THE EFFECT OF A MATERNAL LOW PROTEIN DIET ON PANCREATIC GLUTOKINASE ACTIVITY

by

Wendy Elizabeth Heywood

A thesis submitted for the degree of doctor of philosophy in the
University Of London

Gastroenterology Unit
Institute of Child Health
30 Guilford St
London WC1N 1EH
Abstract

The work presented in this thesis provides evidence that a maternal low protein (LP) diet during gestation and lactation periods in rats 'programs' pancreatic β-cell glucokinase (GK) the glucose sensor and glucose induced insulin secretion response in newborn, suckling and adult offspring. Pregnant female rats were divided into 3 groups, group A (20%) was kept on a normal protein diet, group B on a LP (6%) diet during pregnancy and lactation and group C on a LP diet during pregnancy and a normal protein diet during lactation. The glucose-induced total insulin secretion response and peak insulin secretion was markedly reduced in group B newborn and 3-week old suckling pups compared with group A controls which is a result of poor nutrition. The insulin secretion also showed an altered secretory response in group B adults and group C 3-week old and adult stage pups compared with group A. The GK protein levels were reduced in newborn, 3-week old pups and adult rats from both group B and C. The GK enzyme exhibited interesting changes in enzyme activity to convert glucose to glucose-6-phosphate particularly in the kinetic reaction parameters pertaining to the enzymes affinity for glucose (K_m values) and maximal reaction velocity (V_max). Poor nutrition reduced GK protein enzyme activity and K_m values. The prenatal and postnatal LP diet appears to have a permanent programming effect by increasing the GK affinity for glucose and decreasing the reaction velocity indicating that the critical period of programming of GK's function is after birth during the postnatal weaning period since the adult offspring of group B when fed a normal protein diet showed no reversal in the K_m values of the enzyme. Similar experiments on adult offspring of group C which were fed a normal protein diet during weaning and after showed normalisation of the GK K_m values for glucose but still exhibited a permanent reduction in V_max. In conclusion low protein diets during both pregnancy and weaning have immediate and sustained irreversible effects on glucose homeostatic mechanisms of a mother's offspring. Fetal and early life infantile nutrition programs pancreatic β-cell function, with poor nutrition predisposing to diabetes in later life.
ACKNOWLEDGEMENTS

I would like to acknowledge the support from my supervisors Professor Peter Milla and Dr Keith Lindley and their supervision throughout the course of this project and I would like to thank Dr Nasi Mian for critical advice and support in writing this thesis.

I would also like to thank the Child Health Research Appeal Trust who provided the financial support and funding for this project.

Finally I am indebted to mum and dad and my boyfriend Jason for their patience and support for which this thesis would not have been possible.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AcCoA</td>
<td>Acetyl Coenzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>Akt</td>
<td>serine/threonine protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Biconinic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl_{2}</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complimentary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene regulated peptide</td>
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<tr>
<td>CO_{2}</td>
<td>Carbon dioxide</td>
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<tr>
<td>CpG</td>
<td>cytosine phosphodiester-guanosine</td>
</tr>
<tr>
<td>ddH_{2}O</td>
<td>Double distilled water</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNAse</td>
<td>Deoxyribonulease -1</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>E</td>
<td>embryonic</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>eIF2B</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
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<td>FBPase</td>
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<td>GIP</td>
<td>Gastric Inhibitory peptide</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>GK</td>
<td>Glucokinase</td>
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<td>Glyc</td>
<td>Glycerol</td>
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<td>GR</td>
<td>Growth retarded</td>
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<td>Glycogen synthase kinase 3</td>
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<td>Glucose-6-phosphate dehydrogenase</td>
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<td>HBSS</td>
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<tr>
<td>H₂O</td>
<td>Water</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>ICC</td>
<td>Islet like cell cluster</td>
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<tr>
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<td>Insulin dependant diabetes mellitus</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
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<td>Impaired glucose tolerance</td>
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<td>Magnesium chloride</td>
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<td>Minute</td>
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<td>ml</td>
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<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
</tr>
<tr>
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<td>Millimolar</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<td>Non insulin dependant diabetes mellitus</td>
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</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OAA</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
Pax1  Paired box protein 1
Pax6  Paired box protein 6
pPCCase precursor of propionyl CoA carboxylase β subunit
PDA  Piperazinediacrylamide
PDK1  phosphoinositide-dependant-kinase 1
PDX-1  Pancreatic duodenal homeobox-1
PEP  Phosphoenolpyruvate
PEPCK  Phosphoenolpyruvate carboxykinase
PFK  Phosphofructokinase
PFK-2/FBPase-2  6-phosphofructo-2-kinase/fructose-2-6-biphosphatase
pg  picogram
PI  phosphatidylinositol intermediates
PIP3  PI 3,4,5-triphosphate
PI3K  phosphatidylinositol 3 kinase
PF  Phosphokinase
PKC  Protein kinase C
POD  Peroxidase
Pyr  Pyruvate
SDS  Sodium dodecyl sulphate (laurel sulphate)
Shh  sonic hedgehog
TBS  Tris-Buffered Saline
TCA  Tricarboxylic Acid
TEMED  N,N,N,N′-Tetramethylenediamine
TF  transcription factor
TG  Triacylglycerol
TWEEN20  Polyoxyethylenesorbitan monolaurate
VIP  Vasoactive Intestinal peptide
VNTR  Variable number tandem repeats
WHO  World Health Organisation
w/v  Weight to volume
μm  micromolar
1°  Primary
Programming is a process where by a stimulus or insult, at a critical or sensitive period of development has long lasting or lifelong significance (Lucas-A 1991).

1.1 Introduction

In recent years the significance of metabolic programming in humans has been a growing topic of research. The idea that nutritional, hormonal and metabolic environment provided by the mother may permanently change the structure and physiology of her offspring has been known for a while (Winick & Noble 1966) (Carr-Hill, Campbell, et al. 1987). Evidence of programming in animals by non nutritional influences such as hormonal signals was provided by Angelbeck & DuBrul (1983). A single exogenous dose of testosterone at critical periods of fetal life in female rats resulted in permanently reoriented sexual behaviour whilst a similar dose of testosterone in 20-day old female rats had no effect. This suggested that there is a critical time during which the animal’s sexual physiology is sensitive and could be permanently changed. There are four essential principles, which underlie the concept of programming;

(i) Nutritional (or non-nutritional) manipulations cause different effects at different times in early life.

(ii) Rapidly growing fetuses and neonates are more vulnerable to these manipulations.

(iii) Manipulation in early life has permanent effects.

(iv) The permanent effects include reduced cell numbers, altered organ structure and resetting of hormonal axes.

Nutrition can play a key part in programming events in animals. Early studies used the ‘large and small’ litter model that produced well-nourished and undernourished animals. These studies identified critical stages of development of animal growth where dietary manipulation could have permanent consequences (McCance & Widdowson 1974). Poor nutrition during development has been shown to permanently program the structure and physiology of a range of organs and tissues where effects are latent and do not
become apparent until the animal is mature. For example nutritional status during a critical period of brain development has permanent effects on brain size, brain cell number and performance in rats (Katz, Davies, et al. 1980). In humans low birth weight is associated with reduced cognitive function in children aged 8, through adolescence and into early adulthood (Richards, Hardy, et al. 2001).

Studies of overnutrition in rats induced by litter manipulation showed alterations of hepatic enzyme activities reflecting an increased lipid synthesis in the liver (Duff & Snell 1982). Such studies demonstrate that early diet could play a role in the later development of disease states in humans.

Nutritional programming may explain the high prevalence of NIDDM in human populations that have experienced a transition from an undernourished environment to one of overnutrition, for example in the Pima Indians (Bennett, Rushforth, et al. 1976), Nauruans of the pacific islands (Zimmet, King, et al. 1984) and migrant populations (Cheah JS & Tan BY 1979) (Zimmet, Taylor, et al. 1983) & (Cohen, Stern, et al. 1988).

1.2 Programming And Later Life Complications

The focus of this ensuing study and of many others is to delineate the link between low birth weight and the later development of disease. A plausible link was first highlighted in a key paper by Barker & Hales that has spurred on many further epidemiological studies and introduced the concept of 'programming'. In 1991 Barker and Hales provided evidence that reduced fetal and infant growth was associated with later life complications such as non insulin dependant diabetes mellitus (NIDDM) and impaired glucose tolerance. The study followed up the health status of men born in Hertfordshire between 1920-30 of whose birth weights and weight at one year were recorded in detailed birth records.

Twenty six percent of men whose early life weights were less than average and whose body mass index (BMI) were above average had impaired glucose tolerance and 5% had diabetes. In contrast only 5% of those with average early life weights had impaired glucose tolerance and 2% had diabetes.
The study concluded that impaired glucose tolerance and ischaemic heart disease might be determined by influences, which reduce infant and fetal growth (Hales, Barker, et al. 1991).

Many other studies have been performed that corroborate these findings (Phipps, Barker, et al. 1993) (Lithell, McKeigue, et al. 1996) (McCance, Pettitt, et al. 1994) (Robinson, Walton, et al. 1992) (Yajnik, Fall, et al. 1995). A study in Finland has shown that low weight gain during infancy is associated with increased risk of coronary heart disease. After 1 yr. rapid weight gain is associated with increase in risk but only among boys who were thin at birth (Eriksson, Forsen, et al. 2001) (Phillips, Barker, et al. 1994). Therefore nutrition during gestation and early post-natal life has a programming effect on glucose tolerance. Insulin secretion or insulin resistance in children has been related to childhood size as well as reduced birth weight (Whincup, Cook, et al. 1997). Further studies have found that short prepubertal children with intrauterine growth retardation have an impairment in insulin sensitivity which is a potential marker for early identification and development of late onset NIDDM (Hofman, Cutfield, et al. 1997).

The mechanisms by which reduced birth weight predisposes to NIDDM and cardiovascular disease are not known. A re-setting of major hormonal axes that control growth and development has been observed. One study found that fasting plasma cortisol concentrations were inversely related to birth weight (Phillips, Barker, et al. 1998). Cortisol is a glucocorticoid and has an important effect on basal metabolism, host defence mechanisms, blood pressure and the response to stress and raised cortisol concentrations are related to insulin resistance. Animal and human studies of cortisol concentrations in the offspring of undernourished mothers have shown elevated cortisol in plasma and increased excretion of glucocorticoid metabolites indicating that hypothalamic-pituitary-adrenal axis is re-set by undernutrition during gestation. (Economides, Nicolaides, et al. 1988) (Clark, Hindmarsh, et al. 1996).

1.2.1 Thrifty Phenotype Hypothesis
A thrifty phenotype hypothesis (characteristics displayed by an organism under a particular set of environmental factors, regardless of the actual genotype of
the organism) postulates that poor nutrition during critical periods in fetal and early life, with consequent impaired development of β–cell function is the reason for high incidence of diabetes in the western world (Hales & Barker 1992). This acts as an alternative to the thrifty genotype hypothesis (genetic constitution of an organism or cell, as distinct from its expressed features or phenotype), which proposes that there are diabetogenic genes, which may provide advantage for survival in adverse conditions (Neel 1962). A diagram summarising the effects of programming is given in fig 1,1.

The thrifty phenotype hypothesis includes the possibility of a mechanism known as 'Programming' where if the fetus is exposed to low nutrition, certain mechanisms can be programmed for the organism to accommodate that level of nutrition throughout the rest of its life. This acts as a survival mechanism in a poor nutritional environment but is detrimental when the level of nutrition improves or becomes abundant, as the organism's metabolism cannot adapt to a plentiful supply of nutrients (Desai & Hales 1997).
**Fig 1.1** Overview of developmental stages at which programming effects could operate to produce the metabolic syndrome in adult life. Adapted from Bertram & Hanson 2001.
1.3 Diabetes Mellitus

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in blood, which in turn leads to damage of many of the body's systems, especially the blood vessels and nerves (Report of a WHO Study Group 1994).

Records describing the symptoms of deficient endocrine function have been found as far back as 1500-3000 BC and until the famous discovery of insulin by Banting, Best, Macleod and JB Collip in 1922 sufferers of insulin dependant diabetes eventually died from hyperglycaemia.

Diabetes has become a major health problem in the western world as the incidence has gradually increased over time to reach epidemic proportions. 124 million people worldwide had diabetes in 1997 of which 97% had non insulin dependant diabetes mellitus (NIDDM) and projections for up to 2010 indicate that this figure will rise to 224 million with the greatest potential increase occurring in the developing world (see fig.1,2) (Amos, McCarty, et al. 1997).
Fig 1.2 Numbers of people with diabetes (in millions) for 2000 and 2010 (top and middle values, respectively), and the percentage increase. Adapted from Amos, McCarty, et al. 1997.

1.3.1 Classification

Diabetes mellitus is a homogenous disorder with a variety of causes. The end result being a defect in glucose homeostasis resulting in hyperglycaemia. Diabetes is broadly classified into two forms, Insulin Dependent Diabetes Mellitus (IDDM) or type 1 and the more prevalent ‘Non Insulin Dependant Diabetes Mellitus’ (NIDDM) or type 2. IDDM is more often characterised by an earlier age of onset and usually results from autoimmune destruction of the pancreatic β-cells that synthesise and secrete insulin (Eisenbarth 1986). NIDDM characteristically has an onset in later life, is frequently associated with obesity and is due to insulin resistance with or without insulin deficiency. NIDDM is approximately 90% more prevalent than IDDM and rising numbers of cases are believed to be a result of pronounced changes in environment, human behaviour and lifestyle accompanied by globalisation (Zimmet, Alberti, et al 2001). Other causes of diabetes range from genetic mutations in genes responsible for glucose metabolism (Takeda et al 1993), the metabolic syndrome known as syndrome X which is associated with target tissue resistance to insulin (World Health Organisation 1985) and alterations in the
Introduction Chapter 1: Nutritional Programming & Diabetes Mellitus

Glucose sensing mechanism i.e. Maturity Onset Diabetes of the Young (MODY) (Bell & Polonsky 2001). Impaired glucose tolerance (IGT) is associated with elevated blood glucose levels between normal and diabetic states (see table. 1,1) after a glucose load. IGT affects 200 million people worldwide. People with IGT have a 40% chance of developing NIDDM as IGT is a key stage in the progression to NIDDM and the other 60% either remain IGT or revert back to normal (Zimmet, Alberti, et al. 2001).

<table>
<thead>
<tr>
<th>Glucose concentration (mmol l⁻¹)</th>
<th>Plasma Venous</th>
<th>Whole blood Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>≥ 7.0</td>
<td>&gt; 6.1</td>
</tr>
<tr>
<td>2-h post-glucose load</td>
<td>≥ 11.1</td>
<td>≥ 10.0</td>
</tr>
<tr>
<td><strong>Impaired Glucose Tolerance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>&lt; 7.0</td>
<td>&lt; 6.1</td>
</tr>
<tr>
<td>2-h post glucose load</td>
<td>7.8-11.0</td>
<td>6.7-9.9</td>
</tr>
<tr>
<td><strong>Impaired Fasting Glucose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>6.1-6.9</td>
<td>5.6-6.0</td>
</tr>
<tr>
<td>2-h post-glucose load</td>
<td>&lt; 7.8</td>
<td>&lt; 6.7</td>
</tr>
</tbody>
</table>

*Table 1.1 Values for diagnosis* of diabetes and other types of hyperglycaemia.
*Note that diabetes can be diagnosed in an individual only when these diagnostic values are confirmed on another day (World Health Organisation 1985).

1.3.2 Pathophysiology of Diabetes

To understand the pathophysiology of diabetes it is important to have insight into the mechanisms of insulin secretion and action in controlling glucose homeostasis.

1.3.2.1 The Role of Insulin

Insulin is a key regulatory hormone in mammalian metabolic homeostasis in particular regulating/controlling carbohydrate, lipid and protein metabolism. The body needs to keep a constant blood glucose concentration in order to achieve
euglycaemia. This falls in a narrow range of 3-5mM/L glucose when fasting
(Ashcroft & Ashcroft 1992). After a meal the blood glucose concentrations can
rise transiently to 8-9mM before the body's counter-regulatory control
mechanisms reduce this back to the fasting glucose concentration (see fig. 1,3).
To do this insulin is secreted by the pancreatic β-cells in the islets of
Langerhans and acts by promoting cellular uptake and catabolism of glucose
whilst also inhibiting glucose synthesis in insulin sensitive tissues such as the
liver, kidney and skeletal muscle. Insulin increases the uptake of glucose into
the muscle, adipose tissue, liver and other cells by up regulating cell surface
expression of the facilitative glucose transporter GLUT4 (Klip, Ramlal, et al.
1987). Insulin also increases the rate of glycolysis in muscle and adipose tissue
by an incompletely understood mechanism. Increased glycolysis generates
adenosine triphosphate (ATP) and excess glucose is converted to glucose 6-
phosphate (which is a key intermediary in the glycolytic pathway) and then to
glucose 1-phosphate which is finally converted to glycogen through
glycogenesis and stored in the liver. During fasting, insulin levels are low and
glycogenolysis (with the aid of the catabolic hormone glucagon) occurs,
breaking glycogen back down to glucose-6-phosphate and therefore providing a
source of glucose (Maehlum & Hermansen 1978).
During prolonged fasting glycogen stores are depleted. The organism switches
to an alternative fuel attaining energy by the β-oxidation of fatty acids (FA’s) in
the muscle. The FA’s are derived from the lipolysis (breakdown) of
triacylglycerides that are stored in adipose tissue. When there is increased
blood concentration of FA’s the rate of glucose utilisation decreases in order to
maintain a constant blood glucose concentration. Following feeding, insulin is
again stimulated and switches metabolism back to that of glucose by inhibiting
lipase which is the key enzyme involved in lipolysis (Newsholme & Leech 1983).
Another pathway involved in maintaining a constant blood glucose level is that
of the gluconeogenesis pathway. Gluconeogenesis is a complex pathway
involving more than one tissue. Generally gluconeogenesis is a form of carbon
re-cycling from available carbon sources with the end result being glucose.
These carbon sources primarily include lactate from muscle and liver stores,
glycerol that is released from adipose tissue from the hydrolysis of
triacylglycerol and amino acids. Amino acids can be either glucogenic (e.g. alanine) which means gluconeogenesis will result in glucose production or ketogenic, which means that these amino acids will result in ketone body production (e.g. leucine and lysine). Some amino acids give rise to both glucose and ketone bodies (Leighton, Blomstrand, et al. 1989).

![Control steps of how insulin regulates the blood glucose level.](image)

Fig 1,3. Control steps of how insulin regulates the blood glucose level. Taken from Ashcroft-FM & Ashcroft-SJH 1992. Abbreviations: KB= ketone bodies, gluc= glucose, lac= lactate, aa= amino acids, glyc= glycerol, pyr= pyruvate, TG= triacylglycerol, FFA= free fatty acids.

### 1.3.2.1.1 Insulin Action

The mechanism of insulin signalling is complex and incompletely understood. The insulin receptor (IR) is a trans-membrane protein that is widely distributed among tissues. The receptor consists of two main units the α subunit, which is responsible for the extracellular interaction of insulin and the β subunit, which is intracellular and conveys the initial intracellular signal for the insulin-signalling
cascade (see fig.1,4) (Czech 1985), (Czech, Yu, et al. 1985). When insulin binds to its receptor an intrinsic tyrosine kinase is activated resulting in autophosphorylation of the receptor and enabling intracellular docking proteins to be recruited to the IR. These docking proteins consist of a family of four proteins known as the insulin receptor substrate proteins (IRS-1-4). These proteins are key to the progression of further downstream events in insulin action. IRS-1 and IRS-2 are known to be directly involved in insulin’s metabolic action (White 1998). The roles of IRS-3 and IRS-4 are less clear. It has been proposed that IRS-1 and IRS-2 are scaffolding molecules, which are localised to the cytoskeleton in a regulated fashion. This would enable them to slide along the filaments of the cytoskeleton and interact with the membrane therefore increasing the efficiency of coupling with the IR (Whitehead, Clark, et al. 2000). The IRS proteins when activated bind and activate phosphatidylinositol 3 kinase (PI3K). PI3K generates phosphorylated phosphatidylinositol intermediates (PI) such as PI 3,4,5-triphosphate (PIP3) (Kasuga, Zick, et al. 1982). The PIP3 intermediates in turn recruit other downstream signalling molecules such as protein kinase C (PKC) and a serine/threonine protein kinase (known as Akt) to the membrane. These events facilitate PKC and Akt interaction with upstream regulators such as the phosphoinositide-dependant-kinase 1 (PDK1) which activate PKC and Akt activity (Franke, Yang, et al. 1995). Akt is believed to deactivate glycogen synthase kinase 3 (GSK-3) which leads to the activation of glycogen synthase and thus glycogen synthesis (Cross, Alessi, et al. 1995). Akt activates the translocation of GLUT4 vesicles from their intracellular pool to the membrane allowing increased uptake of glucose into the cell (Bevan 2001).

Other effects of insulin include stimulation of protein synthesis as the inhibited GSK-3 usually inhibits a guanine nucleotide exchange factor that regulates the initiation stage of protein translation eukaryotic initiation factor 2B (eIF2B) (Welsh, Miller, et al. 1998). Insulin resistance has been found to be associated with a decrease in tyrosine phosphorylation of IRS-1 (Whitehead, Clark, et al. 2000), but by which mechanism and how this may be linked with obesity is yet to be elucidated.
**Fig 1.4** Components of the insulin signalling cascade involved in the regulation of glucose metabolism. Indicates inhibitory effect.
1.3.2.2 Metabolic Consequences of Insulin Deficiency

In the absence of insulin there is increased glycogenolysis, gluconeogenesis and lipolysis (see fig.1,5) (Jarrett & Keen 1976). A decrease in the transport of glucose into the cell and decreased glucose utilisation by tissues results in an excess of glucose in the circulation, which is termed ‘hyperglycaemia’. At high concentrations of blood glucose (usually >10mM) the maximum level of renal tubular re-absorption is exceeded resulting in glycosuria (sugar in the urine). This leads to an increase in urine volume by osmotic diuresis, causing dehydration and hence an increased thirst and excessive drinking. Lipolysis is increased rendering an excess of circulating free FA’s of which only a portion can be further catabolised in the tricarboxyl acid (TCA) cycle. Excess free FA’s are converted to ketone bodies resulting in ‘hyperketonemia’. If left untreated metabolic acidosis ensues resulting in coma and death (Murray, Granner, et al. 1996).
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**Fig 1.5** Effects of severe untreated insulin deficiency (diabetic ketoacidosis).

- **Insulin Deficiency**
  - \(\downarrow\) Glucose uptake by cells
  - \(\uparrow\) Glycogenolysis
  - \(\uparrow\) Gluconeogenesis
  - \(\uparrow\) Plasma glucose
  - \(\uparrow\) Renal filtration of glucose & ketones
  - \(\uparrow\) Lipolysis
  - \(\uparrow\) Plasma free fatty acids
  - \(\uparrow\) Ketone synthesis
  - \(\uparrow\) Plasma ketones
  - \(\uparrow\) Plasma concentration of H⁺ (acidosis)
  - \(\downarrow\) Osmotic diuresis
  - \(\uparrow\) Sodium & water excretion
  - \(\downarrow\) Plasma volume
  - \(\downarrow\) Arterial blood pressure
  - \(\downarrow\) Brain blood flow
  - \(\downarrow\) Brain dysfunction, coma and death
1.3.2.2.1 Insulin Dependant Diabetes Mellitus (IDDM)

IDDM is normally an immune mediated disease characterised by the presence of autoantibodies, self-reactive T-cells (MacCuish, Irvine, et al. 1974) and an association with the major histocompatibility complex (MHC) haplotypes human leukocyte antigen (HLA)-DR4 and HLA DR3 genes (Vadheim, Rotter, et al. 1986). Although the presence of these genes is associated with IDDM their presence does not always give rise to IDDM. Twin studies do not show a 100% concordance (Todd 1999) and the mouse model for IDDM (non-obese diabetic strain (NOD)) also does not show a 100% chance of development of the disease (Wicker, Todd, et al. 1995). It is believed that an environmental factor also has a part to play in the pathogenesis of IDDM. The influence of environment has been demonstrated using the NOD mouse showing that there is an increased susceptibility to IDDM development (almost 100%) in mice that are from a germ-free environment whilst there is a decrease in IDDM in mice deliberately infected with a variety of pathogens (Todd 1991). Studies in humans have also shown that multiple infections during the first few years of life are linked with a decreased risk of IDDM (Gibbon, Smith, et al. 1997). These findings correlate with the fact that the incidence of IDDM has increased and so has the level of sanitation and healthcare within the last 30 years. Two main genetic loci have been found that contain susceptibility genes for IDDM, ‘IDDM1’ is the MHC region on chromosome 6 where HLA DR4 and DR3 genes are found. The other locus IDDM2 is a region containing a set of variable number tandem repeats (VNTR) upstream of the insulin gene (INS) on the short arm of chromosome 11. VNTR’s are regions of DNA that contain a variable number of repeated sets of base pairs within an intron and are variable from person to person making VNTR analysis a useful tool in genetic fingerprinting. In the case of the IDDM2 locus there is an allele containing a VNTR of which there are three classes. Class 1 consists of 26-53 repeats that appears to be linked with a susceptibility to IDDM. Class II is very rare but the class III allele (140-210 repeats), appears to confer a form of resistance to IDDM. The reasons for this are currently being studied but seem to be related to the levels of insulin mRNA transcription in the thymus which is one of the key organs for the development of self antigen recognition (Vafiadis, Ounissi-Benkalha, et al. 2001).
Non Insulin Dependant Diabetes Mellitus (NIDDM)

Non insulin dependant diabetes mellitus is clinically heterogenous. Unlike IDDM a level of insulin secretion still occurs. NIDDM is commonly associated with a reduced sensitivity to insulin by peripheral tissues (DeFronzo 1988). One important factor associated with NIDDM particularly with insulin resistance is obesity. Obese subjects in every population have a higher incidence of NIDDM and countries that have the higher incidence of obesity have the highest incidence of the disease (Modan, Karasik, et al. 1986). NIDDM is prevalent in certain populations for example the people of the pacific island of Nauru. These people comprise the 'fattest people in the world' (International obesity taskforce 2000) with 77% of men and women classed as obese and 40% of Nauru's population have NIDDM (Zimmet, Dowse, et al. 1990).

Other findings in NIDDM include decreased β-cell function and insulin secretion (Cerasi, Luft, et al. 1972). There is a diminished or absent first phase insulin release, abnormal pulsatility of insulin secretion, increased secretion of pro-insulin like molecules and progressive failure of compensatory insulin release (Evan AJ & Krentz AJ 2001) (Cerasi 1988) with an early abnormal oscillatory insulin secretion observed in early development of NIDDM (O'Rahilly, Turner, et al. 1988).

NIDDM is usually caused by a combination of insulin resistance and β-cell dysfunction. Whether one or the other is the cause of the other is currently a matter of debate.

One theory is that a diminished insulin response as observed in glucose intolerant and NIDDM patients precedes a glucose intolerant and mild diabetic state (Cerasi 1988). By the time this is established β-cell function is found to be more impaired than peripheral insulin responsiveness to insulin (insulin resistance). Another event must occur to further diminish insulin secretion or reduce insulin sensitivity; such factors that can bring about these events are increasing age, physical inactivity and obesity. This leads to a further hyperglycaemic state which if it reaches a critical level further impairs the β-cell by glucose toxicity thus worsening the situation and resulting in NIDDM (Ashcroft & Ashcroft 1992). There appears to be no specific genetic cause for late onset NIDDM but it is widely believed that there may be susceptibility.
genes, which with a combination of environmental factors such as age and obesity can result in NIDDM (see fig.1,6). The only susceptibility loci found so far are NIDDM1 and NIDDM2, which have only been found in Mexican-American (Hanis, Boerwinkle, et al. 1996) and Finnish populations respectively (Mahtani, Widen, et al. 1996) but not in any other ethnic population. One popular theory that is discussed in more detail in chapter 3 is the fetal origins hypothesis put forward by Hales, Barker, et al. 1991 where altered fetal and early life development will predispose an individual to the development of later life complications such as heart disease, elevated blood pressure and diabetes.

**Fig 1,6** Pathogenesis of non-insulin dependant diabetes mellitus.
1.3.2.2.3 Maturity Onset Diabetes of the Young

The genetic basis of a form of diabetes known as 'maturity onset diabetes of the young' (MODY) is well understood. This form of diabetes is characterised by non-ketotic diabetes mellitus, an autosomal dominant mode of inheritance, onset usually before 25 years of age and frequently in childhood and a primary defect in β-cell function. There are 6 forms of MODY with different genes implicated (see table 1,2) The most common gene mutation causing MODY is in the glucokinase gene which is responsible for MODY2 (Velho, Froguel, et al. 1992), although mutations in Hepatic nuclear factor –1alpha (HNF-1alpha) (Stoffel & Duncan 1997) and HNF-4alpha (Yamagata, Furuta, et al. 1996) are also described.

Over 100 mutations have been described in MODY2 patients with a variety of changes including missense, nonsense and base pair deletions. In most cases the catalytic activity of glucokinase (GK) is affected with reported decrease in the $V_{\text{max}}$ (maximal activity) and $K_m$ values (measure of substrate binding). Also some mutations result in protein instability of GK (Burke, Buettger, et al. 1999).
<table>
<thead>
<tr>
<th>MODY Type</th>
<th>Gene</th>
<th>Clinical features of heterozygous state.</th>
<th>Molecular basis</th>
<th>Clinical features of homozygous state.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODY1</td>
<td>HNF-4α</td>
<td>Diabetes, microvascular complications, reduced serum triglycerides &amp; apolipoproteins</td>
<td>Abnormal regulation of gene transcription in β-cells, defect in metabolic signalling of insulin secretion, β-cell mass or both</td>
<td></td>
</tr>
<tr>
<td>MODY2</td>
<td>Glucokinase</td>
<td>Impaired fasting glucose, impaired glucose tolerance, diabetes, normal proinsulin to insulin ratio</td>
<td>Defect in sensitivity of β-cells to glucose due to reduced glucose phosphorylation, defect in hepatic storage of glucose as glycogen.</td>
<td>Permanent neonatal diabetes requiring insulin treatment</td>
</tr>
<tr>
<td>MODY3</td>
<td>HNF-1α</td>
<td>Diabetes, microvascular complications, renal glycosuria, increased sensitivity to sulphonylurea drugs, increased proinsulin to insulin ratio</td>
<td>Abnormal regulation of gene transcription in β-cells, defect in metabolic signalling of insulin secretion, β-cell mass or both</td>
<td></td>
</tr>
<tr>
<td>MODY4</td>
<td>IPF-1</td>
<td>Diabetes</td>
<td>Abnormal transcriptional regulation of β-cell development and function.</td>
<td>Pancreatic agenesis, neonatal diabetes requiring insulin treatment</td>
</tr>
<tr>
<td>MODY5</td>
<td>HNF-1β</td>
<td>Diabetes, renal cysts, other renal development abnormalities, progressive non-diabetic renal dysfunction renal insufficiency &amp; failure, in females internal genital abnormalities</td>
<td>Abnormal regulation of gene transcription in β-cells, defect in metabolic signalling of insulin secretion, β-cell mass or both</td>
<td></td>
</tr>
<tr>
<td>MODY6</td>
<td>NeuroD1</td>
<td>Diabetes</td>
<td>Abnormal transcriptional regulation of β-cell development and function.</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1.2. MODY related genes and the clinical phenotypes associated with mutations in the genes. Taken from (Fajans, Bell, et al. 2001).*
1.3.2.3 Treatment and Therapy
Exogenous insulin therapy is required to control IDDM. Insulin is administered by injection, although other delivery systems including an aerosolised spray (Laube 2001) are being investigated. Islet cell transplantation has also been performed successfully in a small group of patients (Shapiro, Lakey, et al. 2000). NIDDM is treated by dietary restriction and to increase insulin sensitivity and decrease insulin requirement, drugs that stimulate insulin secretion for example sulphonylureas or increase insulin tissue sensitivity i.e. thiazolidinediones are used (Hauner 2002). Some patients with this form of diabetes mellitus require exogenous insulin therapy (Mauvais-Jarvis, Andreelli, et al. 2001).

1.3.2.4 Long Term Complications & Health Impact
Complications of poorly controlled diabetes include cardiovascular disease (myocardial infarction and stroke) and microvascular disease resulting in nephropathy, retinopathy and polyneuropathy (Ashcroft & Ashcroft 1992) (Donnelly, Emslie-Smith, et al. 2000).

The World Health Organization (WHO) estimates that four to five percent of health budgets are spent on diabetes-related illnesses.
A person with diabetes incurs medical costs that are two to five times higher than those of a person without diabetes. This is due to more frequent medical visits, purchase of supplies and medication, and the higher likelihood of being admitted to a hospital.
Not only are there the costs to the individual to consider but also the direct costs to the healthcare system, the indirect costs to society (i.e. loss of productivity resulting from disability, sickness absence, premature retirement or premature death). And the intangible or psychosocial costs which have the greatest impact on the lives of people with diabetes and their families, and include stress, pain and anxiety. Life expectancy and quality of life can be significantly reduced by diabetes with an average reduction of life expectancy by 15 years (World Health Organisation 1985).
1.3.3 Summary

In view of the epidemiological evidence of worldwide diabetes incidence it is imperative that the ensuing diabetes mellitus epidemic is addressed and action and awareness is taken. IDDM is less prevalent than NIDDM but the incidence is rising therefore further understanding of how IDDM develops is required. So far genetic elements have been found but these themselves require another possibly environmental factor to induce the disease. NIDDM is the form of diabetes that causes most concern as the numbers of people affected are steadily rising and there is a predicted surge of NIDDM for the developing world. There is no known genetic cause for NIDDM and our current understanding suggests that the cause is largely environmentally related. A “western lifestyle” associated with obesity may lead to the development of insulin resistance which in addition to β-cell dysfunction renders the hapless individual diabetic. The causes of insulin resistance and β-cell dysfunction are not yet fully understood but one possible mechanism is related to programmed metabolism during early life. Mechanisms of insulin action via the insulin receptor and insulin secretion may play an important role in elucidating the causes of insulin resistance and β-cell dysfunction.
1.4 Animal Studies
Epidemiological studies can only tell us the outcome in humans but to look further into programming in more detail an animal model is required. This has lead to many animal studies being performed using the rat model to investigate the effects of dietary manipulation during gestation and the suckling period.

1.4.1 Low Protein Diet Rat Model
The programming effects of low protein during early development are of significance as this model may apply to developing countries and explain the high incidence of NIDDM in recently developed countries.

1.4.1.1 Effects on Pancreas Morphology
Snoeck, Remacle, et al. 1990 studied the effect of a low protein (LP) diet on the fetal rat pancreas. Fetal rats were procured from LP and control diet fed mothers. β-cell proliferation was decreased as well as islet size and islet vascularisation which led Snoeck et al to conclude that a LP diet during development alters the structure of the fetal rat pancreas. Further studies following the LP offspring into adulthood on a normal diet again showed that the fetal pancreas had not changed but there was a reduction of islet insulin content resulting in decreased fasting plasma insulin concentrations (Dahri, Snoeck, et al. 1991). Smaller islets (but numerous) were observed in adult offspring of prenatally LP fed mothers (see fig. 1,7a). However pre & postnatal LP offspring showed fewer larger islets with a decrease of percentage of islet tissue & β-cells per pancreas (see fig. 1,7b). All LP groups exhibited an increase in irregularly shaped islets and a decrease of β-cells within the islets (Berney, Desai, et al. 1997).
Petrik and colleagues studied β-cell turnover and found that the LP fetal and weanling rat β-cells had a prolonged G1 phase of the cell cycle. Increased apoptosis was observed at all stages of development in LP rats. mRNA levels of Insulin like growth factor II (IGF-II) which is a known survival factor were found to be decreased in suckling rats. Hence a LP diet alters the development of the pancreas by changing the balance of β-cell replication and apoptosis in fetal and neonatal life, which contributes, to the smaller size of islets (Petrik, Reusens, et al. 1999).

1.4.1.2 Effects on Islet Function
Insulin secretion is impaired by a low protein diet through out gestation and the suckling period (Swenne, Crace et al. 1987) and many other studies have confirmed this. Wilson & Hughes 1997 found that offspring of adult’s fed LP diets showed reduced insulin secretion, which was more, pronounced in a sub group fed high fat diets. This indicates that poor early nutrition leads to impaired pancreatic β-cell function that persists into adult life (programmed). Alone this is not sufficient to produce NIDDM but an inability to respond to a highly palatable
high fat diet may tip the balance towards impaired glucose tolerance (Wilson & Hughes 1997).

Wilson et al found that after feeding with sucrose, glucose utilisation and oxidation and ATP levels were normal in the LP groups but the insulin secretory response to keto-isocaproate and tolbutamide were decreased. These results suggested that the malfunction might lie within the ion channel steps of the insulin secretory pathway as glucose metabolism and ATP generation appeared normal (Wilson & Hughes 1998).

Another study examined the effect of a high fat (HF) diet on islets of LP reared rats (recovered at weaning) showed that the diet (given for 8 weeks during adulthood) induced impaired glucose tolerance. Lipolysis was enhanced in these rats by the administration of norepinephrine. Rats that were fed LP then HF showed a significant increase of lipolysis compared with controls. The effect of varying concentrations of administered insulin was looked at. The LP-HF rats did not show a decrease in lipolysis until the highest dose of insulin was given. It therefore appears that increased supply and utilisation of FA’s from increased lipolysis underline the rapid decline in insulin stimulated-glucose disposal and illustrate a decrease of antilipolytic response of insulin in adipocytes (Holness & Sugden 1999).

Work on fetal rats (taken at 21.5 days gestation) from LP fed mothers showed a decrease in islet insulin secretion when stimulated with amino acids or cAMP and also to metabolic and non-metabolic secretagogues. However the response seemed to be restored by stimulation with barium which permeates the membrane through Ca^{2+} channels and directly triggers exocytosis of insulin indicating that the defect in LP fetal islets is to do with Ca^{2+}. Insulin secretion was also restored by stimulation with cytochalasin-B, which acts by changing the \( \beta \)-cell microfilamentous cell network involved in the last step of the insulin secretion cascade. Therefore according to Cherif et al 2001 the defect in insulin secretion induced by a LP diet through development could lie within the exocytosis step of insulin secretion (Cherif, Reusens, et al. 2001). A reduction of \( \beta \)-cell mass in offspring from LP fed mothers during gestation was seen and the animals showed normal plasma insulin responses with borderline glucose tolerance after 12 months.
Rats kept on the LP diet through the suckling period also showed increased apoptosis of β-cells and a profound decrease of insulin and glucose intolerance. This study suggests that perinatal malnutrition impairs β-cell development and with the additional demand from increasing age of the β-cells leads to glucose intolerance (Garofano, Czernichow, et al. 1999).

Latorraca et al investigated the effect of a LP diet on the early steps of insulin action on rats that were on a LP diet throughout gestation, suckling and after weaning as well as only through gestation and suckling. They also found that insulin secretion was impaired in both LP groups and there was enhanced insulin sensitivity particularly in rats kept on the LP into adulthood. Insulin receptor (IR) levels were significantly increased and the phosphorylation of IRS-1 with IR was increased as well as an increase in the association of IRS-1 with the lipid metabolising enzyme PI3-K. This study demonstrates that decreased insulin secretion induced by the LP diet is associated with enhanced tissue sensitivity to insulin (Latorraca, Reis, et al. 1998).

1.4.1.3 Effects on Liver Function
Programming of glucose metabolism has also been demonstrated in the liver. Burns et al 1997 found that perfused livers of rats from mothers fed a LP diet through gestation and suckling produced more glucose from an exogenous dose of lactate. In LP livers glucose formed from lactate in the periportal region of the liver was not efficiently taken up by distal perivenous cells which gave rise to an increase of glucose levels. GK activity in the distal perivenous cells was greatly reduced in LP rats. The intralobular distribution of phosphoenol pyruvate carboxykinase (PEPCK) which is a key enzyme involved in glycogenolysis was not altered in LP rats but increased activity could also contribute to the increased glucose production seen. Suggesting that programming of adult glucose metabolism may operate partly through structural alterations and changes in expression of GK in the perivenous region (Burns, Desai, et al. 1997).
1.4.1.4 Effects on Glycolytic Enzymes

Desai and Hales studied programmed metabolism in rats from protein deprived mothers. Four groups were set-up:

(i) a control group where pregnant dams were fed normal laboratory chow throughout gestation and suckling periods,
(ii) a LP group where the mothers were fed on a LP diet through the same periods,
(iii) a recovery group fed on a protein restricted diet throughout gestation and then put on a control diet through lactation
(iv) a post natal low protein group that was fed on the control diet through gestation but put onto a low-protein diet during lactation.

![Diagram illustrating study by Desai & Hales 1997.](image)

The activity of two hepatic metabolic enzymes were studied in the offspring of these dams, hepatic GK and PEPCK. GK activity was decreased by about 50% and PEPCK activity increased by 100% in the low-protein and recovery groups. The post-natal LP group had activities comparable with the control group (see fig.1,8). These changes were consistent even after the offspring were weaned onto normal lab chow for 11 months. The mRNA levels of both enzymes mirrored the results indicating that programming may occur at the gene level (Desai, Byrne, et al. 1997a).
Subsequently Desai and Hales examined the effect of highly palatable diets on offspring from mothers fed either on normal lab chow or low-protein diets during gestation and lactation (see fig. 1,9).

12 weeks after birth, hepatic GK levels were significantly decreased in the group from mothers fed a LP diet and then normal lab chow when compared with the control group. Both highly palatable diet groups exhibited an increase in GK activity although less so in offspring of LP mothers. PEPCK activity was increased in the LP group, later fed on normal lab chow compared with the control group and levels were both decreased in both highly palatable groups. Groups fed the highly palatable diet had increased plasma insulin concentrations with the highest levels seen in male rats fed the LP than highly palatable diet. This may indicate there is a different response to maternal protein-restricted diet between the sexes as it is noted in humans that men tend to be more insulin resistant than women (Phillips, Barker, et al. 1994). From these two studies it was concluded that the hepatic enzymes GK and PEPCK activities are altered at some critical period of development during gestation. (Desai, Byrne, et al. 1997b).
1.4.1.5 Lipid Metabolism

Lipid metabolism is also programmed in rats. Lucas et al. (1996) conducted a LP rat study with mothers fed LP before birth, from birth to weaning and pre and post partum. At 6 months of age plasma cholesterol, HDL-cholesterol and triacylglycerol (TAG) concentrations were 20-50% lower in offspring from mothers fed LP during gestation and suckling periods and in the suckling only offspring group. Only a decrease in plasma TAG's was found in the gestation period LP group. This study showed that both pre and perinatal nutrition were the sensitive periods involved in cholesterol and TAG metabolism programming (Lucas, Baker, et al. 1996).

1.4.2 Studies Using High Carbohydrate Diet Manipulation

High carbohydrate diet may also program metabolism a scenario likely in western countries where 'high carbohydrate food is found in abundance. The high carbohydrate rat or HC rat model has been used to study programming effects induced in the fetal and suckling periods, with a variation to study the suckling period known as the ‘pup in a cup’ model. This is where 4-day-old rat pups are reared in styrofoam cups floating on a temperature controlled water bath. These rats are fed via intragastric cannulas introduced nonsurgically and raised on an HC milk formula. The HC diet induces hyperinsulinemia within 24hrs which persists into adulthood even after the HC rat is weaned onto a normal diet. Pup body weights remain similar to controls during the suckling phase but do develop obesity into adulthood. Smaller islets are seen in neonatal HC rats that have a higher insulin secreting capacity than normal islets (Petrik, Srinivasan, et al. 2001). Increased insulin synthesis and gene expression of preproinsulin with also increased mRNA levels of pancreatic transcription and regulatory factors that are involved with pancreatic organogenesis for example pancreatic-duodenal homeobox factor-1(PDX-1) were also observed (Song, Srinivasan, et al. 2001). The finding that PDX-1 is increased in the HC rat is of interest as this DNA-binding protein has been proposed to be modulated by glucose and then via a phosphorylation cascade increases insulin gene transcription (Macfarlane, Smith, et al. 1997). Therefore PDX-1 may play a role in the cellular adaptations as well as onset and maintenance of hyperinsulinemia in the HC rat. Along with the hyperinsulinemia there is
increased glucose sensitivity and decreased $K_{ATP}$ channel activity with subsequent increased $Ca^{2+}$ activity (Komatsu, Schermerhorn, et al. 1997). Many of these metabolic changes in the suckling rat are found to persist into adulthood and with chronic hyperinsulinemia there is an increase in body weight leading to full-blown obesity. These data show that a HC milk formulae substituting normal mothers high fat milk elicit significant alterations in islet functions. An important consequence of this programming was the transmission of the females HC rat phenotype to her progeny even though the pups themselves did not undergo any HC diet manipulation. Cross breeding experiments showed that it was only the HC females that transmit these traits implying that intrauterine experience is involved (Vadlamudi, Kalhan, et al. 1995).

1.5 Possible Mechanisms of Programming
How specific tissues are permanently affected by their nutritional biochemical environment during development giving them a sense of 'memory' is yet to be elucidated. The possible mechanisms that could cause a programmed effect are reviewed by Waterland & Garza (1999) and include:

1) induced variations in organ structure,
2) alterations in cell number,
3) clonal selection,
4) metabolic differentiation

1.5.1 Organ Structure
Gross morphologic alterations occurring during organogenesis such as organ vascularisation, innervation and changes in positioning of different cell types within an organ have also been implicated. This mechanism may permanently affect an organism's metabolism by the ability of individual cells to generate and respond to external signals within the organism through organ vascularisation that would affect the cells responses to blood-borne nutrients or hormonal signals. During organogenesis the fate of cells depends on external signals from adjacent cells and morphogen gradients that originate from other sites. Therefore local concentrations of nutrients, metabolites, or both could modulate this. One example is retinoic acid a derivative of vitamin A, which serves as an
important regulatory role during normal organogenesis by binding to ligand-activated transcription factors that regulate genes involved in morphologic development (Sucov, Lou, et al. 1996).

1.5.2 Cell Number
Alterations of cell number is the simplest mechanism of those proposed which could result in programming of metabolism. During development organs can either increase in size by cell hyperplasia or cell hypertrophy. The rate of cellular proliferation is directly dependent on nutrient supply and maybe indirectly dependent on nutritional status via hormonal signals that control cell proliferation. Therefore limited or excessive nutrients during critical periods of growth that affect rates of cell division may lead to permanent changes in cell number, regardless of later nutrient availability. For permanently reduced islet size and reduced β-cell mass have been observed as a result of a low protein diet in early development by Snoeck, Remacle, et al. (1990).

1.5.3 Clonal Selection
Cellular proliferation in all organs involves the initial multiplication of a finite population of founder cells that are not necessarily identical to each other. As cellular proliferation proceeds individual cells will differentiate (by genetic and environmental influences) distinguishing them from others in subpopulations of rapidly dividing cells. These distinguishing characteristics may offer selective advantages to cells competing for nutrients. The two similar heterogeneous populations of rapidly dividing cells may develop distinct metabolic characteristics as a result of diverse micro-environmental conditions for instance if the nutrient environment is deficient in structural fatty acids, cells with a slightly more efficient lipogenic pathway could disproportionately populate a tissue. Therefore it is possible that variations in nutritional status during development could lead to permanent alterations in the cellular composition and hence the metabolism of tissues and organs.
1.5.4 Metabolic Differentiation

The metabolic states of cells and gene expression can be influenced by epigenetic factors that may mediate metabolic programming.

- DNA binding proteins or transcription factors in combination, control gene expression to regulate efficiently thousands of different genes in a cell-specific manner. Most genes are not controlled by a single transcription factor but by a combination of them. This may form a developmental cascade that establishes cell specific patterns of gene regulatory proteins which early nutrition can influence.

- Chromatin structure is highly correlated with gene expression (Shemer, Birger, et al. 1996). Non active DNA is packaged into condensed histones. Active DNA however is in the open configuration of the histones enabling genes to be transcribed and this is specific to the cell type i.e. genes for insulin secretion will only be found in an open chromatin configuration in the β-cell. The regulation process that 'remembers' what areas of DNA should be kept open when passing DNA to progeny is of interest as histones and DNA binding proteins must detach from DNA so that DNA polymerase can replicate it. This mechanism is not fully understood but a current model involves specific histone modification such as acetylation, which could act as a marker for DNA that needs to remain in a transcriptionally active configuration. So a specific chromatin configuration established during development could be generated through cell turnover and thus early metabolic conditions could modulate histone post-translational modifications and thereby early nutrition may have a lasting effect.

- Areas of increased density of the dinucleotide sequence guanine—phosphate diester—cytosine are known as CpG islands. Methylation of cytosine nucleotides in CpG islands is highly correlated with gene expression. The specific pattern of cytosine methylation varies among cells in different tissues and the methylation pattern is maintained through DNA replication (Eden & Cedar 1994). The rat PEPCK gene is undermethylated in liver and kidney tissue enabling expression but is hypermethylated in all other tissues where PEPCK is not expressed (Benvenisty, Mencher, et al. 1985). If early nutrition affects gene expression during differentiation, cell
specific DNA methylation may be influenced and could also be a candidate mechanism for programming.

1.6 Summary
Epidemiological evidence provides a strong argument for the thrifty phenotype hypothesis and the concept of programming. This is of increasing interest in the quest for an explanation to the rising numbers of NIDDM cases throughout the world. Animal studies have provided an avenue to examine how dietary manipulation during critical periods of early development affects metabolism. The glucose metabolism is clearly affected by early nutritional experience. The cause of an impaired insulin secretory response needs to be investigated. It would be of interest to look at GK, which acts as the glucose sensor in the β-cell which can influence overall β-cell metabolism. To pinpoint the timing the programming of GK during early development, it would be pertinent to determine the effect of a low protein diet on GK enzyme levels and activity during different stages of development.
Chapter 2
Pancreatic Function and Development

2.1 Islets & β-cells
Insulin is produced and secreted by the endocrine β-cells of the pancreas. β-cells are found within clusters of endocrine cells first described by Paul Langerhans in 1869 and which are known as Islets of Langerhans. Islets contain four different cell types, which secrete different hormones involved in metabolism. Mature islets are generally spherical in appearance; their size and number vary among species. In the rat they range from 50-500 μm in size (Lacy & Greider 1972). The smaller islets are more numerous than larger islets but larger islets (>150μm) take up approximately 50% of total islet volume (Hellman 1970). A cartoon of a pancreatic islet is shown in fig.2,1. The four types of cell that make up an islet are the α-cells which generally comprise about 25% of islet cells, β-cells which make up 70%, δ-cells that make up <5% and F-cells which are found in small amounts (Orci & Unger 1975). β-Cells form the core of the islet whilst all the other cells form the mantle (Hellman 1967).

Fig 2,1. Diagram of a pancreatic islet (islet of Langerhans) and surrounding acini.
The α-cells secrete glucagon which acts in an opposite fashion to insulin, stimulating glycogenolysis and glucose mobilisation. The β-cells secrete insulin, δ-cells secrete somatostatin which inhibits secretion of a number of hormones including growth hormone and insulin and regulates gastrointestinal secretomotor events and also acts as a neurotransmitter in the central nervous system. F-cells secrete pancreatic polypeptide the exact function of which is unknown (Ashcroft & Ashcroft 1992).

### 2.1.1 Islet Cell Interaction

β-Cells have also been found singly within the pancreas but the glucose responsiveness of these single cells is lower than β-cells in islet units. This is due to a 'co-operativity' between the β-cells in the islet by structures known as 'Gap junctions' which allow electrical coupling between cells. These junctions result in an intercellular form of transport where ions and small molecules can be transferred between cells (Meda, Kohen, et al. 1982). They are not fixed anatomical structures but are dynamic formations that disassemble and reassemble in gap junction particles (In't Veld, Pipeleers, et al. 1986). There are also interactions between islet cells through secretory products where insulin release can be influenced by other pancreatic hormones (Pipeleers 1984). The presence of gap junctions highlights the significance in the organisation of the islet, the clustering of cells creating a highly effective functional unit of cells that can work to an optimal efficiency when acting together. A unit of β-cells can respond quicker and with an amplified response by the expeditious communication of signals between cells provided by the gap junctions. The vascular supply is another important feature of the pancreatic islet. The islet capillary system establishes close contacts with the majority of β-cells and with each islet bordered on at least two sides by a capillary (Bonner-Weir 1988) this provides the effective procurement of islet secretory products into the bloodstream. Following the arterioles into the islet a rich autonomic innervation of nerve fibres provides a neurological communication network (Pearse 1982).
2.2 Insulin Molecular Structure

Insulin itself is a highly conserved protein of 5.6KDa and comprises of two chains; the A-chain (21 amino acids) and the B-chain (30 amino acids) that are connected by two disulphide bonds.

Insulin is initially translated into a single chain precursor in the β-cell known as preproinsulin. Preproinsulin (110 amino acids) is further cleaved into proinsulin which is 86 amino acids (11.98KDa) in the endoplasmic reticulum from which proinsulin is packaged into secretory granules. Inside the granules proinsulin is again cleaved by endopeptidases to finally form insulin (Docherty, Carroll, et al. 1983) (Docherty, Hutton, et al. 1984). Insulin is then secreted from the pancreatic β–cell through the exocytosis of vesicles packaged with insulin.
2.3 Insulin Secretion

The initiation and regulation of insulin secretion is primarily controlled by the β-cell’s metabolism and glucose concentration. Cells contain two main secretory pathways: the constitutive and the regulated. It is believed that the default pathway for secretory proteins is the constitutive route. Insulin however is secreted almost entirely via the regulated pathway (>99%) to which it is directed to by specific signals (Rhodes & Halban 1987). ‘Secretagogues’ which include hormones, neurotransmitters and other nutrients can also influence insulin secretion in concert with circulating glucose or influencing each other to either potentiate or inhibit insulin release. They also act as signals for β-cell gene
expression, protein biosynthesis and cell proliferation (Deeney, Prentki, et al. 2000). Some of these factors are summarised in fig 2,3.

**Fig 2,3** Modulators of insulin secretion. VIP = Vasocactive-intestinal peptide, GIP = Gastric inhibitory peptide, CGRP = Calcitonin-gene-related peptide.

### 2.3.1 Insulin Secretion Mechanism

The secretion of insulin from the pancreatic β-cell has been thoroughly investigated and the widely accepted mechanism (see fig. 2,4) described here is unique because the signals that stimulate insulin release are derived from the intracellular metabolism from glucose rather than generated from a ligand-receptor interaction (Malaisse, Sener, et al. 1979) (Meglasson & Matschinsky 1986).
2.3.1.1 Glucose Transport

The first step of insulin secretion is when glucose enters the β-cell by non-rate limiting facilitated diffusion. This occurs via the GLUT2 transporter, which is a trans-membrane protein that has a high $V_{max}$ value. This means it has a high capacity to transport glucose into the β-cell. The $K_m$ value of GLUT2 is 50mM glucose meaning uptake of glucose by the β-cell resembles that of the liver. The GLUT2 transport protein belongs to a family of structurally related glucose transporters that form 3 classes, the formerly known GLUT1-4, the fructose transporters GLUT5, 7, 9 & 11 and GLUT 6, 8, 10 & 12. GLUT transporters 1-5 are well known and characterised (see table 2.1) (Gould & Bell 1990) but the others have only recently been described (Joost & Thorens 2000).

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Appx. $K_m$ for glucose mmol/liter</th>
<th>Tissue distribution</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT-1</td>
<td>20</td>
<td>Widely expressed high levels in brain, erythrocytes, and endothelial cells</td>
<td>Constitutive glucose transporter</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>42</td>
<td>Kidney, small intestine epithelia, liver, pancreatic β-cells</td>
<td>Low-affinity glucose transporter, has a role in sensing glucose concentrations in islets</td>
</tr>
<tr>
<td>GLUT-3</td>
<td>10</td>
<td>Neurons, placenta</td>
<td>High affinity glucose transporter</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>2-10</td>
<td>Skeletal muscle, cardiac muscle, adipose cells</td>
<td>Insulin responsive glucose transporter</td>
</tr>
<tr>
<td>GLUT-5</td>
<td>NA</td>
<td>Small intestine, sperm, kidney, brain, adipose cells, muscle</td>
<td>Fructose transporter, very low affinity for glucose</td>
</tr>
</tbody>
</table>

Table 2.1 Characteristics of the five facilitated-diffusion glucose transporters. Adapted from Shepherd & Kahn 1999.

2.3.1.2 Glucose Phosphorylation

The first step towards insulin secretion in the β–cell involves the glucose uptake into the cell and its entry into glycolysis. This step is widely accepted as the
glucose sensing mechanism for insulin secretion in β-cells (Matschinsky, Liang, et al. 1993) (Terauchi, Sakura, et al. 1995) (Efrat, Tal, et al. 1994). Glucose is phosphorylated to glucose-6-phosphate by either one of two enzymes, the high affinity Type III hexokinase which has a low $K_m$ value of 0.1-4mM for glucose and which is saturated at all glucose levels and the low affinity glucokinase (Lynedjian, Mobius, et al. 1986). This step and glucokinase is discussed further later in this chapter.

### 2.3.1.3 Ion Channels & Secretion

The pancreatic β-cell is electrically active. The β-cell has plasma membrane bound ion channels, which regulate electrical activity. Glucose induced oscillations in β-cell membrane potentials vary with extracellular glucose concentrations. Two ionic conductances pivotal to the process of insulin secretion are the ATP regulated potassium (K-ATP) channel, which is sensitive to the ATP/ADP ratio, and the L-type Ca$^{2+}$ channel (Ghosh, Ronner, et al. 1991). Metabolism of glucose by glycolysis increases β-cell intracellular ATP concentration. An increase in the intracellular ATP/ADP ratio brings about closure of K-ATP channels increasing intracellular K$^+$ causing depolarisation of the β-cell. In turn depolarisation activates a voltage dependent Ca$^{2+}$ channel allowing Ca$^{2+}$ entry into the cell. An increase in intracellular [Ca$^{2+}$] promotes exocytosis of the insulin secretory granule (Malaisse-Lagae, Mathias, et al. 1984).

### 2.3.1.4 β-cell Electrical Activity

Insulin secretion is pulsatile. The bursts of insulin secretion occur as a consequence of changes in the flux of glycolysis. One of the main regulators of glycolysis is phosphofructokinase (PFK) as the allosteric regulation of this enzyme results in bursts of ATP production which can cause alternating opening and closing of ATP-sensitive K$^+$ channels, leading to the observed oscillations in membrane potential and Ca$^{2+}$ influx in pancreatic beta-cells, and may also have downstream effects on exocytosis (Tornheim 1997). The purpose of pulsatile insulin secretion is not fully understood but may be related to more effective regulation of metabolic and exocytotic events as prolonged
exposure to glucose may lead to desensitisation of cell signalling (Berridge & Galione 1988). Pulsatile insulin secretion has been noted to have a greater hypoglycaemic effect than constant secretion (Matthews, Naylor, et al. 1983) as Individuals with NIDDM lose pulsatile insulin secretion in vivo and in vitro (Polonsky, Given, et al. 1988).

**Fig. 2.4** Diagram illustrating how β-cell glucose metabolism initiates insulin secretion.
2.3.2 Insulin Secretory Patterns

The exocytotic release of insulin from the β-cells after glucose stimulation shows a general secretory pattern. When stimulated there is an immediate discharge which is termed as the first or early phase response and usually peaks after 2-3 min and lasts from 6-10 min before secretion decreases. This is then immediately followed by a gradual increase in the rate of insulin release and is known as the second or late phase. With continual glucose stimulation over a period of a few hours a third phase can be observed where the insulin release begins to decline to a lower steady state rate (see fig 2,5) (Grodsky & Bolaffi 1992). The third phase is a diminished insulin response following extended stimulation with glucose. Accelerated glycolysis results in prompt increase of ATP/ADP ratio causing the closure of the K-ATP channel. This depolarises the cell and activates the Ca\(^{2+}\) channels. The abrupt influx of extracellular Ca\(^{2+}\) could be the initiating signal for immediate granule exocytosis while a sustained insulin response requires further mobilisation of intracellular pools of [Ca\(^{2+}\)]. Evidence points to the interaction of extra- and intracellular Ca\(^{2+}\) recruitment with several amplifying couplers in the generation of the late phase insulin response (Zawalich & Rasmussen 1990).

Further work has led to the identification of a readily releasable pool of insulin containing granules that rapidly discharge during the first phase of glucose stimulated insulin release and is primed by refilling of a reserve pool of insulin during the second phase which appears to be the rate limiting step (Daniel, Noda, et al. 1999). The metabolic mechanisms controlling insulin secretion are complex and interrelated however the relative contribution of these mechanisms to the different phases of insulin release has not yet been established. Typical phasic insulin release in response to extracellular and intracellular glucose levels is depicted in fig.2,5.
2.4 Glucokinase

Glucokinase (GK) was first identified in 1966 in rat liver. GK is a type IV hexokinase which catalyses the first step in glycolysis, the conversion of glucose to glucose-6-phosphate. The hexokinases are a family of related enzymes present in most eukaryotic cells with four types found in mammalian cells.

GK has a much lower affinity (demonstrated by its high $K_m$ value) than its other counterparts (hexokinases I-III) (Grossbard & Schimke 1966), meaning that glucokinase can work at around 50% maximal rate at normal glucose concentrations leaving it with the capacity to work at much higher glucose concentrations. This is important in initiating insulin secretion and reducing the blood glucose levels after a meal.
2.4.1 Glucokinase Isoforms

Unlike its counterparts the ubiquitous housekeeper hexokinase's, GK is only found in two important metabolic organs the 'liver and the β-cells of the pancreas' (Ashcroft & Randle 1970) (Matschinsky & Ellerman 1968). Although GK is the same in liver and β-cells there appears to be tissue specific regulation which is achieved by the presence of two tissue specific promoters for the GK gene. The liver promoter lies contiguous to the GK gene and is regulated by insulin and glucagon, and expression is dependent on nutritional status, as activity decreases during fasting and increases after feeding (Iynedjian 1993). The β-cell promoter lies further upstream, but does not appear to be regulated by the hormones or other effectors that regulate the liver promotor. The β-cell
GK mRNA and protein levels are maintained at a constant level during prolonged starvation or following an oral glucose load (Lynedjian, Pilot, et al. 1989).

Fig 2,7 GK gene in the rat. Top line: exon-intron organisation of the gene. □ represent exons. Arrows represent start of transcription initiation. Line 2 and 3 show pre mRNA splicing pancreatic and liver specific transcription. ▲ Indicates start and termination codons.

Glucose is the physiological mediator of GK activity, concentration and glucose responsiveness. Islets cultured for 7 days in a low glucose medium showed reduced GK activity and insulin secretion whilst islets cultured in a high glucose medium for 7 days showed increased GK activity and insulin secretion indicating that glucose can affect the glucose responsiveness of GK in islets (Liang, Najafi, et al. 1990). Glucose has also been confirmed to regulate GK protein content as well as GK activity using western blotting techniques (Chen, Hosokawa, et al. 1994).

Whilst the two GK isoenzymes come from the same gene they differ in their first 14-16 amino acids because tissue specific leader exons make their primary structure distinct. The reason for this difference is unknown, as kinetic properties of purified liver and islet forms appear indistinguishable (Vischer, Blondel, et al. 1987).
2.4.2 GK Regulation

Pancreatic GK gene expression is regulated by insulin, of which controls its own transcription. A recent interesting study found that although insulin regulates both its own and pancreatic GK gene expression there is a degree of specificity. There are two isoforms of the insulin receptor that result from alternative mRNA splicing of the insulin proreceptor transcript the A type and the B type. They are distinguishable by the A type lacking 12 amino acids at the C-terminus of the α-chain of the receptor (Ebina, Ellis, et al. 1985). The functional significance of these two isoforms is unclear as no insulin mediated effect has been reported that discriminates signalling via the A or B type receptors. But it has been found in the β-cell that insulin promotes the transcription of its own gene via the insulin receptor type A and through subsequent signalling molecules promotes transcription of the pancreatic GK gene via the B type insulin receptor and through other subsequent signalling molecules (see fig 2,8). These two isoforms of the receptor therefore provide a selectivity of insulin action (Leibiger, Leibiger, et al. 2001). One advantage to this would be to provide a means of preferentially expressing either insulin or GK but this remains to be elucidated.

![Diagram](image-url)

**Fig 2,8** Selective activation of insulin and GK gene transcription by selective insulin signalling via A and B type insulin receptors. Adapted from Leibiger B, et al, 2001.
GK activity as described above is based on glucose concentration. A liver GK regulatory protein has recently been found (termed GKRP) which controls GK activity by protein-protein interaction. The binding of GK to GKRP is competitive with glucose but favoured if GKRP is liganded with fructose-6-phosphate (F-6-P). Release of GK from GKRP is favoured however when GKRP is liganded with fructose-1-phosphate (F-1-P) hence hepatic GK activity is increased after the ingestion of fructose containing carbohydrates because absorption of fructose leads to an increase in the ratio of hepatic F-1-P to F-6-P (Van Schaftingen & Davies 1991). The mechanism by which GKRP inhibits GK is by binding to GK, then the GKRP-GK complex translocates to the nucleus therefore trapping GK and preventing it from catalysing glucose to G6P (Toyoda, Ito, et al. 1997). GKRP has not been found in islet β-cells (Malaisse, Malaisse-Lagae, et al. 1990). The possibility of a β-cell GK regulatory protein was first proposed by (Tiedge, Steffeck, et al. 1999) who found that GK interacts with an as yet unidentified protein factor capable of modulating its activity. Further studies have analysed binding motif sequences from cDNA libraries of GK. The protein structure of three such proteins has recently been described. Munoz-Alonso, Guillemain, et al. 2000 describe a dual specificity phosphatase termed GK associated phosphatase (GKAP) which phosphorylates recombinant GK enhancing its activity. The GKAP protein has been detected in liver and pancreas. Shiraishi, Yamada, et al. 2001 describe another novel GK regulator in β-cells a precursor of propionyl CoA carboxylase β subunit (pβPCCase) which becomes functional in fatty acid metabolism in the mitochondria. pβPCCase was found to decrease recombinant GK $K_m$ and increase GK $V_{max}$. A third protein that has a high binding affinity for GK has been described in liver and islets that showed a complete homology to the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK-2/FBPase-2) (Baltrusch, Lenzen, et al. 2001). The biological significance of these newly described GK associated proteins has yet to investigated but they could play crucial roles in GK regulation and the β-cell response to glucose.
2.4.3 GK the Glucose Sensor

The GK reaction (the first step in glycolysis) has been widely accepted as the glucose sensing mechanism for the initiation of insulin secretion. Evidence supporting GK as being the glucose sensor includes:

i) The capacity of glucose transport is at least 100 fold in excess of glycolysis which is sufficient to equalise the extracellular and intracellular glucose concentrations within seconds of any blood glucose changes therefore pointing to an intracellular glucose-sensing device. A decrease of > 90% of glucose transport has to occur to have an effect on β-cell glucose metabolism (Tal, Liang, et al. 1992).

ii) The reaction kinetics of GK are such that it can effectively control glycolysis in the range of 3-15mM glucose. This is indicated by the $K_m$ and the positive cooperativity of GK with its substrate glucose.

iii) The GK reaction is the rate-limiting step for glycolysis, controlling the overall flux of glycolysis. Insulin secretion parallels that of β-cell metabolism (Matschinsky, Liang, et al. 1993).

iv) Thiazolidinediones which are synthetic ligands of peroxisomal proliferator receptor-gamma (PPAR-gamma) are used to improve peripheral insulin sensitivity and glucose stimulated insulin secretion in pancreatic β-cells in diabetics. A study to explore the role of PPAR-gamma in glucose sensing found that the β-cell specific GK promoter contains a peroxisomal proliferator response element (PPRE). The PPAR-gamma heterodimer binds to the response element and activates the β-GK promoter in β-cells as well as non-β-cells (Kim Hi, Cha, et al. 2002).

v) A strong piece of evidence to confirm that GK is the glucose sensor in β-cells is the finding of two activating mutations in the GK gene in families affected with familial hyperinsulinism. In these families the GK has a higher affinity for glucose therefore at low/normal blood glucose concentrations GK is working at a higher maximal capacity initiating insulin secretion and subsequently causing hypoglycaemia in these patients (Glaser, Kesavan, et al. 1998) (Christesen 2002).

Usually mutations in the GK gene have resulted in decreased or null activity of GK leading to the development of NIDDM that has its onset during childhood.
and as previously mentioned is known as MODY2 (Takeda, Gidh-Jain, et al. 1993).

2.5 Pancreatic Development

Knowledge of the normal development of the pancreas and the ontogeny of islet gene expression may be important for an understanding of the programming process.

2.5.1 General Development of the Pancreas

In the mouse the pancreas first appears as an évagination on the dorsal surface of the primitive gut endoderm at around embryonic (E) day 9.5. Shortly after a pancreatic bud arises which undergoes rapid growth and the epithelial cells form protrusions that lead to a highly branched network covered in mesenchymal tissue. Over the next 10 days cells within the primitive epithelium develop the ductal, endocrine and acinar cells of the pancreas. The dorsal and ventral lobes of the pancreas fuse at around E18.5. Isolated endocrine cells migrate from the epithelial network into the surrounding mesenchyme where they form islets. Differentiation of islet cell types does not occur until towards the end of gestation. Islets continue to mature after birth during which they develop their ability to sense and regulate insulin secretion (Edlund 2001). In the human (see fig. 2,9) the pattern of development is very similar although timing is different, initial évagination of the primitive duodenum occurs at about 30 days. With endocrine cells appearing at 8 weeks, acinar cells at 12 weeks and ductal cells at 18 weeks gestation, with fusion of the dorsal and ventral lobes by 7 weeks. As in the mouse there is continuing maturation of the endocrine and exocrine tissue after birth and during the 1st year of life (Skandalakis & Gray 1994).
2.5.2 Pancreatic Cell Differentiation

The differentiation of the different cell types in the pancreas is controlled by a signalling cascade of transcription factors (TFs) that are expressed at specific timed intervals throughout development. Many of these transcription factors contain homeodomains that are recognised motifs of a helix loop helix confirmation within their protein structure. One key signal implicated in the generation of the pancreas from the gut endoderm and generated by the notochord is sonic hedgehog (shh). The absence of shh, which itself is controlled by other TF’s determines which part of the gut endoderm will form the pancreas (Hebrok, Kim, et al. 1998). The next step in pancreatic development is bud formation and evagination of the pancreatic primordia. The three main TF’s involved here are forkhead box a2 (Foxa2) which is also known as hepatic.
nuclear factor 3β (HNF3β), pancreatic duodenal homeobox-1 (PDX-1) and homeobox protein (HB9). PDX-1 plays a critical role in growth and differentiation of the pancreatic buds that appear on the dorsal and ventral sides of the gut endoderm. Mice devoid of PDX-1 fail to form a pancreas (McKinnon & Docherty 2001). PDX-1 appears to define a compartment of the gut endoderm foregut that specifies the dorsal and ventral pancreas. Differences between the development of the dorsal and ventral lobes of the pancreas are controlled by HB9. Islet-1 transcription factor (Isl1) is expressed early in pancreatic development and appears to have two functions: development of the dorsal mesenchyme (which is needed for the development of the exocrine pancreas) and differentiation of islet cells (Ahlgren, Pfaff, et al. 1997).

The second phase of TF expression commits endoderm derived cells towards an endocrine or exocrine lineage. Neurogenin 3 (ngn3) is believed to define an islet progenitor cell and act as a determination factor for an endocrine cell lineage and is largely controlled by Delta/Notch signalling on adjacent epithelial cells (Apelqvist, Li, et al. 1999). Notch is a transmembrane receptor and delta a transmembrane ligand, the expression of which is controlled by ngn3. Notch is activated by delta (expressed by a neighbouring cell) and in turn activates Hes-1 (Hairy and enhancer of split 1) (Beatus, Lundkvist, et al. 1999). Hes-1 inhibits expression of ngn3 and hence NeuroD1 (Neurogenic differentiation factor 1) another endocrine cell differentiation factor which inhibits delta (see fig. 2,10). In this manner cells differentiating into endocrine cells inhibit neighbouring cells following the same differentiation pathway forcing them to pursue a non-endocrine fate.
Fig.2.10 Role of Delta/Notch signalling in controlling endocrine versus exocrine cell differentiation. (a) Expression of ngn3 stimulates NeuroD1 and drives the cell towards an endocrine fate. (b) Ngn3 also activates Delta, which in turn activates Notch within an adjacent epithelial cell. (c) Notch activates Hes-1, which acts as a repressor of ngn3 and NeuroD1. This cell is then driven towards an exocrine fate.

The next phase in pancreatic development is differentiation into islet cell subtypes (see fig. 2,11). TF's implicated in this process include Nkx2.2 (Na(+)/K(+)/Ca(2+)-exchange protein 2.2) and Nkx6.1 (Na(+)/K(+)/Ca(2+)-exchange protein 6.1) which are β-differentiation factors (Jensen, Serup, et al. 1996). Pax1 acts as a β-cell determination factor whereas Pax6 acts as an α-cell determination factor (Docherty 2001). The final stages of islet formation occur within the mesenchyme where the endocrine precursor cells migrate from the ductal epithelium and into the adjacent extracellular matrix to form islets. This event is controlled by the integrin family of cell adhesion molecules.
2.5.3 Islet Cell Growth During Fetal And Neonatal Development In The Rat

Kaung (1994) studied the growth dynamics of pancreatic islet cell populations during fetal and neonatal development in rats between 18 days gestation and 28 days after birth.

As in all mammals the pancreas forms from the gut endoderm and endocrine cells first appear as single or small clusters of cells within the epithelium. As
their numbers increase they separate from the exocrine tissue migrating into the mesenchyme to form islets (Pictet et al., 1976).

In the mouse at 18 days gestation islet cells are seen scattered as either single cells or irregularly arranged clusters of cells with $\alpha$ (glucagon producing) & $\beta$ (insulin producing)-cells being the most prominent. During the last four days of gestation there is a phase of rapid cell proliferation. Acinar tissue differentiates and by 22 days gestation islet cell arrangements are seen as in adults. Following birth there is a shrinkage of the exocrine cell area until day 4. Thereafter a slow gradual rise in all pancreas cell populations is observed up to day 28 after birth.

Fig. 2.12 [Kaung (1994)] illustrates the islet growth pattern. This study shows that islet development continues after birth therefore nutritional status concerning islet development is important pre and perinatally.

**Fig 2.12** Growth patterns of insulin, glucagon, pancreatic polypeptide & somatostatin cell populations. Total quantity or volume (mm scan) of each islet cell population from 18 day fetal to 28 day neonatal ages. Taken from Kaung, 1994.
2.5.4 Islet Growth and Development in the Adult

There is a low replication rate of β-cells after birth. β-Cell mass is dynamic and is regulated to maintain euglycemia and at any one time the number of β-cells is determined by the balance of cell renewal and cell loss (Finegood, Scaglia, et al. 1995).

A morphometric study of autopsied human pancreas from NIDDM and non-diabetic patients showed that in both groups the β-cell mass was increased by 40% in obese subjects compared with lean subjects, suggesting a compensatory growth of β-cell mass with the increasing insulin resistance seen with obesity (Kloppel, Lohr, et al. 1985). The β-cell has a finite life span and finally undergoes apoptosis (Scaglia, Cahill, et al. 1997). If β-cell replication equals that of β-cell apoptosis then the pancreas could be considered as a slowly renewed tissue. The alterations involved for compensatory mechanisms are a change in the replication rate, cell death rate and change in the rates of differentiation from precursor cells remaining in the adult pancreatic ducts (neogenesis) and also regulation of cell size/volume ‘hypertrophy’. Hypertrophy of β-cells would allow for increased gene expression without cell division. One example of the varied mechanisms of compensation and expansion of the β-cell mass in the adult rodent is the Zucker fatty (fa/fa) rat. One colony of Zucker (fa/fa) rats exist where the male rats develop diabetes at 9 months of age but the females do not become diabetic at all. There is an intrinsic defect in the β-cells of this colony compared with lean littermates of the other non-diabetic Zucker fatty rats. Female (fa+/fa-) Zucker fatty rats compared with their lean littermates (fa+/fa+) at 12 weeks old have increased body weight and increased β-cell mass. However females were compensated and had only slightly impaired glucose tolerance unlike the male counterparts. In addition both β-cell size and replication were increased compared with lean animals. Therefore hypertrophy played an adaptive role in the Zucker rat. Additionally rats were treated with dexamethasone, which has direct as well as peripheral effects on the β-cell. From this treatment the lean Zucker females compensated with increased β-cell mass with increased β-cell hypertrophy and increased replication. But the female Zucker diabetic fatty rats that were adequately compensated before treatment became diabetic as the already hypertrophied β-
cells did not increase further in size, their replication was enhanced but due to increased apoptosis the β-cell mass did not increase (Pick, Clark, et al. 1998). Hypertrophy is a rapid response to an increased demand with an arrest in the G1 phase of the cell cycle, which allows a sustained amplification of gene expression without completing the cell cycle. However the drawback to hypertrophy is that the hypertrophied cell is more vulnerable to apoptosis explaining the increase of apoptosis in the Zucker diabetic fatty females. One other mechanism for increasing β-cell number/mass is neogenesis, evidence for this has been found in obese humans and animal models. It is thought that stem cells within the pancreatic ducts have the ability to adopt any pancreatic cell phenotype (Bonner-Weir 2000).

2.6 Developmental Aspects of Metabolism
Pancreatic endocrine cells also follow a well defined ontogeny of functional development which in part reflects the changing metabolic demands during development. The diet of fetal and newborn rats encompasses three stages. Fetal stage where the main energy source is glucose from the mother. After birth nutrition comes from the mother's milk and finally at weaning energy comes from solid carbohydrate food. With differing forms of nutrition a varied metabolic response is required.

During gestation the fetus receives a constant supply of nutrients via the placenta for maintenance of basal metabolism, substrates, energy for growth and fuel stores. After birth the constant supply of nutrients stops and the neonate must adapt to the change in nutrient availability by metabolic adaptation. The neonate has to adapt to this cessation of constant nutrition as well as to a change from intravenous to enteral nutrition and also from using glucose as its main energy substrate to fat from milk. During gestation relatively constant based secretion of insulin promotes growth as well as laying down fuel stores such as glycogen in the liver and fat in adipose tissue. After birth secretion of insulin is intermittent and counter regulatory hormones embark on inducing enzymes that can change the neonates metabolism by releasing the previously built up fuel stores (Hawdon 1998).
2.6.1 Intra-uterine Growth Retarded (IUGR) Infants.
IUGR infants are at particular risk from hypoglycaemia as placental insufficiency of nutrient transfer affects the laying down of glycogen and fat stores and IUGR fetuses even have to mobilise substrates from structural tissues such as muscle. They also have an impaired hormonal and metabolic response, which is related to the degree of growth retardation (Hawdon & Ward Platt 1993). Pre-term growth retarded (GR) infants also appear to suffer from antenatal/perinatal hypoglycaemia and stress. The degree of growth retardation is also correlated with high blood lactate levels in full term infants at birth and in the immediate post-natal periods. IUGR infants are unable to mount a sufficient endocrine and ketogenic response to low blood glucose (Hawdon, Weddell, et al. 1993). This may be related in some part to the patterns of insulin secretion in neonates. Blood glucose induced insulin secretion is not as tightly regulated in neonates as it is with older children and adults. Preterm and IUGR infants are unable to mount a sufficient counter-regulatory ketogenic response to low blood glucose which is due to a failure to switch off insulin secretion which persists for up to several weeks after birth (Aynsley-Green, Hawdon, et al. 1997).

2.6.2 Development of Glucose Responsiveness in Rats
Fetal rat pancreases at 19.5 day’s gestation are responsive to glucose but show a different pattern of insulin secretory response to 3-day-old rats. Fetal islets show a monophasic pattern of insulin release with a lower overall release in comparison with the biphasic pattern of release observed in 3-day-old rat pups (fig 2,13).
During a period of six days (19.5-day-old fetus to the 3-day-old pup) the secretory response to glucose develops from monophasic to biphasic or ‘adult’ like. The amplitude of insulin secretion also increases with age in suckling rat pups with a large increase between 14 and 21 days when the rats are beginning to wean and eat solid food (Hole, Pian-Smith, et al. 1988). Bliss et al observed that glucose induced insulin secretion more than doubled in 20.5 day old fetal islets when compared with 19.5 day old islets. When fatty acid oxidation was inhibited with 2-bromostearate in the 19.5-day fetal islet the glucose stimulated insulin secretory response was more than tripled. Whilst 20.5-day fetal islets were unresponsive to fatty acid oxidation inhibition as the insulin secretion pattern in 20.5-day-old islets was still monophasic. This implies that fatty acid oxidation is a major metabolic pathway in the 19.5 day fetal islet and inhibiting this pathway allows increased glucose metabolism and increased glucose signalling to the $K_{ATP}$ channel (as the changes could be blocked by $Ca^{2+}$ antagonists) (Bliss & Sharp 1994).

### 2.6.2.1 Observations in Fetal Glucokinase

Hepatic glucokinase (GK) does not appear in rat liver until the weaning stage at two weeks after birth (Walker & Holland 1965). At this time the change from maternal milk (high fat and low carbohydrate) to laboratory chow (high
carbohydrate and low fat) is accompanied by increases in plasma insulin and glucagon, which control gene expression of GK as the administration of glucose to 1 or 10 day old suckling pups induces hepatic GK gene expression (Bossard, Parsa, et al. 1993).

Pancreatic GK, unlike hepatic GK, is detected in in fetal rat β-cells and α-cells at the earliest time the islet develops which is day 16 of gestation (Pictet, Clark, et al. 1972) and protein levels of GK are still regulated by glucose (Tu 1999). Underdeveloped fetal islets or islet like cell clusters secrete insulin poorly in response to glucose (Dudek, Freinkel, et al. 1980) (Freinkel, Lewis, et al. 1984) but synthesize, store and secrete insulin in response to other insulin secretagogues that increase cyclic cAMP levels (Mourmeaux, Remacle, et al. 1989). These differences can be explained by insufficient glucose metabolism in the fetal islet. GK activity was investigated in fetal islets by Tu & Tuch (1996), who found that fetal GK still acts as the glucose sensor and the inability of fetal islets to properly secrete insulin was not due to deficient GK activity or glycolysis. They found that human fetal pancreatic islets from 17-19 weeks gestation showed no difference in GK protein content and $K_m$ value. $V_{max}$ was reduced but this could be due to a reduction of β-cell numbers in fetal islets in comparison with adult islets. When cultured in medium containing high glucose levels GK activity increased 3.7 fold as well as a 1.8 fold increase in glucose utilisation. However the enhanced activity did not lead to an improved ability of the β-cell to secrete insulin when exposed to glucose (Tu & Tuch 1997).

These observations on fetal GK activity are however disputed by other reports suggesting that GK activity is lowered in fetal islets and is responsible for poor fetal insulin secretion (Hughes 1994) (Tiedge & Lenzen 1993) (Taniguchi S 2000). Therefore the role of fetal GK in deficient glucose stimulated insulin secretion in fetal islets is not fully understood.
2.7 Summary
The morphological and functional development of the endocrine pancreas is complex and incompletely understood. Human studies suggest that insulin plays a vital role in the development of the newborn's metabolic machinery. Studies in the rat have highlighted the importance of fatty acid oxidation in β-cell metabolic control in utero. But the importance of GK activity in fetal insulin secretion is disputed.
Study Hypothesis

It has been postulated that glucose intolerance in later life may be as a consequence of programming or entrainment of metabolism in early life by nutrition. If so this may result from either a defect in the secretion of insulin by the pancreatic β-cells or the development of peripheral insulin resistance. It would therefore be of interest to investigate the development of the mechanism that controls insulin secretion and whether there is evidence of programming or entrainment of this mechanism by manipulating nutrition in fetal life. The first step of glycolysis (the conversion of glucose to glucose-6-phosphate) is believed to be the glucose sensing mechanism that controls insulin secretion and this is controlled by the activity of pancreatic glucokinase (GK). Therefore if insulin secretion is programmed it is likely to involve the pancreatic GK protein. Glucokinase has been shown in a previous study to be programmed in liver metabolism (Desai et al., 1997) by maternal protein malnutrition. Therefore it was hypothesised that the activity of pancreatic glucokinase may also be programmed during early life, in such a way that activity is permanently reduced or its function is impaired. Thus in later life when nutrition is abundant the permanently reduced activity or function of glucokinase would lead to the inability of the pancreatic β-cell to appropriately respond to a glucose stimulus thus resulting in an inappropriate insulin secretion response.

Aim of the study

The aim of this study is to determine whether programming of glucokinase in the rat pancreatic β-cell occurs as a consequence of nutritional manipulation in fetal life. Thie study was carried out by determining the amount and activity levels of glucokinase in relation to insulin secretion capacity. The study was undertaken on newborn, weaned pups and adult rats born from rat dams fed either protein sufficient (control) or a protein restricted diet.
Chapter 3

Rat Pancreatic Islet Isolation

3.1 Introduction

To carry out this study an effective method of isolating the pancreatic islets from rats was required. This method was developed from the techniques described by Lacey & Kostianovsky (1967) and Gotoh et al., (1987). It is based on the principle of digestion of collagenous fibrous material and separation of the pancreas.

3.2 Method

3.2.1 Dissection

The rat is killed using a schedule one method and placed on its back, the outer layer of skin is removed around the stomach. Crosscuts are made to expose the abdominal cavity. The tip of the sternum is removed and the chest is manipulated to release the liver to allow it to be flipped back on itself and held out of the way using a towel/cloth. The pancreatic-biliary duct can then be clamped at the duodenal end and the common bile duct clamped at the liver end. The duct is cannulated between clamps toward the liver end using a needle and cannula. 5mls of 1mg per ml collagenase type XI (Sigma Chemicals. Co) solution in Hanks balanced salt solution (HBSS) with 6ul Deoxyribonuclease I (DNAse) (Amersham Pharmacia Biotech.) is infused into the pancreatic duct. Once the pancreas is inflated the cannula and clamps are removed and the inflated pancreas is carefully dissected out. To dissect out the pancreas, a cut is made starting at the stomach then around part of the duodenum until it meets the mesentery, at the small intestine. It can be suspended by the spleen, which is then cut off as shown in Fig 3,1. The pancreas is placed into 10mls of cold HBSS and kept on ice until digestion.
3.2.2 Digestion
As much HBSS as possible is decanted from the pancreas suspension and topped with warm (37°C) HBSS. The suspension is incubated at 37°C for approximately 20mins depending on the collagenase. To stop digestion, warm HBSS is decanted off and topped up with cold HBSS and the suspension is kept on ice. The pancreas is then shaken vigorously for approximately 1min. and the digest is filtered through a mesh to remove undigested material. The digest is examined using a dissecting microscope for islets.
For subsequent homogenisation, sonication and tissue culture, islets are picked out of the digest and transferred to fresh HBSS and re-picked into the relevant buffer solutions.

3.3 Factors Important for Digestion
Appearance and abundance of islets is generally used to judge the effectiveness of a digestion. In contrast to exocrine tissue, islets appear as bright spherical orbs differing in size.
A good digestion can release up to a thousand islets however a proportion of islets are too small. The islets that were more than 100\(\mu\)m in size were used as they were easier to see and pick out. 300 or more islets can be obtained from one adult rat.

The choice of collagenase is important as the activity of the collagenase differs between different types and batches. Two different collagenases Type XI from ‘Sigma Chemicals. Co’ and collagenase from ‘Serva Electrophoresis’ were initially used but the Sigma collagenase was found to be less damaging to the islets.

There are a number of factors, which were important in ensuring a good yield of adequate islets. These are as follows....

- Control of the excessive release of DNA. The digest was often found to be clumped and sticky making it very difficult to collect islets. This was due to the over digestion of the exocrine tissue by the collagenase which released DNA from the tissue therefore DNAse was introduced into the collagenase preparation.
- The appropriate size of the container for incubation of the pancreas the volume of HBSS (usually 10ml), and careful dissection of the pancreas are also important.
- To avoid hypoxia, the HBSS buffer is gassed with 95% O\(_2\), 5% CO\(_2\) prior to the dissection procedure.
- An energy substrate for the viability of islets therefore a low concentration of glucose in the buffer is required (2.5mM).
- The digest can be centrifuged and washed if required however this introduces the risk of mechanical damage to islets. Other methods for isolating islets from a digest use a ficoll gradient but this technique was not used at it was found easier to distinguish islets when in the digest.

### 3.4 Isolation of Islets From Neonate Rat Pups

New-born pups are too small to perform the dissection procedure usually required for islet isolation. Therefore the pancreas is carefully dissected out,
and placed into a solution of HBSS with 0.8/mg collagenase type V (Sigma Chem. Co.). This is a weaker form of collagenase, which has a less aggressive action on the smaller tissue. The pancreases are pooled from a litter, minced using dissecting scissors and incubated at 37°C for 8min whilst being shaken every 2-3 min. To stop digestion the preparation is placed on ice. The preparation can be examined for islets straight away and the preparation does not require filtration because of small amount of starting material. This protocol is similar to that used by Tu & Tuch, (1996).

3.5 Islet Viability
To ensure that the islets have survived the isolation process they can be tested for viability by using a Trypan blue exclusion stain test. The principle of this test is that dead cell membranes will be more permeable to the trypan blue than live cells (viable cells). Thus live cells exclude Trypan blue and dead cells will take up trypan blue and display a blue colour.

3.5.1 Trypan Blue Staining of Pancreatic Islets
A random sample of islets in HBSS is picked to test. The islet preparation is mixed with an equal volume of 0.4% Trypan blue solution (Sigma Chem. Co). Islets are picked out onto a slide and viewed using a light microscope at x10 magnification. As the islets consist of clusters of large numbers of cells it is best to estimate a percentage of dead cells per islet by the appearance. If more than 10-15% of the islet is stained it was classed a non-viable islet. Due to time taken to photograph islets it was not possible to show a photo of a viable islet but islets at two varied degrees of staining are shown (figs 3.2 and 3.3).
Fig. 3.2 Photo (x10) of adult rat isolated pancreatic islet with moderate trypan blue staining app. 20%.

Fig. 3.3 Photo (x10) of adult rat isolated pancreatic islet with considerable trypan blue staining app. 70%.
Viability tests were performed for each digestion to assess the state of the preparation and whether the islets were useable. If more than 10% of the preparation had non-viable islets, then the digestion was considered unsuccessful.
Chapter 4
Measurement of Insulin Secretion

To perform a comparative study of insulin secretory patterns on isolated pancreatic islets a perifusion system devised by Warnock, Ellis, et al. (1998) was used.

4.2 Principle of the Perifusion System
The perifusion method used here is an adaptation from Holmes et al., (1995) and Clayton et al., (1990) and is designed to study the insulin secretory response of isolated pancreatic islets. Islets are placed in a chamber that is connected to a continuous flow of incubation media. The media can be changed by a switch mechanism so that the islets can be exposed to a different stimulus or exposed to a different factor such as an inhibitor, or to alternating glucose concentrations. Aliquots are taken of the media at various time intervals and assayed for insulin content. The insulin response is then plotted and analysed. The perifusion system has proved a useful tool to researchers investigating the function and viability of islets particularly in the field of islet transplantation where islets are subjected to different treatments (Holmes, Clayton, et al. 1995) or microencapsulation of islets (Clayton, London, et al. 1990) to improve and safeguard their function in transplantation.

4.3 Method

4.3.1 Perifusion
One litre of Krebs Ringer Bicarbonate (KRB) solution (135mM NaCl, 3.6mM KCl, 10mM HEPES pH7.4, 2mM NaHCO3, 0.5mM MgCl2, 1.5mM CaCl2 and 0.1% BSA) is made. 350mls of the KRB solution is used to make up 16mM glucose. The 16mM glucose KRB solution is used to make 500mls of 1.5mM glucose. During the perifusion procedure these solutions are gassed (95% O2: 5%CO2) at 37°C in a water bath. The principle of the apparatus is shown in the diagram in Fig.4.1. The tubing and chambers are primed with 1.5mM glucose. Glass wool is packed into each chamber but not packed too tightly so to prevent an airlock. Once everything is set up 50 adult islets (and 70 neonate & 3-week
old) are placed into the chamber, these islets need to be dispersed throughout the chamber. 1.5mM glucose solution is pumped at a flow rate of 1ml/min through the system and left for 1 hour to allow the islets to settle and achieve a basal level of insulin release. 1ml aliquots are taken at timed intervals (0, 10, 19, 26, 28, 30, 32, 34, 44, 54, 64, 74, 79, 85, 90, 100, 110, 120, 130 and 140 minutes). The media is switched to high glucose at the end of the 19th min and is switched back to low glucose at the end of the 79th minute. After the experiment the chambers are checked by examining the chamber contents under the stereomicroscope for the presence of islets to ensure that not too many have been lost through the system.
**Methods Chapter 4**

**Measurement of Insulin Secretion**

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**Fig. 4.1** Diagram of Islet perifusion technique set up.

**Fig. 4.2** Photograph of basic islet perifusion set-up. Note: Water bath not present.
4.3.2 Insulin Measurement

The 1ml aliquots of the collected incubation media are assayed for insulin concentration using an Enzyme-Linked Immunosorbent Assay (ELISA). This is available as a prepared kit from Crystal Chem Co. USA. The protocol is briefly described below and depicted in fig. 4.3.

1. The insulin in the sample is simultaneously bound to mouse anti-insulin monoclonal antibody coated on a 96 microwell plate.
2. Anti-insulin antibody of guinea pig serum is added to the well. This complex is immobilised on the microplate well.
3. A horseradish peroxidase (POD)-conjugated anti-guinea pig antibody is added that binds to the guinea pig anti-insulin antibody part of the complex.
4. The complex is then detected by a colorimetric reaction from the HRP conjugate and the absorbance read at 492nm.

Two species specific anti-insulin antibodies are used to detect the insulin so as to prevent the binding of POD-conjugate to unbound mouse anti-insulin antibody on the microwell wall.

1. Microplate well coated with Mouse Anti-insulin monoclonal antibody.
2. Bound mouse antibody anti-insulin antibody/insulin complex.

Continued on the following page
Methods Chapter 4  Measurement of Insulin Secretion


Horse radish peroxidase (POD)-conjugated anti-guinea pig antibody

4. Bound POD-conjugate complex

O-Phenylenediamine Substrate Solution.

5. Bound POD-conjugate antibody complex detected and measured at 492nm.

Fig. 4,3 Diagram illustrating the principle of the insulin ELISA used for Islet insulin secretion studies.
A rat insulin standard is provided with the ELISA kit. Dilutions are made and a standard curve is performed on all ELISA plates.

![Graph showing insulin standard Log curve for insulin ELISA kit.](image)

**Fig. 4.4** Insulin standard Log curve for insulin ELISA kit. Data points represent the mean from duplicate assays.

Insulin concentration is estimated from the standard curve (fig.4.4) and plotted against time. The area under the curve is calculated for each glucose concentration.

### 4.4 Method Validation

To evaluate the efficiency of the method a number of experiments were performed to investigate the time lag effect of glucose concentration on insulin secretion by the islets and to check that insulin was not sticking to the plastic tubing.
4.4.1 Glucose Concentration Gradient

The design of the apparatus causes a time lag of app. 2 mins before an altered glucose concentration solution reaches the islets and 4 mins for the media to travel to the collection point. This needs to be taken into account when analysing the results.

To investigate the time it took for the glucose concentrations to completely change, an experiment to monitor the glucose concentration in the chambers was performed where glucose concentrations were switched from 1.5mM to 16mM and 16mM to 1.5mM.

4.4.1.1 Procedure

Aliquots of 0.25-0.5ml were taken from inside the chamber every two minutes. These aliquots were assayed by a hexokinase glucose 6-phosphate dehydrogenase method which is available as a manual glucose testing kit (Randox Laboratories Ltd) and the results were plotted against time.

The kit utilises a spectrophotometric assay reaction. The assay involves the phosphorylation of glucose by hexokinase and the further oxidation of the product by glucose-6-phosphate dehydrogenase which is coupled with the reduction of Nicotinamide adenine dinucleotide (NAD) to Nicotinamide adenine dinucleotide-H (NADH). The absorbance of NADH is measured by a spectrophotometer and reflects the concentration of glucose from the sample that was converted in the reaction as the stoichiometry of the reaction is 1 mole for 1 mole both for glucose to 6-phosphogluconate an for NAD to NADH, as shown in the following reaction.

\[
\text{Hexokinase} \\
\text{Glucose + ATP} \rightarrow \text{Glucose-6-Phosphate}
\]

\[
\text{Glucose-6-Phosphate Dehydrogenase} \\
\text{Glucose-6-Phosphate} \rightarrow 6\text{-Phosphogluconate} + \text{NAD} + \text{NADH}
\]
A standard curve for glucose concentration estimation is shown in fig. 4,5.

**Fig. 4.5** Glucose standard curve as estimated by hexokinase-glucose-6-phosphate dehydrogenase method. Data represent the mean of triplicate samples ± SEM.
4.4.1.2 Result

As seen from fig 4.6 it takes 8 minutes for a complete glucose concentration switch. However the islets start responding after a certain threshold is reached which is about >7mM glucose (Flatt & Lenzen 1994) and this is reached after 110 seconds. A consistently observed feature of islets is a delay of 1-2 mins from the addition of glucose to the start of β-cell electrical activity and insulin secretion (Bertram & Pernarowski 1998). Islets should start responding after about 4 minutes with an additional lag of 4 minutes before aliquots can be collected. Readings for high glucose should be taken after 8 min. The switch from 16mM to 1.5mM glucose takes 10 mins to complete the switch but drops below 7mM after 2 mins. Therefore islets will not start to respond until after this time period with the additional time lag of 4 min, for low glucose after a 16mM to 1.5mM switch samples should therefore be collected 10-12 min after the switch.
4.4.2 Insulin Concentration Gradient

To further validate this technique similar experiments to the glucose concentration change experiments were performed using insulin. This experiment was designed to find out how freely insulin moves through the system and also to check whether insulin was sticking to the inside of the plastic tubing.

4.4.2.1 Procedure

A solution of 5ng/ml of porcine insulin (CP Pharmaceuticals Ltd) was made in KRB solution. The perifusion apparatus was set up as described previously (fig. 4,1) and primed with insulin free KRB solution. At time 0 the chamber was switched to the 5ng/ml insulin solution and allowed to run for 15mins. 1ml aliquots were collected at the end of the system at 0,5,7,9,11,13,15 minutes. At the 15th minute the chamber was switched back to the insulin free KRB solution and run for a further 15mins with aliquots taken at 20,22,24,26,28,30min. The aliquots were assayed by the same kit used to assay the perifusion samples and insulin concentration was estimated using a porcine insulin standard curve and plotted against time.
**4.4.2.2 Result**

The pattern of insulin concentration profile as shown in fig.4,7 corresponded to the time course of glucose infusion as shown in fig.4,6 in section 4.1.2. From the time of the switch the 5ng/ml insulin takes 12 min before a change is observed at the collection point, then it takes a further 8mins for a full concentration change (time to peak) to be observed. It takes 5-7mins before a decline in insulin concentration is registered and 15mins for a complete change to be observed after a switch to the KRB solution. It appears that the full insulin concentration does flow through the system without a significant amount being lost due to sticking to the plastic tubing. During the islet perifusion experiments, insulin originates from within the chamber containing the islets and not from the switch therefore it takes less time to reach collecting point than indicated by this experiment. It takes more than 15mins before insulin will return to baseline from 5ng/ml but this time is variable in the perifusion system as the peak insulin
secretion rates differ but never producing more than 4.5ng/ml (total for islets in system). This time would be increased to by the time it takes for a relevant change of glucose in the chamber (approx. 2mins according to the glucose change experiment) and the time for the islets to stop secreting insulin. Therefore it should take no more than 20mins for a change in insulin levels in the perifusion system to be detected.
Chapter 5
Measuring Pancreatic Glucokinase (GK) Activity

5.1 Aims
The GK assay was set up to measure the pancreatic GK activity from the islets pooled from litters from all groups of rats. This assay measures the activities of both GK and HK. Data obtained was analysed to determine the Michaelis Menten kinetics of the reaction and to obtain the parameters $V_{\text{max}}$ which is the maximal velocity of the enzyme reaction and the $K_m$ value which is substrate required for half maximal velocity and is an indicator of the affinity of the enzyme for its substrate. By measuring GK activity it will determine if there is a decrease in activity and/or affinity of GK in islets from offspring exposed to nutritional deficiency.

5.2 Principle of the Assay
The method for this assay was originally developed by Joshi & Jagannathan, 1968 for measuring hexokinase and was further modified by Trus et al., 1981 for the measurement of GK in pancreatic islets. Glucokinase activity can be measured by the fluorometric measurement of NADH. The assay involves a two step reaction. Step one is the conversion by glucokinase of glucose to glucose-6-phosphate. Glucose-6-phosphate is then converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PD). This reaction requires NAD as a cofactor and thus the second step is coupled to the reduction of NAD to NADH. As the stoichiometry of these reactions are 1:1 moles of the reactants and the second reaction is coupled to NAD to NADH mole for mole; one mole of glucose will yield 1 mole of 6-phosphogluconate and 1 mole of NADH as shown in fig. 6.1. Thus the activity of GK can then be measured by quantitating the production of NADH, which reflects the quantity of glucose converted to glucose-6-phosphate. To determine the GK activity it is also necessary to determine HK activity. HK has a higher affinity for glucose and is best measured at low glucose concentrations (0.03-0.5mM), GK however has a lower affinity for glucose and cannot be detected at low concentrations therefore GK is measured at higher glucose concentrations (5-100mM). The activity of HK is determined and...
Methods Chapter 5  

Measuring Pancreatic Glucokinase Activity

subtracted from the activity measured between 5-100mM to obtain GK activity.

\[
\text{GK} \quad \text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}
\]

\[
\text{NAD}^+ \quad \text{dehydrogenase}
\]

\[
\text{NADH} \quad \text{6-phosphogluconate}
\]

Fig. 5.1 Diagram illustrating GK assay enzyme reaction.

5.3 Method

5.3.1 Sample Preparation

Rat pancreatic islets are collected using a dissecting microscope. Approximately 200-300 islets are placed in 160\(\mu\)l of homogenising buffer (20mM \(K_2\)HPO\(_4\), 1mM EDTA, 110mM KCl, 5mM DTT, pH 7.7) and homogenised on ice by 100 manual strokes in a glass homogeniser. The homogenate is then centrifuged at 12,000 x g for 10min. The supernatant is separated, used for the assay and later assayed for protein content using the BCA protein determination kit.

5.3.2 Assay procedure

Ten different concentrations of glucose are assayed. Five in the range 0.03-0.5mM and five in the range 5-60mM. The assay is performed by the addition of 4\(\mu\)l of supernatant to 100\(\mu\)l of assay reagents (50mM Hepes-HCL, 100mM KCl, 7mM MgCl\(_2\), 10mM DTT, 0.5mM NAD\(^+\), 5mM ATP, 0.05% BSA & 0.7U/ml G6PD (Sigma Chemicals. Co) pH 7.7). Two blanks are used per assay a substrate blank (without substrate) and a reagent blank (without ATP). An NADH standard curve is run in parallel at G6P concentrations 0.5-8mM. The reaction mixture is incubated for 90mins at 30\(^\circ\)C and the reaction
stopped by the addition of 900μl of 500mM Na(CO₃)₂ pH 9.4. Fluorescence of NADH is measured at 340nm emission and 460nm excitation.

5.3.3 Determination of Activity
The values of the blanks are subtracted to give a final fluorescence result. This can be converted to specific activity (velocity) units of nmols/mg-protein/hr using an NADH standard curve that uses G6P as a substrate and protein assay result.

![Fluorescence units x10 vs Glucose-6-Phosphate mM](image)

**Fig. 5.2** NADH standard curve showing point to saturation. Data points are taken as the average from triplicate assays.

The conversion of glucose to glucose-6-phosphate is equi-molar where one mole of glucose is converted to one mole of G6P. Therefore a standard curve of the first reaction can be used to quantify the amount of glucose catabolised. A standard curve was performed to find the saturation point for G6PD (see fig.
5.2) which appears to be around 1500 fluorometric units. For the assays a standard curve between 0.5-8mM glucose-6-phosphate was used. The assay procedure for the standard curve is similar to the routine enzyme assay except that no tissue is used and G6P is used instead of glucose.

5.3.4 Protein Concentration Determination

In order to quantitate the specific velocity of the GK enzyme reaction the sample protein concentration needs to be determined. This is obtained by a spectrophotometrical reaction using a bicinchoninic acid (BCA) method. The BCA kit from 'Sigma Chemical company' was used. The BCA kit is based on the principle that Cu(II) is converted to Cu(I) by protein in a concentration dependant manner. The BCA is a highly specific chromogenic reagent for Cu(I) forming a purple complex with an absorbance at 562nm. When estimating a samples protein content a standard curve of 5-50ug BSA (Bovine serum albumin) is performed in parallel and the unknown sample concentration can be read off from this. The assay was linear over this range.

![Absorbance @562nm vs Protein ug](image)

**Fig. 5.3** Protein Assay standard curve. Data represent means of duplicate assays.
Methods Chapter 5  Measuring Pancreatic Glucokinase Activity

In the case of assaying islet homogenates a standard curve of 1.5-30ug was used. As the protein content is low, tissue homogenate was assayed directly and not diluted.

5.4 Enzyme Kinetics

It is important to study the reaction kinetics of enzymes since this analysis provides valuable information on the mechanism of action of an enzyme. It gives an insight into the role of an enzyme under the conditions that exist in the cell and the response of an enzyme to changes in the concentrations of metabolites under physiological conditions.

Michaelis-Menten kinetics is used to study enzymes that can be saturated and can form a 'steady state'. It assumes that a short-lived enzyme-substrate complex (ES) which soon yields the enzyme and the product (E + P) is in a steady state where the rate of its formation equals the rate of its breakdown. The maximal velocity and hence activity of an enzyme reaction is calculated as the $V_{\text{max}}$ and the substrate concentration to give half maximal velocity is termed as the $K_m$ and is a measure of an enzyme's affinity for its substrate. It is very difficult to determine the limiting value of $v$ (i.e. $V_{\text{max}}$) directly from a plot of velocity ($v$) against substrate concentration ([S]) and therefore $K_m$ cannot readily be determined in this way either. To overcome these difficulties the Michaelis-Menten parameters $V_{\text{max}}$ and $K_m$ are best deduced by a number of transformations of the Michaelis-Menten equation (given below) which result in convenient graphical representations (fig.5,4).

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]
Fig. 5.4 Examples of (a) dependence of velocity (v) on substrate ([S]) (b) Lineweaver-Burke equation and plot (c) Eadie-Hofstee equation and plot and (d) Hanes-Woolf equation and plot for pancreatic GK. Adapted from Price & Stevens (1989).

Three of the most recognised plots are the Lineweaver-Burke plot, which is also known as the double reciprocal plot where 1/v is plotted against 1/[S]. This is commonly used but the regression line can be easily influenced by...
measurements at low substrate concentrations, which depending on the reaction can be the most sensitive to error. The Eadie-Hofstee plot is a plot of $v$ vs. $v/[S]$ and the Hanes-Woolf plot is a plot of $[S]$ vs. $[S]/v$. The Hanes-Woolf plot is often preferred over the Eadie-Hofstee as it does not involve the use of the error containing variable ($v$) in both axes and is approved in a paper published by Matschinsky et al., 1998 in the determination of kinetic parameters for glucokinase in tissue preparations.

As islet GK works at higher glucose concentrations compared with HK (see fig. 2, 6) GK activity is measured at high glucose concentrations 5-60mM. To determine GK activity the HK activity, which will also be present at higher concentrations, needs to be measured at low concentrations 0.03-0.5mM. Once $V_{\text{max}}$ has been estimated for HK this can be subtracted from the specific activity determined at the high glucose concentrations to obtain the specific activity for GK.

Islet GK $K_m$ has been previously measured within a range of 6-12mM glucose (Trus, Zawalich, et al. 1981) (lynedjian-PB 1993) (Matschinsky, Glaser, et al. 1998) whilst hexokinase $K_m$ value has previously been measured at 0.028 ± 0.016mM glucose (Trus, Zawalich, et al. 1981). GK $K_m$ can be determined by the intercept at the x-axis and $V_{\text{max}}/1/$slope on the Hanes-Woolf plot. GK activity is variable depending on the method used for determination and is best analysed with a control when being compared.
Chapter 6
Detection of Glucokinase Protein From Pancreatic Islets

6.1 Aim
In order to investigate if GK activity is affected by a decrease in GK enzyme protein as a result from the low-protein diet, a method to detect and quantify GK protein was set up. Western blotting was used to quantitate GK protein for comparison between the control and experimental groups. A liver sample was used as a control on all blots as liver is easy to obtain and all samples were quantitated as a percentage of the liver control by measuring the optical density. The final results of the experimental groups are expressed as a percentage of the control group optical density.

6.2. Principle of Western Blotting
Western blotting is a method used to detect the presence of a specific protein. The principle of western blotting involves Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of a protein sample. Protein/s are then blotted onto a membrane, which is probed with an antibody specific to the protein of interest. The bound antibody can then be detected using a secondary conjugated antibody which itself can be detected using chemiluminescence.

The method used here has been adapted from the procedures described by Gremlich et al., (1997) & Chen et al., (1994).
Fig. 6.1 Diagram illustrating the principle of Western Blotting.

### 6.2.1 Electrophoresis

Electrophoresis is the process of moving charged molecules in solution by applying an electric field across the mixture. Molecules in an electric field separate with a speed dependant on their charge, shape and size. They are separated within a matrix and in the case of protein separation acrylamide is used. As molecules are forced through the gel by applied voltage, larger molecules are retarded by the gel more than smaller molecules. Generally most SDS PAGE require a stacking gel which is a lower acrylamide concentration gel that is prepared on top of a resolving gel and allows the proteins to concentrate in a thin zone or a "stack" of which when they reach the resolving gel they will "unstack" and separate (Amersham Pharmacia Biotech 1999).
6.2.2 Blotting

For analysis based on antibody reactivity, the separated molecules need to be free of the polyacrylamide gel matrix. This can be done by blotting, where molecules in the gel are eluted onto a membrane filter that binds the molecules as they emerge and the proteins remain on the surface of the membrane where they are accessible to immuno-detection. The transfer is accomplished by using electrophoresis between the sandwiched gel and membrane.
6.3 Method

6.3.1 Sample Preparation

300-500 islets were picked into 100ul of sonicating buffer (5% SDS, 80mM Tris-HCL pH 6.8, 1mM phenylmethylsulphonyl fluoride, 0.2mM N-ethylmaleimide & 5mM EDTA pH 7.7) and sonicated for 1.5 min in a siliconised Eppendorf tube. The protein content of the islet sonicate is estimated by using the BCA protein assay kit. Prior to electrophoresis the samples are aliquoted into the required concentrations and treated with a loading buffer (1.5mMTris-HCL pH6.8, 20% glycerol, 10% SDS, 0.2mM DTT & bromophenol blue (pinch) ). If samples were too dilute they were dried down using a speed vacum at manufacturers settings (Savant Instruments) for 30-60 mins depending on sample volume. The sample could then be reconstituted using ddH$_2$O in the volume required and vortexed.

7.3.2 Electrophoresis

The islet sonicated samples are resolved on a 10% polyacrylamide gel. The gel apparatus is set up as follows:
### Methods Chapter 6

**Detection of Glucokinase Protein**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide (national diagnostics)</td>
<td>33.3%</td>
<td>13.4%</td>
</tr>
<tr>
<td>1.5mM Tris-HCL pH 8.8</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>0.5mM Tris-HCL pH 6.8</td>
<td></td>
<td>25%</td>
</tr>
<tr>
<td>5mM Na$_2$S$_2$O$_3$</td>
<td>0.5%</td>
<td></td>
</tr>
<tr>
<td>10% SDS (w/v)</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Bis-Acrylamide (PDA)</td>
<td>7.8mM</td>
<td></td>
</tr>
<tr>
<td>Piperazinediacrylamide</td>
<td></td>
<td>5.5mM</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>39.2%</td>
<td>60%</td>
</tr>
</tbody>
</table>

Sonicate the gel preparation then add

1% APS (w/v) | 1% | 0.5%  
TEMED | 0.05% | 0.05%

Mix quickly, pour and wait to set.

**Table 6.1** Reagents required for SDS PAGE gel setup.

The resolving gel is prepared and allowed to set first for 30mins. The stacking gel is prepared when the resolving gel has set and poured on top of the resolving gel. Combs are inserted and the gel is left to set for another 30mins. The set wells are aspirated with tank buffer (0.2mM Glycine, 0.03mM Tris-HCL, 0.1% (w/v) SDS) and protein samples are loaded. As soon as the samples have settled (app. 10mins) the gels are loaded into the gel tank topped up with buffer and set on 60volts, 30mA per gel until the sample line has run through the stacking gel then voltage is set to 250volts with amps remaining fixed.

#### 6.3.3 Blotting

When the blue sample line has run off the gel, it is placed into blotting buffer (0.2mM Glycine, 0.03mM Tris-HCL, 0.1% (w/v), 20% methanol) for 10mins. The gel is sandwiched with a nitrocellulose membrane between filter paper and sponges. The proteins are then electroblotted onto a nitrocellulose
Methods Chapter 6

Detection of Glucokinase Protein

membrane at 300 mA 25volts for 1-1.5hrs. Following transfer the membrane is blocked in blocking buffer (5% non-fat dry milk/0.05% Tween 20 in 20mM Tris-buffered saline (TBS) pH 7.4) overnight at 4°C. The membrane is then incubated with a 1: 1250 dilution of sheep anti-rat GST-GK-fusion protein antibody in blocking buffer (gift from Dr. M. Magnuson, Vanderbilt University USA) in TBS for 1-2 hrs. The membrane is then washed in wash buffer (0.5% Tween 20 in TBS) and incubated with a streptavidin linked horse radish peroxidase conjugate (Amersham Pharmacia Biotech) in a 1:1500 in TBS dilution for 1 hr. The membrane is washed again and rinsed in TBS. The proteins can be visualised using the Enhanced chemiluminescence (ECL) technique where the membrane is incubated for 1min in a mixed ECL solution and photographed.

The principle of the ECL technique is that the light emitting chemical ‘luminol’ is oxidised by HRP (which is attached to the secondary antibody) and hydrogen peroxide (see fig. 6,1). Immediately following oxidation the luminol is in an exited state which then decays to ground state via a light emitting pathway. ECL is achieved by performing the oxidation of luminol by HRP in the presence of chemical enhancers such as phenols. This increases the light output approximately 1000 fold and extending the time of light emission.

The GK band is determined by checking with a rainbow molecular weight marker (Amersham Pharmacia Biotech) (Fig. 6,4) which contains different colour dyed molecular weight marker proteins. The rainbow marker can be transferred onto the blotted membrane and checked with the photograph of detected bands to determine an estimate of the protein size.
Methods Chapter 6  Detection of Glucokinase Protein

Fig. 6.4 Picture of Rainbow molecular weight marker. Protein size indicated in KiloDaltons (KDa).

6.4 Method Validation

6.4.1 Primary Antibody Biotinylation

The GK antibody used incurred a considerable degree of background on the Western blots, therefore the method was modified to reduce the high background. This involved the biotinylation of the primary antibody. Labelling the 1° antibody with biotin increases sensitivity. Streptavidin conjugated with HRP is used to detect the biotinylated 1° antibody as streptavidin has a high affinity for biotin. The protein concentration of the antibody is determined by use of the BCA protein assay kit. Biotinylation is performed by using a kit from Amersham Pharmacia Biotech. 2-2.5mgs of GK antibody in 40mM bicarbonate buffer is incubated with biotinylation reagent (Amersham) at room temperature for 1hr and the biotin-antibody complex is
eluted from unbound biotin using gel filtration. A 90% recovery of antibody is assumed. The method is illustrated in fig.6.5.

![Diagram illustrating principle of Biotin-streptavidin system.](image)

**Fig. 6.5** Diagram illustrating principle of Biotin-streptavidin system.

### 6.4.2 Primary Antibody Concentration Determination

To obtain a good result with minimum background it was important to determine the optimal concentration of 1° GK antibody. A strip experiment was performed where three different concentrations of islet sonicate were blotted and detected on three strips of membrane, each strip was incubated with a different dilution of GK antibody (fig. 6.6).
Fig. 6.6 Strip experiment with three dilutions of GK antibody with three different amounts of islet protein 20, 40, & 60 ug.

From the comparison of the GK band and background, a dilution of 1:2500 was routinely used.

6.4.3 Sample Protein Content Determination
To be able to compare band intensity among the three groups it was important to find out what would be the ideal content of islet sonicate to run. Therefore a standard curve was constructed with increasing concentration of islet sonicate protein (fig.6,7 and fig.6,8).
It is important to run enough islet sonicate protein so that a decrease of up to 50% can be detected as similar studies in the liver have noticed this degree of reduction of liver GK (Desai, Byrne, et al. 1997) & insulin secretion (Wilson & Hughes 1998).
According to the standard curve (fig 6,6) optical density levels out after 20μg protein concentration. Therefore to be able to detect a decrease of up to 50% (10μg) 20μg protein concentration was considered to be an ideal amount to run.

6.4.4 Liver Control
To be able to compare between blots (only 7-8 samples can be run per gel) a standard was required. The ideal standard was a liver homogenate sample, as enough homogenate could be easily obtained for all experiments from one sample. To find out how much liver homogenate to run, a standard curve was constructed (fig.6,9 and fig.6,10).

![Liver homogenate standard curve](image)

**Fig. 6,9** Liver homogenate standard curve.

![Blot of increasing amounts of liver homogenate](image)

**Fig. 6,10** Blot of increasing amounts of liver homogenate.
Detection of Glucokinase Protein

According to the standard curve OD appears to level out after 60\(\mu\)g protein concentration. So it was decided to use 40\(\mu\)g of liver sample on all blots.
Plan of the study

Wistar rats were studied at three stages of development: at birth (newborn group), at three weeks old when they are weaned and in adulthood, which will be from age 6 weeks or more.

Readings were taken from observations of pooled islets from litters. Readings from animals at the newborn stage are taken from two litters, as the number of islets from one litter was insufficient. Litters were maintained at 8 animals per litter. A final result consisted of an average of 5 readings. Therefore each reading consisted of 8 animals with results from 40 animals representing the final observation.

Yields of islets from these pools varied according to stage of development with less islets observed from animals from maternal LP fed mothers. Offspring from control fed diets would yield approximately 50-70 useable islets per newborn rat pup, three week old animals would yield approximately 200-300 islets per animal and adults would yield up to 500-700 islets per animal.

There were three groups of rats as outlined in the following diagram:

Group A, the control group consists of offspring at birth, 3 weeks of age and adults from mothers fed a normal diet.

Group B, consists of offspring at birth, 3 weeks of age and adults from mothers fed a low protein diet from first day of pregnancy and were kept on low protein diets throughout gestation and suckling periods. The adult group are weaned onto a normal diet at three weeks of age.

Group C consists of offspring at 3 weeks of age and adults from mothers fed a low protein diet only during pregnancy.
Islets were isolated and pooled for each group of rats (A, B & C) and at each time point (birth, suckling and adulthood) with approximately

- 50-70 pooled islets used for measuring insulin secretion.
- 200-300 islets were used for Hexokinase and glucokinase assays.
- 400-500 islets were used for measuring GK protein.

**Diet**

The mothers were placed onto the relevant diets at day 1 of pregnancy and fed *ad libitum*. It was not felt necessary to pre-feed the mothers before day 0 on the low protein diets as the period of gestation that appears to be vulnerable to programming in rats is the last trimester (Bertin, Gangnerau, et al. 1999) and not the first two trimesters (Portha, Kergoat, et al. 1995).

A 6% protein diet was used as the low protein diet as this is considered the most severe restriction resulting in approximately a 70% decrease of protein intake compared with a 20% protein control diet (Latorraca, Reis, et al. 1998).
The composition of these diets used in the present work is based on the study by Langley 1994 and the diets were prepared by Dyets Inc USA in pellet form. The composition of the diet is shown in the following table.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/Kilogram of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control diet</td>
</tr>
<tr>
<td>Caesin</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch</td>
<td>412</td>
</tr>
<tr>
<td>Fibre</td>
<td>50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>206</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>5</td>
</tr>
<tr>
<td>AIN-76 Mineral mix</td>
<td>20</td>
</tr>
<tr>
<td>AIN-76 Vitamin mix</td>
<td>5</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
</tr>
</tbody>
</table>

Table of control and low protein diet composition.

Weight gain was monitored twice weekly in pregnant rat dams and suckling infants, then monitored weekly after weaning.

Please note there are summary tables of data at the end of chapter 9.
Chapter 7  
Newborn Results

7.1 Experimental Design
Twenty-two pregnant female Wistar rats were used to study the programming of pancreatic GK at the newborn stage. As islet numbers were a lot smaller in neonatal pups (40-100 per pancreas (Tu J 1999) compared with adults (app. 500-682 islets per pancreas (Gotoh, Maki, et al. 1987) two litters were required for a set of studies at this age. These included measurement of insulin secretion by the perifusion of islets, measurement of GK activity and quantification of GK protein. Twelve pregnant rat dams were placed onto the 6% protein diet at day 0 of pregnancy. Food and water was provided ad libitum.

7.2 Animal Weight Gain
Mothers were weighed at least twice per week and offspring were weighed at birth.

7.2.1 Maternal Weight Gain

<table>
<thead>
<tr>
<th>Maternal Diet (%) protein</th>
<th>n (Pregnant females)</th>
<th>Weight gain in pregnancy (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>16</td>
<td>91.72 ± 3.59</td>
</tr>
<tr>
<td>6%</td>
<td>32</td>
<td>78.24 ± 3.77 *</td>
</tr>
</tbody>
</table>

Table 7.1. Maternal weight gain. Data shown as mean ± SEM for n observations * P<0.03 Weight taken from day 1 to 21 of pregnancy.

Mothers on a low protein diet put on 14.7% less weight than mothers on the 20% control diet. This may be attributed in part to the decrease in food consumption as mothers on the 20% diet ate on average 29-30g/day whilst mothers on the 6% protein diet ate 22-23g/day.
### 7.2.2 Litter Sizes And Weight Of Pups

<table>
<thead>
<tr>
<th>Maternal Diet (% Protein)</th>
<th>Litters</th>
<th>Litter Size (no. pups)</th>
<th>Pup n</th>
<th>Birth weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>24</td>
<td>8.75 ± 0.58</td>
<td>88</td>
<td>5.3 ± 0.06</td>
</tr>
<tr>
<td>6%</td>
<td>33</td>
<td>9.39 ± 0.52</td>
<td>150</td>
<td>5.1 ± 0.05*</td>
</tr>
</tbody>
</table>

**Table 7.2.** Litter sizes and weight of pups. Data shown as mean ± SEM for n observations. * Indicates significant difference to 20% protein diet pups P<0.008.

Pups born from mothers fed on a 6% protein diet were 4.3% lighter than pups born to mothers fed on the 20% protein diet. There was no difference in litter sizes between groups.

### 7.3 Total Plasma Protein

To confirm a decrease in protein intake, blood samples were taken from rats at the time of study and assayed for total protein content. Approximately 0.5ml of blood was collected in lithium heparin tubes (Sarstedt UK) and centrifuged for 5mins at 855g. 100-200ul of plasma was eluted from the top layer. Due to inability to collect sufficient blood from a single newborn pup, blood was pooled from litters of newborn rats. The plasma was diluted to a 1:8 dilution and analysed using the BCA protein concentration determination kit (as mentioned earlier in chapter 5.3.4).
Fig. 7.1 Total plasma protein for newborn rats. Data represent mean ± SEM for group A (n=9) and group B (n=15).

Although there was a decrease in birth weight there was no significant difference in total plasma protein between the newborn groups. One possible explanation for this is that the mothers own plasma protein is being utilised in order to maintain fetal plasma protein.
7.4 Insulin Secretion

Approximately 70 newborn rat islets were used to determine insulin secretion by the perifusion method as described in chapter 5. These were the islets that were first picked out from the digestion and were carefully deposited in glass wool within the chambers.

As mentioned earlier the lag time (the time for the media to be collected from the chambers) is indicated here in fig.7,2.

Fig. 7,2 Newborn rat Insulin secretion in response to 16mM. Group A, Group B. 1.5mM and 16mM indicate glucose concentration. Data represent mean + SEM n=4. Time of switch ------ lag period.
There appeared to be no difference in the basal insulin secretion between the two groups of islets. However there was a significant decrease in both peak insulin and total insulin secretion (as observed by measurement of the area under the curve using a computer program) after stimulating the islets with glucose in group B compared to group A. The peak insulin release is the highest concentration of insulin secreted while the islets were exposed to 16mM glucose. As there is a lag time between exposure and collection the peak is considered to be between 19 to 100 minutes.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total insulin release (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>1404 ± 225.4</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>399.7 ± 151.4*</td>
</tr>
</tbody>
</table>

Table 7.3. Total insulin release from newborn rat pancreatic islets. Data shown as mean ± SEM for n observations. * Indicates significant difference to group A pups P< 0.011. Coefficient of variation for group A =32.1%

Total insulin release is decreased by 71.5% in group B newborns. This observation is consistent with a notable reduction of maximal peak secretion (73.9%), which is the highest rate of insulin secreted during the high glucose stimulation period.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Maximum peak secretion pg/ml/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>41.58 ± 7.345</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>10.86 ± 4.337*</td>
</tr>
</tbody>
</table>

Table 7.4 Maximum peak secretion of newborn rat pancreatic islets. Data shown as mean ± SEM for n observations. * Indicates significant difference to group A pups P< 0.011.
The time taken to reach peak secretion was also followed. In group A the response showed peak whilst the secretory response tended to plateau in group B much earlier on, which is probably due to the reduced insulin output. The data was taken for either the peak value or the start of the plateau and time was taken from the switch from low to high glucose.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Time to maximal secretion (Mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>67.00 ± 5.05</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>31.50 ± 2.5*</td>
</tr>
</tbody>
</table>

Table. 7,5 Time to maximum peak secretion of newborn rat pancreatic islets. Data shown as mean ± SEM for n observations. * Indicates significant difference to group A pups P< 0.0007.

**7.4.1 Conclusion**

The insulin secretion data between islets from the two groups of newborn pups conclusively show that a low protein diet during pregnancy reduces the glucose stimulated insulin secretory response of freshly isolated pancreatic islets and results in a reduction in total insulin release by approximately 71.5%. Other observations about the insulin secretion response show that this decrease is accompanied by a general reduction of maximal insulin secretion rate by 73.9% a decrease similar to the peak insulin secretion. As the group B response is decreased so much, the time to reach maximal insulin secretion rate occurs 35.5 minutes sooner giving the appearance of a plateaued response instead of a peaked one.
7.5 Hexokinase & Glucokinase Activity

Approximately 200 islets were used per assay. Islets were considerably fewer in number and smaller than adults and the islet homogenate samples were small therefore hexokinase assays were performed in duplicate and GK assays were performed in triplicate where possible. To obtain the GK activity kinetic parameters $K_m$ and $V_{\text{max}}$, fluorescence readings were quantitated to glucose concentration using an NADH standard curve. These values could then be averaged and specific velocity calculated.

![Graph](image)

**Fig. 7.3** Newborn group hexokinase activity result. Hanes-Woolf plot illustrating $K_m$ and $V_{\text{max}}$ obtained from neonate HK assays. — Represents group A, —— represents group B n=6.

The Hanes-Woolf plots express the $K_m$ as the intercept at the X-axis and the $V_{\text{max}}$ is $1/\text{slope}$. Hexokinase results showed no statistical difference in maximal velocity or $K_m$ between groups A and B.
Results Chapter 7

Newborn Results

Fig. 7.4 Newborn group glucokinase activity result. Hanes-Woolf plot illustrating $K_m$ and $V_{\text{max}}$ obtained from GK assays. --- Represents group A, — represents group B n=4. * indicates P<0.02.

The GK results show that the $K_m$ values are significantly reduced in the low protein group B, however there appears to be no statistical difference in maximal velocity between the two groups.

7.5.1 Conclusions

Hexokinase activity is not affected in the newborn offspring of rat mothers fed a low protein diet as well as no observed reduction of GK maximal velocity. However even though the amount of newborn tissue was barely adequate the results do show that the GK $K_m$ values are very much reduced indicating that the GK affinity for the substrate was approximately 5 fold greater in newborn offspring of mothers fed a low protein diet throughout their gestation.
7.6 Glucokinase Protein Concentration

Approximately 300-400 newborn rat islets were isolated for sonication to be blotted and probed for GK protein content. The islets of group B offspring were less in number than those in group A and protein obtained from each litter was smaller in amount. This was to be expected as previous studies (Snoeck, Remacle, et al. 1990) & (Dahri, Snoeck, et al. 1991) have shown that islet size and number are reduced in newborn rats from mothers fed a low protein diet. With a low amount of islet tissue GK protein was difficult to detect in group B even when the amount of the total tissue protein was doubled from 20μg to 40μg. However GK from group A was detectable with the same amount of protein as in group B. As shown in fig.7,5 the GK protein content of islets in group B was markedly and significantly reduced compared to group A.

![Fig. 7,5](image)

**Fig. 7,5** Representative GK bands from newborn groups A and B.
7.6.1 Conclusions

These results show that there is a 71.8% decrease of GK protein in the offspring of mothers fed a low protein diet. However this is not concurrent with the observation that there was no reduction in maximal activity of GK. The insulin secretion experiments however are in accord with the Western blot results as there is a decrease in insulin output of 71.5% with a similar reduction of GK protein. Why this is not also reflected by the GK assay is not certain but may pertain to the low amount of tissue from the newborn rat islets and the potential for experimental error.
8.1 Experimental Design
Fifteen litters of 8 pups per litter were used to study the programming of pancreatic GK at the weaning stage. Islet numbers and size were increased in the 3-week rats but had still not reached adult proportions. Three groups of 5 litters were set up into groups A, B & C as mentioned earlier. As with the newborn rat groups the islets were used for measurement of insulin secretion by perifusion, measurement of GK activity and quantification of GK protein. Rat dams from group A & B were kept on their respective diets until offspring were 3 weeks of age whilst group C rats were placed onto the 20% protein diet from the 6% protein diet at birth of their litters. Food and water were ad libitum.

8.2 Weight Gain
Pups were weighed the day they were born and then at least twice weekly for three weeks (see fig. 8,1).
Pups from mothers fed a 6% protein diet during pregnancy (groups B&C) were lighter compared with the control group until after day 6 when group C pups began to follow the control groups pattern of weight gain. Group B was significantly lighter throughout the whole 3 weeks of suckling. This was also apparent by the appearance of the pups, which were smaller in group B. One other observation of note was that more mothers in group B ate one or more of her pups early on during the suckling period.
8.3 Plasma Protein Results

0.5ml of blood was collected in lithium heparin tubes by severing the aorta after the pancreas had been removed.

**Fig. 8.2** Total plasma protein in 3 week weaning groups. Data represent mean + SEM. * = p<0.0001 ** = p<0.02.

There is a 20% decrease in total plasma protein in the group B pups compared with group A and a 13.4% increase of total plasma protein in group C compared with group A. One possible reason for this is an increase in diet consumption in order to ‘catch up’ in growth.
8.4 Insulin Secretion

Approximately 70 islets were used per perifusion and were treated as described earlier for the newborn group experiments.

As observed with newborn rat islets in fig.7.3, there appeared to be no difference in the basal insulin secretion between the islets from the three groups. There was a significant decrease in total insulin secretion after stimulating the islets with glucose in group B as shown in Table 8.1.
Table 8.1. Total insulin release of pancreatic islets from 3-week weanling pups. Data shown as mean ± SEM for \( n \) observations. * Indicates significant difference to group A pups \( P<0.05 \). Coefficient of variation for group A= 29.2%. # represents significant difference between variances calculated by the F-test \( p<0.05 \).

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Total insulin release (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>1019 ± 132.8</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>602 ± 118.7 *</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>1084 ± 340.4 #</td>
</tr>
</tbody>
</table>

Fig. 8.4 Scattergram of results from table 10.1. Illustrating difference of variation of groups.

There is a significant decrease of insulin secretion by about 40.1% in the group B pups whilst there seems to be no difference in total secretion of group C pups. But there is a difference in the spread (indicated by the large standard error value) in the results from group C which cancels out any significance between mean results when compared with group B. The spread of data seems to show that there are two populations within this group, one that behaves like
group A and another like group B. A test to check for a significant difference in variances was performed and did prove that there was an unusual spread of results for group C.

Fig. 8.5 Three week group C insulin secretion results. Data represent means + range of duplicate results. ▲ Group C glucose responsive population (n=3), ▼ group C glucose unresponsive population (n=2, bar indicates range). ——— Time of switch ———— lag period.

From the data on individual group C it can be noted that there are at least two litters that behave like group B and in fact do not appear to respond to the switch back to low glucose.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Maximal peak secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>33.11 ± 6.59</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>13.56 ± 2.89 *</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>27.07 ± 4.59</td>
</tr>
</tbody>
</table>

Table 8.2. Maximal peak secretion of pancreatic islets from 3-week weanling pups. Data shown as mean ± SEM for n observations. * Indicates significant difference from group A and group C pups P< 0.04.
The maximum amount of insulin that the islets are able to secrete is also decreased in group B (59.2%) compared to groups A and C.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Time to peak secretion (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>49.4 ± 8.55</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>39.8 ± 12.76</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>58.4 ± 17.37</td>
</tr>
</tbody>
</table>

Table 8.3. Time to maximal insulin secretion results. Data indicate mean ± SEM.

There is no significant change in the time to peak secretion among the three groups, analysis was difficult as groups B & C have large variances caused by peak secretion readings occurring well after the islets were switched back to a low glucose concentration. This does not happen at all in group A and is indicative of a lack of control of insulin secretion within groups B and C.

8.3.1 Conclusions

Pups from mothers fed a low protein diet throughout pre and postnatal development have a decreased insulin response to glucose with a lack of insulin output and maximal insulin secretory capacity. This is not entirely reflected by group C who only received inadequate protein during prenatal development. There are indications of two different populations in this group one of which there is a lack of control of insulin secretion and a trend towards a reduced maximal insulin secretory capacity and the other which shows normal glucose induced insulin secretion response. The reason for this is unknown as the litters were treated the same and all rats were of the same Wistar strain.
8.5 Hexokinase & Glucokinase Activity

Approximately 200-300 islets were isolated and homogenised for each assay as with the newborn rat assays. The islets were small although not quite as small as in the newborns. Assays were performed in duplicate for HK and in triplicate for GK.

\[
V_{\text{max}} = \frac{V}{[S]} \\
K_m = 0.13 \pm 0.02 \\
K_m = 0.11 \pm 0.04 \\
K_m = 0.1 \pm 0.02
\]

Fig 8.6 Hanes-Woolf plot illustrating \(K_m\) and \(V_{\text{max}}\) obtained from HK assays of 3 week weanling pups. — represents group A, — represents group B. — represents group C. Data represent means $\pm$ SEM. * indicates difference from group A \(p<0.05\) \(n=4-6\)

The only change in HK activity appears to be in group C (see fig. 8.6) with a 1.5 fold increase in \(V_{\text{max}}\). This may be due to a compensatory effect from a reduced GK activity.
These results show that the maximal velocity of GK is significantly decreased in both groups B & C (see fig. 8,7). The $K_m$ value is decreased only in group B corresponding with results obtained with newborn rats which showed again that group B $K_m$ was decreased.
8.5.1 Conclusions
Maximal velocity of hexokinase activity is increased 1.5 fold in group C. The reason for this is unknown but may be an attempt to compensate as group C’s GK activity is decreased. Or it may be as with the newborn rat results that there was insufficient islet tissue, however this is not apparent in group B. The GK $V_{\text{max}}$ data show a significant decrease in groups B & C compared with group A. As with the newborn rat results, a significant decrease of GK $K_m$ in group B was indicative of a change in functional substrate binding ability of GK.
8.6 Glucokinase Protein Concentration

Approximately 400-500 islets were isolated for sonication to be blotted and probed for GK protein content. The group B offspring pancreas size and islets were less than their group A counterparts as reported previously in 21 day old low protein fed pups by Petrik, Reusens, et al. 1999. This time 20μg protein samples of islet homogenate were applied on SDS-PAGE. The results obtained were doubled to give a 1:1 ratio against the liver control as 40μg of liver control was used. Western blots of the samples are shown in fig. 8,8 and the analysis of the islet GK protein concentration as a percentage of liver control is shown in fig.8,9.

![Fig. 8,8](image)

Fig. 8,8 Representative GK bands from islet tissue from 3-week groups A and B & C.

![Fig. 8,9](image)

Fig. 8,9 Three week weanling rats islet glucokinase protein content illustrated as a percentage of a 40μg liver control GK band. Data represent mean + SEM.

* indicates P<0.03 from group A.
8.6.1 Conclusions

The Western blot data show a definite and significant decrease of GK protein content in islets from groups B (65%) & C (81.8%). Although there appeared to be less GK protein in group C than group B there was no significant change between the groups. This observation correlates with the maximal GK activity results, which shows that there was a significant decrease in activity in groups B & C. A change in $K_m$ was also observed in group B. Weanling pups from mothers fed a low protein diet during prenatal development seem to show a decrease in GK enzyme with reduced maximal activity. However even though the insulin secretion response appears adequate when stimulated with glucose the pattern itself does not seem to conform to group A indicating possible changes in the glucose sensing mechanism. Weanling rats that were currently fed a low protein diet and received a low protein diet through pre and perinatal development show a decrease in insulin secretion accompanied with a reduction of GK activity and a decreased affinity for glucose.

In relation to the newborn rat results it appears that the decreased insulin secretion is a consequence of the animals current nutritional state (as also observed in group B 3-week results) as group C animals do not continue this trend. However there appears to be some controlling effect during prenatal development as the insulin secretion response is not uniform with the control group A. The decreased GK $K_m$ value noticed in group B at the newborn stage is not a permanent effect as the recovery group do not show this decrease. The Western blot data however show a permanent decrease of GK protein (71.5% of control) decrease at the newborn stage does not change after the switch in diet as the 3 week group pups still have reduced GK protein (81.8% of control).
9.1 Experimental Design
As before with the 3-week old group fifteen litters of 8 rats per litter were used to study if the diets administered during their early development have had a permanent programming effect on GK. Islet numbers and size were greater in the adults making analysis easier. Although rats receiving under nutrition during pre and perinatal development are reported to have a 40% decrease in pancreas density (Dahri, Snoeck, et al. 1991). As with the 3-week old rats the adults were allocated into the three groups A, B, & C. After 3 weeks from birth at the weaning stage, all animals were placed onto normal standard laboratory chow which was provided ad libitum, until 6-8 weeks of age at which time islet insulin secretory response, GK activity and GK protein content were studied.
9.2 Weight Gain
Rats were weighed twice weekly from birth. After weaning they were weighed only once weekly until the day of study.

![Graph of weight gain for Adult study groups A, B, & C.](image)

**Fig. 9.1** Weight gain for Adult study groups A, B, & C. Data are means ±SEM. Significance by one-way ANOVA *p<0.0001.

The profiles of weight gain are similar to those observed in the 3-week group. At 21 days and after weaning the rate of increase in weight in all groups is as expected as the animals are no longer receiving milk from their mothers. The rate of weight gain is similar in groups A & C after 21 days but group B appears to have a slower weight gain and is generally 40% lighter than groups A & C.
9.3 Plasma Protein Results

0.5ml of blood was collected by severing the aorta. Plasma was separated and assayed for total protein content and the results are shown in Fig. 9.2.

There was no significant decrease in total plasma protein concentration in the group B animals compared with group A. There was an increase in protein concentration in group C (16.5%). This was also observed at 3 weeks. There was no difference in plasma protein between group B & C but this could be due to a large spread of results in group B. This was confirmed by an F-test to see if the variances are significantly different. Why group C has a high plasma protein concentration even though their weight is on par with group A is unknown.
9.4 Insulin Secretion Result

Fifty adult islets were perifused instead of 70 and experiments were performed as stated previously.

The results indicate a split in the responses in groups B & C (see fig.9.3) this is due to the animals that do not respond to the switch back to low glucose.

To illustrate the altered secretory response in groups B & C, the individual data for each group is displayed (fig.9.4).
Fig. 9.4 Individual adult group perifusions shown to illustrate loss of regulated secretion in groups B & C.
In the adult islets there is no difference in baseline insulin secretion, total insulin secretion or peak insulin secretion between the three groups. There is a trend to a lower total insulin release in group C however this is not significant (see table 9.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total insulin release (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>1716 ± 353.0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>1996 ± 286.4</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>1362 ± 395.4</td>
</tr>
</tbody>
</table>

Table 9.1 Total insulin release for adult groups. Data represent mean ± SEM. Coefficient of variation for group A = 46%.

There is also no significant difference in peak insulin secretion and maximal peak insulin secretion between groups (table 9.2).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Maximal peak secretion Pg/ml/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>50.74 ± 8.28</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>63.93 ± 4.65</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>52.46 ± 11.76</td>
</tr>
</tbody>
</table>

Table 9.2 Maximal peak secretion for adult groups. Data represent mean ± SEM.

It is apparent that groups B & C do not behave in a uniform fashion compared to group A. The spread of group B & C data is large, therefore an unpaired t-test is not the appropriate test to use. In order to determine if there were significant differences in variance the data was subjected to the F-test.
Table 9.3 Time to maximal secretion of adult group perifusion experiments. Data represent means ± SEM. # p < 0.001 ## p < 0.0003 significance of difference in variances according to F-test.

The large variation in time of maximal peak secretion indicates a difference in the pattern of insulin secretion in groups B & C (table 9.3 and fig. 9.5). This suggests a change in the glucose sensing mechanism in the \( \beta \)-cells.

9.4.1 Conclusions
Within the adult rat group C there appears as with the 3 week old animals two different populations of animals. Some islets do not respond to the switch back to a low glucose concentration and these islets continue to increase secreting
insulin. It is unlikely that the observed differences are due to experimental variation as all experiments were performed the same way. Therefore these erratic secretion patterns are likely to be the result of the diet manipulation and variation between the subsets of animals.

9.5 Hexokinase & Glucokinase Activity
Approximately 250-300 isolated islets were homogenised and assayed in duplicate for HK assays and triplicate for GK assays. The results were plotted as given in fig.9,6 and fig.9,7.

![Graph](image)

**Fig. 9,6** Hanes-Woolf plot illustrating $K_m$ and $V_{max}$ obtained from HK assays of adult rat islets. --- Represents group A, --- represents group B. --- represents group C. Data represent means $\pm$ SEM * indicates difference from group A *p<0.05 **p<0.0005 n=4-5

There was a significant decrease in maximal velocity of HK but no change in $K_m$ in groups B & C compared to group A.
Fig. 9.7 Hanes-Woolf plot illustrating $K_m$ and $V_{max}$ obtained from GK assays of adult rats. — Represents group A, — represents group B. — represents group C. Data represent means ± SEM * indicates difference from group A p<0.003 **p<0.05 n=4-6

As observed from the Hanes-Woolf plot for GK the $V_{max}$ is significantly reduced in both groups B & C and only group B has a reduction in the $K_m$ value.

9.5.1 Conclusions

The adult group results are very interesting as they clearly show a decrease of GK maximal velocity in both recovery groups B (65%) & C (66.4) as well as a decrease of $K_m$ in group B (78.4%). A decreased $K_m$ in group B was also observed at the 3 week weanling stage but its not clear whether this was a result of the current low protein diet or of programming by the prenatal low protein diet.
9.6 Glucokinase Protein

Approximately 450-550 islets were isolated, sonicated and samples containing 20μg protein were analysed by Western blotting (fig.9.8, fig.9.9, fig.9.10 and fig.9.11).

Fig. 9.8 Representative islet tissue GK bands from adult groups A and B & C.

Fig 9.9 Adult rat islet glucokinase protein content illustrated as a percentage of a 40μg liver control GK band in a 1:1 ratio. Data represent mean + SEM indicates P<0.02 **p<0.004 from group A.
To investigate the possibility that the low protein diet causes a general decrease in islet cell protein the optical density of other protein bands was also measured. The western blots also display a few bands of other proteins of unknown identity. To find out whether it was only the GK band that was reduced in optical density two other bands were also measured within the adult group samples.

Fig. 9,10 Representative Western blot of liver standard GK, depicting non-GK protein bands that occur both in liver and islet sonicate samples.

Fig. 9,11 Graph depicting differences in optical density between groups for bands 1 & 2 from islet protein western blots (n=3-4).
9.6.1 Conclusions

GK protein was again significantly lower in groups B (66.5%) and C (82%) at the adult stage compared with the control group A as measured by GK band density. However the data between groups B & C are not significantly different. This trend is similar to that observed in the 3 week old rats thus indicating that a decrease in GK protein in both groups B & C is a permanent effect. There appears to be no difference in optical intensity of bands 1& 2 that were measured between groups from the adult Western blots. This indicates that the reduction of GK protein in not due to a general reduction of β-cell proteins.

The adult data conclusively show a permanent change in insulin secretion in rats that exclusively received a low protein diet prenatally. It can also be noted that there are probably two populations of islets within groups B & C, one that conforms to a typical secretion pattern and another that seems to lack a switch off response as the insulin secretion rate continues to rise. The reason for this is unknown, as it would indicate a difference between litters and not individual animals.

In the prenatal and perinatal low protein rats GK $K_m$ value is consistently reduced. GK protein and maximal activity are reduced from birth in all animals receiving a prenatal low protein diet. Rats raised on the low protein diet during the perinatal stage (suckling period) not only show similar results as animals only reared on low protein prenatally, which would be expected. This further insult is enough to permanently reduce the GK $K_m$ value. This would result in the inability of the enzyme to metabolise glucose effectively at high glucose concentrations. Therefore inadequate nutrition modelled by a low protein diet administered to pregnant rat dams is enough to invoke a permanent alteration of glucose metabolism in the adult offspring which can be exacerbated to a more severe change by continued administration of inadequate nutrition during perinatal development as well.
9.7 Summary

The data discussed are shown in summary in the following tables.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Group</th>
<th>Peak Insulin Secretion (Pg/ml/islet/min)</th>
<th>Maximal Insulin Secretion (Pg/ml/islet)</th>
<th>Time to peak Insulin secretion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>A</td>
<td>1404±225.4</td>
<td>41.58±7.345</td>
<td>87.00±5.05</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>399.7±151.4*</td>
<td>10.86±4.337</td>
<td>51.50±2.50</td>
</tr>
<tr>
<td>3wk</td>
<td>A</td>
<td>1019±132.8</td>
<td>33.11±6.589</td>
<td>69.40±8.548</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>602.2±118.7*</td>
<td>13.56±2.893*</td>
<td>59.80±12.76</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1084±340.4*</td>
<td>27.07±4.588*</td>
<td>78.40±17.37</td>
</tr>
<tr>
<td>Adult</td>
<td>A</td>
<td>1716±353.0</td>
<td>50.74±8.275</td>
<td>87.80±2.200</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1996±286.4</td>
<td>63.93±4.646</td>
<td>82.40±16.05</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1362±395.4</td>
<td>52.46±11.76</td>
<td>86.40±22.85</td>
</tr>
</tbody>
</table>

Table 9.4 Insulin Secretion Parameters Summary Table

* Indicates significant difference between means with group A.

# Indicates significant difference between variances with group A.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Group</th>
<th>% of Liver Control</th>
<th>% of group A</th>
<th>% decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>A</td>
<td>30.53±5.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8.62±5.83*</td>
<td>28.2%</td>
<td>71.8%</td>
</tr>
<tr>
<td>3wk</td>
<td>A</td>
<td>233.1±52.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>81.72±23.16*</td>
<td>35%</td>
<td>65%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>42.49±10.62*</td>
<td>18.2%</td>
<td>81.8%</td>
</tr>
<tr>
<td>Adult</td>
<td>A</td>
<td>292.7±48.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>97.98±29.28*</td>
<td>33.5%</td>
<td>66.5%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>53.04±20.57*</td>
<td>18%</td>
<td>82%</td>
</tr>
</tbody>
</table>

Table 9.5 GK protein content summary Table

* Indicates significant difference between means with group A.
<table>
<thead>
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<th>Development Stage</th>
<th>Group</th>
<th>Hexokinase</th>
<th>Glucokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>Newborn</td>
<td>A</td>
<td>0.065 ± 0.017</td>
<td>3.84 ± 0.691</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.072 ± 0.024</td>
<td>5.35 ± 0.954</td>
</tr>
<tr>
<td>3 Weeks</td>
<td>A</td>
<td>0.133 ± 0.021</td>
<td>4.818 ± 1.39</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.103 ± 0.03</td>
<td>6.88 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.109 ± 0.02</td>
<td>12.39 ± 3.51*</td>
</tr>
<tr>
<td>Adult</td>
<td>A</td>
<td>0.104 ± 0.024</td>
<td>11.25 ± 1.54</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.097 ± 0.025</td>
<td>4.51 ± 2.29</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.119 ± 0.027</td>
<td>2.42 ± 0.37*</td>
</tr>
</tbody>
</table>

Table 9.6 GK Assay parameters summary table. * Indicates means significant from group A.
9.7.1 General Conclusions

The results conclusively prove a reduction of GK enzyme from birth in prenatally fed low protein rats with a decrease of 65-82% for groups B & C. This reduction of GK enzyme however cannot be corroborated by a lack of GK activity in newborn rats but can be at the 3 week & adult stages groups B & C corresponding to the decreased enzyme levels. Therefore GK activity is reduced in offspring from mothers fed a low protein diet during pregnancy and this is a result from the reduction of GK enzyme. The affinity GK has for glucose, which is measured by the $K_m$ value, is only affected in group B rats. Group C rats do show a slight decrease of $K_m$ from controls but not enough to be significant. So the functional ability of GK is only affected in offspring from mothers fed low protein pre and postnatally.

The apparent effect these observations have on insulin secretion can be seen by the adult perifusions. Although the reduction of GK activity does not significantly reduce insulin output it does however have a bearing on the control mechanisms of insulin secretion. There appears to be no difference between adult groups B & C therefore this is likely due to the reduction of GK activity.

The study overall shows that programming of GK activity occurs during gestation and can be exasperated by further under nutrition during the first 3 weeks of life. The effect of having a permanently reduced $K_m$ value will result in the inability of GK to appropriately metabolise glucose. This in itself could result in a slower clearance of glucose in the blood, which could result in glucose intolerance under prolonged exposure to an over than adequate diet.

If the group B & C adult group litters that did not respond to a decreasing concentration were to continue to behave this way then the animals would become hyperinsulineamic. This is not seen in a diabetic state as it is the lack of an insulin response that causes NIDDM so it is likely that the levels would eventually start to decline.
Chapter 10
Discussion

The hypothesis of "the fetal origins of adult disease" proposed by Hales & Barker in 1991 postulates that conditions, most likely nutritional, "program" the fetus for the development of cardiovascular disease, non insulin dependant diabetes and other metabolic disorders in adulthood (Hales, Barker, et al. 1991). The thrifty phenotype hypothesis proposes that the epidemiological associations between poor fetal and infant growth and the subsequent development of type 2 diabetes and the metabolic syndrome result from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism. These changes include reduced capacity for insulin secretion and insulin resistance which, combined with effects of obesity, ageing and physical inactivity, are the most important factors in determining type 2 diabetes (Hales & Barker 2001).

The hypothesis was further extended by Desai & Hales (1997) who used a rat model where rat dams were fed a low protein diet during pregnancy and/or lactation and demonstrated a permanent growth retardation in the offspring. These authors also observed permanent programming of liver metabolism as reflected by changes in the activities and reaction kinetics of hepatic enzymes of glycolysis and gluconeogenesis pathways (glucokinase and phosphoenolpyruvate carboxykinase). Therefore at the onset of the present project it was considered pertinent to investigate the programming of GK in the pancreatic β-cell in a rat model, as the β-cell plays a cardinal role in glucose homeostasis and is also the site of insulin synthesis, storage and secretion. This study could have important implications in the later development of NIDDM, depending on the alterations in the programming of the glucose sensing mechanism during fetal and neonatal development.

Effect of maternal low protein on insulin secretion
The present study clearly demonstrated that a maternal low protein diet during gestation and lactation periods significantly reduces insulin secretion in the offspring. The insulin secretory response was reduced in group B newborns
by 71.5% and by 40% in the 3-week old group B offspring. Insulin secretion was also altered in the offspring born of mothers fed a low protein diet during gestation but then placed onto a normal diet during lactation as shown by offspring obtained from group C (1019 ± 132.8 pg compared with control group A 1084 ± 340.4).

The data from group B & C adults (3-4 weeks old after 3 weeks of weaning during the suckling period) also showed a difference in time to reach peak insulin secretion; B= 62.4 ± 16.05 min, C= 86.4 ± 22.85 compared with the control group A 67.8 ± 2.2. Although the secretion of insulin is not decreased there is a change in the uniformity of the secretory response.

**Effect of maternal low protein diet on GK protein level and activity**

The protein levels of glucokinase enzyme were also decreased in all groups fed a prenatal low protein diet at all stages (newborns and 3-week old offspring). This was found to be a permanent effect and it is highly likely that the down regulation of GK protein synthesis or the GK mRNA synthesis occurs at or just before birth. The reduced level of GK protein observed in groups B & C at 3-weeks and adulthood may also be considered a contributory factor for the low $V_{\text{max}}$ values observed in these animals. The GK from rats on a low protein diet (groups B newborn & 3-week old) showed both reduced $K_m$ and $V_{\text{max}}$ values. There is also a degree of correlation between these data and insulin secretion indicating that whilst the animal is in a state of malnourishment it does not require GK to work optimally at higher glucose concentrations but can function at lower concentrations hence the reduced $K_m$ value. This effect is not permanent in newborn rats as the $K_m$ value normalises when the animal is placed onto a normal diet after birth as observed in the case of group C rats. A prenatal and postnatal low protein diet appears to have a permanent effect on the substrate binding ability of GK (as evident from the permanently reduced $K_m$ value in group B adults) therefore indicating that the critical period for the programming of GK’s affinity for glucose is after birth during the suckling period. The altered secretory capacity of islets from prenatally malnourished rats could be directly or indirectly linked with the reduction of GK $V_{\text{max}}$. If the GK activity is decreased then it is possible that other mechanisms involved in insulin secretion may be up-
regulated in order to compensate the effect. However these may not result in the typical phasic response seen in normal glucose stimulated insulin secretion. The changes in the $K_m$ value as a result a pre and postnatal low protein diet appear to be permanent in the case of group B rats, on the other hand prenatal low protein diet induced reduction of the $K_m$ value is rectified by a normal diet from birth. The mechanisms of changes in the $K_m$ of GK and the ability of GK to bind with glucose are not clear as yet. It may involve a change in the protein conformation of the GK enzyme molecule with respect to its active site or a change in the interaction with another protein involved with GK regulation or with a cofactor required for GK activity.

The expression level of GK protein was examined by Western blotting of islet homogenate by immunodetection of GK protein using a sheep anti-rat GST-GK-fusion protein antibody. Liver homogenate was used as control. The GK antibody was raised against liver GK but reacts with islet GK as well. There is a level of cross-reaction with non GK proteins. Smaller proteins detected could very well be GK protein fragments of degraded GK protein and larger proteins may represent HK I-III. The HK proteins are nearly twice the size of GK (100.3-102.5 KDa) but there is approximately 48-49% amino acid sequence homology of GK to HKI-III (Nishi, et al 1988).

During fetal development until birth or immediately before birth the fetal pancreatic (in particular) and hepatic (in general) maturation is not necessary because of the fetus dependence on the mother. So the maternal LP diet status compared with normal maternal diet has a dampening or depressive effect on the programming of gene activation, gene expression and protein synthesis for all fetal genes and proteins, which are not immediately required for the intrauterine survival of the fetus.

The programmed reduction of GK protein has proven to occur in this study but whether it is only GK that is permanently reduced or if it is an effect on all proteins in the β-cell was not entirely addressed in this study. Several pieces of evidence suggest that not all proteins were affected. Plasma protein results were monitored and the total plasma protein was only reduced in those
groups that were currently on a low protein diet. The present observation on
the body weight and weight gain in the animals and previous studies on the
permanent growth pattern of these animals (Desai & Hales 1997), suggest
that a reduction in general protein synthesis is a contributing factor in
maternal low protein diet model studies.

Other bands that consistently occur on GK Western blots were looked at and
there appeared to be no change in band intensity between the groups.
Therefore it would appear that a low protein diet does not cause a general
decrease of β-cell proteins.

Implications from the present investigation and proposals for future
investigations
Glucokinase belongs to a family of hexokinases (I-IV), and is also known as
hexokinase IV or D. In mammals the two principal organs that contain
abundant amounts of GK are liver and pancreatic islet β-cells and detectable
amounts of GK occur in the brain (Lynch et al 2000). Although hepatic and
pancreatic β-cell hexokinases are products of structurally related genes, their
developmental regulation and enzymatic kinetic properties are distinctly
different. In comparison with hepatic GK, relatively little information is
available on the pancreatic β-cell GK. As described in the preceding section of
the discussion, the main finding of the present investigation clearly
demonstrates that the low protein maternal diet during gestation and lactation
periods has a permanent effect on the levels of synthesis of GK and alters the
kinetic properties of the enzyme in the newborn as well as the adult offspring.
How and when GK is programmed in liver and pancreatic cells and its
subsequent role in the later development of NIDDM in man in relation to
reduced birth weight needs detailed investigation. Future studies should be
designed to elucidate the developmental time table and the molecular
processes and mechanisms involved in the GK programming. These studies
should also keep in view the basic ontogenetic and phylogenetic differences
between the animal model such as rat and human.
In the rat studies, the critical period for GK programming appears to be during
gestation therefore it would be of relevance to look in depth at this
development stage. It has been suggested by Hughes (1994), Tiedge & Lenzen (1993) and Taniguchi (2000) that fetal glucose metabolism is immature with reduced glucose induced insulin secretion of isolated islets as a result of reduced GK activity. However other work has disputed this finding (Tu & Tuch 1997). Therefore it is quite likely that the programming events which influence the GK protein synthesis and regulation of its affinity for glucose may occur during the maturation of islets and a reduced maternal protein diet may have a permanent effect on the function of β-cell specific processes involved in the regulation of the GK activity. The present study provides some support to this hitherto unknown mechanism. Similarly additional programming events may continue after birth which may affect the GK enzyme.

To understand this it will be important to unravel the mechanisms of GK regulation, which are still poorly understood. At the protein level it is known that liver and pancreatic GK are controlled differently. It has been shown that the functional activity of liver GK is regulated by the GK regulatory protein (GKRP) (Van Scaftingen & Davies 1991). The regulation of pancreatic GK by a similar mechanism is as yet not fully understood. It may involve interaction with the glucokinase associated protein (GKAP) (Munoz-Alonso, Guillemain, et al. 2000), pβPCCase (Shiraishi, Yamada, et al. 2001) and or PFK-2/FBPase-2 (Baltrusch, Lenzen, et al. 2001). It is possible that these proteins themselves may be programmed thus affecting GK activity or substrate binding ability and protein-protein interaction. If it is only GK protein itself that is programmed then it must occur through some mechanisms which not only affect the folding conformation of the molecule but also have a permanent pressure and lasting effect on the GK molecules to undergo parallel conformation during the adult period. Conformational changes resulting from natural mutations or by experimental mutagenesis of the GK gene have been shown to affect its glucose binding affinity.

For instance in some cases a MODY2 mutation causes a decrease in the GK $K_m$ for glucose increasing the enzyme affinity for glucose (Davis, Cuesta-Munoz, et al. 1999). On the other hand the result of another mutation such as
E279Q has an opposite effect in the sense that it increases $K_m$ values and reduces the GK affinity for glucose (Moukil et al. 2000). Moukil et al. (2000) also studied the regulatory properties of human islet GK by site-directed mutagenesis and showed that certain mutations increased the affinity for glucose by two fold (K296M) and six-fold (Y214A) and combining the two mutations with N166R resulted in a 50-fold decrease in the half-saturating substrate concentration ($S_{0.5}$) value, which became comparable to the $K_m$ of hexokinase II. The location of N166, Y214 and K296 positions in the three dimensional structure of GK suggests that these mutations act by favouring closure of the catalytic cleft. The mutations changed the affinity for glucose and for the competitive inhibitor mannoheptulose in parallel, where as they barely affected the affinity for N-acetylglucosamine. A small reduction in the affinity for the regulatory protein was observed with mutations of residues on the smaller domain and in the hinge region, confirming the bipartite nature of the binding site for the regulatory protein. The K296M mutant was found to have a three-fold decreased affinity for palmitoyl CoA. This effect in addition to that observed for the E279Q mutant (MODY mutation), indicated that the binding site for long-chain acyl CoA is located on the upper face of the larger domain. It is interesting to mention here that in the present investigation all group B rats (newborn, 3-week old and adult) born of mothers who were on a low protein diet during gestation and lactation periods, the islet GK affinity showed a 6-fold increased affinity for the glucose (see summary table 9,6). How could low protein maternal diet affect the folding of the GK molecule is a matter of speculation at present. Dhabi et al. (1997) have made some interesting observations on the endoplasmic reticulum (ER) chaperone gene expression in the liver of mice kept on calorie restricted (CR) diets. The authors have reported that in hepatocytes of CR-mice there was a general decrease of the mRNA and protein levels of a number of ER molecular chaperones. The mRNA response to diet occurred reproducibly only in liver and not in adipose, brain, heart, kidney, lung, muscle or small intestine. Thus the expression of nearly all ER chaperones responded rapidly and specifically to dietary energy in mice. These studies are important in the sense that they prove that dietary status can have a significant impact on basic and fundamental molecular processes.
such as protein folding which in turn can affect the biological properties and
activity of the molecules. There is no such work reported on islet GK and the
effect of maternal dietary status. GK is a cytoplasmic protein and like other
cytoplasmic proteins requires cytosolic molecular chaperones also known as
heat shock proteins (HSPs) for proper folding and acquiring a three-
dimensional structure. Hence it would be interesting to investigate further the
expression of the heat shock proteins in β-cells in the present rat model.
Further studies on the experimental rat islet GK should also include detailed
investigation on the inhibition kinetics of the enzyme to fully confirm the
changes in its reaction kinetics.

To fully understand the regulation of GK activity or level of GK it would be
important to investigate its transcriptional and translational regulation in the
pancreas. Desai, Byrne, et al. (1997) have performed similar studies on liver
GK, where they demonstrated that liver GK mRNA was reduced therefore
indicating that programming is initiated at the transcriptional level.
As discussed earlier in chapter 2 there are a variety of transcription factors
involved in pancreatic β-cell development (Docherty 2001). Since GK has a
prominent role in glucose metabolism in the whole body, it is likely that there
may be specific transcription factors in the islet for GK gene transcription
during development. Recently the transcription factor forkhead box a2 (Foxa2)
formerly known as HNF3beta has been found to regulate the expression of
genes important for glucose sensing in pancreatic β-cells. Messenger RNA
levels of GLUT2 and GK are drastically reduced after induction of Foxa2 and
loss of Foxa2 leads to up-regulation of HK I and II and GK mRNA expression
(Wang 2002). This transcription factor also controls insulin and glucagon gene
expression in order to maintain fasting blood glucose levels. What is of
interest is the possibility that Foxa2 may transactivate and be the upstream
controller of a number of other pancreatic transcription factors during
embryogenesis (Gerrish 2000) (Duncan 1998). Another factor that is known to
affect GK gene expression is the peroxisomal proliferator receptor gamma
(PPAR-gamma). PPAR-gamma is involved in the regulation of many genes
involved in insulin action, adipocyte differentiation, lipid metabolism and
inflammation (Lenhard 2001) and recently a peroxisomal proliferator response element in the GK β-cell specific promoter has been reported (Kim Hi, Cha, et al. 2002).

These transcription factors could themselves be affected by programming and in effect contribute to the programming of GK. The use of DNA microarray technology may be of some use in analysis of gene expression in β-cells from control and maternally programmed islets. A recent study using this technology on gene expression in the β-cell demonstrates a down regulation of certain genes in rats from small litters (Waterland & Garza C 2002).

A permanently reduced pancreatic GK activity may not necessarily bring on a diabetic state but may lead to a reduced glucose tolerance. This is evident in MODY2 (in which some cases have reduced activity and $K_m$) where the sufferers do not always develop symptoms until later in life and in fact the condition can sometimes go unnoticed. But it is the added factors (i.e. ageing and obesity) in later life that may contribute to the pathogenesis of diabetes. Numerous processes including deterioration of insulin secretion, insulin resistance, glucose intolerance as well as reduction of β-cell mass, occur during ageing in humans and animals (Chen 1988) (DeFronzo 1979) (Reaven 2002) (Wang 1988). The effect of malnourishment during gestation and the weaning period in rats showed that β-cell mass was reduced by 70% at the end of weaning and renutrition from weaning failed to fully restore β-cell mass in young adult rats. Glucose intolerance in later life was observed in these rats and was believed to be due to the additional demand placed by ageing on an already reduced mass of β-cells since the β-cell mass does not expand and apoptosis is increased in ageing (Garofano, Czernichow, et al. 1999). If GK is programmed to work at a lower activity in both the pancreas and the liver then there will be a slower clearance of postprandial blood glucose levels, this could contribute with other factors mainly ageing and obesity, to glucose toxicity of the β-cells resulting in β-cell failure and consequently diabetes. The general rationale of this investigation is the importance of a healthy diet in the prevention of later life complications of which NIDDM is of particular relevance. The importance of the effects of a low protein diet during
development and subsequent change of low to abundant nutrition in later life may have particular impact on the health of migrating populations particularly those from less developed countries and in countries with rapidly expanding economies.

This study has shown that glucokinase, the enzyme responsible for glucose sensing in the pancreatic β-cell is programmed to work at a lower activity due to both reduced amount of enzyme and altered affinity for substrate in response to a low protein diet during pre and post natal development. Further work is required to understand the effect of ageing, obesity and high carbohydrate and high fat diets on GK and the function of the pancreatic β-cell. The timing when the programming of GK occurs needs further studies at different developmental stages. Gene expression of GK should be studied along with other genes of suspected programmed products (i.e. Foxa2 and PPAR-gamma) using a variety of techniques including in-situ hybridisation, DNA microarray technology and proteomics in the developing pancreas.
APPENDIX 1

Statistical methods and presentation adopted in the thesis.

This appendix outlines the standard format used to present and analyse data within this thesis. All statistical tests were performed using a computer program entitled Graphpad prism® by Graphpad software incorporated.

1. Parametric data

Parametric data is data that is continuous and follows a standard ‘bell’ shaped Normal ‘Gaussian’ distribution. It can be accurately described by two parameters – mean and standard deviation (SD). All parametric data in this thesis is presented as mean with error bars representing standard error of the mean (SEM = SD/\( \sqrt{n} \), with \( n \) = sample size) as this improves the clarity of the figures.

Students two sample (unpaired) t-test

Comparisons of two independent normally distributed groups were performed using the Students two-sample t-test. The \( t \) statistic is the ration of the difference of sample means to the standard error of difference of the sample. The mean and SD for each sample are calculated \((m_1, SD_1, m_2, SD_2)\) from which a common SD is then obtained.

\[
s = \sqrt{\frac{SD_1^2(n_1-1) + (SD_2^2(n_2-1))}{n_1 + n_2-2}}
\]

\(s\) is used to calculate the common error of difference between the two means.

\[
SEM = s \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}
\]

The \( t \) value can be calculated and a probability value \((p)\) obtained from \( t \)-tables.
\[ t = \frac{(m_1 - m_2)}{SEM} \]

A large \( t \) indicates that the difference between the two groups means is larger than what would be expected from sampling variability and therefore the null hypothesis, that there is no difference, can be rejected. It should be noted that \( t \)-tests do not tell you anything about the size of the difference.

It is common practice for \( p \) values less than 0.05 to be called significant whilst those > 0.05 are sad to be not significant. However it is important to note that \( p \)-values are probabilities and there is no sudden changeover to values being likely or unlikely at some point of the distribution.

**F test to compare variance, from unpaired \( t \) test**

A standard \( t \) test assumes the two groups have equal variances. To test this assumption, the variance of each group (the variance equals the standard deviation squared) and the statistic \( F \), which equals the larger variance divided by the smaller variance are calculated. The degrees of freedom for the numerator and denominator equal the sample sizes minus 1. From \( F \) and the two df values, a \( P \) value is given that answers this question: If the two populations really have the same variance, what is the chance that you'd randomly select samples and end up with \( F \) as large (or larger) as observed in the experiment. If the \( p \) value is small, then you can conclude that the variances (and thus the standard deviations) are significantly different.

**One way ANOVA**

Where comparisons are made between more than two independent groups of data and there is no natural ordering to those sets, then the one-way analysis of variance is an appropriate way of determining whether the means of the groups differ more than could be expected by chance. The result of an ANOVA will be given as a \( p \)-value. This is the probability of obtaining differences in the means of the sample groups as large as those observed in the current samples if the population means of all the groups were identical.
2. Correlation and regression analysis

Correlation

Correlation is the method of analysis used when studying the possible association between two continuous variables. The degree of correlation is calculated as the correlation coefficient (r) which is a number between -1 and 1 which measures the degree to which two variables are linearly related. If there is perfect linear relationship with positive slope between the two variables, we have a correlation coefficient of 1; if there is positive correlation, whenever one variable has a high (low) value, so does the other. If there is a perfect linear relationship with negative slope between the two variables, we have a correlation coefficient of -1; if there is negative correlation, whenever one variable has a high (low) value, the other has a low (high) value. A correlation coefficient of 0 means that there is no linear relationship between the variables. For continuous data correlation is quantified by the Pearson's correlation coefficient.

Regression line analysis

Regression line analysis describes the relation between two continuous variables and allows the prediction of the value of one variable for an individual when only one set of variables is known. The least squares regression line can be described by the equation y = ax + b with a = the intercept at the y axis and b the slope. This line explains a proportion of the variability in the dependent variable (y) and the amount of unexpected variability. A means of assessing the goodness of fit of the line is to calculate the proportion of the total variation explained by the model. This can be done by considering the sum of the squares explained by the regression as a percentage of the total sum of squares. This is called $r^2$ (being the square of the correlation coefficient (r) of the two variables). Therefore $r^2$ expresses the % variance of the total data that can be explained by the dependant variable being determined.
Appendix 2

Individual results of insulin secretion, GK activity and GK protein content of all groups.

Insulin Secretion

Fig. A,1 Newborn group insulin secretion results.
Fig. A.2 3-Week group insulin secretion results.
Fig. A.3 Adult group insulin secretion results
Fig. A.4 Hexokinase & Glucokinase assays for newborn group results. HW plots = Hanes Woolf plots

Newborn Group A HK Assays

Newborn Group A HK HW Plots

Newborn Group A GK Assays

Newborn Group A GK HW Plots
Table A.1: Newborn rat group assay kinetic parameters obtained using the Hanes Woolf Plot.

<table>
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<tr>
<th>Reading</th>
<th>Hexokinase</th>
<th>Glucokinase</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>$K_m$</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.96</td>
<td>0.076</td>
</tr>
<tr>
<td>2</td>
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<td>0.016</td>
</tr>
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<td>3</td>
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<td>0.057</td>
</tr>
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<td>0.022</td>
</tr>
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<td>5</td>
<td>0.97</td>
<td>0.104</td>
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<td>6</td>
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Fig. A.5 3-Week group hexokinase & glucokinase assay results

3Wk Group A HK Assay

3Wk Group A HK HW Plots

3Wk Group A GK Assays

3Wk Group A GK HW Plots
Table A.2 3 week group HK and GK assay parameters obtained using the Hanes-Woolf plot.

<table>
<thead>
<tr>
<th>Reading</th>
<th>Hexokinase</th>
<th>Glucokinase</th>
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<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>Km</td>
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<td>0.148</td>
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<td>0.08</td>
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<td>2</td>
<td>0.99</td>
<td>0.056</td>
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<tr>
<td>3</td>
<td>0.98</td>
<td>0.057</td>
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<tr>
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<td>5</td>
<td>0.98</td>
<td>0.247</td>
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</tbody>
</table>

C

|         | $r^2$    | Km | Vmax | $r^2$ | Km | Vmax |
|---------|------------|-------------|
| 1       | 0.99    | 0.071 | 5.44 | 0.99 | 5.178 | 0.5032 |
| 2       | 0.99    | 0.08  | 25.06| 0.99 | 7.91 | 2.42 |
| 3       | 0.99    | 0.08  | 8.79 | 0.95 | 4.187 | 0.353 |
| 4       | 0.99    | 0.12  | 14.68| 0.68 | 25.2 | 1.118 |
| 5       | 0.97    | 0.153 | 7.994| 0.98 | 8.68 | 0.605 |

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Fig. A6 Adult group hexokinase & glucokinase results.

Adult Group A HK Assays

Adult Group A HK HW Plots

Adult Group A GK Assays

Adult Group A GK HW Plots
Table A.3 Adult HK and GK assay parameters as determined by the Hanes Woolf plot.

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<th>Glucokinase</th>
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Western Blot results

GK protein bands were measured using an optical density program Scion Image® and quantitated as a percentage of a liver control that was run on the same blot.

<table>
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<th>Group</th>
<th>Reading</th>
<th>% of Liver Control</th>
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<tr>
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<tr>
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<tr>
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<tr>
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Table A.4 Western blot results.
References


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