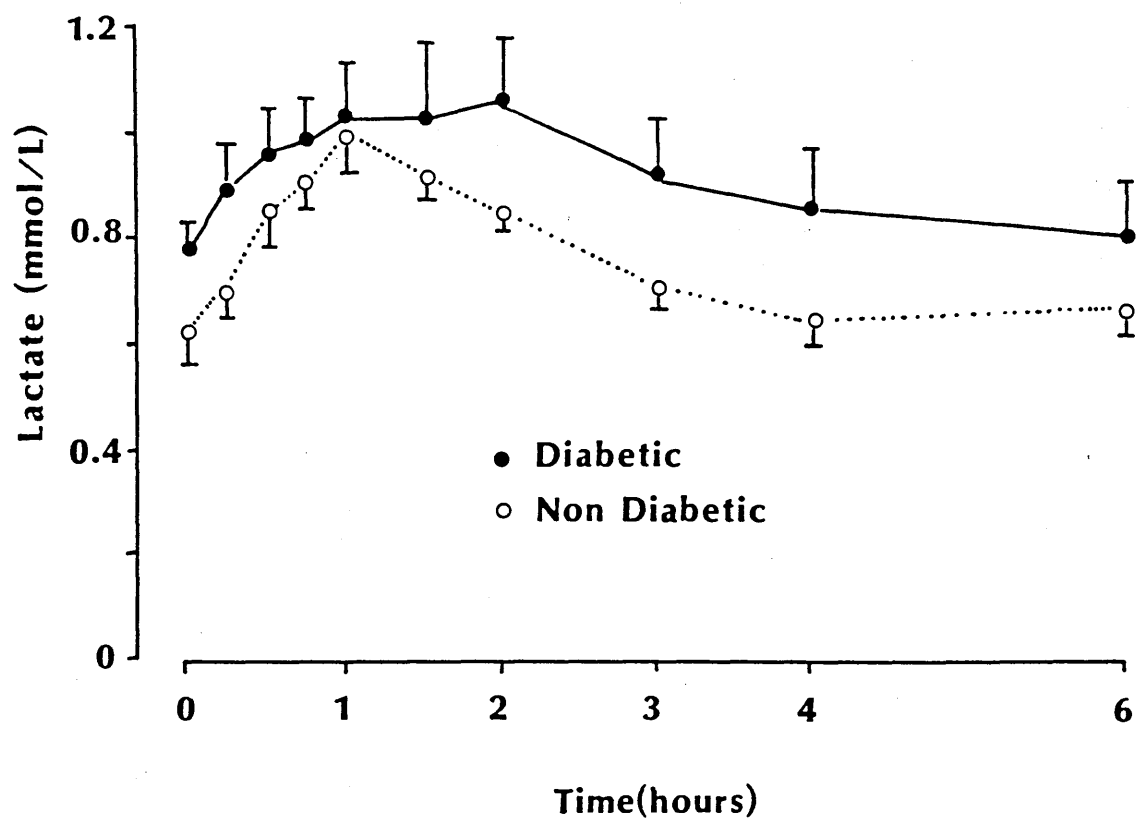
Figure 7.30   Diabetic: Lactate profile
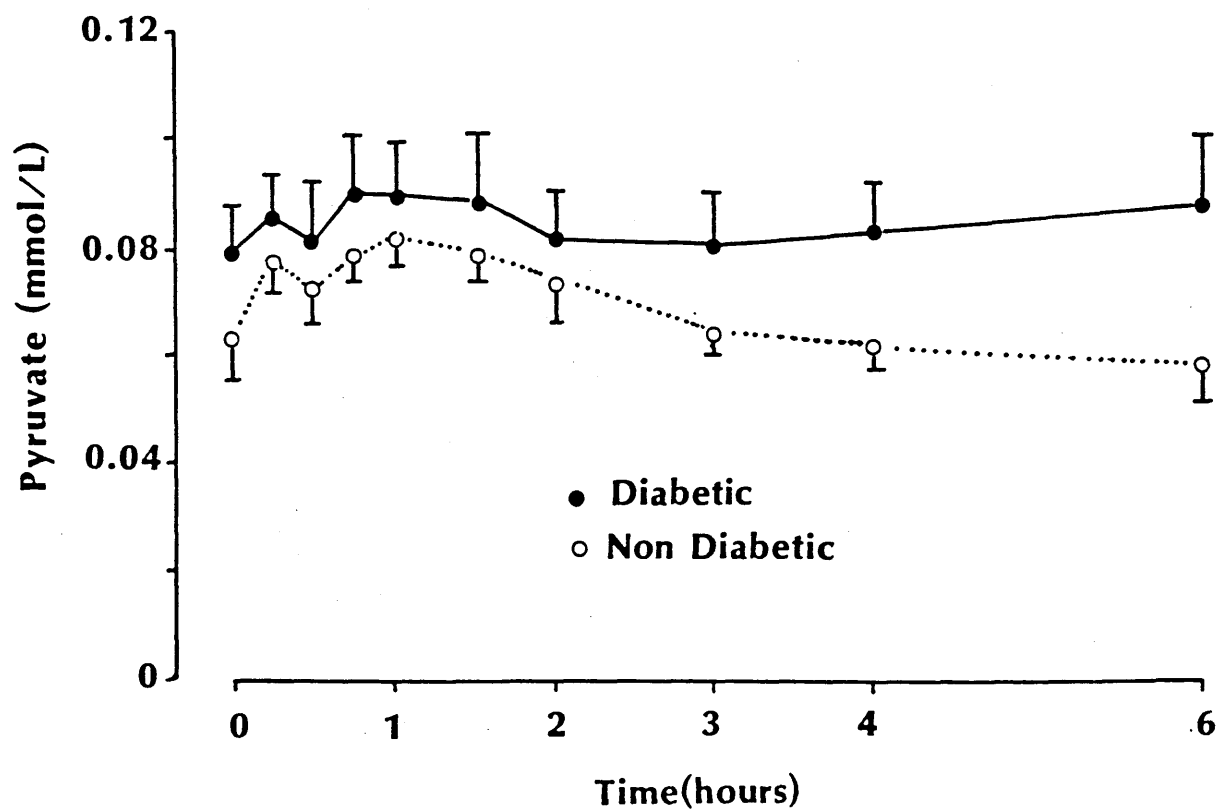
Figure 7.31   Diabetic: Pyruvate profile
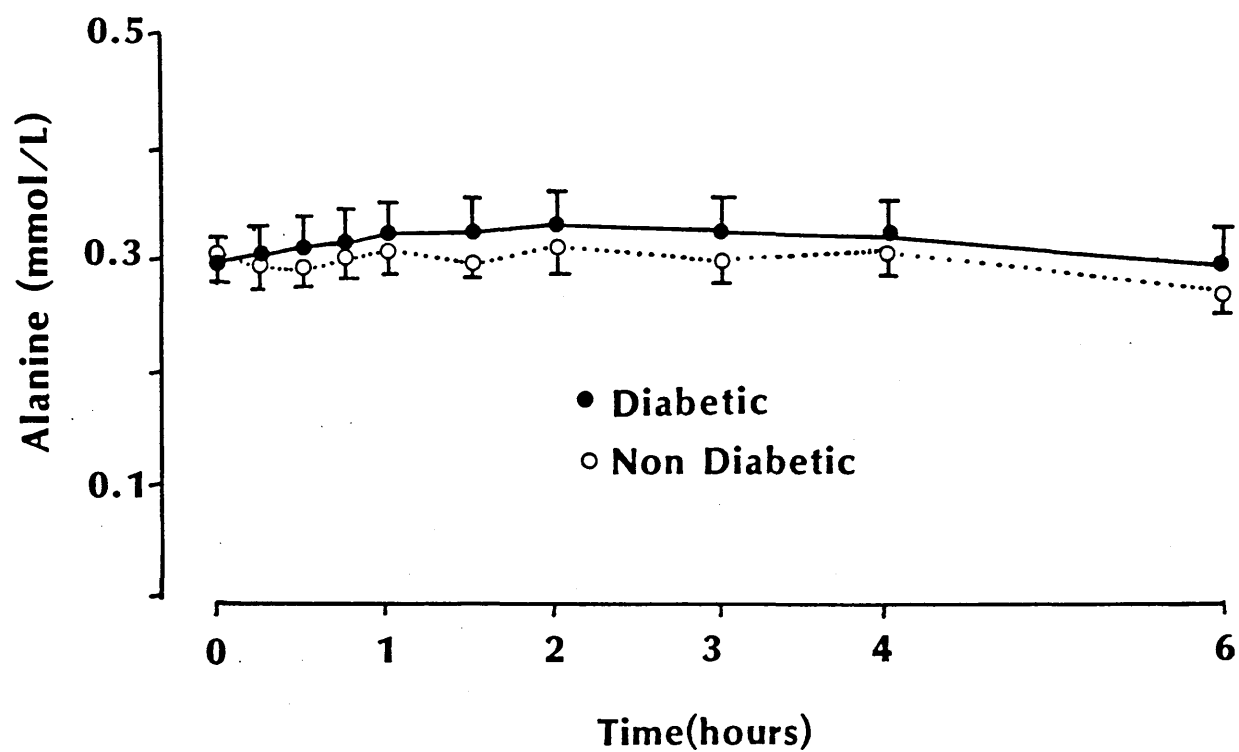
Figure 7.32   Diabetic: Alanine profile
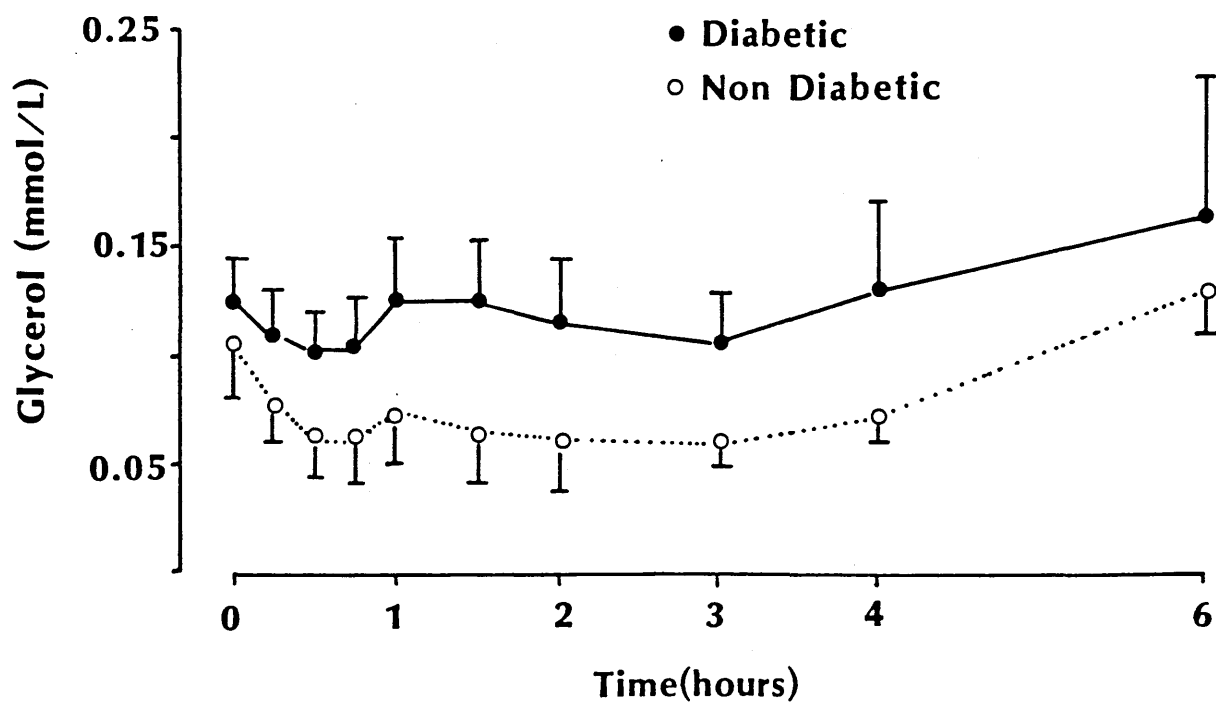
Figure 7.33   Diabetic: Glycerol profile
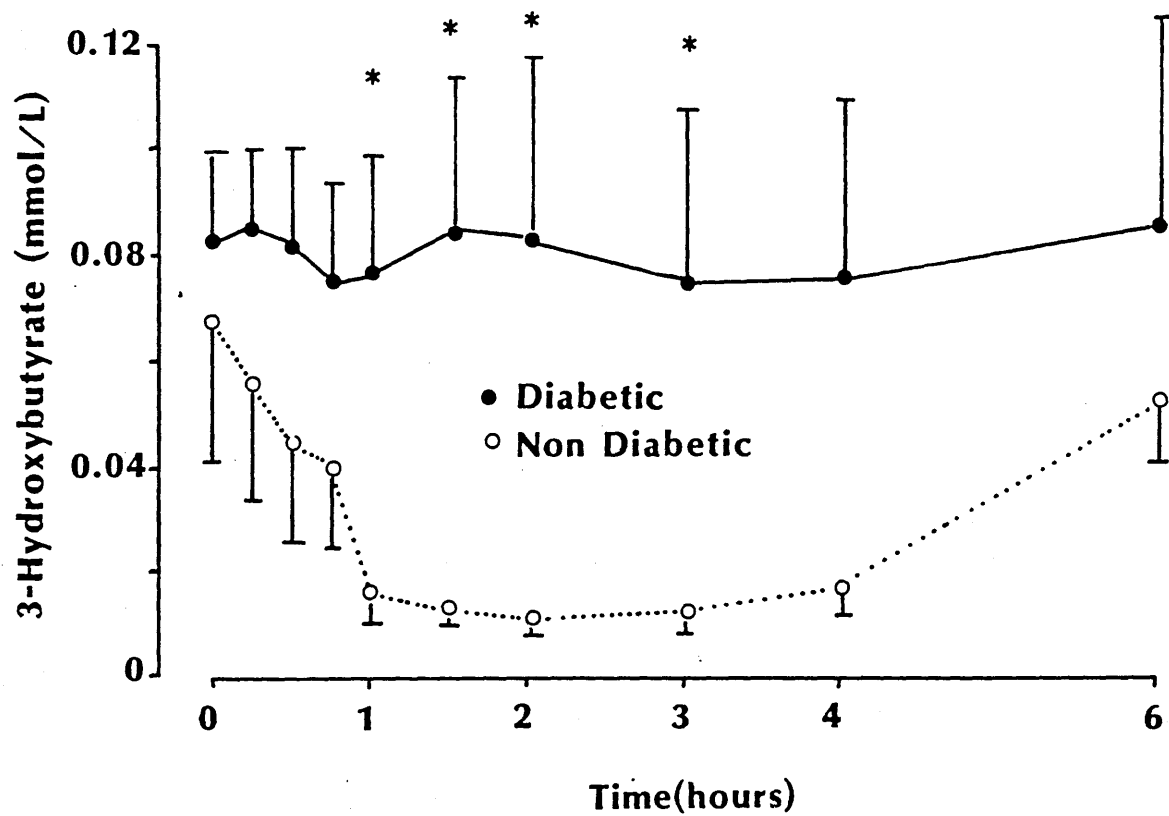
Figure 7.34   Diabetic: 3-Hydroxybutyrate profile

* $p < 0.05$
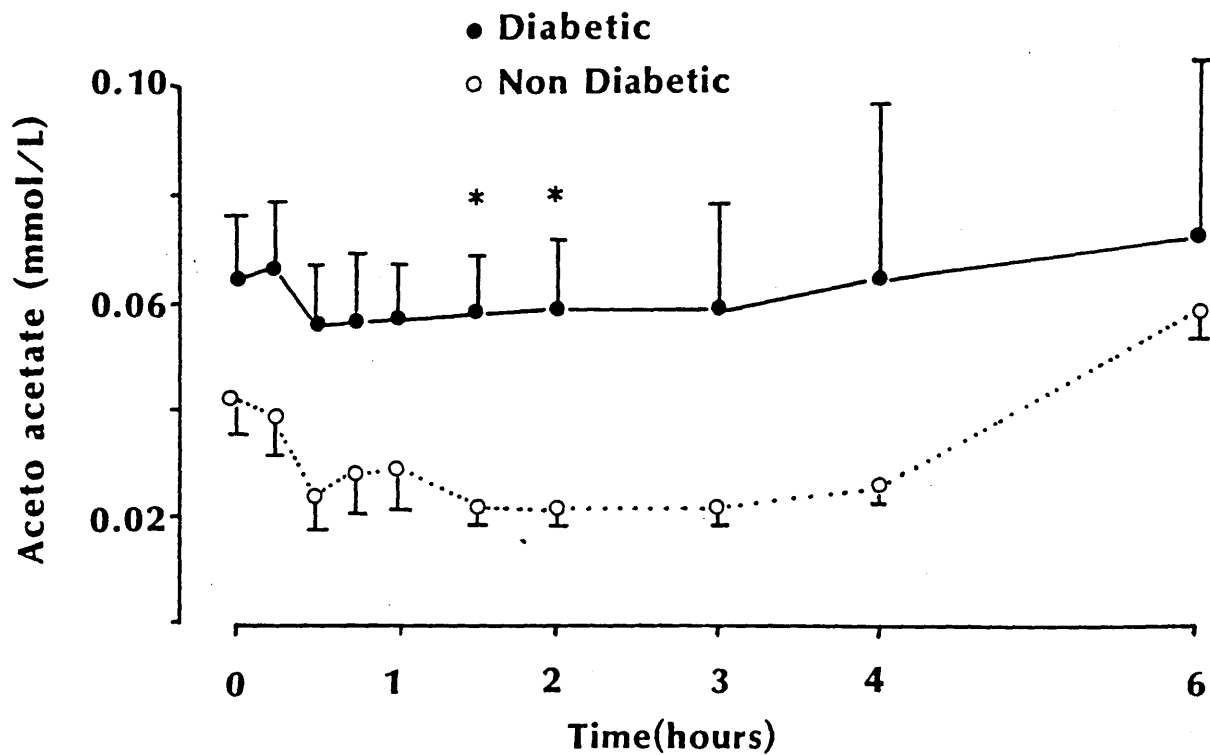
Figure 7.35   Diabetic: Acetoacetate profile

not statistically significant due to the large variation in basal concentrations. However, diabetics had significantly higher (p<0.05) 3-hydroxybutyrate concentrations between 1 and 3 hours than non-diabetics.

Acetoacetate: Acetoacetate (Figure 7.35) responded similarly to 3-hydroxybutyrate. In diabetics acetoacetate remained persistently elevated throughout the hypertonic exchange. In contrast non-diabetics showed a decrease (p<0.05) within the first hour. There was marked variation in acetoacetate concentrations in the diabetic group particularly towards the end of the cycle. Significant differences (p<0.05) were found between the groups at 1 to 2 hours of dialysis dwell.

The 3-hydroxybutyrate:acetoacetate ratio remained essentially normal in diabetics (Table 7.VI). In contrast the non-diabetics (i.e. hypertonic H group) showed a decrease in the ratio between 1 and 3 hours of the cycle due to a relatively greater fall in 3-hydroxybutyrate.

Glycerol: Glycerol (Figure 7.33) showed no change during the dialysis cycle in diabetics and concentrations remained persistently elevated. In contrast the non-diabetics had a small but significant fall (p<0.05) in glycerol in the first half of the cycle. There was, however, no significant difference in glycerol concentrations between the two groups throughout the dialysis dwell.

A summary of the hormone and metabolite changes in diabetics in the post absorptive (fasting) state and during a high dextrose peritoneal dialysis exchange are shown in Table 7.VIII. As previously

	FASTING		DURING CAPD	
	NON DIABETIC	DIABETIC	NON DIABETIC	DIABETIC
Glucose	↑	←	←	←
Insulin	↑	(↑)	→←	↑
C-Peptide	←	→	→	↑
Glucagon	←	←	↑	↑
Growth Hormone	↑	↑	↑	↑
Cortisol	↑	↑	←	←
Lactate	↑	↑	←	↑
Pyruvate	↑	↑	↑	↑
Alanine	↑	↑	→	↑
3OH-Butyrate	↑	←	←	↑
Acetoacetate	↑	←	→	↑
Glycerol	↑	←	→	↑

TABLE 7. VIII

Summary of hormone and metabolite changes in diabetics.
Comparison of non-diabetic and diabetic patients in fasting
state and effect of CAPD. Changes in non diabetics in fasting
state are compared to controls.

← increase, ↓ decrease, → little or no change

stated, the non-diabetics were patients in the hypertonic (H) CAPD subgroup.

In the fasting state glucose was increased and c-peptide was decreased in diabetics compared to non-diabetics on CAPD. Glucagon was marginally increased in both diabetics and non-diabetics though not significantly different from healthy subjects. Fasting growth hormone, cortisol, lactate, pyruvate and alanine were unchanged in both CAPD groups, while basal ketone bodies and glycerol were increased in diabetics.

During the hypertonic (42.5 g/1) dextrose peritoneal load the changes due to glucose occurred generally in the early part of the cycle. Blood glucose increased but serum free insulin, c-peptide and glucagon remained unchanged in diabetics. Growth hormone and cortisol were unaltered in both groups. Lactate showed a small increase but pyruvate and alanine did not change in diabetics. In contrast to non-diabetics the ketone bodies and glycerol were not suppressed by peritoneal glucose in diabetics.

5. DIALYSATE, GLUCOSE AND ULTRAFILTRATION

The ultrafiltration volume (UFV) was calculated from the difference in weight between the pre- and post-dialysis fluid. Glucose and lactate were also measured in the dialysate pre- and post-exchange. The ultrafiltration volumes and dialysate glucose concentrations are shown in Figure 7.36 (values are mean and SEM). The isotonic (15 g/1) solution had a measured pre-dialysis glucose of 81.0+0.7 mmol/1 and a post-dialysis glucose of 24.3+2.2 mmol/1. The ultrafiltration volume was 150+50 ml for the isotonic group. The pre-dialysis glucose concentration for the hypertonic (42.5 g/1) solution was 224.6+52.4 mmol/1 and the post-dialysis concentration was
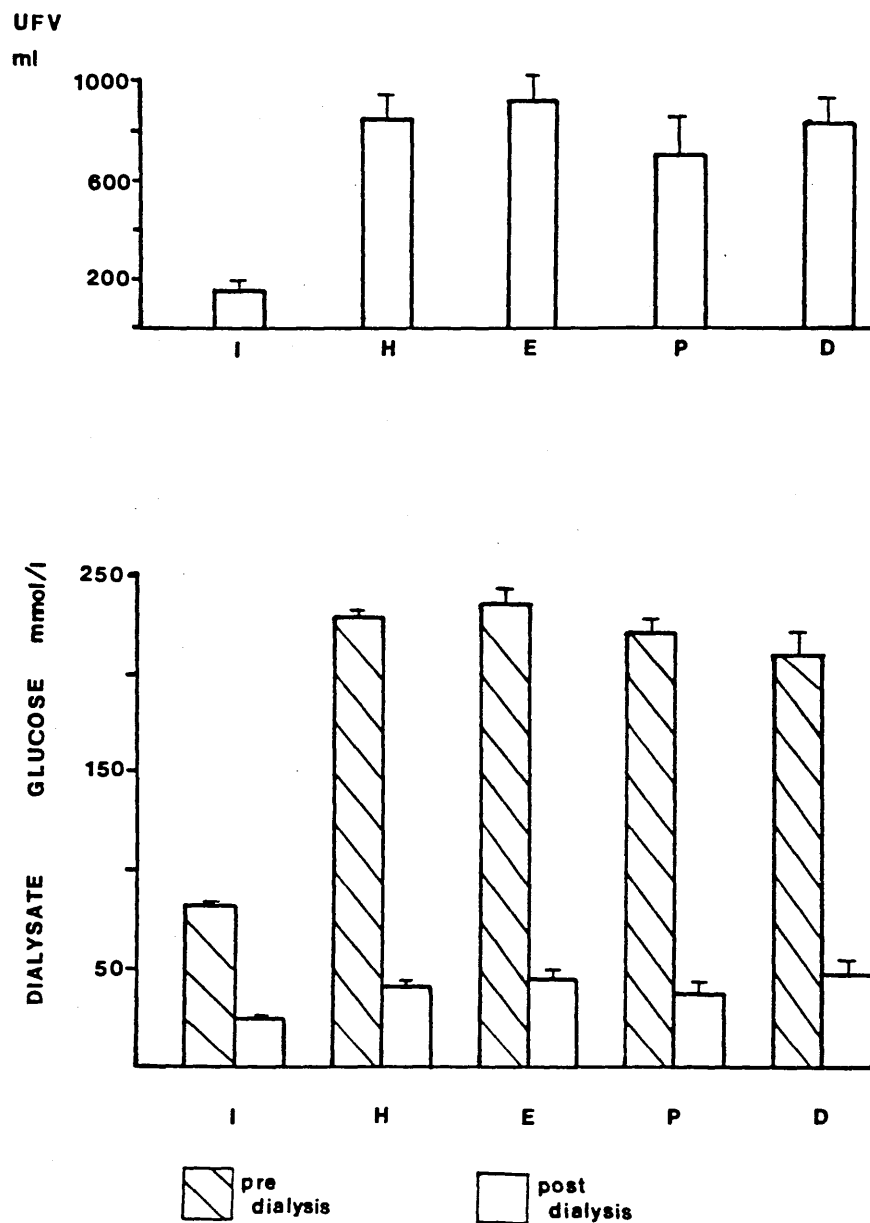
Figure 7.36

Ultrafiltration volumes and dialysate glucose
concentrations in each CAPD sub-group

42.5+2.0 mmol/l. These values were the mean of 30 hypertonic

solutions. The mean ultrafiltration volume for the hypertonic groups

varied between 678 ml and 923 ml for the 6 hour dwell. Dialysate

effluent glucose concentrations were similar in all hypertonic groups

and although the ultrafiltration volume was lower in the peritonitis

group this was not significantly different from the others.

It should be noted that a "2 litre" dialysis fluid bag contained

marginally more than 2000 ml. A 2 litre 1.5% (isotonic) bag weighed

about 2100g and a 2 litre 4.25% (hypertonic) bag weighed about 2150g.

This data was derived from over 100 separate bag weighings including

the 42 bags used in this study. However, the marginal excess does not

affect the results and was consistent throughout.

The amount of glucose and lactate absorbed from 2 litres of

dialysis fluid over a 6 hour cycle is shown in Table 7.IX (values are

mean and SEM). The isotonic fluid (15 g/l dextrose) resulted in 21g

(70%) of glucose being absorbed while the hypertonic solutions (42.5

g/l dextrose) resulted in absorption of between 65 and 71g of glucose

(76-84%). Lactate absorption, 6.6-6.9g (84-88%), was uniform in all

patients.

Changes in total body weight was a relatively insensitive marker

of ultrafiltration capacity over a single dialysis cycle. The

decrease in body weight and measured ultrafiltration volume are shown

in Table 7.X. No significant correlation was found between change in

body weight and ultrafiltration volume.

Ultrafiltration showed considerable variability within each group.

Individual ultrafiltration volumes are plotted against the amount of

glucose absorbed over 6 hours (Figure 7.37). Apart from the obvious

difference between the isotonic and hypertonic fluids, ultrafiltration

varied from individual to individual even though the amount of glucose

absorbed was fairly constant (approximately between 60 and 80g with

hypertonic solutions). No significant linear correlation was found

between the quantity of glucose absorbed and ultrafiltration capacity

for any one group or even all groups combined.

GROUP	GLUCOSE (g)	ABSORPTION (%)	LACTATE (g)	ABSORPTION (%)	UFV (ml)
Isotonic	21.0 (0.9)	69.9 (2.9)	6.7 (0.1)	85.9 (1.2)	150 (50)
Hypertonic	69.8 (2.1)	82.1 (2.4)	6.9 (0.1)	87.8 (1.4)	842 (109)
New	68.0 (3.3)	80.0 (3.9)	6.8 (0.1)	86.1 (1.1)	855 (118)
Elderly	69.4 (1.6)	81.7 (1.9)	6.7 (0.1)	85.5 (0.9)	923 (104)
Peritonitis	70.9 (2.9)	83.4 (3.4)	6.9 (0.2)	87.6 (1.9)	678 (165)
Diabetic	65.7 (3.4)	77.3 (4.0)	6.9 (0.2)	87.9 (2.6)	835 (110)
OPD	64.9 (1.5)	76.4 (1.8)	6.6 (0.1)	83.7 (1.3)	813 (151)

TABLE 7. IX   Dialysate glucose and lactate absorption and ultrafiltration

Dialysis fluid: volume 2 litres, dwell 6 hours
Isotonic solution (15 g/l dextrose), hypertonic solution
(42.5 g/l dextrose); lactate 3.9 g/l (35 mmol/l) in all
solutions.
Values are mean and (SEM)

GROUP	WEIGHT (Kg)	UFV (ml)
I	− 0.08 (0.08)	150 (50)
H	− 1.17 (0.21)	842 (109)
N	− 1.17 (0.21)	855 (118)
E	− 0.92 (0.16)	923 (104)
P	− 0.92 (0.08)	678 (165)
D	− 1.00 (0.26)	835 (110)
OPD	− 1.00 (0.26)	813 (151)

TABLE 7. X   Change in body weight and ultrafiltration volume after 6 hour dialysis cycle
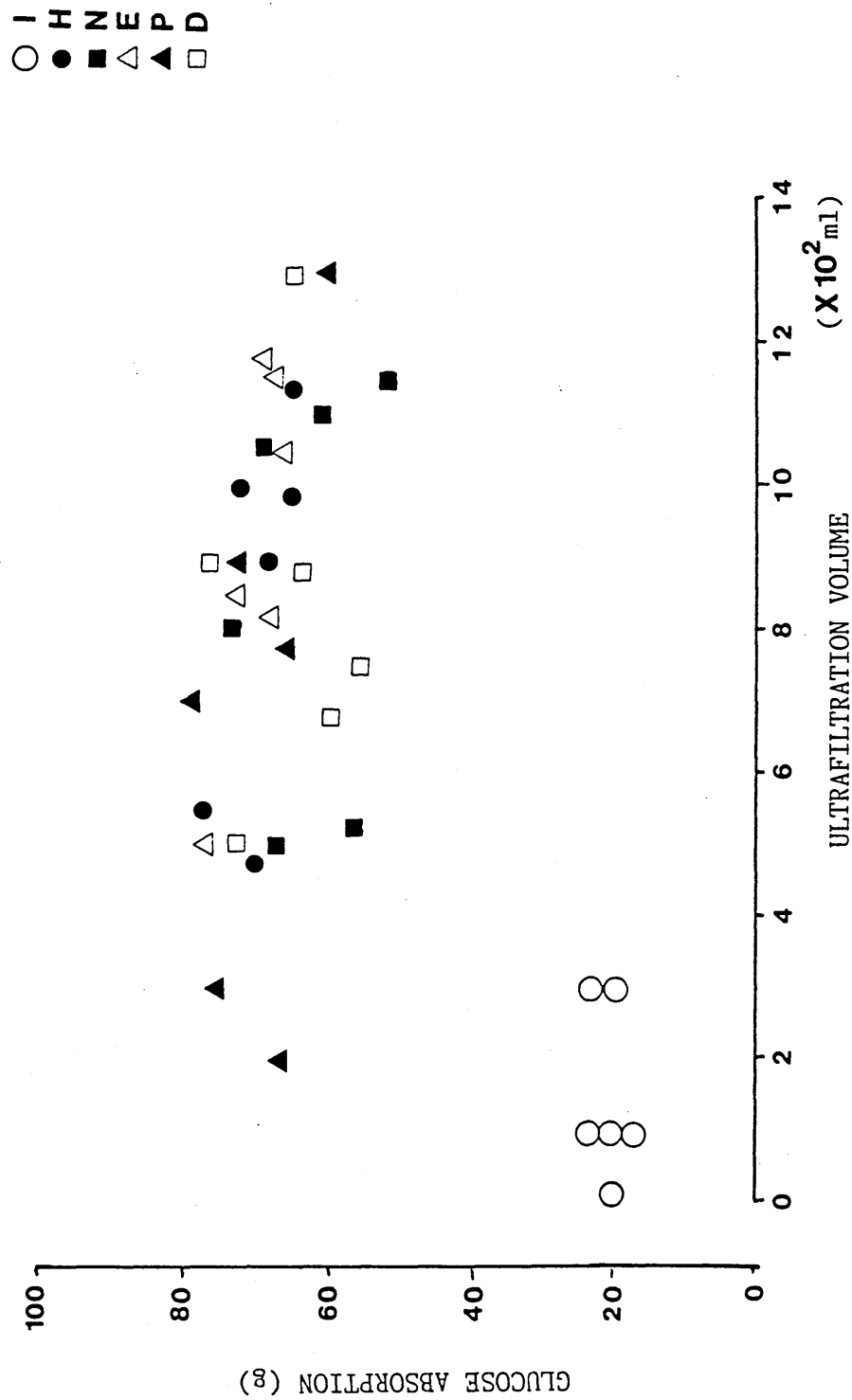
Figure 7.37 Ultrafiltration volume and glucose absorption in each CAPD subgroup

DISCUSSION

The study is discussed in 5 sections in a similar format to the results. Firstly, some comments on the design of the study followed by discussion of the metabolic variables in the post-absorptive state and then during the peritoneal dialysis cycle. The penultimate section discusses diabetics on CAPD and finally comments about dialysate changes and ultrafiltration.

1. GENERAL

Glucoregulatory hormones and intermediary metabolites were investigated in a 'mixed uraemic population' in a fasting state to determine if any specific changes were due to the type of dialysis rather than uraemia per se. Metabolic variables were assessed in CAPD during a 6 hour dialysis cycle to determine the effects of peritoneal glucose absorption. The CAPD population was divided to evaluate factors such as age, diabetes and peritonitis which might induce further metabolic changes.

This work was performed at a large centre for CAPD but the number of patients suitable for investigation was nevertheless limited. To date no large, in depth, metabolic study has been published on CAPD. The few published reports of hormone and/or metabolite changes in CAPD have been limited to 5 patients in each study. The variability of metabolite and hormone concentrations even in normal healthy subjects is well recognised (Foster et al., 1978) and thus some caution is needed in interpretating changes in the metabolic variables.

Values of hormones and metabolites tended to have a normal or slightly skewed distribution but log transformation was not performed in the analysis. Comparison between the groups was done by analysis of variance and p values were derived using the pooled standard

deviation for all groups. However, accepting the statistical restrictions comparison between the sub-groups was necessary to assess specific clinical factors. The possibility of a type I error is increased by the numerous variables and patient groups studied.

2. FASTING STATE

Hyperlipdaemia was present in all uraemic patients. Fasting serum total triglycerides were markedly increased in all patients, but total cholesterol although increased in all was only significantly elevated in CAPD patients. HDL-cholesterol was also uniformly suppressed in uraemia. These results are in agreement with many previous studies (Olefsky et al., 1974; Sanfellipo et al., 1978; Cattran et al., 1976; Ramos et al., 1983). In this study higher cholesterol and triglyceride concentrations were found in the CAPD population compared to other uraemic patients. The difference could not be readily explained by age or body weight and there was no correlation with duration of CAPD therapy. Cholesterol and triglycerides have been reported to fall after 1 year on CAPD (Lindholm et al., 1983), which may indicate a metabolic adaption to the glucose load. It has been shown that the total carbohydrate intake in some CAPD patients is regulated by the spontaneous reduction of the oral carbohydrate intake (Von Baeyer et al., 1983). In the present study lipid status was assessed at a single point in time and in view of the well known individual variability in blood lipids, a longitudinal type study would be more appropriate to evaluate the effect of CAPD on hyperlipidaemia. Nevertheless, the marked hyperlipidaemia particularly in CAPD (partly carbohydrate induced and accompanied by glucose intolerance - type IV) is a major risk factor in atherosclerosis. If one then considers other factors, such as

hypertension and possibly hyperglycaemia and hyperinsulinaemia (Keen et al., 1965), uraemic and particularly CAPD patients are strong candidates for ischaemic heart and other vascular diseases.

In the post-absorptive state (after a 12 hour fast) blood glucose was normal in uraemic patients. This has been well documented (DeFronzo, 1973, 1978b). However, recent studies on CAPD patients have shown increased fasting glucose concentrations (Heaton et al., 1983, 1985), but in these studies the patients were not truly fasted for 12 hours as they had peritoneal dialysis overnight prior to the study. Glucose absorption from the overnight exchange could explain the increased blood concentrations. This was seen in the present study in patients who had an overnight exchange. CAPD is normally a continuous 24 hour process, but for comparison with other groups a basal reference state is necessary and thus CAPD patients had both an oral and peritoneal overnight fast prior to the study.

Fasting insulin has been reported as being increased, normal and decreased in uraemia. Normal basal levels have been found by many (Spitz et al., 1970; Roth et al., 1973; DeFronzo, 1978b), while others have recorded basal hyperinsulinaemia (Hutchings et al., 1966; Briggs et al., 1967; Horton et al., 1968). In the present study fasting serum insulin was increased in all uraemic groups compared to controls, but the difference was small and in most cases not significant. Haemodialysis patients had marginally higher insulin than non-dialysis uraemics and CAPD patients. Recent studies continue to show conflicting data. Haemodialysis patients after a 12 hour fast had insulin levels higher though not significantly different from controls (Dumbauld et al., 1983), while undialysed chronic renal failure patients had significantly higher insulin concentrations (Ricanati et al., 1983). However, on reviewing these studies the mean

fasting serum insulin concentrations were similar, but control insulin levels varied and this may have accounted for the different interpretations. In CAPD basal hyperinsulinaemia was found in 5 patients in each of Heaton et al. (1983, 1985) studies, but these patients did not have a true fast as they had peritoneal dialysis prior to the study. Interestingly, in the current study one of the two subgroups to have a significant increase in basal insulin was the group who had overnight peritoneal dialysis. Thus this may, in part, explain some of the divergence of data. Normal fasting insulin (Armstrong et al., 1985) and low fasting insulin concentrations (Von Baeyer et al., 1983) have also been observed in CAPD. Each of the above studies in CAPD evaluated only 5 patients. Larger studies are obviously needed. The low basal insulin concentrations were explained by peritoneal clearance of insulin (Von Baeyer et al., 1983), but in the present study no significant difference was found between haemodialysis and CAPD patients.

Thus with these conflicting reports what is the basal insulin state in uraemia and CAPD? There is certainly individual variability and perhaps some patients have normal and some increased basal insulin concentrations depending on their degree of glucose intolerance (DeFronzo, 1978b). In the present study 30 CAPD patients, in a truly fasted state had a mean basal insulin concentration within the normal reference range, which was not significantly different from controls. Four individuals had basal concentrations just out with the normal range. Thus, on balance, basal insulin was normal in CAPD. In addition to individual variability, standards of radioimmunoassay are important and it is not feasible to simply compare absolute concentrations of insulin found in different studies as the quality of standard and anti-sera are bound to vary. It is interesting that

earlier studies found hyperinsulinaemia more frequently than later
ones. Currently available assays are more sensitive, specific and
precise compared to methods of 10-15 years ago, when cross-reactivity
with proinsulin was a major problem and tended to overestimate serum
insulin concentrations. In the present study the assay method used a
double antibody technique with inter- and intra-assay coefficients of
variation of between 5% and 8%. The assay was part of the
supraregional assay service (SAS) and subject to stringent quality
control procedures. There was no significant cross-reactivity with
proinsulin.

C-peptide was grossly elevated in all uraemics and confirms the
findings of Chapter 6 on beta cell function. Elevated c-peptide
concentrations have been documented in uraemia (Jaspan et al., 1977)
and increased levels have also been reported in CAPD (Wideroe et al.,
1984). Raised circulating plasma c-peptide concentrations are due to
impaired renal degradation, the kidney being almost exclusively
responsible for metabolism and excretion (Jaspan et al., 1977).
Interestingly, haemodialysis patients had higher concentrations than
both non-dialysed chronic renal failure and CAPD patients but the
difference was not significant. It is possible that residual renal
function and peritoneal clearance of c-peptide may have contributed to
the small difference between the uraemic groups. It has been
suggested that CAPD may increase proinsulin production which could
lead to an increase in c-peptide (Wideroe et al., 1984). However,
although proinsulin concentrations are increased in uraemia due to
impaired renal degradation (Katz et al., 1973) increased production is
unlikely in CAPD as serum insulin concentrations were normal.
Furthermore, cross-reactivity between c-peptide and proinsulin during
the assay was less than 10% using the c-peptide antiserum M1230 (see

Chapter 5).

Fasting plasma glucagon was increased in all uraemic groups with significant differences observed in undialysed chronic renal failure, haemodialysis and some of the CAPD subgroups. Hyperglucagonaemia in uraemia has been documented and is largely due to reduced renal clearance (Emmanouel et al., 1976; Kuku et al., 1976) as pancreatic secretion is normal in uraemia (Lefebvre and Luyckx, 1975). However, in uraemia there is also a marked increase in proglucagon and other glucagon intermediates which are not biologically active (Emmanouel et al., 1976). The assay used in this study specifically measured c-terminal (pancreatic) glucagon but some interference with the biologically inactive 9000 Dalton precursor cannot be excluded. Although elevated, relatively lower concentrations of glucagon were found in CAPD patients than other uraemics. After overnight dialysis basal glucagon was also found to be increased in three small studies (Heaton et al., 1983, 1985; Armstrong et al., 1985), in agreement with results in this study. The small difference between CAPD patients and others may be due to peritoneal clearance of pancreatic glucagon.

Growth hormone concentrations were markedly variable in the uraemic population studied. Generally, no significant differences were observed in comparison with controls although haemodialysis patients and some CAPD patients had raised fasting concentrations. Growth hormone has been reported as normal (Marumo et al., 1979) and increased (Saaman and Freeman, 1970; Orskov and Christensen, 1971) in uraemia. In CAPD normal fasting growth hormone has also been observed (Von Baeyer et al., 1983; Heaton et al., 1985). However, the value of isolated concentrations is minimal and growth hormone status is best assessed by a dynamic test. The differences found in this study between haemodialysis and CAPD patients is small and probably

unimportant, although better middle molecule clearance of growth hormone (MW 21000) by CAPD is a possible explanation.

Fasting plasma cortisol was increased in all uraemic patients, but significant elevation was found in the chronic renal failure and haemodialysis patients but not in CAPD patients. Only a marginal increase was found in CAPD in agreement with others (Heaton et al., 1985). Considerable controversy exists regarding the pituitary-adrenocortical axis in uraemia. Although normal diurnal variation in plasma cortisol is described, frequent irregular bursts of cortisol secretion occurs interspersed with periods of non-secretion (Weitzman et al., 1971). Thus major variations in plasma cortisol can occur depending on the time of sampling. Cortisol conjugates accumulate in the plasma of uraemics and can cross-react with cortisol antibodies. This may lead to gross over-estimation (Nolan et al., 1981). Changes in cortisol binding protein which is reduced in uraemia can also influence plasma cortisol concentrations (Rosman et al., 1984). Thus, it is not surprising that conflicting results have been reported. Normal (Nolan et al., 1981) and increased (Wallace et al., 1980) fasting cortisol have been described. It is interesting, but purely speculative, that CAPD patients, who in this study had lower cortisols than other uraemics, may be able to clear cortisol conjugates via the peritoneum thus lowering the cortisol components measured by radioimmunoassay.

Patients new to CAPD had similar hormone concentrations as those well established on the technique. Elderly patients and those with peritonitis had similar fasting profiles as other CAPD patients. The use of an overnight dialysis cycle did affect some variables, such as increasing blood glucose, insulin, c-peptide and cortisol. Non-dialysed chronic renal failure patients differed from CAPD

patients in having higher glucagon and cortisol concentrations, while haemodialysis patients tended to have a generally increased profile with raised c-peptide, glucagon, growth hormone and cortisol. Although the difference between CAPD patients and other uraemics was usually small peritoneal clearance of middle molecules may have played a role.

Data on intermediary metabolites in uraemia are sparse and only one group has studied metabolites in CAPD (Heaton et al., 1983, 1985). Thus frequent reference is made to these studies which assessed metabolic profiles during a dialysis cycle and also 24 hour metabolic rhythms in 2 separate groups of 5 patients on CAPD. Values for circulating intermediary metabolites in healthy subjects have been documented (Alberti et al., 1975; Foster et al., 1978) and the mean and normal reference range for the variables measured in this study are given in the Appendix 1. The values are similar to those of Foster et al. (1978), who used the same semi-automated enzyme fluorometric assays as in this study. These authors found considerable variation in intermediary metabolites even in overnight fasted, resting, apparently healthy subjects. In the present study the precision of the assay was good. The inter- and intra-assay coefficients of variation were under 5% for all metabolites except 3-hydroxybutyrate which had a coefficient of variation of about 12%. Acetoacetate and pyruvate were assayed by a manual method, within 48 hours of sampling, due to their relative instability on storage. Precision was again satisfactory with coefficients of variation of about 5% (see Chapter 5).

The principle behind the assay of intermediary metabolites depends on changes in fluorescence resulting from alterations in NADH concentrations produced by enzyme action. Fluorescence that is

non-enzymatic must be taken into account to increase the accuracy of analysis. Serum from uraemic patients has a 'native' fluorescence, which correlates with the level of serum creatinine and can also be affected by drugs (Hadjivassiliou et al., 1984). Thus all samples were run with blanks (that is, omitting the enzyme-coenzyme stage in the assay), to exclude non-enzymatic or native fluorescence due to uraemia. Values for healthy control subjects were similar to previously reported normals (Alberti et al., 1975; Foster et al., 1978).

The three gluconeogenic precursors lactate, pyruvate and alanine were not significantly different from controls. The lactate:pyruvate ratios were also normal. Fasting blood lactate and pyruvate concentrations have been widely reported as normal in chronic renal failure, haemodialysis, intermittent peritoneal dialysis and after renal transplantation (Campanacci et al., 1968; Attman et al., 1979; Parrish, 1981; Nakao et al., 1982). In contrast, elevated fasting lactate has been observed in haemodialysis and CAPD (Dumbauld et al., 1983; Heaton et al., 1985) although pyruvate was normal. The reason why lactate was increased while pyruvate was normal in these latter studies is unclear, as they are theoretically in equilibrium. Fasting blood alanine was normal in all uraemic patients in this study and is in agreement with others (Randerson et al., 1981; Deferrari et al., 1983). In contrast, elevated basal alanine has also been reported in dialysis patients (Dumbauld et al., 1983; Heaton et al., 1985). However, in the study by Dumbauld et al. the control group had 'low' alanine concentrations compared to the usually accepted normal values. The circulating concentration of alanine depends on the balance between production and uptake. Increased hepatic uptake has been reported by some (Rubenfeld et al., 1978) and normal uptake by others

(DeFronzo and Felig, 1980). Thus, it is possible that both normal and increased alanine can occur in renal failure depending on the balance between production, utilization and uptake as well as other metabolic and hormonal events.

The ketone bodies, 3-hydroxybutyrate and acetoacetate, as well as glycerol which was used as an index of lipolysis, were generally increased in the fasting state in uraemia. 3-Hydroxybutyrate was higher in all uraemic patients compared to controls, but significant increases were observed only in the non-dialysis chronic renal failure group and the elderly CAPD patients. Data in the literature is again conflicting. 3-Hydroxybutyrate was increased in chronic renal failure (Ricanati et al., 1983), normal in haemodialysis (Dumbauld et al., 1983) and decreased in CAPD (Heaton et al., 1985). It is also documented that fasting ketone bodies increase with age (Foster et al., 1978) and in the present study the elderly patients had higher basal concentrations than the other CAPD patients. The effect of overnight dialysis in one study (Heaton et al., 1985) may have contributed to suppression of ketogenesis as the patients were not in a 'true' fasted state, thus explaining the lower basal ketone body concentrations. Acetoacetate was found to be normal in CAPD (Heaton et al., 1985) and decreased in haemodialysis (Dumbauld et al., 1983). Like 3-hydroxybutyrate, acetoacetate in the present study was higher in uraemic patients, but significant increases were found only in CAPD patients and primarily due to the elderly subset of patients. Acetoacetate is elevated in healthy elderly subjects (Foster et al., 1978). Bearing in mind the possible suppressive effect of overnight peritoneal glucose absorption on ketone bodies, the basal concentrations of both 3-hydroxybutyrate and acetoacetate observed by Heaton et al. are not too disimilar from the data in the present

study. Fasting glycerol concentrations in the current study were not significantly different between either chronic renal failure or haemodialysis patients and controls. CAPD patients tended to have higher glycerol concentrations particularly the elderly. Glycerol was found to be normal in CAPD (Heaton et al., 1985) but increased levels are documented in healthy elderly subjects (Foster et al., 1978; Davidson et al., 1979; Jackson et al., 1982). Allowing for possible suppression of lipolysis due to overnight peritoneal glucose absorption, the difference between the 'normal' glycerol concentrations found by Heaton et al. and the 'increased' glycerol found in the present study is small, particularly if the age factor is also considered.

Patients new to CAPD had similar metabolite profiles as those well established on CAPD. Peritonitis did not have any specific influence on basal levels but elderly patients tended to have higher ketone bodies and glycerol, which is well recognised in healthy elderly subjects.

3. PROFILES DURING CAPD

Glucoregulatory hormones and intermediary metabolites during a peritoneal dialysis cycle has been studied to date by only two groups. Hormone (Heaton et al., 1983; Armstrong et al., 1985) and intermediary metabolites (Heaton et al., 1983) were measured during low and high glucose exchanges in 5 patients in each study. The possible effect of age, peritonitis and diabetes has not been reported. Diabetics will be discussed in the next section.

The change in blood glucose in response to both low and high dextrose dialysis solutions in this study confirms the findings of others (Heaton et al., 1983; Armstrong et al., 1985). Isotonic

solutions induced only a marginal increase in blood glucose over 6 hours, while hypertonic solutions caused a significant increase in the first hour of dwell and blood glucose returned to basal values by 4 hours. Elderly patients behaved similarly to younger patients and new patients produced the same response as those established on CAPD. Patients with peritonitis had an exaggerated increase in blood glucose. There was an increase in the rate of blood glucose elevation and a higher peak due to the increased permeability of the peritoneum due to infection. This increase in permeability has been previously documented by several groups (Rubin et al., 1981a; Verger et al., 1984). However, the total net amount of glucose absorbed from the dialysate was similar to non-infected patients using hypertonic solutions by the end of the 6 hour cycle. Thus during peritonitis the rate of glucose absorption, but not the quantity of glucose absorbed is increased. This may reduce the ultrafiltration capacity by decreasing the osmotic gradient across the peritoneum, resulting in the need for more hypertonic or more frequent exchanges to control fluid balance. Furthermore, the increased permeability of the peritoneum can affect the pharmacokinetics of intraperitoneal drugs (McIntosh et al., 1984) and can also lead to greater protein losses (Rubin et al., 1981b).

The serum insulin response mimicked that of glucose, reaching a maximum within 1 hour and returning to basal concentrations by 4 hours. Small increments were seen with 1.5% dextrose solutions but a 3-fold rise in insulin occurred with the 4.25% dextrose fluid. This data is in agreement with others (Heaton et al., 1983; Armstrong et al., 1985). Elderly and patients new to CAPD did not differ from those established on the technique, but the insulin response was further increased during peritonitis though not significantly. The

relatively higher insulin response in peritonitis corresponds to the
increase rate of glucose absorption. The effect of other hormones and
peptides of the 'enteroinsular axis' such as somatostatin and
gastrointestinal polypeptide are unknown in CAPD.

Hypertonic solutions induced a marked increase in c-peptide due to
pancreatic stimulation by the peritoneal glucose load. The rate of
rise was slower than that of glucose and insulin in keeping with the
lower metabolic clearance rate (Jaspan et al., 1977). The maximum
response was obtained by 2 hours in agreement with c-peptide kinetic
studies (Wideroe et al., 1984). The rate of decline of c-peptide was
slow, in comparison with insulin, due to the lower metabolic clearance
rate and its almost exclusive renal degradation. C-peptide did not
return to basal concentration with hypertonic solutions by the end of
the 6 hour cycle. This might suggest accummulation of c-peptide, but
peritoneal clearance does occur (Wideroe et al., 1984) and during
isotonic exchanges c-peptide returns to basal levels by 4 hours.
Elevated c-peptide appears to have no metabolic effect (Faber and
Binder, 1977; Jaspan et al., 1977). In peritonitis both the rate of
rise and peak response was greater, though not significantly
different, from other non-infected patients using hypertonic
solutions. This exaggerated response is due to increased pancreatic
stimulation caused by the increased rate of glucose absorption due to
increased permeability of the peritoneum. Elderly patients had a
similar c-peptide response suggesting that beta cell function,
assessed by both the insulin and c-peptide response, is no different
from younger uraemics. This is in agreement with data from
non-uraemic elderly subjects (Jackson et al., 1982).

During the dialysis cycle hyperglucagonaemia persisted, although
there was an initial fall in the first hour of dwell corresponding to

the period of maximum glucose absorption and insulin secretion. This transient fall in glucagon, although small, was more marked during high dextrose cycles. This is in agreement with the findings of others (Heaton et al., 1983; Armstrong et al., 1985). Elderly patients and those with peritonitis had a similar response to other CAPD patients. Thus despite the increased rate of glucose absorption and beta cell stimulation glucagon was not suppressed further. In the study by Armstrong et al. (1985) higher glucagon concentrations were observed in all his 5 patients. This may reflect the presence of proglucagon. In the present study a c-terminal specific assay was used which measured pancreatic glucagon and concentrations were similar to those of Heaton et al. (1983).

The normal response of a fall in growth hormone concentration following glucose administration is not seen in uraemia. A paradoxical rise during glucose tolerance testing has been observed (Wright et al., 1968; Saaman et al., 1970). In haemodialysis high levels persist (Spitz et al., 1970) and a paradoxical rise is found after a glucose challenge (Swenson et al., 1974). During the procedure of haemodialysis growth hormone concentrations fall (Hansen et al., 1979), possibly due to the absorption of acetate from the dialysis fluid, as i.v. infused acetate inhibits growth hormone secretion (Schmitz et al., 1982). In CAPD patients during a dialysis exchange there was considerable variation in growth hormone concentrations, but levels were essentially normal and did not significantly change despite both glucose and lactate absorption. This is in agreement with others (Von Baeyer et al., 1983), but contrasts with the paradoxical rise reported in intermittent peritoneal dialysis (Gahl et al., 1980). The reason for the latter difference is unclear. Theoretically, CAPD might affect growth

hormone secretion and clearance. The constant glucose absorption over 24 hours may lead to suppression of growth hormone secretion (Heaton et al., 1985). Alternatively, protein losses from the dialysate may lead to malnutrition. Malnutrition is associated with raised growth hormone (Pimstone et al., 1968) and an inverse relationship between serum albumin and growth hormone has been observed (Wright et al., 1968). Furthermore, growth hormone (MW 21,000) is likely to cross the peritoneal membrane and may contribute to lower concentrations in CAPD. Whether a balance between the tendency to hypersecretion and increased excretion exists is not clear, but in CAPD no paradoxical rise in growth hormone has been reported.

Plasma cortisol did not change during the CAPD cycle and concentrations were essentially normal. Little is known about cortisol secretion in CAPD, but a higher 24 hour secretion was found by Heaton et al (1985) due largely to reduced nocturnal suppression. This is, in contrast, to observations found in chronic renal failure and haemodialysis patients who maintained a normal circadium rhythm (Wallace et al., 1980). As previously discussed, CAPD may clear cortisol conjugates via the peritoneum, reduced cortisol binding to albumin and relative hypoalbuminaemia may also contribute to lower cortisol in CAPD than in other forms of uraemia. Interpretation of cortisol concentrations in uraemia remains complex. In this study no apparent change occurred in response to hyperglycaemia and hyperinsulinaemia, suggesting that cortisol is not involved in the glucoregulatory hormone control during CAPD, although a 'permissive' role for cortisol cannot be ruled out.

Blood lactate increased significantly from normal fasting concentrations to peak levels in 1 hour in all CAPD patients. A similar rise was seen with both low and high dextrose solutions and,

therefore, was independent of glucose concentration. The blood

lactate increase was most likely due to the dialysate lactate, which

was similar in both isotonic and hypertonic solutions, rather than

secondary to increased glycolysis. The blood lactate profile in new

and elderly patients was similar to the other non-infected CAPD

patients and returned to baseline by 4 hours. In peritonitis blood

lactate increased markedly in the first hour of dwell. This could be

explained by, either a greater rate of lactate absorption from the

peritoneal fluid due to increased permeability of the peritoneum, or

from increased glycolysis due to the greater rate of glucose

absorption and exaggerated hyperinsulinaemia. Possibly both

mechanisms play a role, although the increased rate of dialysate

lactate absorption was probably the major factor. The amount of

lactate absorbed during the 6 hour cycle was constant, in all groups,

at about 7g or 28g per day which is much less than the estimated

endogenous production of 120g per day and maximum utilising ability of

330g per day (Krebs et al., 1975). Thus dialysate lactate is unlikely

to have any significant clinical effect on lactate homeostasis.

Furthermore, the blood lactate rise was relatively transient and

values of greater than 3 mmol/l were observed only in a few

individuals with peritonitis. These levels are not high enough to

induce lactic acidosis ($>$5 mmol/l). Lactate normally increases

temporarily after exercise but is readily metabolized and utilized

particularly by the liver. Higher lactate concentrations after

exercise have been found in uraemics compared to healthy controls by

some (Parrish et al., 1981), while others (Nakao et al., 1982)

observed lower concentrations in uraemic patients. However, it is

unlikely that either lactate or glucose in dialysis fluid per se could

induce lactate acidosis under normal conditions. A recent case report

of lactic acidosis (Conte et al., 1986), the first documented in CAPD, cited lactic acid coma, but this patient had concomitant hepatic failure, which was the most likely predisposing factor. In the present study, all patients had normal liver function and blood lactate concentrations returned to basal levels within 4 hours, again evidence that lactate was not accumulating.

Pyruvate, which theoretically is in equilibrium with lactate, increased in the first hour of the cycle and was also independent of the dialysate glucose concentration. In peritonitis a more marked increase in pyruvate occurred in parallel with changes in blood lactate. It is more likely that dialysate lactate absorption was responsible for the rise in blood pyruvate, but again stimulation of glycolysis and suppression of gluconeogenesis by hyperinsulinaemia cannot be excluded. The lactate:pyruvate ratio was normal in all patients except those with peritonitis. In the latter group the ratio increased due to a relative increase in lactate. The only other data on blood lactate and pyruvate during CAPD (Heaton et al., 1983) showed that basal blood lactate was high but remained unchanged during the dialysis cycle, while normal basal pyruvate became elevated during hypertonic dialysis. These results are not easily explained.

Blood alanine did not show any significant overall change during the peritoneal exchange. There was a small rise in the isotonic, elderly and peritonitis groups. This is not surprising as hyperinsulinaemia can induce endogenous production by glycolysis and suppress utilization by gluconeogenesis. However, this small rise was seen with low glucose solutions but not with all high glucose solutions. Nevertheless, although the alanine profile did not alter significantly a suggested trend similar to the other gluconeogenic precursors was observed. Alanine, in theory, is in equilibrium with

lactate, through its equilibrium with pyruvate (Krebs et al., 1975; Alberti and Nattrass, 1979). Thus dialysate lactate might also induce a rise in alanine. Patients with peritonitis had a small but marginally greater relative rise than the other patients using hypertonic solutions. This could be explained by, either increased glycolysis and suppression of gluconeogenesis by raised ambient insulin levels, or secondary to raised blood lactate induced by dialysate lactate absorption. It is not possible to clarify which was the most important pathway. Blood alanine concentrations, although higher, were unchanged with CAPD in the study of Heaton et al. (1983). Alanine production and utilization have been observed to be increased (Rubenfeld et al., 1978) and also normal (DeFronzo and Felig, 1980) in renal failure. In the present study blood alanine showed no obvious change despite the ambient hyperinsulinaemia and dialysate lactate absorption.

Ketone bodies, 3-hydroxybutyrate and acetoacetate were suppressed by peritoneal glucose absorption, the effect being more marked with high glucose dialysate. Dialysate induced hyperglycaemia stimulates insulin release and suppresses gluconeogenesis and ketogenesis. During the latter half of the dialysis cycle, when blood glucose was returning to basal concentrations, suppression of ketogenesis was less marked with isotonic than hypertonic dialysate. The 3-hydroxybutyrate:acetoacetate ratio was essentially normal during the CAPD cycle as both ketone bodies fell in parallel, but in the middle of the cycle at the point of maximum suppression of ketogenesis the ratio increased due to a relatively greater suppression of acetoacetate. Similar though less obvious changes were observed by Heaton et al. (1983). Although elderly patients had higher basal levels of ketone bodies the decrement was similar to other groups

using hypertonic exchanges. The presence of peritonitis despite increasing the rate of glucose absorption and did not induce any further change in ketone body concentrations.

Glycerol, an indicator of free fatty acid status, was suppressed during the dialysis cycle in all patients in a similar manner to the ketone bodies. The changes were mainly in the early part of the cycle corresponding to the period of maximum hyperinsulinaemia which inhibited lipolysis. The suppression of glycerol and free fatty acids in response to oral glucose has been long recognised in uraemia (Roth et al., 1973). In CAPD, non esterified fatty acids, glycerol and ketone bodies correlated with each other, although glycerol did not significantly differ from controls in the study of Heaton et al. (1983).

4. <u>HORMONE AND METABOLITE CHANGES IN DIABETICS ON CAPD</u>

No data on hormone or metabolite changes in diabetics on CAPD have been reported. The metabolic consequences of diabetes, uraemia and peritoneal glucose absorption may be superimposed on each other. In this study exogenous insulin (normally given by addition to the peritoneal fluid bag immediately prior to instillation) was omitted during the dialysis cycle. Thus, this study gave the opportunity to investigate the metabolic changes unopposed by insulin. Although all 6 patients were insulin treated diabetics, 2 did have some residual pancreatic function manifested by high basal c-peptide concentrations.

Hypercholesterolaemia, hypertriglyceridaemia and low HDL-cholesterol were found in the diabetics but it was not possible to conclude whether uraemia, CAPD or diabetes was responsible, as each is a predisposing factor to abnormal lipid metabolism.

a)   Fasting state

In the fasting state (that is, no overnight dialysis, a 12 hour oral fast and no insulin for 12 hours) blood glucose was as expected higher than both healthy controls and non-diabetic CAPD patients. Serum free (unbound) insulin, although not strictly comparable with total serum insulin (Nakagawa et al., 1973) was quantitatively similar to the 'hypertonic' CAPD group, which was used as the control CAPD group in this study. Fasting c-peptide was higher than expected for type I diabetics and this can be explained by the presence of 2 patients who were 'type II-insulin treated' diabetics. Type II diabetics frequently have normal or raised c-peptide concentrations (Unger and Foster, 1985) and in the presence of uraemia impaired renal degradation can markedly increase the c-peptide concentration (Jaspan et al., 1977). Hence caution is needed in interpreting isolated c-peptide concentrations which may be elevated due to uraemia rather than significant residual beta cell function (see Chapter 6). Fasting plasma glucagon concentrations were higher, though not significantly, than healthy controls but similar to non-diabetic CAPD patients. Absolute and relative hyperglucagonaemia are recognised in diabetes (Unger and Foster, 1985), but it is unclear from the study whether the raised fasting glucagon was due to diabetes or uraemia. However, it is interesting that diabetics and uraemics on CAPD had very similar concentrations. The role of growth hormone in diabetes is still uncertain, but raised growth hormone concentrations are seen in poorly controlled diabetes and can cause hyperglycaemia and hyperketonaemia (Press et al., 1984). Increased growth hormone may also induce insulin resistance in non-diabetics, by impairing both insulin supression of hepatic glucose and peripheral utilisation (Rizza et al., 1982). However, in this study fasting growth hormone did not

significantly differ from non-diabetic uraemics.

Although cortisol is capable of increasing blood glucose, cortisol infusion in healthy subjects failed to increase glucose production and even during prolonged fasting plasma cortisol remained normal (Shamoon et al., 1981). In this study fasting plasma cortisol concentrations were normal in diabetics as well as non-diabetics on CAPD. It is suggested that cortisol has no direct short term role in the control of glucose in diabetics (Cryer, 1984).

The gluconeogenic precursors lactate and pyruvate have been reported to be normal (Nosadini et al., 1985) and increased (Alberti et al., 1975) in diabetics on insulin. In type II diabetics improved glycaemic control by diet has resulted in lowering fasting lactate pyruvate and alanine (Sheppard et al., 1983). In the present study, in the relative absence of insulin, fasting lactate and pyruvate were similar to both healthy controls and non-diabetic uraemic patients. Fasting values were similar to those observed by Sheppard et al. (1983). Fasting alanine is usually normal in diabetics (Capaldo et al., 1984; Nosadini et al., 1985) and basal concentrations in this study confirmed that diabetics on CAPD were not any different from either healthy controls or non-diabetic uraemics. The fasting alanine concentrations were similar to those found by others (Potter et al., 1982; Sheppard et al., 1983). Fasting ketone bodies and glycerol are significantly elevated in both type I and II diabetics (Alberti et al., 1975; Sheppard et al., 1983; Nosadini et al., 1985). The cardinal factor affecting lipolysis and ketogenesis in the fasting state is the balance between insulin and glucagon. In this study elevation of 3-hydroxybutyrate, acetoacetate and glycerol were observed in diabetics compared to healthy subjects. However, ketone body concentrations in diabetics on CAPD were similar to non-diabetic

uraemics and fasting glycerol was also similar to non-diabetic CAPD patients although higher than other uraemics. Thus, it is not clear whether the rise in ketone bodies and glycerol, due to augmentation of ketogenesis and lipolysis, in the post absorptive state was due to diabetes or uraemia.

b) During CAPD

Diabetics had a progressive rise in blood glucose over the first 90 minutes of dialysate instillation and then reached a plateau, the hyperglycaemia being unopposed due to the lack of insulin. Serum free insulin remained unchanged throughout the period of peritoneal glucose absorption. Two patients (type II insulin treated diabetics) did show a small, but insignificant rise in free insulin which might have suggested some residual pancreatic function. This is, in contrast, to the non-diabetic CAPD patients who had a significant rise in total serum insulin. Mean c-peptide concentrations were relatively increased but remained unchanged during the dialysis cycle. Raised mean concentrations were due to 2 patients having high c-peptide levels, while the others had negligible concentrations. The elevated levels of c-peptide may be explained, in part, by some residual pancreatic function, but largely by impaired renal degradation of the peptide. However, as c-peptide concentrations did not change despite the dialysis induced hyperglycaemia it can be concluded that no clinically significant beta cell reserve existed in any of the diabetics.

A pure carbohydrate meal normally causes suppression of glucagon and stimulation of insulin but signals other than the absorbed glucose may be responsible for the alpha and beta cell peptide changes. Somatostatin is a strong suppressor of glucagon and may play a role in

response to an oral carbohydrate load (Unger and Foster, 1985).
Gastrointestinal polypeptide, which is released during a carbohydrate
meal, is a stimulus to insulin secretion. When the insulin response
is adequate (as in non-diabetics) the stimulating effect of
gastrointestinal polypeptide upon glucagon is not apparent because of
concomittant suppression of glucagon by insulin. However, in
diabetics the insulin response is blunted and the glucagon response to
an oral carbohydrate load becomes paradoxically positive. This may be
a consequence of the unoppossed actions of gastrointestinal
polypeptide on the αcells of the pancreas (Unger and Foster, 1985).
In this study diabetics showed no change in glucagon while
non-diabetics had a fall in response to the dialysate glucose
absorption. It is not known whether peritoneal glucose absorption
stimulates the enteroinsular axis although most of the glucose is
absorbed into the portal circulation. The actions of somatostatin and
gastrointestinal polypeptide are unknown in CAPD.

As previously discussed, the role of growth hormone in diabetes is
unclear but several reports link increased plasma growth hormone with
poorly controlled diabetes (Vigneri et al., 1976; Press et al., 1984).
The small group of diabetics in this study had good glycaemic control,
assessed by glycosylated haemoglobin measured colorimetrically.
Growth hormone did not show any change during the dialysis cycle in a
similar manner to non-diabetics. Thus, neither raised concentrations
due to the presence of diabetes or a paradoxical rise due to uraemia
(Orskov et al., 1971) were observed during peritoneal glucose
absorption. Whether prior good glycaemic control or the specific
effect of CAPD prevented changes in growth hormone is unclear. Growth
hormone probably has a permissive rather than glucoregulatory role in
diabetes (Cryer, 1984).

Cortisol, like growth hormone, probably has a permissive rather than regulatory role in diabetes (Cryer, 1984). Thus, it is not surprising that no change in cortisol occurred during peritoneal glucose absorption, even in the absence of exogenous insulin. Normal plasma cortisol concentrations were observed in both diabetics and non-diabetics throughout the dialysis cycle. It is possible, though speculative, that peritoneal removal of cortisol conjugates could explain the normal values found in all CAPD patients, in contrast to, elevated concentrations in non-dialysed chronic renal failure and haemodialysis patients.

Lactate concentrations are usually normal (Nosadini et al., 1985) or slightly increased (Capaldo et al., 1984) in diabetics with 'average' glycaemic control. The normal response after oral glucose or a meal would be to increase lactate due to increased glycolysis modulated by insulin release. However, in this study despite the lack of insulin, blood lactate had a small, though significant increase in diabetics during the first hour of dwell similar to non-diabetics. The most likely explanation for the rise in blood lactate was dialysate lactate absorption rather than an increase in endogenous lactate via glycolysis. It was also observed that the decline in blood lactate was slower in diabetics suggesting suppression of lactate utilization due to insulin deficiency.

Pyruvate was marginally but not significantly higher in diabetics in the fasting state in agreement with Nosadini et al. (1985). However, during peritoneal glucose absorption pyruvate did not change in diabetics although increased in non-diabetics. The lack of change in pyruvate may be explained by the failure to suppress gluconeogenesis and inhibition of glycolysis due to insulin insufficiency. Although, theoretically, lactate and pyruvate are in

equilibrium the dialysate lactate absorption would explain the greater change in lactate than pyruvate.

Alanine is usually normal in well controlled diabetics (Potter et al., 1982; Capaldo et al., 1984; Nosadini et al., 1985) but several reports of raised blood alanine have been documented, particularly after pancreatectomy (Barnes et al., 1975; Del Prato et al., 1985). In this study diabetics had normal alanine concentrations which did not change with peritoneal glucose absorption. This might be due to lack of insulin stimulation with failure to increase glycolysis and suppress gluconeogenesis, but the non-diabetics who had a significant hyperinsulinaemia also failed to induce a significant increase in alanine, although some patients did show a small rise compatible with insulin mediated glycolysis.

Ketogenesis and lipolysis are under integrated control and represent a link between carbohydrate and lipid metabolism. Ketone bodies accumulate in plasma during fasting and uncontrolled diabetes and the initiating event is a change in the molar ratio of glucagon to insulin. Insulin deficiency triggers the lipolytic process liberating free fatty acids and glucagon appears to be the primary hormone involved in fatty acid oxidation and the formation of ketone bodies (Miles et al., 1980; Foster and McGarry, 1982).

Normally ketone bodies and glycerol are suppressed by glucose loading, but in this study diabetics in contrast to non-diabetics failed to suppress 3-hydroxybutyrate and acetoacetate. This can be explained by insulin deficiency and relative glucagon excess. Glycerol did not change from fasting concentration in diabetics despite glucose absorption whereas non-diabetics had a significant fall in the early part of the cycle. Again, the imbalance between insulin and glucagon explains the failure to suppress lipolysis in

diabetics.

5. DIALYSIS FLUID AND ULTRAFILTRATION

In view of the potential hazards from excessive dialysate glucose absorption, such as hyperlipidaemia and accelerated altherosclerosis it is prudent to limit the number of hypertonic exchanges, but many patients require at least one high dextrose cycle per day. On a typical regimen of 3 isotonic (1.5% dextrose) and 1 hypertonic (4.25% dextrose) cycles, it is estimated from this study that 130g of glucose is absorbed per day from the peritoneal cavity. This is in agreement with data from others (Nolph et al., 1979; Grodstein et al., 1981). Although the rate of glucose absorption varies between individuals, reflecting individual peritoneal characteristics the total amount of glucose absorbed was quite uniform. In 30 CAPD patients using 4.25% dextrose solutions (2 litres) glucose absorption was between 60 and 80g over a 6 hour dwell, but the ultrafiltration volume varied considerably.

Ultrafiltration depends on many factors such as the state of hydration, residual renal function, catheter mechanics, integrity of the peritoneum and the osmotic gradient between blood and dialysate (Nolph, 1983). Nolph et al. (1981) postulated that ultrafiltration takes place at the proximal side of the peritoneal capillary network and glucose absorption at the distal end of the network. The proximal end has a higher hydrostatic pressure and there is low solute permeability due to 'tight' ($10A^{o}$) cell junctions. The distal end has lower hydraulic pressure and wider cell separation ($40A^{o}$). This theory suggests different sites for glucose absorption and water removal. It has also been shown that ultrafiltration capacity can be severely reduced while solute (glucose) transfer remains unaffected

(Manuel, 1983). Thus, it is not surprising that no significant correlation was found between the amount of glucose absorbed from the dialysate and net ultrafiltration.

Hyperglycaemia per se may reduce the osmotic glucose gradient and decrease the ultrafiltration capacity, but in this study diabetics who had persistent hyperglycaemia did not show reduced ultrafiltration. In contrast, patients with peritonitis had reduced ultrafiltration. In peritonitis the rate of dialysate glucose absorption increased and this had been previous verified by others (Rubin et al., 1981a; Verger et al., 1984). However, the total amount of glucose absorbed over the 6 hour cycle was the same as in other patients using similar dextrose solutions. Overall, glucose absorption and ultrafiltration were similar in new and established patients, as well as the elderly and diabetics using the same concentration of glucose in the dialysate. Thus the rate of glucose absorption as well as the concentration of glucose is important in respect to ultrafiltration. The method of assessing ultrafiltration in this study was crude, but easily applicable to any patient, especially at an out-patient clinic. More accurate kinetic models for solute transfer and ultrafiltration measurement have been designed (Spencer and Farrel, 1986). Although more applicable for research purposes, further information on the viability and integrity of the peritoneum needs to be obtained to determine the durability of CAPD.

Dialysate volume measurements based on dilution of high molecular weight marker molecules have shown that with a 2 litre hypertonic exchange (3.86% dextrose) the ultrafiltration volume peaked at 3.0-3.3 litres at 3 hours and, thereafter, reabsorption took place and the ultrafiltration rate varied between 25 ml/min and -1.5 ml/min (Pyle et al., 1981). Thus at the start of the dialysis cycle the glucose

gradient and ultrafiltration rate are highest. These fall as the
dwell time lengthens and after long dwells glucose may diffuse back to
the dialysate and water may be reabsorbed.

Peritoneal lactate absorption was constant in all groups and
amounted to 6.8g during a 6 hour cycle or about 28g per day. As
previously discussed, this adds little to the normal endogenous
lactate production of about 120g/day (Krebs et al., 1975). During
peritonitis, the rate of lactate absorption increased but the rise in
blood concentration was not sufficient to induce lactic acidosis and
the effect was only temporary. Thus dialysate lactate, in the
presence of normal liver function, does not adversely affect lactate
homeostasis. Lactate in commercial dialysis solutions is present in a
racemic configuration. The lactate measured in this study did not
distinguish between the L(+) and D(-) species, but the clearance of
lactate from the peritoneal cavity is relatively stereospecifc. The
L(+) isomer has a greater clearance from the peritoneum than the D(-)
and the latter is less readily metabolised (Rubin et al., 1982).
However, the difference in clearance between the two isomers is only
about 2.5 ml/min and probably not clinically significant.

SUMMARY

Hyperlipidaemia was observed in all uraemic patients, but hypercholesterolaemia and hypertriglyceridaemia were most marked in CAPD and particularly diabetics on CAPD. This emphasises the role of glucose intolerance, hyperinsulinaemia and carbohydrate induced hyperlipidaemia and the risk of coronary heart disease in patients with end stage renal disease.

In the fasting state glucose and insulin were normal although haemodialysis patients tended to have marginally increased insulin concentrations. C-peptide was grossly elevated in uraemia due to impaired renal degradation. Glucagon was generally increased, while cortisol was normal in CAPD patients though increased in other uraemics. Growth hormone was essentially normal in most uraemic patients. The gluconeogenic precursors, lactate, pyruvate and alanine were normal in the post-absorptive state, but ketone bodies and glycerol tended to be higher in uraemia particularly in the elderly CAPD patients.

Hormone and intermediary metabolite profiles during a peritoneal dialysis cycle generally showed greater changes with the higher dextrose solutions. Peritoneal glucose absorption produced hyperglycaemia which induced hyperinsulinaemia and an increase in c-peptide. Glucagon decreased while growth hormone and cortisol were unchanged. Lactate and to a lesser extent pyruvate increased during the cycle. This was largely due to absorption of dialysate lactate as the increase appeared to be independent of dialysate glucose concentration, although some endogenous production of lactate and pyruvate from glycolysis cannot be excluded. Alanine was unchanged throughout the cycle. Ketone bodies and glycerol were suppressed during peritoneal glucose absorption. Thus dialysate glucose

absorption causes hyperglycaemia and the concomitant hyperinsulinaemia stimulates glycolysis, inhibits gluconeogenesis and suppresses ketogenesis and lipolysis. Peritonitis increases the permeability of the peritoneum and enhances the rate of glucose absorption which induces a more marked hyperinsulinaemia, although glucagon was not further suppressed. The elevated lactate and pyruvate during peritonitis was most likely due to increased dialysate lactate absorption, but increased endogenous production due to hyperinsulinaemia cannot be excluded. Ketogenesis and lipolysis were not further suppressed by peritonitis. Elderly patients who had higher basal concentrations of ketone bodies and glycerol had similar profiles as other patients.

Hormones and intermediary metabolites in diabetics on CAPD were deranged with the deprivation of exogenous insulin. Peritoneal glucose absorption produced persistent hyperglycaemia and no beta cell response. Glucagon, growth hormone and cortisol were unchanged. Lactate increased despite the absence of hyperinsulinaemia due to dialysate lactate absorption, while pyruvate and alanine were unaltered during the cycle. Ketone bodies and glycerol were not suppressed despite the ambient hyperglycaemia due to the lack of endogenous and exogenous insulin. Thus insulin deficiency and relatively normal or increased glucagon failed to stimulate glycolysis, while gluconeogenesis, ketogenesis and lipolysis were maintained.

Glucose absorption from the peritoneal dialysate in general terms is similar to an oral glucose load, although the role of the entero-insulin axis is not known in CAPD. The essential role of insulin in controlling metabolic substrates during CAPD is clearly shown. During CAPD hyperinsulinaemia stimulates glycolysis and

inhibits gluconeogenesis, ketogenesis and lipolysis. It should be noted that normally the CAPD patient is never truly fasted as dialysis normally continues 24 hours per day, every day. It is estimated that about 130g of glucose per day is absorbed from the dialysis fluid, in addition to the oral carbohydrate intake. It is, therefore, important that caloric and dietary intake is regularly reviewed and ideally an individual dietary prescription should be formulated to minimise obesity and hyperlipidaemia.

The long term sequelae of the metabolic consequences remain to be seen, but the risks, particularly of vascular disease have to be balanced against the alternatives. Although renal transplantation is, undoubtedly, the preferred mode of treatment for end stage renal disease it is not suitable or available for all patients. Haemodialysis is also not suitable or available for all patients and hypertension and fluid balance are usually better controlled by CAPD. The other alternative is CAPD, thus the metabolic effects and potential consequences of CAPD need to be assessed in perspective.

CHAPTER 8

GLYCOSYLATED AND CARBAMYLATED HAEMOGLOBIN IN URAEMIA

Glycosylated haemoglobin, as an indicator of integrated long term blood glucose control, in diabetics with normal renal function is well established clinically and biochemically (Gabbay et al., 1977; Jovanic et al., 1981). Recently, a possible pathophysiological significance has been suggested between the formation of non-enzymatic glycosylation of proteins and the complications of diabetes mellitus (Cohen, 1986). This has been discussed in Chapter 4. Glycosylated haemoglobin status in uraemia is complex and controversial with reports of elevated, normal and reduced concentrations.

Elevated concentrations of haemoglobin A_1 (HbA_1) and HbA_{1c} in non-diabetic uraemic patients has been frequently documented (De Boer et al., 1980; Graf et al., 1980; Kovarik et al., 1981; Fluckiger et al., 1981). Normal concentrations of total glycosylated haemoglobin in the red cell have been reported (Fluckiger et al., 1981; Nath et al., 1982) in renal failure. Reduced concentrations of HbA_1 have also been observed in uraemia (Dandona et al., 1979; Freedman et al., 1982). Haemoglobin A_1 has been reported to be higher in uraemic diabetics than non-uraemic diabetics (Kumar et al., 1983), but others have found no difference between similar groups (Panzetta et al., 1983).

Several explanations have been forwarded to account for the elevation of glycosylated haemoglobin in renal failure such as co-existing hyperglycaemia and glucose intolerance (Casparie et al., 1977; Stanton et al., 1978). However, many more recent studies have found no association with impaired glucose metabolism in non-diabetic uraemic patients (Graf et al., 1980; Kovarik et al., 1981; Nath et al., 1982; O'Regan et al., 1982; Saloranta et al., 1986). Evidence that a direct effect of uraemia is responsible, is supported by the

correlation between HbA_1 and plasma creatinine (Kovarik et al., 1981) and urea (DeMarchi et al., 1983a), although the latter authors favour the role of acidosis as the cause of increased glycosylated haemoglobin. Interference in the measurement of HbA_1 by some non-glucose adduct such as urea has been demonstrated (Fluckiger et al., 1981). These authors suggest that haemoglobin carbamylation results from urea-derived cyanate and this may be responsible for the rise in glycosylated haemoglobin in uraemia. Reduced concentrations of glycosylated haemoglobin in renal failure have been postulated to be due to the reduced red cell life span (Freedman et al., 1982).

Objectives

In view of the complex and conflicting data in the literature this study aimed to reassess and clarify the subject of glycosylated haemoglobin in uraemia. The objectives of the study were:-

1. To evaluate glycosylated haemoglobin in a large uraemic population including non-dialysis, dialysis and transplant patients, as well as diabetics with and without renal failure.

2. To compare different methods of measuring glycosylated haemoglobin (from the same blood sample);

    a) by cation chromatography to detect HbA_1 and specific components HbA_{1c} and HbA_{1a+b}.

    b) by a specific chemical method using colorimetry to detect the total glycosylation in the red cell.

3. To measure carbamylated haemoglobin simultaneously with glycosylated haemoglobin and to investigate the possible associations.

4. To clarify the role of glycosylated haemoglobin in uraemia and in diabetics with renal failure.

1. Patients

    Over 200 subjects were studied to evaluate glycosylated and carbamylated haemoglobin in a wide spectrum of renal disease. Nine groups of subjects were investivated. The abbreviations for each group are used throughout the Chapter.

Group	n
1 Control (healthy volunteers)	20
2 PRD (primary renal disease)	22
3 CRF (chronic renal failure)	40
4 CAPD (continuous ambulatory peritoneal dialysis)	24
5 HD (haemodialysis)	31
6 Tx (transplants with normal renal function)	16
7 Tx-RF (transplants with renal failure)	14
8 D (diabetics with normal renal function)	22
9 D-RF (diabetics with renal failure)	24

    A further 9 haemodialysis patients were studied pre- and post-dialysis to assess the effects of the procedure. Patients in the PRD group had proven underlying renal disease (mainly glomerulonephritis, polycystic kidney disease or tubulointerstitial disease) with normal blood urea and creatinine. Impairment of glomerular filtration rate and proteinuria were not excluded. The CRF group consisted of patients with wide degrees of renal impairment from mildly elevated creatinine to end stage renal disease, but none of the group were on dialysis. The CAPD and HD groups comprised patients established on dialysis for at least 6 months. Transplant patients

with normal renal function had normal blood chemistry but impairment of the glomerular filtration rate and proteinuria were not excluded. Transplants with renal failure had persistently elevated urea and creatinine but were managed without dialysis. Diabetics with normal renal function (14 type I, 8 type II) had normal urea and creatinine but milder degrees of diabetic nephropathy, such as abnormal glomerular filtration and proteinuria, were not excluded. Diabetics with renal failure (20 type I, 4 type II) had elevated urea and creatinine and 8 patients in this group were on dialysis.

2. Study criteria

None of the patients had a blood transfusion within 3 months of the study or any detectable haemoglobinopathy. Liver disease, alcoholism, jaundice and recent alcohol ingestion were all exclusion criteria. Some of the patients had hyperlipidaemia but this was not formerly screened or excluded. Drug therapy was not altered for this study. All transplant patients took prednisolone and either cyclosporine A or azathioprine. Uraemic patients were taking many drugs but the only exclusion was aspirin or antibiotic medication at the time of the study. None of the patients were diabetic other than the two diabetic groups.

3. Protocol

Random venous blood samples were taken from subjects usually during out-patient clinic visits and after informed consent. Blood was taken for the simultaneous measurement of glucose, full blood count, urea, electrolytes, creatinine, liver function tests, glycosylated and carbamylated haemoglobin. Blood for glycosylated haemoglobin was allowed to stand for 24 hours at $4^{\circ}C$ to reduce the

'labile' fraction in diabetics. None of the diabetics had severe
hyperglycaemia at the time of sampling. To assess the effect of
haemodialysis blood was taken immediately pre-dialysis (i.e. 48 hours
after last dialysis) and post-dialysis (after a 4 hour session).
Arterial blood for H^+ ion concentration and bicarbonate was taken from
the arterial line in the 9 patients investigated during haemodialysis.
Ethical committee approval was obtained for this study.

4. Assays

Glycosylated haemoglobin was measured by 2 distinct methods.
Firstly, total glycosylation in the red cell by the thiobarbituric
acid - colorimetric method (Fluckiger and Winterhalter, 1976) with
modifications as described in Chapter 5. Red cells were washed, lysed
and hydrolysed to split off the glycosyl moiety which converts to
5-hydroxymethylfurfural (5HMF) (as shown in Figure 4.2). The 5HMF was
then measured colorimetrically following reaction with thiobarbituric
acid. Within and between batch coefficients of variation were 3.5%
and 6% respectively.

Secondly, cation chromatography using minicolumn commercial kits
estimated HbA_1 and the specific components HbA_{1c} and HbA_{1a+b}. The
kits were from BioRad laboratories and the methods are fully described
in Chapter 5. The chromatographic methods used whole blood
haemolysate which was applied to a weakly acidic cation exchange
resin. An elution/developing reagent was then added to the column
which separated the fast moving components. By using specific buffers
the components were separated. The within and between batch
coefficients of variation were approximately 3% and 6% respectively.

Carbamylated haemoglobin was estimated by quantifying the amount
of valine (isopropyl) hydantoin released from the acid hydrolysis of

globin. Valine hydantoin was then measured by gas liquid chromatography. The method was adapted from Manning et al. (1973) and Fluckiger et al. (1981) with modifications as described in Chapter 5. The reaction is represented in Figure 8.1. All samples were assayed in duplicate and between assay variation was 12%.

5. Statistics

Parametric tests were used as glycosylated and carbamylated haemoglobin had a normal distribution. In view of the multiple groups investigated analysis of variance was used for comparison between groups. In calculating the 't' statistic the "combined standard deviation" was employed. Comparison between pre- and post-dialysis data was done using the paired 't' test. Correlations were sought by linear regression analysis. Analysis of ratios were done by the Mann-Whitney non-parametric test.
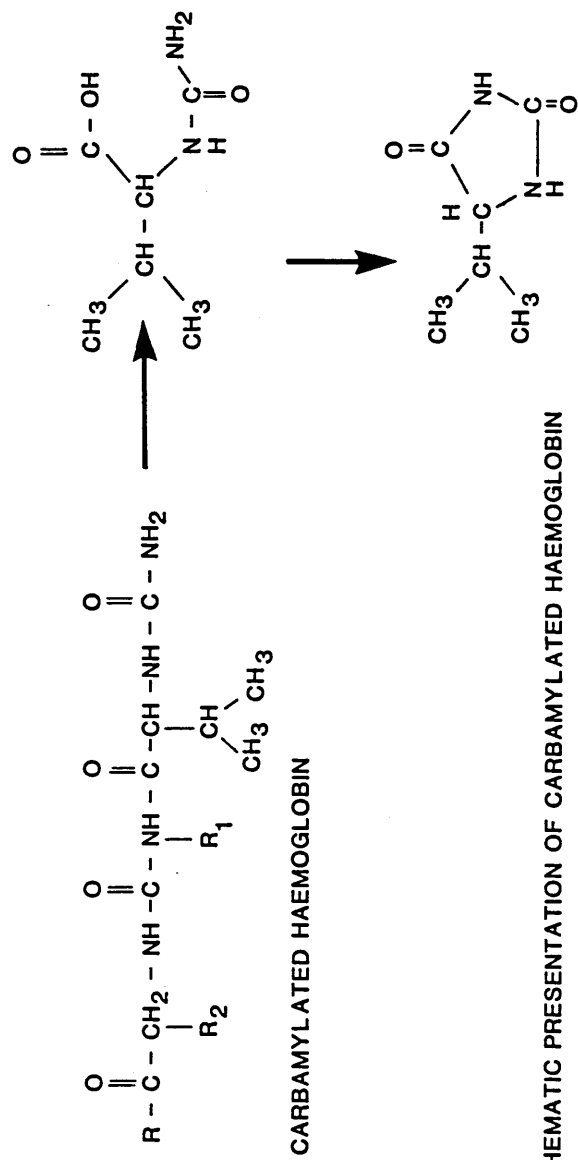
CARBAMYLATED HAEMOGLOBIN

5 – isopropyl hydantoin

(2,4 – imadazolidine – dione)

SCHEMATIC PRESENTATION OF CARBAMYLATED HAEMOGLOBIN

Figure 8. 1

RESULTS

1. General data

Sex and age (mean and SD) are shown for each group in Table 8.I. The control group were the youngest and the CAPD group the oldest, but most of the other groups were, of comparable age, between 40 and 50 years. Haemoglobin, packed cell volume (PCV) and serum albumin are shown in Table 8.II (mean and SEM). Haemoglobin and PCV were decreased in all groups with renal failure ($p<0.001$) compared to controls, while diabetics and transplants with normal renal function had similar values to controls. Albumin was normal in all groups except the CAPD group which had a lower albumin concentration ($p<0.05$).

Blood urea, serum creatinine and plasma bicarbonate (mean and SD) are shown in Table 8.III. The primary renal disease (PRD), transplant (Tx) and diabetic (D) groups had normal urea and creatinine concentrations while all the other groups had increased urea and creatinine ($p<0.001$). Bicarbonate was statistically reduced in all the renal failure groups compared to controls ($p<0.01$).

Random blood glucose (mean and SEM) values are shown in Table 8.IV. Glucose concentrations were normal in all but the 2 diabetic groups which had significantly increased concentrations ($p<0.001$). Most diabetics with or without renal failure had random glucose concentrations under 12 mmol/l and all were below 16 mmol/l.

2. Glycosylated haemoglobin

Glycosylated haemoglobin measured by the colorimetric method was evaluated in about 100 patients prior to this study. This confirmed that glycosylated haemoglobin by this technique (GHb) was elevated

	Group	n	Sex M	F	Age
1	C	20	9	11	33.1 (7.4)
2	PRD	22	13	9	41.7 (8.9)
3	CRF	40	25	15	52.2^{c}(8.3)
4	CAPD	24	12	12	57.7^{c}(11.8)
5	HD	31	18	13	44.0^{b}(7.9)
6	TX	16	7	9	38.8 (9.7)
7	TX–RF	14	12	2	40.6 (10.9)
8	D	22	9	13	46.5^{b}(9.9)
9	D–RF	24	16	8	49.8^{b}(10.2)

Age – values are mean and (SD)
p values refer to comparison with controls
b – p <0.01

Table 8. I   c – p <0.001   Clinical Data

Sex and Age

	Group	n	Haemoglobin g/dl	PCV %	Albumin g/1
1	C	20	14.6 (0.3)	43.8 (0.9)	47 (0.6)
2	PRD	22	14.7 (0.2)	43.9 (0.6)	42 (1.2)
3	CRF	40	10.8^{c}(0.3)	32.4^{c}(1.0)	40 (0.7)
4	CAPD	24	9.6^{c}(0.3)	28.8^{c}(0.9)	36^{a}(0.9)
5	HD	31	9.0^{c}(0.4)	27.7^{c}(1.2)	43 (1.0)
6	TX	16	14.0 (0.5)	42.0 (1.3)	43 (1.0)
7	TX – RF	14	12.5^{b}(0.5)	37.0^{c}(1.4)	41 (0.8)
8	D	22	14.0 (0.3)	42.1 (1.0)	41 (1.4)
9	D – RF	24	11.0^{c}(0.5)	33.7^{c}(1.5)	38 (1.2)

Values are mean and (SEM)
p values refer to comparison with controls
Table 8. II   a – p <0.05
b – p <0.01   Clinical Data
c – p <0.001

Haemoglobin
and Albumin

	Group	n	UREA (mmol/l)	CREATININE (μmol/l)	BICARBONATE (mmol/l)
1	C	20	5.1 (1.1)	89 (20)	27.7 (6.2)
2	PRD	22	5.8 (1.2)	97 (21)	27.8 (5.9)
3	CRF	40	24.7^c (3.9)	521^c (82)	22.3^b (3.5)
4	CAPD	24	20.3^c (4.1)	998^c (204)	23.7^b (4.8)
5	HD	31	21.3^c (3.8)	967^c (174)	23.4^b (4.2)
6	TX	16	6.9 (1.7)	111 (28)	26.6 (6.7)
7	TX-RF	14	13.0^c (3.5)	277^c (74)	24.3^b (6.5)
8	D	22	5.6 (1.2)	87 (19)	26.7 (5.7)
9	D-RF	24	20.1^c (4.1)	466^c (95)	25.2^a (5.1)

Values are mean and (SD)
p values refer to comparison with controls
a — $p < 0.05$
b — $p < 0.01$
c — $p < 0.001$

Table 8. III   Clinical Data
Urea, Creatinine, Bicarbonate

	Group	n	Glucose (mmol/l)
1	C	20	4.0 (0.2)
2	PRD	22	3.8 (0.1)
3	CRF	40	4.1 (0.1)
4	CAPD	24	4.5 (0.2)
5	HD	31	4.4 (0.2)
6	TX	16	4.1 (0.2)
7	TX – RF	14	4.1 (0.3)
8	D	22	9.4^c (0.8)
9	D – RF	24	7.3^c (0.7)

Table 8. IV   Values are mean and (SEM)   Clinical Data
p values refer to controls
c — $p < 0.001$ Glucose

GLYCOSYLATED HAEMOGLOBIN – COLORIMETRIC METHOD

	Subjects	n	GHb (mmol HMF/mol Hb)
A)	Controls	17	35.9 (0.7)
B)	Uraemia	60	37.0 (0.4)
	CRF	13	38.3 (0.9)
	CAPD	31	35.8 (0.9)
	HD	16	37.1 (0.8)
C)	Diabetes	22	50.6 (2.2)
	No Uraemia	4	55.8 (4.2)
	CRF	5	55.0 (3.3)
	CAPD	9	49.3 (3.8)
	HD	4	43.5 (4.7)

Values are mean and (SEM)

A   v   B   NS
A   v   C $p < 0.001$
B   v   C $p < 0.001$

Table 8. V
Glycosylated Haemoglobin measured by the TBA – colorimetric
method in pilot study.

only in diabetics. Results are shown in Table 8.V (mean and SEM).
Non-diabetic uraemic patients and healthy controls had similar GHb
concentrations, whereas diabetics had significantly higher
concentrations ($p<0.001$).

Glycosylated and carbamylated haemoglobin (CHb): Table 8.VI shows the
values of glycosylated haemoglobin (GHb, HbA_1, HbA_{1c}, HbA_{1a+b}) and
carbamylated haemoglobin for 6 groups. The PRD, Tx and Tx-RF groups
are discussed later. Values are mean, (SEM) and [95% confidence
intervals]. Comparison of these results are shown below.

HbA_1: Figure 8.2a shows the scatter of HbA_1 values for each group
(transplants are shown later) and Figure 8.2b shows the mean, 95%
confidence interval and statistical difference between the groups.
HbA_1 was elevated in all 3 non-diabetic uraemic groups and in both
diabetic groups. The haemodialysis (HD) group had a lower HbA_1 than
the other non-diabetic uraemic groups. Both diabetic groups had
significantly higher HbA_1 than non-diabetics ($p<0.001$). The mean
level of HbA_1 was similar in both diabetics groups.

HbA_{1c}: Figure 8.3a shows the scatter of HbA_{1c} values and Figure 8.3b
shows the mean, 95% confidence interval and statistical difference
between the groups. The 3 non-diabetic uraemic groups (CRF, CAPD, HD)
had increased HbA_{1c} values compared to controls although there was
some overlap. Statistically there was no difference between the HD
group and controls although the other non-diabetic uraemic groups had
significantly increased HbA_{1c} concentrations. The diabetic groups had
significantly increased HbA_{1c} compared to controls and non-diabetic
uraemic subjects ($p<0.001$). There was no difference between the

GROUP	n	A_1 (%)	A_{1c} (%)	A_{1a+b} (%)	GHb (mmol HMF/mol Hb)	CHb (ngHMl/mg globin)
CONTROL	20	7.81 (0.13) [7.55 – 8.07]	5.25 (0.09) [5.06 – 5.43]	1.53 (0.04) [1.46 – 1.61]	36.7 (0.5) [35.7 – 37.7]	26 (2) [23 – 29]
CRF	40	9.56 (0.22) [9.12 – 10.00]	6.20 (0.14) [5.92 – 6.49]	2.04 (0.07) [1.90 – 2.17]	37.8 (0.4) [36.9 – 38.7]	137 (10) [116 – 158]
CAPD	24	9.50 (0.18) [9.13 – 9.87]	6.13 (0.14) [5.84 – 6.42]	2.25 (0.06) [2.12 – 2.37]	36.8 (0.7) [35.4 – 38.3]	127 (8) [111 – 144]
HD	31	8.82 (0.13) [8.56 – 9.07]	5.45 (0.10) [5.24 – 5.67]	1.88 (0.44) [1.79 – 1.97]	37.0 (0.6) [35.8 – 38.2]	100 (5) [89 – 111]
DIAB	22	11.86 (0.43) [10.97 – 12.75]	8.27 (0.26) [7.74 8.81]	2.12 (0.07) [1.96 – 2.27]	51.5 (1.9) [47.6 – 55.4]	27 (2) [22 – 32]
DIAB-RF	24	11.87 (0.50) [10.84 – 12.90]	7.73 (0.32) [7.08 – 8.39]	2.31 (0.09) [2.12 – 2.50]	49.6 (1.9) [45.6 – 53.6]	105 (11) [83 – 127]

Values are mean, (SEM), and [95% confidence intervals]

Table 8. VI   Glycosylated and Carbamylated Haemoglobin
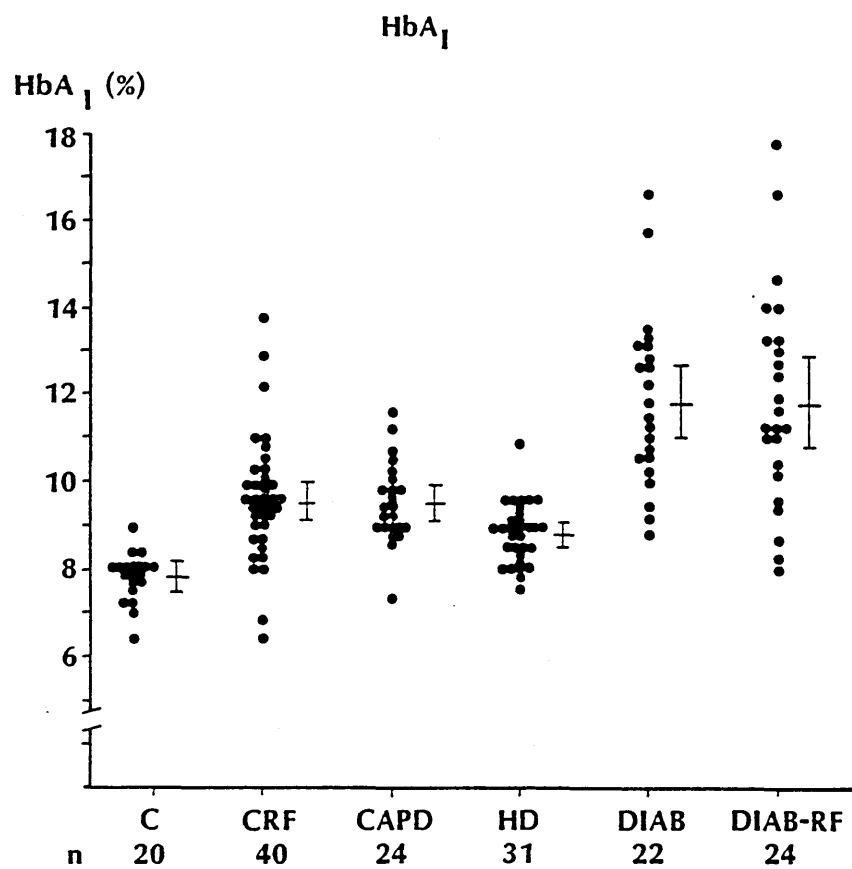
HbA₁

HbA₁ (%)

Figure 8. 2a Scatter Plot
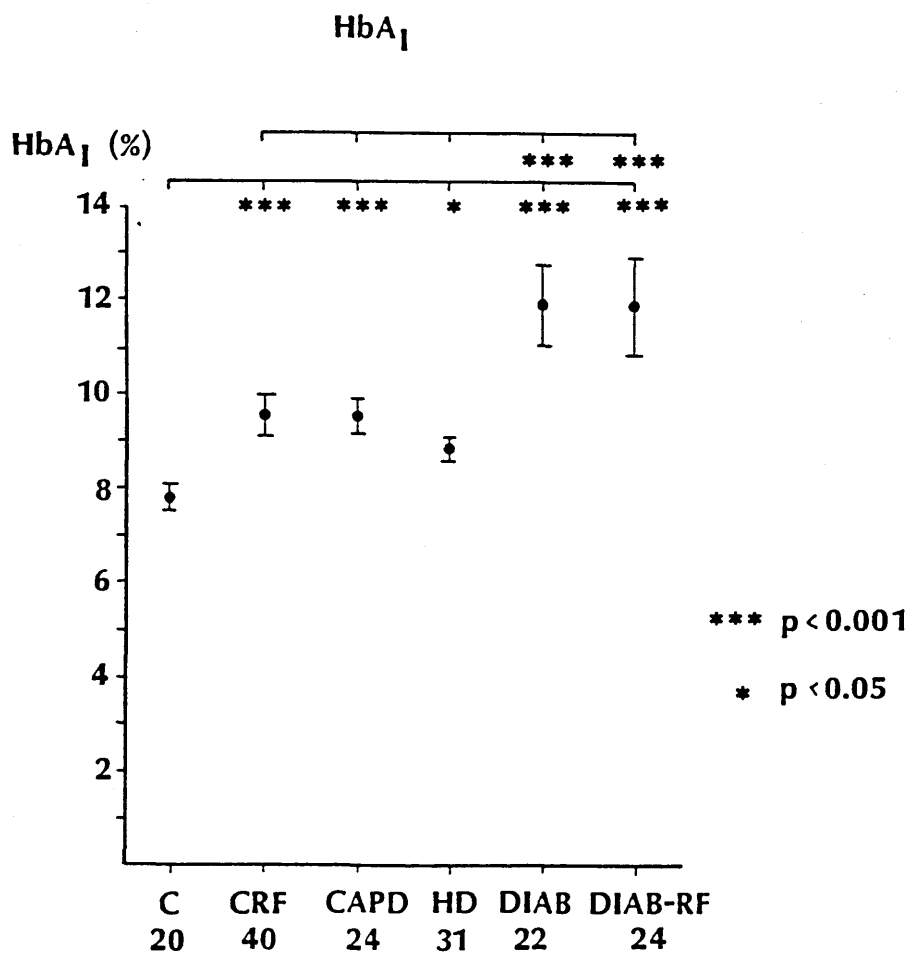
HbA₁

HbA₁ (%)

Figure 8. 2b   Values are mean and 95% C.I.

HbA$_{IC}$

HbA$_{IC}$ (%)
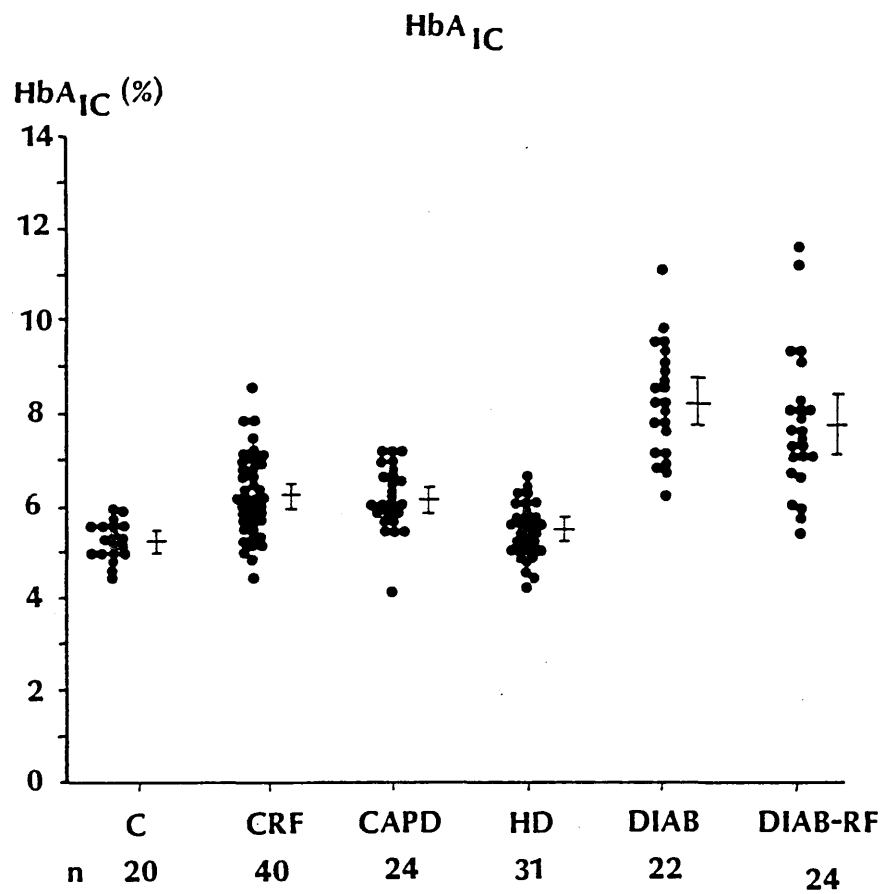
Figure 8. 3a   Scatter Plot

HbA$_{IC}$

HbA$_{IC}$ (%)

*** p < 0.001

** p < 0.01
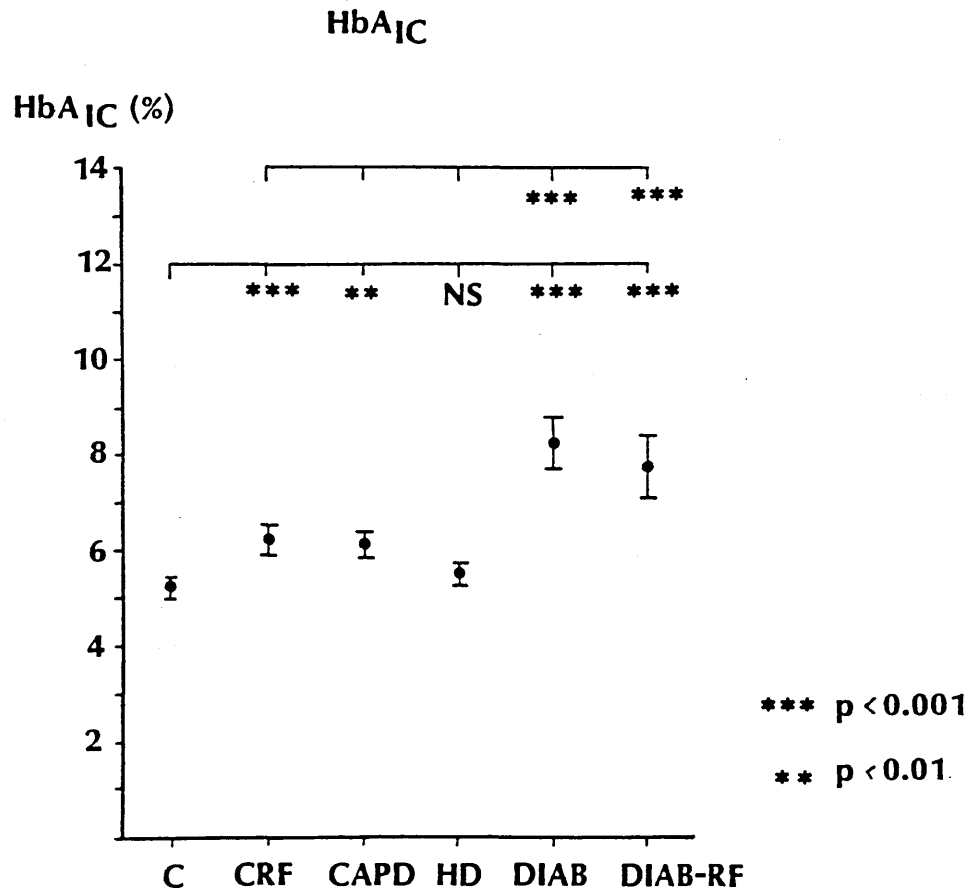
Figure 8. 3b   Mean and 95% C.I.

diabetic groups.

HbA_{1a+b}: Figure 8.4a shows the scatter of HbA_{1a+b} values and Figure
8.4b the mean, 95% confidence interval and statistical difference.
HbA_{1a+b} was significantly increased in all uraemic and diabetic groups
compared to controls ($p<0.001$). The scatter of HbA_{1a+b} values was
similar in both uraemia and diabetics. The HD group had the lowest
mean HbA_{1a+b} concentration of the uraemic groups and was significantly
lower than the CAPD group ($p<0.001$) but not significantly different
from the CRF group. Diabetics with renal failure had a marginally
higher mean than diabetics with normal renal function but the
difference was not significant. Diabetics with renal failure had
similar HbA_{1a+b} concentrations as the CAPD group and both were higher
than the CRF group.

GHb: Figure 8.5a shows the scatter of GHb values and Figure 8.5b the
mean, 95% confidence interval and statistical difference.
Glycosylated haemoglobin measured by colorimetry was elevated only in
the presence of diabetes ($p<0.001$). There was no difference between
diabetics with normal or impaired renal function. GHb was similar in
non-diabetic uraemics and controls.

CHb: Figure 8.6a shows the scatter of CHb values and Figure 8.6b the
mean, 95% confidence interval and statistical difference. Although
there was a wide scatter of CHb concentrations there was no overlap
between individuals with renal failure and normal renal function. All
uraemic groups including diabetics with renal failure had gross
elevation of CHb ($p<0.001$). The HD group had a lower mean CHb
concentration than the CRF and CAPD groups ($p<0.01$), but this was not
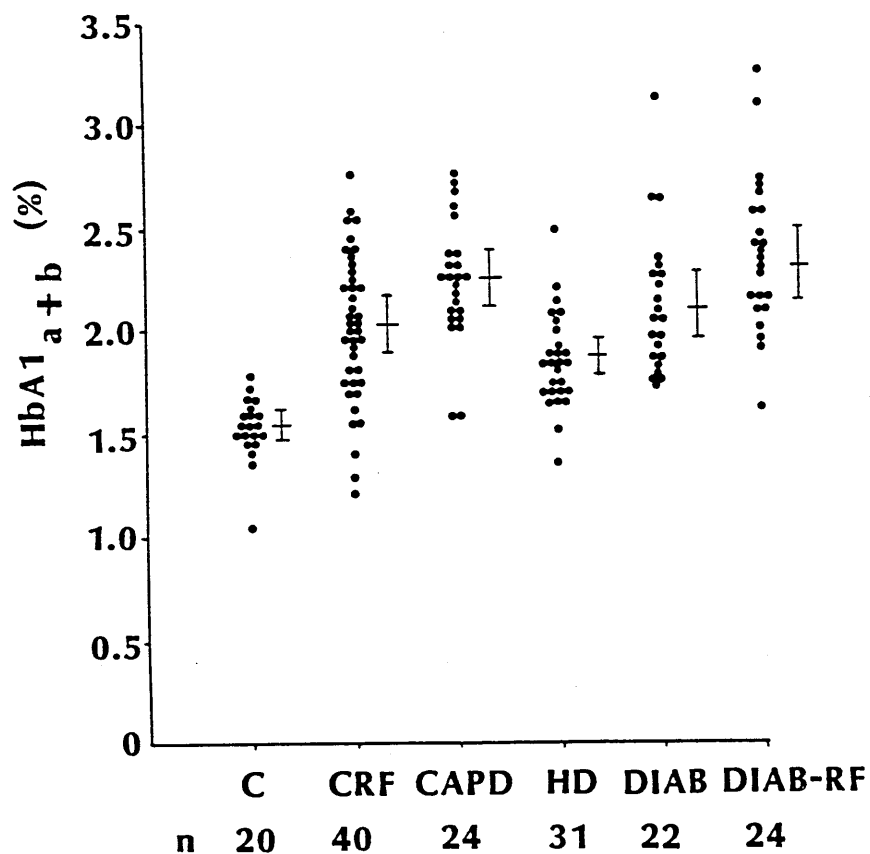
Figure 8. 4a   Scatter Plot
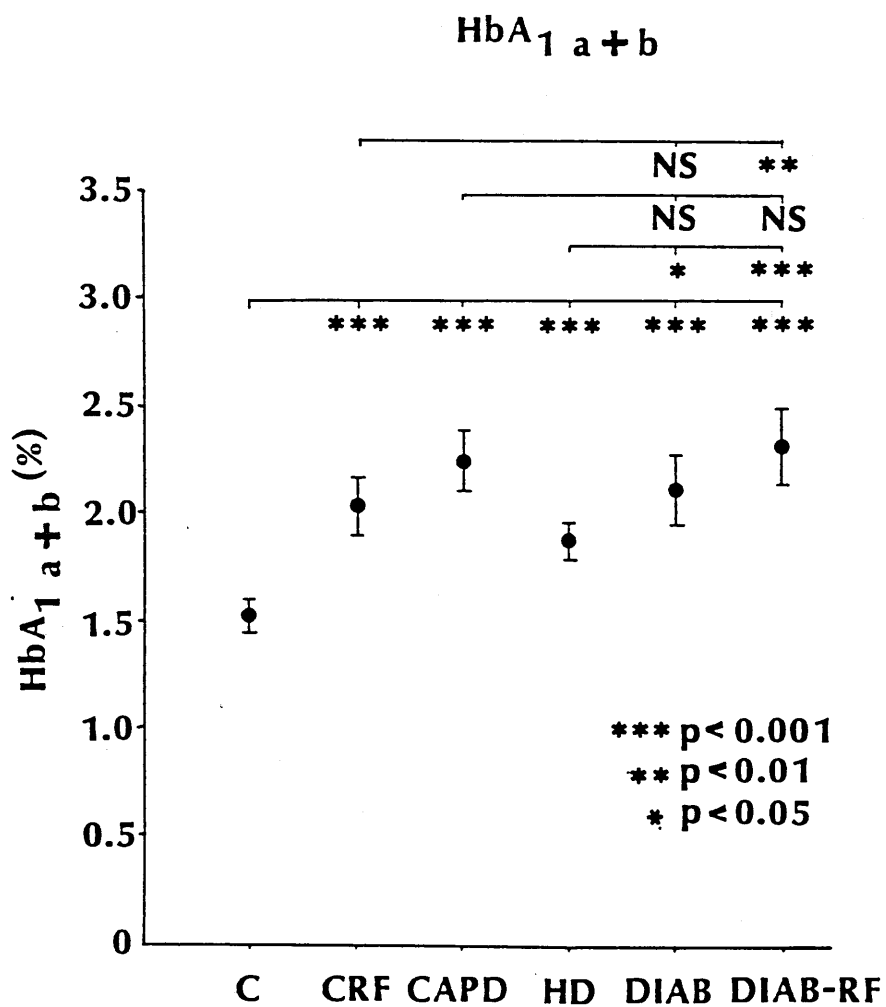
Figure 8. 4b   Mean and 95% C.I.

GLYCOSYLATED HAEMOGLOBIN

GHb
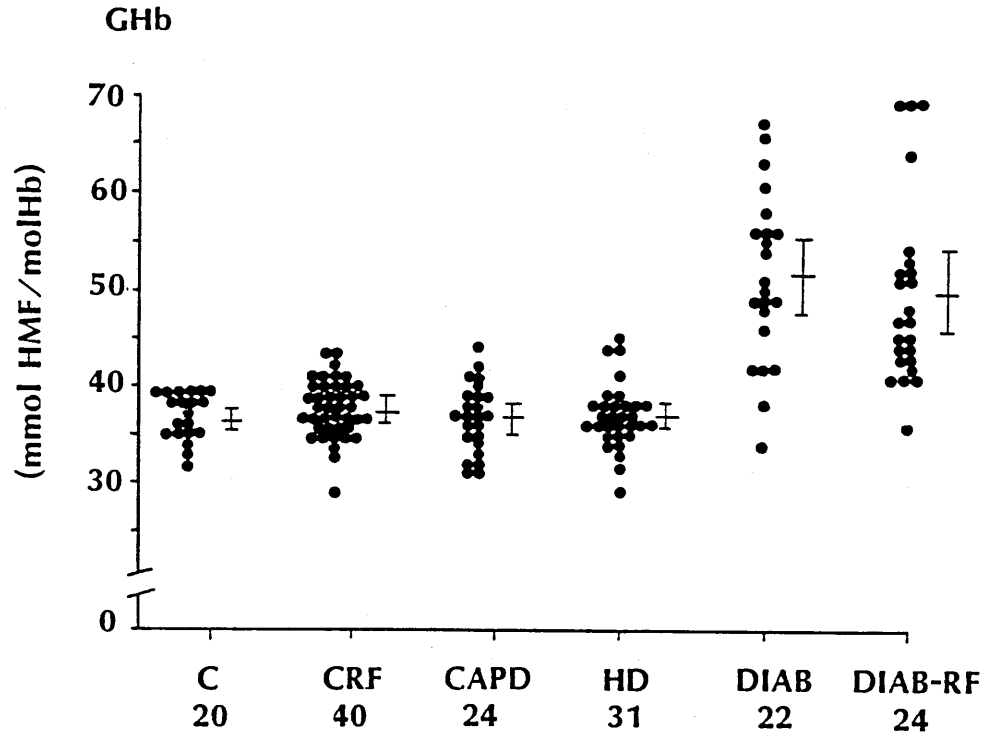
Figure 8. 5a   Scatter Plot

GLYCOSYLATED HAEMOGLOBIN

*** p < 0.001
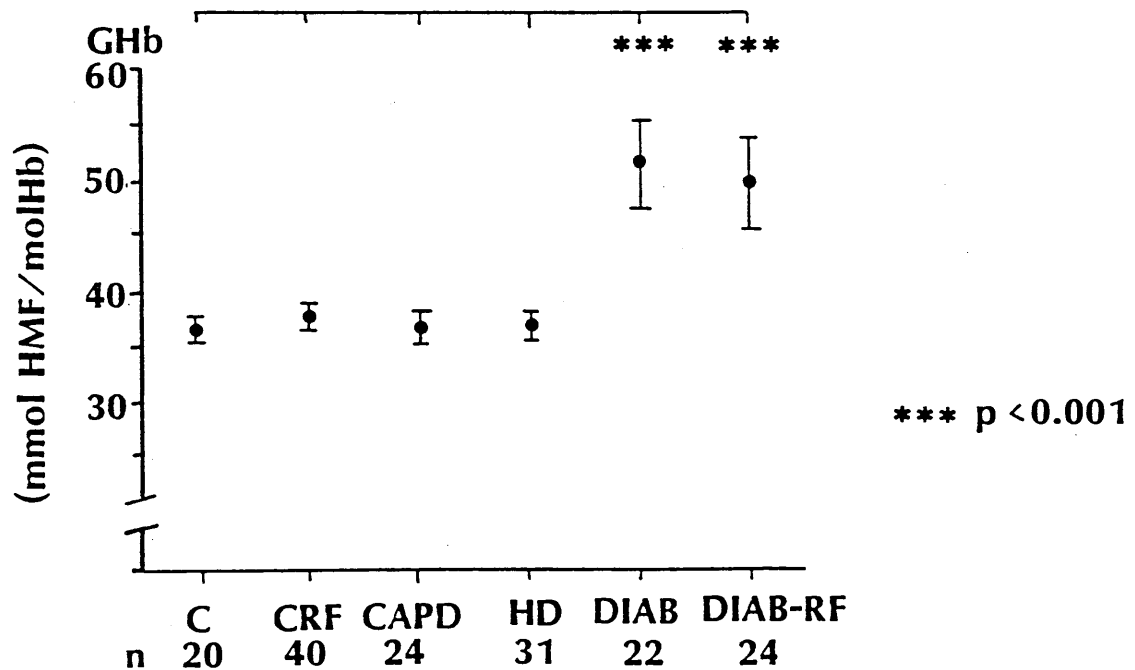
Figure 8. 5b   Mean and 95% C.I.

CARBAMYLATED HAEMOGLOBIN
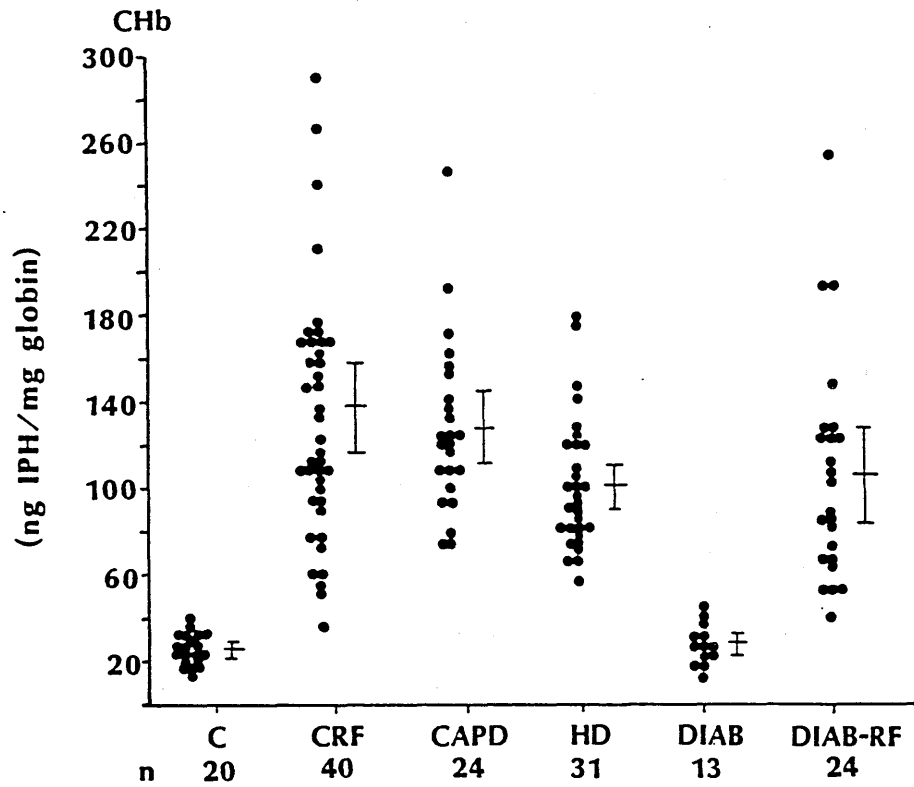
Figure 8. 6a   Scatter Plot

CARBAMYLATED HAEMOGLOBIN
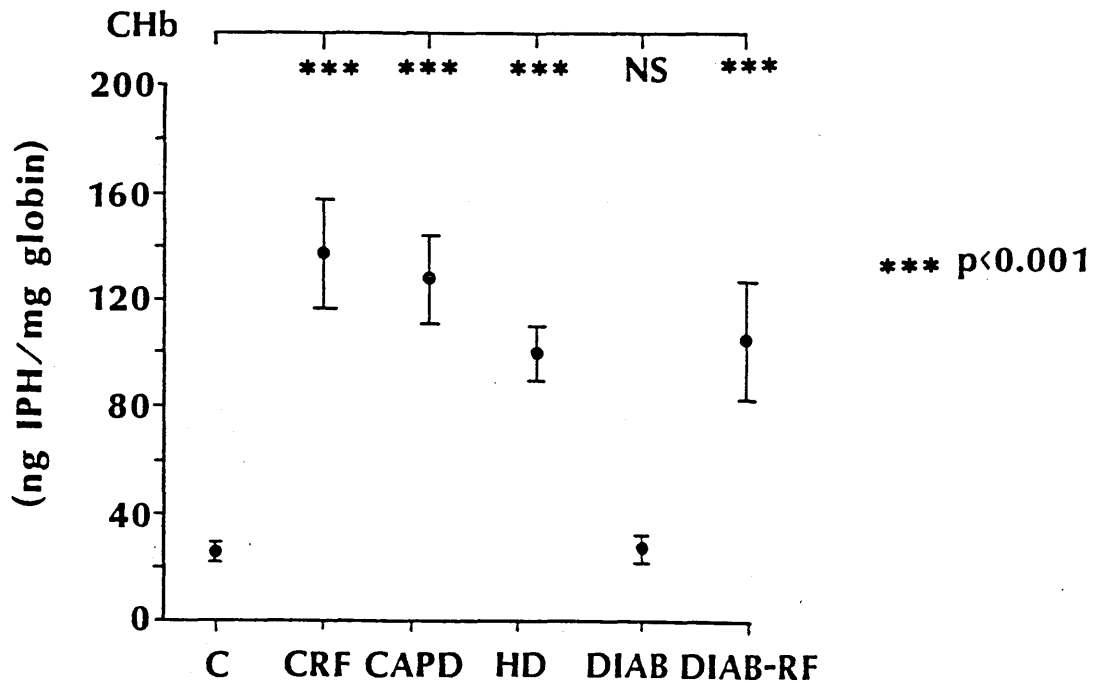
Figure 8. 6b   Mean and 95% C.I.

statistically different from diabetics with renal failure.

3. <u>Comparison of glycosylated haemoglobin methods</u>

Glycosylated haemoglobin measured by colorimetry (GHb) and by the minicolumn cation chromatographic techniques (HbA_1, HbA_{1c}, HbA_{1a+b}) were compared and are shown in Figures 8.7, 8.8 and 8.9. Figure 8.7a shows the correlations of HbA_1 and GHb and Figure 8.7b the correlation of HbA_{1c} and GHb in diabetics with normal renal function. A strong and significant positive linear correlation was found in both comparisons. Linear regression analysis was carried out in diabetics with renal failure comparing HbA_1 (Figure 8.8a) and HbA_{1c} (Figure 8.8b) with GHb. A strong linear correlation was found for both methods. Comparison between HbA_{1a+b} and GHb in diabetics with normal renal function is shown in Figure 8.9a and Figure 8.9b shows the correlation between HbA_{1a+b} and GHb in diabetics with renal failure. Linear regression analysis shows that the correlation was less strong in both diabetic groups with the HbA_{1a+b} fraction compared to the HbA_1 and HbA_{1c} fractions, although a significant correlation was found in all. No significant correlations between GHb and HbA_1 or HbA_{1c} or HbA_{1a+b} were found in any of the non-diabetic uraemic groups.

Comparison of the microcolumn methods for each fraction are shown in Table 8.VII. The correlation coefficients for each patient group are shown with the strongest correlation in diabetics with and without renal failure. Generally the best correlation was found in comparing the HbA_1 component with the HbA_{1c} fraction.

The ratio of HbA_{1a+b}/HbA_{1c} for each group is shown in Table 8.VIII. The values are mean and (SD). The ratio HbA_{1a+b}/HbA_{1c} was significantly increased in all non-diabetic uraemics compared to the control group due to a relatively greater increase in the HbA_{1a+b}

DIABETIC

HbA$_1$(%)

y = 2.25 + 0.187x
r = 0.817
n = 22
p < 0.001

10   30   50   70 GHb
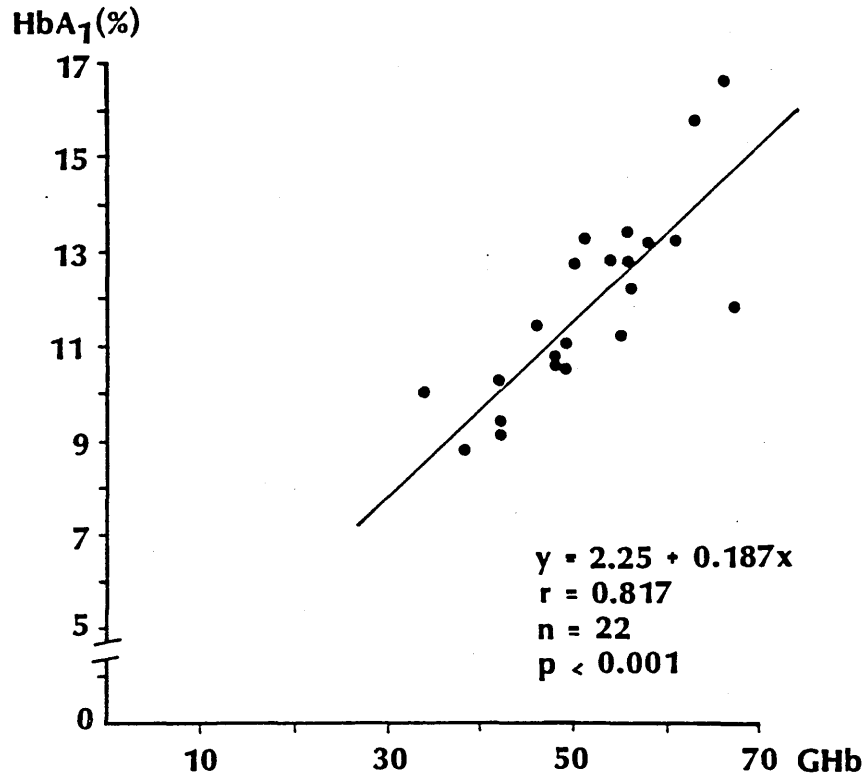
Figure 8. 7a **(mmol HMF/mol Hb)**   Correlation between HbA$_1$and
                                        GHb in non uraemic diabetics

DIABETIC

HbA$_{1C}$ (%)

y = 2.66 + 0.109x
r = 0.795
n = 22
p < 0.001

10   30   50   70   GHb
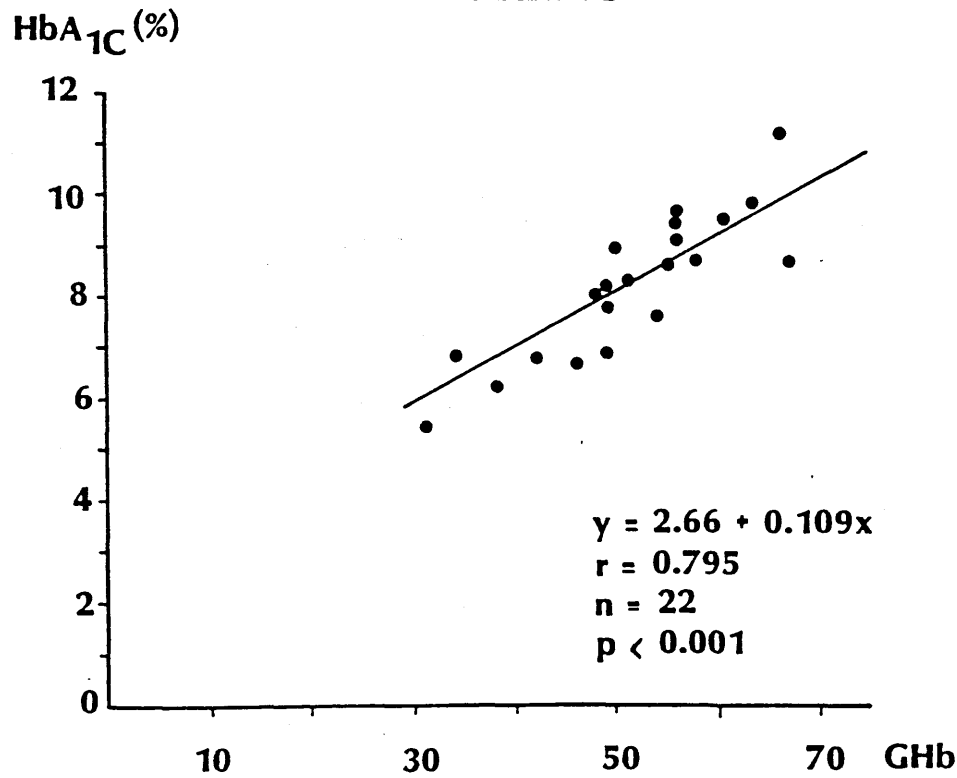
Figure 8. 7b   **(mmol HMF/molHb)**   Correlation between HbA$_{1C}$and
                                         GHb in non uraemic diabetics

DIAB-RF
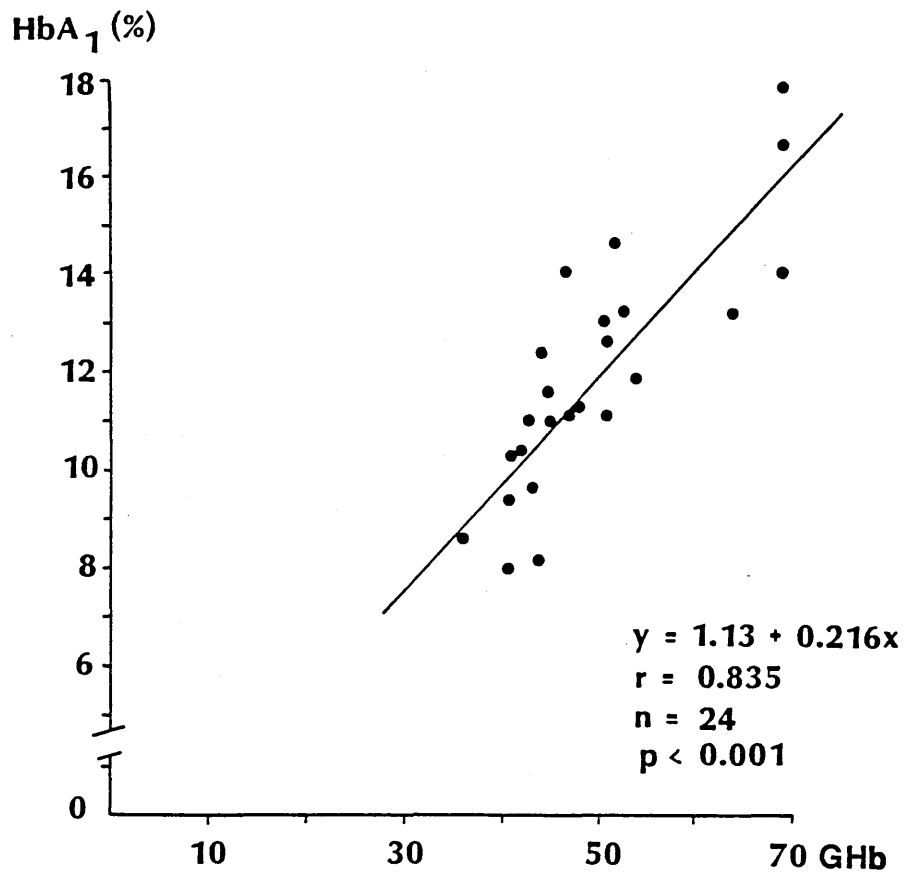
Figure 8. 8a   (mmol HMF/mol Hb)

Correlation between HbA_1 and GHb in diabetics with renal failure

DIAB-RF

Figure 8. 8b

Correlation between HbA_{1c} and GHb in diabetics with renal failure

DIABETIC
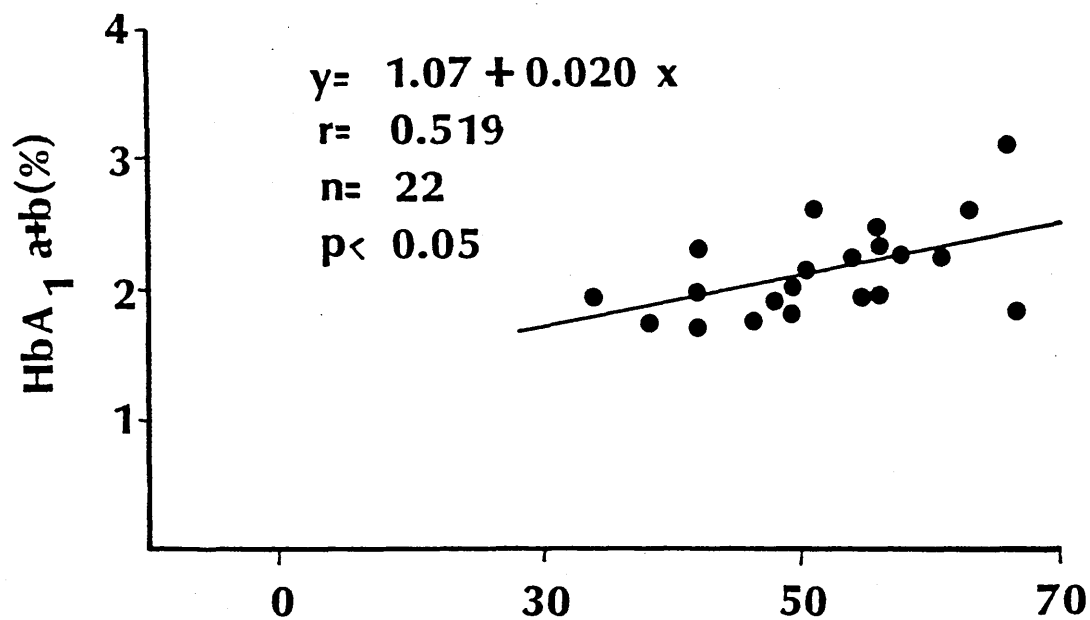
Figure 8. 9a
Correlation between HbA_{1a+b} and GHb in non-uraemic diabetics

DIAB-RF
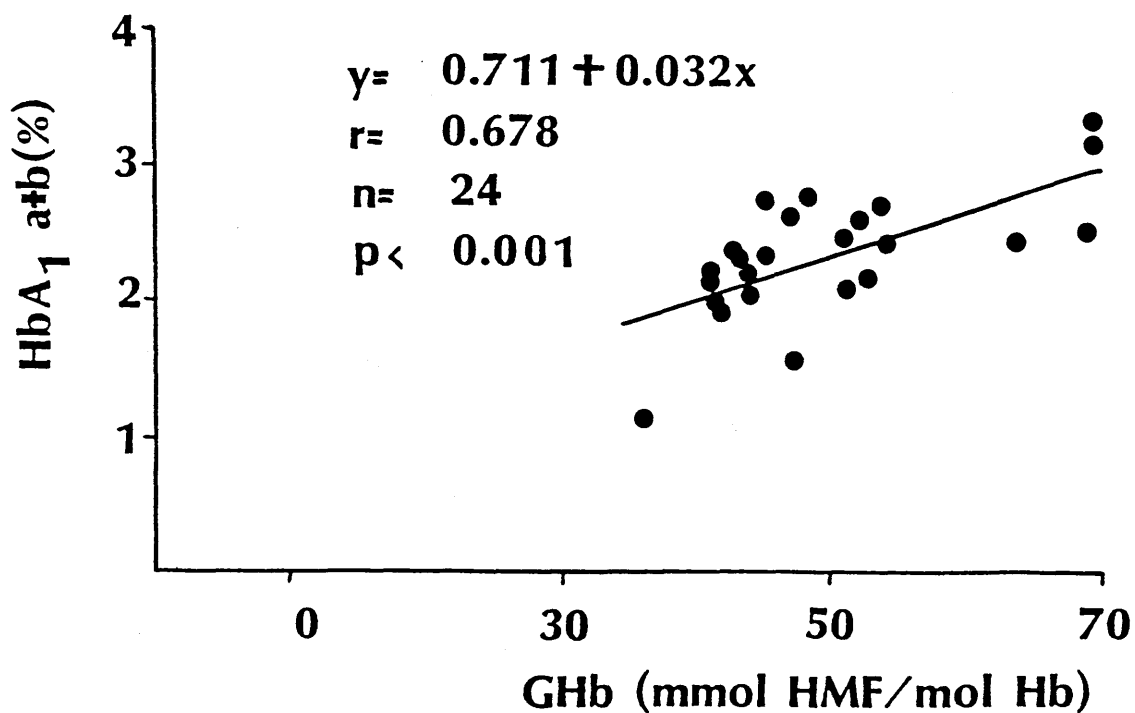
Figure 8. 9b

Correlation between HbA_{1a+b} and GHb in diabetics withrenal failure

Table 8. VII

Correlation coefficients (r) of

HbA$_1$ and subfractions

GROUP	A$_1$vA$_{1c}$	A$_1$vA$_{1a+b}$	A$_{1c}$vA$_{1a+b}$
C	0.731	0.707	0.749
CRF	0.518	0.539	0.712
CAPD	0.724	0.696	0.555
HD	0.475	0.388	0.318
DIAB	0.857	0.829	0.675
DIAB-RF	0.889	0.763	0.799

Comparison of ion exchange chromatographic methods

Table 8. VIII

Ratio of HbA$_{1a+b}$/HbA$_{1c}$

GROUP	n	HbA$_{1a+b}$/HbA$_{1c}$	p
C	20	0.292 (0.021)	
CRF	40	0.328 (0.049)	<0.01
CAPD	24	0.368 (0.039)	<0.001
HD	31	0.347 (0.046)	<0.001
DIAB	22	0.257 (0.033)	<0.01
DIAB-RF	24	0.301 (0.042)	NS

Values are mean and (SD)

p values refer to comparison with controls

Identical levels of significance were obtained
by both Mann Whitney test and Analysis of
Variance

fraction. Diabetics with normal renal function had a significantly
lower ratio due to a relatively greater increase in the HbA_{1c}
components. Diabetics with renal failure had a ratio similar to
controls, even though the absolute quantities of both HbA_{1a+b} and
HbA_{1c} increased.

4. Glycosylated haemoglobin and renal function

Comparison of glycosylated haemoglobin (HbA_1, HbA_{1c}, HbA_{1a+b}, GHb)
and both urea and creatinine were sought by linear regression
analysis.

HbA_1 and urea showed no significant correlation in any of the
groups except for the HD group ($r=0.502$, $p<0.01$). All the other
groups had an r value below 0.2. HbA_1 and creatinine showed no
correlation in any of the groups except controls ($r=0.710$, $p<0.001$).

HbA_{1c} and urea showed no correlation in any of the groups with the
exception of a modest correlation with the CAPD group ($r=0.460$,
$p<0.05$). HbA_{1c} and creatinine did not show any correlation in any of
the groups.

HbA_{1a+b} and urea were observed to have a correlation in the CRF
($r=0.331$, $p<0.05$) and CAPD ($r=0.422$, $p<0.05$) groups but not in any of
the others. HbA_{1a+b} and creatinine were correlated in the CAPD group
($r=0.473$, $p<0.05$) but not in the others. Neither urea nor creatinine
showed any correlation with GHb in any of the groups. Overall none of
the glycosylated haemoglobin components consistently correlated with
urea or creatinine in any of the groups.

Glycosylated haemoglobin did not correlate with random blood
glucose, total haemoglobin, sex or age in any of the groups.

5. Carbamylated haemoglobin and renal function

Carbamylated haemoglobin (CHb) expressed as isopropyl hydantoin (IPH) was similar in controls (Table 8.VI) to both diabetics and patients with primary renal disease (Table 8.XIII). The 'normal' concentration of CHb was 26 (range 23-29) in 20 healthy controls. Combining the above 3 groups a non-uraemic reference range was determined in 64 patients: mean=27, range 22-32 ng IPH/mg globin.

Comparison of CHb concentrations and renal function are show in Table 8.IX and Figures 8.10 and 8.11. The correlation coefficients and p values between CHb and both urea and creatinine are shown in Table 8.IX. A stronger correlation was found between CHb and urea than between CHb and creatinine. The strongest correlation was found in undialysed patients with chronic renal failure. Controls showed a weak correlation but diabetics with normal renal function showed no correlation. Figure 8.10a displays a significant positive linear correlation between CHb and urea in the CRF group and Figure 8.10b shows the correlation between CHb and creatinine in the CRF group. The comparison between CHb and urea for diabetics with renal failure is shown in Figure 8.11. The CAPD group was found to have a stronger correlation between CHb and both urea and creatinine than the haemodialysis (HD) group.

CHb and bicarbonate: No significant correlation was found between CHb and bicarbonate in any of the groups with the exception of the CAPD group (r=-0.411, p<0.05), which had a mean bicarbonate level within the normal range.

Table 8. IX

Correlation of Carbamylated Haemoglobin
and Renal Function

GROUP		UREA		CREATININE	
	n	r	p	r	p
CONTROL	20	0.551	<0.05	0.521	<0.05
CRF	40	0.752	<0.001	0.624	<0.001
CAPD	24	0.552	<0.01	0.467	<0.05
HD	31	0.440	<0.05	-0.022	NS
DIAB	13	0.483	NS	0.445	NS
DIAB-RF	22	0.682	<0.001	-0.065	NS
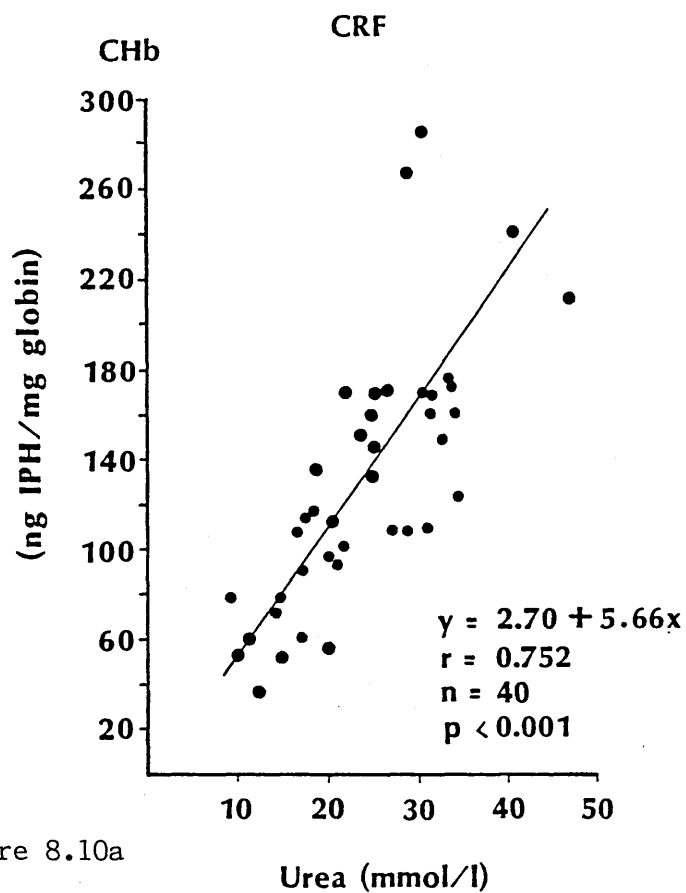
Figure 8.10a

Correlation between carbamylated haemoglobin
and urea in chronic renal failure
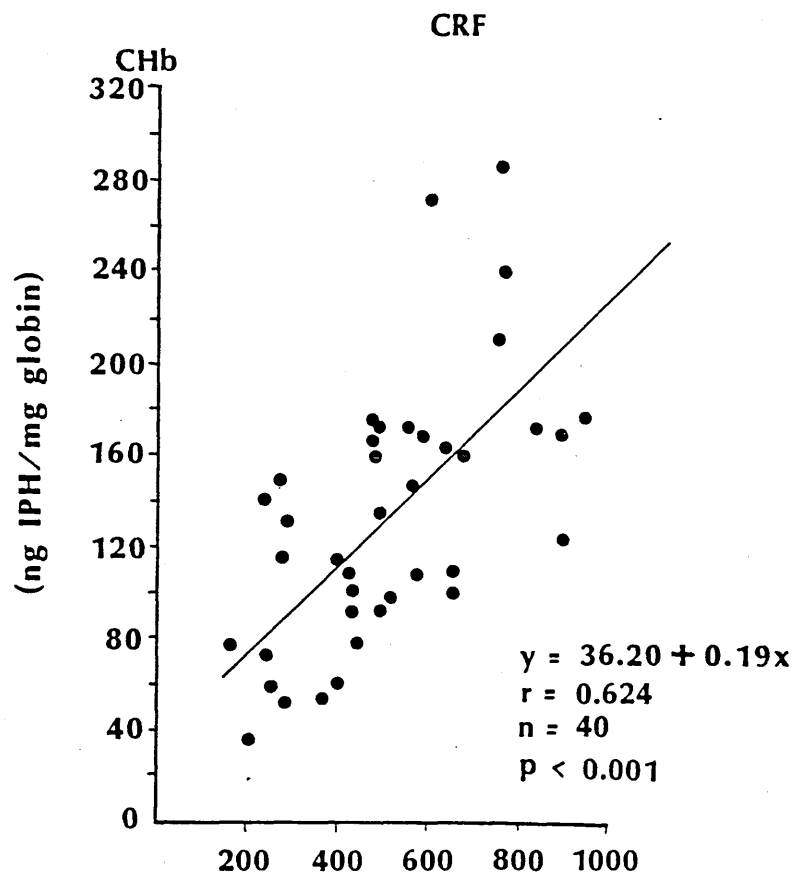
Figure 8.10b   Creatinine (μmol/l)

Correlation between carbamylated haemoglobin and
creatinine in chronic renal failure

DIAB-RF

$y = 1.62 + 5.30x$
$r = 0.682$
$n = 24$
$p < 0.001$
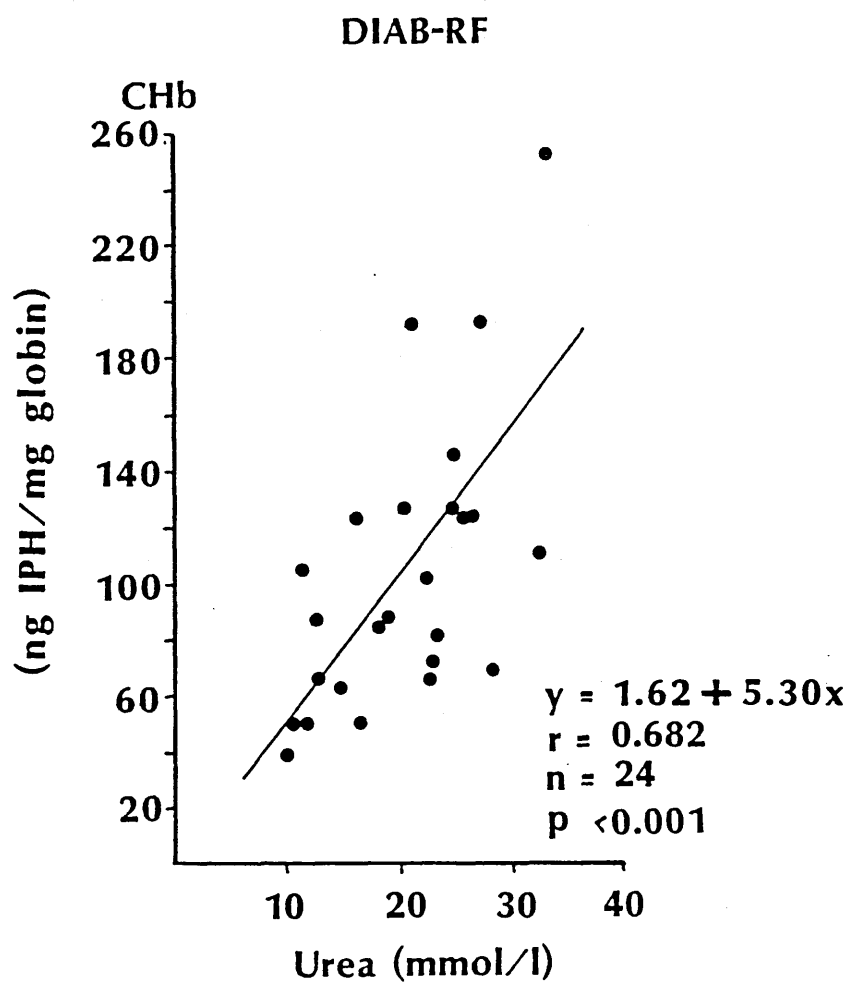
Figure 8.11   Correlation between carbamylated haemoglobin and urea in diabetics with renal failure

CHb and haemoglobin: There was no correlation between CHb and total haemoglobin (or PCV) in any of the groups.

CHb and glucose: No correlation between CHb and random blood glucose was observed in any of the groups.

6. Carbamylated and glycosylated haemoglobin

CHb and HbA_1: Plots showing the correlation between HbA_1 and CHb are shown in Figure 8.12. In the CRF group (Figure 8.12a) and the CAPD group (Figure 8.12b) a significant positive linear correlation was found between the 2 variables. A weak correlation ($r=0.355$, $p<0.05$) was found in the HD group but no significant correlation was found between HbA_1 and CHb in diabetics with renal failure (Figure 8.13).

CHb and HbA_{1c}: HbA_{1c} and CHb showed a significant linear correlation in CRF patients ($r=0.605$, $p<0.001$) as displayed in Figure 8.14. A moderate correlation was found in the CAPD group ($r=0.434$, $p<0.01$). No correlation was found between the variables in either the HD ($r=0.042$) or the diabetic renal failure groups ($r=0.178$).

CHb and HbA_{1a+b}: HbA_{1a+b} and CHb showed a significant positive correlation in CRF and CAPD patients as seen in Figures 8.15a and 8.15b. The HD and diabetic renal failure groups showed no correlation (HD: $r=0.233$; D-RF: $r=0.248$).

CRF

HbA$_1$(%)

$$y = 8.33 + 0.009x$$
$$r = 0.425$$
$$n = 40$$
$$p < 0.01$$

40 80 120 160 200 240 280 320 360 CHb
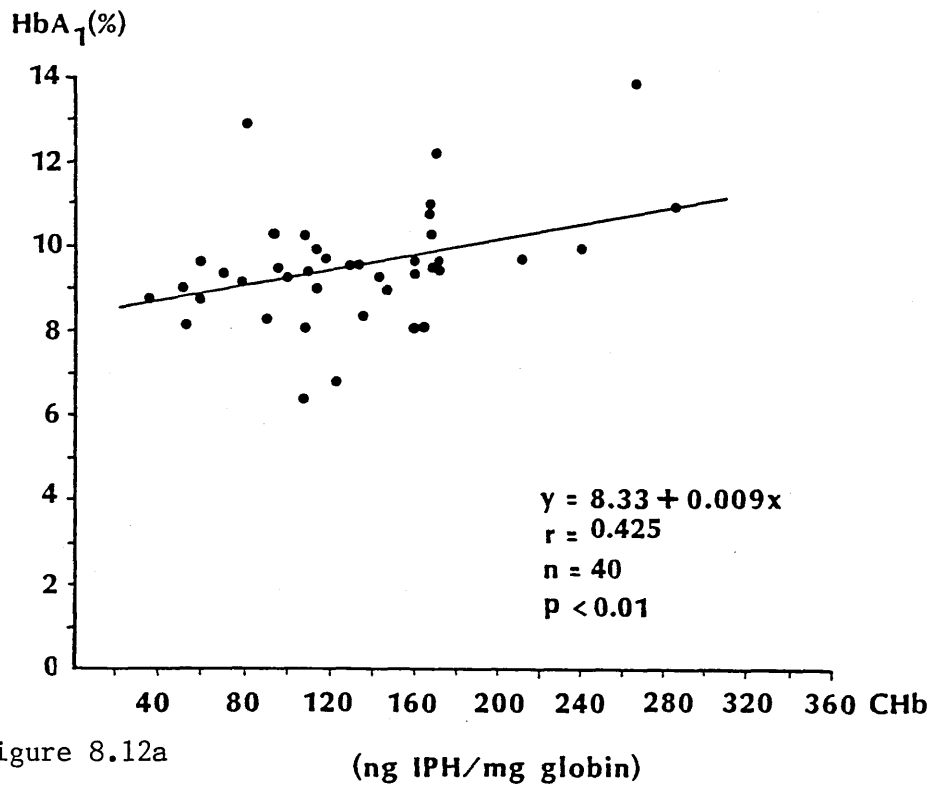
(ng IPH/mg globin)

Figure 8.12a

Correlation between HbA$_1$ and carbamylated haemoglobin
in chronic renal failure

CAPD

HbA$_1$(%)

$$y = 7.90 + 0.012x$$
$$r = 0.563$$
$$n = 24$$
$$p < 0.01$$

20 60 100 140 180 220 260 300 CHb

(ng IPH/mg globin)

Figure 8.12b

Correlation between HbA$_1$ and carbamylated haemoglobin
in CAPD

DIAB-RF

HbA$_1$(%)

y = 10.7 + 0.011x
r = 0.238
n = 24
p = NS

CHb
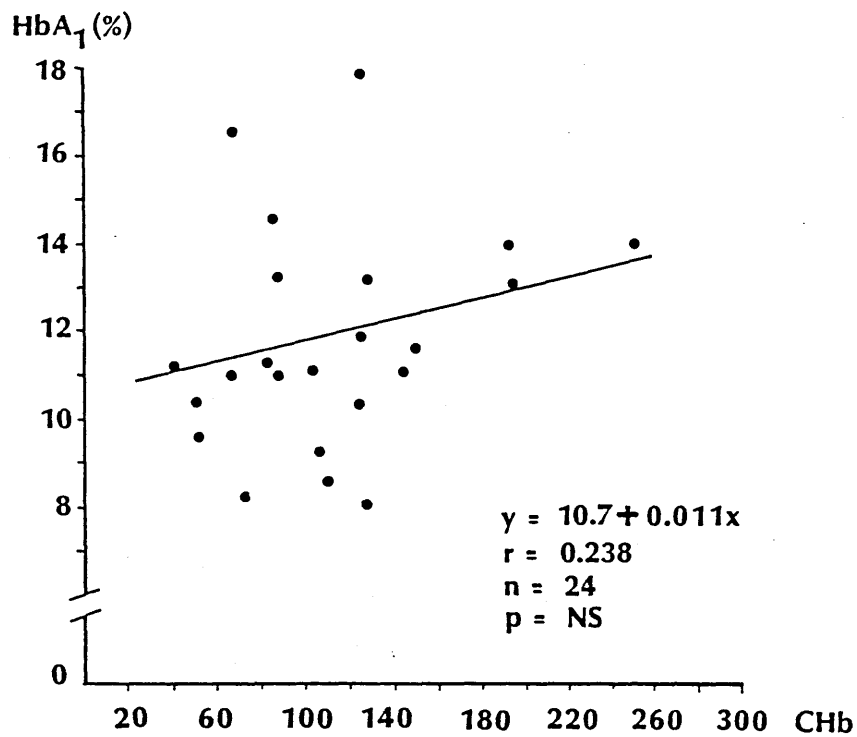
Figure 8.13   (ng IPH/mg globin)

Correlation between HbA$_1$ and carbamylated haemoglobin
in diabetics with renal failure

CRF

HbA$_{1C}$ (%)

y = 5.07 + 0.008x
r = 0.605
n = 40
p < 0.001

CHb
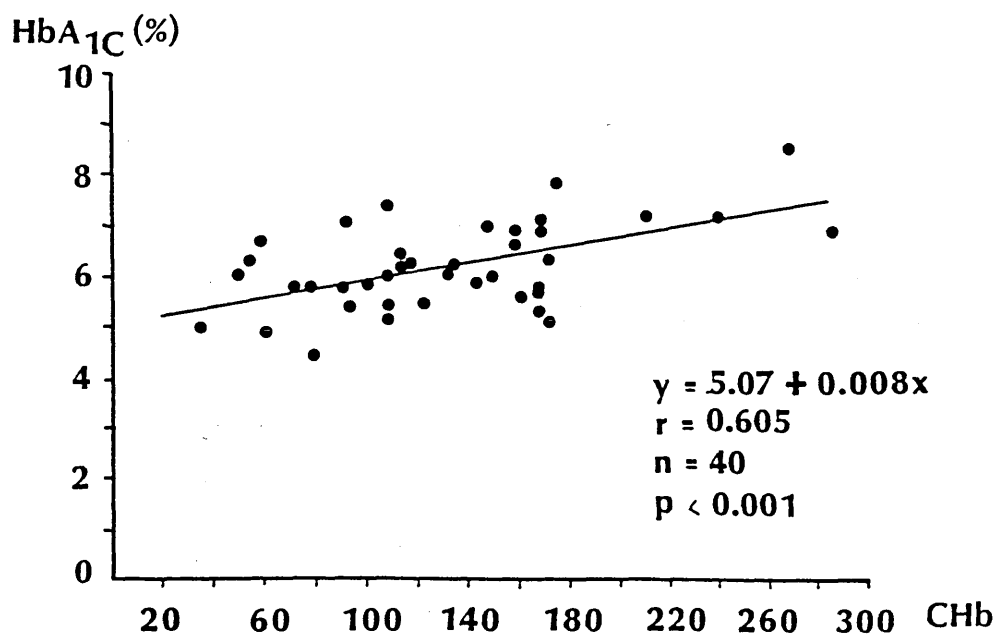
Figure 8.14   (ng IPH/mg globin)

Correlation between HbA$_{1c}$ and carbamylated haemoglobin
in chronic renal failure

CRF

$$y = 1.58 + 0.003x$$
$$r = 0.519$$
$$n = 40$$
$$p < 0.001$$
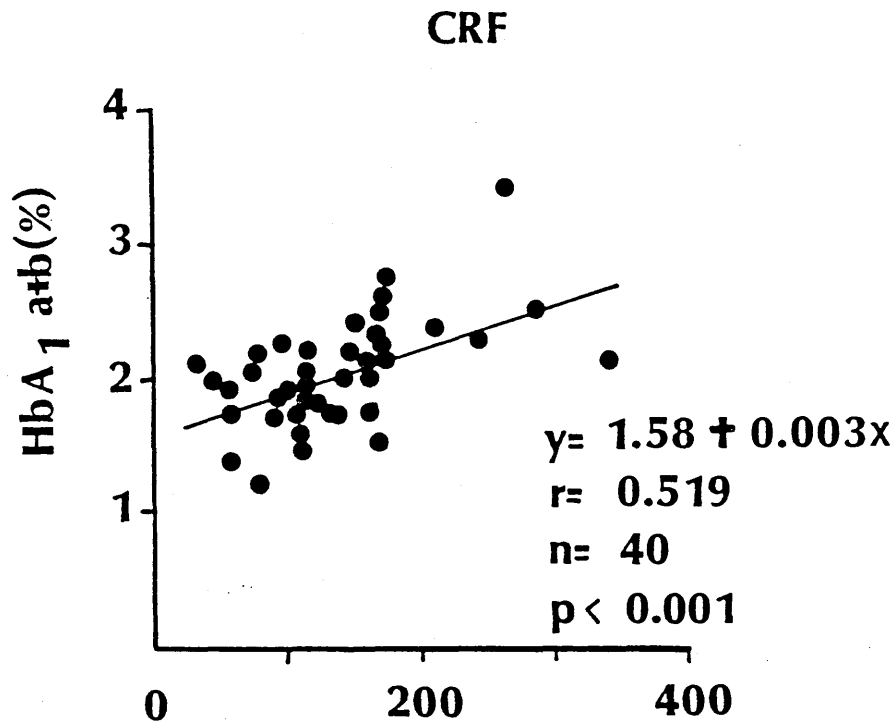
Figure 8.15a **CHb (ng IPH/mg globin)**

Correlation between HbA_{1a+b} and carbamylated
haemoglobin in chronic renal failure

CAPD

$$y = 1.72 + 0.004x$$
$$r = 0.560$$
$$n = 24$$
$$p < 0.01$$
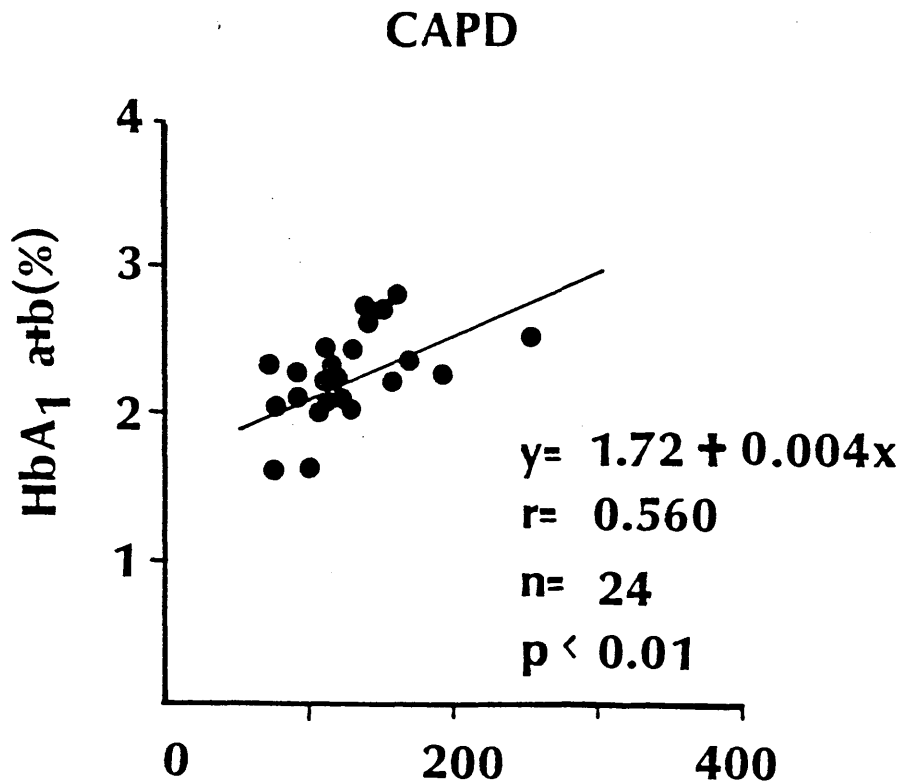
Figure 8.15b **CHb (ng IPH/mg globin)**

Correlation between HbA_{1a+b} and carbamylated
haemoglobin in CAPD.

CHb and GHb: There was generally no correlation between glycosylated haemoglobin measured by colorimetry and CHb. The only exception was a weak correlation in the CRF group (r=0.341, p<0.05). No correlation was observed between GHb and CHb in diabetics with renal failure (r=0.174).

.Overall there was a correlation between CHb and the chromatographic measured components of glycosylated haemoglobin but not with the colorimetric measured species. This relationship was seen in non-diabetic uraemic patients, particularly the CRF and CAPD groups, but not in diabetics with renal failure.

The absolute values for glycosylated and carbamylated haemoglobin were shown in Table 8.VI and a summary of the relative change, in each variable for each patient group, compared to the control group is shown in Table 8.X. HbA_1 increased by about 12 to 20% in the non-diabetic uraemic patients compared to a 50% increment in diabetics. HbA_{1c} showed more variation but less relative change in uraemics while diabetics had a rise of about 50%. HbA_{1a+b} was increased by over 50% in uraemic diabetics but all the uraemic groups showed a relatively greater increase in the HbA_{1a+b} fraction than the HbA_{1c}. GHb showed negligible change in non-diabetics while both diabetic groups showed an increment of 35 to 40%. CHb had a 2 to 4-fold increase in all uraemic patients including diabetics with uraemia.

7. The effect of haemodialysis

Several variables were measured in 9 haemodialysis patients immediately pre- and post- a 4 hour dialysis session. The paired 't' test was used to compare the changes. The results are shown in Table

Table 8. X

Relative change in glycosylated and
carbamylated haemoglobin

	HbA_1 %	HbA_{1c} %	HbA_{1a+b} %	GHb %	CHb %
CRF	22.4	18.1	33.3	3.0	427
CAPD	21.6	16.8	47.1	<1	385
HD	12.9	3.8	22.9	<1	285
D	51.9	57.5	38.6	40.3	4
D-RF	52.0	47.2	51.0	35.1	304
Tx-RF	11.4	9.3	19.0	4.6	235

Values are the percentage change (increase) in comparison
to controls.

Table 8. XI

The effect of haemodialysis

	Pre Dialysis	Post Dialysis	P
Glucose	4.2 (3.9 – 4.6)	6.9 (5.6 – 8.1)	< 0.001
A_1	9.0 (8.3 – 9.8)	9.1 (8.6 – 9.6)	NS
A_{1c}	5.3 (4.9 – 5.7)	5.3 (4.9 – 5.7)	NS
A_{1a+b}	1.9 (1.7 – 2.1)	2.0 (1.7 – 2.2)	NS
GHb	38.3 (35.2 – 41.4)	36.8 (34.7 – 38.9)	NS
CHb	111 (84 – 139)	100 (79 – 120)	NS
Urea	26.9 (23.3 – 30.5)	12.9 (9.2 – 16.5)	< 0.001
Creatinine	1043 (928 – 1159)	568 (516 – 622)	< 0.001
H+	43.4 (40.1 – 44.6)	36.1 (32.4 – 39.8)	< 0.01
Bicarb	22.2 (21.4 – 23.1)	25.8 (24.5 – 27.0)	< 0.001

n = 9
Values are mean and (95% C.I.)
P–comparison between pre and post dialysis

Table 8. XII

Correlation between Glycosylated/Carbamylated
Haemoglobin and Bicarbonate/H^+ concentrations

	GROUP	n	r	p
$HbA_1 vBic$	C	20	0.238	NS
$HbA_1 vBic$	CRF	40	0.141	NS
$HbA_1 vBic$	CAPD	24	-0.545	< 0.01
$HbA_1 vBic$	HD	31	-0.425	< 0.05
$HbA_1 vH^+$	HD	8	0.221	NS
$HbA_1 vBic$	DIAB-RF	24	-0.044	NS
$HbA_{1c} vBic$	CRF	40	0.033	NS
$HbA_{1c} vBic$	CAPD	24	-0.441	< 0.05
$HbA_{1c} vBic$	HD	31	-0.191	NS
$HbA_{1c} vH^+$	HD	8	-0.086	NS
$HbA_{1c} vBic$	DIAB-RF	24	0.205	NS
$HbA_{1a+b} vBic$	CRF	40	0.094	NS
$HbA_{1a+b} vBic$	CAPD	24	-0.576	<0.01
$HbA_{1a+b} vBic$	HD	31	0.026	NS
$HbA_{1a+b} vH^+$	HD	8	-0.396	NS
$HbA_{1a+b} vBic$	DIAB-RF	24	-0.197	NS
GHbvBic	CRF	40	-0.070	NS
GHbvBic	CAPD	24	-0.386	NS
GHbvBic	HD	31	-0.095	NS
$GHbvH^+$	HD	8	0.282	NS
GHbvBic	DIAB-RF	24	-0.160	NS
CHbvBic	CRF	40	-0.234	NS
CHbvBic	CAPD	24	-0.411	<0.05
CHbvBic	HD	31	-0.254	NS
$CHbvH^+$	HD	8	0.232	NS
CHbvBic	DIAB-RF	24	0.102	NS

8.XI (values are mean and 95% CI). Blood glucose (random) and plasma bicarbonate increased (p<0.001), while hydrogen ion concentration (p<0.01), blood urea and serum creatinine decreased (p<0.001). Glycosylated haemoglobin (HbA_1, HbA_{1c}, HbA_{1a+b}, GHb) and carbamylated haemoglobin did not change.

8. The effect of acidosis

Comparison between plasma bicarbonate and both glycosylated haemoglobin and carbamylated haemoglobin in general showed no significant correlation. Correlation coefficients are shown in Table 8.XII. A weak but persistent correlation was found in the CAPD group but the bicarbonate concentration was essentially normal. As a group the CRF patients were more 'acidotic' but no correlation was found. There was no correlation between H^+ ion concentration in the HD group and either glycosylated or carbamylated haemoglobin.

9. Glycosylated and carbamylated haemoglobin in renal transplantation

This part of the study evaluated 4 groups of subjects: controls (as previously described), patients with underlying renal pathology but normal renal function i.e. primary renal disease (PRD), renal transplant subjects with normal renal function (Tx) and renal transplants with failing graft function (Tx-RF).

a) General data

Age and sex are shown in Table 8.I; total haemoglobin, PCV and albumin in Table 8.II; and urea, creatinine and bicarbonate in Table 8.III. Patients in the PRD group were similar to controls for all variables. Transplants with normal renal function had normal total haemoglobin, PCV, albumin, urea, creatinine and bicarbonate.

Transplants with renal failure had reduced total haemoglobin, PCV and bicarbonate and increased urea and creatinine. Random blood glucose (Table 8.IV) was normal in all groups.

b) Glycosylated and carbamylated haemoglobin

Glycosylated haemoglobin (HbA_1, HbA_{1c}, HbA_{1a+b}, GHb) and carbamylated haemoglobin are shown in Table 8.XIII. Values are mean (SEM) and [95% CI]. Figure 8.16 shows the chromatographic measured components of haemoglobin A_1, i.e. HbA_1, HbA_{1c} and HbA_{1a+b} for each of the 4 groups. Values are mean and 95% CI, and statistical differences are also shown. The PRD group had similar HbA_1, HbA_{1c} and HbA_{1a+b} values as the control group. Transplants with normal renal function had an increase in HbA_1 concentration ($p<0.05$) but HbA_{1c} and HbA_{1a+b} were similar to controls. Transplants with renal failure had an increase in HbA_1 ($p<0.01$), HbA_{1c} ($p<0.05$) and HbA_{1a+b} ($p<0.01$).

Glycosylated haemoglobin measured by colorimetry (GHb) is shown in Figure 8.17 (values are mean and 95% CI) for each of the 4 groups. There was no change in GHb and all groups were similar to controls.

Carbamylated haemoglobin is shown in Figure 8.18 with the scatter of values, mean, 95% CI and statistical difference between the groups. The control and PRD groups were similar but transplants with normal renal function, although they generally overlapped with the control group, had a few individuals with elevated concentrations of CHb and the mean of the Tx group was significantly increased ($p<0.05$). Transplants with renal failure showed a wide scatter and the mean CHb concentration was grossly elevated ($p<0.001$).

Table 8. XIII   Glycosylated and Carbamylated Haemoglobin in Renal Transplantation

Group	n	A_1 (%)	A_{1c} (%)	A_{1a+b} (%)	GHb (nmol HMF/mol Hb)	CHb (ng HMF/mg globin)
PRD	22	7.9 (0.18) [7.62 - 8.36]	5.35 (0.15) [5.04 - 5.66]	1.63 (0.06) [1.51 - 1.75]	36.0 (0.6) [34.7 - 37.2]	29 (3) [22 - 35]
TX	16	8.41 (0.20) [7.97 - 8.84]	5.40 (0.19) [4.98 - 5.81]	1.66 (0.05) [1.56 - 1.79]	37.7 (1.1) [35.3 - 40.1]	43 (4) [36 - 50]
TX-RF	14	8.70 (0.28) [8.09 - 9.30]	5.74 (0.23) [5.25 - 6.23]	1.82 (0.08) [1.66 - 1.99]	38.4 (1.1) [36.1 - 40.6]	87 (14) [58 - 117]

Values are mean, (SEM), and [95% confidence intervals]

HAEMOGLOBIN A
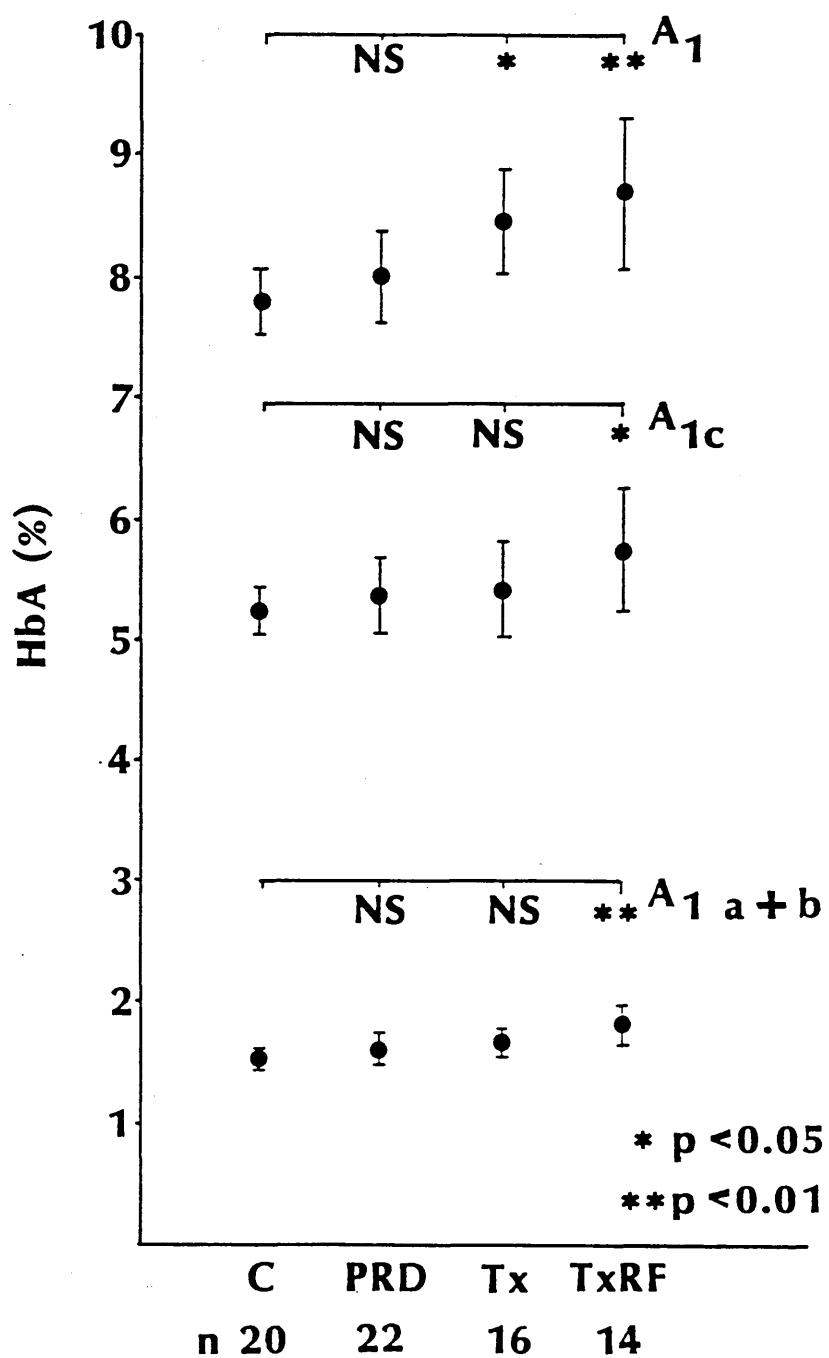
Figure 8.16 HbA_1, HbA_{1c}, and HbA_{1a+b} in Renal Transplantation

Glycosylated Haemoglobin
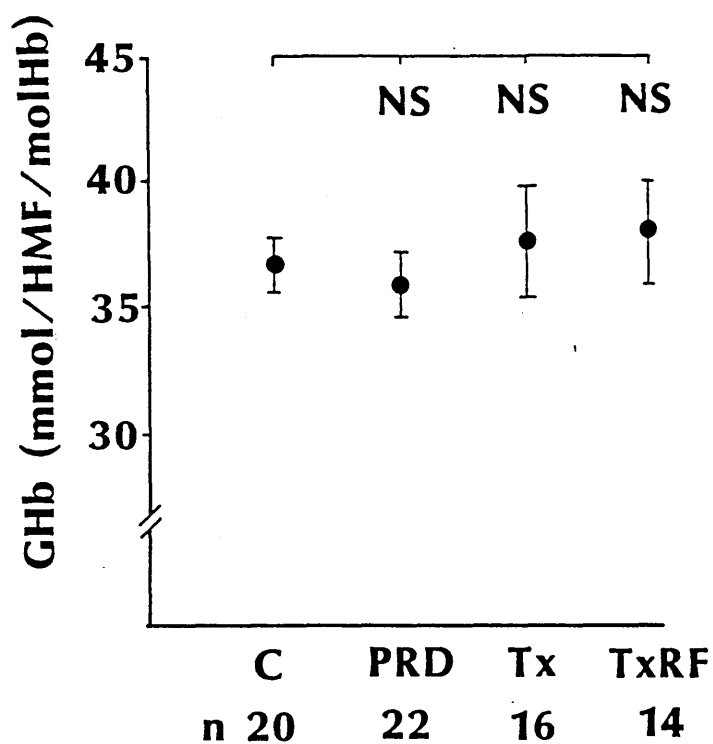
Figure 8.17   Glycosylated haemoglobin in Renal Transplantation

Carbamylated Haemoglobin
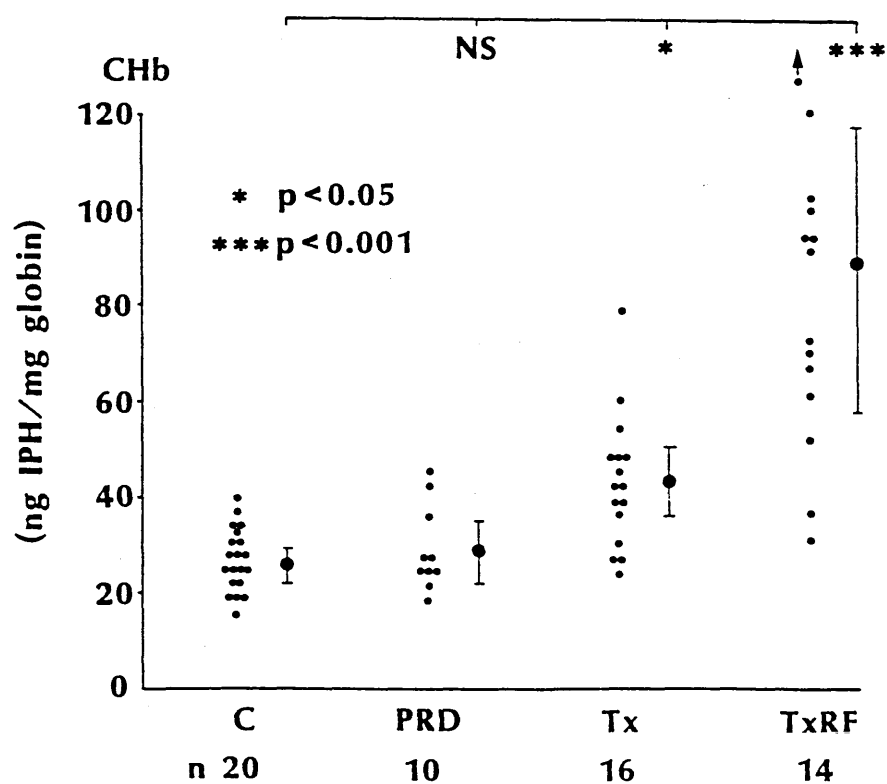
Figure 8.18   Carbamylated Haemoglobin in Renal Transplantation

c) <u>Renal function and other associations</u>

HbA_1, HbA_{1c} and GHb did not correlate with either urea or
creatinine in any of the groups. HbA_{1a+b} showed a significant
correlation (r=0.602, p<0.05) with urea but not with creatinine in the
Tx-RF group. The other groups showed no correlation with HbA_{1a+b}.
CHb was found to have a strong positive linear correlation with urea
in the transplants with renal failure (r=0.874, p<0.001) and is
plotted in Figure 8.19. The other groups did not show any correlation
between CHb and urea. Transplants with renal failure also showed a
correlation between CHb and creatinine (r=0.589, p<0.05) but this was
not observed in the other groups.

Comparison of bicarbonate with HbA_1, HbA_{1c} or HbA_{1a+b} did not show
any correlations between the variables in any of the groups.

In transplants with renal failure CHb and HbA_{1a+b} showed a strong
correlation (r=0.761, p<0.01) but correlations of CHb with HbA_1
(r=0.471, p-NS) or HbA_{1c} (r=0.634, p<0.05) were less obvious. The
other groups did not show any correlation between CHb and glycosylated
haemoglobin.

The ratio of HbA_{1a+b}/HbA_{1c} in transplants with renal failure was
significantly higher than controls (p<0.01). The other groups showed
no change in the HbA_{1a+b}/HbA_{1c} ratio.

Tx-RF
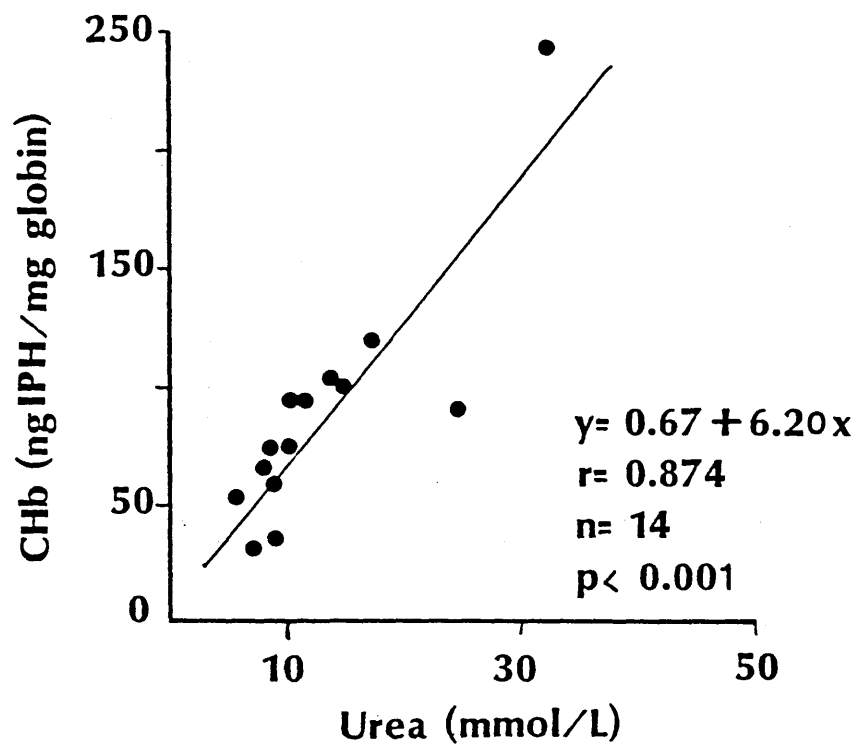
Figure 8.19

Correlation of carbamylated haemoglobin and urea in
transplants with renal failure

DISCUSSION

The aims of this study were to evaluate glycosylated and carbamylated haemoglobin in uraemia and attempt to clarify the confusing and conflicting data in the literature. In this large study, over 200 patients with varying degrees of renal impairment were studied. Different methods of measurement and several components of glycosylated haemoglobin were assessed and the association with carbamylated haemoglobin examined.

This study showed elevation of chromatographically determined glycosylated haemoglobin (HbA_1, HbA_{1c} and HbA_{1a+b}) and normal colorimetrically measured glycosylated haemoglobin in non-diabetic uraemic subjects. The increased formation of carbamylated haemoglobin was confirmed and showed a parallel rise with HbA_1 and its sub-fractions. This investigation provides strong evidence that this non-glucose adduct of haemoglobin is responsible for the interference and 'false' elevation of glycosylated haemoglobin measured by ion-exchange column chromatography. Carbamylation of haemoglobin was strongly influenced by the degree of uraemia but was detectable in non-uraemic subjects. Diabetics with renal failure had elevated glycosylated haemoglobin independent of assay method and increased carbamylated haemoglobin, but the latter did not correlate with glycosylated haemoglobin.

1. Study design

This study was designed to measure simultaneously, i.e. from the same blood specimen, the chromatographic components of HbA_1, total glycosylation of the red cell, carbamylated haemoglobin and other biochemical variables in a population of uraemic and diabetic patients. This enabled a comparison to be made between the components

of glycosylated haemoglobin and carbamylated haemoglobin in individuals.

A number of situations can cause interference with the measurement of chromatographically determined glycosylated haemoglobin (Gabbay et al., 1983) and these have been discussed in Chapter 4. In the study protocol several exclusion criteria were implemented to reduce interfering factors. None of the subjects had a recent blood transfusion (within 3 months of the study), haemoglobinopathy, jaundice or had recent alcohol, antibiotic or aspirin ingestion. The presence of hyperlipidaemia was, however, not excluded and as most subjects were uraemic or diabetic or both, interference from lactescence cannot be ruled out. Lactescent plasma due to hyperlipoproteinaemia has been shown to elute with the HbA_1 fraction (Dix et al., 1979a). In reality, it would be very difficult to find a population of uraemic or diabetic patients without some degree of hyperlipidaemia. The determination of total glycosylation in the red cell by a direct chemical method (colorimetry) circumvents most interfering factors, but shortened red cell life span can reduce the concentration of glycosylated haemoglobin independent of methodology. In uraemia red cell survival is generally accepted to be reduced in undialysed chronic renal failure (Shaw, 1967), haemodialysis (Fried et al., 1978) and CAPD patients (Hefti et al., 1983). However, by the simultaneous measurement of both chromatographic and colorimetric determined glycosylated haemoglobin the net effect of reduced erythrocyte life span should be minimised.

In view of the multiple groups of patients investigated and the numerous variables measured the risk of a type-I error is increased and thus some caution has to be used in the statistical interpretation of the results.

2. Methodology

Much of the confusion about glycosylated haemoglobin is related to nomenclature and methodology. This has been discussed in Chapters 4 and 5. In this study two separate cation exchange minicolumn chromatographic methods were used. One measured HbA_1 (i.e. total HbA_{1a+b+c}) and the other measured the specific fractions HbA_{1c} and HbA_{1a+b}. These methods based on the principle of a net change in charge were quite similar, with different fractions being eluted by changing the buffer solution. The assays used whole blood haemolysates, were sensitive to temperature changes, required a high degree of operator skill to achieve reproducibility and precision, and were relatively expensive. All assays were performed in a water bath with a constant ambient temperature of $26^{o}C$. Glycosylated haemoglobin was also measured by the thiobarbituric acid – colorimetric method, whereby furfural compounds are generated from the ketoamine linked carbohydrate moieties upon acid hydrolysis and quantified colorimetrically after reacting with thiobarbituric acid. This direct chemical method measures the total glycosylation in the red cell and has the advantages of being unaffected by non-glucose adducts, aldimine intermediates and is inexpensive to run, but has the drawbacks of having no universally accepted standard, hydrolysis conditions must be carefully controlled for precision, and is a time-consuming, labour intensive procedure compared to the minicolumn kits.

3. Haemoglobin glycosylation

In this study HbA_1 was significantly increased in non-diabetic uraemic patients. This is in general agreement with many others (DeBoer et al., 1980; Graf et al., 1980; Kovarik et al., 1981). HbA_1

was found to be increased by 13 to 20% in uraemics compared to controls. The increment was greater in non-dialysed chronic renal failure and CAPD patients than haemodialysis patients. By comparison diabetics independent of renal function had a greater rise in HbA_1, showing a 50% increase.

HbA_{1c} has been less frequently measured in non-diabetic uraemics, but the elevated concentrations found in this study are in agreement with others (Graf et al., 1980; O'Regan et al., 1982; Saloranta et al., 1986). HbA_{1c} increased proportionately less in uraemics (less than 20%), in contrast to increments of 40 to 50% in diabetics.

The HbA_{1a+b} fraction was increased in diabetic and uraemic patients. HbA_{1a+b} has been reported to be increased in uraemia (Fluckiger et al., 1981; Lantz et al., 1981) but no elevation of this fraction was found in diabetics with uraemia (Saloranta et al., 1986). In the present study HbA_{1a+b} increased by between 20 and 50% in non-diabetic uraemic patients, by about 40% in diabetics with normal renal function and by 50% in diabetics with renal failure.

Glycosylated haemoglobin measured by colorimetry was unchanged by uraemia, both in this study and in the pilot study (unpublished data) and was only increased in the presence of diabetes. This is in agreement with the findings of others (Fluckiger et al., 1981; Nath et al., 1982). Thus differences in methodology can explain some of the confusion regarding glycosylated haemoglobin in uraemia. Furthermore, the effect of reduced red cell life span, which could conceivably suppress glycosylated haemoglobin concentration independent of methodology, was not seen in the colorimetric estimation as non-diabetic uraemic patients had similar values to control subjects. Colorimetrically measured glycosylated haemoglobin (GHb) was, by chance, similar in both diabetics with and without renal impairment.

This finding allows further comparison between the methods and renal function in diabetics.

Comparison of the methods of measuring glycosylated haemoglobin showed an excellent correlation between the colorimetric and chromatographic techniques for diabetics, independent of renal function, and particularly for the HbA_1 and HbA_{1c} components. HbA_{1a+b} was found to have a strong correlation with the colorimetric method in diabetics with renal failure, while diabetics with normal renal function showed a weaker, though statistically significant correlation. No correlation was found between the 2 different methods in non-diabetic uraemic patients. This latter finding is in agreement with others (Fluckiger et al., 1981; Oimomi et al., 1981).

The data from this investigation suggests that either method of estimating glycosylated haemoglobin can be used to monitor blood glucose control in diabetics with renal failure. The colorimetric method is unaltered by uraemia and may be the preferred method, but it has other limitations as previously discussed. The excellent correlation between colorimetry and chromatography suggests that HbA_1 or HbA_{1c} is still useful and its rapid estimation does allow the result to be available more readily and is particularly suitable for out-patient management. The assumption that since uraemic patients have elevated HbA_1 or HbA_{1c} then diabetic uraemic patients would have false elevation of glycosylated haemoglobin must be questioned. There is little data in the literature specifically on glycaemic control in uraemic diabetics, but data extracted from 2 studies show conflicting results. Kumar et al. (1983) found HbA_1 to be higher in uraemic diabetics than non-uraemic diabetics, but clinically the latter were better controlled and the number of patients studied was small. Thus the difference may be due to the glycaemic rather than the uraemic

effect. In contrast, Saloranta et al. (1986) found no difference between uraemic and non-uraemic diabetics in HbA_1 or HbA_{1c} concentrations measured chromatographically. In the present study the similar GHb measurements by colorimetry in the uraemic and non-uraemic diabetics gave an independent assessment that both groups had similar glycaemic control uninfluenced by uraemia. Comparison of the chromatographic components showed that HbA_1 and HbA_{1c} were also similar. This provides further evidence that chromatographically measured glycosylated haemoglobin is still a useful clinical measure of glycaemic control in uraemic diabetics.

The ratio of chromatographically determined HbA_1 components demonstrated that the HbA_{1a+b} fraction was more influenced by uraemia and the HbA_{1c} fraction by diabetes. This has been previously suggested by others (Fluckiger et al., 1981; Oimomi et al., 1981). In diabetics with renal failure both fractions were proportionately raised and thus the ratio was unchanged.

HbA_1 and components had a weak, but significant correlation with urea in some of the non-diabetic uraemic groups, but the relationship was quite variable. The HbA_{1a+b} fraction correlated with urea in non-dialysed chronic renal failure and CAPD patients but not in haemodialysis subjects. Diabetics with renal failure showed no association between glycosylated haemoglobin concentration and renal function. Thus, overall no consistent significant correlation was found between either random (at time of sampling for glycosylated haemoglobin) urea or creatinine and glycosylated haemoglobin. A weak but significant correlation between HbA_1 and creatinine (Graf, 1980; Kovarik et al., 1981), between HbA_1 and urea (Oimomi et al., 1984; Saloranta et al., 1986) have been reported, while others have found no correlation (Nath et al., 1982; O'Regan et al., 1982). Furthermore,

others have reported a significant correlation between both urea and

the time-averaged concentration of urea and HbA_1 in non-dialysed

chronic renal failure patients but no correlation in haemodialysis

patients (DeMarchi et al., 1983a; DeMarchi et al., 1983b). This

variable response was seen in the present study and may be explained

by the difference in uraemic status between haemodialysis patients,

who have wide fluctuations in urea concentration, and other uraemics

who tend to have a more stable level of blood urea. This will be

discussed later in relation to carbamylated haemoglobin.

All glycosylated species of haemoglobin showed no correlation with

random glucose, total haemoglobin, age or sex in any of the groups

including diabetics. It is well recognised in diabetes that

glycosylated haemoglobin reflects the time-averaged concentration of

glucose rather than random glucose estimations. However, as

previously discussed, there is ample evidence against glucose

intolerance or hyperglycaemia being the explanation of elevated

glycosylated haemoglobin in uraemia.

4.   Haemoglobin carbamylation

Carbamylated haemoglobin was estimated by detecting the amount of

isopropyl hydantoin released after hydrolysis of globin. Increased

concentrations of carbamylated haemoglobin were demonstrated in all

patients with renal impairment. Non-uraemic diabetics and patients

with underlying renal pathology but normal urea and creatinine, had

similar concentrations as control subjects. These results confirm the

only previous report of direct measurement of haemoglobin

carbamylation in uraemia (Fluckiger et al., 1981). These workers

detected valine hydantoin from the eluate of three haemoglobin peaks

(HbA_1, HbA_{1a+b} and HbA_{1c}) of a uraemic haemolysate chromatographed on

a macrocolumn. Although they did not detect valine hydantoin in non-uraemic haemolysate a highly significant correlation was found between the amount of valine (isopropyl) hydantoin released from haemoglobin and the time-averaged urea concentration in uraemic patients.

In the present study modification of the assay, (detailed in Chapter 5) increased its sensitivity and isopropyl (valine) hydantoin release was detected in non-uraemic subjects. A normal range can now be established. In 64 patients with normal renal function a mean value of 27 ng IPH/mg globin with a 95% confidence interval of 22 to 32 ng IPH/mg globin was found. Patients with overt renal failure had gross elevation with increases of 200 to over 400% and there was no overlap between uraemics and non-uraemics. Carbamylated haemoglobin had a significant correlation with urea and creatinine in uraemic patients. Urea estimations in this study were taken from a single random sample, whereas Fluckiger et al. found a strong correlation using time-averaged urea concentrations in a small group of patients. Carbamylated haemoglobin has been more extensively studied in Sickle Cell disease and the carbamylation mechanism is represented by an irreversible covalent second-order kinetic reaction (Uvelli et al., 1978; Uvelli et al., 1980). Most studies report in vitro carbamylation characteristics, but it is likely that in vivo the urea derivative isocyanate forms a stable adduct with the N-terminal amino group on the haemoglobin chain (Manning et al., 1974), analogous to the non-enzymatic glycosylation reaction. Although there are some differences in the biochemical characteristics and binding sites between carbamylated haemoglobin and glycosylated haemoglobin they, nevertheless, share several common features. Thus one can postulate the analogy, between urea and carbamylated haemoglobin in uraemia and

glucose and glycosylated haemoglobin in diabetes. In both situations low molecular weight reactants, urea-derived isocyanate and glucose, are present in limited amounts, but as both reactions with haemoglobin are irreversible the modified haemoglobin accumulates in the red cell throughout its life span. Hence in both conditions haemoglobin behaves as a reporter molecule.

In this investigation carbamylated haemoglobin significantly correlated with chromatographically determined glycosylated haemoglobin in uraemic patients. This is in agreement with the in vitro findings of raised HbA_1 in normal erythrocytes incubated with cyanate (Oimomi et al., 1984). Non-dialysed chronic renal failure and CAPD patients showed the strongest correlation with HbA_1, HbA_{1a+b} and HbA_{1c}, but haemodialysis patients tended to have a weaker and more variable correlation and diabetics with chronic renal failure showed no correlation. The generally weaker association in haemodialysis patients can be explained by the fluctuating concentrations of urea, which reach a peak pre-dialysis and trough post-dialysis. These oscillations will influence the time-averaged urea concentration and a random urea estimation is not representative. In contrast, most of the non-dialysed chronic renal failure and CAPD patients had a relatively more stable urea concentration which was more representative of the time-averaged urea. The lower carbamylated haemoglobin in haemodialysis patients also explains the lower HbA_1, HbA_{1c} and HbA_{1a+b} concentrations generally observed in relation to other uraemic patients. It is possible that reduced red cell life span, due to haemolysis in the extracorporeal circulation, may have contributed to lower values in haemodialysis patients. However, there was no direct evidence to support this and colorimetrically measured glycosylated haemoglobin which is equally dependent of red cell life

span was also normal.

The combination of glycosylation and carbamylation of haemoglobin was observed in diabetics with renal failure. In these patients carbamylated haemoglobin correlated with urea but not with HbA_1 and sub-fractions. HbA_1 and sub-fractions correlated with colorimetrically determined glycosylated haemoglobin (the latter reflecting time-averaged glucose concentrations and unaffected by uraemia). From the molecular point of view, it is possible for both reactions to occur together and although they share the common binding site at the N-terminal valine residue, the glycosyl moiety is attached predominantly to the β chain (Bunn et al., 1979) while the carbamyl moiety is distributed to both α and β chains with a slight preference for the α chain (Manning et al., 1974; Nigen et al., 1974). Several other binding sites for both adducts have been identified but this detail is beyond the scope of this discussion.

The combination of both reactions in uraemic diabetics may initially appear antagonistic. However, the colorimetrically determined glycosylated haemoglobin was similar, in both diabetics with and without renal failure and the similar HbA_1 and HbA_{1c} values confirm that the latter measurements reflect the glycaemic rather than the uraemic effect in diabetics with renal failure. The marginal difference in HbA_{1a+b} between the diabetic groups would be in keeping with a small carbamylation effect. In non-diabetic uraemic patients all 3 components HbA_1, HbA_{1c} and HbA_{1a+b} were increased, but the HbA_{1a+b} sub-fraction was relatively more increased, which is in partial agreement with Fluckiger et al. (1981) who detected most of the carbamylated haemoglobin (valine hydantoin) from the HbA_{1a+b} fraction. Carbamylated haemoglobin did not show any correlation with age, sex or total haemoglobin concentration.

The effect of reducing urea and creatinine concentrations and correcting the metabolic acidosis during a 4 hr haemodialysis session did not induce any acute change in either glycosylated or carbamylated haemoglobin. The latter are formed by a non-enzymatic process taking weeks or months and dependent on the life span of the red cell as well as the ambient glucose or urea concentration.

It has been suggested that uraemic acidosis plays a major role in the elevation of glycosylated haemoglobin (DeMarchi et al., 1983a; DeMarchi et al., 1983b). This was based on data showing correlations between HbA_1 and either arterial blood pH or plasma bicarbonate. In this present study no correlation was found between glycosylated haemoglobin and either H^+ concentration or plasma bicarbonate and is in agreement with others (Panzetta et al., 1983).

Generally, patients with a primary renal disease or a renal transplant but normal renal function, showed the same features as control subjects. However, carbamylated haemoglobin was elevated in a few transplant individuals who had rejection episodes or were recovering from post-transplant acute tubular necrosis over the preceding 3 months. This reflects the retrospective marker effect of carbamylated haemoglobin. Transplant patients with failing graft function were similar to other chronic renal failure patients and demonstrated elevation of carbamylated haemoglobin, HbA_1, HbA_{1c} and particularly HbA_{1a+b}. The association between carbamylated haemoglobin and HbA_{1a+b} was reaffirmed and both variables showed a strong correlation with both urea and creatinine.

5. <u>Red cell life span</u>

Reduced concentrations of HbA_1 in uraemia have been reported by one group (Dandona et al., 1979; Freedman et al., 1982) who explained

their findings by assuming shortened red cell life span was responsible. However, this was not measured in the study. As previously discussed, reduced erythrocyte survival is well documented in uraemia, but no studies have been published that directly measure red cell life span in conjunction with glycosylated haemoglobin in uraemia. Glycosylated haemoglobin measured by colorimetry is dependent on red cell life span and the finding that all uraemic patients had normal values is evidence, albeit indirect, that red cell survival in uraemia does not have a significant effect on the measurement of glycosylated haemoglobin. Decreased glycosylated haemoglobin has been demonstrated in haemolytic but not non-haemolytic anaemia (Panzer et al., 1982). Thus the normochromic normocytic anaemia of uraemia should not affect glycosylated haemoglobin concentrations. There is no data available on carbamylated haemoglobin and erythrocyte survival in uraemia, but it is interesting that in Sickle Cell disease cyanide was used therapeutically and the resultant carbamylated haemoglobin prolonged sickled red cell life span and increased oxygen affinity (Cerami et al., 1971; Gillette et al., 1974). The increase in red cell life span reflected the degree of carbamylation (Milner and Carache, 1973; Gillette et al., 1974). It might be postulated that the formation of carbamylated haemoglobin could prolong red cell survival in uraemia and this may also directly affect glycosylated haemoglobin. However, this is purely speculative and further studies are necessary to elucidate any relationship between carbamylated haemoglobin and red cell life span.

The analogy between non-enzymatic carbamylation in uraemia and non-enzymatic glycosylation in diabetes should create further avenues of research. The possible clinical and pathophysiological role of non-enzymatic carbamylation warrants further investigation. Data from

this study suggests that carbamylated haemoglobin could be a useful clinical indicator of the 'uraemic state' as it appears to reflect the time-averaged urea concentration. This could be clinically valuable in several situations such as differentiating acute from chronic renal failure, detecting the timing and progression of chronic renal failure, assessing compliance in protein diet restriction therapy and evaluating the efficiency of dialysis procedures. The pathophysiological role of non-enzymatic carbamylation could have wider implications, if it can modify other proteins analogous to non-enzymatic glycosylation. Diabetes and uraemia share several common pathological complications, such as vascular disease, lipoprotein abnormalities and neuropathy and it is tempting to speculate that non-enzymatic modification of proteins may be implicated. Thus non-enzymatic carbamylation warrants further investigation in uraemia to assess both its potential clinical usefulness and possible pathophysiological significance.

SUMMARY

In a large number of patients with a wide spectrum of renal disease glycosylated haemoglobin was increased in uraemia when measured chromatographically but not colorimetrically. All components of HbA_1 increased, particularly HbA_{1a+b}. Carbamylated haemoglobin was detected in all subjects, but was grossly elevated in renal failure and paralleled the rise in HbA_{1a-c} and correlated with the severity of uraemia. Glycosylated haemoglobin increased in both assay methods in diabetics independent of renal function, but carbamylated haemoglobin was elevated only in uraemic diabetics. HbA_{1c} appeared to be more influenced by diabetes and HbA_{1a+b} by uraemia. Although the colorimetric assay may in some situations be the method of choice being unaltered by uraemia, it is suggested that HbA_1 or HbA_{1c} is still useful in monitoring glycaemic control in uraemic diabetics. The role carbamylated haemoglobin requires further study, but the current findings suggest that it may reflect the recent mean uraemic state and thus analogous to diabetes and glycosylated haemoglobin. Thus, carbamylated haemoglobin may act as a retrospective indicator of integrated uraemic control. The concept of non-enzymatic carbamylation of proteins may also have some pathophysiological significance and warrants further investigation.

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NORMAL VALUES AND REFERENCE RANGE

	Mean	Reference Range	Source
INSULIN	5.0	2.8-13.5 mu/1	1
C-PEPTIDE		0.18-0.52 nmol/1	1
GLUCAGON	70	60-80 pg/ml	2
GROWTH HORMONE	2-10	<10 mu/1	3
CORTISOL		140-610 nmol/1	3
GLUCOSE	5.0	2.2-6.7 mmol/1	4
LACTATE	0.638	0.041-1.136 mmol/1	4
PYRUVATE	0.064	0.029-0.144 mmol/1	4
LACTATE/PYRUVATE RATIO	10.8	7.0-14.5	4
ALANINE	0.304	0.206-0.449 mmol/1	4
3-HYDROXYBUTYRATE	0.046	0.010-0.217 mmol/1	4
ACETOACETATE	0.032	0.007-0.160 mmol/1	4
3-HYDROBUT/ACACETATE RATIO	1.3	0.4-4.8	4
GLYCEROL	0.053	0.031-0.093 mmol/1	4
TOTAL CHOLESTEROL		3.8-7.0 mmol/1	5
TOTAL TRIGLYCERIDES		0.5-2.2 mmol/1	5
HDL-CHOLESTEROL		1.0-1.7 mmol/1	5
GLYCOSYLATED HAEMOGLOBIN (colorimetry)		29-39 mmol HMF/molHb	5
HbA_1	7.6	7.3-8.0 %	6
HbA_{1c}	6.1	5.9-6.3 %	6
HbA_{1a+b}	1.5	0.9-2.3 %	6
CARBAMYLATED HAEMOGLOBIN	27	22-32 ng IPH/mg globin	6

Values from sources 1, 2, 4 and 5 refer to normal subjects after an overnight fast

Source

1. Department of Medical Biochemistry, University of Newcastle: values are for normal subjects after overnight fast.

2. Prof. Orskov, Kommune Hospitalet, Aarhus, Denmark.

3. Supraregional Assay Service, Royal Victoria Infirmary, Newcastle: cortisol values are for 9 am specimens.

4. Adopted by Department of Therapeutics, University of Sheffield, from Forster, K.J., Alberti, K.G., Hinks, L. et al., 1978. Values are for whole blood.

5. Department of Clinical Chemistry, Royal Hallamshire Hospital, Sheffield.

6. Work from this thesis: a) Values for HbA_1, A_{1c} and A_{1a+b} are similar to Biorad Ltd. reference ranges, b) carbamylated haemoglobin values have not been previously published: values taken directly from this thesis.

APPENDIX 2

1. Free Insulin Concentrations:

    Polyethylene Glycol Solution (PEG)

    The measurement of free insulin in diabetics was performed after

    removal of insulin antibodies by precipitation with PEG solution.

    a) Buffer   3.1g/l   Boric Acid

                    3.7g   KCL

                    0.24g/l   Thiomersal

                    39.7ml   0.1 M NaOH

        and made up to 1 litre with deionised water.

    b) Take 200 ml buffer and add 1g Bovine Serum Albumin (BSA)

        (Armour) and make up to 400 ml with deionised water.

    c) Polyethylene glycol (PEG) 6000 (British Drug Houses). Add

        30 g PEG to 70 g of the buffer/BSA solution (pH 7.2-8.0) to

        give the final PEG solution.

        Specimen: 0.25ml fresh serum was added to 0.25ml PEG/buffer

        solution, centrifuged at 3000 rpm for 30 minutes at $0^{o}C$ and

        the supernatant was stored at $-20^{o}C$ until assayed.

2. Due to the instability of pancreatic glucagon, blood was taken

    immediately into the proteinase inhibitor, aprotinin. The

    'preservative' solution contained:

    Disodium EDTA (Sigma) 372 mg, Aprotinin (Trasylol, Bayer) 5ml

    (100,000 Kallikrein Inactivator Units) made up to 10 ml with

    fresh deionised water.

    Specimen: 0.25ml of EDTA/Aprotinin was added to a plain glass

    tube and kept at $0^{o}c$ prior to the addition of 2.5ml of whole

    blood. After separation the serum was stored at $-20^{o}C$.

Work from this thesis has been published, mainly in abstract form, but several papers have or are being currently submitted for publication:

1. Pancreatic Beta Cell Function in Non-Diabetics with Renal Failure. Scottish Renal Association, Glasgow, April 1985.

2. Pancreatic Beta Cell Function in Non-Diabetics with Renal Failure. XXII Congress of the European Dialysis Transplant Association, Brussels, June, 1985. Published in Proceedings of EDTA 1985;22:1067-1070.

3. Glycosylated and Carbamylated Haemoglobin in Uraemia. XXIII Congress of the European Dialysis Transplant Association, Budapest, June 1986. Abstract in Nephrology, Dialysis and Transplantation 1986;1(2):p88.

4. Glucose and Intermediary Metabolism in CAPD. XXIII Congress of the European Dialysis Transplant Association, Budapest, June 1986. Abstract in Nephrology, Dialysis and Transplantation 1986;1(2):p115.

5. Glycosylated and Carbamylated Haemoglobin in Uraemia. Scottish Renal Association, Glasgow, October 1986. Abstract in Nephrology, Dialysis and Transplantation 1987;2(1):p59.

6. Hormones and Metabolites in CAPD. IV Congress of the International Society for Peritoneal Dialysis, Venice, June 1987. Abstract in Peritoneal Dialysis Bulletin 1987;7(supp):S71.

7. Glycosylated Haemoglobin in CAPD. IV Congress of the International Society for Peritoneal Dialysis, Venice, June 1987. Abstract in Peritoneal Dialysis Bulletin 1987;7(supp):S70.

8. Pancreatic Beta Cell Function in CAPD, submitted to Nephrology, Dialysis and Transplantation, July 1987.

9. Glucoregulatory Hormones and Intermediary Metabolism in Diabetics on CAPD. Xth International Congress of Nephrology, London, July 1987. Abstract in Congress Abstract Book, p199.