A STUDY OF THE FACTORS WHICH MAY INFLUENCE THE ISOLATION OF PORCINE ISLETS OF LANGERHANS

By

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STATEMENT OF ORIGINALITY

This dissertation has been produced from my own individual work at The University Of Leicester Department of Surgery From 1993 to 1996

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CONTENTS

DEDICATION

To Anna for all her encouragement and support
and to our daughters Mia and Sofi

To my parents for all their help over the years

Success is to be measured

not so much by the position that one

has reached in life as by the obstacles

which he has overcome while trying

to succeed.

Booker T. Washington

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PUBLICATIONS AND PRESENTATIONS FROM THIS THESIS

Principle publications

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ABBREVIATIONS

AD	Anno Domini
ADP	Adenosine diphosphate
AEP	Artificial endocrine pancreas
AMP	Adenosine monophosphate
AN	Autonomic neuropathy
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BC	Before Christ
BSA	Bovine serum albumin
C	Complement
cDNA	Copy deoxyribosenucleic acid
c.i.	Confidence interval
CIT	Conventional insulin therapy
CP	Chronic pancreatitis
CsA	Cyclosporin A
CSII	Continuous subcutaneous insulin infusion
CSSA	Cross sectional surface area
CVD	Cerebrovascular disease
DCCT	Diabetes Control and Complications Trial
DIC	Disseminated intravascular coagulation
DKA	Diabetic ketoacidosis
DMSO	Dimethyl sulphoxide
DN	Diabetic nephropathy
DNA	Deoxyribose nucleic acid
DNR	Diabetic neuropathy
DSG	15 Deoxyspergualin
ESRF	End stage renal failure
FA	Fatty acid
FAAF	2-furanacryloyl-L-phenanalyl-L-phenanalanine
FAAL	2-furanacryloyl-L-alanyl-L-lycine
FALGPA	2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine
g/day	Gram per day
GAD	Glutamic acid decarboxylase
GFR	Glomerular filtration rate
HB A1c	Glycosylated haemoglobin
HBSS	Hanks balanced salt solution
HCL	Hydrochloric acid
HDL	High density lipoprotein
HES	Hydroxethylstarch
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HOC	Hyperosmolar citrate solution
IAA	Insulin autoantibody
ICA	Islet cell antibody
ICC	Islet cell clusters
ICSA	Islet cell surface antibody
IDDM	Insulin dependent diabetes mellitus
IEQ	International islet equivalent
IFN	Interferon
IIT	Intensified insulin therapy
IL	Interleukin
IPITA	International Pancreas and Islet Transplant Association
KB	Ketone bodies
Kd	Kilodalton
Kg	Kilogram

LDL	Low density lipoprotein
LTC	Low temperature culture
MAA	Mannuronic acid alginate
MEM	Minimal essential medium
mg/day	Milligram per day
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
µm	Micrometer
MMF	Mycophenolate mofetil
mmol/l	Millimole per liter
mRNA	Messenger ribosenucleic acid
NAD	Nicotinamide adenine dinucleotide
NIDDM	Non insulin dependent diabetes mellitus
NO	Nitric oxide
°C	Degrees centigrade
PAK	Pancreas after kidney
PBS	Phosphate buffered saline
PFNA	Preformed natural antibody
PTA	Pancreas transpant alone
PVD	Peripheral vascular disease
SGE-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SPK	Simultaneous pancreas kidney
TcR	T cell receptor
TNF	Tumour necrosis factor
TOOS	Sodium N-ethyl-N-(2hydroxy-3-Sulfopropyl)-m-toluidine
UAE	Urinary albumin excretion
UK	United Kingdom
ULEIC	University of Leicester solution
USA	United States of America
UW	University of Wisconsin solution
VLDL	Very low density lipoprotein
VPT	Vascularised pancreas transplant
WHO	World Health Organisation

ABSTRACT

The recent multi-centre Diabetes Control and Complications Trial has definitively demonstrated the beneficial effect of intensified subcutaneous insulin therapy in slowing the progression and preventing the development of the chronic irreversible secondary complications of diabetes. Nevertheless, this puts the patient at a three times greater of developing severe hypoglycaemia suggesting that an alternative treatment should be found.

Such a treatment could be pancreas transplantation but it is a major surgical procedure with a high morbidity and mortality, whereas an islet transplant can be performed under a local anaesthetic and is much less invasive. Also, with the current worldwide shortage of human organ donors, investigators have been forced to seek an alternative source of donor tissue. One such source could be the porcine pancreas but porcine islet isolation is well known to be extremely variable. The object of this thesis was to improve upon some of the current methods of porcine islet isolation and to identify some of the reasons for its variability.

One of the most important factors effecting porcine islet isolation is the number of islets within the pancreas and this was assessed using computer image analysis and demonstrated significant differences between pancreata in terms of the percentage CSSA density. A large proportion of the work for this thesis was committed to the collagenase digestion phase of porcine pancreata. Part of this work was to develop a cold storage solution (ULEIC), specifically for porcine islet isolation. I also looked at the distribution of the collagen substrate (Types I, III, IV, V and VI) within the pancreas though no differences were apparent between pancreata but collagen type VI was the most predominant throughout. Furthermore, it is feasible that the pancreas is also digested by endogenous pancreatic enzymes released during the dispersion phase, as well as by collagenase, but little is known of their activation within the digestion circuit. Again, this showed extreme variability between isolations. Lastly, if porcine islet isolation were to be routinely used for xenotransplantation, there are many concerns over the infective potential of porcine islets but this can be minimised by using specific antibiotic regimens and careful aseptic isolation techniques. Overall there are many factors limiting porcine isolation, the reason for its variability appears to be multifactorial.

CHAPTER ONE

DIABETES MELLITUS

1.0 Introduction

1.1a The millenia before insulin

Diabetes mellitus is an affliction of antiquity, the word 'diabetes' is derived from the Greek 'διαβητης ' meaning 'to run through a siphon', first quoted by Aretaeus of Cappadocia (81-138 AD). He became one of the first to be familiar with some of the symptoms of diabetes based upon observations that' *patients never stop making water but the flow is incessant, as if from the opening of aqueducts. .....More over life is disgusting and painful; thirst unquenchable.'* (Adams, 1856). However, it was not until the Papyrus Ebers of 1500BC were discovered at El Assassif, Thebes, Egypt in 1862 by the German George M. Ebers (1837-1898) that some remarkably primitive descriptions of diabetes were first recognised in modern times. Also inscribed on this document was a prescription for the treatment of 'over abundant urine' which consisted of bones, wheat grains, fresh grits, green lead earth and water (Papaspyros, 1964).

The Old Sanskrit texts by Susruta from India (400 BC) similarly made reference to 'madhumeha' (urine of honey). Galen (129-199 AD), the Greek philosopher and physician also described a disease likely to be diabetes 'diarrhoea of urine'. Oriental physicians of China and Japan from the 2nd and 3rd centuries had also seen the passage of copious urine and noticed its associated sweetness (Wong and Wu, 1932). Avicenna (960-1037 AD), an eminent Arabian physician, also described gangrene, carbuncles and collapse of sexual function in combination with the 'sweetness of urine' (Barach, 1928).

Heralding the 'diagnostic era' of the disease in 1674 Thomas Willis, re-discovered the sweetness of urine, observed in ancient Greece some 1,000 years earlier. He hypothesized that this was due to sugar passing from the blood into the urine, thus dismissing previous allegations that the organ of default was the kidney. He then went on to distinguish diabetes mellitus from diabetes insipidus, the latter being deficient of anti-diuretic hormone (Willis, 1674). One hundred years on it was generally accepted that glycosuria was a characteristic feature of the disease and consequently William Cullen introduced the term 'mellitus' (from the Latin and Greek meaning 'honey') so as to differentiate from diabetes insipidus (Cullen, 1787).

During the 19th century Thomas Cawley originally suggested an association between pancreatitis and diabetes (Cawley, 1788). Shortly after the discovery by Paul

Langerhans of pancreatic 'clusters of cells' in 1869, Lancereaux (Lancereaux, 1881) and Frerichs (Frerichs, 1884) unequivocally twined diabetes with pancreatic pathology. It was then left to Von Mering and Minkowski (Von Mering and Minkowski, 1890) who showed, without doubt, that pancreatectomy in dogs induced a *'diabetic syndrome'*. A few years later Laguesse (Laguesse, 1893) summized that perhaps the 'islets of Langerhans', a description he proposed, secreted an unidentified substrate capable of reducing blood glucose which prevented this diabetic syndrome.

Some fifty years later the successful isolation of this 'blood sugar lowering hormone' by Banting and Best in July of 1921, marked an unprecedented step towards the treatment of diabetes mellitus. By administering crudely prepared extracts from canine and fetal calf pancreata, they successfully ameliorated the diabetogenic state in pancreatectomized dogs and subsequently in humans in January of 1922 (Banting and Best, 1922; Banting *et al.* 1922). Their primitive extracts, originally called 'isletin', were later to be called 'insulin' (Latin, 'insula' meaning island) a term which had already been used by Jean de Meyer (De Mayer, 1909), although at that time its existence was still purely speculative. Finally, during 1938 the source of insulin secretion, from the islet beta cell, was finally discovered (Richardson and Young, 1938).

1.1b Definition

The hallmark of diabetes mellitus is hyperglycaemia provoked by a chronic multi-systemic disorder of carbohydrate, lipid and protein metabolism. The absolute definition proposed by the World Health Organisation (WHO; WHO Study Group, 1985) states that diabetes is diagnosed when patients have two fasting venous plasma glucose levels in excess of 7.8mmol /l or a random sample with a level greater than 11.1 mmol/l. If doubt exists a formal 75 grams oral glucose tolerance test with a 2 hour post meal venous plasma glucose level in excess of 11.1 mmol /l would confirm the diagnosis.

1.1c Classification

The WHO have standardised the diagnostic criteria for diabetes and adopted a classification essential for treatment regimes (WHO Study Group, 1985; 1994). The current WHO classification distinguishes two major subclasses, Type 1 and Type 2 diabetes mellitus, by the necessity to administer exogenous insulin in the former Type.

Insulin dependent diabetes mellitus (IDDM) or Type 1 diabetes mellitus is widely accepted to be an autoimmune disease characterised by beta cell destruction (Bottazzo *et al.* 1974; Eisenbarth, 1986). This results in absolute insulin deficiency

CLASSIFICATION OF DIABETES MELLITUS

A Clinical classes

Diabetes mellitus

* Insulin dependent diabetes mellitus

* Non-insulin dependent diabetes mellitus

(a) non-obese
(b) obese

*Malnutrition related diabetes mellitus.

*Others associated with conditions and syndromes:

(i) pancreatic disease
(ii) disease of hormone aetiology
(iii) drug induced or chemical
(iv) abnormalities of insulin or receptor
(v) genetic syndromes
(vi) miscellaneous

*Impaired glucose tolerance

(a) non obese
(b) obese
(c) associated conditions or syndromes.

*Gestational diabetes mellitus.

B Statistical risk classes (normal glucose tolerance but increased risk to diabetes mellitus)

*Previous abnormality of glucose tolerance (latent diabetes)

*Potential abnormality of glucose tolerance.

Table 1.1 Classification of diabetes mellitus

with a tendency to develop ketosis and a dependence on exogenous insulin in order to maintain life. In contrast, Type 2 or non-insulin dependent (NIDDM) diabetic patients, although often asymptomatic and not prone to developing ketosis, do not depend on insulin to control hyperglycaemia but require additional insulin if diet alone or oral

hypoglycaemic drugs are not effective. Overall, patients with IDDM constitute less than 10% of all persons with diabetes (Selby *et al.* 1990).

There are other classes of diabetes mellitus and other allied categories of glucose intolerance which are included in the WHO classification *(Table 1.1 p.4)*. For example, malnutrition diabetes which occurs primarily in undernourished children from developing countries and gestational diabetes which presents or is first recognised during pregnancy. Another distinct category is impaired glucose tolerance. This is not diabetes mellitus *per se*, but is merely a deviation outside the normal range of blood glucose seen in healthy adults. There are many other types of diabetes that are associated with certain conditions and syndromes but the principles of this thesis concern the transplantation of insulin producing tissue in juvenile onset Type 1 diabetic patients.

1.1d Epidemiology and incidence

Diabetes mellitus is one of the most common chronic diseases with an overall prevalence in the white Caucasian population of approximately 2 %. Current geographical trends reflect the genetic susceptibility between markedly different populations throughout the world (Karvonen *et al.* 1993). Unfortunately, in contrast to the extensive epidemiological studies performed mainly in Europe and USA (United States of America), there is a lack of conclusive data from the African continent.

It is now appreciated that the highest incidences of Type 1 diabetes mellitus appear in the countries of the Northern Hemisphere among them Scandinavia and USA. Those of the Southern Hemisphere, particularly Japan and Korea, have some of the lowest recorded incidences (Diabetes Epidemiology Research International Group, 1988). Throughout the world the distribution of diabetes, greater than that of any other autoimmune disease is reflected by race. Caucasians are more vulnerable in comparison to those of Black descent (Lorenzi *et al.* 1985; Lipman, 1993) and those of oriental descent (Lorenzi *et al.* 1985; Wong *et al.* 1993), that live in the same communities. IDDM is most commonly diagnosed during the ages 10 to 14 years with the lowest prevalence being from birth to 4 years (Green *et al.* 1992). Controversy still surrounds any predisposition between males or females to developing IDDM. The previous notion that there is a slight male preponderance was not statistically proven in the recent multi-centre Eurodiab Ace Study, although it has been argued that there is perhaps a slight male excess in areas of high incidence (Tuomilehto *et al.* 1992).

Within the European community there is a wide 10-fold variation of incidence. Incidence rates also vary between countries that are adjacent. For example a recent analysis of four Baltic countries shows that the incidence is increasing in Finland and Lithuania while in Latvia and Estonia it is stable (Padaiga *et al.* 1997). Finland has the highest overall rate anywhere in the world *(Figure 1.1* ; 35.3 cases per 100,000 population; Tuomilehto *et al.* 1992) in striking contrast to the age adjusted incidence of Macedonia (southern republic of former Yugoslavia) where there are an estimated 2.45 cases per 100,000 (Kocova *et al.* 1993). This "cold spot" is also in keeping with the 4.6 cases per 100,000 incidence observed in the neighbouring area of northern Greece (Green *et al.* 1992). This pattern of variation is also seen across the Atlantic Ocean with the highest incidence of 23.9 cases per 100,000 reported on Prince Edward Island (Canada) contrasting with 0.6 cases per 100, 000 in Mexico. With respect to the UK the Eurodiab Ace Study has reported an incidence of 16.4 cases per 100,000 in and around the Oxford region (Green *et al.* 1992) consistent with previous studies quoting 13.5 to 13.7 per 100,000 in other areas of the UK (Metcalfe and Baum, 1991; Staines *et al.* 1993).

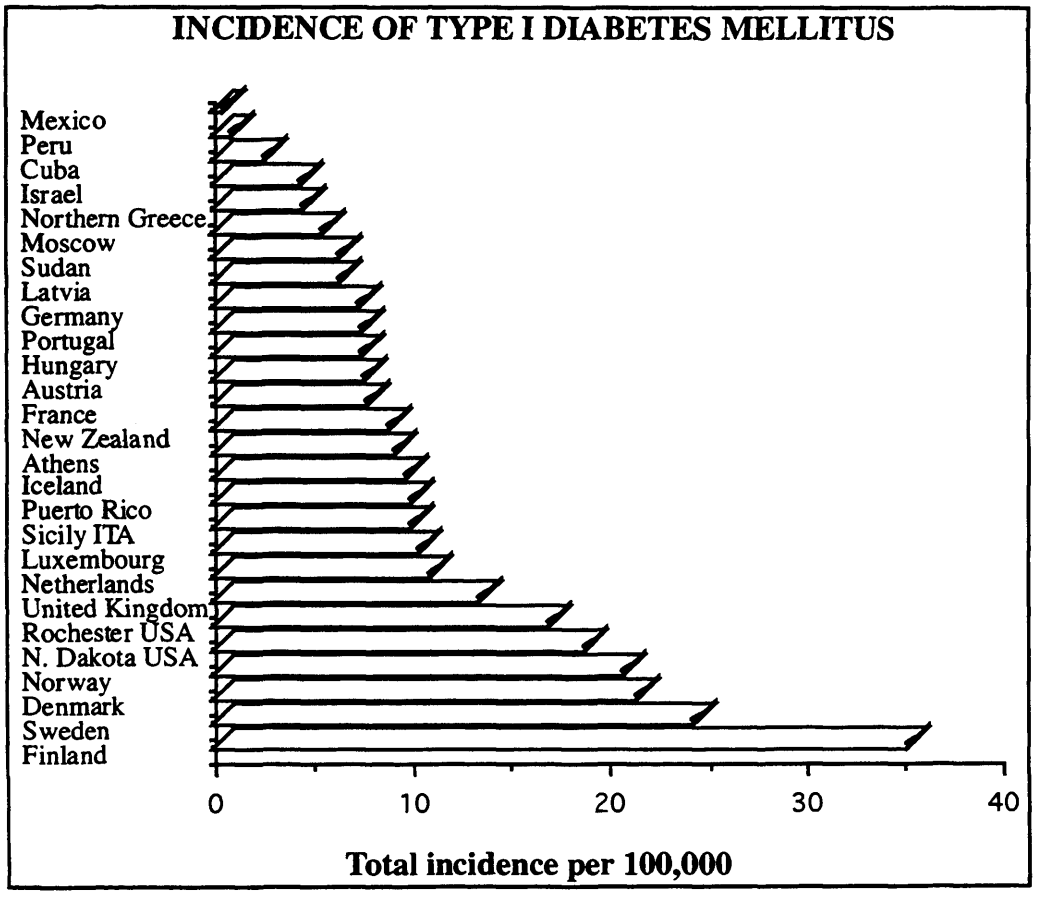

Figure 1.1 Incidence of worldwide Type 1 diabetes mellitus

Current trends have suggested that Type 1 diabetes mellitus is on the increase (Bingley and Gale, 1988; Burden *et al.* 1989; Gardner *et al.* 1997), especially in males.

This increasing trend has been highlighted by a study in Sardinia where a 29-fold increase between the late 1930's and the 1960's birth cohorts was shown (Songini *et al.* 1993), contrary to the generally lower incidence rates seen in the rest of Southern Europe (Green *et al.* 1992). Nonetheless, it has been proposed that the life time incidence is not increasing and that these changes are perhaps attributable to the disease merely manifesting itself at a younger age in genetically susceptible individuals (Kurtz *et al.* 1988). In summary, it can be deduced that Type 1 diabetes is still a significant cause of morbidity in our society and although insulin has reduced the acute metabolic complications, its palliative effect has failed to stop the incidence of complications from increasing throughout the world.

1.2 The islets of Langerhans and insulin biosynthesis

1.2a The islets of Langerhans

The islets of Langerhans are ovoid clusters of cells scattered throughout the pancreas *(Figure 1.2 p.8)*. There are up to 2 million islets per non-diabetic human pancreas (Saito *et al.* 1978) making up 2% of all pancreatic tissue. The rest of the pancreas serves an exocrine function involving the synthesis, storage and secretion of digestive enzymes.

Human islets may vary in size from only a few cells to 5,000 with individual diameters ranging from 40 to 400 μm (Weir and Bonner-Weir, 1990). The size of other mammalian islets are also of comparable size (Ulrichs *et al.* 1994a). The volume density of islets in both pig and human pancreata are greater in the tail (splenic) than the head of the pancreas (Saito *et al.* 1978), decreasing in number as one progresses towards the duodenal flexure (Petkov *et al.* 1971; Marchetti *et al.* 1990). The distribution of endocrine cell types within the islets, and throughout the pancreas, is not random but represents a precise organ topography. Islets within the head, tail and body of the human pancreas contain proportionately more beta cells in comparison to more polypeptide secreting cells in islets of the duodenal portion (Atkinson and Maclaren, 1993).

A fine capsule of reticular fibres, predominantly of collagen, surrounds both human (van Deijnen *et al.* 1994) and porcine islets (Ulrichs *et al.* 1994a; van Deijnen *et al.* 1994) separating them from neighbouring exocrine tissue. In the case of isolated porcine islets only half have a detectable capsule, (van Deijnen *et al.* 1994) accounting for their increased fragility during islet isolation (Ulrichs *et al.* 1994a). A more detailed

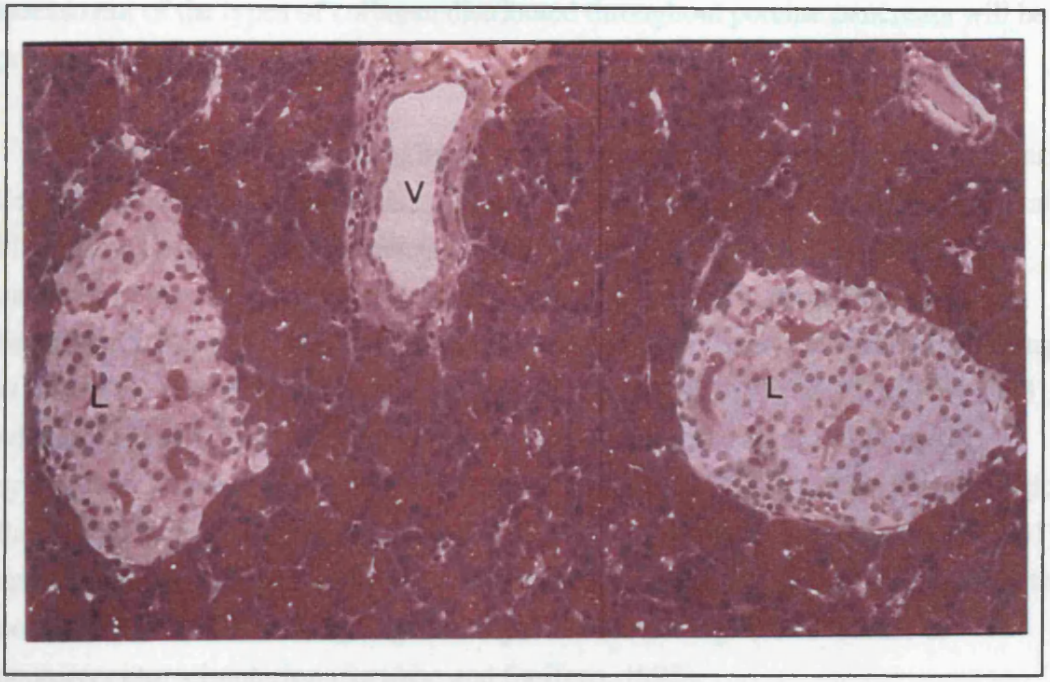

Figure 1.2 Haematoxylin and eosin stain of human islets of Langerhans (Islet-L; V-venule)

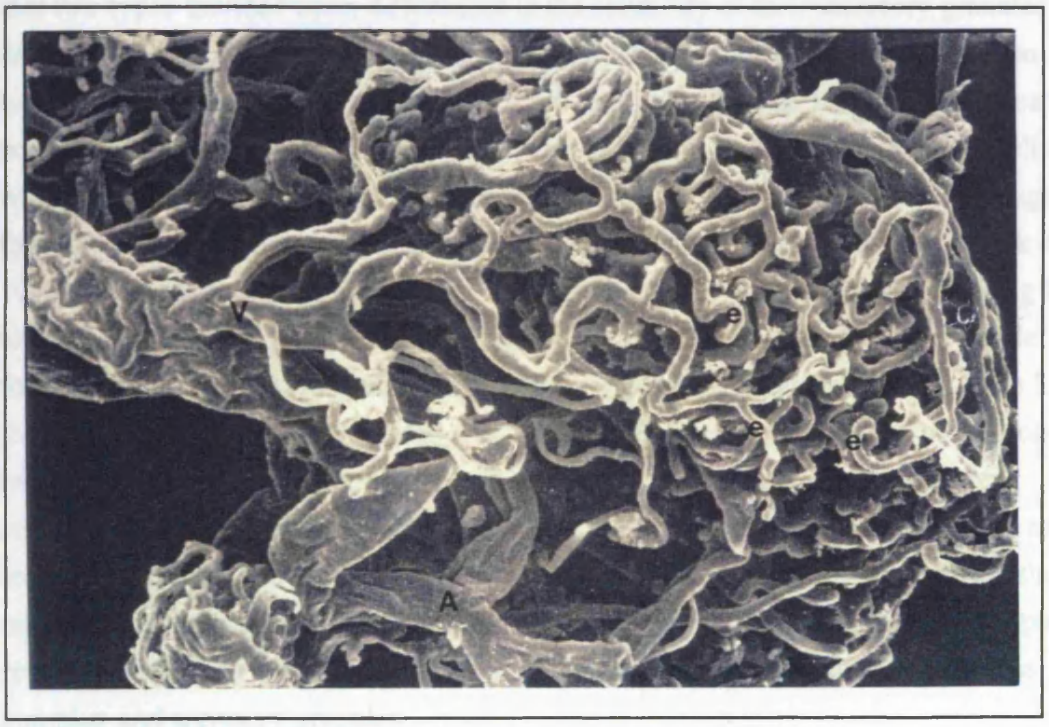

Figure 1.3 A scanning electronmicrograph demonstrating the extensive vascularity of islets (V-venule; A-arteriole; e-end capillary)

assessment of the types of collagen distributed throughout porcine pancreata will be presented in Chapter Five.

The islets are surrounded by an extensive labyrinth of anastomosing capillaries lined with fenestrated endothelium *(Figure 1.3 p.8)* requiring up to 10% of the total blood flow to the pancreas (Bencosme and Pease, 1958; Lifson *et al.* 1980). The direction of blood flow is to the beta cell core and then to the periphery, hormones being directly secreted into the portal vein, thus optimising hepatic metabolism (Stagner *et al.* 1988). The nerve supply of cells within an islet (Strubbe and Steffens, 1993), which can reinnervate following transplantation (Korsgren *et al.* 1993), are of sympathetic origin via the splanchnic nerves (Fyles *et al.* 1986) and parasympathetic via the vagus nerve (Malaisse Lagae *et al.* 1967). Studies on porcine (Holst *et al.* 1981) and human (Ahren *et al.* 1986) islet neuroendocrine secretion have also suggested the possibility of a noncholinergic (peptidergic and serotonergic) mechanism of neuroreceptor stimulation (Strubbe and Steffens, 1993).

The cells within an islet have been divided into four types based upon their morphology and function *(Figure 1.4 p.10).* In 1907, Lane (1907) subdivided islet cells into two types founded upon differences in the solubility of their secretory granules and he designated these as alpha and beta cells. Subsequent studies and improvements on histological staining revealed other cell types: the delta (Bloom, 1931) and pancreatic polypeptide secreting cells (Larsson *et al.* 1975). The products of the different cell types within an islet are insulin from the beta cell (Hartroft and Wrenshall, 1955), glucagon from the alpha cell (Baum *et al.* 1962) and somatostatin from the delta cells (Polak *et al.* 1975). Beta cells, concentrated in the core, are the most numerous cell accounting for 60 to 80% (Stefan *et al.* 1982; Weir and Bonner-Weir, 1990) of all cells within an islet followed by polypeptide secreting cells in the periphery making up a further 10 to 35% (Malaisse Lagae *et al.* 1979; Stefan *et al.* 1982). More recently, a degree of heterogeneity has been demonstrated amongst the beta cell population because of their different responses to insulin secretion (Pipeleers, 1992). Except for the specific structure of the secretory granules, the ultrastructure of human islets do not markedlydiffer from those of other mammalian islets (Marchetti *et al.* 1990). They resemble that of any polypeptide producing cell with a diffuse network of rough endoplasmic reticulum, polysomes, golgi complex and secretory granules.

1.2b Insulin biosynthesis

In 1955 Frederick Sanger's group (Brown *et al.* 1955) first described the primary sequence of porcine insulin consisting of an alpha chain of 21 residues and a beta chain of 30 residues, covalently linked by two disulphide bonds. Remarkably the same pattern existed in humans, the only difference being threonine at position 30 on its beta chain *(Figure 1.5 p.11)*.

Figure 1.4 Electronmicrograph illustrating alpha, beta and delta cells within an islet.

A breakthrough in the understanding of insulin biosynthesis came when Donald Steiner (Steiner and Oyer, 1967) discovered large quantities of a protein, difficult to distinguish from insulin, within the beta cells of a rare islet cell adenoma. Subsequent studies revealed this protein to be a precursor of insulin, called proinsulin. This single polypeptide chain contained a connecting, biochemically inert fragment given the name c-peptide (Rubenstein *et al.* 1969). This fragment is cleaved off and secreted along with insulin from the islet beta cells. As with slight differences in human insulin, the porcine c-peptide differs from that of human c-peptide at 11 amino acid residues (Chance *et al.* 1968). This distinct c-peptide sequence would undoubtedly be

advantageous after porcine islet xenotransplantation, allowing differentiation of xenografted beta cell function from that of residual human beta cell function.

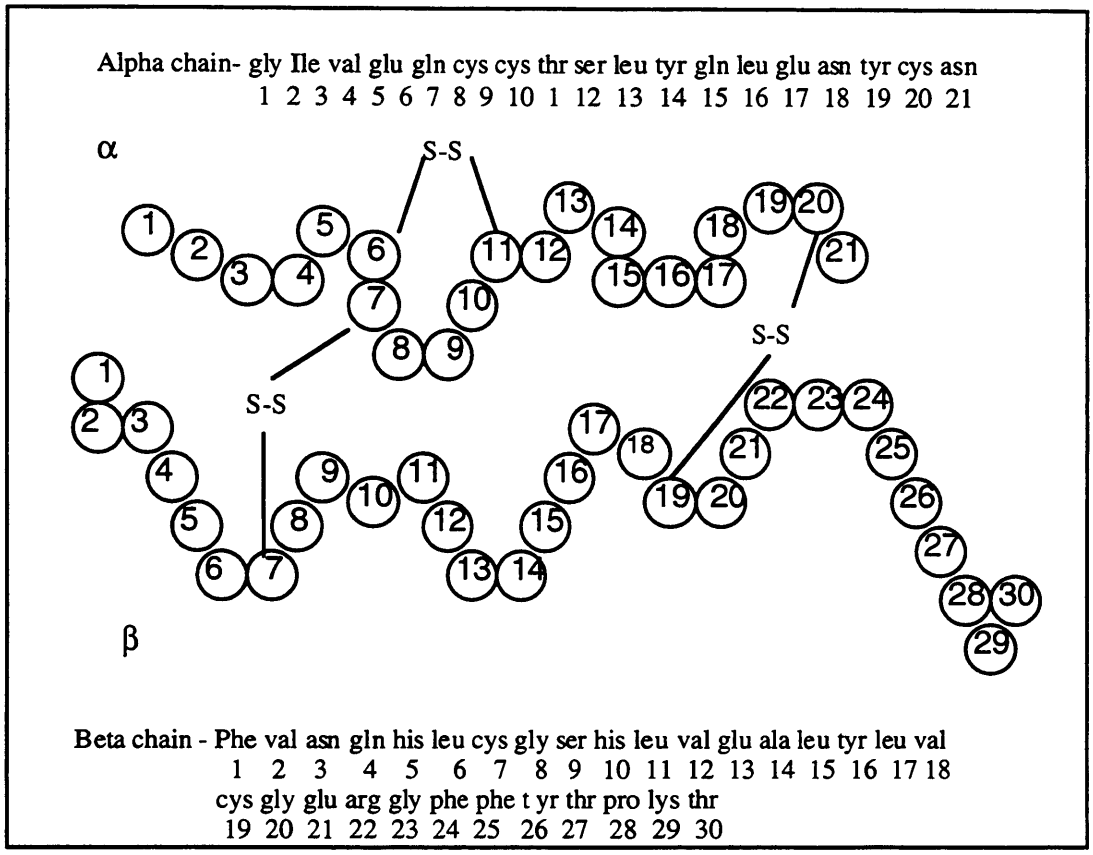

Figure 1.5 The primary sequence of human insulin

Subsequent studies have revealed that proinsulin is not the earliest form of the hormone, preproinsulin derived from translation of mRNA is its precursor. This is rapidly discharged into the rough endoplasmic reticulum. After removal of a receptor sequence, the newly formed proinsulin is transported in microvesicles to the trans golgi network where proteolysis of its connecting peptides begin (Orci, 1984). Two endoproteases, PC2 and PC3 are involved in the processing of proinsulin to yield 32-33 and 65-66 split proinsulin. Removal of basic amino acide pairs produces des 31-32 and des 64-65 proinsulin respectively (Sobey *et al.* 1989). Trypsin and carboxypeptidase-B like enzymes (Kemmler *et al.* 1971) remove the c-peptide, forming insulin molecules which coprecipitate with zinc (Emdin *et al.* 1966) to form a three dimensional hexamer of insulin microcrystals within the secretory granule (Blundell *et al.* 1972; Steiner, 1973). The insulin molecules in mature storage granules are then secreted by exocytosis along with its cleaved c-peptide *(Figure 1.6 p.12)* when the membrane of the granule fuses with the plasma membrane of the beta cell after stimulation by glucose (Lacy, 1970).

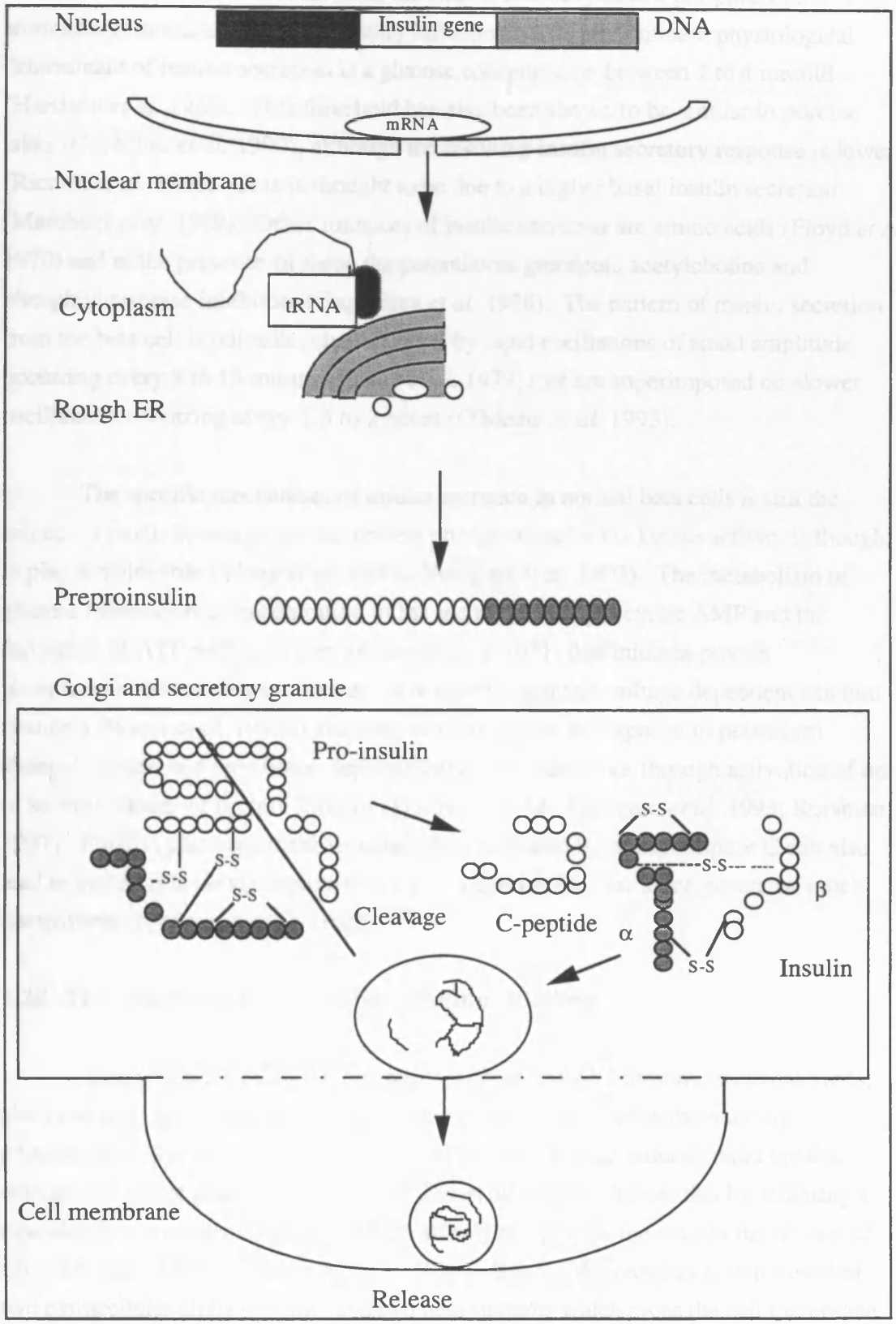

Figure 1.6 Insulin processing and secretion

1.2c Physiology of insulin secretion

The secretion of insulin from the beta cells is subject to a pot-pourri of stimulatory, modulatory and inhibitory influences. The predominant physiological determinant of insulin secretion is a glucose concentration between 2 to 4 mmol/l (Harrison *et al.* 1985). This threshold has also been shown to be similar in porcine islets (Crowther *et al.* 1990), although the resulting insulin secretory response is lower (Ricordi *et al.* 1986). This is thought to be due to a higher basal insulin secretion (Marchetti *et al.* 1989). Other initiators of insulin secretion are amino acids (Floyd *et al.* 1970) and in the presence of these the potentiators glucagon, acetylcholine and phosphodiesterase inhibitors (Gagerman *et al.* 1978). The pattern of insulin secretion from the beta cell is pulsatile, characterized by rapid oscillations of small amplitude occurring every 8 to 15 minutes (Lang *et al.* 1979) that are superimposed on slower oscillations occurring every 1.5 to 2 hours (O'Meara *et al.* 1993).

The specific mechanism of insulin secretion in normal beta cells is still the subject of much investigation but protein phosphorylation via kinase activity is thought to play a major role (Wang *et al.* 1993a; Newgard *et al.* 1993). The metabolism of glucose increases the concentration of the second messenger cyclic AMP and the formation of ATP within an islet (Ashcroft *et al.* 1971) that initiates protein phosphorylation via protein kinases. It is also thought that voltage dependent calcium channels (Wang *et al.* 1993a) allowing calcium influx, in response to potassium channel closure and membrane depolarization, may also work through activation of one of several classes of protein kinases (*Figure 1.7 p.14* ; Newgard *et al.* 1993; Rorsman 1997). Finally, phosphoinositide metabolism mediated by phospholipase C can also lead to insulin release via protein C activity, a concept that has since generated much controversy (Montague *et al.* 1985).

1.2d The physiology of insulin receptor binding

Along with the catabolic hormones such as growth hormone, corticosteriods, glucagon and catecholamines, insulin forms a vital component in maintaining physiological fasting blood glucose concentrations. Insulin induces rapid uptake, storage and use of glucose (anabolic) in almost all tissues. It does this by initiating a cascade of events after binding to a high affinity receptor on the plasma membrane of cells (Olefsky, 1990; Becker and Roth, 1990). Briefly, the receptor is composed of two extracellular alpha subunits and two beta subunits which cross the cell membrane (Jacobs and Cuatrecasas, 1981). The sequence of events after insulin receptor binding appears to be mediated by protein phosphorylation (Frattali *et al.* 1992), particularly of

serine, threonine and tyrosine kinase residues, but after this process the exact second messenger of postreceptor binding is still unknown (Garvey and Birnbaum, 1993; Stralfors, 1997) but is thought to be via a signal transduction step involving the insulin receptor substrates IRS-1 and IRS-2 (Duckworth *et al.* 1997).

To prevent persistent hyperglycaemia insulin induces glucose storage as glycogen mainly in muscle and liver by the activation of glycogen synthetase (Dent *et al.* 1990). Adipose tissue takes up glucose to produce fatty acids and triglycerides which can then be oxidised to form energy. If there is an excess of glucose entering hepatocytes it can be converted to fatty acids which are then packaged as triglycerides for transport to adipose tissue. Insulin also promotes active transport of amino acids into cells for protein metabolism. Finally, the action of insulin is antagonised by glucagon through inhibition of gluconeogenesis and glycogenolysis.

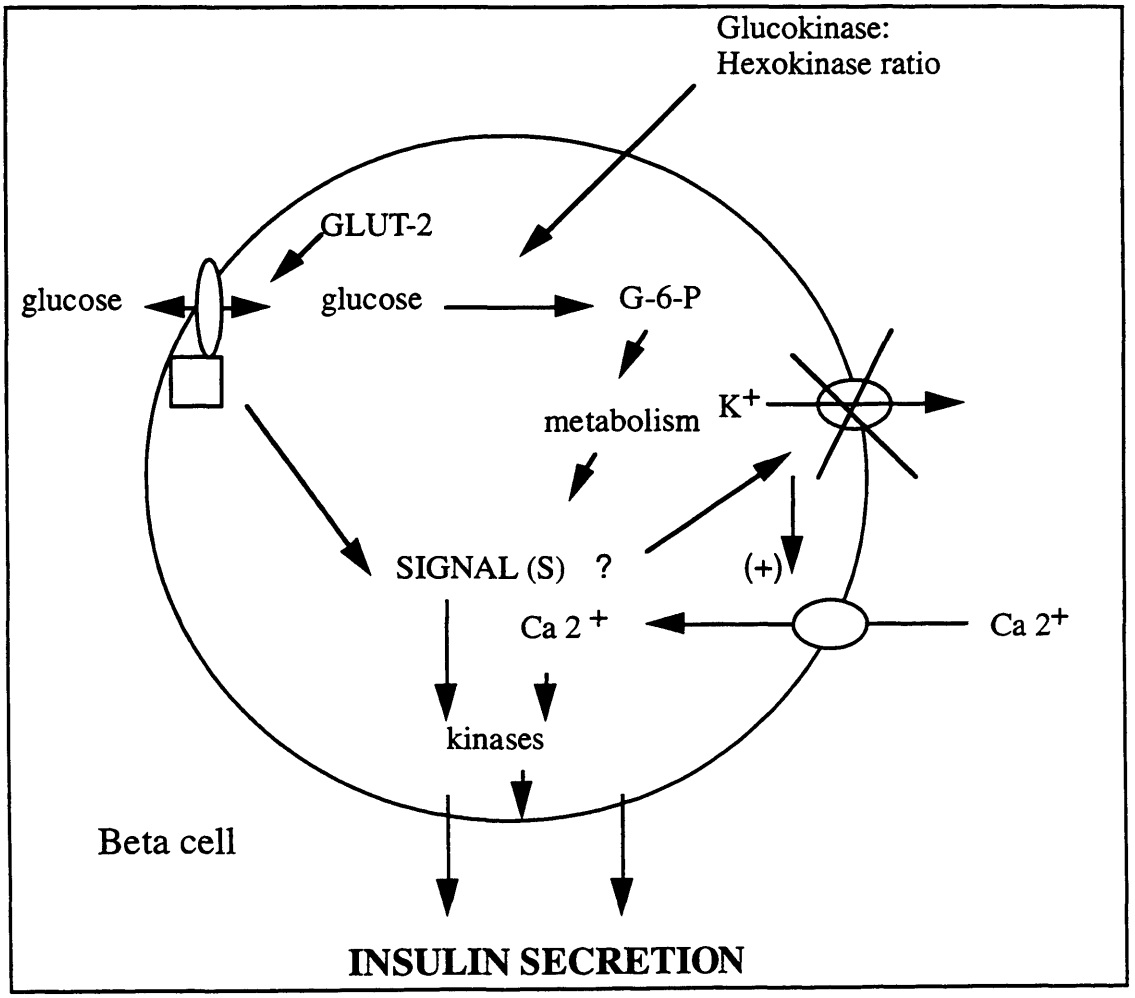

Figure 1.7 Biochemistry of insulin secretion

After insulin receptor binding the uptake of glucose is initially limited due to the impermeability of the plasma membrane to glucose. This is overcome by the availability of sodium-glucose transporters and facilitative transporters comprising low density microsomes or transmembrane transporters. These exist in five isoforms GLUT 1 to 5. The concentrations are cell dependent, GLUT-1 being expressed in most cells, GLUT-4 predominantly in adipose and muscle (Bell *et al.* 1990) whereas GLUT-2 is found most frequently in the beta cells of pancreatic islets (Newgard *et al.* 1993). Insulin plays a key role in glucose transport by augmenting redistribution of these isoforms to the cell surface during hyperglycaemic states enabling rapid intracellular glucose transport (Devaskar and Mueckler, 1991). Glucose is then phosphorylated by hexokinase II forming glucose 6-phosphate which is the substrate of many metabolic pathways *(Figure 1.7 p.14)*.

1.3 Aetiology of IDDM

1.3a Viral pathogens

It has long been speculated that there could be a causal relationship between viral illness and the onset of diabetes (Harris, 1899; Gundersen, 1927; Szopa *et al.* 1993). Accumulating epidemiological evidence has confirmed an increase in the incidence of diabetes comparable to epidemic proportions (Rewers *et al.* 1988; WHO DIAMOND, 1992). This evidence is based upon the seasonal variation of diagnosis where some studies have shown a less frequent rate of diagnosis during the cooler winter months (Bingley and Gale, 1988; Wagenknecht *et al.* 1991; Tuomilehto *et al.* 1992; Green *et al.* 1992) but others have shown this to be an age related and geographical phenomenon (Lévy-Marchal *et al.* 1995). In conjunction with the well known ability of common human viruses to infect and destroy human islet cells, the possibility of viral pathogens being a prerequisite for the onset of the disease has been continually hypothesized.

Coxsackie virus, picornavirus, has commonly been documented to be associated with the onset of IDDM. Epidemiological studies in favour of Coxsackie virus have shown a significant excess of diabetic patients with antibody to Coxsackie B4 (Gamble *et al.* 1969; Banatvala *et al.* 1985) and B5 (Wagenknecht *et al.* 1991) when compared to healthy controls. However, an association with insulin autoantibodies (IAA) has not been supportive of these findings (Karjalainen *et al.* 1988). More convincingly, the viral strains B4 (Yoon *et al.* 1979) and B5 (Champsaur *et al.* 1980) have been isolated from human pancreata of patients after the onset of IDDM and *in vivo* evidence suggests that B4 infection can lead to an impaired secretion of insulin

(Szopa *et al.* 1989). Further evidence supportive for Coxsackie virus stems from studies which implicate molecular mimicry between Coxsackie B and the beta cell glutamic acid decarboxylase enzyme, thus a T lymphocyte response against Coxsackie B may eventually destroy pancreatic beta cells (Solimena and De Camilli, 1995).

The pathogenesis of diabetes has also been linked to congenital rubella infection. This has been strengthened by clinical and experimental reports of up to 20% of children with Congenital Rubella Syndrome, later developing diabetes (Menser *et al.* 1978). An explanation for this predisposition could be because of the virus possibly residing in T lymphocytes which may then be capable of triggering an autoimmune process (Rabinowe *et al.* 1986).

There have been periodic reports which have implicated mumps (Kremer, 1947; McCrae, 1975) as a cause for diabetes but with an illness so common the connection is difficult to prove. Notwithstanding this view, studies have also failed to show any association between the presence of islet cell antibodies (ICA) and antecedent mumps infection (Bodansky *et al.* 1986; Scherbaum *et al.* 1991) or between infection and the later onset of Type 1 diabetes (Karjalainen *et al.* 1988). Now, with the widespread introduction of the mumps, measles and rubella vaccine, the risk for diabetes may decrease and this has been substantiated by a fall in mumps antibody levels in diabetic children (Hyöty *et al.* 1993).

Other less frequently reported viruses in association with diabetes are Cytomeglovirus (CMV; Ward *et al.* 1979), echoviruses and Epstein-Barr virus (EBV). In a previous epidemic of Echo-4 virus in Cuba it was shown that 36% of those affected developed ICA (Uriarte *et al.* 1987) but again these findings are inconclusive. Also, Foulis *et al.* (1997) were not able to identify either CMV, EBV or enteroviruses in 29 pancreata that were analysed in patients dying from recent onset Type 1 diabetes, suggesting that an acute or persisting infection at the time of diagnosis is unlikely.

In summary one can assume that viruses may be diabetogenic in man and perhaps possess the ability to precipitate subtle immunological changes that could provoke the onset of an autoimmune attack but at present their overall importance is still unclear.

1.3b Genetic susceptibility genes

The familial nature of diabetes has been appreciated for many years but the specific mode of inheritence is still unknown. Evidence for a genetic determinant in the

aetiology of IDDM arises because of observations of incomplete concordance (30 to 55%) between identical twins (Tattersall and Pyke, 1972; Barnett *et al.* 1981), recently reviewed by Hawkes, (1997). Recent confirmation of a genetic determinant also comes from a Danish study where probands were found to have at least one first degree diabetic relative in 25% of all cases (Lorenzen *et al.* 1994).

During the early 1970's associations between IDDM and the MHC class I antigens HLA-B8 and HLA-B15 (Singal and Blajchman, 1973; Nerup *et al.* 1974) were substantiated. Stronger correlations have since been demonstrated with the class II antigens HLA-DR3 and DR4 (Platz *et al.* 1981; Johnston *et al.* 1983). Those individuals expressing both DR3 and DR4 the risk of developing IDDM is fourteen times greater (Cudworth and Wolf, 1982). Not surprisingly, at least one of these class II antigens can be found in 80% of Type 1 diabetic patients, whereas in the general population the prevalence of these antigens is only 30 to 50% (Krolewski *et al.* 1987). Not only does the HLA system confer susceptibility to IDDM, it can also confer resistance to IDDM through HLA-DR2 and to a lesser extent DR5, being rare among Type 1 diabetic patients (Sachs *et al.* 1980; Thomson *et al.* 1988). These aforementioned antigens cannot be the sole determinant of susceptibility because the incidence of IDDM is not reflected by the same variability in trends of HLA-DR3 and DR4 amongst different populations. For example, in contrast to Caucasians, DR-3 and DR-9 are associated with IDDM in Chinese, whilst DR-4 and DR-9 are more common in Japanese insulin dependent diabetic patients (Tait and Harrison, 1991).

Recently, IDDM has been shown to be associated with another class II allele, the DQ gene. It has been proposed that the amino acid on the DQb chain at residue 57 is important (Todd *et al.* 1987), in that those expressing non-aspartic acid are associated with a 50 to 100 times greater risk of IDDM (Trucco, 1992) and those expressing aspartate (Asp) are relatively protected from IDDM. When Asp-57 exists rather than a noncharged amino acid at point 57, a salt bridge forms in the MHC α chain thus adversely affecting the recognition of the HLA complex by T lymphocyte clones (Brown *et al.* 1988). These changes could then determine susceptibility or resistance to autoimmune disease. Furthermore, other investigators have suggested that DQ molecules having arginine at position 52 on the α chain in conjunction with non-asp at 57 on the β chain have a more significant predisposition to IDDM (Khalil *et al.* 1990).

From this overview it can be seen that the current role of genetics in the aetiology of IDDM is still unfolding and very complex. Indeed, recent studies in humans have shown two chromosome regions to be associated with and linked to

IDDM: the MHC HLA region on chromosome 6p21 (IDDM 1; Morton *et al.* 1983) and the insulin gene region on chromosome 11p15 (IDDM 2; Julier and *et al.* 1991). Futher studies are still ongoing and suggest that a proportion of the familial clustering unaccounted for by IDDM 1 and IDDM 2 is caused by at least three other genes located at chromosome 11q (IDDM 4) and 6q (IDDM 5). There are probably no other genes with the exception of IDDM 1 that have a major influence ; therefore, polygenic inheritance is indicated with a major locus at the MHC (Davies *et al.* 1994). The importance of these studies may at some point in the future allow the identification of children who are predisposed to developing IDDM. Ultimately, this may allow the initiation of therapeutic treatments sufficiently early in the course of the disease that could prevent the complications seen in later life.

1.4c Toxins and dietary factors

There are very few reports of toxins inducing diabetes in humans. There have been several case reports of accidental insulin dependence induced by vacor ingestion, a rodenticide (Karam *et al.* 1980). Tuomilehto *et al.* (1990) have postulated that high concentrations of caffeine or its metabolites have a toxic effect on intrauterine development of the pancreatic beta cells in genetically susceptible individuals. Cows milk protein has also been implicated as a possible trigger of autoimmune diabetic disease (Karjalainen *et al.* 1992), recently being reviewed by Hammond-McKibben and Dosch, (1997). It is thought to increase the risk of Type 1 diabetes by 1.5 times (Gerstein, 1994) but its role as a universal cause is disputed by others (Dahlquist *et al.* 1990).

1.4 The pathogenesis and pathophysiology of IDDM.

1.4a Autoantibodies and antigens of IDDM

IDDM is brought about by a process causing a selective destruction of the insulin producing beta cells of the pancreas, generally agreed to be an autoimmune phenomenon (Eisenbarth, 1986). The aetiological factors, both endogenous or exogenous, that are capable of inducing beta cell autoimmunity, continues to elude researchers (Lernmark *et al.* 1993).

The first evidence to suggest that Type 1 diabetes mellitus had an autoimmune pathogenesis was by the demonstration of cytoplasmic ICA in patients with a polyendocrine autoimmune disease (Bottazzo *et al.* 1974). Subsequent studies confirmed the presence of these ICA in up to 70 to 80% of newly diagnosed IDDM

patients. Unfortunately, ICA are not specific for diabetes occurring in at least 0.1 to 0.5% of healthy controls (Bruining *et al.* 1989; Riley *et al.* 1990; Karjalainen, 1990), but generally they still serve as the major marker for IDDM (Lernmark *et al.* 1993). Many IDDM patients also have antibodies which react with the surface of pancreatic beta cells, called islet cell surface antibodies (ICSA). Their sensitivity and specificity remain to be defined (Lernmark *et al.* 1993) because of variability between assays (Scherbaum, 1992).

In parallel with ICA, another important Type 1 diabetic autoantibody has been described, the 64 kDa antibody (Baekkeskov *et al.* 1982). Almost a decade later its autoantigen was identified as glutamic acid decarboxylase (GAD) (Baekkeskov *et al.* 1990). Antibodies to GAD are predictive for the future development of Type 1 diabetes (Bärmeier *et al.* 1991), appearing in 70% of newly diagnosed patients (Christie, 1996). They are more persistent throughout the course of the disease (Christie *et al.* 1990a) but it is still not clear how specific GAD antibodies are as a marker for subsequent diabetes (Roll *et al.* 1994). Evidence for lymphocyte directed immunity towards GAD has been shown to be strongly associated with insulin dependence (Atkinson *et al.* 1992). They are also thought to be beta cell specific within an islet (Christie *et al.* 1990b), whereas ICA can react with all the endocrine cells within an islet (Bottazzo and Doniach, 1978).

Autoantibodies specific to insulin and proinsulin (Kuglin *et al.* 1988) have also been related to diabetic patients who have previously not taken insulin. It is also possible to identify these antibodies long before the onset of the disease (Palmer *et al.* 1983) but may not be a useful marker in older age groups (Christie, 1996). Further studies have confirmed IAA to be present in approximately 50% of newly diagnosed patients (Landin Olsson *et al.* 1992). Nevertheless, the predictive value of IAA seems low, especially in the absence of ICA (Ziegler *et al.* 1989; Atkinson and Maclaren, 1993), with up to 22% of ICA negative individuals having circulating IAA versus 29% of ICA positive relatives (Dean *et al.* 1986). These IAA are, therefore, essentially considered of secondary importance as a humoral marker for IDDM (Palmer, 1987). A recent study by Petersen *et al.* (1997) also suggests this, where there was no difference in the prevalence of ICA in either dizygotic or monocygotic twins (with 1 twin being diabetic), suggesting that environmental factors are important in the pathogenesis of diabetes. Further studies are still necessary to establish the mechanism by which IAA develop and their usefulness in predicting the onset of IDDM (Greenbaum and Palmer, 1991; Lernmark *et al.* 1993).

In summary, IAA and ICA have been extensively studied but with the more recent identification of multiple autoantigens such as GAD, carboxypeptidase H

(Castano *et al.* 1991), islet gangliosides, 37kd (Baekkeskov *et al.* 1982) and 52kd molecules (Karounos and Thomas, 1990) additional predispositions to diabetes may become apparent and their role being further evaluated once more reliable specific assays are developed (Pugliese and Eisenbarth, 1992).

I.4b Histological changes of IDDM

There is an overall reduction of approximately 80% of insulin producing cells at the time of clinical onset of Type 1 diabetes mellitus (Kloppel *et al.* 1984). The disease process is slow; some of the immune and metabolic changes can occur many months, or even years, before the onset of clinical diabetes (Srikanta *et al.* 1984). The rate of beta cell loss is unknown but it is likely to be considerable, especially in younger patients (Wallensteen *et al.* 1988). The decrease in beta cell mass has been shown to be directly proportional to the duration of disease with only a few beta cells surviving after a prolonged chronic course of destruction. The alpha, delta and pp cells always remain intact, the exocrine pancreas is only rarely involved with mild degrees of interstitial fibrosis and atrophy (Gepts and De Mey, 1978).

The histopathological findings described by Gepts in 1965, gave the first suspicion that the clinical onset of Type 1 diabetes may be associated with an auto-immune destruction. He described the infiltration of pancreatic islets by mononuclear cells (Gepts, 1965) in young newly diagnosed patients, becoming known as insulitis, thought to be present in up to 80% of all new cases (Foulis *et al.* 1986).

The infiltration of beta cells is patchy. Islets containing insulin are over 20 times more likely to be affected compared to only 1% of islets affected which are deficient in insulin (Foulis *et al.* 1986). These changes are, therefore, consistent with autoimmune attack selective for those islets containing insulin. The cellular composition of the infiltrate comprises a mixture of mononuclear cells, T helper ($CD4^+$) and T suppressor lymphocytes ($CD8^+$), B lymphocytes and macrophages. Of these, $CD8^+$ cells make up the majority (Bottazzo *et al.* 1985). The T lymphocytes of the infiltrate express HLA class II molecules as well as being positive for IL-2 receptors indicating that these cells are perhaps initiating an immune response specific to beta cells (Foulis, 1991).

1.4c Mechanisms of immunological destruction of beta cells

Distinguishing self from non-self is fundamental to the normal functioning of the immune system (Male *et al.* 1991). For T lymphocytes, this discrimination is in a precarious balance between the extremes of nonresponsiveness to autoimmunity. It is

thought that the gradual destruction of beta cells is by a sequence of events similar to those which eliminates foreign antigens (Parham, 1990).

The initial event in an immune response is the processing of foreign antigen by macrophages, dendritic cells or B lymphocytes. These cells, collectively called antigen presenting cells (APC) then present foreign antigen in association with MHC class I or II molecules. T lymphocytes of the $CD4^+$ phenotype respond to foreign antigen in association with MHC class II molecules, expressed by macrophages and dendritic cells, which leads to clonal proliferation. These cells, in turn, by producing cytokines can initiate the proliferation of $CD8^+$ cytotoxic T lymphocytes and antibody producing B lymphocytes (Male *et al.* 1991; *Figure 1.8*).

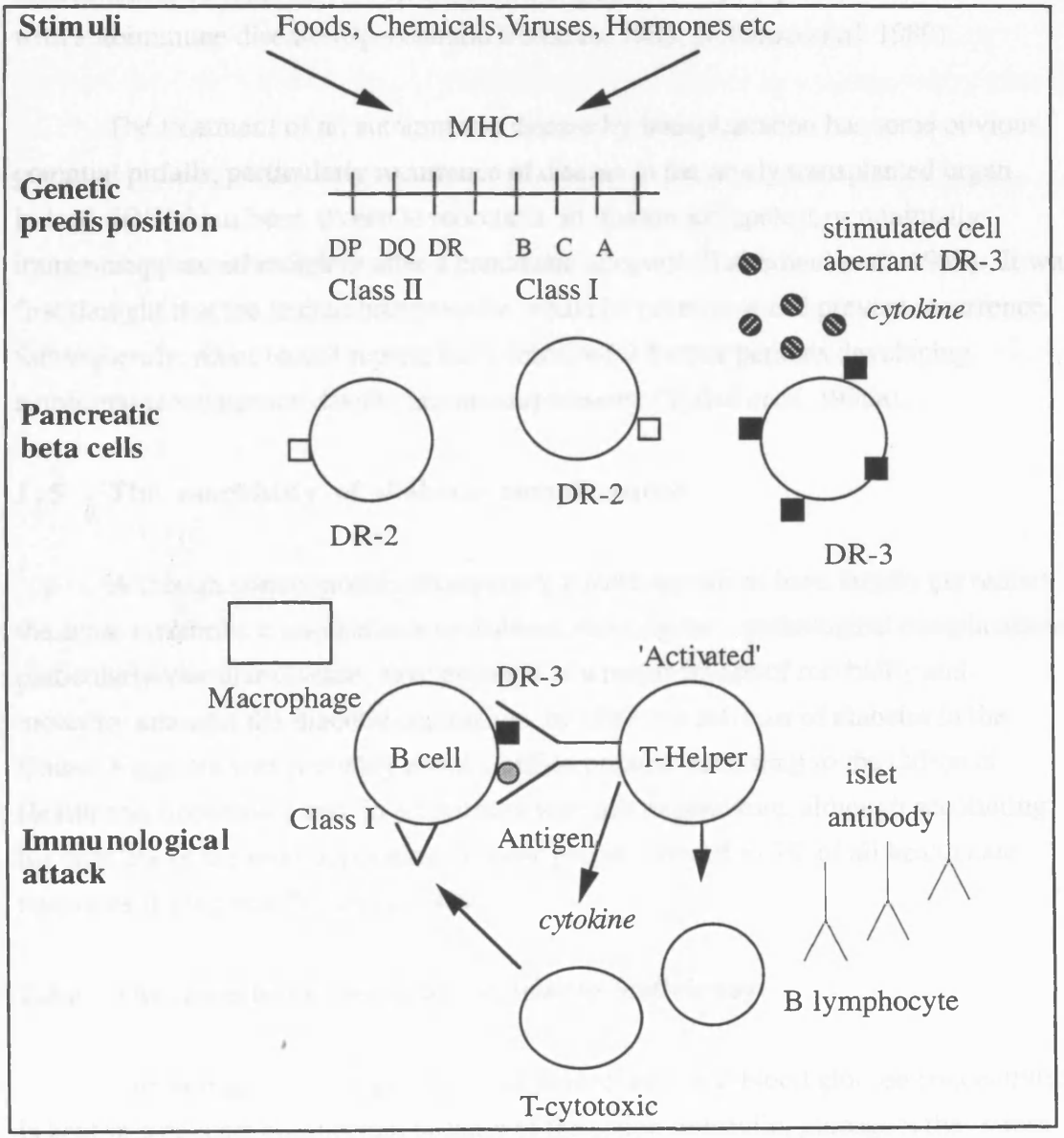

Figure 1.8. A proposed mechanism for beta cell destruction

Pancreatic islet cells do not normally express MHC class II molecules but recently the aberrant expression of MHC class II molecules upon cells subjected to autoimmune attack, including pancreatic beta cells, has been observed (Bottazzo *et al.* 1985; Foulis and Farquharson, 1986). The specificity of the autoimmune attack against beta cells can thus be explained by the aberrant class II expression, as it is absent on the other cells within an islet. Despite this hypothesis, the exact sequence of events is still to be confirmed but it has been suggested that the beta cell having become an MHC class II APC is now capable of presenting their own surface autoantigens to T lymphocytes (Bottazzo *et al.* 1983) initiating immune attack in genetically predisposed individuals *(Figure 1.8).* However, whether this initial induction of autoimmune T lymphocytes leads to autoimmune disease would depend on other factors such as abnormalities of suppressor T lymphocytes which have been reported to also coexist with autoimmune disease (Spencer and Bottazzo, 1988; Bottazzo *et al.* 1989).

The treatment of an autoimmune disease by transplantation has some obvious potential pitfalls, particularly recurrence of disease in the newly transplanted organ. Indeed, IDDM has been shown to reoccur in an immunocompetent or minimally immunosuppressed recipient after a pancreatic allograft (Sutherland *et al.* 1989). It was first thought that the immunosuppression would be protective and prevent recurrence. Subsequently, more recent reports have described 2 further patients developing autoimmune recurrence despite immunosuppression (Tyden *et al.* 1996a).

1.5 The morbidity of diabetic complications

Although conventional subcutaneous insulin injections have largely prevented the acute metabolic complications of diabetes, the long-term pathological complications, particularly vascular disease, have emerged as a major source of morbidity and mortality amongst the diabetic population. In 1989, the full cost of diabetes in the United Kingdom was probably about 1 billion pounds, according to the Office of Health and Economics and, in accordance with this expenditure, although accounting for only 2% of the total population, diabetic patients need 4 to 5% of all health care resources (Laing and Williams, 1989).

1.5a The metabolic sequelae of insulin deficiency

The normal physiology of glucose homeostasis and blood glucose concentration is kept in a delicate equilibrium because of the action of insulin, glucagon, the "stress hormones"; cortisol, adrenaline, noradrenaline and growth hormone. During the fasting state insulin inhibits lipolysis, the formation and breakdown of ketones,

gluconeogenesis, glycogenolysis and proteolysis. These anti-catabolic actions control the rate of breakdown of energy stores, with the absence of insulin upsetting this homoestasis and leading to an enhanced catabolic state (Alberti, 1989).

The insulin deficiency of a newly diagnosed diabetic patient and the relative excess of both glucagon and catecholamines, results in increased glycogenolysis and gluconeogenesis. There is excessive breakdown of adipose tissue (lipolysis) forming non-esterified fatty acids (FA) which, in the state of insulin deficiency, can enter liver mitochondria forming the ketone bodies (KB) acetoacetate and 3-hydroxybutyrate (Krentz and Nattrass, 1991). Skeletal and cardiac muscle can utilize FA and KB from the lipolysis of energy stores. Most other tissues can utilize glucose produced from gluconeogenesis and glycogenolysis but not in the absence of insulin. However, the brain has the capacity to use glucose as an energy source despite insulin deficiency. Nevertheless, the accumulation of glucose and KB manifests as a clinical entity known as 'diabetic ketoacidosis'(DKA). KB are organic acids which dissociate to produce hydrogen ions at a rate that outstrips the buffering capacity of the body causing a metabolic acidosis. This increases the risk of cardiac arrythmias and respiratory depression (Basu *et al.* 1993). Additional intake of glucose aggravates this process further (Keckes, 1993). Eventually the persisting hyperglycaemia leads to glucosuria, polyuria and dehydration. If fluid intake is not maintained, the hyperosmolarity causes disturbed consciousness with eventual coma (Berger and Keller, 1992) and death if exogenous insulin is not given.

DKA is the largest single cause of death in diabetic patients under 20 years of age in the UK carrying an average mortality rate of approximately 7% (Krentz and Nattrass, 1991). The incidence in many European countries is increasing in the under 5 years age group (Metcalfe and Baum, 1991). In a prospective population based study of 219 newly diagnosed diabetic children, it was estimated that 16% were in severe DKA (pH less than 7.10) with 10% being mildly affected at presentation. Severe DKA was significantly more common in children under 5 years of age with one patient dying (Pipeleers, 1992). DKA remains an uncommon disease in childhood but it has probably doubled in incidence in many European countries over the past 20 years (Bingley and Gale, 1988), especially in the under 5 age group (Metcalfe and Baum, 1991). It is particularly important in this very young group where presentation is more acute and diagnosis difficult (Pipeleers, 1992) that treatment is instituted immediately.

Not only does insulin deficiency cause DKA but in middle age or elderly patients diabetes may present as hyperosmolar non-ketotic coma characterized by

marked hyperglycaemia with KB being absent. The precise mechanism of this absence is currently unknown but it carries a mortality of 30% (Berger and Keller, 1992).

Despite the improvements in general medical care since the advent of insulin, it can still be seen that the acute metabolic complications of diabetes can still cause death if diagnosis is delayed.

1.5b Microvascular

It has now been shown, without doubt, that the microvascular complications of diabetes are associated with long-term hyperglycaemia (DCCT, 1993). The pathological hallmarks of microvascular disease are thickening of the capillary basement membrane (Ashton, 1949; Williamson *et al.* 1988), with increased capillary permeability (Feldt-Rasmussen, 1986), along with abnormalities of blood flow (Patel *et al.* 1992), blood viscosity (Barnes *et al.* 1977) and platelet function (Tooke, 1987).

Retinopathy

The development of eye disease is a major concern to patients with IDDM. Diabetic retinopathy is the commonest cause of blindness in the UK in people under 65 years of age (OPCS, 1989). It has been estimated that the yearly incidence of diabetic induced blindness is 3.3 per 100,000 population or 1,600 new cases for England and Wales (Moss *et al.* 1988).

The characteristic, well described lesions of retinopathy can be detected clinically by fundoscopic examination *(Figure 1.9 see over).* Essentially they consist of background (non-proliferative) and proliferative changes. The earliest manifestation of *background retinopathy* is capillary dilatation followed by retinal non-perfusion as the vessels gradually occlude. As the disease progresses pericytes are lost from the capillary wall leading to microaneurysm formation (Orlidge and D'Amore, 1987), flame and blot haemorrhages and finally retinal ischaemia. Sometimes these pathological changes can also cause profound oedema known as diabetic maculopathy. Finally, all these background features invariably result in increased capillary permeability with deposition of hard exudates and when deposited around the fovea blindness ensues (Michels *et al.* 1990).

The continued capillary changes resulting in retinal ischaemia eventually lead to new vessel formation, termed *proliferative retinopathy.* Sometimes the changes of proliferative retinopathy are preceded by "cotton wool spots" (McCleod, 1975),

venous and arterial abnormalities (pre-proliferative changes) such as dilatation and narrowing respectively. Proliferative changes can cause haemorrhage and with the addition of the increased permeability of these new vessels predispose to macular oedema. After a chronic course these changes initiate blindness because of inevitable retinal detachment as there is traction of fibrous adhesions formed between the vitreous and retina. Other causes of blindness in diabetic patients can be due to extensive new vessel formation within the iris and complicating thrombotic (neovascular) glaucoma. Diabetes also commonly predisposes to the development of cataracts in later adult life (Michels *et al.* 1990). In the Framingham Eye Study (Leibowitz *et al.* 1980) senile lens changes were consistently more frequent in diabetics than non-diabetics, with cataracts arising because of the accumulation of impermeable polyols within the epithelium of the lens (Kohner *et al.* 1991).

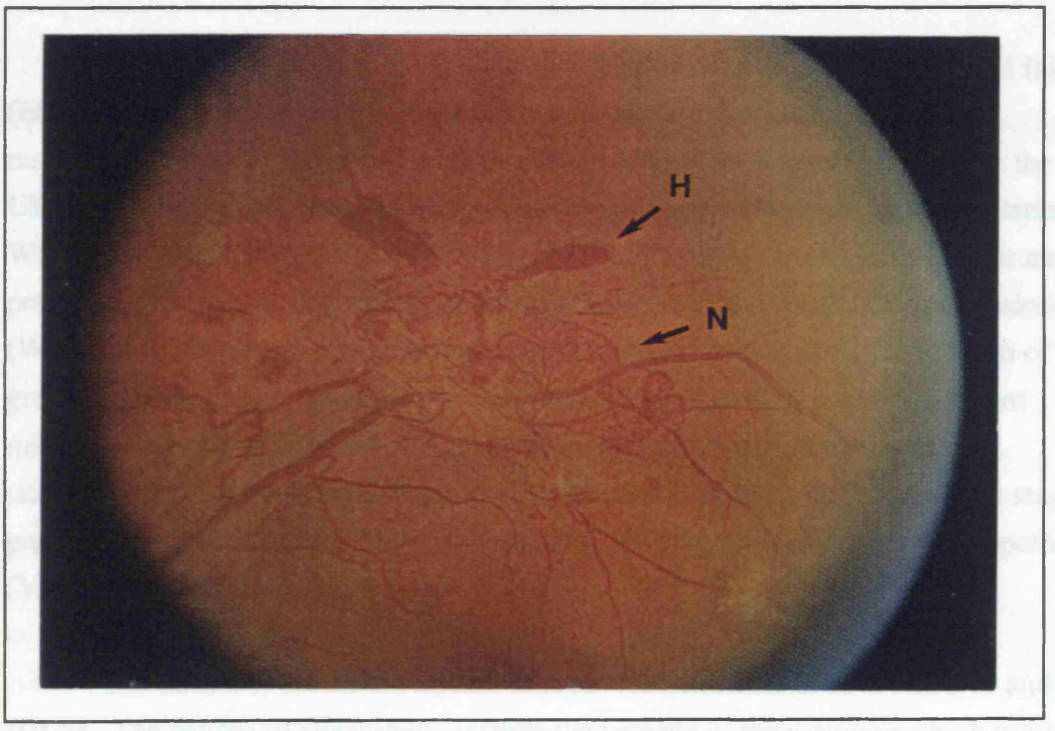

Figure 1.9. Fundoscopic changes of diabetic retinopathy (H-haemorrhage; N-neovascularisation)

The true prevalence of background retinopathy amongst diabetic patients is unknown but it is undoubtedly related to the duration of diabetes (Marshall *et al.* 1993). Previous American studies have implicated that almost all long-term diabetic patients have evidence of background changes (Klein *et al.* 1984; Goldstein *et al.* 1993), although European studies suggest only about 80% are affected (Stephenson and Fuller, 1994; Sjølie *et al.* 1997), probably because of better diabetic control. Even with the high prevalence of background retinopathy, the progression to proliferative

changes, after over 30 years of diabetes, is between 36% to 75% of patients (Klein *et al.* 1984; Stephenson and Fuller, 1994). Visual loss secondary to maculopathy is also severe with up to 50% of people with initially good vision rapidly deteriorating after 5 years of follow-up (British Multicentre Study Group, 1983).

From this review it can be deduced that the prognosis for patients with diabetic eye disease is unfavourable but, fortunately, most of these patients can be treated by laser photocoagulation as the mainstay of treatment (Diabetic Retinopathy Study Research Group, 1987). Nonetheless, until the specific mechanism inducing these pathological changes is discovered, progression to blindness will not be prevented even with today's conventional insulin therapy.

Nephropathy

Diabetic nephropathy (DN) is the most common cause of end stage renal failure (ESRF) in the western world (United States Renal Data Systems, 1990), being responsible for up to 25% of all diabetic related deaths (Moloney *et al.* 1983) in the UK. DN was first recognised as a specific complication of diabetes by Kimmelstiel and Wilson in 1936 (Kimmelstiel and Wilson, 1936). The characteristic clinical features are persistent proteinuria, decreasing glomerular filtration rate (GFR) and hypertension (Watkins, 1990). Persistent proteinuria is defined as a urinary protein excretion of greater than 0.5g/day. However, this is most often preceded by a silent incipient microalbuminuria (less than 300mg/day) of many years duration that remains undetected with conventional Albustix (Deckert and Grenfell, 1991), but at this stage is predictive of those patients who may eventually develop clinically overt nephropathy (Viberti *et al.* 1982).

The term DN covers the myriad of renal complications of both NIDDM and IDDM. The degree of albuminuria reflects the severity of renal disease which is also thought to be directly proportional to the duration and control of diabetes (DCCT, 1993; Coonrod *et al.* 1993). The overall incidence of DN is higher in males than females (Andersen *et al.* 1983), being less common in Caucasians (Samanta *et al.* 1991; Freidman, 1993). Most, but not all diabetic patients, will eventually develop DN (Watkins, 1990) if albuminuria is present at the onset of Type 1 diabetes mellitus (Ammann *et al.* 1984). Previous studies have shown the prevalence of DN to be of about 40% after 40 years duration of diabetes, with two thirds developing renal failure (Andersen *et al.* 1983; Krolewski *et al.* 1985; Watkins, 1990). However, a large Leicestershire study suggested that persistent or intermittent proteinuria occurred in only 17% of juvenile onset diabetic patients (McNally *et al.* 1990). In the light of these

findings, a Swedish study has since confirmed an overall reduction in the cumulative incidence of nephropathy, after 25 years of diabetes, from 28% to 5.8% in successive cohorts with these results perhaps reflecting improved glycaemic control (DCCT, 1993; Bojestig *et al.* 1994).

The histopathological features of diabetic nephropathy occur in the glomerulus. There is generally an increased glomerular volume which can easily be recognised at the time of diagnosis of diabetes (Mogensen *et al.* 1979). This is thought to be due to basement membrane thickening (Bergstrand and Bucht, 1959), mesangial enlargement , hyaline deposits (Mauer *et al.* 1992) and global glomerular sclerosis of renal tissue (Kimmelstiel and Wilson, 1936) as the disease progresses. These changes initiate the relentless decline in function of the nephron with the emergence of proteinuria and elevation of blood pressure.

Persistent albuminuria not only signifies renal disease but is also associated with generalized vascular damage (Jensen and Deckert, 1992). It is not surprising, therefore, that patients with proteinuria also have a greater than ten times increased risk of coronary heart disease (Borch-Johnsen and Kreiner, 1987; Jensen and Deckert, 1992), along with an increased risk of developing proliferative retinopathy (Kofoed-Enevoldsen *et al.* 1987). With these extensive pathological changes, approximately 50% of those patients starting treatment for ESRF will die within 3 to 7 years (Andersen *et al.* 1983; Krolewski *et al.* 1985; Cameron, 1992); the cause of death being attributable to a combination of uraemia and ischaemic myocardial and cerebrovascular disease (Moloney *et al.* 1983). With multi-organ involvement of diabetes, the management of established DN requires a multi-disciplinary team (Drury and Watkins, 1993). Initial maintenance regimes require dialysis, the viable long-term treatment option for most patients if an opportunity for renal transplantation is not offered. The outcome after transplantation has progressively improved during the last decade with five year patient survival increasing to 70% and 67% for graft survival (Drury *et al.* 1989; UKTSSA, 1997).

Neuropathy

Diabetic neuropathy (DNR) is an important cause of morbidity often leading to foot ulceration and amputation (Walters *et al.* 1992). It is not a single entity but comprises a number of different syndromes each with a unique spectrum of clinical and subclinical manifestations (Vinik *et al.* 1992). The precise classification is still not agreed but essentially comprises reversible (distal sensory symptoms and conduction

abnormalities) and established (focal, multi-focal and mixed) neuropathies (Thomas, 1991).

Patients usually present a diverse range of symptoms which are of a slow and insidious onset. In brief, those of sensory neuropathy comprise tingling, hyperaesthesia, cramps and frank pain (Thomas, 1991; Veves *et al.* 1993) that mainly affects the legs and feet. These features also exacerbate any pre-existing diabetic foot ulceration and neuropathic arthropathy. In contrast, diabetic amyotrophy, presents with sudden severe pain accompanied by quadricep wasting but sensory loss rarely occurs (Garland, 1955). Autonomic neuropathy (AN), as distinct from sensory neuropathy, often presents vague and unrecognised symptoms, consisting of diarrhoea, postural hypotension, bladder symptoms, disturbed gastric motility (Abrahamsson, 1995), impotence and again is a prerequisite to the development of foot ulceration (Vinik *et al.* 1992). Finally, focal cranial nerve palsies are also commonly described, particularly those of III, IV, VI and VII nerve origin (Thomas, 1991).

The pathological changes of DNR have been determined from nerve biopsies, and essentially consist of axonal degeneration, demyelination along with changes in the perineurium, epineurium and Schwann cells, (Johnson *et al.* 1986). The aetiology of this heterogenous disorder is still the subject of much debate but there is now good evidence to suggest that the accumulation of sorbitol in peripheral nerves is associated with myoinositol depletion and depression of sodium ion and potassium ion ATPase which leads to nerve dysfunction and damage (Ward, 1985).

Attempts to estimate the overall prevalence of DNP vary widely, as many studies show differences in inclusion criteria and study size. Estimates of 10 to 100% have been quoted (Vinik *et al.* 1992; Ziegler *et al.* 1993) with no clear predisposition to sex (Young *et al.* 1993). One of the largest studies concerning the prevalence of DNR stems from a study performed in Brussels by Pirart and colleagues. Pirart summised that the prevalence of DNP was 7% in those patients with diabetes of 1 year duration in comparison with 50% with 25 years duration (Pirart, 1978). More recently, however, it has been suggested that 66% of all IDDM will have some degree of neuropathy, the most common form being polyneuropathy in 54% (Dyck *et al.* 1993).

In general the treatment options for DNR are very limited due to the elusive nature of its specific aetiology. Management regimes include general measures including tight diabetes control and specific measures such as symptomatic, palliative and supportive care (Vinik *et al.* 1992). However, in the presence of autonomic neuropathy patient prognosis is very poor carrying a 25 to 50% mortality rate (Ewing *et al.* 1976).

1.5c Macrovascular

Risk factors

The relative risk of developing macrovascular complications such as heart, cerebrovascular or lower limb ischaemia in diabetic patients is high. These complications are secondary to the pathological affects of generalised atherosclerosis, similar to that seen in non-diabetic patients, but the severity is often much worse (Colwell *et al.* 1993) with a 60% mortality in diabetic patients (Hsueh and Anderson, 1992).

Endothelial cell injury is an initiating event in the development of atherosclerosis. The arterial endothelium is chronically damaged not just by diabetes but also because of the simultaneous presence of independent risk factors such as hypertension (Garcia *et al.* 1974; Williams, 1991), smoking (Kesson and Slater, 1979) and unfavourable lipid profiles (Garcia *et al.* 1974). This injury leads to smooth muscle proliferation, lipid accumulation and platelet aggregation forming an atheromatous plaque which gradually occludes arterial lumens (Davies, 1993).

Abnormalities of plasma lipids and lipoprotein concentrations are common in both IDDM and NIDDM. In general those who are untreated or inadequately treated have elevations in serum triglyceride levels conferring increased susceptibility to accelerated atherosclerosis (Ginsberg, 1991). In support of this, research has shown that patients with poor diabetic control may have excessive glycosylation of extravascular matrix proteins such as collagen, capable of binding lipoproteins that participate in the deposition of atherosclerotic plaques (Brownlee *et al.* 1985; Colwell *et al.* 1993). Unfortunately, conventional insulin treatment leads to unphysiological insulin blood profiles, with variable periods of hyper and hypoinsulinaemia, together adversely affecting lipoprotein profiles (Eschwege and Fontbonne, 1992)

Ischaemic heart disease

Premature cardiovascular mortality is a well recognised complication of diabetes (Garcia *et al.* 1974; Giles and Sander, 1989). The majority of long-term diabetic patients succumb to cardiovascular sequelae, accounting for up to 75% of all diabetic deaths, in comparison to 25% who die of renal disease (Marks and Krall, 1971; Moloney *et al.* 1983). Diabetes destroys and distorts arterial walls increasing the risk of acute thrombus formation within the coronary arteries manifesting itself as myocardial ischaemia or infarction (Sniderman *et al.* 1992). Overall, these pathological changes

leave diabetic patients with a two to four times greater risk of dying from myocardial infarction, compared to healthy non-diabetic patients (Sniderman *et al.* 1992). In addition, if they survive patients are more likely to suffer reinfarction (Kornowski *et al.* 1993) thereafter, but the risk can be reduced if their diabetes is intensively treated (Malmberg *et al.* 1997).

Widespread areas of infarction and fibrosis are common in diabetic heart disease and when sufficiently extensive, heart failure will ensue (Sniderman *et al.* 1992), especially in the presence of hypertension (Giles and Sander, 1989). Irrespective of this, it is important not to forget that diabetes accounts for the largest proportion of patients requiring dialysis (Drury *et al.* 1989); therefore, in addition to heart disease, most have anaemia and an arteriovenous dialysis fistula together worsening the already compromised ventricular function. Finally, diabetes has a propensity to disrupt the autonomic nerve supply of the heart causing tachycardia, postural hypotension, silent ischaemia or infarction (Faerman *et al.* 1977). Some patients benefit from coronary artery bypass surgery although current figures indicate conflicting results (Van Brussel *et al.* 1993; Weintraub *et al.* 1993), but until improved methods of metabolic control have been found no other option exists.

Cerebrovascular disease (CVD)

Diabetes afflicts the central nervous system in many ways. It increases the risk of CVD and seizures. Over zealous treatment with insulin causes cognitive dysfunction (Ryan *et al.* 1993) and brain damage (McCall, 1992). It also alters blood flow kinetics (MacCleod *et al.* 1994), clotting (Fuller, 1984) and can induce chronic encephalopathy (McCall, 1992).

Diabetes confers a two to six times increased risk of stroke with up to 25% of all diabetics having some evidence of CVD (Kannel and McGee, 1979; McCall, 1992). Females are significantly more affected especially in the fifth and sixth decades (Bell, 1994). Several previous studies have shown an increase in both short-term and long-term mortality in diabetic patients with CVD (Asplund *et al.* 1980; Cox and Lorains, 1986). This increase in mortality is thought to be twofold; firstly, in the presence of hyperglycaemia cerebral oedema is more pronounced, increasing irreversible cerebral ischaemia and poor outcome (Berger and Hakim, 1986); secondly, those patients with poorly controlled glucose are more likely to have generalised atheroma in all the vasculature of the brain (McCall, 1992), along with more systemic diabetic complications such as renal failure. In the presence of renal disease the risk of stroke is further enhanced (Abbot *et al.* 1987) because of associated hyperlipidaemia (Keane *et*

al. 1988), hypertension (Selby *et al.* 1990), increased platelet aggregation and blood clotting abnormalities (Tooke, 1987).

Prevention of CVD in diabetic patients requires early recognition and treatment, particularly of coexisting hypertension. In the diabetic population, smoking, obesity, hyperlipidaemia and glycaemic control have not been shown to be independent risk factors for CVD but because of their indisputable association with atherosclerosis, it would be prudent not to treat these factors aggressively (Bell, 1994). Nevertheless, despite aggressive rehabilitation, 27% of all patients presenting with CVD have a raised glycosylated haemoglobin (HbA_{1c}) and previously unrecognised glucose intolerance (Asplund *et al.* 1980; Gray *et al.* 1989). This predicts a grave prognosis and suggests that there are still many patients who would benefit from diabetic treatment if the disease were to be discovered in time.

Peripheral vascular disease (PVD)

In view of the multi-systemic nature of diabetes, it is not surprising that a large proportion of diabetic patients suffer from arterial insufficiency of the lower limbs *(Figure 1.10).* It is an especially onerous condition having serious implications

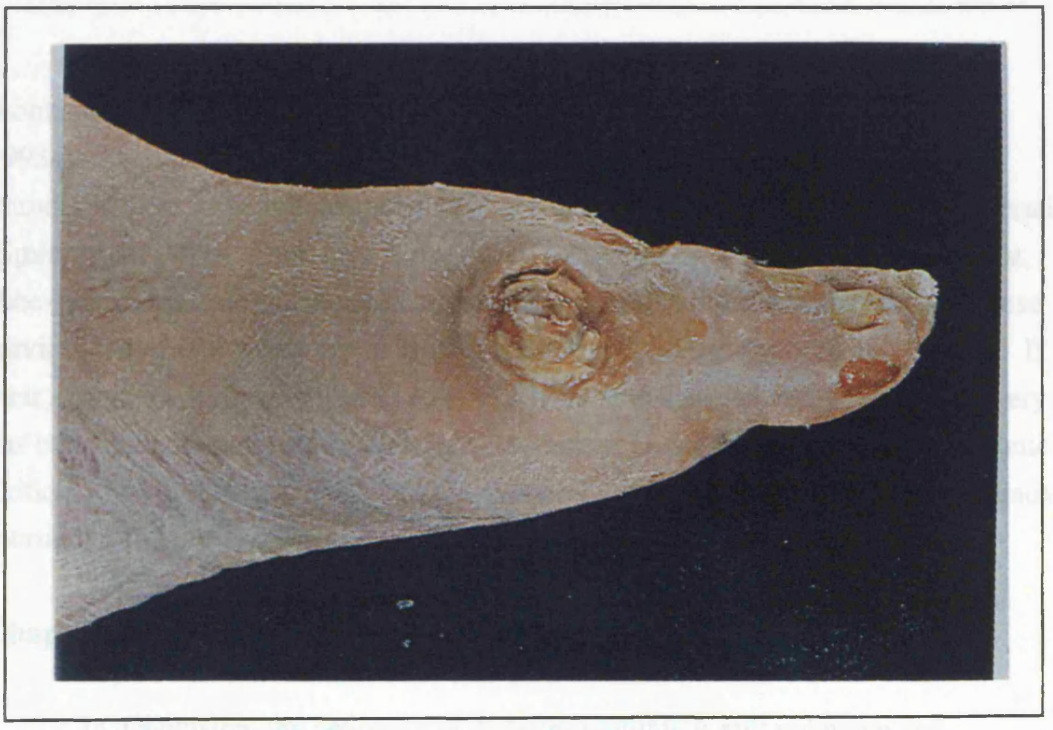

Figure 1.10 Diabetic foot disease with skin necrosis and ulceration

regarding dependency, morbidity and mortality. The symptoms of PVD are progressive often presenting with intermittent claudication, rest pain, tissue necrosis or

gangrene and, with the addition of diabetic neuropathy, patients very often suffer from chronic foot ulceration. This is because of persistent infection in areas of pressure necrosis and minor trauma caused by patients having an inability to feel pain (Levin, 1993).

PVD has been widely shown to be more prevalent in diabetics than non-diabetic patients (Fowkes *et al.* 1992). The distribution of atherosclerotic lesions are different from those of non-diabetic patients in that diabetics develop a unique tendency of below knee insufficiency with the vessels of the foot often being spared (LoGerfo and Coffman, 1984). These features, along with the duration of diabetes (Moss *et al.* 1992) and persistent neuro-ischaemic ulceration, predisposes to lower limb amputation (Siitonen *et al.* 1993). The age adjusted incidence of limb amputation in diabetic men is estimated to be 349 cases per 100,000 in comparison to 34 cases per 100,000 in non-diabetic age matched controls (Siitonen *et al.* 1993). Studies have also demonstrated that diabetic patients account for 39% of all amputees and it is more disturbing that 15% were previously undiagnosed diabetic patients (Deerochanawong *et al.* 1992). Patient prognosis after amputation is poor having a 10% perioperative mortality rate with survivors only having a median life expectancy of 22 months (Deerochanawong *et al.* 1992).

As the proportion of elderly subjects in the population increases, the foot problems of diabetic patients are becoming a major health problem (Siitonen *et al.* 1993a). This cohort of patients commonly have prolonged hospitalisation and immobilisation. It is estimated that 15% of diabetic patients will develop foot ulceration (Smith *et al.* 1987). Their care is integrated with close liaison between chiropodist, shoe-fitter, nurse, physician and surgeon, although it is not helped by some of these services being suboptimal in the United Kingdom (Deerochanawong *et al.* 1992). If their symptoms cannot be treated conservatively then vascular reconstructive surgery has been recommended as the treatment of choice (European Consensus For Chronic Critical Limb Ischaemia, 1992) but the overall outlook is still poor with a perioperative mortality of about 18% (Sayers *et al.* 1993) .

Chapter summary

In conclusion, the aetiology of diabetes mellitus is still unknown and conventional subcutaneous insulin therapy cannot prevent, slow or reverse the complications of diabetes mellitus. Recent evidence is beginning to suggest that tighter control of blood glucose may reduce diabetic complications. This has resulted in an intense search by investigators to find a better alternative form of treatment; the outcome

of which will undoubtedly have a major impact on diabetic treatment in the future and therefore the current progress in this field will be reviewed in some detail in the following chapter.

CHAPTER TWO

THE TREATMENT OF DIABETES MELLITUS

PART ONE

Conventional diabetic treatment and its limitations

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2.1b Insulin injection therapy

2.1c Alternative insulin delivery

2.1d Artificial endocrine pancreas

2.1e Immunotherapy

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2.2 **Clinical studies**

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PART TWO

Diabetic control by pancreas and islet transplantation

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PART ONE

Conventional treatment of diabetes and its limitations

2.1 Exogenous insulin therapy

2.1a Insulin preparations and their manufacture

Shortly after Banting and Best's discovery of insulin in the early 1920's insulin preparations were commercially manufactured. Despite early products being of poor quality and very impure they were shown to prevent the acute metabolic complications of diabetes and dramatically reduced mortality.

For many years porcine and bovine insulins were the mainstay of diabetic treatment but these preliminary preparations met with great controversy after reports of immunological allergic reactions against extracts of animal pancreata (Tuft, 1928), inferring that human insulin should be used. It was thought that the use of human insulin would not produce high titres of neutralising insulin antibody and patients would not, therefore, develop allergic reactions (Lowell, 1944; Yalow and Berson, 1961) but allergic problems do still occur but are currently found in less than 1 % of de-novo treated patients (Schernthaner, 1993).

The first human insulin preparations were manufactured from cadaveric human pancreata but were found to be therapeutically inadequate (Nicol and Smith, 1960). Early unsuccessful attempts to synthesize human insulin involved treating porcine insulin with trypsin or carboxypeptidase A to produce a *humanized* form (Markussen, 1977). Further improvements in this technique eventually led to the production of "semi synthetic " human insulin by Morihara *et al.* (1979).

At present most insulin is produced biosynthetically. This technique involves insertion of a synthetic human insulin gene sequence into a micro-organism such as *E coli* . This is done by exploiting a plasmid vector, the whole process being known as recombinant DNA synthesis (Goeddel *et al.* 1979). The micro-organism freely expresses the desired sequence culminating in biosynthetic human insulin secretion (Chance *et al.* 1981). A variety of different recombinant techniques have been described either by using insulin alpha and beta chain combinations (Goeddel *et al.* 1979) or by conversion of proinsulin (Johnson, 1982).

The introduction of highly purified synthetic insulin has not entirely solved the problem of immunogenicity but more recent efforts have concentrated on making insulin more suitable for subcutaneous insulin injection, leading to the development of

insulin analogues (e.g. Lispro®). Briefly, analogues remain as monomers or dimers in the subcutaneous space thus facilitating their diffusion but the problem with highly purified synthetic insulins is that they form dimers or hexamers which limit their diffusional properties (Holleman and Hoekstra, 1997). Currently, there are hundreds of commercially available insulin preparations which are simply classified as short, intermediate or long acting based on their duration of activity.

2.1b Insulin injection therapy

As with most hormones, insulin secretion is precisely controlled by feedback mechanisms producing a unique diurnal plasma insulin profile. It consists of acute high peaks of insulin, after meals, superimposed upon a pulsatile basal insulin secretion. This poses an intriguing problem for clinicians in that the perfect treatment of IDDM would be to administer exogenous insulin in such a way that precisely mimics this diurnal insulin profile, but as yet is impossible to do successfully.

Subcutaneous administration of insulin in the thigh has been the preferred route of delivery for many years but chronic use can cause lipohypertrophy, known to be detrimental to overall insulin absorption (Kølendorf *et al.* 1983). The thigh is preferred because it produces the most constant rate of absorption, with the smallest peaks of plasma insulin and has the lowest patient to patient variation (Binder, 1969; Berger *et al.* 1982). However, the ideal method of insulin administration would be directly into the portal vein which is, of course, impractical.

A further problem of subcutaneous insulin delivery is its variability of absorption from different sites because of fluctuations in temperature and other intrinsic factors adversely affecting blood flow (Binder *et al.* 1984; Rönnemaa and Koivisto, 1988). The method of administration is also critical. It has been shown that regions commonly used for administration have less subcuticular fat than is assumed and, in the case of accidental perpendicular injection insulin, is more likely to be delivered directly into muscle in preference to the subcutaneous layer where it is more rapidly absorbed (Frid *et al.* 1988).

During the early 1980's daily urine glucose provided the patients with an indication of how good their diabetic control was. Although still of value, this method has been shown to be confounded by the time lapse between hyperglycaemia and micturition, variation in the renal threshold for glucose (Johansen *et al.* 1984) and the inability to distinguish between normoglycaemia and hypoglycaemia (Amiel, 1993a). In 1978 colourimetric enzymatic assays for glucose were developed making glucose

measurement possible by taking blood from a pinpricked finger (Ikeda *et al.* 1978). With this more precise monitoring, it became theoretically possible to perhaps achieve longer periods of normoglycaemia.

During the last decade there has been a rekindled interest in trying to achieve near normoglycaemia. Many modern insulin treatments are now centred upon intensified insulin injections (IIT). Most regimes are tailor made depending on the type of diabetes, its duration, age, patient lifestyle, metabolic stability and the presence of diabetic complications. Ordinarily, older regimes constituted the use of twice daily injections of both short and intermediate acting insulins given before breakfast and evening meals. One of the disadvantages of this technique is that soluble insulins produce peaks at 90 to 120 minutes after injection (Berger *et al.* 1982), contrasting the peaks at 30 to 60 minutes observed in non-diabetic individuals (Vaag *et al.* 1990). Moreover, the final intermediate acting injection of the day may produce rising hyperglycaemia the following morning which, if increased, may revert to early morning hypoglycaemia (Bolli *et al.* 1993). Newer intensified regimes involve splitting the evening dose between an evening meal and immediately before bedtime. This delays peak insulin action ensuring sufficient insulin to maintain fasting normoglycaemia, but minimizing the risk of rebound hyperinsulinaemia (Amiel, 1993b). As a consequence of attempting to achieve physiological (near normoglycaemia) insulin secretion by IIT, patients have to exert considerable treatment compliance existing on a fine threshold of developing hypoglycaemia due to increased insulin administration (DCCT, 1993). This has its disadvantages as continued hypoglycaemic episodes over many years carries a significant morbidity (Ryan *et al.* 1993). Over a period of time patients lose adrenergic warning symptoms that normally alert the patient to hypoglycaemic attacks (Cryer, 1993) and in their absence hypoglycaemia may prove to be fatal (Grimaldi *et al.* 1990; Amiel, 1993a). With some of these problems in mind there has been an increased need to develop alternative insulin delivery that firstly achieves near normoglycaemia and secondly is more patient friendly.

2.1c Alternative insulin delivery

Continuous insulin infusion

The first continuous insulin infusion devices to be developed administered insulin intravenously (AEP *vide infra*) via a portable pump but even after switching to the more modern continuous subcutaneous insulin infusion devices (CSII) long-term use still carried a risk of infection (Vaan Faassen *et al.* 1989), septicaemia (Chantelau *et al.* 1987) and thrombosis (Irsigler and Kritz, 1979).

These pumps control glucose by producing a basal pre-set insulin concentration in conjunction with additional patient controlled ('open looped') prandial boosts (Pickup *et al.* 1978). Nevertheless, as with any artificial system there are drawbacks, the most serious being accidental dislodgement of the device predisposing the patient to ketoacidosis (Pickup *et al.* 1982; Knight *et al.* 1985). Several large randomized controlled trials have demonstrated superior glycaemic control over conventional insulin injections (Lauritzen *et al.* 1983; KROC, 1985) but doubt exists over case selection as these patients were probably supervised in a highly motivated academic unit. As a consequence, in the routine outpatient setting, results have been less favourable (Marshall *et al.* 1987). The major disadvantages of this technique are, firstly, high doses of insulin are required to maintain sufficient portal delivery of insulin to maintain normoglycaemia, (Eaton *et al.* 1980) preceding to peripheral hyperinsulinaemia, which promotes atherogenesis (Stout, 1987) and, secondly, glycaemic control in teenagers, who are most likely to develop recent onset IDDM, have variable success rates and require intensive supervision as compliance is poor (Schriffen *et al.* 1984). Disconnection rates in this group have been estimated to be as high as 80% (Brink and Stewart, 1986).

Non-injection insulin delivery

Pharmaceutical companies have for years attempted to find alternative methods of administering insulin without the need for unpleasant injections. Intranasal delivery, first attempted in 1935 (Major, 1935), has since become a favoured technique but is not routinuely used. Effective mixtures of insulin, with the addition of absorption enhancers, have recently been manufactured but large quantities of insulin are still required because of a low bioavailability and its extremely variable rate of absorption (Gizurarson and Bechgaard, 1991; Saudek, 1993).

Similar patterns of variability and low bioavailability have been demonstrated with intrapulmonary insulin delivery with preliminary studies showing promise (Laube *et al.* 1993). The use of rectal insulin suppositories have also been tried (Yamasaki *et al.* 1981) but probably meets with less social acceptance than a subcutaneous injection. Simplistically, the best and most convenient method of insulin delivery would be orally but previous attempts have been unsuccessful as insulin is degraded by gut proteases having a poor final rate of absorption (Spangler, 1990; Home, 1991).

2.1d Artificial endocrine pancreas (AEP)

Investigators have also developed an AEP. These integrate a glucose autoanalyser and pump capable of controlling precise insulin delivery (Kadish, 1964). Improvements have allowed devices to sense glucose delivery, interpret levels and deliver an appropriate dose of either insulin or dextrose solution (Albisser *et al.* 1974; Pfeiffer and Kerner, 1985; Albisser *et al.* 1986). Although an AEP takes up a lot of space it can control blood glucose but there are still doubts over their long-term clinical use. Generally, they are only reserved for managing diabetes during childbirth (Nattrass *et al.* 1978) and cases of severe ketoacidosis in a minority of patients (Pfeiffer and Kerner, 1981).

Investigators have endeavoured to improve implantable and programmable insulin pumps (Blackshear *et al.* 1970). Currently, most implantable pumps have insulin reservoirs of approximately 15 to 30 ml and are powered by fluorocarbon liquids (Buchwald and Rhode, 1992). Early trials have indicated that perhaps there is some success in applying this method as a treatment for IDDM (Irsigler *et al.* 1981; Selam *et al.* 1992).

Other disadvantages of the AEP, particularly 'open loop' systems is the necessity to carry a cumbersome portable pump. This can be overcome by using a 'closed loop' implantable delivery pump that provides continuous intraperitoneal insulin administration (Selam *et al.* 1990). Insulin reservoirs can then be refilled transcutaneously. The rate of ketoacidosis is significantly less than with 'open loop' systems, as the device is surgically implanted. More importantly implantable devices have also been shown to be safe and effective in maintaining near normoglycaemia (Hagmüller and Kritz, 1983; Broussolle *et al.* 1994), without severe hypoglycaemia but they are limited by catheter occlusion (Dunn *et al.* 1997).

2.1e Immunotherapy

Recently investigators have tried to identify methods which will slow beta cell autoimmune destruction. In view of the autoimmune pathogenesis of diabetes immunosuppressant drugs were the first to be tried. Azathioprine and Cyclosporin A (Cs A) have been shown to be effective in delaying destruction of beta cells and prolonging remissions in a series of studies (Colman and Eisenbarth, 1987; Canadian European Randomized Control Trial Group, 1988; Jenner *et al.* 1992; Skyler and Rabinovitch, 1992), but results are variable. Newer alternatives have also been tried, the most common being FK 506 (Thomson *et al.* 1993). Nevertheless, all these drugs

are non-specific, highlighting serious ethical implications. Although insulin has prolonged the life expectancy of diabetic patients, albeit with long-term complications, immunosuppressants in contrast have toxic unacceptable systemic side effects, including that of the beta cell, without justifiably preventing the development of IDDM.

Other therapeutic options have been considered (Skyler and Marks, 1993), including anti-inflammatory drugs, free radical scavengers and recombinant insulin like growth factors (Bondy *et al.* 1994). Most recent attention has been focused on nicotinamide after a series of studies from New Zealand. It has been proposed that nicotinamide induces remission in newly diagnosed IDDM and delays the onset in those patients that are already antibody positive (Elliott and Chase, 1991). Studies have demonstrated nicotinamide to be a useful therapeutic adjunct to insulin in the early phase of IDDM increasing the rate of clinical remission and improving most metabolic parameters of beta cell function (Pozzilli *et al.* 1994; The IMDIAB Study Group, 1995). Its mode of action is still controversial but it is thought to increase the NAD pool and inhibit poly (ADP-ribose) polymerase (Pociot *et al.* 1993) which are mechanisms implicated in DNA repair of defective pancreatic beta cells (Eizirik *et al.* 1993; Pozzilli and Andreani, 1993).

More recently a meta-analysis conducted under the auspices of the International Diabetes Immunotherapy Group has demonstrated a therapeutic effect of nicotinamide in preserving beta cell function when given at the time of IDDM diagnosis (Pozzilli, 1996). Taken together, these studies, although encouraging, have not been confirmed elsewhere so results from the multi-centre European Nicotinamide Diabetes Intervention Trial (ENDIT) are eagerly awaited.

2.1f Gene therapy

There are a number of ways in which gene therapy could be applied to diabetes mellitus. Firstly, one potential advance would be to target the immune system thus preventing autoimmune destruction of beta cells. Secondly, using genetic technology the insulin secreting capacity could be replaced by introducing an active insulin gene into tissues *in vivo*, not necessarily pancreatic, or by engineering cells *ex vivo* that could be transplanted e.g. hepatocytes. Recent studies have focused on injection of retroviral vectors harbouring insulin cDNA. When taken up by hepatocytes insulin expression has led to prevention of ketoacidosis in streptozotocin treated animals (Koloddka *et al.* 1995). Nevertheless, there are many problems associated with gene therapy that still need to be solved. Unfortunately, after *in vivo* delivery of insulin genes, there are currently no safe methods of preventing inappropriate insulin release

from ectopic tissues or the random manner of gene integration within the genome (Docherty, 1997) both factors limiting its routine application.

2.2 Clinical studies of insulin therapy

2.2a Retrospective studies

The relationship between chronic hyperglycaemia and the development or progression of microvascular and macrovascular complications has been vigorously contested for many years. The reliability, validity and reproducibility of early studies trying to prove this hypothesis have been reviewed in detail by Kaplan and Feinstein (Kaplan and Feinstein, 1973). They summised that previous therapeutic trials were subject to many statistical inconsistencies and were misleading.

For example, it is difficult to retrospectively differentiate between poorly controlled from well controlled diabetes. Not until 1980 were patients able to self-monitor their daily fluctuations of blood glucose, supplemented by additional HbA_{1c} measurements, thus allowing some discrimination between poor and good glucose control. In an ideal world the best control group for any retrospective study, comparing the effect of hyperglycaemia on diabetic complications, would be a non-treated group who were not taking insulin. This is not ethical and therefore the only patient cohorts that can be compared, are those patients who were subsequently poorly controlled against those well controlled. In parallel with these early studies, many authors included both IDDM and NIDDM patients even though the incidence and pathogenesis are known to differ widely between the two (Kaplan and Feinstein, 1973).

One of the first retrospective studies (Johnson, 1960) compared the effects of a strictly controlled diabetic population with a loosely controlled group of patients. It was shown that the frequency of nephropathy and advanced retinopathy was significantly lower in those with intensive insulin therapy but the two groups did not match for duration of diabetes. Interestingly, intensive insulin regimes used in the tightly controlled patient group caused a higher frequency of hypoglycaemic comas (Johnson, 1960). Later, Pirart published observations from a remarkable retrospective study involving over 4,000 patients across 26 years of follow up. Patients were assigned, somewhat arbitrary to today's strict standards, to groups with differing levels of glycaemic control being good, fair or poorly controlled. Their chosen endpoints of glycaemia control were glycosuria, the presence of ketotic symptoms together with random fasting, and postprandial blood glucose levels. Despite the study having many subjective limitations several features were consistent; retinopathy, nephropathy and

neuropathy were all simultaneously present in a significant proportion of patients with diabetes and the degree of severity was inversely proportional to the degree of glycaemic control (Pirart, 1978). Similar conclusions were drawn from the Joslin Clinic where the prevalence of nephropathy, retinopathy and arteriopathy increased with diabetic duration, especially in those patients with poor control, being 10 to 15% in those well controlled and 70 to 80% in those poorly controlled (Keiding *et al.* 1952).

Other observations, using glycosylated haemoglobin as an index of diabetic control, have been shown to correlate well with diabetic complications. That is with increasing HbA1c levels patients are more likely to have severe grades of retinopathy (McCance *et al.* 1989). Raised HbA1c is not only associated with retinopathy but also with worsening neurological signs as documented after motor and sensory nerve studies (Duck *et al.* 1991). Although inconclusive, these studies do suggest that there is a strong correlation between high glycosylated haemoglobin levels, particularly in the preceding years of diabetic diagnosis, and the subsequent progression of chronic diabetic complications.

With the limitations of retrospective studies and the ability to utilize current technology for assessment of metabolic control and detection of complications, there has been a resurgence of interest in randomized prospective clinical trials. Subsequently, investigators have recently demonstrated that there is a conclusive relationship between hyperglycaemia and the severity of chronic diabetic complications.

2.2b Prospective studies

Retinopathy

Many randomized controlled clinical trials have been performed during the last two decades, the five best known being Aarhus (Beck-Nielsen *et al.* 1985), Steno (Lauritzen *et al.* 1983; Lauritzen *et al.* 1985), KROC (KROC, 1985), Oslo (Brinchmann-Hansen *et al*.1992) and Stockholm trials (Reichard *et al.* 1993).

All these studies have shown improved Hb A1c after IIT (mostly with CSII) but not to within normal limits. Interestingly, retinopathy worsened in the initial months of treatment in the intensified groups and steadily progressed in those with conventional treatment. This initial deterioration was not observed in all studies, being absent in Aarhus, but retinopathy in general was less severe in their study cohort. After 7 years of follow up the Oslo Study reported that patients treated with CSII had less progression of retinopathy (Brinchmann-Hansen *et al.* 1992), supporting the results

from the Stockholm Study Group. It was noted that a small proportion of patients in these two studies still required photocoagulation and it took over seven years for the advantages of intensified treatment to become statistically significant. This can perhaps be explained by most patients having clinically evident retinopathy at the onset of the study and failure of intensive therapy to reverse these changes is, therefore, not surprising. Finally, a recent meta-analysis of 16 randomized trials confirmed that IIT has a significant effect on the overall progression of diabetic retinopathy (Wang *et al.* 1993b), but patients are still at a higher risk of hypoglycaemia as a consequence of improved diabetic control (Reichard and Pihl, 1994).

Neuropathy

Measuring the effect of DNR in relation to diabetic control is difficult due to its unclear aetiology and method of assessment. Current conventional assessment is done by electrophysiological testing of peripheral nerves and it is thought to be a reliable correlate of morphological abnormalities found in sural nerve biopsies (Duck *et al.* 1991). Nevertheless, it must be remembered that nerve conduction velocities may be acutely altered by short-term therapeutic changes in diabetic treatment regimes (Gregersen, 1968) and perhaps, as a result of these difficulties, very few studies have been performed. Slight improvements in motor nerve conduction velocities after IIT, over periods from six weeks to five years have been demonstrated, concluding that evolution of DNR may be related to poor glycaemic control (Pietri *et al.* 1980; Ehle and Raskin, 1986; Ziegler *et al.* 1991).

The Oslo Study Group, after only two years of follow up, showed patients treated by CSII had significant improvements in motor nerve conduction velocities (Dahl-Jorgensen *et al.* 1986). After eight years these findings were further confirmed by higher Hb A1c levels in patients with the most severe neuropathic progression, evidenced by slower conduction velocities in the peroneal, sural and tibial nerves (Amthor *et al.* 1994). The Stockholm Group assessing the same lower limb nerves, with the addition of vibration and temperature thresholds, also verified lower conduction velocities in those patients having conventional treatment, in comparison to those who were intensively treated. Nonetheless, there were no differences demonstrable between the two differently treated groups assessed for vibration, perception and temperature threshold (Reichard *et al.* 1993).

Nephropathy

At the time of diagnosis of IDDM, glomerular filtration rate and kidney size have been directly related to glucose control (Christiansen *et al.* 1982), with improved glycaemic control ameliorating both. Evidence arising from epidemiological studies has shown that poor glucose control has a strong correlation with the development of clinical nephropathy in IDDM (Andersen *et al.* 1983; Krolewski *et al.* 1985).

There have been many prospective randomized controlled trials examining the effects of glycaemia in association with diabetic renal complications (KROC, 1998; Beck-Nielsen *et al.* 1985; Feldt-Rasmussen, 1986; Dahl-Jorgensen *et al.* 1986; Reichard and Rosenqvist, 1989). However, many of these studies have been of small magnitude, lacked careful inclusion criteria, had inadequate assessment of both albuminuria and renal function along with considerable variation in their duration of follow-up. Discrepancies of this nature certainly undermining any positive findings from these studies (Selby *et al.* 1990). Furthermore, the role of other factors in the pathogenesis of DN, including genetics (Tuomilehto *et al.* 1995), hypertension and smoking cannot be controlled but these factors did not appear to influence the final outcome in a recent study by Reichard and colleagues (Reichard *et al.* 1993), where DNR developed more frequently in those patients treated with conventional insulin regimes.

The original two year study by Feldt-Rasmussen and colleagues (Feldt-Rasmussen *et al.* 1991) was extended to eight years of patient follow-up in an attempt to improve upon the previously published short-term studies. It was shown that those patients allocated to CSII had significantly improved glycaemic control during the extended duration in marked contrast to the findings over the first two years. Clinical nephropathy was diagnosed more often in those conventionally treated than in those with insulin infusion (100% *versus* 22%) suggesting that studies of longer duration were required if a definitive outcome was to be sought.

Similar findings were seen after four years of follow up in the Oslo study. Urinary albumin excretion (UAE) was reduced in the intensively treated group when compared to conventional treatment. Nevertheless, the reduction was not statistically significant, the difference observed being only very slight. After seven years those patients with more than 300 mg per 24 hour UAE had significantly higher Hb A 1c levels than those conventionally treated, while those with Hb A1c <8.5% had improved UAE suggesting that long-term normoglycaemia is needed to improve overall renal function (Dahl-Jorgensen *et al.* 1988). The findings from the Aarhus study group were

less convincing in the preliminary stages (Beck-Nielsen *et al.* 1985) where, after one year UAE was only slightly improved in the CSII, in contrast to those conventionally treated. Four years later there was still no statistical difference between the two different treatment groups where UAE remained low in both.

The Diabetes Control and Complication Trial (DCCT)

The DCCT was a multi-centre double blind randomized controlled trial undertaken by the USA National Institute of Health (DCCT, 1993). A total of 1,441 patients were enrolled in the trial who had IDDM of 1 to 15 years duration. Patients were randomly allocated either to receive IIT administered by an external insulin pump or by three or more daily insulin injections, or by conventional insulin treatment (CIT) entailing twice daily insulin injections. The patients were followed for a mean of 6.5 years with the premise of answering two important questions; firstly, would IIT prevent the development of retinopathy and other diabetic complications in patients with no evidence of disease (primary prevention) and secondly would it affect the progression of early diabetic disease in those who already had it (secondary prevention) ?

The cumulative incidence of retinopathy in the two primary prevention treatment cohorts was similar until approximately 36 months. From five years onward the cumulative incidence in the IIT group was approximately 50% less than in the CIT group. The IIT group had a lower adjusted mean risk of retinopathy in the primary prevention cohorts by 76% *(Table 2.1).* As with other studies patients in the secondary prevention IIT cohort, who already had clinical evidence of retinopathy, had an initial deterioration in retinopathy over the first year of IIT but a lower cumulative incidence was observed after 36 months and continued for the rest of the study. Overall IIT reduced the average risk of progressive retinopathy by 54% in the secondary prevention cohort and a 56% reduction in the need for photocoagulation.

	Primary Prevention	**Secondary Prevention**
Retinopathy	76%	54%
Nephropathy	34%	43%
Neuropathy	69%	57%

Table 2.1 The effect of IIT in reducing the risk of developing diabetic complications in DCCT patient cohorts

In both primary and secondary preventative cohorts microalbuminuria developed in fewer patients intensively treated than in the CIT group, reducing the mean

adjusted risk by 34% in the primary prevention cohort, as opposed to 43% in the secondary prevention cohort. Similar improvements were seen in those patients assessed with neuropathy. In those patients with no evidence of neuropathy, IIT reduced the appearance at five years by 69% and in those with clinical neuropathy at baseline, the risk of progression was reduced by 57%. Finally, when all the major cardiovascular and peripheral vascular events were combined, IIT reduced the risk of macrovascular disease by 41% but this was not a statistically significant finding (DCCT, 1993).

Offset against the 'near normoglycaemia' achieved with IIT patients were at a two to three fold increased risk of developing severe hypoglycaemia (DCCT, 1993) and, in addition, patients were more likely to gain weight (Caplan, 1992). Nevertheless, after publication of this study, it became clear that patients with IDDM should be treated by IIT with the goal of maintaining their glycaemic status as close to the normal range that was safely possible. Although the intensive therapy in this trial was carried out by expert diabetologists in a highly motivated setting, it was possible to implement IIT and careful monitoring. However, because of the risks of inducing hypoglycaemia, especially in those with hypoglycaemic unawareness, its feasibility becomes questionable (Cryer, 1994). On the basis of these results (DCCT, 1993), it can be concluded that IIT delays the onset and slows the progression of diabetic complications on all accounts, but whether this can be achieved safely by exogenous insulin is debatable; therefore, one can summise that if physiological insulin secretion could be achieved perhaps chronic diabetic complications will be prevented by normoglycaemia and at present the only realistic way of attaining this goal is by the transplantation of insulin secreting tissue.

PART TWO

Diabetic control by pancreas and islet transplantation

2.3 Vascularised pancreas transplantation (VPT)

2.3a Surgical techniques

The rationale for VPT was realised in the late sixties when surgeons at Minnesota (USA) accepted the hypothesis that with the persistence of diabetes, DN could not be cured by renal transplantation alone. Particularly with steriod immunosuppression, to prevent organ rejection, postoperative diabetes was, and still is, difficult to control and correction of both uraemia and diabetes by one operation by simultaneous kidney-pancreas (SPK) transplantation seemed a logical, yet controversial idea. The first human kidney-pancreas transplant was performed by Kelly and Lillehei,

for a diabetic recipient with ESRF in December 1966. This initial experience, although eventually succumbing to graft pancreatitis, still managed to maintain a brief period of normoglycaemia (Kelly *et al.* 1967).

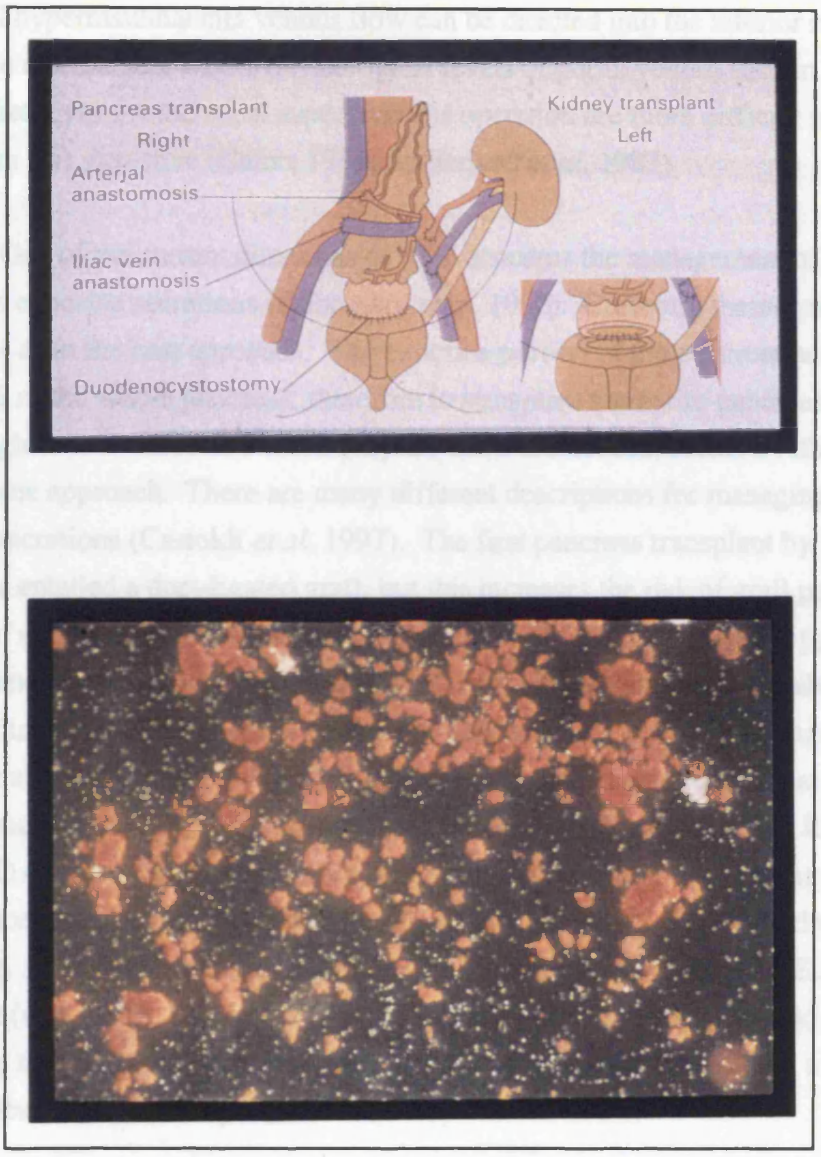

Figure 2.1 Complexity of VPT versus islet transplantation

Over 8,800 VPT have been reported to the International Pancreas Transplant Registry (IPTR) since 1966 (Sutherland and Gruessner, 1997). The majority have been performed in the USA and are more commonly SPK transplants (90%). More recently, the whole pancreas rather than a segment of distal pancreas has been grafted, with the presumption of increasing islet cell mass, reducing blood loss and exocrine leakage. The vasculature of the pancreatic graft is generally anastomosed to the recipients iliac vessels with the graft being placed intra-peritoneally *(Figure 2.1)*, although many variations of VPT have been described (Castoldi *et al.* 1997). In the situation of pancreatico-iliac anastomoses, insulin is secreted into the systemic venous

system. This causes hyperinsulinaemia, that is also aggravated by steriod immunosuppression causing peripheral insulin resistance and promotes atherogenesis (Fontbonne *et al.* 1991; Eschwege and Fontbonne, 1992). In an attempt to avoid peripheral hyperinsulinaemia venous flow can be directed into the inferior mesenteric vein or the splenic vein where physiological levels of portal venous insulin have been demonstrated, yet the technical aspects of this operation are more difficult making this application less attractive (Calne, 1984; Sutherland *et al.* 1987).

One of the current dilemmas of VPT concerns the management of the pancreatic exocrine secretions (Dubernard *et al.* 1992). Currently there is no universal agreement as to the best approach. The exocrine portion of the pancreas accounts for up to 98% of the whole pancreas; therefore to transplant the entire pancreas, in order to maintain glucose homeostasis when only the islets are needed, seems a rather cumbersome approach. There are many different descriptions for managing pancreatic exocrine secretions (Castoldi *et al.* 1997). The first pancreas transplant by Kelly and colleagues entailed a duct-ligated graft, but this increases the risk of graft pancreatitis because of exocrine secretion obstruction (Kelly *et al.* 1967). The most appropriate and logical manoeuvre for diverting physiological exocrine secretion is by establishing drainage directly into the bowel by pancreaticoenterostomy (enteric drainage-ED). This was originally performed by a duodenoenterostomy, after failed attempts at external drainage via a cutaneous graft jejunostomy (DeJode and Howard, 1962; Lillehei *et al.* 1969). ED of pancreatic grafts is now becoming more popular after falling out of favour since the introduction of bladder drainage (BD) techniques (Sutherland and Gruessner, 1997). The main reason being the high rate of conversion to ED after a BD procedure (up to 20%; Sollinger *et al.* 1993), justifying that patients would perhaps rather take the risk of a higher graft failure rate at the expense of avoiding a second and more difficult conversion operation.

A major innovation in the management of pancreatic exocrine drainage came from Dubernard in Lyon, France. He developed a simple technique that suppressed exocrine pancreatic function yet left islet insulin secretion intact. He did this by obliterating the pancreatic duct by intraductal administration of a synthetic polymer, neoprene (Dubernard *et al.* 1978). It has been shown to have a low complication rate and is still one of the most preferred methods of dealing with exocrine drainage in Europe. In parallel with this development, surgeons from Minnesota preferred employing open duct drainage directly into the peritoneum (Sutherland *et al.* 1980). Initial results were favourable but it was eventually concluded that although exocrine secretions could be absorbed by the peritoneal cavity, and that duct fibrosis occurred over time, some patients were incapable of absorbing secretions via the peritoneum and

therefore developed peritonitis. It then became apparent that the duct occlusion method described by Dubernard had a lower complication rate than the open duct drainage technique described by Sutherland and was probably the safest approach to use (Sutherland *et al.* 1981).

Many other methods of exocrine drainage are still regularly reported. One of the most exciting developments has been the anastomosis of the pancreatic graft to the urinary tract. Gleidman first reported a ureteropancreatic duct anastomosis (Gleidman *et al.* 1973), although most of the credit for the development of urinary exocrine diversion should be given to Sollinger who has reported his experience of over 200 SPK grafts (Sollinger *et al.* 1993). One of the advantages of performing a duodenal to bladder anastomosis is the earlier identification of graft rejection. The use of exocrine BD was a major step forward where (Sollinger *et al.* 1984) rejection, represented by a decline in urinary amylase levels, gave an immunological advantage over the much later rise in hyperglycaemia when reversibility of a rejection response is less likely to be successful. The technique used today has been slightly modified by anastomosing a button of duodenum or by using a short duodenal segment as a conduit between the pancreatic graft and bladder (D'Alessandro *et al.* 1989a), both remain popular (Sutherland *et al.* 1993). Urinary tract complications are a major source of morbidity including; dysuria and urinary tract infections (50%), haematuria, strictures, bladder mucosal metaplastic change and reflux pancreatitis (Sollinger *et al.* 1993; Hickey *et al.* 1997). Other disadvantages have also emerged, including chronic metabolic acidosis and, as a consequence of these complications some groups still favour pancreaticoduodenal grafts with ED. The largest, most recently reported series of ED procedures demonstrates that following experience and refinements in technique complications can be kept as low as 10% (Tyden *et al.* 1996b).

2.3b The affect of VPT on diabetic complications.

It is imperative that VPT normalises diabetic hyperglycaemia (Morel *et al.* 1991a). The rationale for this is that patients must not exchange safe insulin administration for the risks associated with the toxic concomitant immunosuppression necessary to prevent pancreatic graft rejection. This quandary has, to a certain extent, been overcome by performing transplants in those patients already taking immunosuppression for a kidney transplant. In this respect, the addition of a pancreas transplant should confer a benefit to their solitary renal graft. Even so, VPT is still a major operation and not a life saving procedure. To be universally accepted it must be considered safe, technically simple with an acceptable rate of postoperative morbidity and mortality.

Good evidence in favour of whole VPT for diabetic patients for normalisation of glucose homeostasis is limited and has recently been reviewed by Landgraf, (1996). Evidence is most probably questionable because of VPT being undertaken in those with chronic incapacitating diabetic complications that may prove to be irreversible, although up to 90% of VPT recipients report a healthier lifestyle and most remain insulin independent if rejection can be prevented (Nathan *et al.* 1991; Zehr *et al.* 1992; Zehrer and Gross, 1994; Adang *et al.* 1996). Detailed studies of quality of life analysis indicate that patients having SPK grafts have a significantly better quality of life than those having a renal transplant alone but most studies are retrospective and the results still debatable. More specifically a successful pancreas transplant accompanied by cessation of exogenous insulin therapy and liberalisation of diet, decreases the impact and worry of diabetes and improves quality of life. Unfortunately, these studies have yet to be compared to islet transplantation because of the lack of its success (Nathan *et al.* 1991).

Retinopathy

Patients who have had successful SPK, when compared to those whose pancreatic grafts are rejected, gain no additional benefit in terms of improvement in retinopathy, despite their euglycaemic status (Ramsey *et al.* 1988); furthermore, no differences between pancreas/kidney versus kidney alone have been demonstrated. Similar observations have occurred in studies claiming no discernable improvement of diabetic retinopathy after combined kidney-pancreas transplantation (Landgraf *et al.* 1989), where retinopathy benefits by correction of uraemia alone. These disconcerting findings have also been substantiated by others (Zech *et al.* 1991; Bandello *et al.* 1991; Bandello *et al.* 1992). In contrast, Wang *et al.* (1993b) have compared several parameters of diabetic retinopathy. In 43% of SPK recipients regression was documented, compared with 23% having kidney alone. However, 50% of both groups gained no benefit but only 7% of the SPK recipients, as opposed to 27% with a sole kidney graft, had progressive disease. In conclusion this suggests that retinopathy may not benefit from pancreas grafting. It must also be kept in mind that most recipients of a VPT have advanced retinal disease previously treated by photocoagulation which is known to stabilise disease progression, thus confounding any advantage in those having such an operation (The Diabetic Retinopathy Study Research Group, 1987). As photocoagulation has become an established treatment for retinopathy, VPT alone could not be offered as a substitute.

Nephropathy

Immunosuppression that prevents graft rejection is nephrotoxic. This being the case renal function deteriorates more rapidly after sole pancreas transplantation (Morel *et al.* 1991b) than that reported in diabetic patients with overt nephropathy treated with insulin (Parving *et al.* 1983). The assessment of VPT upon nephropathy is perplexing because of there being no easy way of determining renal disease or the risks of developing ESRF, especially after only a few years of diagnosis of IDDM. Moreover, when it is possible to forecast deterioration to overt nephropathy, VPT is unlikely to reverse residual disease (Remuzzi *et al.* 1994). This has been confirmed by a study where patients with established glomerular lesions, who had a pancreatic allograft, failed to have any amelioration of nephropathy after five years of normoglycaemia (Fioretto *et al.* 1993). The Stockholm Group with eight years of follow up demonstrated a significant difference in basement membrane thickness in pancreas after kidney transplants (PAK) as opposed to kidney alone. The overall conclusions from this study was that pancreatic grafting with subsequent normalization of glucose control can prevent or reduce signs of DN in kidney allografts (Wilczek *et al.* 1995). With reference to SPK grafts, a prospective trial has demonstrated less glomerular hypertrophy and mesangial proliferation when kidney recipients were given an additional pancreatic graft (Bilous *et al.* 1989); summising that perhaps VPT is only advantageous in patients with an established renal allograft and confers no additional benefit in those with native kidneys.

Neuropathy

Several studies have demonstrated benefits for patients having PAK grafts in terms of sensory, motor and autonomic neuropathies when compared to those patients with failed grafts or those having no transplant (Kennedy *et al.* 1990; Navarro *et al.* 1990). Likewise, there are reports of milder peripheral polyneuropathy (Martinenghi *et al.* 1997), paraesthesia (Landgraf *et al.* 1989; Solders *et al.* 1992; Naouri *et al.* 1992), autonomic neuropathy (Hathaway *et al.* 1994) and slightly better sensory amplitudes (Vial *et al.* 1992). Improvement is relatively mild but can reduce the risk of death if patients have dysautonomia, particularly with abnormal cardiovascular reflexes (Navarro *et al.* 1990). Although, the duration of follow up in these studies was short, benefit was detectable after only 2 years and, because of similar results being demonstrated after renal transplantation alone, it would be difficult to ascertain whether the improvement was due to the correction of diabetes or uraemia. However, a recent prospective study did not support these findings and showed less improvement in patients in an advanced stage of diabetic polyneuropathy (Müller Felber *et al.* 1993)

after a SPK procedure but it is plausible that results may have been better if the polyneuropathy had been less advanced.

Macrovascular

Finally, there are no controlled studies evaluating the effects of VPT on the multi-systemic macrovascular complications of diabetes. Retrospective data suggests that VPT does not alter the progression of PVD but may in fact accelerate it when compared to uraemic patients having only a renal allograft (Morrissey *et al.* 1997). Results are conflicting, those patients receiving a whole pancreas, in contrast to those receiving a segment, have near normalisation of lipid and lipoprotein profiles (Secchi *et al.* 1991). Nevertheless, very low density lipoproteins particles (VLDL), high density lipoproteins (HDL), and the triglyceride content of low density lipoproteins (LDL) are still higher than in normal subjects (La Rocca *et al.* 1994); their profiles in addition being exacerbated by peripheral hyperinsulinaemia because of the technical aspects of VPT (Eschwege and Fontbonne, 1992). Overall, recipients are, therefore, unlikely to have any regression of atheroma due to their atherogenic profiles.

2.3c Morbidity and mortality

It cannot be denied that the technical failure rate of VPT is high in inexperienced hands. The results of VPT are continually reviewed by the IPTR. For cases performed outside of the USA, there is an 18% technical failure rate in contrast to 12% of those performed within the USA. Figures range between 11 to 39% for all variations of pancreatic transplantation (including SPK, PTA and PAK) performed throughout the world. Patient survival rates at one year for SPK allografts (1994 to 1997) are now 94% with 89% insulin independent, this compares to 90% and 74% respectively, during the 1987 to 1989 era. At five years the actuarial function rate is 65% (Sutherland and Gruessner, 1997). Functional success varies greatly with procedure. SPK allografts fare better than PAK allografts where graft failure can be up to 69% at one year, similar rates of failure occur for PTA if performed outside the USA (Sutherland and Gruessner, 1995). In the recent series of 200 cases reported by Sollinger *et al.* (1993) patients receiving a SPK transplant had a 90% re-admission rate, 84% infection rate (mostly urinary tract infections) and a 27% reoperation rate. The propensity to lose grafts from rejection is more frequent after solitary PTA than in those performed simultaneously with a kidney. A reasonable hypothesis to explain this is that the early recognition of kidney rejection by deteriorating renal function also alerts the surgeon to simultaneous pancreas graft rejection. In general, grafts with BD are also associated with the best patient survival rates.

In summary, VPT offers an alternative treatment to exogenous insulin therapy in those patients with a kidney transplant. The IPTR clearly shows that VPT is a reasonably safe procedure in experienced hands. In general patient survival rates are in the region of 90 to 100%, 80% have a functioning graft and 70% are insulin independent up to 5 years later (Sollinger *et al.* 1993; Sutherland and Gruessner, 1995; Sutherland and Gruessner, 1997). Similarly, long-term metabolic control is impressive (Pfeffer *et al.* 1996). Notwithstanding these encouraging results, the fact that chronic, toxic immunosuppression may offset any potential improvement in quality of life, and the apparent failure to prevent or significantly improve complications with the exception of neuropathy, there is at present no justification for VPT alone other than in patients with extremely labile diabetes that is in itself life threatening or incapacitating. The benefits of VPT compared to insulin therapy are still questionable as its advantages have yet to be proven by a prospectively randomised controlled trial. In order to conclusively demonstrate any benefit patients need to be prospectively randomised to either VPT or IIT.

2.4 Islet transplantation

One of the more appealing alternatives to VPT is transplantation of insulin producing islets of Langerhans. If successful, islet transplantation has several important advantages over VPT. The procedure can be performed with a local anaesthetic and has the potential for immunomodulation (i.e no immunosuppressive drugs) and carries less postoperative morbidity and mortality.

The results of early clinical trials have given hope, but not cure, for many IDDM patients who succumb to the long-term complications associated with diabetes, despite their daily ritual of insulin injections. With the recent confirmation from the DCCT of normoglycaemia reducing the likelihood of developing chronic diabetic complications in later life, there is still a need to develop islet transplantation as a future treatment.

At present three major problems have thwarted human islet transplantation from becoming a successful clinical entity during the 1990's ; firstly, the isolation of intact, viable islets from cadaveric organ donors relies on the enzymatic dissociation of pancreatic extracellular tissue by collagenase, an enzyme secreted by a bacteria called *Clostridium histolyticum.* Unfortunately, there is considerable batch to batch variation of collagenase activity (Johnson *et al.* 1996b) and many other independent variables

concerning islet digestion and purification, which detrimentally affect human islet isolation and, secondly, the reduction of tissue immunogenicity by *in vitro* immunomodulation has proven more difficult than was originally predicted from early experimental animal studies, so much so that allograft rejection still cannot be prevented by conventional immunosuppression; thirdly, most experimental techniques used to assess the viability, immunomodulation techniques, allotransplantation and graft rejection have been based in animal models which do not necessarily correlate to the human situation. Taken together the purpose of this section is to review the current literature of clinical islet transplantation and why it has failed.

2.4a Islet autotransplantation

Diabetes mellitus is a complication after total (100%) or subtotal pancreatectomy (75 to 90%; Ammann *et al.* 1984; Frey and Bodai, 1984; Frey *et al.* 1989; Lambert *et al.* 1987). In most cases (except for malignancy) the indication for this type of operative intervention (combined with an islet autotransplant) is because of unremitting chronic pancreatitis (CP). Alternative less radical operative treatments, in an attempt to preserve both exocrine and endocrine function in those patients with CP, are endoscopic sphincterotomy (Wlliamson, 1988), pancreaticojejunostomy or other drainage procedures (Beger *et al.* 1984; Frey and Bodai, 1984; Bradley, 1987) and coeliac plexus blockade. In the case of recurrent acute on CP, where many patients are young or middle aged, pain often becomes intolerable. It is in this circumstance, as a last resort when all else has failed, that pancreatectomy becomes the treatment of choice. Nevertheless, the advantages of pancreatic resection for CP are soon offset, by its failure to prevent diabetes which is often very labile and difficult to manage, whereas exocrine deficiency can be treated by oral supplementation of exocrine components (e.g. Creon ®)

Although diabetes can be overcome by segmental pancreas transplantation, the risk of recurrent pancreatitis is indeed high because of alcoholic patients continuing to consume alcohol and so total pancreatectomy is theoretically the best treatment option but renders the patient diabetic and afterwards compliance with exogenous insulin regimes is often poor. Therefore, an alternative has been to transplant the patients islets of Langerhans in an attempt to avoid the subsequent diabetic complications and the problems associated with exogenous insulin therapy in this difficult patient cohort.

Clinical islet autotransplantation and insulin independence

Mirkovitch and Campiche were the first to transplant intrasplenic autologous islets in a canine model, (Mirkovitch and Campiche, 1976; Mirkovitch and Campiche, 1977). These initial developments showed promise but shortly after, when attempted in the human situation, insulin independence could not be guaranteed (Hinshaw *et al.* 1981; Morrow *et al.* 1984), although most had been intraportal. At the time this was difficult to understand as similar protocols in the monkey were successful (Gray *et al.* 1986). Approximately 160 islet autotransplants (Islet Transplant Registry (ITR), 1996) have been performed throughout the world *(Table 2.2 p.56)*, the largest published series from Minnesota where 48 cases have so far been reported (Farney *et al.* 1991; Farney and Sutherland, 1992; Farney *et al.* 1992; Sutherland *et al.* 1993; Wahoff *et al.* 1995a). As in most institutions, the authors have performed either subtotal pancreatectomy, in an attempt to preserve some endocrine function, or a more radical total pancreatectomy primarily for pain relief and secondarily to maximize the islet yield for simultaneous autologous islet transplants to allow the best chance for insulin independence. With this in mind data interpretation, particularly the presence of insulin independence, has to be carefully scrutinized. That is because of difficulty in attributing sole insulin secretion to the autologous islet transplant or to the pancreatic segment left *in situ*. Islet autotransplants have been performed in 66 patients having a total pancreatectomy, with 14 of them developing long-term (>3months) insulin-independence *(Table 2.2)*. It is not without mention that of the remainder having a subtotal pancreatectomy, approximately 50% have experienced a similar duration of insulin independence, suggesting that the residual *in situ* pancreatic segment significantly contributes to overall endocrine function. These results are also further confounded by some centres transplanting islets after abnormal preoperative glucose tolerance tests (Memsic *et al.* 1984).

Factors influencing the success and failure of islet autotransplantation

In an effort to to explain the failure of some islet autotransplants, Pydrowski *et al.* (1992) performed detailed studies on insulin secretion using hepatic portal venous sampling. The conclusions from this study suggested that islet autotransplants can maintain insulin independence, normal glucose tolerance tests and Hb A1c levels but cannot necessarily preserve these functions over the patient's lifetime. In the context of subtotal pancreatectomy, it has been shown that the secretion of both the *in situ* residual pancreatic segment and simultaneous dispersed pancreatic islets vary from partial to total insulin contributions. There are a multitude of independent variables

Table 2.2 Results of worldwide autologous islet transplants

Centre/Reference	Series	Graft site	Transient Insulin Indep	Long term Insulin Indep	Complications
Japan (Amemiya, 1994)	8	PV-4 Spleen-1 Hepatic-3	0	0	Nil
Minneapolis, USA (Wahoff *et al.* 1995)	48(27)	PV-46 KC-2	20	12 (7) (1x>10 yrs)	Nil
Los Angeles, USA (Traverso *et al.* 1981)	4(4)	PV	0	0	PV thrombosis GI bleed
Geissen, Germany (Dobroschke *et al.* 1978)	4(3)	PV	0	0	Nil
Baltimore, USA (Cameron *et al.* 1981)	8	PV	3	3 (9-22m)	DIC (1 death) Hepatic infarction
Genoa, Italy (Fontana *et al.* 1994)	24	PV-12 IP-11 IM-1	23	12 (up to 9yrs)	Nil
Berlin, Germany (Lorenz *et al.* 1981)	12(7)	Not documented	4	3 (2) (4-24m)	Nil
Loma Linda, USA (Hinshaw *et al.* 1981)	5	PV	2	2 (7-14m)	Nil
Los Angeles, USA (Weber *et al.* 1978)	1	IM	0	0	Nil
Detroit, USA (Soru and Zaharia, 1972)	3	PV	0	3 4-14m	Nil
Detroit, USA (Toledo-Pereyra, 1983)	8	PV-7 KC-1	1	3 (10w-25m)	Hepatic infarction DIC (2 deaths)
Paris, France (Altman *et al.* 1984)	5(4)	PV	0	1 (32m^^)	Nil
Philadelphia, USA (Dafoe *et al.* 1990)	2(1)	PV	0	1 (22m)	Mesenteric vein thrombosis/ DIC
Pittsburgh, USA (Fontes *et al.* 1992)	2	PV	0	2 (6-15m)	Nil
Geissen, Germany (Hesse *et al.* 1994)	1(1)	PV	0	1(1) (6m)	Nil
Geneva, Switzerland (Morel *et al.* 1997)	9 (3)	PV	3 (6w)	4 (2) (13^^, 6m)	Nil
Madrid, Spain (Arias-Diaz *et al.* 1994a)	1	PV	0	0	Nil
Leicester, England (White *et al.* 1997a)	16 (15)	PV-14 Spleen-2 3-combined	5	2 (2) (12m-30m)	Sp. Infarction PV Thrombosis (2)

( ) Number of total pancreatectomy; ^^ 2/3 pancreatectomy

which can influence the success or failure of islet autotransplantation some of which are also relevant to islet allotransplantation and will therefore, be discussed in more detail in the following section.

In general there is no universal agreement as to the best technique for islet autotransplantation. Some centres prefer to process the islets immediately after pancreatic excision culminating in islet transplantation as little as three hours later (Farney *et al.* 1991). Others elect to use a brief period of *in vitro* culture prior to transplantation (Dafoe *et al.* 1990) which enables islet viability, islet quality, islet losses, the presence of malignancy and any batch contamination that may have been introduced during the isolation procedure, to be identified (Warnock *et al.* 1994). There are both advantages and disadvantages to the latter technique. For example, interim culture can lead to selective exocrine loss and improve overall islet purity and probably islet viability (Weber *et al.* 1976; Weber *et al.* 1978) but, on the other hand, it can be argued that islets do not benefit from *in vitro* culture because of profound islet losses and that *in vitro* viability assessment does not necessarily predict the situation *in vivo* (Socci *et al.* 1992). The role of post-isolation islet culture is undoudtedly more appropriate to islet allotransplantation, because of the reduction in immunogenicity.

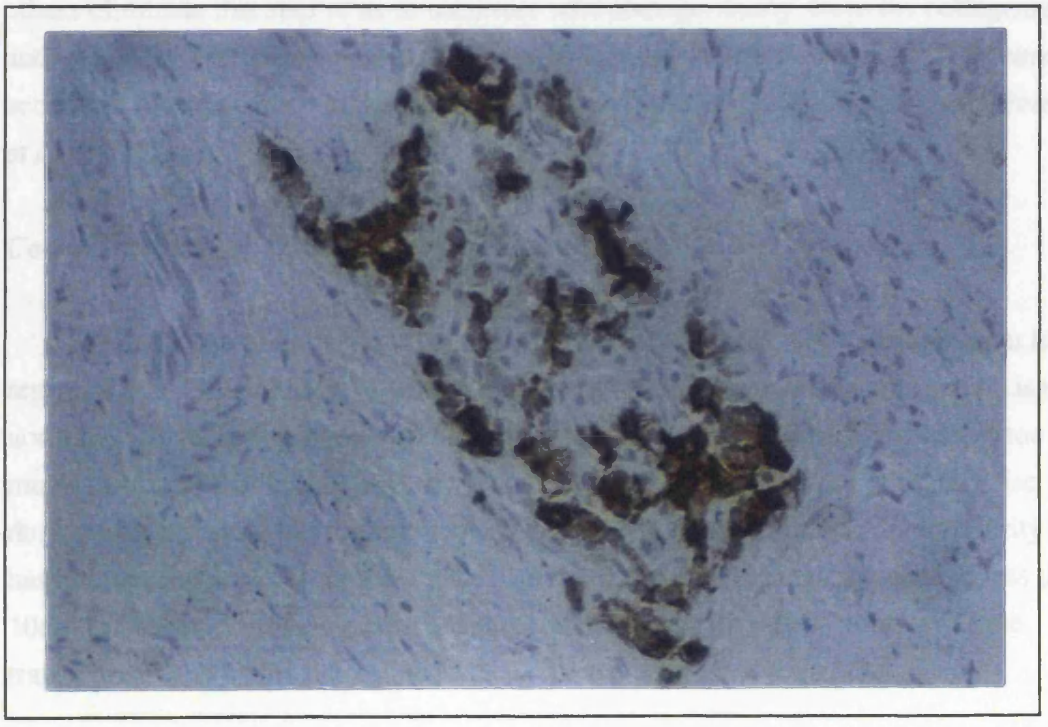

Figure 2.2 Intraportal human islet autograft 4 weeks post-transplant (x40 mag anti-insulin stain)

The majority (80%) of autotransplants have been intraportal *(Figure 2.2.)* , 11 intraperitoneal, 2 in the renal subcapsular space and 6 intrasplenic. Those placed

beneath the renal subcapsule have proved to be unsuccessful. Experimental animal studies have demonstrated that after implantation into the renal subcapsular space, islet function may be compromised, especially in the presence of contaminating pancreatic acinar tissue (Gray *et al* 1988) and because of slower islet revascularisation (Evans *et al.* 1989). Those islets placed directly into the peritoneum failed to prevent diabetes more frequently than those intraportally placed (Fontana *et al.* 1994), probably because of a marginal number of islets being transplanted and the lack of revascularisation; therefore, in the case of islet autotransplantation, the intraportal route is the preferred method of administration as with allograft transplantation, the reasons for which will be discussed in more detail in the following section.

There is no doubt that the number of islets transplanted correlates with insulin independence. Farney *et al.* (1991) state that as little as 109,500 islets (3,000 IEQ/kg patient) can produce insulin independence but long-term independence is more likely when 265,000 (3,500 IEQ/kg patient) islets are administered. Early autotransplants were prepared by a combination of mincing, collagenase digestion and washing. More recently the automated method has been applied (Fontes *et al.* 1992) along with COBE purification (White *et al*, 1997a). Most centres advocate collagenase digestion whilst others eliminate this step so as to minimise islet damage; firstly, from the collagenase and, secondly, from the warm ischaemia which is unavoidable during 37^{o}C *in vitro* activation of collagenase in the automated circuit (Hinshaw *et al.* 1981; Toledo-Pereyra *et al.* 1984b).

Complications of islet autotransplantation

One of the major concerns of treating diabetes by islet autotransplantation is its reported 2 to 3% mortality, in contrast to therapy with exogenous insulin which is not normally life threatening, only in the case of severe hypoglycaemia, because of too much insulin. Most commonly, the pressures within the portal vein gradually rise during islet infusion, but in most cases this is only a transient effect. In a minority this has risen as much as 50 cm (of water) with the mean value being estimated at less than 10cm (of water). However, this is thought to correlate with the volume of tissue transplanted and could also relate to the percentage purity of the graft.

After islet autotransplantation excessively raised portal pressures have in most cases, but not all, been a prerequisite to developing major postoperative complications. Cameron *et al.* (1980) have reported portal pressures of 50 and 60 cm/water in two patients, one of whom survived and the other dying 10 days later as a result of hepatic

infarction (Walsh *et al.* 1982). Both patients had evidence of disseminated intravascular coagulation (DIC) but the infused islets were processed by mincing and collagenase digestion and were not purified. Further reports of DIC have followed from Mittal *et al.* (1981) and Dafoe *et al.* (1990) both groups using the same methods as described by Walsh *et al.* (1982) for islet isolation. Similarly, and perhaps more of a concern, is the report from Memsic *et al.* (1984). They confirmed that raised portal pressures (of up to 45 cm/water) were a prerequisite to *delayed* postoperative complication. Interestingly, their patient made an uneventful postoperative recovery from the pancreatectomy and islet autotransplant until presenting one year later with massive variceal bleeding from the gastro-oesophageal junction, perhaps caused by a gradual portal vein occlusion. It is particularly disconcerting in the light of this report that other late onset complications have been recognised, perhaps also attributable to chronic, insidious portal hypertension, manifesting as a mesenteric venous thrombosis (Dafoe *et al.* 1990). In an effort to reduce the morbidity and mortality of this procedure, investigators have used systemic heparinization and aprotinin (nonselective protease inhibitor) during islet autotransplantation, but this technique has since failed to prevent DIC, hepatic infarction (Toledo Pereyra *et al.* 1984a) and mesenteric vein thrombosis as reported by Dafoe *et al.* (1990). In all of these patients islet infusions were very impure and highly contaminated by exocine tissue hinting that islet purification and *in vitro* culture may prove to be a compulsory undertaking at the expense of islet losses.

There is very little published information regarding the effect of islet autotransplantation upon the portal vein vasculature. Mehigan *et al.* (1980) have studied the consequence of non-automated, unpurified islets on blood coagulation in dogs. It was demonstrated that with the addition of heparin and aprotinin to the suspended islet tissue prior to autotransplantation, there were significantly lower levels of fibrinogen degradation products than in those without. They proposed that tissue thromboplastins produced by pancreatic mincing may be a precursor to DIC. One further important issue relevant to the portal infusion of collagenase digested pancreatic fragments is that it is often accompanied by a transient rise in portal venous pressure and systemic hypotension. Both, theoretically, being mediated by kallikreins released from the exocrine pancreas, although this has not been specifically investigated (Schwartz and Traverso, 1984).

In the presence of these reported complications, islet autotransplantation does offer an alternative to exogenous insulin therapy after pancreatectomy, although some cases can develop major complications even with transplantation of purified islet grafts.

2.4b Islet allotransplantation

To date, 270 islet allotransplants *(Figure 2.3)* have been reported in 22 different centres (Jan 1974 to Dec 1995), the majority of cases being performed in the USA (ITR, 1996). A recent analysis of the ITR demonstrates long-term insulin independence (4 year follow up) in one Type 1 diabetic islet allograft recipient (ITR, 1996). Attempts at transplantation of islets into Type 1 diabetic patients were initiated in Minnesota after successful islet isolation protocols were established in animals (Najarian *et al.* 1977). In a preliminary report, 10 patients, 7 of whom already had a renal allograft, underwent islet allotransplantation. Purified islets were transplanted into an array of different sites; 5 were intraperitoneal, 4 intraportal and 1 in a groin muscle pocket. Contrary to amelioration of diabetes in rodents (Younoszai *et al.* 1970), exogenous insulin was still required for all recipients (Najarian *et al.* 1977). After demonstrating the feasibility and safety of the technique, as no patient developed adverse side effects, it was assumed that failure to reverse diabetes in this series was primarily due to the transplantation of an insufficient islet mass.

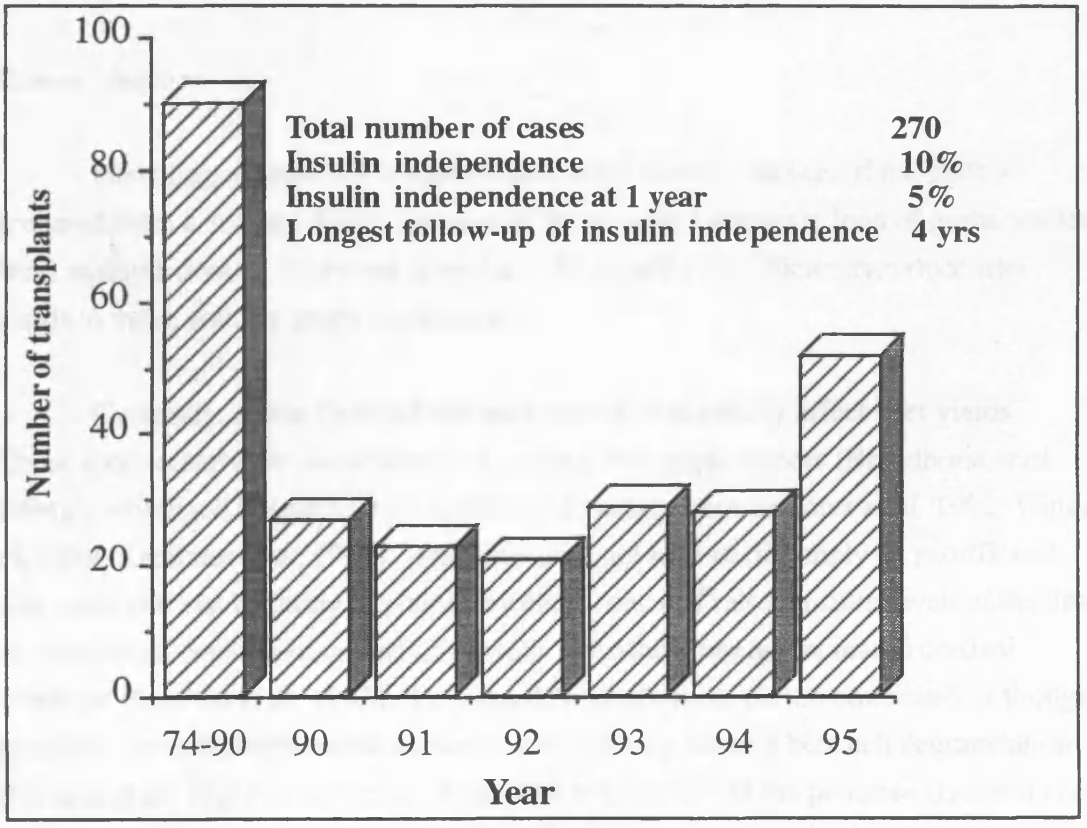

Figure 2.3 Results of islet allografts (ITR, 1996)

Recent reports from Minnesota detail a total of 50 islet allografts have now been performed (ITR, 1996) with long-term insulin independence only recently being

achieved. C-peptide secretion indicative of graft function was more commonly encountered (Younoszai *et al.* 1970), suggesting that perhaps islets were functioning but not secreting enough insulin. Despite islet allotransplantation being an extremely difficult endeavour it has still captured the imagination of other researchers in St Louis (Scharp *et al.* 1991), Milan (Secchi *et al.* 1997), Miami (Alejandro *et al.* 1992), Edmonton (Warnock *et al.* 1992), Pittsburgh (Ricordi *et al.* 1992a), Paris (Altman *et al.* 1992), Leicester (London *et al.* 1992b) and Geissen (ITR, 1996) with insulin independence only being documented in 27 Type 1 diabetic recipients (ITR, 1996).

From this brief summary it appears that the majority of islet allografts fail *(Figure 2.3).* Assessment of grafts performed during the years 1990 through to 1994 indicates patient survival to be 95% at one year, for graft survival 25% (c-peptide >1ng/ml) and 7% insulin independence for greater than 7 days (ITR, 1996). There are many reasons for this failure. Conveniently, they can be subdivided into pre-islet isolation variables, those which may affect the isolation process itself and finally, post-islet isolation variables.

Pre-islet isolation variables

Donor factors

Islet allograft survival is theoretically more likely to succeed if the graft is prepared from a solitary donor, because of the increased antigenic load of grafts pooled from multiple donors. Many variables have the capacity to sufficiently reduce islet yields to make solitary grafts impractical.

Generally, donor factors have been shown to adversely affect islet yields. Those more commonly documented are, young lean organ donors (Brandhorst *et al.* 1994a), prolonged hospitalisation and donor hyperglycaemia (Gores *et al.* 1992; Watt *et al.* 1994; Ketchum *et al.* 1994). The latter does not necessarily imply an insufficient islet mass and can be partly explained by the presence of raised insulin levels at the time of brain death because of underlying insulin resistance through increased cortisol secretion (Masson *et al.* 1993). Prolonged hospitalisation, on the otherhand, is thought to affect islet isolation because of dietary insufficiency causing beta cell degranulation (Ricordi *et al.* 1991) or alteration of the state of hydration of the pancreas (London *et al.* 1990). Alternatively, it can be hypothesised that starvation may lead to acinar insufficiency (Benhamou *et al.* 1994b) which could affect density gradient purification of pancreatic digest, although this concept has not been experimentally proven (Chadwick *et al.* 1993b). Those patients with a significant past medical history of

asthma, hypertension, seizures and phenytoin administration have also been shown to yield fewer viable islets but again these variables have not been thoroughly investigated (Watt *et al.* 1994).

Pancreas procurement

The objectives of pancreas procurement are to keep both warm ischaemia and cold ischaemia (Benhamou *et al.* 1994b; Ketchum *et al.* 1994; Zeng *et al.* 1994) to a minimum and to preserve the pancreatic capsule (Brandhorst *et al.* 1994a). During the retrieval operation a period of pancreatic ischaemia is inevitable because of the pancreas having a lower rate of blood flow relative to other organs and by virtue of its retroperitoneal position (Conti and Cosimi, 1994). However, studies of VPT suggest the pancreas appears to be no more susceptible to ischaemia than any other organ (Marshall *et al.* 1994; Florack *et al.* 1989) whereas warm ischaemic times in excess of only 20 minutes undoubtedly precludes to a poor islet yield (Corlett and Scharp, 1988; Brandhorst *et al.* 1994a). In order to avoid pancreatic warm ischaemia during organ retrieval, a cold *in situ* vascular flush is commonly undertaken. Many different preservation solutions have been tried but it appears that each need to be tailor made to provide optimum success for individual organs.

Usually *in situ* perfusion of the abdominal aorta reduces warm ischaemia of all abdominal organs. It has been shown that when the pancreas is individually perfused via the splenic artery, islet viability is better and less islet degranulation occurs, suggesting that perfusion techniques need to be refined (Raude *et al.* 1991). Recently, the superiority of The University of Wisconsin solution (UW) as an *in situ* vascular perfusate has been proven for the vascularised pancreas (Sollinger *et al.* 1989), liver (Kalayoglu *et al.* 1988) and kidneys (Ploeg, 1990). With respect to islet isolation following pancreatic perfusion with UW, islet yields are less but not significantly less (Kneteman *et al.* 1990a; Kneteman *et al.* 1992). In contrast, a randomised prospective study could not demonstrate any difference in islet yields between those perfused with UW and those that were not (Kneteman *et al.* 1994). Following on from these studies our centre has subsequently focused on the components of UW that interfere with collagenase digestion because of the diminished islet-exocrine cleavage seen after the pancreas is perfused with UW (Robertson *et al.* 1993a; Contractor *et al.* 1994; Contractor *et al.* 1995a). It is now assumed that although UW is currently the most popular preservation solution for most whole organs, some of its components may be deleterious to collagenase digestion of a human pancreas and certain refinements in its composition will need to be made in order to optimize islet yields (Chapter Six).

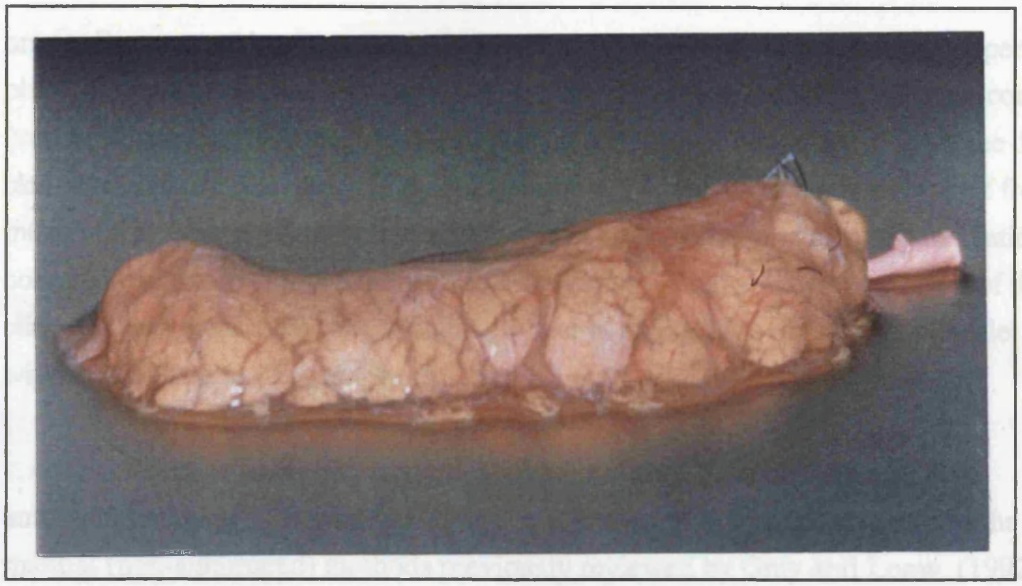

Figure 2.4 Intraductal collagenase distension of a human pancreas

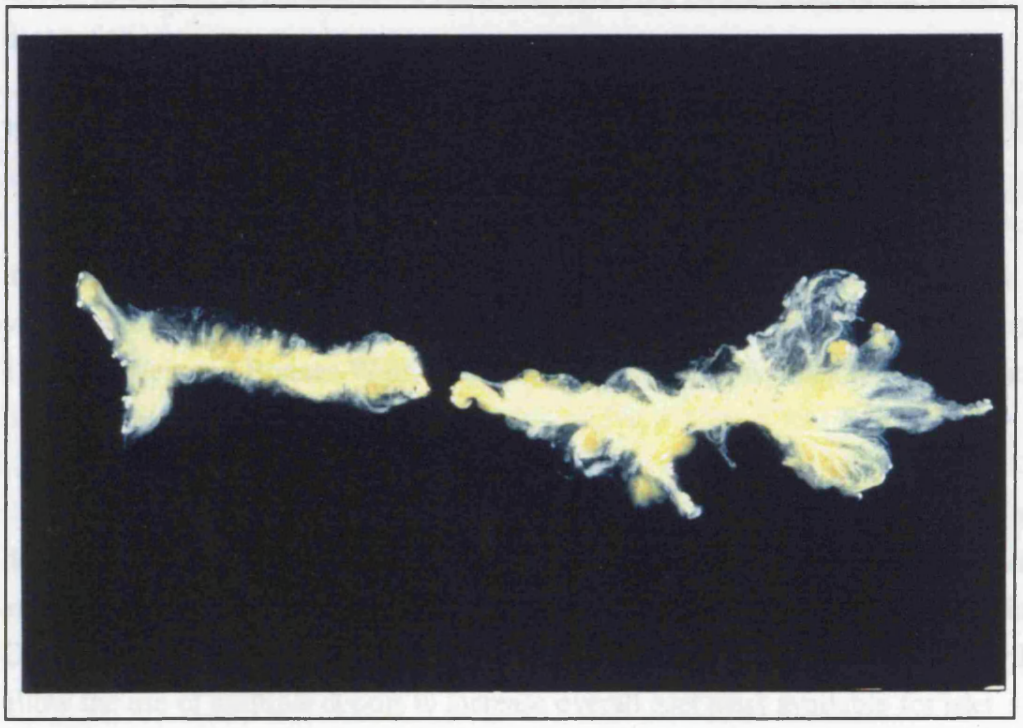

Figure 2.5 Remaining pancreatic ductal system after parenchymal digestion

Isolation variables

Pancreatic dispersion

Although many techniques for islet isolation have been described, most centres use variants of the intraductal collagenase digestion *(Figures 2.4 and 2.5)*

originally reported by Gray *et al.* (1984). The principles of this enzymatic dispersion phase are based upon the fact that the islet-acinar interface has a high collagen content (van Suylichem *et al.* 1987; van Deijnen *et al.* 1992; see Chapter Five). It is the direct cleavage between this interface by collagenase that is crucial to the liberation of free intact viable islets and any subtle differences in collagenase enzymes or pancreatic collagen content may profoundly affect islet yields. A more detailed analysis of the current contentious issues regarding the effect of collagenase digestion upon islet yields will be reviewed in Chapter Three.

After intraductal administration of collagenase, the development of the automated method (Ricordi *et al.* 1988a) was shown to be a major advance to the manual (non-automated) methods previously reviewed by Gray and Leow, (1992). The automated method has now been almost universally accepted as the best method to isolate good islet yields and at the same time allowing careful monitoring of the dispersion process.

Islet purification

After pancreas dispersion the collected digest contains many impurities including acinar tissue, ductal fragments, highly immunogenic lymphocytes and vascular endothelium (Gotoh *et al.* 1986; Gores *et al.* 1986), with islets making up only 1 to 2% (Weir and Bonner-Weir, 1990), thus highlighting the need for a purification phase.

Not all centres purify pancreatic digest. Gores *et al.* (1993) suggest that after density gradient purification, up to 75% of the original islet mass may be lost (Warnock *et al.* 1989b) and the degree of purity thereafter is less than that after islet handpicking. On these assumptions it is hoped that improved immunosuppressive regimens will allow the use of multiple donors to increase overall islet mass available for islet allografts. Presently, crude islet preparations are known to provoke a greater immunological response *in vitro* than purified islets (Ulrichs and Müller-Rucholtz, 1990) but even highly purified dispersed beta cells can still promote an immune response *in vitro* (Stock *et al.* 1991).

Other factors favouring islet purification stem from reports by Gray *et al.* (1988) where exocrine contamination has been shown to impair islet engraftment and *in vitro* islet viability (Sever *et al.* 1992). Whether this may be related to the detrimental effect of highly proteolytic enzymes released from contaminating exocrine tissue is not known and is, therefore, subject to investigation elsewhere (Heiser *et al.* 1994b).

Despite these relatively minor controversies the most compelling evidence in favour of islet purification come from islet autotransplantation studies. Attempts at autotransplantation of unpurified islet grafts, as mentioned previously, have led to three potentially avoidable deaths (Cameron *et al.* 1980; Toledo Pereyra *et al.* 1984). In view of the 300 or so total islet grafts which have been reported to the ITR (1996), a 1% mortality rate seems acceptable but *most importantly* it must not be forgotten that diabetes treated with subcutaneous insulin is not an immediately life threatening disease (except for severe hypoglycaemia) and therefore reports of any fatality is highly disturbing to those involved in clinical islet transplantation.

Post-islet isolation variables

Islet viability

In vitro islet viability can only be a subjective estimation of true *in vivo* function. At present there is no reliable method of absolutely determining that an islet allograft is 100% viable and, if it is, *'how viable'*. Particularly in the event of the pancreas being subjected to injurious warm and cold ischaemia and a traumatic islet isolation process, it is imperative to test islet viability prior to transplantation, but how ?

The most commonly utilised *in vitro* method of determining islet viability, involves the measurement of insulin secretion after glucose stimulation. This can be done *in vivo* or *in vitro*. *In vitro* techniques are based upon perifusion methods first described by Lacy *et al.* (1972), but although they can predict islet function *in vitro*, it has been shown to be an unreliable correlate to *in vivo* function (Socci *et al.* 1992). Many factors can potentially influence the outcome of perifusion including the nature of the perfusate, the temperature of the culture medium and the size of perifused islets. Human islet viability and function *in vivo* can also be assessed using chemically induced diabetic nude mice (Ricordi *et al.* 1988b; Lake *et al.* 1989b) or SCID mice (London *et al.* 1991), although even this technique is subject to variability, because of recipient beta cell regeneration after streptozotocin treatment (Rakieten *et al.* 1963; Movassat *et al.* 1997). It is essential, therefore, to use the subcapsular kidney space as a transplantation site so that the kidney and islet graft can be removed to prove that transplanted islets were maintaining normoglycaemia rather than regenerating beta cells (Lake *et al.* 1988; Lake *et al.* 1989b).

Membrane integrity (London *et al.* 1989b; Gray and Morris, 1987), colormetric assays (Kumar *et al.* 1994) have also been developed but despite being an easy technique they only show whether cells are dead or alive and give no indication of *in*

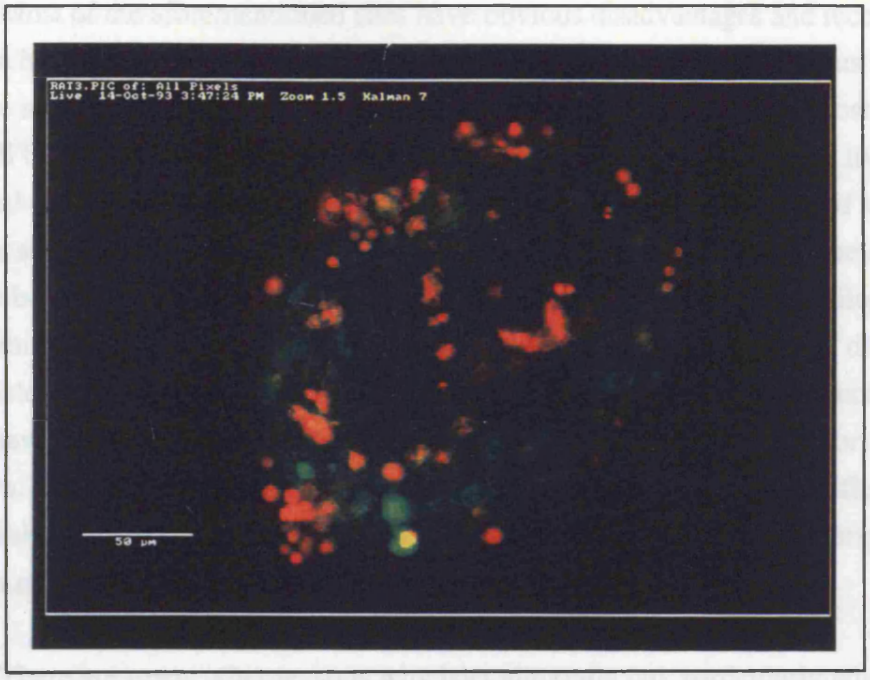

Figure 2.6 Islet viability assessed by fluoroscene diacetate/propidium iodide using confocal microscopy (live cells are green, dead cells are red).

vivo insulin secretion capability. They are also limited by the amount of dye penetration (Amin *et al.* 1995). Similar microscopic analysis has been performed with laser confocal microscopes *(Figure 2.6;* Merchant *et al.* 1993; Amin *et al.* 1995) but from a practical point of view are expensive and again cannot predict *in vivo* insulin secretion. In conclusion, *in vivo* islet function cannot be reliably predicted *in vitro* and can only be assessed after human transplantation. Graft dysfunction may only then become apparent thus significantly contributing to islet allotransplantation failure.

Transplantation site

Islets require a rich vascular supply in order to maintain glucose homeostasis, with this in mind successful transplant sites are limited. Experimental models, particularly rodents, have used an assortment of sites including intraportal (Kemp *et al.* 1973b), splenic (Koncz *et al.* 1976), renal subcapsular space (Toledo-Pereyra *et al.* 1984b and 1984c), peritoneal (Younoszai *et al.* 1970), subcutaneous (Kemp *et al.* 1973a), omental (Rajotte *et al.* 1994), intrathymic (Posselt *et al.* 1991; Barker *et al.*

1991), testicular (Bobzien *et al.* 1983), epiploic (Altman *et al.* 1990), rectum (Lim *et al.* 1994), intrathecal (Hara and Taniguchi, 1994) and intramuscular (Weber *et al.* 1978).

Most of the aforementioned sites have obvious disadvantages and recent attention has been focused on the immunoprivileged sites such as the thymus and testis but these seem impractical in the clinical situation and therefore the three most common sites will be discussed. In 1969 Calne and Davies (1969) proposed that the liver conferred an immunological advantage over other transplanted organs and if kidneys from the same donor were simultaneously transplanted with a liver graft, they were less likely to be rejected (Margreiter *et al.* 1988). Simultaneous islet and liver allografts have been performed elsewhere but the immunological 'liver phenomenon' did not extend islet graft survival (Tzakis *et al.* 1990b), although a subsequent French study has achieved insulin independence for 23 months in one patient. The authors, therefore, suggested that recipients of an islet-liver co-transplant were significantly more likely to maintain graft survival when compared to islet-kidney co-transplants (Altman *et al.* 1992).

There are many other reasons why islet allografts fail, particularly when islets are intraportally transplanted. Most intraportal islet grafts succumb to late failure between 6 months to 2 years after transplantation (London *et al.* 1994). This, to a certain extent, could be attributable to the relative hyperglycaemia of the portal vein known to be detrimental to insulin secretion, graft revascularisation and islet regeneration (Jansson *et al.* 1990; Juang *et al.* 1994) but notwithstanding this view systemic hyperglycaemia is best controlled by direct insulin secretion into the portal vein (Diem *et al.* 1990). The intraportal site also receives high concentrations of toxic metabolites from the gut and inflammatory cytokines. It has been shown that after induction immunosuppression with OKT3 there are raised intraportal levels of TNFα, IL-2, γ-IFN and IL-6 which can incur significant damage to intraportally implanted islets (Burke *et al*.1992; Xenos *et al.* 1992). Similar findings have been demonstrated with intraportal nitric oxide production (Stevens *et al.* 1996) and finally, the delivery of gut endotoxins may also be important. It must also be remembered that the liver is host to many immunocompetent cells, particularly Küpffer cells, a type of macrophage, that when activated have the potential to destroy damaged cells, thus the liver may, in fact, be a potentially hostile site (Davies *et al.* 1997).

The renal subcapsular space is also reported to have immunological advantages over other sites (Reece-Smith *et al.* 1981; Brandlien *et al.* 1987) and offers better growth conditions as well as safety and ease of access for biopsy specimens (one major disadvantage of an intraportally implanted islet graft). In rodents and canine models

renal subcapsule transplants are successful but attempts in pigs (Mellert *et al.* 1993) and humans (Farney *et al.* 1991) have failed with only one recent success being demonstrated in monkeys (Mellert *et al.* 1993). Overall the reversal of hyperglycaemia in this site is somewhat slow when compared to intraportal islet transplantation (Gray *et al.* 1986). The reasons for these poor results are thought to be due to the implantation of impure samples as exocrine contamination can adversely affect graft function (Gray *et al.* 1988). Furthermore, the regulation of blood flow in this area may also be different to that of the liver as the kidney is devoid of vagal innervation thus islet revascularisation may be somehow restricted and physiological function may be compromised (Jansson *et al.* 1990).

The concern of complications after intraportally placed grafts has prompted investigators to search for other implantation sites, among them the spleen. Canine studies have demonstrated better results with intrasplenic islet grafts as opposed to intraportal (Kolb *et al.* 1977). Results in pigs (Hesse *et al.* 1989) and primates (Gray *et al.* 1986) have also been promising. However, of the 15 human intrasplenic islet allografts only 3 have shown evidence of graft function (Hering, 1995) but two intrasplenic islet autotransplant patients from Leicester have developed insulin independence for over one year (White *et al.* 1997a). The main advantages of splenic implantation is that it is a highly vascular organ, possibly provides a more physiological insulin delivery to the portal vein and the splenic venous sinusoids can accommodate large volumes of pancreatic islet tissue.

The effect of transplant site upon islet graft survival is difficult to quantify based on previous clinical studies. This is primarily due to the overall poor performance of islet allografts and the predominance of the intraportal site and therefore further study in this area is urgently needed so as to identify the best site for implantation.

Transplantation of insufficient islets

As with any type of cell, islets show considerable variability in volume and clinical results based on the number of islets transplanted are difficult to compare. It is thought that the mean diameter of a human islet is 150μm and that islet mass should be determined by the number of 150μm IEQ/kg of patient (Ricordi *et al.* 1990a). However, it is only the beta cells which are of concern for reversal of diabetes and an accurate estimation of their number within an islet graft is virtually impossible. The amount of islet tissue needed to reverse diabetes is crucial to its success and has proved particularly difficult to correctly estimate. There have been extensive studies which have tried to determine the number of IEQ needed to reverse diabetes in animals but

only those relevant to the human situation will be discussed. It is thought that an average 70 gram human pancreas contains up to 1.5 million IEQ (Saito *et al.* 1978; London *et al.* 1994), which in an ideal situation would provide enough islets for at least 2 grafts.

The number of islets needed to reverse diabetes can to a certain extent be predicted from previous islet autotransplant studies. After partial pancreatectomy it has been suggested that 5 to 10% of the remaining beta cell mass (200, 000 IEQ) may prevent hyperglycaemia (Child *et al.* 1969) only that the requirements for functional intact transplanted islets are likely to be higher in the presence of the adverse factors outlined above. From the series of autotransplantation studies from Minnesota (Farney *et al.* 1991; Pyzdrowski *et al.* 1992), it appears that 3,000 IEQ/kg patient can maintain normal glucose homeostasis. A similar deduction can been made from the islet transplants performed after abdominal exenteration (Tzakis *et al.* 1990b) where as few as 3,200 IEQ/kg have rendered normoglycaemia in the presence of a diabetogenic immunosuppressive. Of the autotransplants performed in Leicester, five out of seven patients who received approximately 3,000 IEQ/kg developed insulin independence. However, long-term insulin independence (over 2 years) could only be achieved after transplantation of much greater numbers (9,240 IEQ/kg; White *et al.* 1997a).

To date, in the presence of autoimmune diabetic disease after islet allo-transplantation with diabetogenic immunosuppressives, as many as 6,160 to 22,196 IEQ/kg patient have been needed to reverse diabetes (ITR,1996). These figures correlate well with segmental pancreas transplants where a 50% pancreas transplant (up to 750,000 IEQ) can restore insulin independence (7,000 IEQ/kg patient). From this data it can be summised that islet autotransplant and allotransplant recipients require considerably more islets than that predicted from partial pancreatectomy patients; concluding that this is perhaps by virtue of diabetogenic immunosuppressives, inflammatory rejection mediated by cytokines and the peripheral nature of insulin delivery from implantation sites.

Islet engraftment

The revascularisation of transplanted 'avascular' isolated islets (Menger *et al.* 1994) has been investigated by several studies using microsphere technology *(Figure 2.7 p.70).* Following implantation, it is thought that the vascular integrity of islets returns to that seen in the native pancreas but recent evidence suggests that 'reversed revascularisation' may be a problem.

Essentially islet vasculature consists of one or two arterial branches intermingling with a glomerular capillary network draining into individual postcapillary venules (end capillary venules). Revascularisation, takes place from the recipients hepatic artery (Andersson *et al.* 1989), usually during days 10 to 14 after transplantation (Griffith *et al.* 1977; Menger *et al.* 1994). Following either islet allo or autotransplantation patients very often complain of inappropriate hypoglycaemia and it is thought that there is inappropriate local glucagon secretion that lowers the set point for glucose induced insulin secretion, where blood flow is first directed to the alpha cells as opposed to the normal variant where it flows to the beta cells first.

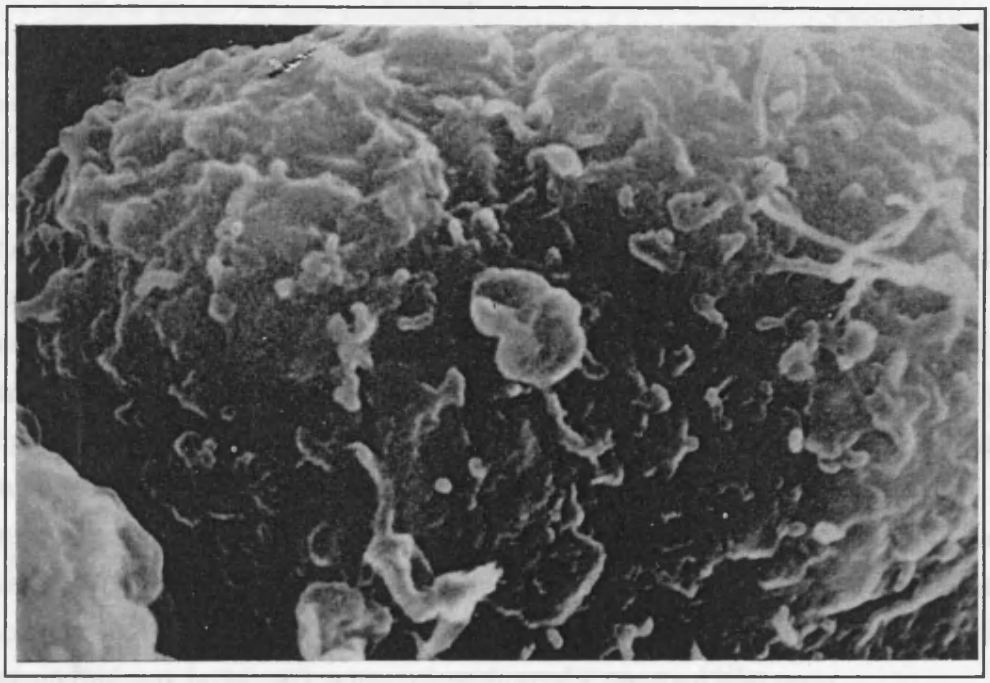

Figure 2.7 A scanning electronmicrograph of an avascular isolated islet

A whole host of factors determine the success or failure of islet engraftment. Firstly, the magnitude of graft size has important ramifications in that large grafts are subject to central necrosis through diffusional anoxia, particularly in an atherosclerotic diabetic patient during the initial period of revascularisation. Microangiopathy prevalent among diabetic kidneys is more likely to deter revascularisation in kidneys than in the liver. Another problem of adult islet transplantation for diabetic recipients is prolonged hyperglycaemia (Gray *et al.* 1989a; Mendola *et al.* 1994) where several studies have shown that less islets are required to reverse diabetes if hyperglycaemia is prevented (Korsgren *et al.* 1989; Yumiba *et al.* 1992). It must be remembered that on the basis of this experimental evidence, conclusions can only be conjectural as human diabetic recipients invariably have *chronic* diabetic microangiopathy which is likely to have an adverse effect on islet revascularisation.

Finally, immunosuppressive drugs, as already outlined, are detrimental to islet grafts perhaps especially during islet engraftment. Studies have documented retarded engraftment in recipients treated with prednisolone (Menger *et al.* 1990b) and Cs A (Merrell *et al.* 1985), although this is unsupported by others (Gores *et al.* 1987). An exception to this rule has been shown with 15-deoxyspergualin (DSG), which in a xenograft model, prevents reduction of capillary density (Menger *et al.* 1990a).

Islet dysfunction

There is increasing recent evidence that suggests islets may be dysfunctional after transplantation. Much can be learnt from islet autograft studies where higher levels of the split products of insulin (e.g intact proinsulin and 32-33 split proinsulin) are reported (White *et al.* 1997b). This may be because of transplantation of islets that are damaged during the islet isolation process, or through transplantation of a marginal islet mass, being a prerequisite to beta cell exhaustion. In this context persistent hyperglycaemia may cause inappropriate beta cell degranulation with the release of premature insulin granules or, alternatively, there may be inefficient processing of the split products that produce insulin and c-peptide as this appears to be a rate limiting step during hyperglycaemia. Another reason for higher insulin split products could be because of porto-systemic overspill by virtue of the intraportal site thus split products bypass hepatic first pass metabolism. It has also been observed in autograft recipients that patients have a blunted first phase insulin response to glucose (White *et al.* 1997b) but again this may be related to transplantation of a marginal islet mass.

Glucagon secretion is also thought to be pathological (*p.70*). It has been well demonstrated after human islet autotransplantation that intrahepatic islets fail to secrete glucagon in response to sustained hypoglycaemia but arginine responses are normal (Kendall *et al.* 1997), although some of these observations appear to be site dependent (Gupta *et al.* 1997). The precise cause for this is not known, it may be due to changes in islet revascularisation, reduction in α cell number or perhaps because of higher local hepatic glucose production that inhibits glucagon secretion.

2.4a Immunology of islet allograft rejection

It is generally agreed that islet allograft failure, in the first instance, is initiated by immune mediated destruction (Scharp *et al.* 1991; London *et al.* 1992b; Ricordi *et al.* 1992b). Rejection episodes of solitary islet allotransplants, where the islets and kidney are from different donors, have been more difficult to identify than for simultaneous

islet and kidney transplants (same donor) where it is assumed that rejection occurs in parallel; islet rejection manifesting as a rise in serum creatinine. In the case of intraportal islet allografts rejection can only be identified by hyperglycaemia or by falling serum c-peptide levels as histological liver biopsies are not routinuely performed because of the risk of haemorrhage and the likelihood of missing islet tissue. It has been suggested that for hyperglycaemia to occur greater than 90% of the islet graft must have been destroyed and restoration of graft function cannot be achieved thereafter (Scharp *et al.* 1991). Moreover, c-peptide levels in the early post-transplant period are suppressed due to supplementary exogenous insulin therapy and therefore cannot be reliably used to determine islet graft rejection at this stage (Villaneuva-Penacarrilo *et al.* 1993). Nevertheless, islet allograft survival improved during 1990 to 1992 but not necessarily leading to complete insulin independence and partial graft function is still only detectable in up to 63% of all cases at one month post-transplant (ITR, 1996).

Since hyperacute allograft rejection can usually be avoided by harvesting islets from a blood group compatible donor (Clayton *et al.* 1993b), the clinical pattern of islet allograft rejection is most commonly by 'acute rejection'. During the afferent phase of graft rejection shed antigenic material passes within the lymphatic system to draining lymph nodes sensitizing antigen specific T lymphocytes. The graft antigens are then processed by host antigen presenting cells (APC) and presented in context of MHC class II molecules to CD 4^+ T lymphocytes (*indirect antigen presentation*). Alternatively, allograft sensitization can occur through dendritic cells within the graft, expressing high levels of MHC class I and MHC class II. These cells migrate from the graft to draining lymph nodes to stimulate an alloreactive response *directly* (Lafferty *et al.* 1983; Stock *et al.* 1991; Shoskes and Wood, 1994).

T lymphocyte proliferation is thought to begin by a random clustering of T lymphocytes around APC *(Figure 2.8 p.73)* and low affinity binding by adhesion molecules on the surface of APC and T lymphocytes (Heeman *et al.* 1994). Activation requires engagement of the MHC class II molecules containing the allogeneic peptide on the APC by the T lymphocyte receptor (TcR). A second signal caused by binding of the B7 molecule of the APC to CD 28 on the T lymphocyte is necessary for T lymphocyte proliferation. Cytokine IL-2 is produced by the T lymphocyte which stimulates more T lymphocyte proliferation via the IL-2 receptor in an autocrine manner. IL-1 released by macrophages and lymphocytes induces IL-2 receptors on the T lymphocytes so acting in a costimulatory manner. CD8+ T lymphocytes stimulated by interaction of the TcR with MHC class I molecules containing allogeneic peptide and high levels of costimulatory molecules, in the presence of CD4+ T lymphocytes seems

to be required for cytotoxic T lymphocyte activation (Male *et al.* 1991; Sayegh *et al.* 1994).

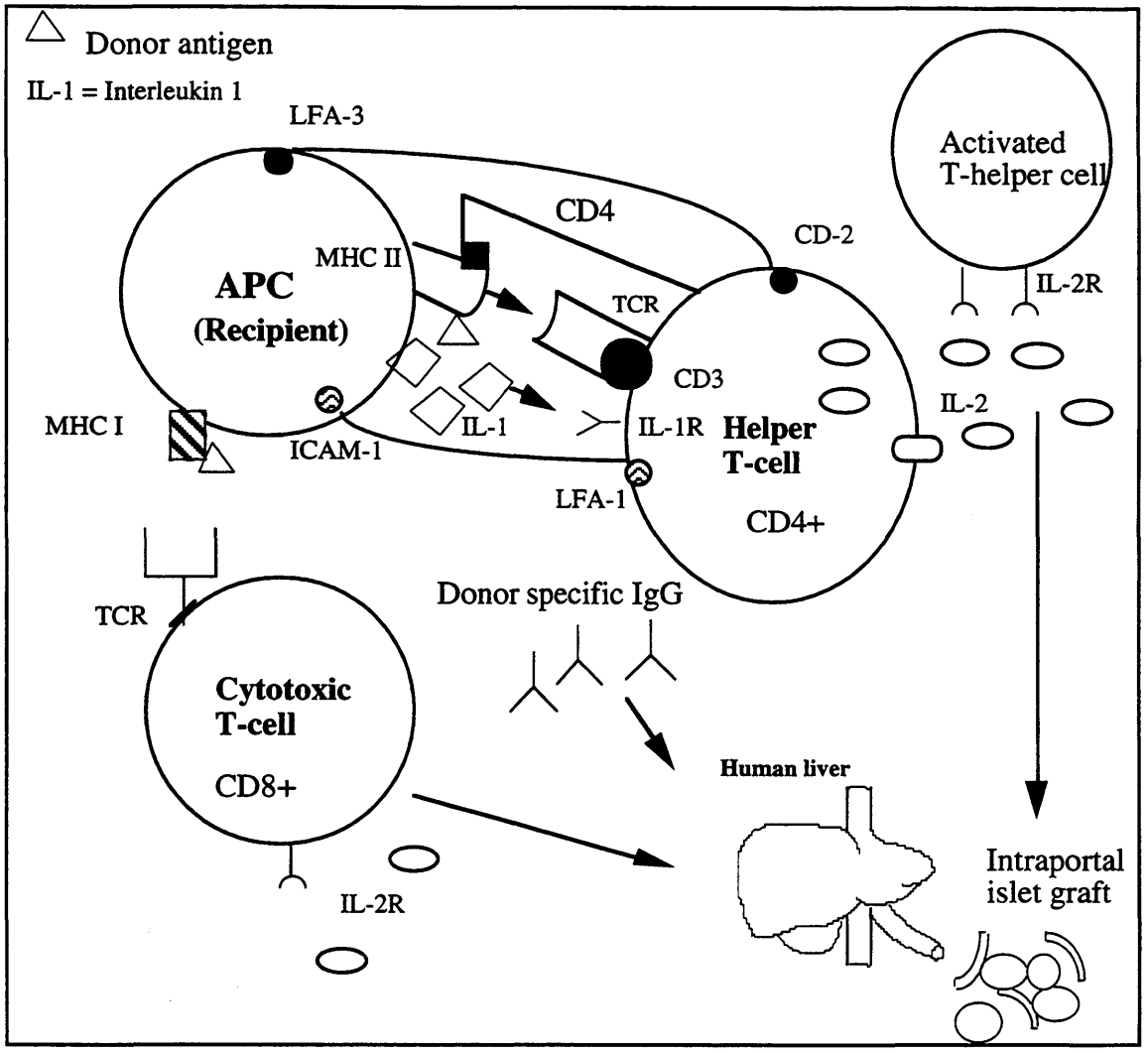

Figure 2.8 A schematic representation of islet allograft rejection (Indirect)

The intraportal site, by virtue of hepatic macrophages, sequester many allogeneic cells. During this process many cytokines and secretogogues are released which influence the tempo of graft rejection. Unfortunately, the cytokines released *(vide infra)* during islet graft rejection are highly toxic and inhibit glucose stimulated insulin release directly (Kawahara *et al.* 1991; Arias *et al.* 1992), thus exacerbating islet graft deterioration during rejection. In an attempt to reduce this detrimental cytokine response, investigators have endeavoured to identify ways to modulate this response *in vivo* by using phosphodiesterase inhibitors such as pentoxifylline. Some of these studies are somewhat contradictory, although pentoxifylline can block the inhibitory responses of TNFα on insulin secretion (Arias-Diaz *et al.* 1994b). Other reports

suggest that pentoxifylline has no effect upon TNFα and IL-6 concentrations (De Vault *et al.* 1994).

Finally, recent attention has also been focused on the role of superoxides, hydroxy free radicals and nitric oxide (NO) production at inflammatory sites. These are highly toxic inflammatory mediators which are known to cause cellular pyknosis and may be the final common pathway culminating in islet cell death (Fantone and Ward, 1982; Langrehr *et al.* 1993). More specifically, NO is produced by activated macrophages after intraportal islet allo or autotransplantation and can elicit islet dysfunction either by itself (Stevens *et al.* 1996) or via IL-1B toxicity (Xenos *et al.* 1994) which, interestingly, has been shown to be inhibited by recipient nicotinamide therapy (Andersen *et al.* 1994).

2.4d Prevention of islet allograft rejection

Immunosuppression for islet allografts

The ultimate aim of immunosuppression is to protect the allograft from immune rejection while reducing side effects on the host and it is not within the scope of this thesis to mention all the side effects of these drugs but those applicable to islet toxicity will be considered and discussed.

It was not until the discovery of 6-mercaptopurine in 1959 by Schwartz and Damashek that graft rejection could be prevented (Schwartz and Damaschek, 1959). With the application of this agent to canine renal allograft recipients, the modern era of transplantation was realised (Calne, 1960). Calne found that this drug had potent immunosuppressive effects and delayed kidney allograft rejection. Although results were variable, a subsequent derivative of 6-mercaptopurine, called azathioprine, produced even better long-term graft survival and with the addition of corticosteriods in 1963 the early immunosuppressive regime, which lasted for nearly 2 decades, was established (Goodwin *et al.* 1963).

The next milestone, and probably the most important, was the clinical use of Cs A, a cyclic peptide metabolite of the soil fungus *Tolypocladium inflatum* (Borel, 1976; Calne *et al.* 1979). After its introduction the results of all organ grafts were improved (Calne, 1992) but as is now well known its side effects, notably nephrotoxicity and malignancy, are a serious problem. Other bacterial products such as FK 506 (Goto *et al.* 1991; Thomson *et al.* 1993) and Rapamycin (Kay *et al.* 1989) have since been

developed and their modes of action recently being reviewed. Research into other novel immunosuppressive drugs continues, some of the more effective are thought to be DSG and mycophenolate mofetil (MMF; Dickneite *et al.* 1986).

Biological preparations have also been used to combat allograft rejection. The early crude heterologous antilymphocyte globulins had a significant impact in transplantation but they were rather non-specific and toxic (Woodruff and Andersen, 1963). Later with the discovery of monoclonal antibodies, by Kohler and Milstein (1975), results for steriod resistant rejection were improved. Unfortunately, the natural human immune response to these mouse antibodies eventually leads to neutralisation of their effectiveness, although in parallel with the developments in genetic engineering the 'foreign-ness' of monoclonal proteins has been eliminated making humanized versions available for clinical trials (Winter and Milstein, 1991).

The strategy to prevent islet allograft rejection in recipients of a previous renal allograft is based upon triple immunosuppression regimes (azathioprine daily, prednisolone daily, Cs A twice daily) plus 10 days of ATG or OKT3 (London *et al.* 1992b; Secchi *et al.* 1997). Other centres also use nicotinamide and pentoxifylline, in combination with immunosuppression, though their benefits have not been proven scientifically (Bretzel *et al.* 1997). By utilising both prednisolone and azathioprine, doses of Cs A can be kept to a minimum thereby reducing its nephrotoxic side effects. Cs A has formed the backbone of immunosuppression after islet allotransplantation (Mintz *et al.* 1988; Scharp *et al.* 1991; Warnock *et al.* 1992) but a report by Gunnarsson *et al.* (1984) revealed undesirable peripheral insulin resistance after segmental pancreas transplantation. This stimulated a series of investigations in the hope of identifying the true extent of Cs A damage upon islet endocrine function. Although most studies have been performed in animal models, Cs A has been shown to impair islet replication (Metrakos *et al.* 1993), engraftment (Merrell *et al.* 1985) and beta cell function by inhibiting insulin secretion of rat islets (Robertson, 1986). In contrast glucagon secretion is not adversely affected (Nielsen *et al.* 1986).

FK 506 has shown favourable results after liver, kidney and thoracic organ transplantation (Starzl *et al.* 1989). As the clinical experience with this drug widened it was shown to be deleterious to glucose homeostasis, but perhaps less than Cs A (Tze *et al.* 1990), as one recipient of an islet allograft with FK 506 immunosuppression has maintained insulin independence for over three years (Carroll *et al.* 1995). Other abnormalities of insulin secretion in human islets have also been demonstrated after azathioprine immunosuppression (Viviani *et al.* 1989) but the diabetogenic effect of prednisolone is much more commonly documented. It is known to impair islet

engraftment (Menger *et al.* 1990b), produce islet exhaustion (through insulin resistance; Morel *et al.* 1992a) and has such a marked diabetogenic effect that often requires exogenous insulin administration to achieve normoglycaemia (Ruiz *et al.* 1973). The reasons for these problems are thought to be a direct consequence of peripheral insulin resistance and abnormalities of insulin secretion (Ekstrand *et al.* 1992). More recently Xenos *et al.* (1993), have conducted *in vitro* experiments investigating the effect of DSG upon human islets. Their initial results showed no association between DSG and altered beta cell function in both human and rodent models (Xenos *et al.* 1993; Jindal *et al.* 1993) and on the strength of these findings DSG has been used in clinical trials (Gores *et al.* 1993) with two out of five allograft recipients becoming insulin independent. Nevertheless, any advantageous effect of DSG *in vivo* is difficult to ascertain with the need for concomitant prednisolone, Cs A and azathioprine administration in order to curtail graft rejection.

Overall one can assume that current immunosuppressive regimes can lead to drug induced diabetes. The true extent of which is difficult to quantify because of the need for multiple immunosuppressive drugs. This suggests that perhaps regimes which have been considered acceptable for vascularised organ transplantation are unlikely to be as successful for islet allotransplantation.

Islet immunomodulation

Snell (Snell, 1957) postulated that MHC class II positive cells 'passenger leucocytes', within allografts (Austyn, 1987) were pivotal in provoking allograft rejection. By exploiting the use of a cellular graft it has become feasible to functionally delete passenger leucocytes expressing MHC class II antigen by a process called immunomodulation, and over the years many investigators have endeavoured to eradicate these cells from allografts prior to transplantation.

Dendritic cells

Lafferty and Prowse (Lafferty and Prowse, 1984) proposed that immunostimulatory passenger leucocytes had the capacity to present alloantigen to T lymphocytes in the same way as other APC and could also deliver a unique activating co-stimulatory signal to both resting T helper and T cytotoxic cells thus eliciting a *direct* rejection response. Islets are known to weakly express MHC class I, (but not class II antigens) but it is the class II antigen expressed constitutively within an islet allograft that is fundamental to initiating the T helper cell response in the *direct* host versus graft rejection cascade. It is proposed that dendritic cells, that strongly express MHC class

I and II antigens, are the passenger leucocytes that can initiate an immune rejection response (Austyn, 1987).

Low temperature culture (LTC)

Initial experiments attempting to delete allografts of passenger leucocytes used pretransplant culture. By culturing mouse thyroid tissue Lafferty *et al.* (1975) demonstrated significantly prolonged transplant survival. They later showed the same phenomenon using mouse islet allografts which was thought to be due to loss of MHC class II expressing endothelium after a period of *in vitro* culture (Parr *et al.* 1980). Lacy *et al.* (1979b) extended Lafferty's original culture conditions by using LTC at 24 ^{o}C as opposed to 37^{o}C. Rat islet allografts subsequently survived for longer periods after LTC in CMRL 1066 (Lacy and Finke, 1991). This response was thought to be because of the loss of class II MHC antigen bearing cells (perhaps endothelial cells lost during *in vitro* culture) as following the intraperitoneal injection of class II bearing peritoneal cells in previously established islet allograft recipients (Lacy *et al.* 1979a) allograft rejection shortly ensued. Having realised the efficacy of LTC upon rodent and canine islets (Warnock *et al.* 1989a; Dabbs *et al.* 1991), other studies upon human islets have been less convincing. Scharp *et al.* (1991) have performed human allogeneic islet transplants after seven days LTC rendering one patient insulin independent. Nevertheless, the validity of any beneficial effect upon immunogenicity by LTC can only be conjectural in the presence of immunosuppressive drugs. Investigators have only shown the apparent advantage of LTC upon the immunogenicity of human islets when transplanted into nude mice (Ricordi *et al.* 1988b) and human islets dispersed into single cells assessed *in vitro* (Stein *et al.* 1994). The relevance of these latter studies to clinical islet allotransplantation are, therefore, speculative.

Gamma irradiation

Since the early immunomodulation experiments many groups have used other protocols to functionally delete dendritic cells in the hope of reducing islet immunogenicity. Anderson and Warner (1976) first demonstrated the toxic effect of gamma irradiation to subpopulations of lymphocyte cell lines, in contrast to islet tissue which is relatively irradiation resistant (Dobelower and Milligan, 1984). The effect of gamma irradiation is mediated by the ionisation of cellular molecules and the formation of damaging free radicals (Duncan and Nias, 1977). Preliminary studies of gamma irradiation as an immunomodulation protocol for isolated rat islets were first performed by Heinzman *et al.* (1983), demonstrating insulin release was impaired by doses of 25

Grays. Further protocols have been repeated with hamster (Tsubouchi *et al.* 1981) and mouse islets (Kanai *et al.*1989). James *et al.* (1989) also achieved long-term reversal of diabetes following allogeneic transplantation of rat islets pretreated with 2.5 Grays along with low dose Cs A . The beneficial effect of gamma irradiation has also been demonstrated by Stegall *et al.* (1990), they thought the immunomodulatory effect was secondary to a quantitative loss of donor interstitial MHC class II bearing cells. With regard to gamma irradiation of human islets current data suggests that up to 15 Grays (Gainer *et al.* 1994) can be used for immunomodulation although its capacity for a down regulation of an alloimmune response does not occur when assessed *in vitro.*

Ultraviolet irradiation

Additional strategies for depletion of passenger leucocytes have also included the use of direct ultraviolet irradiation. Ultraviolet light can inhibit lymphocyte mediated proliferation in the mixed lymphocyte reaction (Lindahl-Kiessling and Safwenberg, 1971), this graft pretreatment presumably inactivated passenger leucocytes to a greater degree than islet cells. In contrast to gamma irradiation, ultraviolet irradiation does not ionise atoms or molecules but loses energy to matter in the form of electron excitation primarily by disrupting DNA, thus reducing viability and inhibiting initiation of alloreactive responses. Hardy *et al.* (1984) published a comprehensive series of experiments confirming this hypothesis by producing prolongation of both allogeneic and xenogeneic rat islets after immunomodulation *in vitro* by UV-B irradiation at 1,000 J/m^2 but their results were markedly strain dependent. More recently UV-B irradiation of human islets has allowed islet function to be retained and reduced immunogenicity at 300 J/m^2 when transplanted into athymic mice (Benhamou *et al.* 1994a), although other investigators have successfully reversed hyperglycaemia with human islets transplanted into nude mice after 700J/m^2 irradiation (Dellagiacoma *et al.* 1992).

Despite the success of murine immunomodulation experiments consistency has proved difficult in some strain combinations unless the recipient received a short course of immunosuppression. For example, in the case of immunomodulation of rat islet allografts, recipient immunosuppression is a prerequisite to success. This latter finding supporting the concept of *indirect* antigen presentation. Taken together, any immunosuppressive protocol attempting to reduce graft rejection by leucocyte depletion must also include a method to revoke indirect presentation of donor alloantigen by recipient APC that are capable of inherently expediting T helper cell proliferation. In this context T helper cells are not specific for transplanted tissue, they may act as helper cells to B lymphocytes leading to the production of graft specific antibody (Lafferty and

Prowse, 1984). However, indirect antigen presentation is considered to be a much less efficient process than direct antigen presentation but the significance of indirect presentation has received support from experiments in large animals and *in vitro* studies using a mixed lymphocyte islet co-culture (Stock *et al.* 1989b).

MHC immunomodulation

The influence of strain combinations and their variable efficacy have led to a search for other factors influencing graft immunogenicity. Other studies have suggested that islet expression of MHC class I antigen affects longevity after allotransplantation (Osorio *et al.* 1994). Thus Markmann *et al.* (1990) compared outcome after allotransplantation of isolated rat islets cultured at either 24°C or 37 °C. They found both temperatures produced a comparable reduction in APC cells, but that 24°C markedly reduced islet endocrine cell MHC class I expression. The immunomodulatory affect was, therefore, assumed to be because of down regulation of islet MHC class I expression. However, the effect of MHC class I expression could also be overcome by the simultaneous use of anti-MHC class I antibody (Stock *et al.* 1989a). Other monoclonal antibodies used to avert graft rejection experimentally are anti-class II (Faustman *et al.* 1981), anti-dendritic (Faustman *et al.* 1984) and anti LFA-1 (Gotoh *et al.* 1994).

From this short synopsis some broad based conclusions can be drawn; firstly, with a few exceptions, the majority of the experiments concerning islet immunomodulation have been performed in rodents and the relevance of these to clinical islet transplantation is uncertain. There is a difference in the distribution and nature of MHC class II expression, both pig and human islets contain MHC class II bearing cells of endothelial origin whereas in the rat the endothelial cells are MHC class II negative (Shienvold *et al.*1986) and, secondly, despite the passenger leucocyte hypothesis, it has become apparent that MHC class I expression on endocrine cells is also important. The relevance of these findings needs to be investigated and the wealth of experimental work on islet immunomodulation in animal models needs to be urgently transposed to the human situation.

Immunoisolation

The idea of preventing the immune system from gaining access to cells by means of encapsulation is more than 40 years old when a diffusion chamber was developed to study the nature of homograft resistance (Prehn *et al.* 1954). A reappraisal by Lim and Sun in 1980 described a method for encapsulation of isolated

islets of Langerhans, effectively immunoisolation. This technique offers many advantages over the AEP (Friedman, 1989), which is susceptible to thrombosis (Tze *et al.* 1980), calcification (Klomp *et al.* 1983) and infection (Lim and Sun, 1980; *Figure 2.9*).

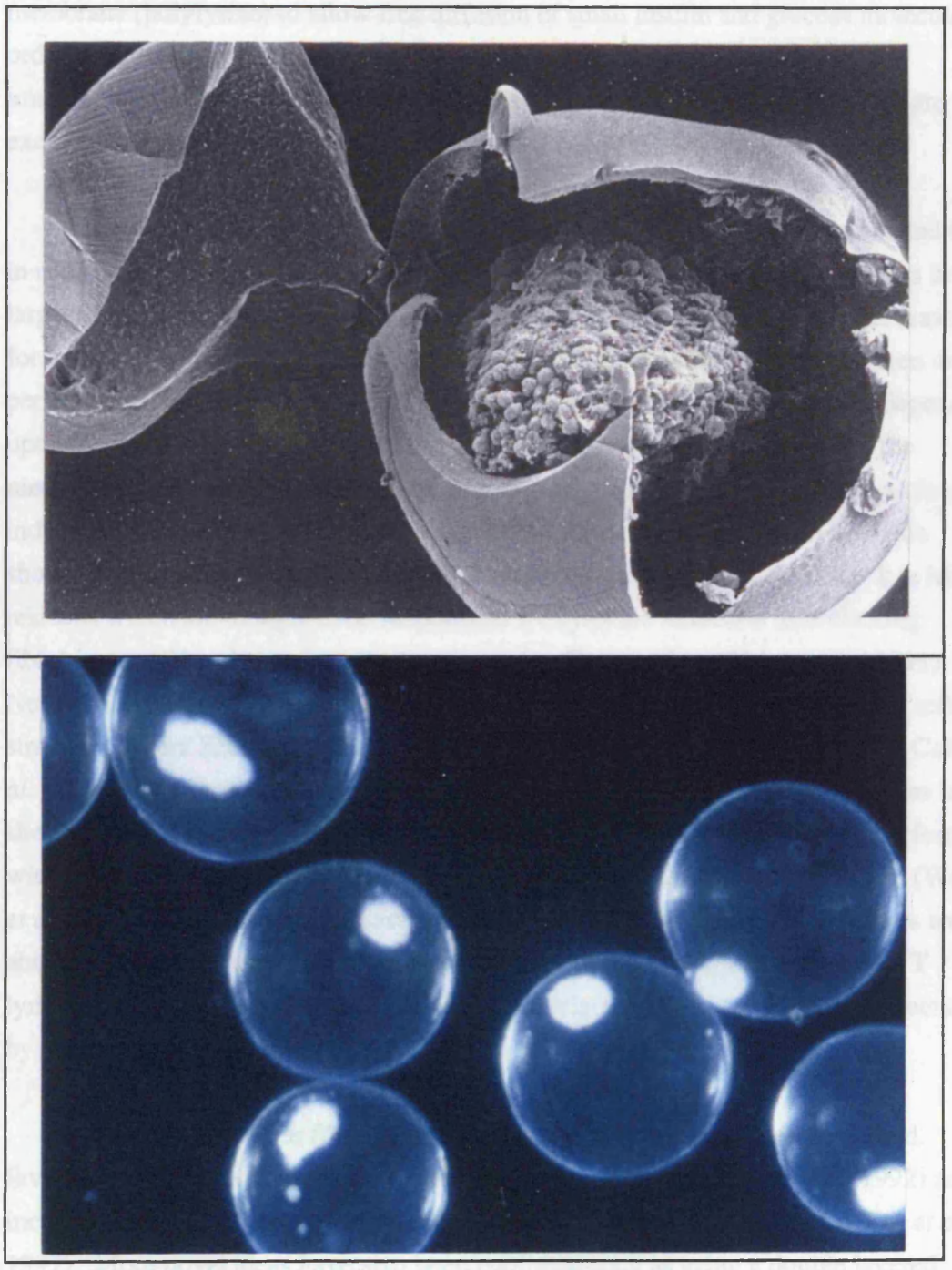

Figure 2.9 Encapsulated islets of Langerhans

The prototype islet capsule was alginate based (Lim and Sun, 1980); a potentially biocompatible material 93% of which is water based (Goosen *et al.* 1985).

The process of encapsulation entails the suspension of sodium alginate which is sprayed into a dish of calcium chloride using a droplet device (London *et al.* 1989a). When exposed to calcium ions the droplets solidify, with the islets being entrapped within a calcium alginate gel. The droplet is then coated with a semi-permeable membrane (polylysine) to allow free diffusion of small insulin and glucose molecules in order to maintain normoglycaemia. The larger immunoglobulins and other immunocompetent cells capable of mediating allogeneic or xenogeneic rejection are excluded.

Despite successful reversal of diabetes after transplantation of encapsulated islets in rodents (Calafiore *et al.* 1986; Petruzzo *et al.* 1991; Calafiore, 1992), attempts in larger animals have more commonly led to failure (Soon-Shiong, 1992). The reasons for this are two fold; firstly islet microencapsulation graft failure has largely been due to pericapsular fibrosis (Clayton *et al.* 1993a). The problem of fibrosis may be dependent upon the immunostimulatory capacity of the alginate and polylysine capsule, the mechanical and chemical capsular stability and the propensity for the outer capsule to induce cellular adherence (Soon-Shiong, 1992). One component which has been shown to provoke pericapsular fibrosis is mannuronic acid alginate (MAA). It is MAA residues which are thought to be responsible for cytokine induction thus eliciting fibroblast proliferation and marked pericapsular fibrosis (Soon-Shiong *et al.*1991). Nevertheless, this hypothesis has not been reproduced in other centres and may reflect strain dependent fibrotic reactions, the site of implantation (Clayton *et al.* 1991; Cole *et al.* 1992) or the degree of alginate purity (Goosen *et al.* 1985). Secondly, studies have shown increased survival after islet or recipient pretreatment. This has been performed with monoclonal anti-CD 4^+ antibodies (Weber *et al.* 1990b), UV-B irradiation (Weber *et al.* 1991) and immunosuppressive drugs. This implies that encapsulated islets are able to release antigens which may be capable of stimulating cytokine release, a T lymphocyte response and fibrosis obviating the original concept of immunoprotection by islet encapsulation.

Methods to reduce fibrosis and graft failure have recently been described. Investigators have tried to reduce the size of the islet capsule (Zekorn *et al.* 1992) and increase the purity of the alginate preparations (Zimmerman *et al.* 1992; De Vos *et al.* 1997). More novel ideas have also been published such as using a double layered vascular prosthesis (Calafiore, 1992) and the macroencapsulation of microencapsulated islets by using an alginate gel matrix (Lanza *et al.* 1991). Unfortunately, the problems of graft failure are still largely unsolved but this has not deterred the start of clinical trials in the USA where a patient has been reputed to be insulin independent after transplantation of allogeneic microencapsulated human islets (Soon-Shiong *et al.* 1994).

2.5 Islet Xenotransplantation

2.5a The evolution of clinical xenotransplantation

Xenotransplantation is the transplantation of organs or tissues from one species to another. This subject has recently generated a great deal of interest, mainly because of the current lack of human organ donors *(Figure 2.10).* The reason is not just experimental but is the only answer to the lack of organ donors. As the new millenium approaches, the substantial advances in medical research have provided an ever increasing pool of patients who are kept alive by conservative treatment e.g. dialysis for renal failure (UKTSSA, 1997).

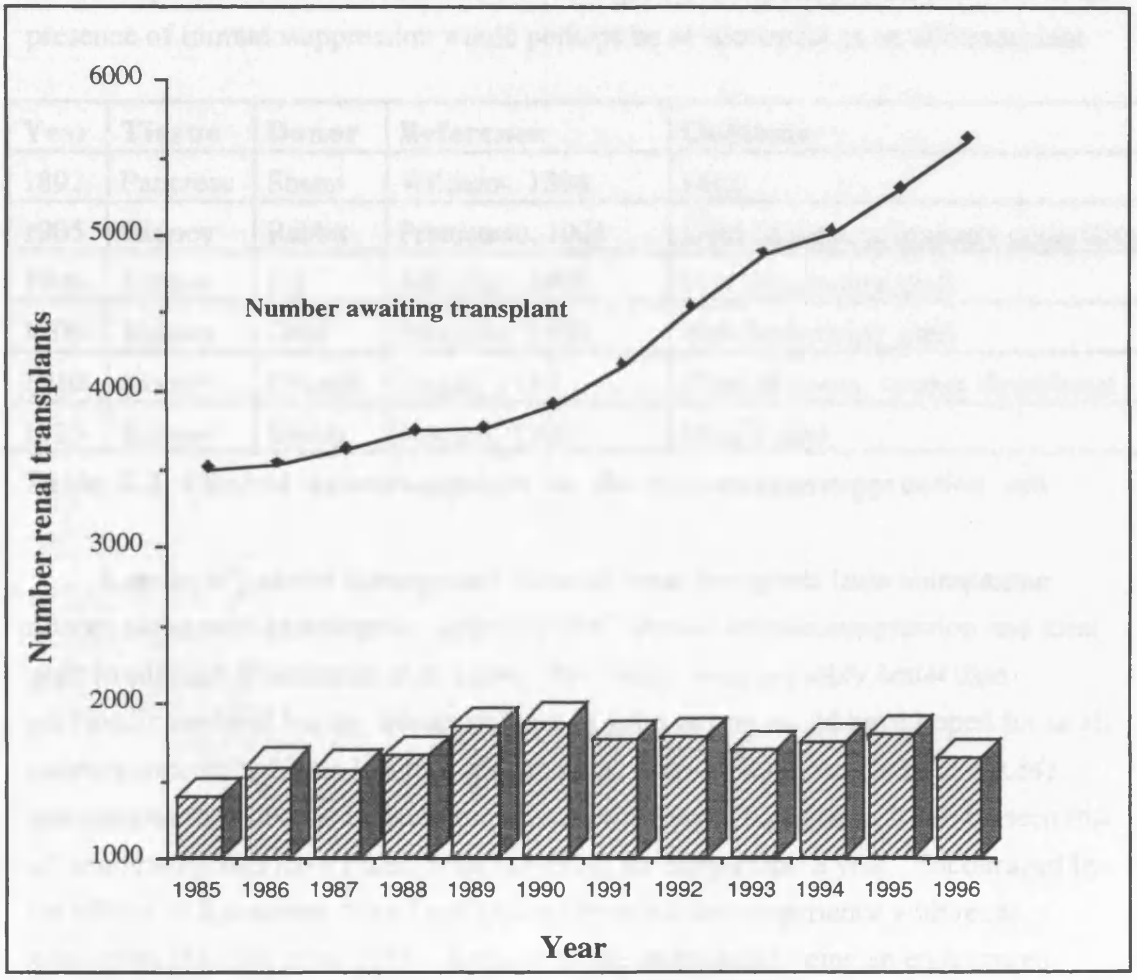

Figure 2.10 A graph illustrating the lack of renal organ donors (UKTSSA, 1997)

Clinical xenotransplantation can be traced back as far as 1892. Ironically this was an islet xenograft performed in a 15 year old child, who received pancreatic fragments from a sheep (Williams, 1894). Prior to this Boronio described the failure of skin grafts between cows and horses in 1804 (Kuss and Bourget, 1992). Since then

there have been a vast number of publications demonstrating successful xenotransplantation in animals but for the purpose of this brief review the evolution of clinical xenotransplantation will be presented and discussed.

Results of early xenotransplants, previously called heterotransplants, were daunting *(Table 2.3)*. Most xenografts in the absence of immunosuppression succumbed to almost immediate graft rejection, the nature of which was unknown. In the early sixties, following Medawar's description of the fundamental principles of skin allograft rejection (Medawar, 1944), along with the advent of immunosuppressive regimes and the difficulting in obtaining cadaveric donors, surgeons resumed clinical xenotransplantation. It was assumed that an organ from a closely related species in the presence of immunosuppression would perhaps be as successful as an allotransplant

Year	Tissue	Donor	Reference	Outcome
1892	Pancreas	Sheep	Williams, 1894	Died
1905	Kidney	Rabbit	Princeteau, 1905	Died 16 days, pulmonary congestion
1906	Kidney	Pig	Jaboulay, 1906	Non functioning graft
1906	Kidney	Goat	Jaboulay, 1906	Non functioning graft
1910	Kidney	Primate	Unger, 1910	Died 32 hours, venous thrombosis
1923	Kidney	Sheep	Neuhof, 1923	Died 9 days

Table 2.3 Clinical xenotransplants in the preimmunosuppression era

A series of patients subsequently received renal xenografts from chimpanzee donors along with azathioprine, actinomycin C, steriod immunosuppression and local graft irradiation (Reemtsma *et al.* 1964). The results were arguably better than previously reported but the impact was not as good as one would have hoped for as all patients succumbed after less than nine months. The table below *(Table 2.4 p.84)* summarises the published clinical experience with organ xenografts. It can be seen that all xenotransplants have failed, none surviving for longer than a year. Encouraged by the efforts of Reemtsma, Starzl and Millard reported their experience with renal xenografts (Millard *et al.* 1985). Because of the chimpanzee being an endangered species and in short supply the more distantly related baboon was their organ donor of choice. As predicted using similar immunosuppressive regimens survival was short lived and less than that achieved by Reemstma using chimpanzee donors. After these early efforts, despite ending in failure, some very important conclusions were drawn; firstly, it became evident that organs from a different species could maintain life in a human recipient; secondly, graft rejection could be reversed in a similar way to allograft rejection suggesting a similar pathophysiology and, thirdly, as with early allografts

which at the time had limited survival, although are now greatly improved, perhaps leads one to assume that xenograft survival is likely to improve over forthcoming years.

Reference/Surgeon	Tissue	Donor	No	Outcome
Reemtsma *et al.* 1964	Kidney Kidney	Chimp Monkey	12 1	< 9months 10 days
Hitchcock *et al.* 1964	Kidney	Baboon	1	4.5 days
Starzl *et al.* 1964	Kidney	Baboon	6	<2 months
Hume, 1964	Kidney	Chimp	1	1 day
Hardy *et al.* 1964	Heart	Chimp	1	Non-function
Traeger *et al.* 1965	Kidney	Chimp	3	2 months
Millard *et al.* 1985	Kidney	Chimp	2	4 months
Cooley *et al.* 1968	Heart	Sheep	1	Non-function
Cortesini *et al.* 1969	Kidney	Chimp	1	1 month
Ross, 1969	Heart	Pig	1	Non-function
Marion, 1969	Heart	Chimp	1	Non-function
Starzl, 1989	Liver	Chimp	3	< 14 days
Barnard *et al.* 1977	Heart Heart	Chimp Baboon	1 1	4 days Non-function
Bailey *et al.* 1985	Heart	Baboon	1	20 days
Starzl *et al.* 1993b	Liver	Baboon	2	< 70 days
Starzl *et al.* 1993c	Liver	Pig	1	1 day
Czaplichi *et al.* 1992	Heart	Pig	1	<24 hours
Groth *et al.* 1994	Islets	Pig	10	Non-function

Foetal xenografts omitted (Schumakov *et al.* 1987; Benikova *et al.* 1987).

Table 2.4. Clinical xenotransplantation in the immunosuppression era.

During the seventies despite fervent experimental research, reports of clinical xenotransplants were sporadic and it was not until 1984 that a new era of clinical xenotransplantation resumed following the 'Baby Fae' baboon to human heart xenograft. It was assumed that because of the immunological immaturity of the neonate recipient, improved immunosuppression with Cs A, and advances in intensive care medicine, the patient would survive for a longer duration than had been previously reported. Initially the child made good progress, but had a forlorn outlook surviving for 20 days, unfortunately succumbing to progressive xenograft rejection (Bailey *et al.* 1985). It became quite clear that xenotransplantation was the only solution and an urgent requirement in the light of the recent decline in human organ donors.

The transplantation of human foetal islets into recipients with insulin dependent diabetes has also been conducted over the last decade with even less success than adult islet allotransplantation. A clinical trial of porcine to human, intraportal foetal islet like cell clusters (ICC) has been commenced in Sweden (Korsgren *et al.* 1992; Andersson *et al.* 1992; Groth *et al.* 1994). All patients in this series, now 10 in total, had received previous renal allografts with standard triple immunosuppressive therapy. In the first five patients porcine c-peptide could only be detected in the recipients' urine, the longest period in one patient was for 289 days (Groth *et al.* 1993; Tibell *et al.* 1994). No porcine c-peptide could be identified in any patients serum and insulin requirements remained unchanged throughout.

Further reports of clinical xenografts have followed from Poland (Czaplichi *et al.* 1992) and Pittsburgh (Starzl *et al.* 1993b; Starzl *et al.* 1993c). Starzl's group most notably have performed a baboon to human liver transplant in a 35 year old patient with Hepatitis B and HIV infection (Starzl *et al.* 1993b). This choice of donor was selected because of the resistance of baboon livers to hepatitis B infection. The liver transplant was performed according to conventional surgical techniques supplemented with FK 506, prednisolone and cyclophosphamide immunosuppression with the patient surviving for 70 days. In contrast the Polish experience was less encouraging the recipient of a porcine heart xenograft survived less than 24 hours (Czaplichi *et al.* 1992). Interestingly, both xenografts had little evidence of rejection. In the case of the liver xenograft the recipients hepatic profiles became those of the baboon with no obvious adverse affects. It is conceivable in this case, however, that the increased xenograft survival may have been attributable to the HIV virus alone which is known to deplete CD 4^+ T lymphocytes capable of abrogating a rejection response. However, a preoperative *in vitro* analysis suggested immunocompetence prior to immunosuppression and transplantation but further attempts in HIV positive liver recipients have also had prolonged allograft survival, although this was not proven to be statistically significant (Tzakis *et al.* 1990a).

2.4b The pig as potential organ donor for man

If the immunological problems of organ rejection could overcome, a xenograft would have to retain similar anatomical, physiological and morphological characteristics to those of the recipient. In general terms the most obvious choice of animal would be the large non-human primates such as the chimpanzee. There is close resemblance in the physiology between these two species and, genetically, in the structure of the DNA (Hammer, 1991). Nevertheless, chimpanzees are almost extinct and reproduce far too

slowly leaving one to consider an animal donor that is phylogenetically unrelated (Balner, 1974).

Practicality and ethical issues

Ethical considerations and cost effectiveness are salient features of our current medical bureaucracy and therefore an animal that could perhaps become the donor of choice is porcine. There would be fewer ethical constraints if this animal were to be used, as it is freely slaughtered for human consumption (Caplan, 1992). The pig has several advantages over other animals of comparable size if it were to be considered a suitable organ donor (Cooper, 1992). The pig requires less space to breed and is an easy and inexpensive animal to feed when compared to primates, sheep, cattle and goats (Niekrasz *et al.* 1992; Caplan, 1992). It also has a high reproductive capacity having oestrus cycles every three to four weeks producing large litters of up to 20 piglets which reach sexual maturity at 6 months (Niekrasz *et al.* 1992).

Islet isolation

There are now many reports concerning non-human islet isolation. In 1990 Ricordi introduced a modified automated method for porcine islet isolation (Ricordi *et al.* 1990c) that was adapted from his original description used for the human pancreas in 1988 (Ricordi *et al.* 1988a). Some modifications were necessary so as to overcome the fragility of porcine islets during the digestion phase. Using a gentle shaking technique 5,000 to 10,000 islets/g of tissue were isolated and is currently the most reproducible technique as yet for obtaining high porcine islet yields (Toomey *et al.* 1993). With respect to the size of an adult porcine pancreas, these yields suggest that there is still room for improvement. If porcine islet digestion and purification could be further advanced, a single pancreas could perhaps provide enough islets for several diabetic recipients. Other techniques have also been reported, Calafiore demonstrating similar yields with a slightly different technique using a multi-enzyme digestion phase (Calafiore *et al.* 1990).

Reports from other non-human species are less favourable. Lacy first isolated bovine islets but the technique has been improved by Hering *et al.* (1989) and most recently by Marchetti *et al.* (1995b; 2,743 IEQ/g). However, bovine islet yields are still much less in comparison to porcine islet yields. Other investigators have isolated islets from the canine pancreas (Alejandro *et al.* 1990; Cattral *et al.* 1991; Warnock and Rajotte, 1988; van der Burgh *et al.* 1994), obtaining 2,000 to 5,000 islets/ g but it must be remembered that canine islet isolation could not be routinuely performed because of

ethical constraints. Other investigators have isolated islets from cynomolgus monkeys (Gray *et al.* 1986; Stegall *et al.* 1989), baboons (Rastellini *et al.* 1994), rabbits (Hamelmann *et al.* 1994) and chickens (Giannarelli *et al.* 1994) but in view of the small number of islets obtained and ethical constraints, these animals should only be reserved for experimental work. Likewise, the widely used rodent pancreas for islet isolation is completely impractical in the clinical sense because of the small size of each pancreas and the need for many donors. In summary, therefore, in terms of islet yield the porcine pancreas would appear to be the most suitable species for clinical xenotransplantation, providing the large numbers of islets necessary for multiple, viable functioning islet xenografts.

Physiology and biochemistry

Not only is the pig practically and ethically suitable as an animal donor, it is the classic species that has provided exogenous insulin replacement for human diabetic patients for many years. As previously described, porcine islet neuroendocrine secretion (Holst *et al.* 1981; Ahren *et al.* 1986; Strubbe and Steffens, 1993) and glucose threshold (Crowther *et al.* 1990) are similar to those of human islets, although the biphasic insulin secretory responses are lower (Ricordi *et al.* 1986). Despite this slight disparity the pig can still maintain fasting blood sugar levels in the range seen in humans (Philips and Panepinto, 1985). Blood constituents are one of the most important physiological parameters to consider when trying to identify a suitable animal donor. The function of any transplanted organ relies on the delivery of nutrients and cells of haemopoetic origin. In terms of quantity the number of erythrocytes, white blood cells and total protein content all differ significantly between widely disparate species. In more finite detail it has been shown that human erythrocytes are much larger than those of other animals and coupled with the high viscosity of human blood may interfere with the microcirculation in a newly xenografted organ *(Table 2.5 p.88* ; Hammer, 1989b). Fortunately, although considered important for other organ transplants such as experimental renal and liver xenografts, this aspect may be of less a concern with highly purified transplanted islets that neovascularise from the host. The initial phase of neovascularisation from host derived endothelium (Calcinaro *et al.* 1992) gives a cellular transplant one distinct advantage over a whole organ transplant in that it should protect the islet graft from immediate hyperacute vascular rejection as binding between donor endothelium and immunocompetent cells may not take place. Nevertheless, most animals including man produce preformed natural antibodies (PFNA) without any prior porcine antigen exposure (Boyden, 1964; Hammer, 1989b). In the situation of islet xenotransplantation, human PFNA have been shown to bind vigorously not only to vascular endothelium but also endocrine, exocrine and ductal

tissue thus capable of mediating hyperacute graft rejection (Eckstein *et al.* 1992). In general, humans display relatively low levels of noncytotoxic PFNA to chimpanzees and baboons being closely related, in comparison to higher levels against widely disparate species such as porcine.

Species	Viscosity (cP)	Erythrocytes N^O*size(mm)		Leucocytes x 10^3 ml	Blood Groups
Man	4.7	5.0	7.2	7.0	4
Horse	4.1	7.5	5.5	9.0	8
Cattle	4.6	6.0	5.7	8.0	12
Sheep	4.3	10.0	5.1	8.0	8
Pig	**5.9**	**6.5**	**6.1**	**12.0**	**15**
Dog	4.7	6.0	7.3	12	7

*10^6/ml

Table 2.5 Haematological considerations between species

Evidence suggests that pigs are generally of blood group A or O, or Type 1 (absence of A or O). Chimpanzees on the other hand express O or A with no identifiable B or AB groups. These blood types do offer the possibility of a universal donor. However, only 18% of chimpanzees express blood group O, and in an animal that is already in short supply a donor match is more likely to be found in the more abundant pig (Andersen, 1962; Reemtsma and Benvenisty, 1991). In the case of baboons they exhibit an AB blood group system, rarely group O (Socha *et al.* 1984), as opposed to the ABO in humans which poses severe limitations (Chiche *et al.* 1993) but although blood typing has to be done if xenotransplantation were to be considered the expression of the antigen is not strong on the erythrocyte (Wiener and Moor-Jankowski, 1969). Domestic farm animals resemble the Rhesus system more than the ABO system seen in man and primates. Therefore, no reaction occurs in cattle and sheep after the first mismatched transfusion. Horses, dogs and pigs need to be tested for blood group compatibility before xenotransplantation can be considered (Hammer, 1991).

Calne was one of the first to draw attention to what he called "milieu interieur" of the recipient and whether the recipient's metabolic conditions would be satisfactory for good function (Calne, 1970). Minor differences in human pH or biochemical parameters could have a profound effect on xenograft function. Consequently, biochemical and physiological parameters have been studied in considerable detail for some porcine organs (Hannon *et al.* 1990) but little is known of the pancreas. One can

only summise from insulin secretion studies that porcine islets can maintain human glucose homeostasis (Crowther *et al.* 1990; Davalli *et al.* 1993; Marchetti *et al.* 1994). From the experience of pig livers providing *ex vivo* support for humans in fulminant hepatic failure (Chari *et al.* 1994) and *in vivo* function in primates (Calne *et al.* 1968; Cooper *et al.* 1988), initial findings do lend some encouragement to islet xenotransplantation. Nevertheless, evidence favouring long-term graft survival may be organ specific. It has been shown that porcine kidneys when perfused with human whole blood do elicit increased vascular resistance, platelet aggregation and immunoglobulin deposition whereas this is quantitatively reduced for a porcine liver (Douglas, 1970; Otte *et al.* 1990).

In conclusion, evidence suggests that biochemically a porcine pancreas could probably provide islets that could function successfully in a human recipient. Porcine insulin can clearly control human glucose levels, insulin secretion and regulation is very similar to that of the human.

Infection and disease transmission

The pig can harbour many bacterial, viral, fungal and protozoal organisms. Undoubtedly, some micro-organisms could easily be transferred with a transplanted organ procured from a primate and the question of whether they could be eradicated or prevented from causing infectivity is raised (Douglas, 1970). The answer, although expensive and time consuming, could be by using gnotobiotic animals (Miniats, 1984) but advances in chemotherapy, like those seen in immunology, suggest that infections may be overcome.

A more questionable dilemma is how to rapidly screen organs for infection. For example, both human and non-human primates habour not only their own viruses but may also be infected with viruses that are not of primate origin which only become of concern when they are latent in a new immunocompromised host. There are still many unclassified viruses, particularly of the simian class. Many are still to be isolated from non-human primates and consequently little is known of their infective potential or transmission (Chiche *et al.* 1993). Current laboratory procedures are not adequate in detecting the presence or guaranteeing the absence of a virus, accordingly the potential for transmission is then increased. By using animal donors, particularly non-human primates, the risks of transmitting viral infections are greater than with porcine. The baboon is resistant to the majority of human viruses which is not the case for the chimpanzee (Chiche *et al.* 1993).

The xenograft recipient is not only at risk from activation of latent infection because of immunosuppression, but susceptibility to an infectious micro-organism habouring in the grafted organ may also be enhanced. It has generally been voiced that most viral infections in humans after transplantation are from latent infections that reactivate (Ho, 1977), and in the context of xenotransplantation latent infections will probably occur.

Not only should infection be considered but it is important to consider other pathological processes that may be transferred. It has been estimated that within the porcine population neoplasms occur in about 0.005% whereas in cattle it is thought to be slightly higher (USDA, 1972). These comparatively small figures would be somewhat higher if pigs were allowed to survive to middle age. It has been estimated that malignant lymphoma is possibly the most common neoplasm in porcine species contributing to 46% of all cases (Plummer, 1956) followed by embryonal nephromas and multiple melanomas (Niekrasz *et al.* 1992). In conclusion, despite meticulous assessment, it is possible that neoplasms could be picked up but as with any other occult malignancy 100% detection rates are unlikely.

2.5c Islet xenograft rejection

After preliminary laboratory investigations by Perper and Najarian (Perper and Najarian, 1966) whole organ xenografts were seen to be rejected more rapidly between phylogenetically distant donors and recipients (dog to pig) than with closely related species (chimpanzee to human). At the time it was proposed that the presence of cytotoxic antispecies antibodies within the organ recipient mediated organ xenograft rejection. Based upon these observations the descriptive terms *concordant* and *discordant* pairs were used to differentiate between xenografts with prolonged graft survival to those rapidly rejected (Calne, 1970).

There are many aspects of xenograft rejection which still need to be clarified. It is thought that a concurrent interplay between PFNA, complement activation and cell mediated immunity, induce xenograft rejection. These individual components will be discussed herein with the discordant pig to human model in mind.

Preformed natural antispecies antibodies (PFNA) and their target antigens

The role of xenoreactive PFNA in the pathogenesis of xenograft rejection has been the subject of controversy for many years. Many animals have circulating

antibodies that react with cells and tissues from totally unrelated species without any previous antigenic immunisation.

PFNA and their target antigens are not well characterized and are quite different to sensitised antibodies, which are most commonly IgG. Studies have shown that most (up to 100%) humans possess PFNA against various pancreatic cell types, particularly of vascular endothelium (Eckstein *et al.* 1992; Schaapherder *et al.* 1993; Oriol *et al.* 1993; Ulrichs *et al.* 1994b) and it has been estimated that 0.1% to 0.2% of all B lymphocytes spontaneously produce them (Kunori *et al.* 1992) particularly the CD 5^+ subset (Turman *et al.* 1991; Platt *et al.* 1991).

The aetiology of PFNA is unknown but characterization of the immunoglobulin subclasses has shown a preponderance of IgG2 in some cases (Ross *et al.* 1993) but IgM predominates. In healthy humans this is produced in response to polysaccharide antigens and IgG2 commonly binds with antigens composed of carbohydrate polymer moieties that form the backbone of bacterial cell walls (Hammarstrom and Smith, 1986). Therefore, PFNA may represent unpredictable cross reactivity between antibodies directed against common bacterial antigens, subsequently confirmed by lower concentrations of PFNA in animals raised in a gnotobiotic environment (Hammer and Hingerle, 1992). PFNA are found in all mammalian species, although in variable concentrations, and it is unlikely that their origin is from antigenic stimulation alone and this may merely be a physiological development of gamma globulin (Hammer *et al.*1992) that provides early defence against microbes before induced responses can occur. This typical broad pattern of reactivity, low affinity and high avidity for antigen is, therefore, inkeeping with their IgM isotype predominance (Markmann and Barker, 1995).

PFNA in humans have been shown to react with many 'polyreactive' (Geller *et al.* 1993) glycoproteins (Ratner *et al.* 1992; Platt and Bach, 1991; Platt *et al.* 1994). It is well recognised that PFNA bind avidly to oligosaccharide determinants having a terminal galactose and alpha linkage to unfucosylated hexose (Gal α-1,3-Gal; Galili *et al.* 1984; Galili, 1993). This epitope is the target of 75% of all PFNA and up to 1% of all circulating antibodies (Vaughan *et al.* 1993) and from previous publications this epitope is present on both porcine pancreatic ductal tissue and on islet tissue (Oriol *et al.* 1993; Boucher *et al.* 1996). It is possible that Gal α-1,3-Gal may be present in other swine species as expression differs considerably from animal to animal (Geller *et al.* 1994). Gal α-1,3-Gal is not expressed in old world monkeys and apes because of a mutation in galactosyltransferase that renders it nonfunctional (Markmann and Barker, 1995). In an attempt to elucidate the nature of PFNA-antigen binding to isolated islets,

the immune response to porcine foetal ICC xenografts has also been assessed (Kumagai Braesch *et al.* 1992). It was found that PFNA bound strongly with porcine peripheral blood lymphocytes but not to porcine foetal ICC. Unfortunately, with respect to adult porcine islet isolation, no definite conclusions can be drawn as immature tissue provokes a less vigorous immune response than adult porcine tissue (Ulrichs *et al.* 1994b).

The binding of human PFNA to donor xenogeneic tissue, particularly vascular endothelium, triggers the activation of the complement cascade which mediates hyperacute graft rejection (HAR), similar to that seen in ABO incompatible grafts. The magnitude of HAR is still largely unrealised, even in allotransplants as it is prevented by using ABO compatible grafts or transplantation after a negative cross match between donor and recipient.

Complement activation

Complement components provide a potent non-specific defence against foreign xenogeneic tissue. In short, the combination of events are generated via two different pathways called *classical* or *alternative* *(Figure 2.11 p.93).* As alluded to it is thought that PFNA bind to antigenic porcine epitopes via their Fc region binding with C1q which activates the 'classical' complement pathway. In contrast to the classical pathway the alternative pathway can be activated in the absence of PFNA (Johnston *et al.* 1992) by a selection of damaged tissues of bacterial origin, polysaccharides, as well as IgA or IgE (Reid, 1983), thus bypassing C1, C2 and C4. Evidence supporting the role of both pathways in xenograft rejection stems from studies where PFNA have either been blocked or reduced in concentration yet graft rejection still rapidly occurs (Johnston *et al.* 1992). The terminal sequence of events in both pathways results in the formation of a C5 convertase complex yielding a membrane attack complex (C5b-9n) that penetrates plasma membranes causing cellular death (Kim *et al.* 1987). Many byproducts of the complement cascade also participate in tissue destruction by stimulating the production of potent inflammatory mediators such as cytokines, free radicals, histamine and prostaglandins (Baldwin *et al.* 1995).

Experimental evidence supports the hypothesis that PFNA mediate discordant *hyperacute* xenograft rejection via the complement cascade by causing endothelial activation (Platt and Bach, 1991). Recent studies have portrayed a variety of immunological events both *ex vivo* (Kirk *et al.* 1993) and *in vivo* (Starzl *et al.* 1993b; Makowka *et al.* 1994). If porcine organs are perfused with human blood there is

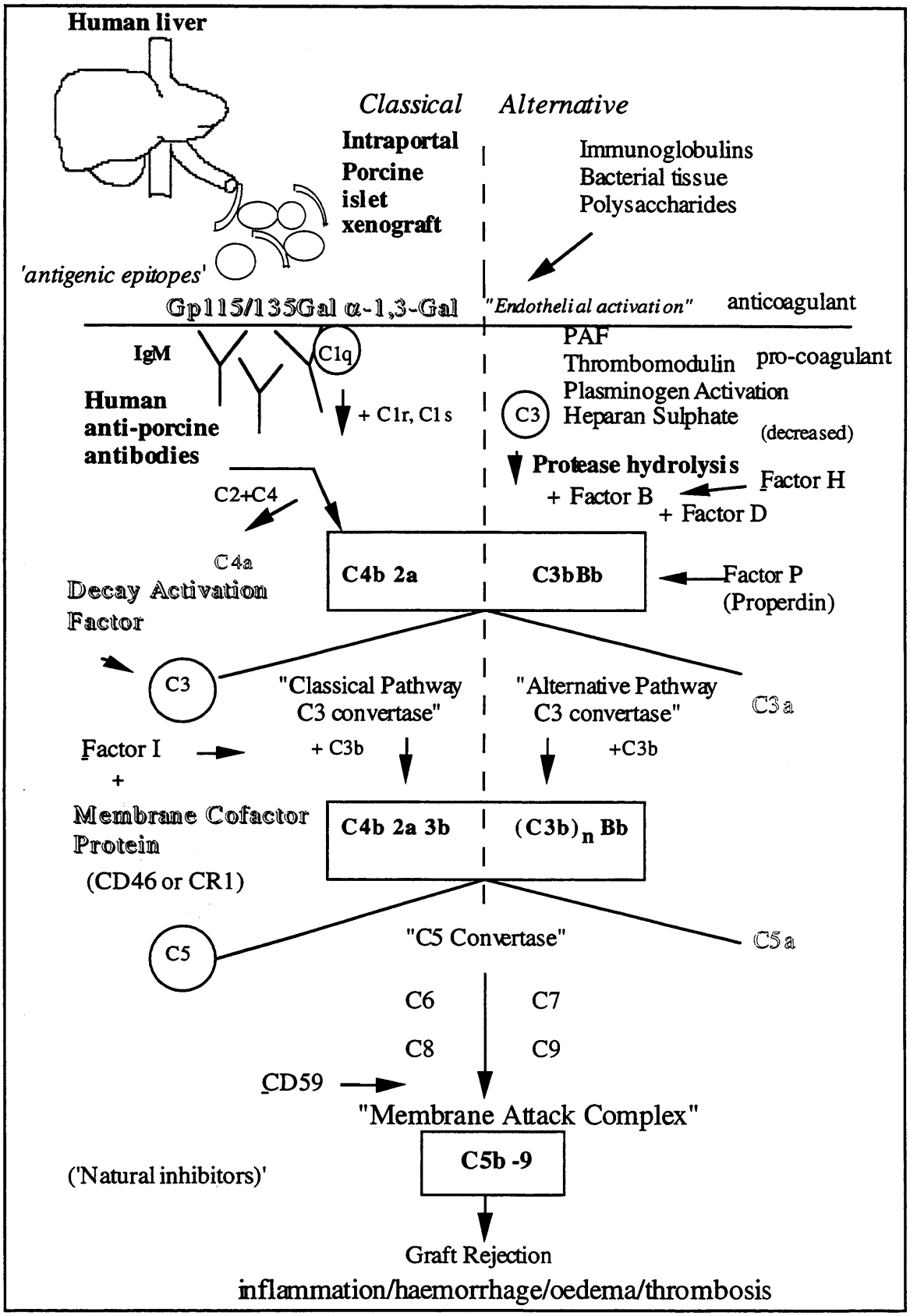

Figure 2.11 Schematic diagram of a xenograft rejection response

marked vascular deposition of IgM, IgG, fibrinogen, C3, C1q along with infiltration by macrophages, T lymphocytes, B lymphocytes and natural killer cells (Dalmasso and Platt, 1993; Kirk *et al.* 1993; Tuso *et al.* 1993). Further to this, C3 complement deposition and complement activation occurs in porcine pancreatic sections when incubated with human serum (Eckstein *et al.* 1994). Studies have also confirmed complement activation by observations that discordant grafting induces a precipitous decrease in serum complement due to sequestration (Starzl *et al.* 1993b; Makowka *et al.* 1994), accumulation in rejecting xenografts, and by administration of complement inhibitors such as cobra venom factor (depletes C3 and C5; Gewurz *et al.* 1967), C1 inhibitors (Dalmasso and Platt, 1993) and decay accelerating factor (Dalmasso *et al.* 1991).

Exposure of endothelial cells to cytokines, e.g. TNFα or other inflammatory mediators causes structural and metabolic changes. Endothelial activation results in the conversion to a procoagulant environment through the release of tissue factor, plasminogen activator inhibitor that decreases *in vivo* cell surface thrombomodulin activity (Platt and Bach, 1991). Platelet adhesion ensues after elaboration of platelet activation factor and upregulation of endothelial leukocyte adhesion molecules (Heeman *et al.* 1994; Platt and Bach, 1995). The action of PFNA and complement also causes a receptive loss in heparan sulphate binding mediating an anticoagulant action via anti-thrombin III. These series of events lead to platelet aggregation, fibrin generation and neutrophil adhesion causing interstitial haemorrhage, inflammation, oedema and thrombosis together causing hyperacute rejection within minutes to hours after xenogeneic transplantation (Bach, 1991). Whether this endothelial activation by complement is PFNA dependent in all donor recipient combinations, still remains to be conclusively proven (Bach, 1991) and that the alternative pathway may also lead to endothelial activation and consequent xenograft rejection in the absence of PFNA (Antibody Independent Complement Activation; Platt and Bach, 1991; Dunning *et al.* 1994; Platt and Bach, 1995).

Cell mediated xenograft rejection

Cell mediated xenograft rejection *(Figure 2.12 p.95)* is in most instances of secondary importance in whole organ xenografting because of the overwhelming damage caused by PFNA. In the case of islet xenograft rejection it may be of primary importance. It is recognised that transplanted islet grafts neovascularize from the host; therefore, in the absence of donor endothelium, it is debatable as to whether PFNA/ endothelium/ complement interactions will induce islet xenograft rejection in a

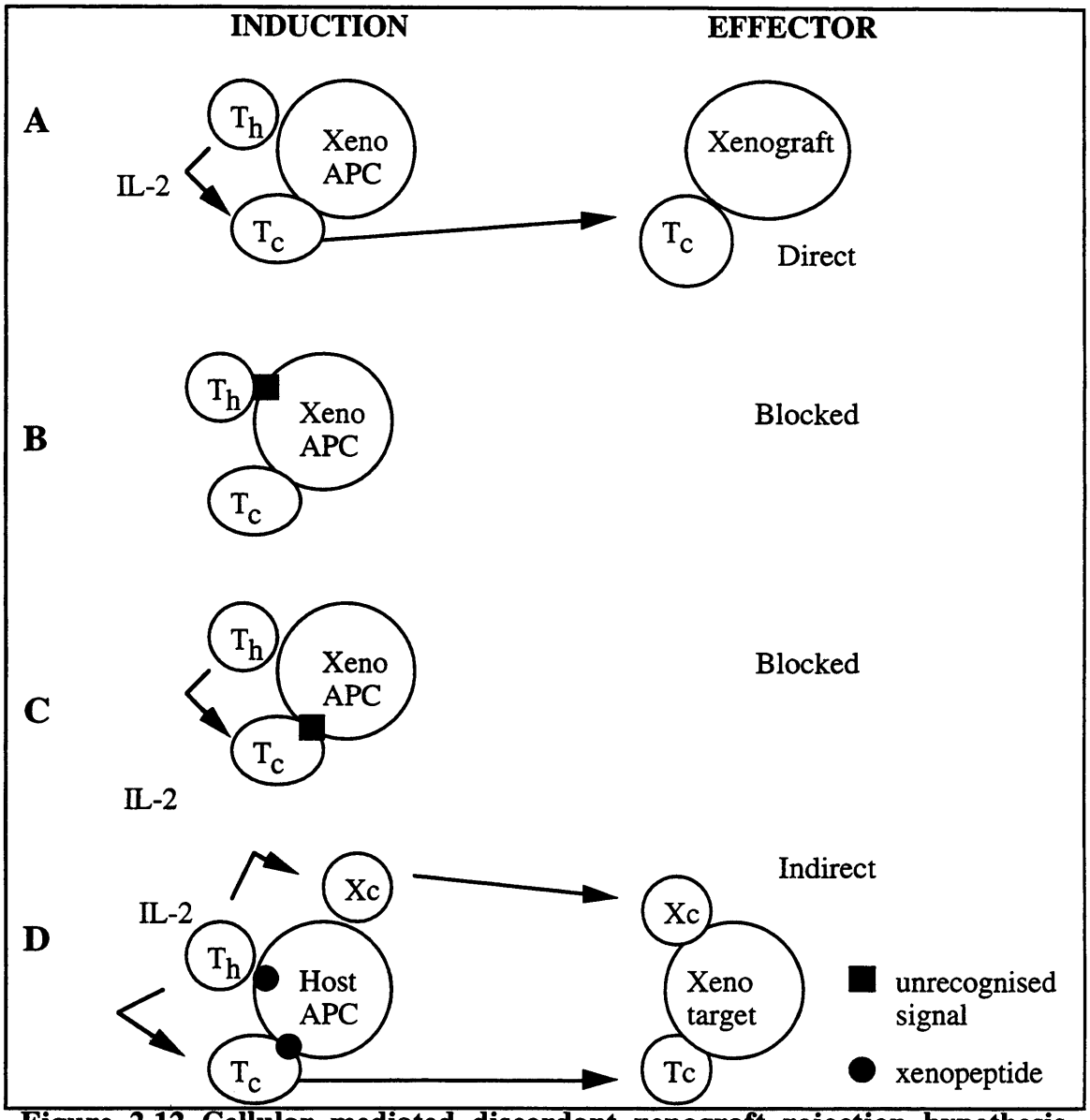

Figure 2.12 Cellular mediated discordant xenograft rejection hypothesis. If Th and Tc can recognise xenoantigens directly on xenogeneic cells (A) then Th will activate Tc specific antigens on somatic cells of the xenograft and the graft will be rejected by Tc. If both Th (B) and Tc (C) are unable to recognise xenoantigens on xenogeneic cells then Tc specific for antigens on somatic cells of the xenograft will not be activated. Instead, the Tc cells (D) that are activated are specific for xenoantigens in a form not expressed on the somatic cells of the xenograft because they are in association with host MHC molecules. Thus the graft is rejected by Tc independent mechanism involving (Xc) Th dependent, antigen non-specific cytotoxic effector cells

discordant porcine to human model. Contaminating endothelium can also be successfully eliminated in xenogeneic islet grafts by a period of *in vitro* culture prior to transplantation (Parr *et al.* 1980), in contrast to vascularised grafts such as liver, pancreas, kidney and skin. In view of this quandary the studies by Eckstein *et al.* (1992), Schaapherder *et al.* (1993) and Hamelmann *et al.* (1986) have been of critical importance demonstrating that PFNA can react aggressively with most porcine

pancreatic tissues. Overall the most aggressive interaction is between PFNA of diabetic recipients and donor endothelium in contrast to endocrine cells which show less reactivity, but this binding is notably species dependent. In conclusion, the hyperacute rejection seen in discordant pairs by interactions of PFNA and endothelium may be of less importance with islet xenografts, where rejection could be, alternatively, by a concurrent cellular response (Auchincloss, 1988; Deng *et al.* 1994).

The role of cell mediated immunity in islet xenograft rejection (Gill, 1993) is still unclear. In the case of concordant, phylogenetically close pairings, where hyperacute xenogeneic rejection is of less potency, cell mediated graft rejection still occurs (Bailey *et al.* 1985; Starzl *et al.* 1993b). The basis of transplant recognition is the ability to recognise polymorphic residues on allogeneic MHC as 'foreign' but in the case of a *widely divergent* MHC across a species barrier, host CD 4^+ cells may not be able to make this recognition, thus reducing the chance of xenospecific recognition (Woolnough *et al.* 1979; Moses and Auchincloss, 1991). This relative hyporeactivity of divergent T lymphocytes and APC interactions, often lower than corresponding responses to allogeneic antigens (Engelhard *et al.* 1988), can be attributed in part to differences in species-specific signals such as cytokines and accessory molecules, or co-receptor interactions which might not be as efficient for T lymphocyte activation when there is marked species disparity (Gill, 1993). Many studies attempting to confirm T lymphocyte recognition of divergent tissue have produced conflicting results (Irwin *et al.* 1989; Haisch *et al.* 1990). These studies are somewhat difficult to interpret as they have been performed in non-human models and have been applied to *in vitro* mixed lymphocyte co-cultures which do not necessarily predict the same response *in vivo* .

The deficiencies in xenogeneic T lymphocyte-APC interactions can be inferred by considering the direct and indirect pathways of host sensitisation as described in the context of allograft rejection. Direct antigen presentation would be the result of host T lymphocytes recognising and being activated by donor APC elaborating and presenting equivalent xenogeneic MHC molecules along with costimulator activity necessary for T lymphocyte activation. Under widely divergent conditions this species specific reaction is likely to be of limited significance because of antigen disparity of the xenograft relative to the host, functionally blocking the direct xenoantigen recognition and favouring a strong response to shed processed antigens by host APC. The responsive T lymphocytes would not be directly graft specific but specific for graft antigens in association with host MHC class II. This simply means that a divergent xenograft may be weakly immunogenic but strongly antigenic *(Figure 2.12),* in comparison to the host being greatly stimulatory for indirect antigen presentation (Moses and Auchincloss, 1991; Gill, 1993).

Studies supporting the role of indirect antigen presentation arise from the findings that CD 4^+ deficient recipients have extended xenograft survival over allogeneic survival (Gotoh *et al.* 1988), after a period of gamma irradiation, that depletes donor APC necessary for direct antigen presentation. Furthermore, APC depleted murine pancreatic islet allografts were never rejected whereas APC depleted rat xenografts were rejected in murine recipients (Gill and Coulombe, 1992). Nevertheless, Lacy *et al.* (1989) demonstrated the benefit of pretransplant culture that removed graft APC with extended survival of rat, hamster, rabbit and human islets when transplanted into murine recipients along with the use of anti-CD4 monoclonal antibody as the immunosuppressant. The work by Faustman (Faustman and Coe, 1991) also supported a role for direct presentation where human islets coated with masking donor MHC class I antibody had indefinite survival in mice, although islet viability was not documented but can be partly explained by the recognition of human antigen by murine T lymphocytes being defective.

Finally, it is clear that CD 4^+ cells play a dominant role in xenogeneic graft rejection (Moses *et al.* 1990) but different to that of allograft rejection. The exclusive role of the $CD8^+$ subset in T lymphocyte mediated cytotoxicity differs from that seen in the allogeneic situation because $CD4^+$ T lymphocytes can mediate responses to both MHC Class I (Moses and Auchincloss, 1991) and MHC Class II xenogeneic antigens (Greenstein *et al.* 1986). In support of this it has been shown that islet xenografts are acutely rejected in CD8+ deficient mice where allografts have prolonged survival (Gill and Coulombe, 1992; Desai *et al.* 1993).

Taken together it can be extrapolated that the xenogeneic response is predominantly indirect whereas the allogeneic response is directly initiated. However, these studies need to be transposed in larger animal models across greater species differences. The specificity of the effector cells that destroy the graft have still not been completely determined. This is because the antigen is presented with host APC MHC yet the graft expresses xenogeneic MHC therefore graft destruction must be by a non-antigen specific method perhaps involving other cellular pathways. Current methods, therefore, capable of immunomodulating allogeneic grafts are not appropriate for xenogeneic grafts.

2.5d Strategies for preventing islet xenograft rejection

Drug therapy

To date, the strategy of preventing xenograft rejection does not markedly differ from that used to combat allograft rejection after vascularised organ transplantation. Immunosuppression with steriods, azathioprine and Cs A form the backbone of most clinical xenogeneic regimes. Modifications have been made notably by Starzl's group in Pittsburgh. Anti-B lymphocyte type agents such as cyclophosphamide and prostaglandin E1 were used for baboon to human liver xenografts (Starzl *et al.* 1993b). In this situation the xenograft survived for 70 days and evidence suggested that the immune response could be attentuated with a combined anti-B lymphocyte and anti-T lymphocyte therapy. Furthermore, in the recent American (US Multicentre FK 506 Liver Study Group, 1994) and European (European FK 506 Multicentre Liver Study Group, 1994) multicentre trials, FK 506 was shown to be superior to that of Cs A in preventing liver allograft rejection but not renal allografts (Mayer *et al.* 1997) where no additional benefit could be observed suggesting that FK 506 may be better for xenografts.

Experimental models have shown some success in preventing porcine islet discordant xenograft rejection with FK 506 both *in vivo* (pig to rat; Tze *et al.* 1994a) and *in vitro* (pig to human; Borg and Kumagai, 1991). Results with newer immunosuppressives such as MMF (Eugui *et al.* 1991; Figueroa *et al.* 1993) are eagerly awaited because of the known diabetogenic effects of FK 506 (Tze *et al.* 1990). Studies have indicated that suppression of both T and B lymphocytes is needed. Experiments using total lymphoid irradiation and Cs A for suppressing B lymphocyte activity have shown prolonged survival for more than 100 days in contrast to two to five days when each is used alone (Knechtle *et al.* 1990). Experience has also been with a Cs A, prednisolone and azathioprine (Groth *et al.* 1994) combination, after clinical porcine foetal ICC xenotransplants but the prevention of graft rejection was not conclusively demonstrated. However, foetal ICC into primates can be prevented histologically (up to six days post-transplant) using newer regimens of leflunomide, MMF and Cs A (Wennberg *et al.* 1997) but doubts can be raised over the potential toxicity in humans.

The use of DSG has also been extensively pursued in experimental xenografting. DSG inhibits natural antibody production in a discordant xenograft model (Nakajima *et al.* 1989; Tepper *et al.* 1991) disappointingly though not sufficient enough to prevent cell mediated rejection (Nakajima *et al.*1994). Graft survival can be

prolonged by DSG with FK 506 (Hisanaga *et al.* 1992), simultaneous splenectomy (Leventhal *et al.* 1992), or with ATG (Henretta *et al.* 1993). Lastly, DSG is also thought to elicit some form of thymus alteration, but its specific mode of action awaits further clarification (Nakajima *et al.* 1989).

Despite various empirical combination therapies no current regimen has produced an acceptable duration of xenograft survival in larger mammals. Successful studies performed with newer immunosuppressive drugs have been mostly applied to rodent models. In summary, the 'ideal' regime would have to reduce the recipient's PFNA concentration and at the same time manipulate the recipient's complement armourary and cell mediated response *in vivo*, without being toxic to islet xenografts. Studies to date suggest that highly specific combination therapies will be required for future success by exhibiting both anti-B lymphocyte and anti-T lymphocyte activity without diabetogenic profiles.

PFNA suppression and depletion

Human plasma can be removed in exchange with man made substitutes such as fresh frozen plasma or albumin by a technique called plasmapheresis. This treatment can deplete PFNA levels but repeated exchanges are often required over periods of weeks or months for effective reduction. Alexandre *et al.* (1989) applied this method to a pig to baboon renal xenograft model, prolonging xenograft survival to 22 days. After plasmapheresis, PFNA levels can return to their original levels within 24 hours (Euhler *et al.* 1985) and to combat this other investigators have used simultaneous Cs A, splenectomy or cyclophosphamide (Van de Stadt *et al.* 1988). The relevance of this to islet transplantation has not been investigated.

Another useful appproach is to use *ex vivo* perfusion of human blood into porcine organs or protein columns (Shapiro *et al.* 1990) so as to remove PFNA by sequestration. Immunoaffinity columns with Gal like oligosaccharides absorb serum and PFNA and may become therapeutically useful in the future if the risk of infection is small. After *ex vivo* perfusion another organ taken from the same donor or species can be transplanted into the PFNA depleted patient (Tuso *et al.* 1993). This application is still experimental, attempts in a clinical setting have been tried but have not prolonged survival (Czaplichi *et al.* 1992) and no studies have been applied to islet xenotransplantation.

Having temporarily removed PFNA by plasmapheresis, the xenograft is protected from early destruction as the rebound rise of PFNA will not, in all cases,

cause HAR (Fischel *et al.* 1991). For example, an ABO incompatible renal allograft after a temporary depletion of antibodies can sometimes survive for long periods (Palmer *et al.* 1989). This phenomenon has been called *graft accommodation* (Bach *et al.* 1991). The precise mechanism of which is unknown. A series of explanations have been proposed including new antibodies being of a different isotype or specificity to endothelial antigens, altered or lost expression of xenoantigens or due to a lack of unresponsiveness of endothelial activation.

Plasmapheresis or *ex vivo* organ perfusion is limiting as it not only removes PFNA but has a tendency to reduce non-pathogenic immunoglobulins. Other complications can arise such as graft thrombosis, anaphylactic reactions and viral transmission through repeated exchanges (Bach *et al.* 1991; Otte *et al.* 1992).

Encapsulation

O'Shea and Sun were the first to demonstrate long-term survival (144 days) in a concordant (rat to mice) islet xenograft model (O'Shea and Sun, 1986) and subsequently encapsulation has proven to prolong survival (51 days) in a discordant model (dog to mice) with minimal cellular reaction (Weber *et al.* 1990a). Success in larger mammals has been more difficult to achieve until recently. The feasibility of encapsulation and transplantation of porcine islets into cynomolgus monkeys (Zhou *et al.* 1994) or rodents (Sun *et al.* 1993) has been reported with 75% of rodent recipients reversing hyperglycaemia. With respect to the primate model only four cynomolgus monkey recipients were assessed. Although porcine c-peptide was detected in all four monkey recipients only one remained insulin independent (360 days; Zhou *et al.* 1994).

One of the advantages of islet encapsulation prior to xenotransplantation was thought to be its ability to exclude large immunoglobulins such as PFNA from the xenogeneic tissue thus preventing HAR. More recent investigation suggests that this may not be the case as graft survival can still be prolonged (< 10 weeks) in a porcine islet to mouse recipient when the islets are encapsulated in uncoated, PFNA permeable, microspheres. These findings perhaps suggesting that when rejection occurs there is also a significant cell mediated response (Lanza *et al.* 1995) and that exclusion of PFNA from islet tissue is not critical for islet xenograft survival.

Immunomodulation

Immunomodulation protocols have mostly been developed in allograft experimental models as previously described. Most xenogeneic immunomodulation

protocols have been in rodent (concordant) models. Graft survival has been significantly inferior to those achieved in allograft models in both UV-B irradiated concordant (Rossi *et al.* 1992) and discordant (primate to mice) models (Chabot *et al.* 1987) supporting the role of indirect antigen presentation. Similar results have been demonstrated with gamma irradiation and ALS (rat to mice; Gotoh *et al.* 1988). Immunomodulation of porcine islets with either UV-B irradiation or LTC have not been described. A study by de Villa *et al.* (1992), showed no restoration of normoglycaemia after gamma irradiation of porcine islets at 250 grays because of an acute irradiation injury prior to transplantation.

Genetic manipulation

With the realisation that complement is critically involved in hyperacute xenograft rejection recent lines of investigation have used recombinant DNA technology to produce complement inhibition. Attempts have been made to transfect recipients with cDNA for donor decay accelerating factor (DAF) or membrane cofactor protein (MCP) genes. Preliminary experiments in a mouse to human model inhibited lysis by human complement (White *et al.* 1992). On the basis of these encouraging results the production of transgenic animals expressing human complement regulatory products such as DAF or MCP, may serve as organ donors for human recipients, with the donor organs perhaps being resistant to HAR. Even after elimination of HAR by averting the deposition of PFNA and complement activation it is plausible that graft rejection may still occur by an antibody response seen in concordant xenograft rejection but may be suppressed by anti-B lymphocyte immunosuppressives as well as conventional anti-T lymphocyte therapy.

Subsequent studies at Cambridge have led to the production of human complement regulatory proteins expressed within transgenic pigs (Langford *et al.* 1994; White *et al.* 1995b). Initial results have shown extreme variation of expression amongst the herd, but 67% of pigs express the transgene in skin, liver, spleen, lung and heart. Primate studies have demonstrated survival of porcine hearts transgenic for human DAF for up to three weeks, although high levels of immunosuppression were required and led to adverse side effects in the recipient (White *et al.* 1995a). Detailed analysis of expression of the transgene within isolated islets are eagerly awaited.

As already alluded to the 1,3 galactose epitope is the major xenoantigen expressed on porcine cells. Interestingly, if pig α-1,3-galactosyltransferase that creates this xenoantigen could be eliminated from a porcine organ then xenograft survival in human recipients may be prolonged. Researchers in Australia have cloned the gene for

the pig α 1,3-galactosyltransferase (Dabkowski *et al.* 1994). The transferase enzyme galactosylates lactobiose to produce Gal-α1,3-Gal expression and it would, therefore, seem appropriate to knock out this gene in pigs by homologous recombination. Unfortunately, this novel idea although solving one problem creates another in that, by knocking out this gene other sub-terminal sugars are exposed that also bind PFNA. An alternative approach known as "transferase dominance" has been shown to induce a more favourable pathway in rodents, in that lactosamine can accept fucose in preference to galactose, under the influence of H-transferase, instead of α-1,3-galactosyl-transferase thus producing α 1,2-fucosyltransferase (Cohney *et al.* 1997). This essentially reduces the expression of Gal α 1, 3-Gal, its place being substituted by fucose that can resist PFNA binding and hopefully PFNA mediated xenograft rejection.

It must be emphasised that most, but not all human IgM PFNA, are directed to this xenoantigen (Dabkowski *et al.* 1993) and it is unlikely that HAR will be totally prevented by this highly specific targetting. If the severity of the response could be reduced then this advance may help to induce xenospecific tolerance during the early transplant period. Alternatively, a better approach would be to deplete the MHC complex from all tissues providing suitable "blind organs" but because of the highly polymorphic nature of MHC molecules monoclonal antibody therapy will not be the solution and perhaps genetic manipulation may be the only answer.

2.5e Xenotolerance

The process of graft stabilisation involves a change in the graft, thereby the inherent vulnerability of the graft to rejection decreases over time, known as graft accommodation or to altered immune reactivity in the host, known as immunological tolerance (Billingham *et al.* 1953). By achieving immunological tolerance between the transplant recipient and the graft toxic immunosuppressives could be withdrawn. There are many experimental models of allogeneic and xenogeneic tolerance, particularly in rodents but tolerance in patients has remained an elusive goal.

Current methods for achieving tolerance are by pretreatment of donor tissue (immunomodulation), immunosuppression and monoclonal antibody therapy. Other efforts have involved the infusion of donor lymphoid cells derived from blood, spleen or bone marrow, either with or without total body irradiation (Ildstad and Sachs, 1984; Li *et al.* 1994). Despite these experimental concepts tolerance has still not uniformly been reproduced in large animals and limited non-specific immunosuppression is always needed (Calne *et al.* 1994).

The exchange of migratory leucocytes between the donor transplant and the recipient with consequent long-term cellular chimerism, is the basis for acceptance of all whole organ allografts and xenografts as proposed by Starzl (Starzl *et al.* 1992), although a causal relationship between donor cell chimerism and long-term graft acceptance has not been proven. It is assumed that the bi-directional migration of lymphocytes between transplanted organs and its recipient is crucial to tolerance induction. If a delicate balance is not achieved, graft rejection or graft versus host disease may occur that would require a period of immunosuppression as treatment. Preliminary studies have been performed in Pittsburgh where liver allograft recipients have survived seven to thirteen years without immunosuppression (Starzl *et al.* 1993a). The most convincing evidence of chimerism comes from female recipients of male allograft organ donors where Y-chromosomes have been demonstrated in the tissues of female recipients, presumably due to donor cell migration. With this in mind the liver, because of its dense constituency of migratory leucocytes, has been proposed as the most 'tolerogenic' organ (Calne *et al.* 1969). Strategies to achieve similar effects in kidney, heart and islet allografts have been by perioperative infusion of lymphoreticular cells obtained from the donor's bone marrow or spleen (Ildstad and Sachs, 1984) although islet grafts have not been prolonged even in the presence of a high level of xenogeneic chimerism (Zeng *et al.* 1992).

Chimerism has likewise been demonstrated after baboon to human liver xenotransplantation, although once again graft survival was short (Starzl *et al.* 1993c). Whether failure was because of surgical difficulities or inadequate immunosuppression is not known but newer immunosuppressive drugs or improved techniques at abrogating rejection by PFNA elimination may allow better success in the future. Current regimes utilising FK 506 with simultaneous cyclophosphamide, DSG, MMF do not permit consistent reproducible xenograft survival (Starzl *et al.* 1993b) and cellular chimerism.

Another interesting strategy to avert graft rejection altogether but not in the case of whole organ transplantation, would be to transplant islets into an immunopriveledged site such as the thymus, testis or within the central nervous system. These sites are unique in that most lack lymphatic drainage preventing graft sensitisation. If recipients receive ATG to remove peripheral T lymphocytes prior to receiving a thymus islet allograft then prolonged graft survival can be achieved (Posselt *et al.* 1990). Prolonged extrathymic islet xenograft survival has also been demonstrated after intrathymic inoculation with MHC class II bearing bone marrow cells suggesting that it is the MHC class II bearing cells which may be of importance in the acquisition of unresponsiveness (Mayo *et al.* 1994). It is thought that the thymus gland crucial to T

lymphocyte maturation, re-educates regenerating thymocytes so that intrathymic donor cells are seen as *self* as opposed to *foreign* with graft rejection being abolished.

In conclusion, although immunomodulation regimes do prolong survival in some experimental models, it is now arguable that in the case of the dendritic cell depletion approach, the objective should be to promote, not prevent, two way cell migration while at the same time giving treatment to avoid graft destruction immediately after transplantation (Starzl *et al.* 1992). This approach would allow the complete withdrawal of toxic immunosuppressives after tolerance is established. From this review it can be seen that there are still many fundamental problems of whole organ and islet xenograft rejection to be solved and although concordant xenografting may be overcome that of discordant grafting in the case of vascularised organ xenografting is highly unlikely in the foreseeable future but perhaps not so for islet xenografts.

CHAPTER THREE

PORCINE ISLETS

PART A Porcine islet isolation

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3.1b Non-automated methods
3.1c Automated methods
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3.2a Principles and historical development of islet purification
3.2b Density gradient porcine islet purification
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Introduction

Porcine islets were first isolated by Sutherland *et al.* (1974) following successful attempts in rodents (Lacy and Kostianovsky, 1967). The measure of success was determined by *in vivo* viability, islets were reimplanted as an autograft but evidence of normoglycaemia was not proven. Despite this rather crude first description of porcine pancreas processing, many investigators followed suit to try an adapt their own methods to porcine islet isolation. However, many were thwarted by the fragile structure of porcine islets and their susceptibility to ischaemia during the pancreas processing.

PART A

Porcine islet isolation

3.1 Techniques of porcine pancreas dispersion.

3.1a Collagenase distension

Collagenase has played a pivotal role in porcine islet isolation since it was first applied by Sutherland *et al.* (1974). Their technique entailed intraductal pancreatic distension dissolved in cold HBSS solution; a simple method thought to be sufficient enough to allow the disruption of pancreatic acinar tissue. The pancreas was then diced into small pieces and gently shaken, immersed in collagenase solution, allowing liberation of free islets 'cleaved' from the surrounding acinar tissue. The mixture was then washed and aspirated through a 15 G needle and purified on a discontinuous Ficoll gradient (Sutherland *et al.* 1974). Unfortunately, the yield and viability of porcine islets was not published but after pancreatectomy it was subsequently shown that those pigs with an islet autotransplant survived longer than those without suggesting some islet function.

Other isolation techniques were subsequently described both with (Ricordi *et al.* 1986) and without collagenase (Wise *et al.* 1983). Generally, it was thought that the pancreas should be dispersed before collagenase digestion, known to be successful in the rat (Lacy and Kostianovsky, 1967). With the introduction of intraductal collagenase delivery; firstly, in the dog (Horaguchi and Merrell, 1981) and then human (Gray *et al.* 1984), porcine islet yields were improved (Ricordi *et al.* 1986; Warnock *et al.* 1988). Interestingly, when intraductal delivery was then reapplied to the original description of

collagenase digestion in rats, islet isolation was enhanced both in terms of yield and purity (Sutton *et al.* 1986).

3.1b Non-automated methods

Following intraductal delivery of collagenase, the first techniques of pancreatic digestion to be described involved manually teasing the pancreas apart, and indeed in some centres this technique is still used. Biopsies are taken at two to four minute intervals in order to control digestion. At a point when digestion is considered to be optimal the pancreas is cooled to minimise further damage by collagenase activity. The pancreas is then shaken apart (Marchetti *et al.* 1988) or chopped in a tissue macerater (Kiviluoto, 1985; Ricordi *et al.* 1986; Crowther *et al.* 1989), although this is considerably traumatic and damaging to islets. An alternative, less traumatic technique, described by Calafiore *et al.* (1990; Basta *et al.* 1995), is to continually perfuse the gland for approximately 10 minutes at which point no mechanical shaking or teasing is required. In order to separate the digested from the undigested tissue, the remaining tissue can be passed through a series of steel filters or triturated through needles (Gray *et al.* 1984; Kiviluoto, 1985; Marchetti *et al.* 1988). Early methods reported porcine islet yields of only 1,215 islets/g (islets/ g=islets per gram of pancreas; Ricordi *et al.* 1986) following density gradient purification and 400 islets/g (Marchetti *et al.* 1988) after hand-picking. These disappointing yields have since been improved to 2,698 IEQ/g (Toomey *et al.* 1993), 5,718 IEQ/g (Basta *et al.* 1995), and 11,166 islets/g (Calafiore *et al.* 1990) but the latter result must be interpreted with caution as it is only relevant in relation to islet volume which was omitted from this publication and the fact that the islet density was lower than 1.040g/cm^3 reflects a substantial amount of islet fragmentation perhaps falsely elevating the islet count.

On the whole, the non-automated technique is considered to be very traumatic to porcine islets because of their fragility and their marked propensity for dispersion into single endocrine cells. Taken together the general consensus was to develop a new method which was less traumatic and could allow a more controlled pancreatic digestion.

3.1c Automated methods

As described above the poor yields published from non-automated pancreas digestion led investigators to develop a less traumatic method, known as the "automated method". The original description of the automated islet isolation method was by Ricordi *et al.* (1988a). The concept put into practice by the St Louis group

was to provide a continual, controlled, digestion with little human intervention allowing islets to be liberated free from damaging collagenolytic activity. Despite proving to be efficacious in the human, with yields of 4,000 islets/g, the first attempts in the porcine model were far from encouraging (Ricordi *et al.* 1990c).

For the automated method individual splenic lobes from porcine pancreata (60 to 90 g) are placed into a stainless steel chamber (500 ml; *Figure 3.1 p.104*). The automated circuit contains a reservoir of HBSS at 4°C. This circulates with the aid of a peristaltic pump into a heat exchanger raising the temperature to approximately 33 to 37°C necessary to activate the collagenase. As the chamber fills with HBSS the displaced air is collected into a bubble trap. The chamber is agitated manually from side to side two to three times a minute so as to administer less trauma and give a more controlled digestion. When the intrachamber temperature reaches 37°C, the circuit is switched so the HBSS solution bypasses the heat exchanger maintaining a stable temperature at 35 to 37°C as necessary. At this time the steel spheres and collagenolytic activity gradually disperse the pancreas. It is the purpose of the steel spheres to prevent the regular side to side motion of the pancreas within the chamber as it is agitated, thus augmenting pancreatic dispersion as they collide (Horaguchi and Merrell, 1981).

After 10 to 15 minutes digestion both endocrine and exocrine moieties are freely released from the bulk of the pancreas and pass through the fine 350-μm mesh. This tissue can then be assessed via the biopsy port with islets accurately identified as red by dithizone staining (Latif *et al.* 1988). When free 'cleaved' islets are seen, the circuit is switched to phase two and the pancreatic tissue collected free from the collagenase activity within the chamber. Throughout the digestion, samples can be taken to control pancreatic digestion with temperature and flow rates altered accordingly; hence avoiding overdigestion and fragmentation of islet tissue into single endocrine cells. After 45 to 60 minutes the pancreatic tissue exiting the chamber subsides signifying completion of digestion. The digest is then collected in new born calf serum to reduce clumping and inactivate residual collagenase (Wolters *et al.* 1990). It is then pooled and prepared for purification (Ricordi *et al.* 1990b; Ricordi, 1992; Toomey *et al.* 1993).

As already mentioned the increased fragility of porcine islets and the use of a tissue macerater in non-automated methods produced rapid dissociation of porcine islets into single endocrine cells. Briefly, with minor modifications to the original automated method, using larger chambers (370ml *versus* 300ml), bigger outlet ports (6mls *versus* 3ml), increased flow rates (60 ml *versus* 40 ml), less agitation of the digestion chamber (five oscillations every two minutes as opposed to continuous

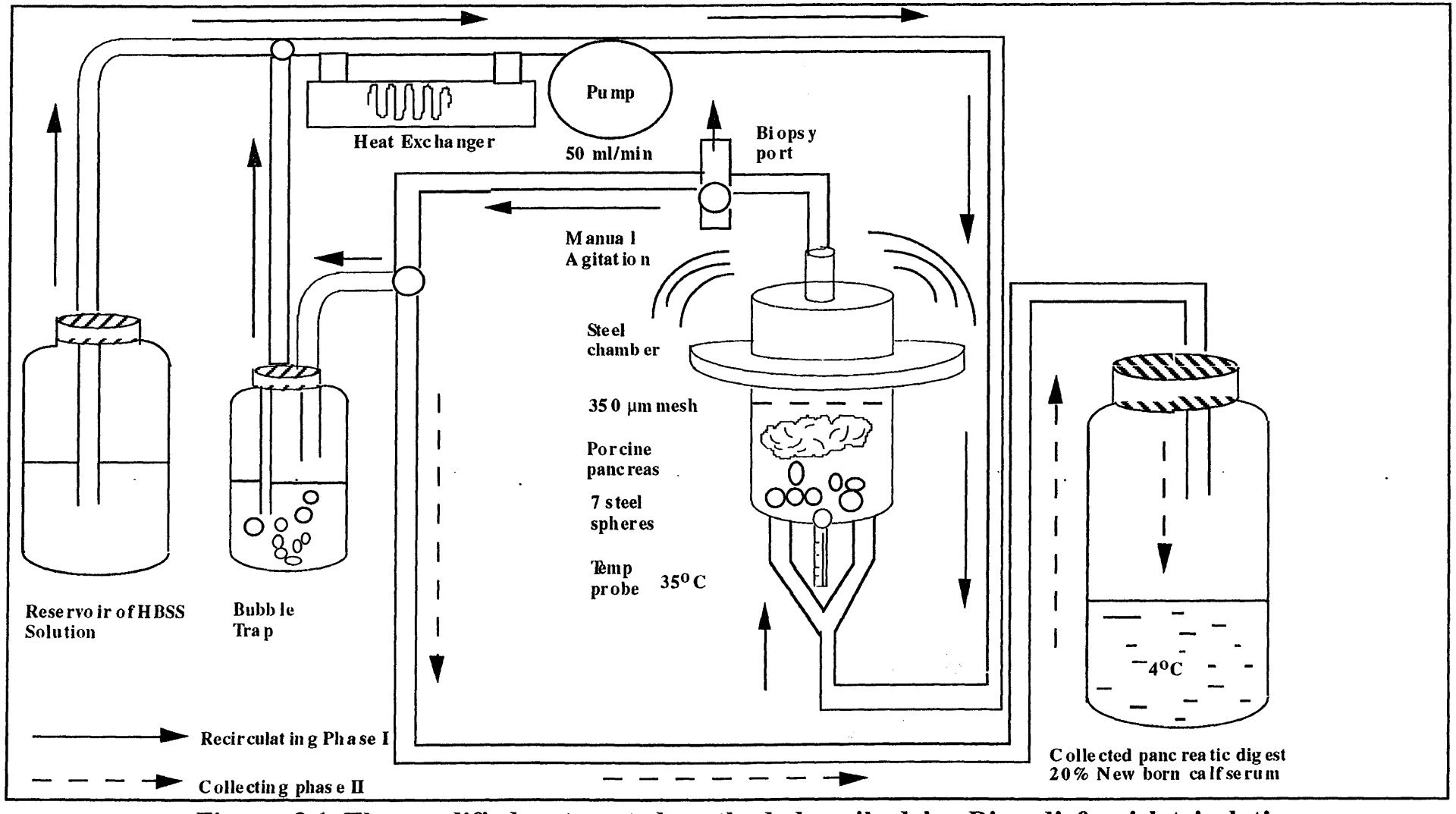


Figure 3.1 The modified automated method described by Ricordi for islet isolation

shaking at 320 oscillations/min), minimising warm ischaemia, significantly more 'whole' islets could be isolated (6,200 IEQ/g prepurification yield; Ricordi *et al.* 1990c). Other investigators practising porcine islet isolation have failed to reproduce Ricordi's originally reported islet yields. Firstly, Finke *et al.* (1991) utilising the automated method produced only 3,264 IEQ/g. Other reported yields after purification range from 377 to 5579 IEQ/g (Marchetti *et al.* 1991; Toomey *et al.* 1993; Kerr-Conte *et al.* 1994; Warnock *et al.* 1995; Hering *et al.* 1995; Prevost *et al.* 1995). Lastly, the superior yields following automated porcine islet isolation, have been confirmed by a prospectively randomized study directly comparing the two different methods. Toomey *et al.* (1993) described highly significant porcine islet yields with the automated method compared to the non-automated technique (1766 IEQ/g *versus* 5875 IEQ/g).

In general the marked discrepancies between centres can be accounted for by differences in many variables *(vide infra)* such as porcine age (Socci *et al.* 1990), strain (Ulrichs *et al.* 1994a; Heiser *et al.* 1994a) collagenase batch (Ricordi, 1992) and warm ischaemia (Ricordi *et al.* 1990b; Kerr-Conte *et al.* 1994) but despite these differences the automated method still remains the 'gold standard' for porcine islet isolation despite its complexity (Kiviluoto, 1985).

3.2 Islet purification

3.2a Principles and historical development of islet purification

The main impetus for cell separation was the need to separate heterogenous populations of cells from the blood and lymphoid systems (Visser and Van Bekkum, 1990). Methods of cell separation are based upon diversities in size, density and the physical properties of their cell surfaces such as electrophoretic and immunomagnetic cell separation techniques.

The process of purification of porcine islets has, in most cases, been adapted from human islet purification. The first method of purification was by handpicking, as described by Ashcroft *et al.* (1971b). Although simple, its large scale application is limited and, subsequently, a year later human islets were purified by density gradient separation (isopycnic) using centrifugation (Ballinger and Lacy, 1972).

Cells can be purified by centrifugation segregating them from other cellular elements of a pancreas because of their differences in density, diameter or sedimentation rates. Velocity sedimentation (VS) separates cells by centrifugation over a predetermined time on a gradient as remote as possible from that of the cells and

separation is based primarily on the differences in diameter rather than density (London *et al.* 1995). In comparison isopycnic separation is achieved by centrifugation at an appropriate force which enables cells to equilibriate at a location within a gradient medium of similar density to the cell (Brakke, 1951). Nevertheless, because of the considerable overlap in size and density between exocrine and islet tissue (Pretlow *et al.* 1975; London *et al.* 1992a) neither application is *ideal* for islet purification. However, it has been suggested (Pretlow and Pretlow, 1982) that isopycnic centrifugation can efficiently purify more dense pancreatic acinar cells from less dense islet cells better than VS despite the overlapping diameters of the two cell types. In summary because of these reasons isopycnic purification has been adopted as the most common technique for islet purification

3.2b Density gradient porcine islet purification

The first description of isopycnic porcine islet purification was by Sutherland *et al.* (1974). Using a discontinuous Ficoll density gradient (range 1.045 g/cm^3 to 1.085 g/cm^3) after centrifugation at 1,000g for 20 mins, it was demonstrated that acinar, ductal and vascular tissue remained in the lower layers of the gradient with porcine islets locating the 1.045 to 1.060 g/cm^3 interface.

A selection of contrasting density gradient media have been described, mostly for rat islet isolation, including sucrose (Lacy and Kostianovsky, 1967), Ficoll (Lindall *et al.* 1969; Scharp *et al.* 1973), dextran (Hehmke *et al.* 1986), BSA (Lake *et al.* 1987) and Euro-Ficoll (Olack *et al.*1991). The most commonly used media for porcine islet purification have been Ficoll (Ricordi *et al.* 1990c; Calafiore *et al.* 1990), Euro-Ficoll (Marchetti *et al.* 1991) or Ficoll-amidotrizoate acid (Hering *et al.* 1990) but these may be superseded in the future by newer iodinated agents such as Nycodenz (Hering *et al.* 1990; Contractor *et al.* 1995b), percoll or iodixanol (Ford *et al.* 1994; Soper *et al.* 1995) the latter offering lower osmolarity and viscosity. Preferences vary from centre to centre. BSA, although reducing cellular aggregation (Shortman, 1972), is susceptible to microbial contamination being of animal origin and on account of this some centres prefer Ficoll. Slight modifications have been described with the addition of Euro-Collins to Ficoll with overall purity being improved by reducing ischaemic injury (Olack *et al.* 1991).

Many factors have led to failure of islet purification. Wall effects, swirling and aggregation are the most disasterous consequences for density gradient separation, especially in a conventional centrifuge (London *et al.* 1995). Purification may also be affected by viscosity, osmolarity (400mOsm/kg/H2O has been shown to be optimal for

porcine) and temperature (4°C for porcine islets and 4 to 22°C for human islets; Chadwick *et al.* 1993c). It is also disconcerting that cellular swelling and acinar degranulation during the isolation process, cannot at present be precisely controlled. In these circumstances tissue density and final purification will in some cases lead to impure samples. This aspect has been the subject of intense research, where in an attempt to reduce ischaemic damage to acinar tissue, both porcine and human pancreatic digest have been stored prior to purification in UW based solutions (Chadwick *et al.* 1993a; Robertson *et al.* 1994).

Until recently large scale porcine islet isolation was performed on discontinuous density gradients. This has major drawbacks. Firstly, there is an interface between two adjacent layers and the density of cells, although different, may come to lie together. This accumulation of cells can lead to aggregation hindering the distribution of cells to other areas of different density (Pretlow and Pretlow, 1982). Perhaps in accordance with this it is not surprising that up to 75% of the total islet tissue can be lost during purification (Socci *et al.* 1991). By virtue that some of these effects can be avoided, by constructing a continuous linear density gradient, some investigators have adapted this to the COBE 2991 *(Figure 3.2 see over page)* processor in the hope of minimising islet losses (London *et al.* 1993). It has been shown that compared to discontinuous gradients, continuous gradients improve human islet purification by 26% (London *et al.* 1995; *Figures 3.3 and 3.4 see over page*). The COBE 2991 originally developed for processing bone marrow (Gilmore *et al.* 1982), has improved upon any other previously described large scale method for purifying islets and when applied to porcine islet isolation 90% purity can sometimes be achieved (personal observation). Because of its design the COBE 2991 processor not only abolishes wall effects and swirling by unloading the gradient without deceleration, but it can process large volumes of pancreatic digest (up to 60mls; Lake *et al.* 1989a) in an enclosed sterile unit. To date discontinuous (Lake *et al.* 1989a) and continuous (Robertson *et al.* 1993b) gradients on a COBE 2991 processor have revolutionised islet purification; firstly human (Lake *et al.* 1989a) then canine (Alejandro *et al.* 1990) and now porcine. There is no doubt that minor changes to the COBE 2991 technique will be described in future, particularly in biochemical modifications of gradient media that could improve islet viability and reduce ischaemic swelling. Currently, large scale density gradient purification produces on average 200, 000 IEQ and is still a limiting factor for allotransplantation. Many islets can still be lost in the impure fractions *(Figure 3.4),* hence investigators have applied secondary purification techniques such as immunomagnetic extraction of exocrine tissue by dynabeads but preliminary experiments have only been applied to rodent models (Davies *et al.* 1995).

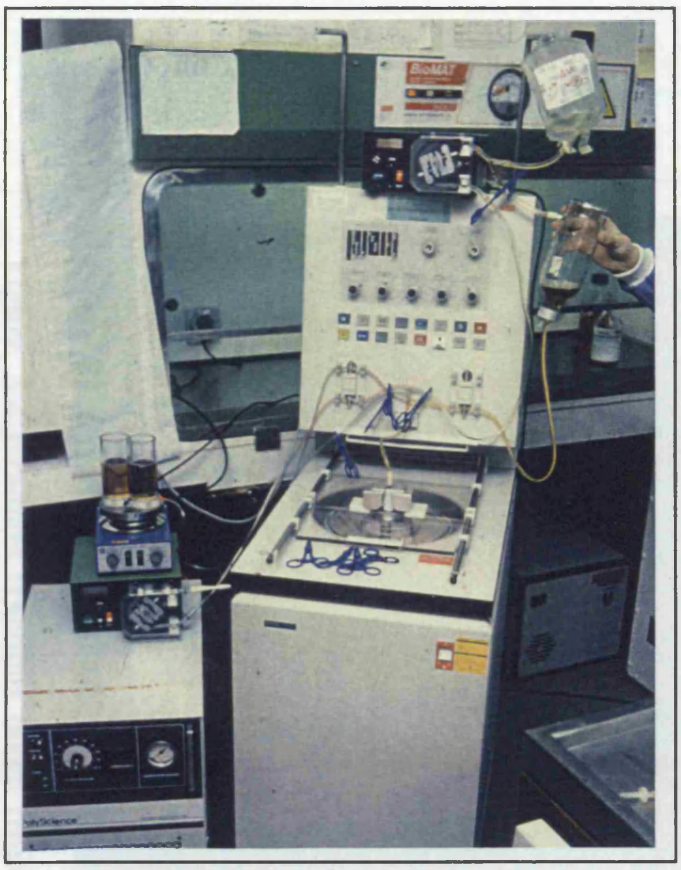


Figure 3.2 COBE 2991 processor for islet purification

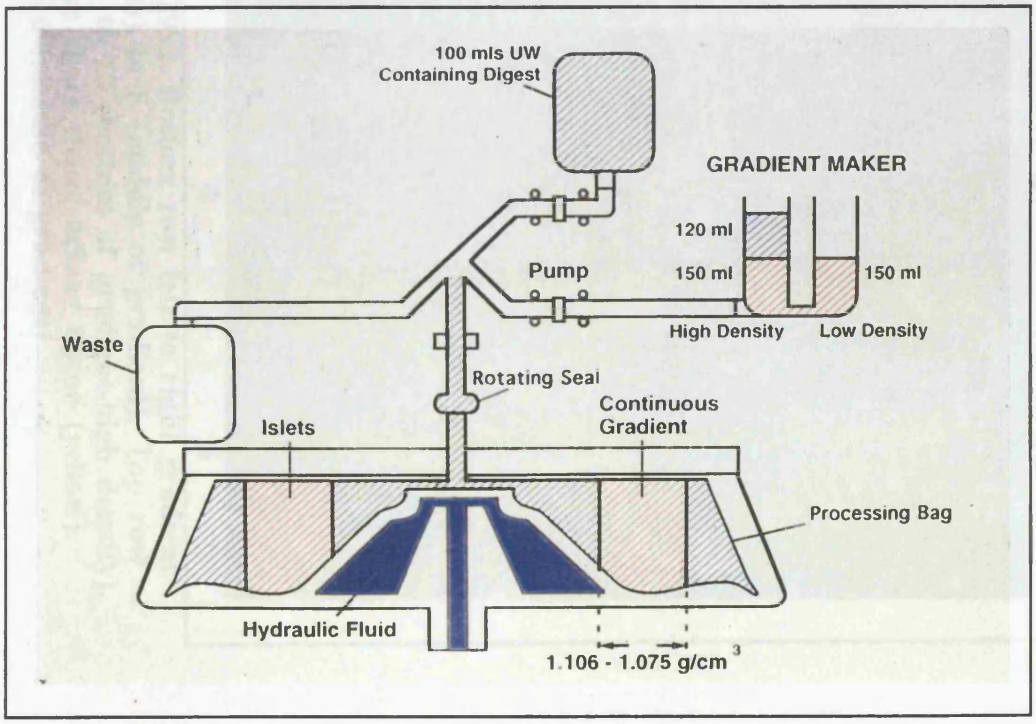


Figure 3.3 Construction of a continuous gradient within the rotating drum of a COBE 2991 processor

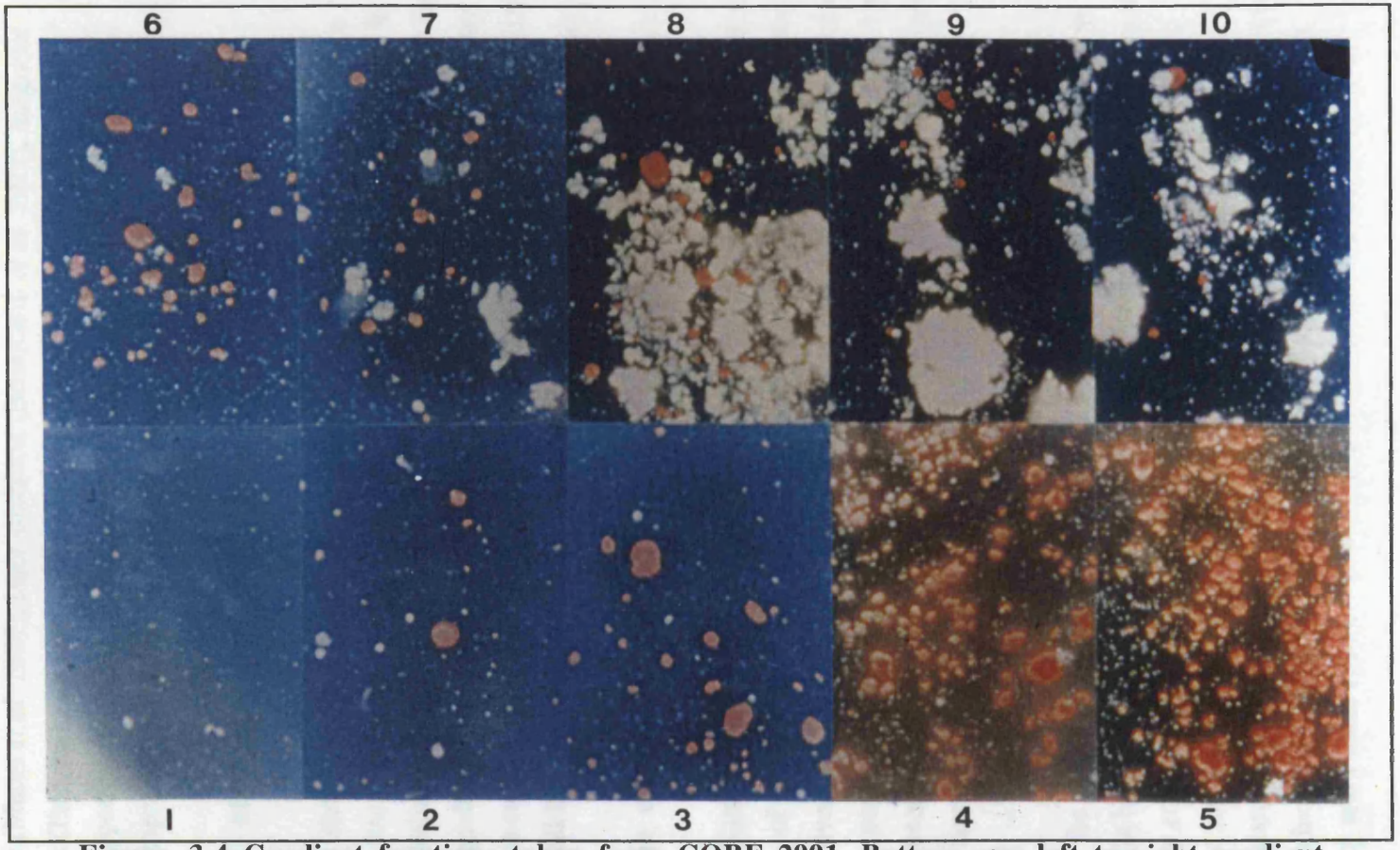


Figure 3.4 Gradient fractions taken from COBE 2991. Bottom row left to right gradient fractions 1 (top of gradient-low density) to 5 (middle of gradient). Top row from right to left gradient fractions 6 to 10 (bottom of gradient-high density). Purified islets (red) are separated from more dense acinar tissue (yellow). Most islets are found in fractions 4 to 6 (middle of gradient)

A number of other promising techniques have been attempted but their yields are still far inferior to the COBE 2991 processor, especially on a large scale basis. These involve the use of velcro (Kuhn *et al.* 1985), gamma irradiation (Nason *et al.* 1986), tissue culture (Matas *et al.* 1977), laser abalation (Brunicardi *et al.* 1992), magnetic microspheres (Fujioka *et al.* 1990), fluorescent-activated cell sorting (Gray *et al.* 1989b) and hypotonic exocrine tissue treatment (Liu *et al.* 1996) all relatively time consuming, impractical ideas that are very expensive.

3.3 Islet storage

After islet isolation, if transplantation is not to be immediately undertaken, a period of *in vitro* storage allows the viability and sterility profiles to be assessed. In addition, as outlined in earlier chapters, immunomodulation can also be performed thus abrogating a graft rejection response. There has been a wealth of literature published concerning the culture of isolated islets of Langerhans (Clayton and London, 1996) but this section will be mainly concerned with culture of porcine islets.

3.3a Tissue culture

The ability to culture individual cells or organs *in vitro* gives an opportunity to study an array of physiological, biochemical and pathological interactions which may be applicable to overall *in vivo* function. Although *in vivo* conditions are not identical to those *in vitro*, physical variables, such as oxygenation, density, osmotic forces, pH and electrolyte concentrations, can be manipulated so as to give an understanding of factors which are advantageous to cellular behaviour and survival *in vivo*.

Since the use of TCM 199 for islet culture by Andersson, (1978) a variety of different media have been used (Holmes *et al.* 1995). Of these RPMI-1640 (Moore *et al.* 1967; Lacy *et al.* 1979b) and CMRL (Parker *et al.* 1957; Lacy and Finke, 1991) seem to be the most commonly used. With the advent of LTC as an *in vitro* immunomodulator, CMRL-1066 has become the most popular culture medium as it was designed to reduce the viability of passenger leucocytes and thus enhance islet graft survival (Lacy and Finke, 1991).

Further support for CMRL-1066 also arose because of the potential toxicity of the high-glucose concentration of RPMI-1640 (11mmol/l) relative to CMRL-1066 (5.5mmol/l; Davalli *et al.* 1992). There is conflicting evidence as to what concentration of glucose should be used. Some reports suggest that 11mmol/l is optimum and others

suggest 5mmol/l but these studies are based upon observations in human islets (Eizirik *et al.* 1992). Studies in porcine indicate higher levels of glucose increases survival but reduces stimulated insulin release and viability (Brandhorst *et al.* 1997). It is thought that at higher glucose concentrations islets become desensitized (Ramirez and Stephens, 1992), have reduced proinsulin and total insulin content (Eizirik *et al.* 1991). At lower concentrations these effects are normalised but may be species dependent. Crowther *et al.* (1990), have described lower insulin secretory responses from porcine islets with a stimulation index of 2 to 2.3 compared to 4 to 10 in humans. Finally, Hegre *et al.* (1993) found that most insulin was released from cultured rat islets when compared to those of dog and pig.

Because of the availability of many commercial media, a recent comparison of 10 of the most commonly used, confirmed CMRL-1066 as the most appropriate for human islet culture (Holmes *et al.* 1995). Reports with respect to porcine islets are conflicting, where Hams F-12 has been shown to be marginally better than CMRL-1066 (Ham, 1965; Holmes *et al.* 1995), although its role is disputed by others (Davalli *et al.* 1993), claiming that medium 199 (5.5mmol/l) offers the best chance of regenerating glucose sensitivity *in vitro*, due to its ATP and xanthine supplementation.

Porcine islets have also been shown to be very susceptible to changes in temperature and pH. It has now been recognised that for optimum viability, temperature should be maintained at 27°C with a pH of 7.3 (Tai *et al.* 1994). Using this technique it has been shown that porcine islet survival could be achieved *in vitro* for many months (personal observation). Further studies suggest that culture of porcine islets at lower temperatures (e.g.22°C) increases islet survival but reduces islet viability. It also lowers insulin metabolism, overall insulin content, 24 hour insulin secretion and the dynamic insulin response to glucose (Brandhorst *et al.* 1995).

Nevertheless, due to the risks of microbial contamination (Corlett *et al.* 1988), hypoxia (isolated islets are avascular; Dionne *et al.* 1993), cytokines, nitric oxide and free radical formation (Vara *et al.* 1995) a duration of one to two weeks should not be exceeded (Marchetti *et al.* 1992c; Tai *et al.* 1994), but an optimum balance needs to be achieved as *in vitro* culture prior to transplantation may improve islet purity through necrosis of acinar tissue (Weber *et al.* 1977).

The oxygen content of media used for islet culture also needs to be considered. To date, little data is available with regard to porcine islets but it has been demonstrated for human islets that the second phase of insulin release is reduced following a period

of culture in hypoxic conditions (Dionne *et al.* 1989). With this in mind there is an urgent need for improving oxygen delivery to cultured islets.

The ideal composition of culture media is still to be realised but most are based on plasma constituents. Many studies have concentrated on the role of serum in tissue culture because of its growth promoting properties (Barnes and Sato, 1980) and effect on insulin secretion (Andersson *et al*. 1974; Andersson, 1978). Notwithstanding this view, serum has extreme variability (London *et al.* 1992c; Tai *et al.* 1994) between batches and standardisation of culture media is, therefore, difficult to achieve. Investigators have since tried to develop serum free culture media (Behboo *et al.* 1994) in an attempt to reduce infection, islet losses and viability, factors which hinder the long-term success of islets transplantation.

Lastly, attempts have been made to 'propagate' islets *in vitro* termed nesidioblastosis. In the adult regenerating pancreas proliferation of the ductal epithelium precedes differentiation of islet endocrine cells *in vivo*. Adult human islet preparations have been cultured in three dimensional collagen gels promoting neogenesis of islets from ductal epithelium *in vitro*. This new concept could increase the mass of endocrine tissue from adult cadaveric pancreata but its mass application is still not known (Kerr-Conte *et al.* 1996).

3.3b Cold storage

Having outlined the established role of cold preservation for whole pancreata it is practical to assume that following isolation, islets may benefit from some form of cold storage at 4°C, in preference to conventional culture at 22 to 37°C. Currently, very little attention has been paid to this concept. Studies have focused on rodent islets cultured in HBSS (Knight *et al.* 1973), RPMI-1640 or CMRL-1066 (Morrow *et al.* 1982) or more preferably UW based solutions, as UW could theoretically reduce ischaemic damage because of its 'intracellular 'electrolyte components. It was subsequently demonstrated that islet viability was maintained for up to 72 hours when stored in histidine-lactobionate or UW based solutions as opposed to 'extracellular based' HBSS that reduced islet viability after only 24 hours (Delfino *et al.* 1993). The challenge is now, therefore, to transpose the use of cold preservation solutions already in universal use for whole organ storage to the preservation of isolated, cellular tissue.

3.3d Cryopreservation

Cellular transplants offer the unique opportunity for indefinite long-term storage. With the unreliability of obtaining a sufficient islet yield from a solitary organ donor, allografts or xenografts may be supplemented with cryopreserved islets pooled from many different individuals. This technique optimizes the critical islet mass necessary to reverse hyperglycaemia thus reducing the likelihood of graft failure secondary to a marginal islet mass being transplanted. With prolonged islet preservation in mind, investigators have applied the technique of cryopreservation to clinical islet transplant programs. Many centres have also supplemented fresh islet allografts with additional, cryopreserved islets (Warnock *et al.* 1991; London *et al.* 1992b) in order to improve long-term insulin independence, such that one patient is documented to have remained insulin independent for at least 15 months (Warnock *et al.* 1992).

Other encouraging reports suggest that cryopreserved islets have an immunological advantage over fresh islet allografts by modulating islet immunogenicity (Coulombe *et al.* 1987) by down regulation of MHC class one expression (Cattral *et al.* 1993), as opposed to MHC class II, which is thought to remain unchanged (Sutton *et al.* 1987). This immunomodulating effect may, in part, be because of acinar tissue loss during the phase of cryopreservation, also aiding islet purification (Bank 1983), but this does not necessarily prolong islet allograft (Cattral *et al.* 1993) or xenograft (Marchetti *et al.* 1995c) survival. It is for these reasons, and with the current lack of organ donors, that tissue banks of purified cryopreserved islets have been set up in the hope of providing enough islets for insulin dependent patients all over the world (Warnock, 1994).

Since the introduction of islet cryopreservation in 1976 (Rajotte *et al.* 1977), many different protocols have been described in rats (Glanville *et al.* 1987), mice (Cattral *et al.* 1993), dogs (Evans *et al.* 1990), pigs (Wise *et al.* 1983; Marchetti *et al.* 1995c) and humans (Warnock *et al.* 1991; London *et al.* 1992b). Techniques vary between species, those applicable to human islets, as described by Rajotte *et al.* (1981), involve the addition of a cryoprotectant such as dimethyl sulphoxide (DMSO) at 22°C (Lakey *et al.* 1994) which is then cooled to 0 °C. The samples are supercooled to -7.5 °C and nucleated with an ice crystal with further cooling at -0.25 °C per minute until -40°C is reached *(Figure 3.5 p.119)*. All the islet samples are then stored in liquid nitrogen (-196°C). Thawing is less technical, involving retrieval of islet samples with rapid thawing at 200 °C per minute and dilution of DMSO with sucrose solution.

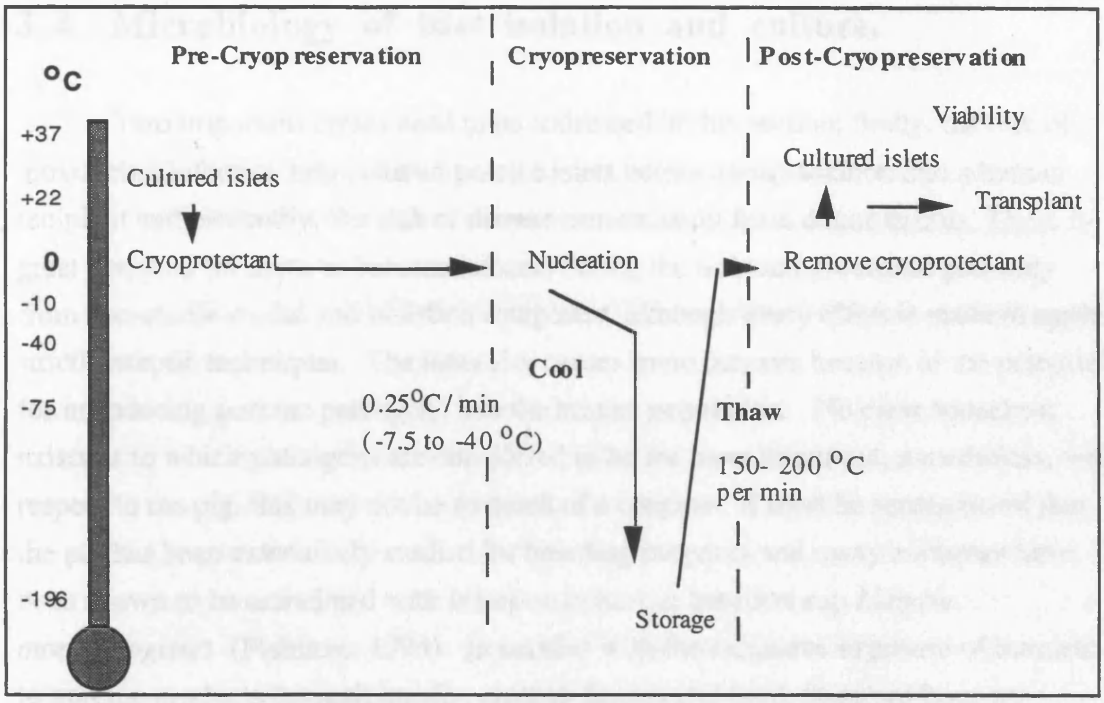


Figure 3.5 Method of islet cryopreservation

Porcine islet cryopreservation was first carried out on mechanically prepared islets (Wise *et al.* 1983). A method similar to that described by Rajotte *et al.* (1981) was used (e.g DMSO), but a more rapid cooling rate was preferred (1 °C per minute). Post-cryopreservation transplantation was undertaken following total pancreatectomy with subsequent islet autotransplantation that normalised hyperglycaemia in only one of six recipients. Failure was deemed to be because of an inability to slow cooling rates and the lack of a standardised method for freezing (Wise *et al.* 1985).

More recently investigators have consolidated the procedure needed for the cryopreservation of adult, purified, porcine islets (Tze *et al.* 1994b; Marchetti *et al.* 1995c). It has been shown that islet morphology and both *in vitro* and *in vivo* viability is similar to non-cryopreserved islets after preservation at -130 °C. It was also noted, as with rodent (Sandler *et al.* 1986) and human (Warnock *et al.* 1987a) islets, that the rate of insulin secretion was significantly higher perhaps due to 'cryo-induced' membrane damage. Post-thawing recovery of porcine islet equivalents was only 66% (Marchetti *et al.* 1995c), but it can be improved by eliminating innocuous overnight islet culture (Lakey *et al.* 1994). In the case of human islets with losses also as high as 30% (Warnock *et al.* 1987a), this is unacceptable, especially with the limited availability of organ donors but for porcine islet xenotransplantation cryopreservation may play a more useful role because of the more readily available porcine pancreas.

3.4 Microbiology of islet isolation and culture.

Two important issues need to be addressed in this section; firstly, the risk of introducing infection into cultured porcine islets before transplantation into a human recipient and, secondly, the risk of disease transmission from donor to host. There is great potential for islets to become infected during the isolation procedure primarily from non-sterile media and isolation equipment, although every effort is made to apply strictly aseptic techniques. The latter risk causes more concern because of the potential for introducing porcine pathogens into the human population. No clear consensus exists as to which pathogens are considered to be the most important, nonetheless, with respect to the pig, this may not be so much of a concern. It must be remembered that the pig has been extensively studied for breeding purposes and many zoonoses have been shown to be associated with infection in human breeders e.g. *Listeria monocytogenes* (Fishman, 1994). In parallel with the extensive exposure of humans to porcine products through insulin, clotting factors and food, there has been no demonstrable serious epidemic relating to transmitted infection. The limitations of this supposition are that the majority of these products have been reliably tested by 'quality

Bacterial	**Yeast and Fungal**
Actinomyces pyogenes	*Aspergillus spp.*
Brucella suis	*Candida spp.*
Campylobacter spp.	*Cryptococcus spp.*
Clostridium spp.	*Histoplasma spp.*
Haemophilus spp.	*Microsporum spp.*
Listeria monocytogenes	*Nocardia spp.*
Mycobacterium avium	*Pneumocystis carinii*
Mycobacterium bovis	
Pseudomonas spp.	
Salmonella spp.	
Staphylococci spp.	
Streptococcus spp.	
Yersinia spp.	

Adapted from references (Marchetti *et al.* 1992b; Damon-Burke *et al.* 1994; Fishman, 1995; Bjoersdorff *et al.* 1995)

Table 1.1 Microbial pathogens of swine origin known to cause infection in humans

control' methods relating to non-viable cells, but this may not be so for xenografting, as a xenograft constitutes viable live cells which probably have the greater potential for pathogen associated transmission; therefore, 'quality control' of viable whole organ, or cellular xenotransplants, is of critical importance and more difficult to assess than was first thought based upon the findings attained from non-viable porcine products (Fishman, 1995).

Microbiological quality control has been recommended as a mandatory screening procedure for clinical islet transplant programs (Carroll *et al.* 1992). Few would disagree, that this is perhaps of more importance for an islet xenotransplantation programme. Even after pancreas digestion and purification, up to 30% of all final islet preparations used for human transplantation are still contaminated (Lloveras *et al.* 1994). Transport fluids from porcine pancreata excised in the abattoir have 100% contamination rates though most microbes can be eradicated by utilising specific antibiotic regimens (Marchetti *et al.* 1992b). Commonly encountered pathogens relevant to porcine islets are *Staphylococci, Corynebacteria*, pseudomonads, *Klebsiella, Enterococci* and Candida spp. (Marchetti *et al.* 1992b; Damon-Burke *et al.* 1994). Various attempts have been made to reduce these contamination rates but will be discussed in more detail in Chapter Eight.

Other microbes have the potential to be pathogenic and need to be considered. For example, viral and protozoan screening tools are very expensive and unreliable, many still remain unclassified, current screening of these pathogens is merely speculative (Bjoersdorff *et al.* 1995). Some porcine viral infections are related to species affecting humans and may be pathogenic e.g. swine influenza virus (Bjoersdorff *et al.* 1995). In a study by Bjoersdorff *et al.* (1995) no contaminating viruses (including pseudorabies virus, diarrhoea virus, porcine parvovirus, herpes simplex, varicella, adenovirus, reovirus, ECHO viruses and Coxsackie viruses) could be identified in porcine foetal ICC. Studies in miniature swine suggest that retroviruses and porcine cytomeglovirus are also important (Fishman, 1995), along with the protozoans, *Toxoplasma gondii* and *Strongloides stercoralis*. Taken together, most investigators have, therefore, assessed the bacterial, yeast and fungal profiles following islet isolation but further investigation is still required for viral and protozoal.

3.5 Porcine islet viability studies

3.5a *In vitro* studies

Many studies have reported *in vitro* islet viability following adult porcine islet isolation (Ricordi *et al.*1986; Mendola *et al.* 1989; Crowther *et al.* 1989; Calafiore *et al.* 1990; Finke *et al.* 1991; Yamaguchi *et al.* 1992; Davalli *et al.* 1993; Kerr-Conte *et al.* 1994; Marchetti *et al.* 1995a; Holmes *et al.* 1995; Brandhorst *et al.* 1995). Insulin release by isolated islets has been mainly tested under static conditions (Ricordi *et al.* 1986; Finke *et al.* 1991; Kerr-Conte *et al.* 1994; Marchetti *et al.* 1995a) in response to alterations in glucose concentrations. Insulin release reflects the function of a single metabolic pathway within islets and may not be truely representative of global islet function. Several studies as previously outlined have indicated poor *in vitro* responses of porcine islets (Crowther *et al.* 1989; Davalli *et al.* 1993) most probably because of islet damage during the isolation procedure. Perhaps, therefore, the only way to truely determine the effect of porcine islet isolation, is to perform porcine islet autotransplantation studies as the ultimate test as studies of allotransplantation and xenotransplantation are confounded by a rejection response that would also be injurious to islets.

3.5b Porcine islet autotransplantation

The first studies of porcine islet autotransplantation were undertaken by Sutherland *et al.* (1974) where survival was approximately for two weeks. Wise *et al.* (1985) using intraportal autotransplantation of cryopreserved porcine islets achieved survival for only three days. Survival was shown to be improved after intraportal autotransplantation without cryopreservation maintaining survival for up to eight weeks (Kiviluoto, 1985) but these islets were unpurified suggesting that the islet mass was perhaps larger.

More recently studies have compared porcine autotransplants at different sites including intraportal, splenic and within the peritoneal cavity (Wahoff *et al.* 1995b). In an effort to reduce ischaemic damage the isolation steps were kept to a minimum and the autografts were unpurified. Intraportal grafts proved to be lethal due to portal hypertension, likewise splenic transplants were also unsuccessful, whereas peritoneal grafts were well tolerated and insulin independence was maintained for up to 30 days but the number of IEQ/kg transplanted were not stated. Similarly Hering *et al.* (1995) maintained survival for up to four weeks using purified autografts but insulin and c-

peptide profiles following an intravenous glucose tolerance test were abnormal. Finally it has been estimated that in order to obtain normoglycaemia following an intraportal purified porcine autograft up to 6,000IEQ/kg are required (Pattou *et al.* 1995).

It can be concluded that isolated porcine islets can secrete insulin in response to hyperglycaemia both *in vitro* and *in vivo* though long-term function is questionable. Many reasons can account for this but it is unlikely that islets remain unscathed during the traumatic isolation procedure. Furthermore, *in vivo* studies are only successful when sufficient numbers of islets are transplanted thus dependent on a good islet yield and *in vitro* studies are unlikely to represent the global functioning of islet cells *in vivo*.

SECTION B

Factors affecting porcine islet isolation

3.6 Donor factors

3.6a Islet size and volume density.

Many studies have endeavoured to estimate the mean diameter of a human islet both in diabetic (Hellman, 1959b; Stefan *et al.* 1982) and non-diabetic pancreata (Hellman, 1959b; Saito *et al.* 1978; Stefan *et al.* 1982). The most detailed analysis was that described by Saito *et al.* (1978) based upon a Weibel distribution analysis of 290 serial tissue sections. From this study, the mean diameter of a human islet was deemed to be 314 μm, but was largely an overestimate because of the exclusion of islets with diameters less than 43μm in the analysis. Similar studies in other species such as rats (Haist and Pugh, 1948; Hellman, 1959a), dogs (Acosta *et al.* 1969; Saladino and Getty, 1972) and monkeys (Thompson and Hussey, 1932) have also been performed but constituted the use of a conventional light microscope. Likewise, analyses of histological sections from porcine pancreata (e.g islet diameters and islet volume density) have also been well described since 1907 (Höcke, 1907; Thomas, 1937; Voigt, 1959).

3.6b Differences in islet volume density between porcine strains

Current knowledge suggests that the number of islets varies between different porcine strains. It seems logical, therefore, that pancreata with a larger volume density of islets needs to be determined (where islet volume density equates to the percentage volume of islet tissue relative to the volume of acinar tissue within a pancreas). German

Large White pancreata (30.7) are thought to have the greatest percentage volume density of islets compared to German Landrace (29.3), Wild Boars (27.3), Piertrain (24.1), Duroc (19.9) or Hampshire (19.4; Ulrichs *et al.* 1994a). Unfortunately, there are limitations to interpretation of this particular data as the results are expressed as relative mean numbers of islets per tissue section rather than islet equivalents and, given that the mean islet diameter differs between strains (e.g. Large Whites 149.3 μm; German Landrace 132.3 μm; Ulrichs *et al.* 1994a), further studies are necessary to determine which is the optimum strain. Despite the marked differences in volume densities, they do not correlate well with islet yields. Piertrain provide the best islet count per gram of pancreas (2,180 islet/g) in contrast to adult hybrids (1,190 islet/g), German Landrace (606 islet/g) and Münich Minipigs (691 islet/g; Heiser *et al.* 1994a), which yield fewer islets per gram (Ulrichs *et al.* 1995). Again, these results are misleading as the counts are expressed as relative islet counts as opposed to the preferred islet equivalent count.

3.6c Donor age

There are marked differences in islet numbers between adult and juvenile pigs. Ulrichs *et al.* (1995) have demonstrated that juvenile pigs have a greater number of absolute islets compared to those of adults but is unsupported by Socci *et al.* (1990). They tested the influence of donor age upon porcine islet yield. Two groups of pigs were compared to those less than six to ten months (juveniles) and those greater than two to three years (adults). Although no significant differences were observed in terms of islet number, the volume of islet tissue per gram of pancreas was significantly greater in the older porcine group (476 mm^3 versus 1190.6mm^3).

3.7 Enzymatic porcine pancreas dispersion

Enzymatic pancreas dispersion is essentially a combination of two processes (e.g. intraductal collagenase delivery and automated digestion), with three important considerations; firstly, the effect of pancreas distension with collagenase; secondly, the effect of pancreatic exocrine enzyme release during the isolation process and, lastly, the configuration of the extracellular matrix. All will be considered and discussed but in some respects there is considerable overlap.

3.7a Pancreatic extracellular matrix.

In 1965 Moskalewski (Moskalewski, 1965) first isolated viable islets by digesting a rodent pancreas with a collagenase. Since these early days, the collagenolytic methods of islet isolation has become the "gold standard" but its

reliability has become extremely unpredictable. This may be in part due to the collagen substrate (London *et al.* 1990) within the pancreas, or due to the inefficiency of the collagenase enzyme itself (Gray and Leow, 1992; Johnson *et al.* 1996b).

Collagen molecules are responsible for the structural integrity of many tissues. It was first assumed that all were identical but nearly 25 years since its recognition, it is now known that 13 distinct types exist, encompassing at least 25 separate genes (Vuorio and De Crombrugghe, 1990). They can be divided into three classes; class one represents types I, II, III, V and XI (Banded Fibre Forming); class two consist of types IX and XII (Banded Fibrillar Type), those of class three consist of IV, VI, VII and X (Independent Banded); whereas the remaining types VIII and XIII are only partially characterized. Briefly, collagen molecules contain a triple helical and globular domain with the size and distribution showing diversity between types (Seifter and Harper, 1970; Burgeson and Nimni, 1992). The extracellular collagen network is constructed from a biosynthetic process involving a precursor called procollagen. This molecule is then cleaved and shortened at its non-helical ends to form collagen (Gross, 1974; Burgeson and Nimni, 1992).

Many questions of collagen biochemistry still remain unanswered. For example, mechanisms controlling the amount and the maturation of collagen deposited in the extracellular pancreatic tissue. Even though the minutiae of steps involved in its intracellular biosynthesis are largely understood, the knowledge of the factors controlling the balance of synthesis and degradation are minimal (Burgeson and Nimni, 1992). From the perspective of islet isolation this knowledge, it would seem, is critical for its consistent success. It is now known that the bulk of collagen is continuously turned over with the most stable fibres being slowly replaced by those newly synthesized (Uitto and Lichtenstein, 1976). This inherent property of collagen could be particularly influenced by patient age (Weiss, 1982) or previous pancreatic pathology (e.g. pancreatitis). It has been suggested by a number of studies (van Suylichem *et al.* 1987; van Deijnen *et al.* 1992) that the inconsistencies of porcine islet isolation may be related to the collagen distribution within the pancreas given that porcine islets have a propensity to disperse into single beta cells during a carefully controlled automated pancreas digestion (Ricordi *et al.* 1990c).

Clostridium histolyticum collagenase is one of the few proteinases capable of degrading the triple-helical region of native collagen under physiological conditions (Seifter and Harper, 1970; Van Wart and Steinbrink, 1981). The presence of collagen molecules at the islet-exocrine interface allows the separation of islets from acinar pancreatic tissue through enzymatic collagenase cleavage at the X-Gly bond of collagen

(Harris and Krane, 1974; Van Wart and Steinbrink, 1981; Johnson *et al.* 1996b). Many variables, other than collagenase, (Gray and Leow, 1992; Johnson *et al.* 1996b) can profoundly influence the success of cleavage, with one of the most important being the integrity of the peri-insular (surrounding the islet) extracellular matrix. The heterogeneity of the extracellular matrix comprises an array of different proteins such as glycosaminoglycans, glycoproteins, fibronectin, elastin and collagen (Weiss, 1982; Uscanga *et al.* 1984; van Deijnen *et al.* 1992). The types of collagen molecules distributed within pancreata is poorly understood with only a few studies pertaining to islet isolation (van Suylichem *et al.* 1987; Ulrichs *et al.* 1994a; van Deijnen *et al.*1992; van Deijnen *et al.* 1994; van Suylichem *et al.* 1995b). Collagen has been identified within the capsule of the native pancreas, between individual lobes (interlobar) and within the lobes (intralobular), within the adventitia of arteries, veins and in the pancreatic ducts (Uscanga *et al.* 1984; Wolters *et al.* 1992; van Deijnen *et al.* 1992; van Deijnen *et al.* 1994; van Suylichem *et al.* 1995b), but these studies are limited to the few anti-collagen monoclonal antibodies available.

The quantity and distribution of collagen fibers have been shown to differ between species (van Deijnen *et al.* 1992). The pancreatic collagen content of the pig, cow and human is significantly greater than that of the rat, most of which is localized within the interlobar and intralobular pancreatic septa (Uscanga *et al.* 1984; van Deijnen *et al.* 1992). When stained with collagen type IV, localisation has been restricted to its incorporation within basement membranes, particularly within the perivascular space of islet capillaries mainly in rat, dog, pig and human (Uscanga *et al.* 1984; van Deijnen *et al.* 1992). The porcine pancreatic capsule has been demonstrated to have little reaction to anti-collagen type IV; it is also intermediate in rats and of weaker intensity in canine pancreata.

Peri-insular staining with collagen type IV is known to be discontinuous and of intermediate intensity in rat, dog and man, in contrast to that of pig which is of very low intensity and very fragmentary (van Deijnen *et al.* 1992). This discontinuity has likewise been demonstrated with scanning electron microscopy and was shown to be characteristic of interruptions through direct islet-acinar contact. This morphology was similar in rat and human pancreata, where islet-exocrine contacts occur frequently with the majority of islets demarcated from acinar tissue by the peri-insular capsule. Those of canine species are markedly different in that canine islets are almost entirely separated from acinar tissue, in contrast to porcine islets which are almost completely deficient of a peri-insular capsule with islets being in direct contact to acinar tissue through cell-cell adhesion (van Deijnen *et al.* 1992).

Similar findings have been demonstrated by Ulrichs *et al.* (1994a) where porcine islets have a capsule composition of at least type I, III and IV. More recently Van Deijnen *et al.* (1994) described the distribution of collagen types I, III and IV within rat, dog, pig and man. The intensity of collagen type I in the peri-insular region, interlobar, intralobular and acinar septa was weak in pig and dog but moderate in rats but collagen type III was markedly more intense though still weak in porcine species. That of type V reacted moderately in rat, dog and man but weakly in pig, particularly within the interlobar and intralobular pancreatic septa.

3.7b Collagenase

There is no doubt that a good collagenase is crucial for islet isolation. Two important concepts concerning collagenase need to be clarified if the established advances of islet isolation are to be superseded (Ricordi *et al.* 1988a; Lake *et al.* 1989a). Firstly, the precise mode and site of action of collagenase in relation to the islet-acinar interface needs to be determined and, secondly, its advantageous components need to be optimised with a *consistent commercial reproducibility.*

Collagenases are secretion endopeptidases assumed to be calcium dependent, zinc metalloproteinases, which have the ability to hydrolyse the triple-helical region of collagen within connective tissues (Seifter and Harper, 1970; Bond and Van Wart, 1984a). The culture filtrate from *Clostridium histolyticum* is the most widely studied proteinase mixture because of the belief that it is the most efficiently known substance for degrading connective tissues (Bond and Van Wart, 1984c). This impure mixture, is composed of a heterogeneity of molecular weight proteins *(Figure 3.6 p.128)*.

Briefly, six collagenases have been described to account for virtually all the collagenase activity with regard to islet isolation (Bond and Van Wart, 1984c). These are conveniently subdivided into two crude classes, called class I and class II based on their different substrate specificities. Both classes hydrolyse collagen and numerous peptides at different rates and sequence specificities. The most widely used peptide is 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA; Bond and Van Wart, 1984a; Bond and Van Wart, 1984c; Heiser *et al.* 1994a). The different class activities are also relatively complementary and synergistically degrade collagen (Van Wart and Steinbrink, 1985). The significance of this two class classification does allow some assessment of the relative proportions of each within commercial products, the ratios of which may differ markedly, thereby accounting for some of its variability of activity during porcine islet isolation (Villani *et al.* 1994). However, to date there are very few studies relating to collagenase activity during porcine islet isolation and with there still

being no reliable substrates available to compare different collagenase activities or purified components, consistent and reproducible comparisons would be very difficult indeed.

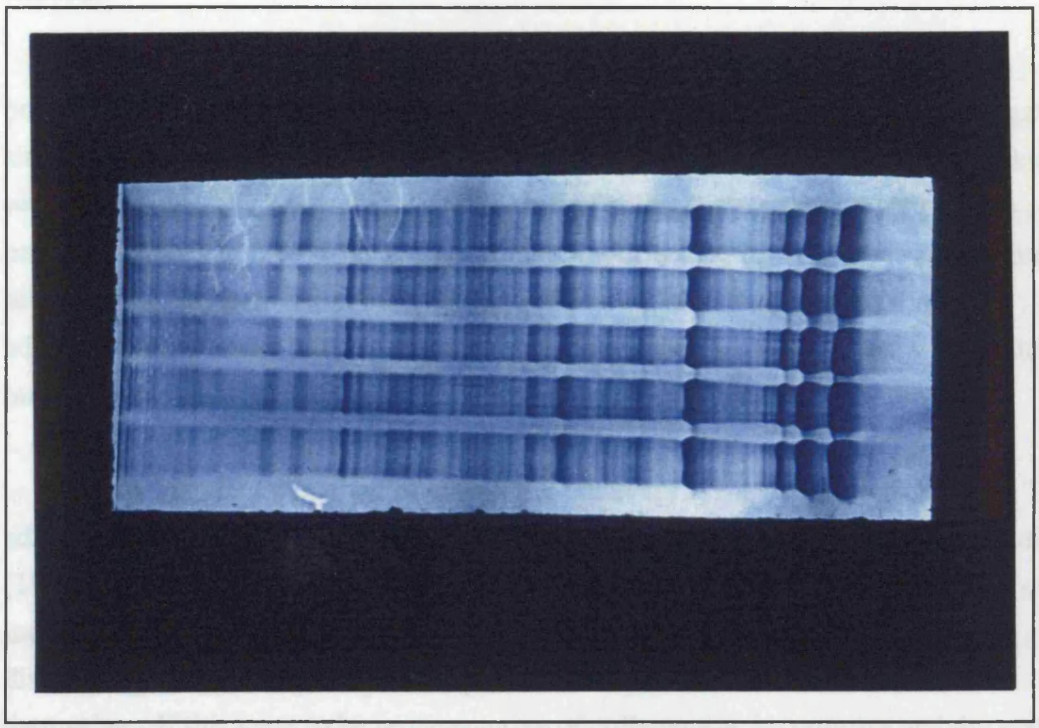


Figure 3.6 SDS-PAGE of collagenase demonstrating all the components with different molecular weights

The classes of collagenase have been the subject of much debate in recent years stimulating numerous attempts at their purification in order to ascertain the vital components for islet isolation (Bond and Van Wart, 1984b). These crude preparations exhibit not only collagenolytic activity but also amidase-peptidase, clostridiopeptidase B (Mitchell and Harrington, 1968), exopeptidase, phospholipase C and non-specific proteinase activities (Mandl, 1971; Bond and Van Wart, 1984c; Van Wart and Steinbrink, 1985). Furthermore, some of the non-specific components of collagenase may be essential for islet isolation as purified class I or class II collagenase samples have been shown to reduce islet yields (Vos-Scheperkeuter *et al.* 1997). One possibility for this could be because of the inherent impurities of collagenase activating pancreatic proteolytic enzymes thus assisting pancreatic dispersion (Traverso and Abou-Zamzam, 1978; Chapter Seven). Studies in the rat (van Schilfgaarde *et al.* 1994) and pig (van Suylichem *et al.* 1995a) support this hypothesis in that the elimination of protease activity and the use of purified enzymes (e.g. Classes I and II alone) is presently a prerequisite to poor islet yields. As with all enzymes, collagenase activity is pH (Khalaf *et al.* 1995) and temperature dependent (Soru and Zaharia, 1972), the effect of which may influence islet isolation (Sutton *et al.* 1990), but the current literature has only

shown significant differences at widely divergent physiological values (Wolters *et al.* 1992).

Attempts have been made to compare different collagenase enzymes upon porcine islet isolation. A comparison of three different commercial collagenases gave similar results in a non-automated system in terms of islet-acinar cleavage but yields were not reported (Villani *et al.* 1994). Purified collagenase enzymes for porcine pancreas dissociation have been tried by Van Suylichem *et al.* (1995a) using a "mini test tube shaking" system but results were inconclusive with there being no clear cut advantage using either purified collagenase alone or purified collagenase with additional purified protease when compared to crude collagenase alone.

The intraductal method of collagenase delivery is the optimum technique. The advantages of intraductal collagenase have been investigated by VanSuylichem *et al.* (1992) by substituting the distribution of collagenase with an intraductal injection of indian ink (van Suylichem *et al.* 1992). It was well demonstrated that the ink distribution was seen not only in the peri-insular space but also within islets themselves. If this were to be representative of collagenase, its protease activity could account for fragmentation and damage by deep intraislet penetration accounting for the estimated 50% loss of islet tissue during isolation (Wolters *et al.* 1992). The pH, temperature (Warnock *et al.* 1988), volume and force (Armstrong *et al.* 1985) of collagenase delivery, may also account for some of these losses, perhaps also relevant to porcine models, where findings in rat pancreata have shown pressures exceeding 50cm water causing duct rupture and tissue oedema. The influence of different loading pressures in the porcine situation has been investigated by Johnson *et al.* (1996a). Each pancreas was distended with crude collagenase and the distribution identified by polyclonal anti-sera to crude collagenase. Normal pressures were shown to be 125 to 200mmHg but surprisingly collagenase entered the islets at pressures as low as 50mmHg.

In conclusion, pancreatic dispersion aims to selectively degrade the extracellular matrix surrounding islet tissue without compromising the islet encapsulating membrane (Wolters *et al.* 1992). However, with respect to the porcine pancreas, a basement membrane is not seen surrounding all (van Deijnen *et al.* 1992;Ulrichs *et al.* 1994a) individual islets, unlike that seen in rat, canine and human where there is a predominant basement membrane. The finding of the relative paucity of peri-insular extracellular matrix in porcine pancreata correlates well with the ease which porcine islets can be liberated during digestion, but still this cannot explain why rat islets are easier to isolate in comparison to human and canine pancreata, despite having similar cell-cell adhesion

(van Deijnen *et al.* 1992). An explanation for this could be due to specific species discrepancies not only in the peri-insular extracellular matrix but also within the acinar tissue (Uscanga *et al.* 1984; Kennedy *et al.* 1987; van Suylichem *et al.* 1987). It is unlikely that any single commercial collagenase will uniformly behave the same in all pancreata which differ in their endocrine-acinar and cell-cell protease sensitivities. Unfortunately, the perplexing dilemma of the correct combination of crude or purified enzymes still eludes all islet laboratories and the companies which manufacture it. Although recent attempts have been made to produce consistent yields for clinical human islet allotransplantation with the introduction of Liberase ® (Gill *et al.* 1995; Linetsky *et al.* 1997). Liberase ® is a purposefully manufactured collagenase; a blend of purified enzymes formulated for the rapid dissociation of intact viable islets. Regrettably, it is very expensive and is unlikely to be routinely used for porcine islet isolation research. In an attempt to overcome this problem, other centres use collagenase in conjunction with elastase, DNAase and calcium (Calafiore *et al.* 1990) but their significance has not been proven and the answer most probably rests with the development of a recombinant collagenase enzyme (Hesse *et al.* 1995).

3.7c Pancreatic exocrine enzyme release.

The collagenase digestion phase of porcine islet isolation releases pancreatic exocrine enzymes (Traverso and Abou-Zamzam, 1978). The majority of these enzymes are called serine proteases, characterized by the reactivity of a serine residue within its active site (Rinderknecht, 1986). The effect of these enzymes upon islet tissue is little understood. It has been proposed that their release potentiates collagenolytic activity and vice versa (Traverso and Abou-Zamzam, 1978) but more recently it has been suggested that they may cause dispersion of islets into single endocrine cells during islet isolation, being a predisposition to poor islet yields (Heiser *et al.* 1994b). Many different exocrine enzymes are released and could potentially influence the integrity of porcine islets given that they are so fragile (Ricordi *et al.* 1990c).

Trypsin is the pivotal enzyme for the activation of most pancreatic exocrine zymogens (Neurath, 1957) *(Figure 3.7 see over).* The major trypsin dependent zymogens secreted *in vivo* from the porcine pancreas are, chymotrypsinogen (Marchis-Mouren, 1965), proelastase (Marchis-Mouren, 1965; Hartley and Shotton, 1971), the procarboxypeptidases A (Folk and Schirmer, 1963; Marchis-Mouren, 1965) and B (Folk *et al.* 1960; Marchis-Mouren, 1965), prophospholipase A_2 (; Marchis-Mouren, 1965; Nieuwenhuizen *et al.* 1974) and lipase through trypsin dependent co-lipase activity (Wieloch *et al.* 1981). Kallikreininogen is also a trypsin dependent zymogen but only accounts for less than 0.4% of pancreatic secretory juice (Rinderknecht,

1986). The other major enzyme secreted from the pancreas is amylase but this is not in a proform and is not trypsin dependent.

Chymotrypsin is activated from a presursor, chymotrypsinogen which is readily activated by trypsin and enhanced in the presence of calcium. It displays a broad range of activity within the pH range 5 to 9. Autocatalytic secondary cleavage of the molecule can also occur but is species dependent. Chymotrypsin has activity directed at the

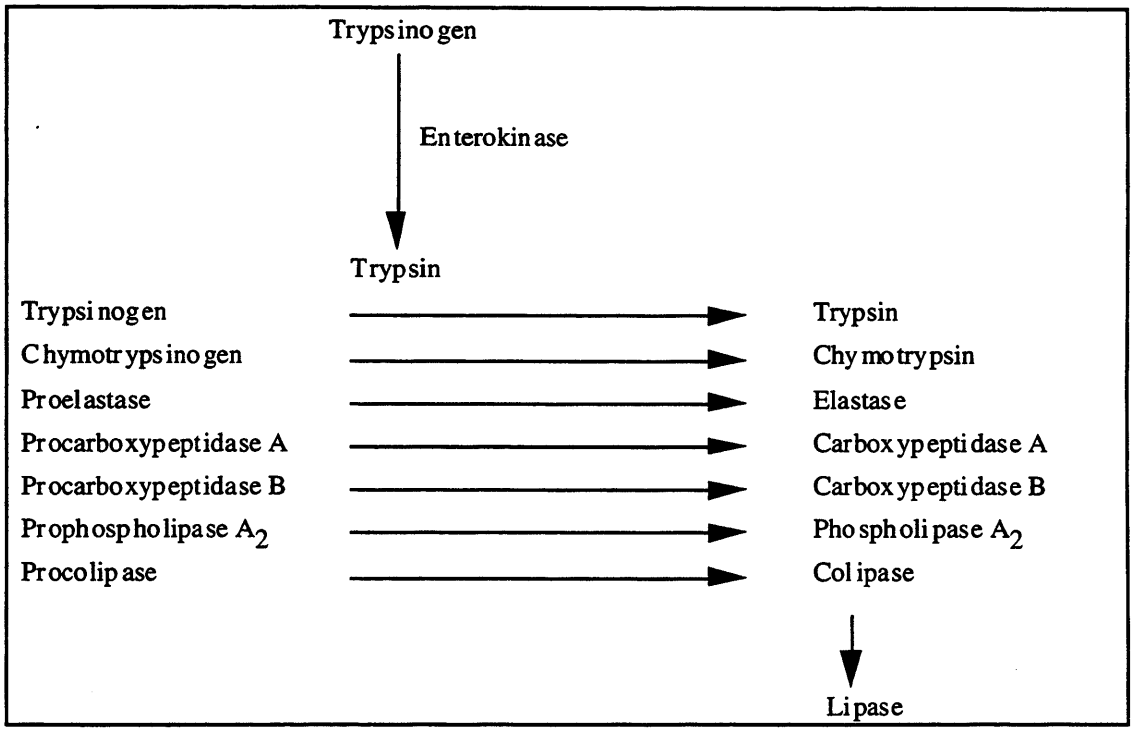


Figure 3.7 Trypsin dependent cascade

carboxyl group of aromatic amino acids phenylalanine, tyrosine and tryptophan (Rinderknecht, 1986) and shows heterogeneity with variable activity (Erlanger *et al.* 1964; Coan and Travis, 1972). Elastase activation occurs as a consequence of cleavage from its precursor, proelastase. It can hydrolyze the scleroprotein elastin and has a low specificity towards different peptide bonds, unlike trypsin and chymotrypsin. The activity is within the range pH (7.5 to 10.5) and it is generally agreed that porcine elastase is three to five times greater than that of human elastase in hydrolysis of elastin (Hartley and Shotton, 1971; Rinderknecht, 1986). Carboxypeptidases are metalloproteinases, as opposed to the serine proteases described above. They contain a zinc atom within their active site. As with the other groups of zymogens they are activated by trypsin (Rinderknecht, 1986). The enzymatic properties of carboxypeptidase A (C-A) have been extensively studied in bovine and porcine models (Peterson *et al.* 1976). This enzyme catalyses the hydrolysis of the carboxyl-terminal amino acids at an optimal pH range of 7.5-8.0 (Petra, 1970). In contrast

carboxypeptidase B (C-B) rapidly hydrolyses peptide bonds to release carboxyl-terminal arginine and lysine (Folk *et al.* 1960).

Phospholipase A2 also exists in its proform within the pancreas. Small amounts of tryspin are capable of activating this zymogen which is also calcium dependent within the pH range 6 to 8. Its activated form has the capability of attacking membrane phospholipids by hydrolysing fatty acids at the 2 position of 1,2-diacyl-sn-glycero-3-phosphocholines, releasing highly toxic lysophospholipids (e.g. lysolecithin) which can further disrupt cellular membranes by their detergent action (De Haas *et al.* 1968; Nieuwenhuizen *et al.* 1974; Rinderknecht, 1986; Mansbach, 1990). Lipase is the most important enzyme for digesting dietary fat. The activity of lipase is restricted to hydrophobic/hydrophilic interfaces and is dependent on the substrate being in a micellar form. The enzyme also acts on fatty acid esters so long as they form emulsions (DeCaro *et al.* 1977). Its enzymatic activity is dependent upon a 'trypsin activated' cofactor, colipase facilitating binding of substrate to enzyme (Larsson and Erlanson-Albertsson, 1981; Larsson and Erlanson-Albertsson, 1991). Colipase is also secreted from the pancreas and is also calcium dependent (Wieloch *et al.* 1981).

Pancreatic zymogens have the potential to be detrimental to islet cells during isolation but the precise damaging mechanism is unknown. Physiologically enterokinase is the all important activating enzyme of the cascade but since no free enterokinase can be found in freshly collected pancreatic secretions (Northrop *et al.* 1948), it is unlikely to play a major role in the activation of exocrine enzymes during islet isolation. Another hypothesis that has been suggested is that activation of pancreatic zymogens during islet isolation could be by commercial collagenolytic activity (Traverso and Abou-Zamzam, 1978). This may be important for pancreas digestion and could be a result of the endogenous autoactivation of trypsinogen through the 'trypsin like activity' of commercial collagenases (personal observation), or through direct activation by collagenase itself. Collagen types IV and V appear to be resistant to vertebrate collagenase degradation but in the presence of activated trypsinogen these types (IV and V) can be degraded, whether this is true of *Clostridia histolyticum* derived collagenase needs to be determined but this mechanism would allow for a better pancreas digestion. In support of this the pancreas contains large amounts of IV and V which is digested during islet isolation and perhaps, therefore, is attributable to endogenous trypsin action or through its trypsin like activity. Furthermore, when collagenase is used in combination with trypsin, good yields are obtained (Henriksson *et al.* 1977) but assumptions from this are limited as the essence of the role of trypsin will only be determined when a "bad" collagenase with low trypsin activity is improved by the addition of trypsin or if endogenous pancreatic trypsin is then activated.

The effects of activated pancreatic enzymes upon the collagenase phase of pancreas digestion could be through a number of different mechanisms. Several previously reported studies have discussed the detrimental effect of exocrine enzymes in relation to pancreatitis where activated enzymes have been incubated with isolated acinar cells (Letko *et al.* 1989; Niederau *et al.* 1995). Letko *et al.* (1989) demonstrated that incubation for four hours with physiological levels of trypsin was not sufficient to destroy isolated acinar cells, but merely induced blebbing. A 100 fold increase in trypsin activity was necessary to kill them. In comparison, chymotrypsin damaged cells at one tenth the activity of trypsin. More recently Niederau *et al.* (1995) applied other exocrine enzymes to assess their damaging effect. Pancreatic elastase in nanomolar concentrations caused marked acinar cell damage after 45 to 90 mintues of incubation though lipase and chymotrypsin caused similar damage at micromolar concentrations. Finally, trypsin, even at millimolar concentrations of trypsin, failed to cause significant damage. Nevertheless, even though these studies give some indication as to the potency of exocrine enzymes in relationship to acinar tissue, they demonstrate that they have the potential to be deleterious to isolated islet cells but further studies are required to confirm this.

Other components of the pancreatic extracellular matrix undergo degradation during islet isolation which are also highly variable. Elastin degradation is likely to be influenced by the activation of endogenous proelastase release. However, activation could be dependent on a number of factors; firstly, by collagenase or, secondly, by endogenous trypsin release leading to a cascade of activation of many other pancreatic zymogens. This process in turn could by affected by pH, temperature (Erlanger *et al.* 1964), pancreatic ischaemia (Warshaw and O'Hara, 1978) and the presence of cofactors e.g.calcium (Wu and Laskowski, 1956; Neurath, 1957; Rick, 1995). It is common practice to immediately reduce temperatures to less than 22°C following collection of liberated islets in order to minimise damage by proteolytic enzymes at higher temperatures (Folk and Schirmer, 1963) thus diminishing their potency. Cofactors such as calcium may also play a role in the activation of pancreatic zymogens and that its presence may enhance this activation (Wu and Laskowski, 1956; Haverback *et al.* 1960; Rick, 1995).

3.8 Pancreas procurement and preservation

Introduction

Most studies relating to porcine islet isolation utilise pancreata obtained from local abattoirs. The use of abattoir obtained pancreata poses many problems on islet

isolation because of the warm ischaemia encountered during the slaughtering process and periods of cold ischaemia endured during the transportation of pancreata to the islet laboratory. Current strategies to reduce the effects of organ ischaemia will, therefore, be reviewed in the following section.

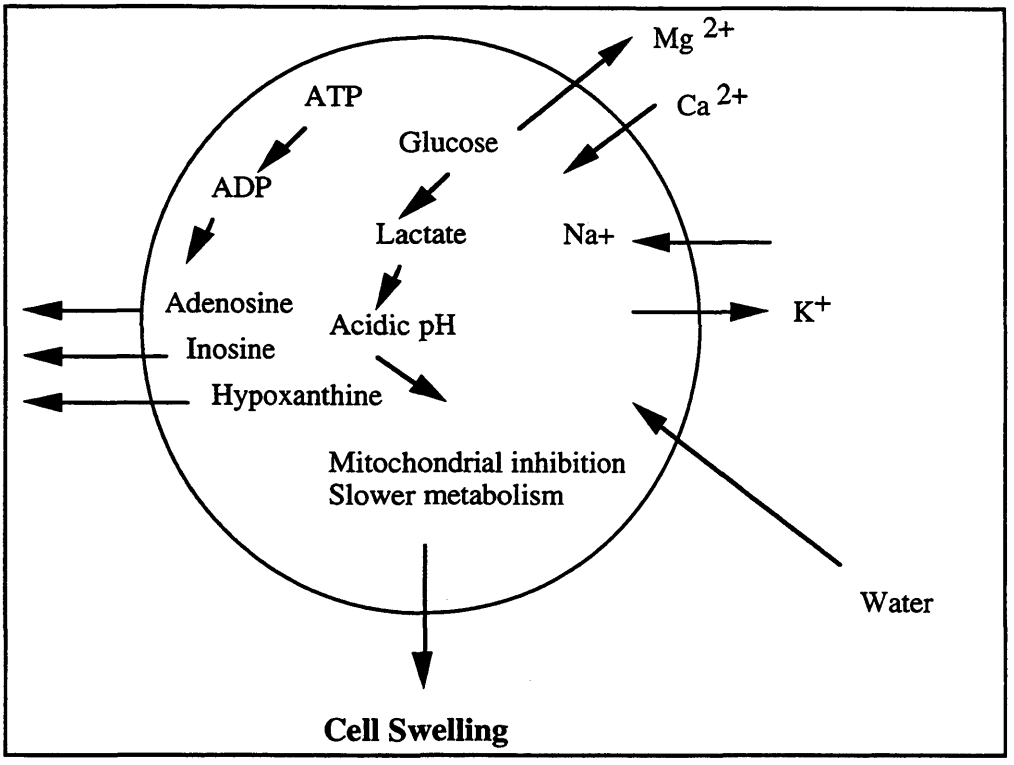


Figure 3.8 Effect of cold ischaemia on mammalian cells

3.8a The importance of organ preservation

The slaughtering process usually entails exsanguination after brain death. Before pancreas excision, a time lapse of up to 15 minutes may have occurred between cessation of cardiac function and total pancreas excision. This period of warm ischaemia is undoubtedly more damaging than cold ischaemia. During this time the metabolism of an organ becomes anaerobic with the depletion of high energy phosphate compounds (ATP). As fuel reserves are consumed, the sodium/potassium ATPase becomes non-functional (Martin *et al.* 1972), cells are then freely permeable to sodium and chloride and they consequently swell *(Figure 3.8).* As anaerobic metabolism continues, lactic acid accumulates causing an intracellular acidosis and lysosomal autolytic activation. Most organs are irreversibly damaged after 90 to 120 minutes of warm ischaemia, although the endocrine pancreas appears to be more tolerant than others (Jones and Trump, 1975), where canine islet function can be maintained after an hour of warm ischaemia (Florack *et al.* 1984). In pig (Ricordi *et al.* 1990b), human

(Brandhorst *et al.* 1994b), canine (Hayward *et al.* 1988) and rat (Ohzato *et al.* 1989) periods of warm ischaemia in excess of 30 minutes severely compromise islet yields.

3.8b Historical advances in organ preservation

The cryopreservation of simple cells or tissues to be frozen indefinitely (Polge *et al.* 1949) was discovered in 1949 but was not as successful with whole organs (Marshall *et al.* 1994). With the application of hypothermic *in situ* vascular perfusion and cold storage (Calne *et al.* 1963), the preservation of whole organs was enhanced and routinuely undertaken before transplantation.

Autolytic enzymatic cellular destruction is temperature dependent and therefore the basis of hypothermic organ preservation is to cool the organ so as to curtail the expenditure of oxygen. Cellular damage is not totally suppressed by hypothermia, merely slowed, ultimately with loss of tissue viability. Furthermore, cooling to less than 10°C arrests sodium/potassium ATPase but not diffusional transport of sodium and chloride and therefore hypothermia causes cellular swelling. The tendency for cellular swelling, which limits organ survival, is dependent upon the intracellular colloidal osmotic pressure (110 to 140 mOsm/Kg) within the cell and the difference outside the cell. The composition of preservation solutions is, therefore, of critical importance. These changes can be altered by adding impermeants to cold storage solutions that provide an osmotic force of equal standing (110 to 140mOsm/kg). Such impermeable anions used in preservation solutions are lactobionate, raffinose and sucrose or, alternatively, chelates such as citrate and magnesium (Belzer and Southard, 1988; Marshall *et al.* 1994). The use of HES has also been described (Jamieson *et al.* 1988a; Wahlberg *et al.* 1989), although controversial, it is thought to benefit by preventing collapse of the vascular network (Kneteman and Wagner, 1992) and reduce the expansion of the extracellular space (Hoffman *et al.* 1983). Finally, with the addition of impermeants such as lactobionate and citrate, hypoxic induced cellular swelling by free chloride diffusion can also be kept to a minimum (Marshall *et al.* 1994).

In order to moderate the adverse effects of acidosis incurred by tissue ischaemia an appropriate acidotic buffer becomes an essential requirement to preservation solutions. Those commonly employed in clinical practice are phosphate (Belzer and Southard, 1988), citrate or histidine (Marshall *et al.* 1994). Following reperfusion the rapid influx of oxygen induces the accumulation of toxic byproducts such as hydrogen peroxide along with unstable free, superoxide and hydroxyl radicals (McCord, 1985). These products are highly toxic and can be cleared by endogenous

scavengers but in the event of ischaemia they accumulate. Scavengers, such as allopurinol, glutathione, and more recently α–tocopherol (Ikeda *et al.* 1994) can be added to preservation solutions so as to minimise their toxic effects, although their capacity for scavenging is still challenged by some (Jamieson *et al.* 1988a; Belzer and Southard,1988; Kneteman and Wagner, 1992).

In conjunction with the pioneering work by Collins (Collins *et al.* 1971), establishing the composition of early organ preservation solutions, hypothermic organ preservation has become common practice throughout transplant centres across the world. It has now been shown, beyond reasonable doubt, that hypothermic preservation with an electrolyte based solution allows kidney storage for greater than 24 hours (Collins *et al.* 1969). These solutions are *intracellular* based, being high in potassium, magnesium and phosphate and low in sodium and chloride content *(vide infra)*. In comparison, solutions with an *extracellular* composition such as HBSS, saline or plasma are now known to be unsuitable for more than four hours cold storage (Jindal and Gray, 1994). This subsequent realisation has had a major impact on clinical practice as organ allotransplants can now be carried out electively as opposed to semi-emergency, allowing more time for accurate tissue matching and providing an opportunity for distant transportation of organs to other potential recipients.

Despite the success of cold storage solutions for kidney; pancreas, heart and liver could only be maintained for six to ten hours. These disappointing findings provided the prime impetus for a renaissance in organ preservation during the early seventies. Published modifications of Collins original work were reported by Ross *et al.* (1976), who initiated the use of hypertonic solutions with citrate and mannitol. Encouraged by its superior role as compared to Euro-Collins in protecting kidney (Lam *et al.* 1988), pancreas (Nolan *et al.* 1983), and liver (Tamaki *et al.* 1986) organ function, it has become the most commonly employed vascular perfusate in the United Kingdom (Lam *et al.* 1988), but worldwide it is now being superseded by more complex solutions (e.g. UW, *vide infra*).

Belzer *et al.* (1988) were the first to utilise more complex components by developing the University of Wisconsin solution (UW). The composition of UW was based upon reversing, and reducing the effects of hypothermia upon cellular swelling, with excellent results for vascularised pancreas for up to 72 hours (Wahlberg *et al.* 1987; D'Alessandro *et al.* 1989b), up to 48 hours for liver (Jamieson *et al.* 1988b) and 72 hours for the kidney (Ploeg, 1990). With these studies in mind, it must be stressed that the successful outcome of preservation for vascularised pancreas may not necessarily correlate with successful islet isolation as enzymatic dispersion in the

presence of a prolonged cold ischaemic time leads to poor islet cleavage and islet fragmentation; hence, cold storage of the pancreas for 12 hours has been shown to reduce islet yields in humans (Kneteman and Wagner, 1992; Lakey *et al.* 1995), pigs (Mellert *et al.* 1991), dogs (Hesse *et al.* 1987a; Munn *et al.* 1989b), and rodents (Munn *et al.* 1989a and 1989b). Despite UW solution having precedence over Euro-Collins (Kneteman *et al.* 1990b) for whole pancreas cold storage before islet isolation, the use of UW for vascular perfusion in preference to citrate based solutions confers no additional benefit when the pancreas is stored for less than six hours (Robertson *et al.* 1993a).

3.8c Porcine pancreas preservation

Some of these different approaches have been applied to porcine islet isolation. Previous studies have attempted to overcome the deleterious effects of warm ischaemia upon porcine islet isolation by procuring the pancreas from a heart beating donor (Ricordi *et al.* 1990b) producing islets yields of 9964 islets/g. This very effective technique is, however, restricted by the availability of a fully equipped operating theatre for anaesthetising such large animals and is not applicable to the majority who retrieve porcine pancreata from local abattoirs.

Other techniques such as cold intravascular perfusion prior to organ excision has proven to be valuable to human islet isolation (Robertson *et al.* 1993a) but when applied to porcine models, it has been shown to culminate in very poor islets yields (148 islets/g; Ricordi *et al.* 1990b). With these studies in mind, others (Ohzato *et al.* 1991) have avoided cold intravascular perfusion altogether by preferring to immediately distend the pancreas with HBSS after excision. This technique in rats has the advantage of maintaining pancreatic ductal integrity allowing a better distribution of collagenase. Following excision is was shown that fresh pancreata were resistant to intraductal pressures up to 500mmHg, as opposed to significantly lower pressures in preserved pancreata where collagenase entered the islets. Histological examination of preserved pancreata after early injection has shown a poorer distribution of collagenase in the peripheral ducts indicative of higher intraductal pressures precluding to an inefficient digestion and islet disruption.

Early pancreas distension has also been performed prior to porcine islet isolation. A prospective study by de Nittis *et al.* (1995) suggested that after early intraductal collagenase injection in HBSS, before two hours cold organ storage porcine islet yields were significantly improved when compared to those injected after cold organ storage (3325 IEQ/g versus 1178 IEQ/g; Socci *et al.* 1995). However, although

good results were obtained using this method it is unlikely to benefit if the pancreas is to be subjected to longer periods (greater than six hours) of cold organ storage because of the adverse effects of the constituents of HBSS. It is a simple saline solution developed in 1948 (Hanks and Wallace, 1949) for the refridgeration of cells and does not contain any cellular impermeants and would, therefore, predispose islets to hypothermic induced cellular swelling. A better approach would be to distend the pancreas with a cold organ storage solution (e.g.UW). This has been successfully attempted in rats and dogs (Munn *et al.* 1989b; Field *et al.* 1989) but other reports suggest limitations in humans (Altman *et al.*1982; Casanova *et al.* 1994) because of collagenase inhibition (Burgmann *et al.* 1992; Robertson *et al.* 1993a; Contractor *et al.*1995a).

In summary, regardless of the preservation solution used, both warm ischaemia and cold storage compromises islet isolation, and it is doubtful that both can be completely avoided during porcine pancreas retrieval. In practice the best results are achieved by minimising warm ischaemia and improving cold storage by distending the pancreas immediately after excision utilising an appropriate cold storage solution. The *ideal* cold preservation solution has yet to be developed but it would undoubtedly be similar to UW, perhaps without the toxic levels of potassium at 37^{o}C, absence of calcium (Dono *et al.* 1992), allopurinol (Kneteman and Wagner, 1992), glutathione (Soru and Zaharia, 1972) and the inhibitory action of HES (Contractor *et al.* 1995a; Burgmann *et al.* 1992), all documented to be deleterious to collagenase digestion.

CHAPTER FOUR

A STUDY OF THE SIZE, DISTRIBUTION AND CSSA DENSITY OF PORCINE ISLETS OF LANGERHANS USING COMPUTERIZED IMAGE ANALYSIS

4.1 Introduction and study aim

Islet isolation greatly depends on the volume density of islets within the pancreas prior to pancreatic dispersion. To date only one study has addressed this issue from the perspective of porcine islet isolation (Ulrichs *et al.* 1995). The assessment of islet size and volume density presents many problems. Islets of Langerhans are regarded as spherical or ellipsoidal organelles scattered randomly within a three dimensional space. The precise quantification of these organelles is impossible using conventional microscopic analysis from histological slides (Lazarus and Volk, 1962), either by simple counting or by applying more complex methods such as point counting (Weibel, 1979). By using a histological tissue section one is presented with an essentially flat profile from which one erroneously interprets three dimensional volume. Previous publications have expressed their results as mean values in terms of the mean diameter of an islet, islet number per cm^2 and percentage islet volume density. However, by using conventional light microscope histological slides, the latter measurement is actually representative of the percentage cross sectional surface area (CSSA) density of islets given that volume density = area density (Weibel, 1973).

By using computerised image analysis, it is now perhaps possible to give a more accurate representation of the total percentage of islet CSSA relative to exocrine tissue within porcine pancreata, contrary to that of islet diameter, which is still rather subjective due to the irregularity in islet margins. The aim of this study was to firstly determine the mean diameter of an islet and the mean CSSA of porcine islets (μm^2) and their frequency distribution in the whole pancreas. An evaluation of these measurements was performed in both juvenile and adults pigs. Currently, there is no universal definition as to what constitutes an adult or juvenile pig. Most studies report adult islet isolation in pigs greater than 12 months (Finke *et al.* 1991; Ricordi *et al.* 1989) and juveniles less than 12 months (Socci *et al.* 1990); therefore for the purpose of this study these two groups were investigated. The differences in CSSA of islets within these two groups were calculated and compared. Following this initial study the total percentage CSSA relative to exocrine tissue (=percentage volume density) was also calculated and the following comparisons made; firstly, a comparison between 5 adults and 5 juveniles, secondly; a comparison within 5 adults and within 5 juveniles and

thirdly; a comparison between the different regions of 5 adult and 5 juvenile porcine pancreata.

4.2 Materials and methods

Animals. Five adult and five juvenile porcine pancreata were excised from Large White sows obtained from a local abattoir (Dawkins International, Congerstone, UK). All pancreatic lobes were resected from the surrounding tissues *(Figure 4.1).* (Pigs were classified as adult if greater than 12 months of age or juvenile if less than 12 months because of random mixing of different herds of approximately the same age during the slaughtering process *see also p.152*).

Pancreatic sampling. The pancreas was cleaned of surrounding connective tissue and then weighed (Ohaus Scale Corporation, USA). Samples (approximately $1cm^3$ in size) were taken from 5 different areas of each pancreas (Area 1 = tail of splenic lobe, Area 2 =body of splenic lobe, Area 3 =head of splenic lobe, Area 4 = posterior lobe and Area 5 = duodenal lobe) and immediately fixed in formalin *(Figure 4.1).*

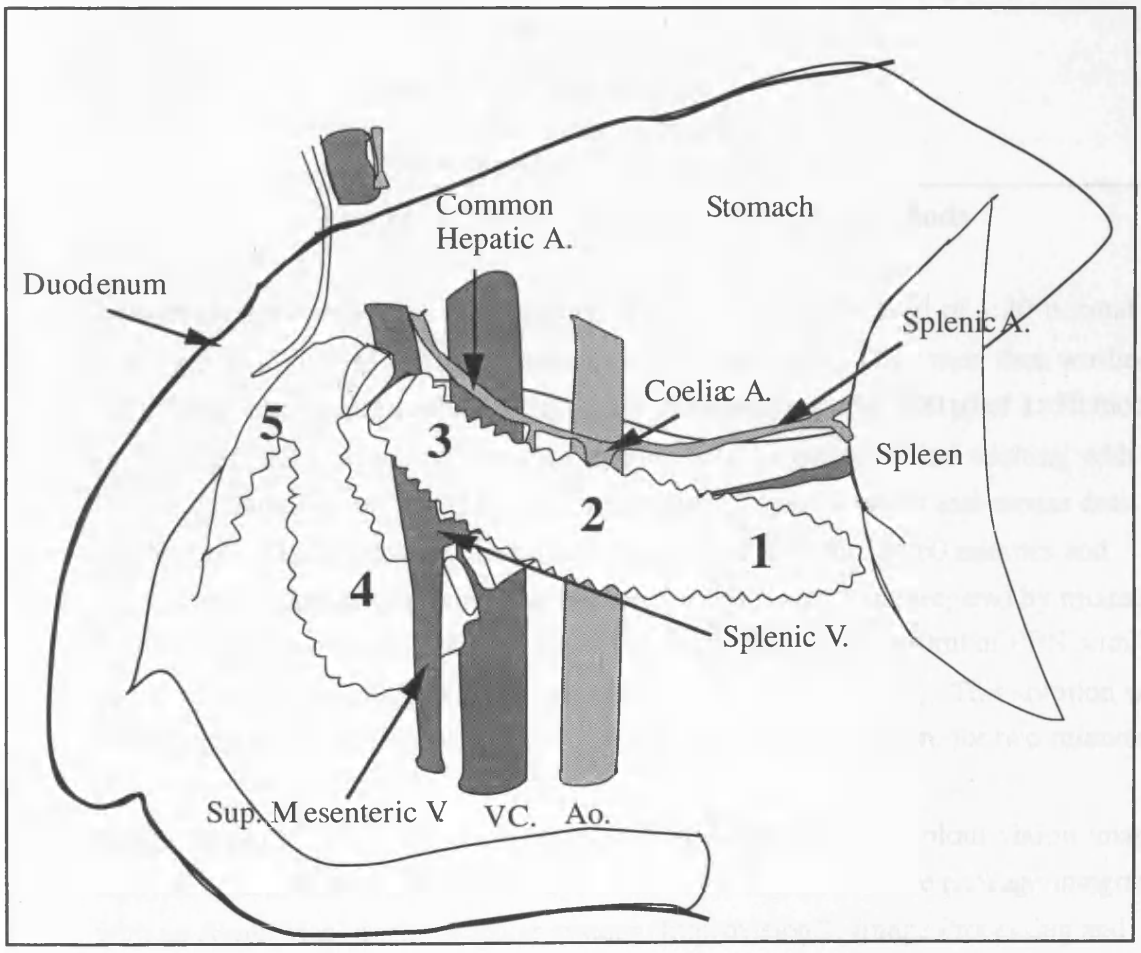


Figure 4.1 Porcine pancreas and areas of tissue sampling (1 to 5)

Slide preparation. Each biopsy was embedded (Shandon Hypercenter XP, Life Sciences International, UK) in parrafin wax (Tissue-Tek III, Sakura, Fine Technical Co Ltd, Japan) and using a microtome (Biocut, Reichert-Jung, Germany) 4 μm sections were cut at random from each of the individual blocks. Sections from three different depths were prepared from each of the 1cm^3 biopsies and mounted on a silane coated slide and dried overnight. The slides were then put into 6% hydrogen peroxide and washed.

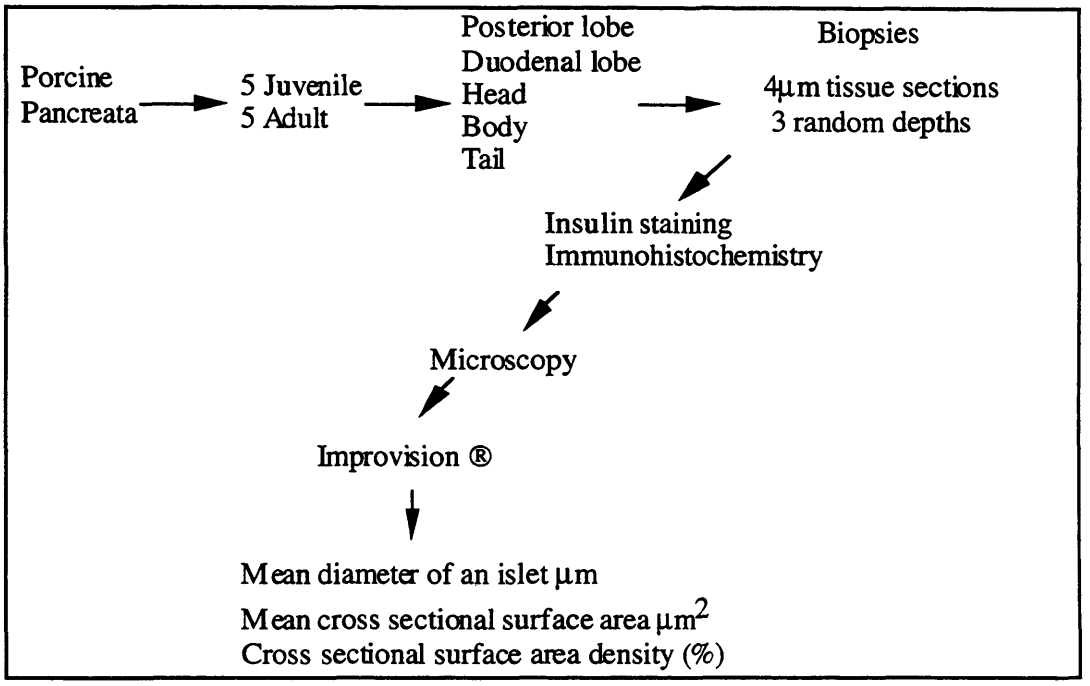


Figure 4.2 Flow diagram of study methods

Immunohistochemistry. Each section was incubated in 100 μl of 1:20 normal rabbit serum (Dako, Glostrup, Denmark) at 4°C overnight. They were then washed with phosphate buffered saline (PBS), prior to overlaying with 100 μl of 1: 50 mouse anti-insulin (HB 124) left at room temperature for 30 minutes. After washing with PBS, a second layer of 1: 400 biotinylated F (ab)2 fragment rabbit anti-mouse anti-insulin Ig G (Dako, Glostrup, Denmark) was added for a further 60 minutes and incubated at room temperature. The immunoperoxide stain was prepared by mixing 5mg of 3,4,3,4 tetra-aminobiphenyl hydrochloride dissolved in 10ml of PBS with 70μl of 3% hydrogen peroxide (Vector Laboratories, California, USA). This solution was filtered and 100μl was added to the surface of the pancreas section for two minutes.

Image analysis. This was undertaken with an Improvision®, colour vision image analysis system that utilised a rasterops PAL video digital software package integrated with an Apple Macintosh computer system (Improvision®, Image Processing and Vision Company Ltd, Coventry, UK; *Figure 4.3 see p.143*). Slides were placed on

the stage of a microscope (Nikon, Diaphot, Japan), the video image was captured and the total visual portion (maximum source rectangle) was calibrated with a stage micrometer to correspond to the number of pixels seen on the image analyser. The periphery of each islet was marked *(Figure 4.4 see p.143)* allowing the number of pixels to be calculated and converted to the islet area in μm^2 .

Within each area (1-5) slides were prepared from 3 different levels and individually assessed. From each level a single high power field (1,466,699 μm^2) was blindly examined by 2 observers. This made a total of 6 high power fields per area and 30 high power fields per pancreas. The number of islets in each high power field varied from 1 to 12. The measurements that were made were the diameter of an islet (μm) and the CSSA of an islet (μm2). The mean diameter of an islet was firstly estimated by recording the mean diameter (≥ 12μm) from the longest and shortest breadth from consecutive islets from each high power field and secondly, by extrapolating the diameter from the mean the mean cut surface area of an islet by using the equation πr^2 (assuming islets are circular) e.g. $\pi(d/2)^2$). The frequency distribution of diameters and CSSA of all islets were plotted and compared between juveniles and adults. Furthemore, the CSSA area of an islet was also compared within adults, within juveniles and the difference between the 2 groups plotted graphically. The percentage islet CSSA density was then estimated by calculating the percentage of total insulin containing islet tissue (from islets with a CSSA ≥ 120 μm^2) relative to exocrine tissue in each maximum source rectangle with a known area of 1,466,699 μm^2. Three comparisons of the total percentage CSSA of islet tissue were made; firstly, a comparison between 5 adults and 5 juveniles, secondly; a comparison within 5 adults and within 5 juveniles and thirdly; a comparison between the different regions of 5 adult and 5 juvenile porcine pancreata .

Statistical analysis. The results are expressed as mean values as reported in other studies (Saito *et al.* 1978; Ulrichs *et al.* 1995) and 95% confidence intervals. Statistical comparison between each pancreas was initially made using the Kruskall Wallis one-way analysis of variance, reported as a P value. Where appropriate differences between pancreata from both juvenile and adult groups were assessed with the Mann Whitney U test, reported as a P value.

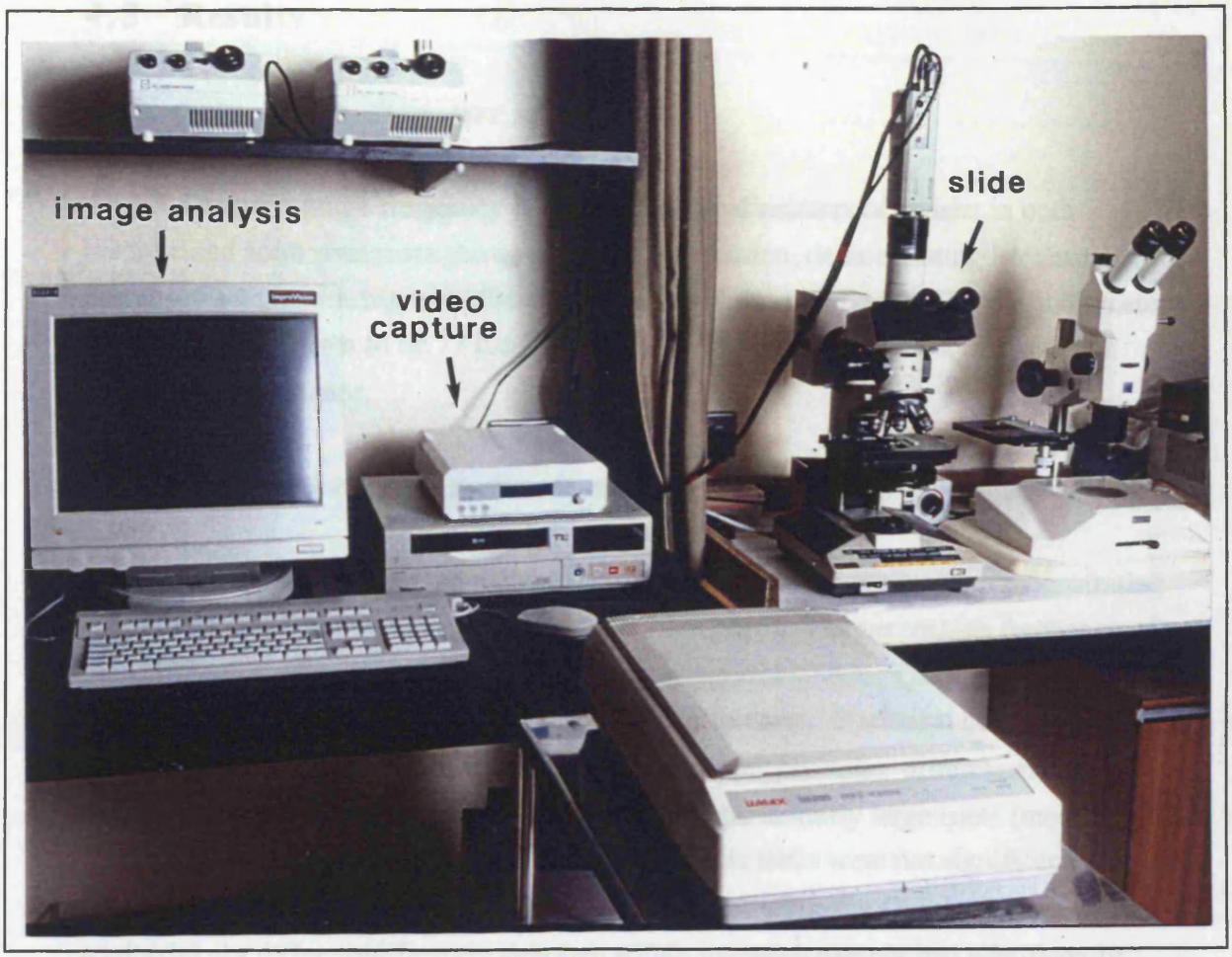


Figure 4.3 Improvision® image analysis system

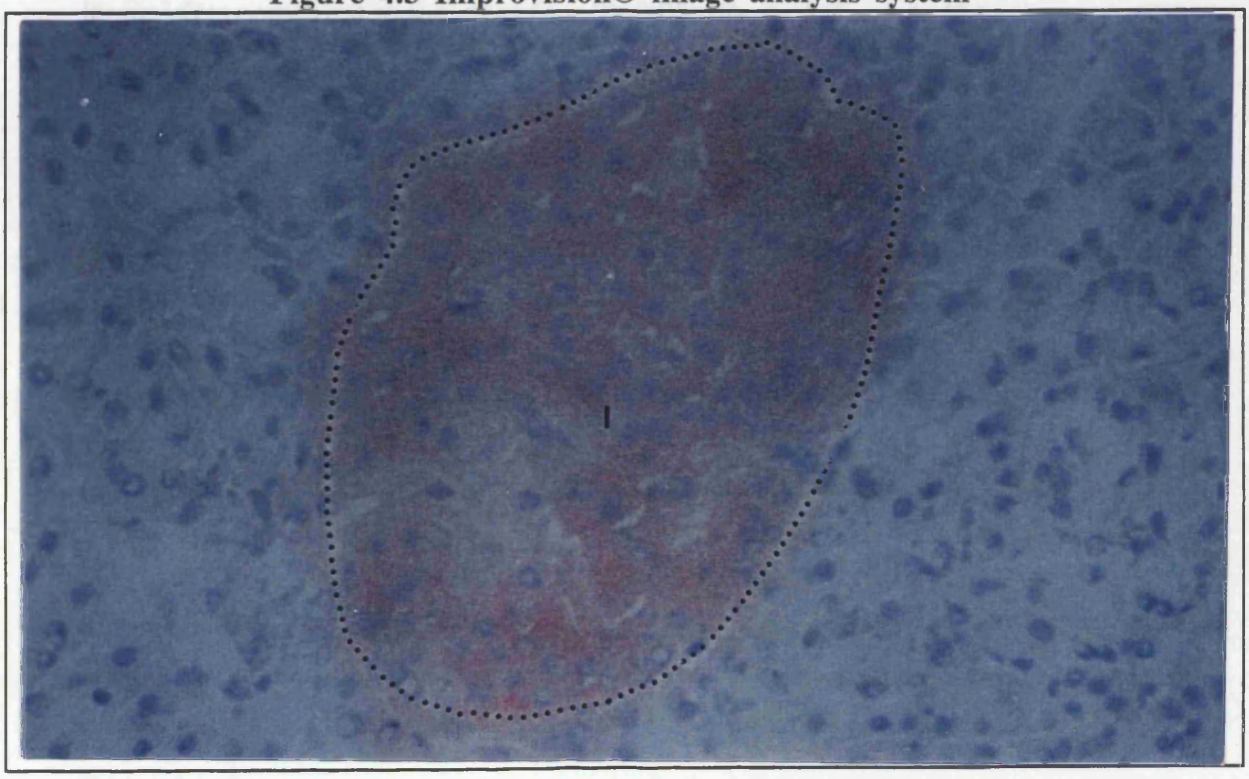


Figure 4.4 Image analysis demarcation of a porcine islet (I) anti-insulin stain

4.3 Results

Differences in the diameters of an islet

The percentage frequency distribution of the diameters of an islet in both juvenile and adult pancreata shows a skewed distribution, demonstrating islet number decreases with increasing islet diameter *(Figure 4.5 see over page)*. The mean diameter of an islet was shown to be 77 μm for adults and 86 μm for juveniles, this was not statistically significant.

Differences in the cross sectional area of an islet

The CSSA of islets were then determined and plotted graphically demonstrating a similar skewed distribution *(Figure 4.6 see over page)*. The percentage frequency of the CSSA of an islet also decreased with increasing islet CSSA. The mean CSSA for adults was 5.04 $x10^3$ μm^2 and 5.99 $x10^3$ μm^2 for females. Statistical comparison was made between the two groups as follows; *adults versus juveniles* differences did exist between adults and juveniles. One juvenile pig had particularly large islets (mean 8.91 x 10^3 μm^2 compared to the other nine although juvenile islets were not significantly larger than adults (P=0.4), *differences within adults and juveniles*; the CSSA of islets within adults did not differ significantly although within juveniles one pig had significantly larger islets compared to the other four (P<0.05; *Figure 4.7 see p.146*).

Finally, by utilising these cross sectional surface areas one can more accurately estimate the mean diameter of an islet by extrapolation from πr^2; for the adult porcine pancreata used in this study the adjusted mean diameter of an islet was 80μm as opposed to 87 μm in juveniles, similar to those calculated by conventional techniques (77 and 86 μm respectively).

Differences in the percentage islet CSSA density

Statistical comparison was made *within adults versus juveniles* where from *Figure 4.8 (see p. 147)* it can be seen that the percentage CSSA density within adults did significantly differ (mean P=0.001). In comparison a statistically significant difference was not demonstrable within the 5 juvenile pancreata (P=0.055). *Difference within areas*; the percentage CSSA densities were not significantly different between the 5 areas of pancreas when adults were compared to juveniles *(Figure 4.9 see p.148).*

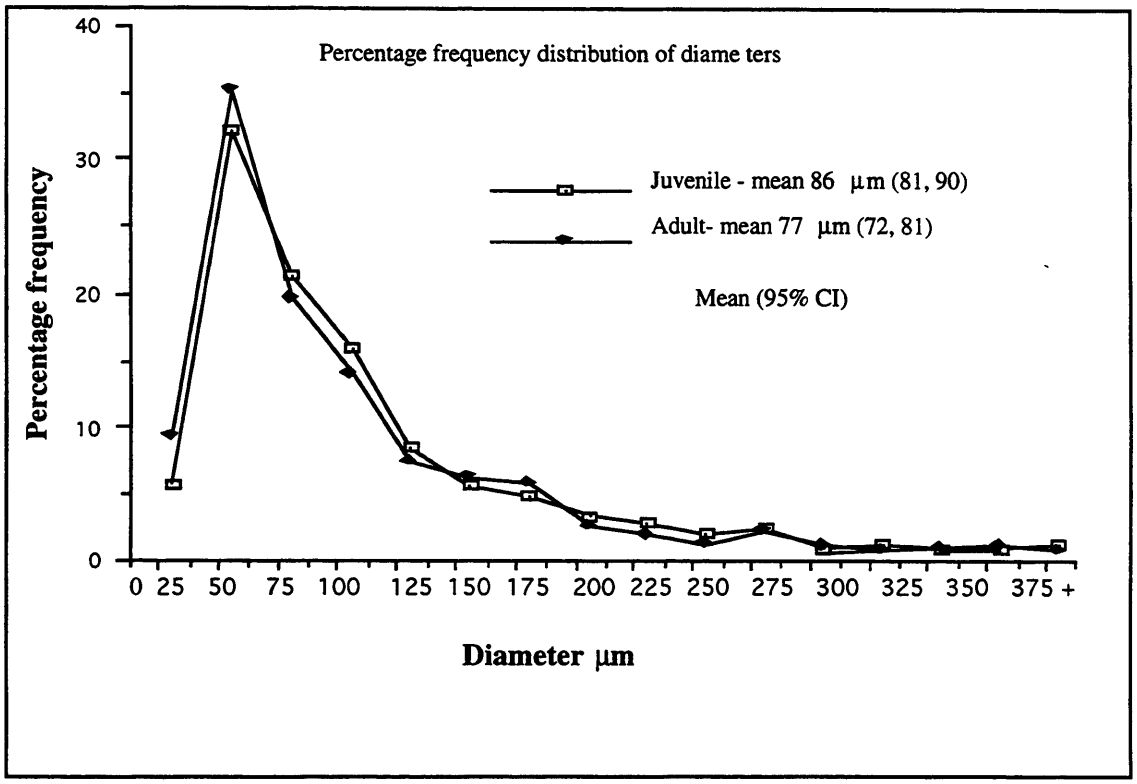


Figure 4.5. Percentage frequency distribution of islet diameters

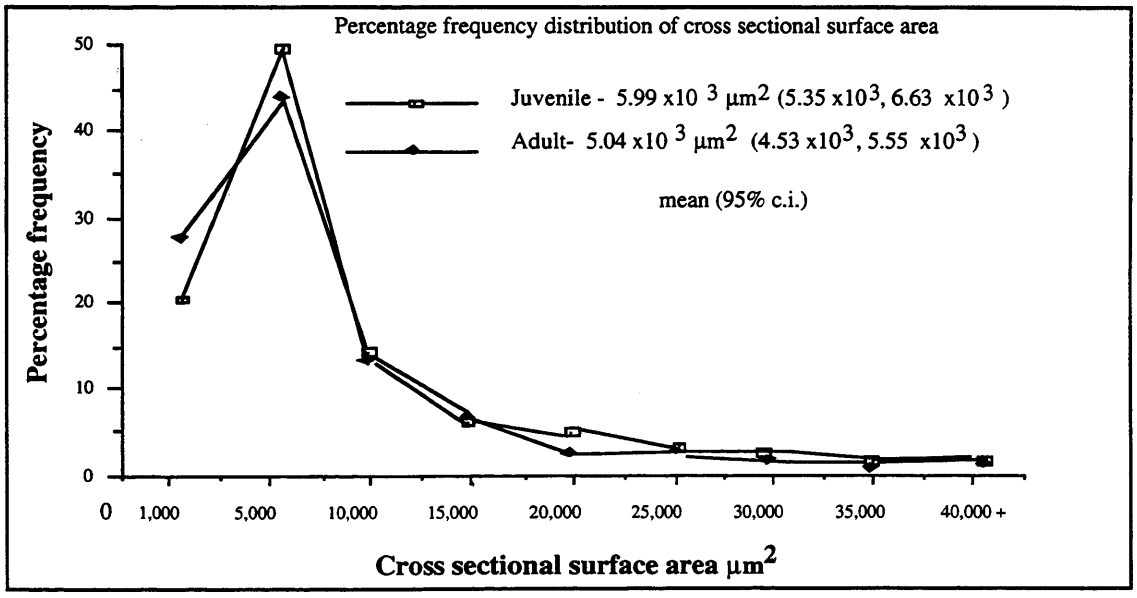


Figure 4.6. Percentage frequency distribution of islet CSSA

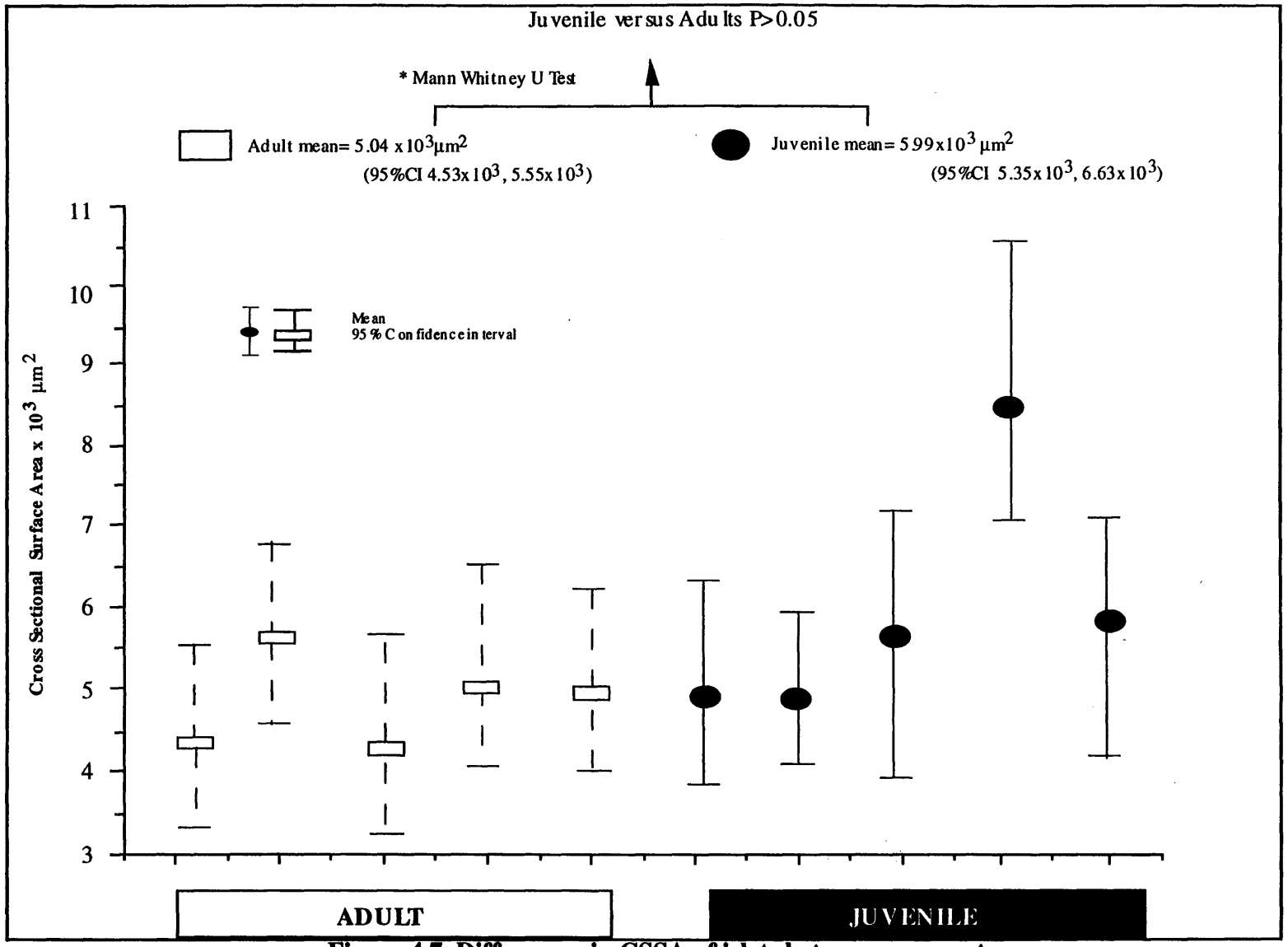


Figure 4.7. Differences in CSSA of islets between pancreata

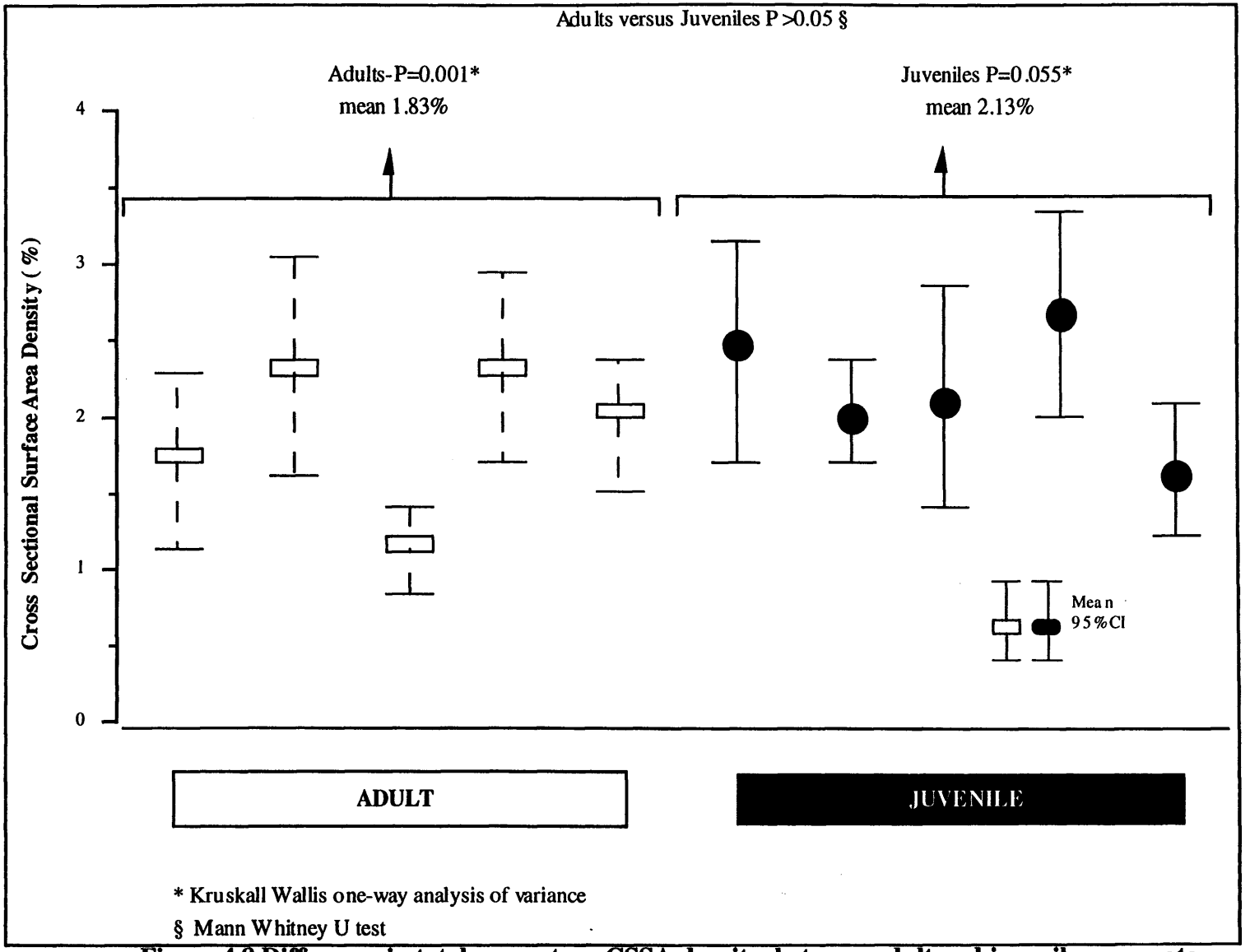


Figure 4.8 Difference in total percentage CSSA density between adult and juvenile pancreata

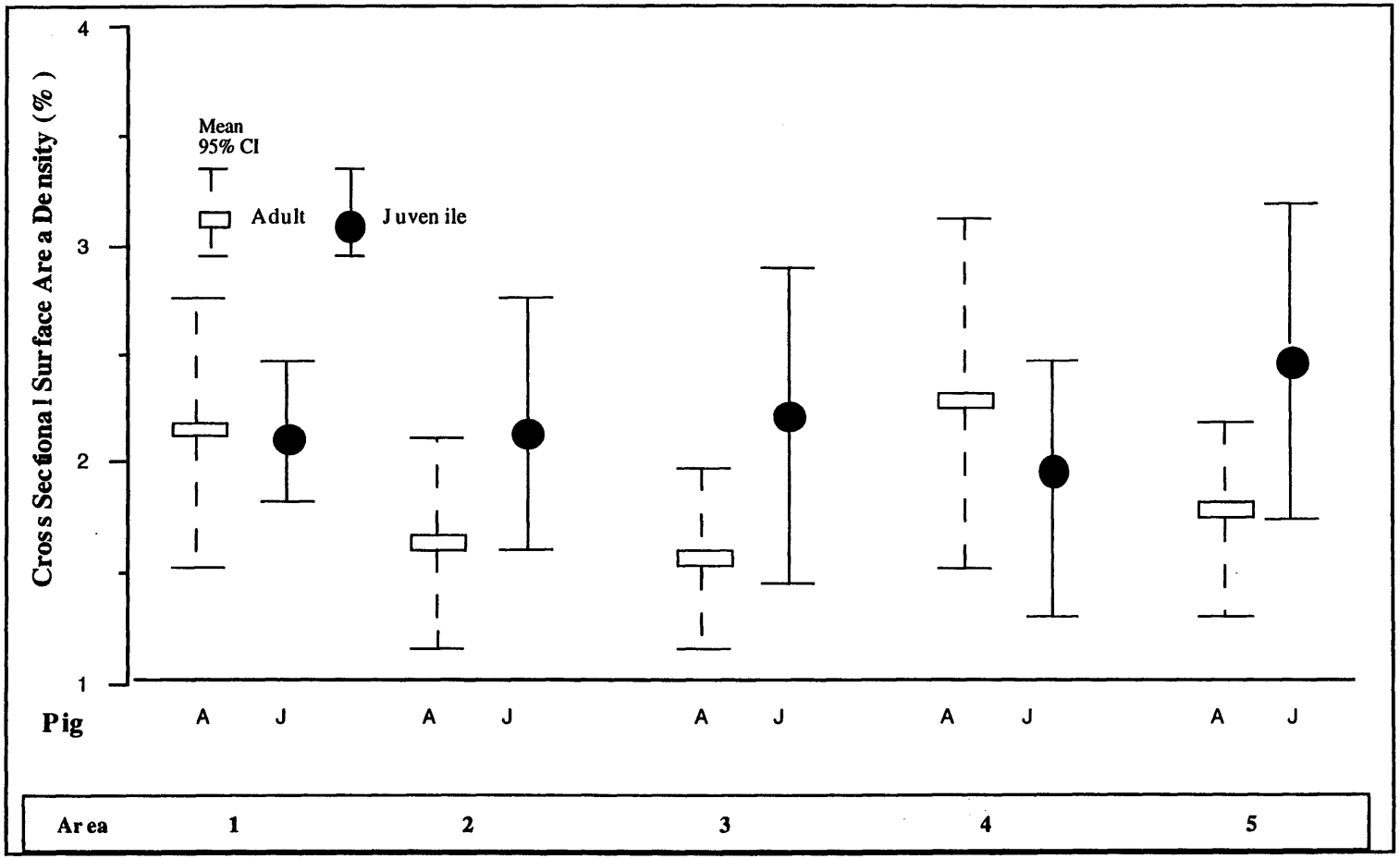


Figure 4.9 **Difference in total percentage CSSA density of islets in different areas of the pancreas in adults (A) and juveniles (J).**

There were also no differences in CSSA density progressing from the tail to the head of the pancreas in adults, similar results were also seen in juveniles. Difference between *adults and juveniles*; overall, the percentage CSSA densities were greater in juvenile pancreata (mean 2.13%) compared to adult pancreata (mean 2.13%) but were not a statistically significant finding (P>0.05; *Figure 4.8 see p.147).*

Discussion

The results in this study contradict the findings of others where juvenile islets were generally smaller than those of adults (Ulrichs *et al.* 1995; Saladino and Getty, 1972). It has been proposed that variables such as age (Saladino and Getty, 1972; Hellman, 1959a) and strain (Ulrichs *et al.* 1995), may influence islet size and number. The ontogeny of porcine islets is little understood (Alumets *et al.* 1983), though it has been shown that at 13 weeks gestation porcine insulin staining of beta cells, is intensely immunoreactive and at this stage of development beta cells are randomly distributed in the growing exocrine parenchyma. At 10 to 13 days after birth, the cells then cluster together representing the formation of small islets of Langerhans. Similarly, glucagon staining alpha cells, are also thought to reach a constant frequency at 13 weeks (Alumets *et al.* 1983), suggesting that by the age of 12 months porcine islets are probably of adult size thus partly explaining the similarities in diameter (80μm adults *versus* 87μm for juveniles). It is also apparent from this study that the use of conventional microscopic techniques in assessing islet diameters gives a good estimate of the true islet diameter. For example, by extrapolating the diameter from the true CSSA (assuming islets are circular) measured by computer image analysis, it was shown that the islet diameters (in both adults and juveniles) were similar when compared to the mean of the largest and smallest islet diameter (Adult-77 μm *versus* 80μm and Juvenile 86 μm *versus* 87μm).

Marchetti *et al*, (1990) and Ulrichs *et al.* (1995), have reported that islet density increases as one progresses from the head to the tail of the pancreas in adult pancreata but were based on relative numbers rather than specific islet volume densities calculated from cross sectional surface area. Significant differences could not be demonstrated within the five different areas of pancreas studied, in either adults or juveniles and this study may, therefore, perhaps represent a more accurate picture of the distribution of the percentage CSSA density within porcine pancreata. More importantly, it was shown that the percentage CSSA density within the duodenal lobe, posterior lobe (range of means 1.49% to 2.27%) and head of the splenic lobe were comparable to those of the body and tail of the splenic lobe (range of means 1.59% to 2.11%). Ulrichs *et al.* also reported similar findings in terms of relative islet numbers and estimated that

up to 50% of the total number of islets were distributed within the duodenal lobes and head portion and that the tail and body made up the other 50% (Ulrichs *et al.* 1995). This suggests that islet yields could be doubled by using the whole porcine pancreas for islet isolation as opposed to just the splenic lobe as is the routine method in most laboratories (Toomey *et al.* 1993; Chapter Six), although yields would also depend on the size of the different lobes.

The mean percentage CSSA density of adult Large White pancreata, in the present study, was 1.83% comparable to other strains reported by others (Ulrichs *et al.* 1995) such as Wild Boar (1.27%), German Landrace (3.38%), Pietrain (1.97%), Belgian Landrace (1.47%) and Duroc (1.95%) that were determined by conventional microscopic techniques. However, a previously unreported finding from the present study were the significant differences (P=0.001) between the mean total percentage CSSA densities between pancreata of adult pigs of approximately the same age and strain but not between juveniles (P=0.055), and one could assume that this would perhaps have a profound influence upon islet isolation and could thus partly explain some of the variability seen between islet yields. In support of this similar significant differences in the insulin content of pancreata have been reported in both human (London *et al.* 1990) and dog (van der Burgh *et al.* 1994) by using insulin extraction. Furthermore, the percentage CSSA density of islets showed an increasing trend in juvenile pigs (2.13%) compared to that of adults (1.83%) and this may be partly due to the slightly larger CSSA (μm^2) of islets seen in juveniles compared to adults (5.99×10^3 *versus* 5.04×10^3 μm^2). Another explanation for the greater CSSA density seen in juvenile pigs could be because of the greater potential for growth of pancreatic acinar tissue in relation to islet tissue (Ulrichs *et al.* 1995). For example, adults have a relatively greater pancreatic mass compared to juveniles by a factor of 1.67, although there is a relatively greater mass of islets in juveniles compared to adults by a factor of 1.57 (Ulrichs *et al.* 1995).

The diversities between pancreata demonstrated in this chapter raise two important issues; firstly, it confirms that poor islet yields may be attributable to a low density of native islets within pancreata prior to its digestion and, secondly, it can now be debated whether current methods for comparing islet yields between different centres is adequate. For instance, the international islet equivalent is described as 150μm (Ricordi, 1991). This arbitrary derived figure is not a suitable predictor of the degree of islet fragmentation during automated porcine pancreas digestion i.e. 'the isolation index' (Prevost *et al.* 1995; Basta *et al.* 1995). This is calculated by dividing the mean volume of an islet from each isolation by the volume of the international islet equivalent assumed to be 150μm in diameter; hence, for porcine pancreata yielding isolated islets

with a mean diameter of 150 μm, the isolation index is one, representative of no fragmentation or dispersion into single beta cells. However, the international islet equivalent has yet to be substantiated by a reliable quantitative scientific method and in the likely situation where the mean diameter of an islet within the pancreas prior to islet isolation is less than 150μm, as is likely for porcine, previous reports of the isolation index following porcine islet isolation are anecdotal (Prevost *et al.* 1995; Basta *et al.* 1995) and the degree of fragmentation speculative. It is of vital importance to express islet yields based on the mean diameter of an islet in each species prior to islet isolation, especially in the situation of porcine islets, which are known to be increasingly susceptible to fragmentation (Ricordi*et al.* 1990c) and have a mean diameter of less than 150μm. With these problems in mind van der Burg *et al.* (1997) has recently suggested that for porcine islet yields those islets less than or equal to 25μm need to be counted, as opposed to only those greater than 50μm, as islet yields will be falsely elevated.

I conclude that some of the variability in porcine islet yields can be partly explained by the significant differences in the percentage islet CSSA density between pancreata prior to porcine islet isolation but this observation alone does not explain the 15 fold differences in porcine islet yields (Chapter Six); therefore the ability to isolate good yields is multifactorial. Furthermore, I suggest the current methods of assessing porcine islet isolation needs to be redefined given that the mean diameter of an adult porcine islet is 80μm.

CHAPTER FIVE

AN INVESTIGATION INTO THE DISTRIBUTION OF DIFFERENT COLLAGEN TYPES WITHIN ADULT AND JUVENILE PORCINE PANCREATA.

5.1 Introduction

Although pancreas digestion using collagenase has become the "gold standard" for islet isolation, it is extremely unpredictable (Johnson *et al.* 1996b). It is well recognized that islet isolation varies between species and it is generally agreed that porcine islet isolation is more inconsistent (Chapter Six), whilst rodent islet isolation is somewhat more predictable (Lake *et al.* 1987). It has previously been suggested in both porcine and rodent pancreata (van Deijnen *et al.* 1992; van Suylichem *et al.* 1995b) that this may be related to both quantitative and qualitative differences in their collagen content and not just to the variability of collagenase. The aim of the present study was to determine the distribution of different collagen types within adult and juvenile porcine pancreata.

5.2 Materials and methods

Animals. Five adult (greater than 12 months) and 5 juvenile (less than 12 months)* Large White porcine pancreata were assessed (Dawkins International, Congerstone, UK). The entire pancreas was resected at the abattoir.

Pancreatic sampling. The pancreas was transported to the islet laboratory in HOC (Travenol, Thetford, UK) at 4°C (60 minutes cold ischaemia) and cleaned of surrounding connective tissue. Biopsies were taken from 5 different areas; area 1= tail of splenic lobe, area 2= body of splenic lobe, area 3=head of splenic lobe, area 4 = posterior lobe and area 5= duodenal lobe (see *Figure 4.1* Chapter Four), each biopsy was then fixed in formalin as previously described in Chapter Four.

Slide preparation and immunohistochemistry. Each biopsy was embedded and tissue sections cut with a microtome (4μm). They were incubated for 45 minutes in pepsin (Sigma Diagnostics, UK; 0.025% in HCL) at 37°C followed by incubation in 6% hydrogen peroxide for 10 minutes. Having washed the section, rabbit serum (1: 20) was overlaid for 10 minutes (Dako, Glostrup, Denmark). The primary collagen monoclonal antibodies were then added. The collagen monoclonal antibodies were

(*see p.140)

against collagen types I, V, VI (Southern Biotechnology Associates Inc, Birmingham, USA), and type IV, (Dakopatts, Denmark). The sections were washed with PBS for 20 minutes and then overlaid with a peroxidase conjugated rabbit anti-goat antibody, or in the case of collagen type IV, a biotinylated rabbit anti-mouse antibody for 30 minutes at room temperature (1: 25) (Dako, Glostrup, Denmark). Those slides originally overlaid with collagen type IV were then washed once again with PBS and then incubated with a Vectastain ® *Elite* ABC (Vector Lab, California, USA) immunoperoxidase staining protocol. All sections were then dehydrated, cleaned and mounted. Other tissue sections that were simultaneously prepared from adjacent tissue sections were controls without the collagen monoclonal antibodies *(Figure 5.1)* Additional sections were stained alternatively with anti-insulin to allow identification of insulin containing islet of Langerhans (as described in Chapter Four)

Evaluation of collagen distribution. Each slide was randomized for assessment by two observers. The definition of collagen distribution was assessed by a modification of that previously used by Van Suylichem *et al.* (1995b; *Table 5.1).* Each slide was analysed by light microscopy (Nikon, Diaphot, Japan) at x 40 magnification. Islets were identified by tissue sections stained for insulin adjacent to the corresponding collagen tissue section. The collagen content was assessed within the interlobar pancreatic septa *(Figure 5.2 p.155),* within the pancreatic lobules (intralobular; *Figure 5.3 and Figure 5.4 p.156*) collagen surrounding the islet (peri-insular; *Figure 5.5 p.157*) and also the collagen within the islet (intraislet capillary tissue; *Figure 5.6 p.157*).

Collagen Distribution				
Score	**Interlobar**	**Intralobular (acinar)**	**Peri-insular**	**Intraislet**
Absent	not identified	not identified	not identified	not identified
One	+	+	<50% circumference	<50% total area
Two	++	++	> 50% circumference	>50% -90% total area
Three	+++	+++	totally surrounding	>90% total area

Table 5.1 Collagen scoring protocol

Statistical analysis. Results are expressed as mean and 95% confidence intervals (c.i.). Comparisons of all parameters between pairs were performed using the Mann-Whitney U test with its significance (adjusted for ties) being expressed as a *P* value.

Specific differences between each of the four different collagens was initially analysed by the Kruskall Wallis one-way analysis of variance.

5.3 Results

Interlobar septa

Within the interlobar septa statistically significant differences were apparent *(Figure 5.7)*, between the different types of collagen in both adults ($p<0.001$) and juveniles ($p<0.001$). This was attributable to the differences between the intensely stained collagen type VI compared to the least intensely stained collagen type I in both adults ($p<0.001$) and juveniles ($p<0.001$). In contrast collagen types IV and V were moderately stained throughout but still significantly different to that of type VI

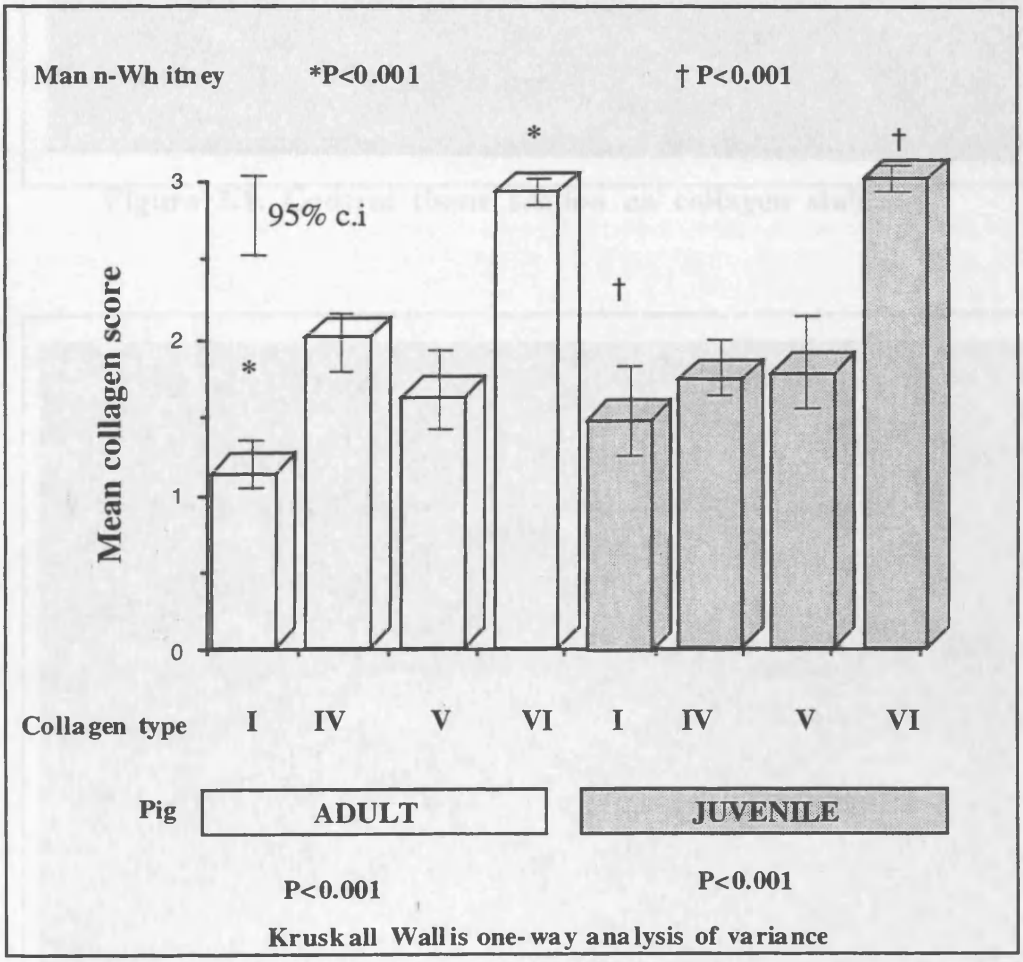


Figure 5.7 Distribution of collagen within the interlobar septa

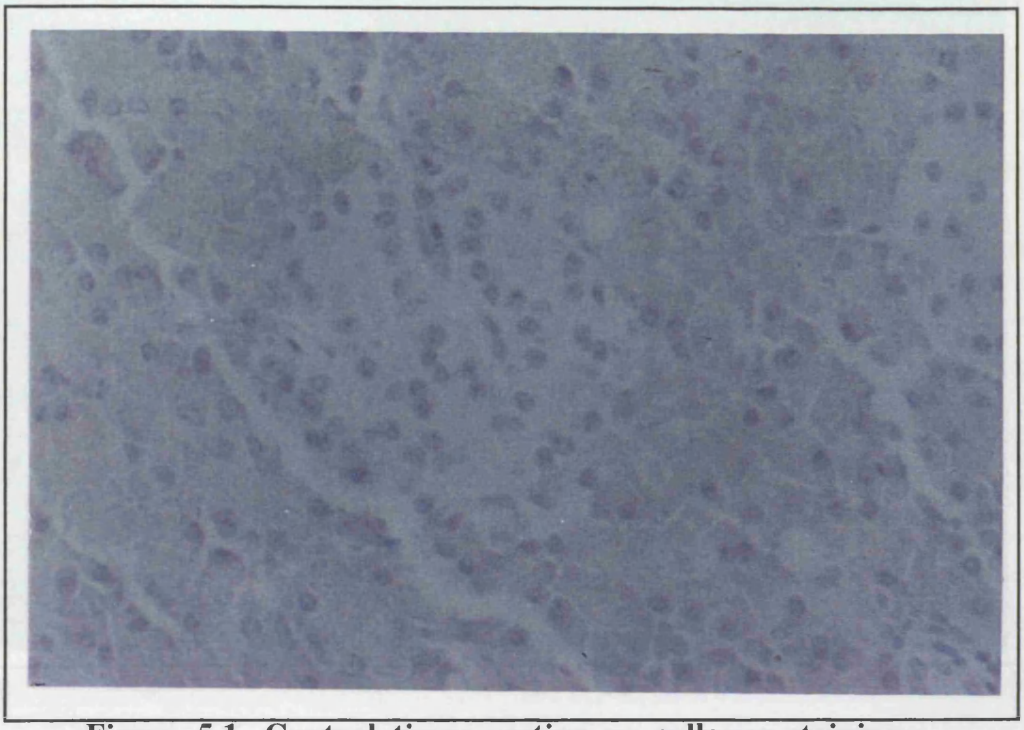


Figure 5.1. Control tissue section no collagen staining

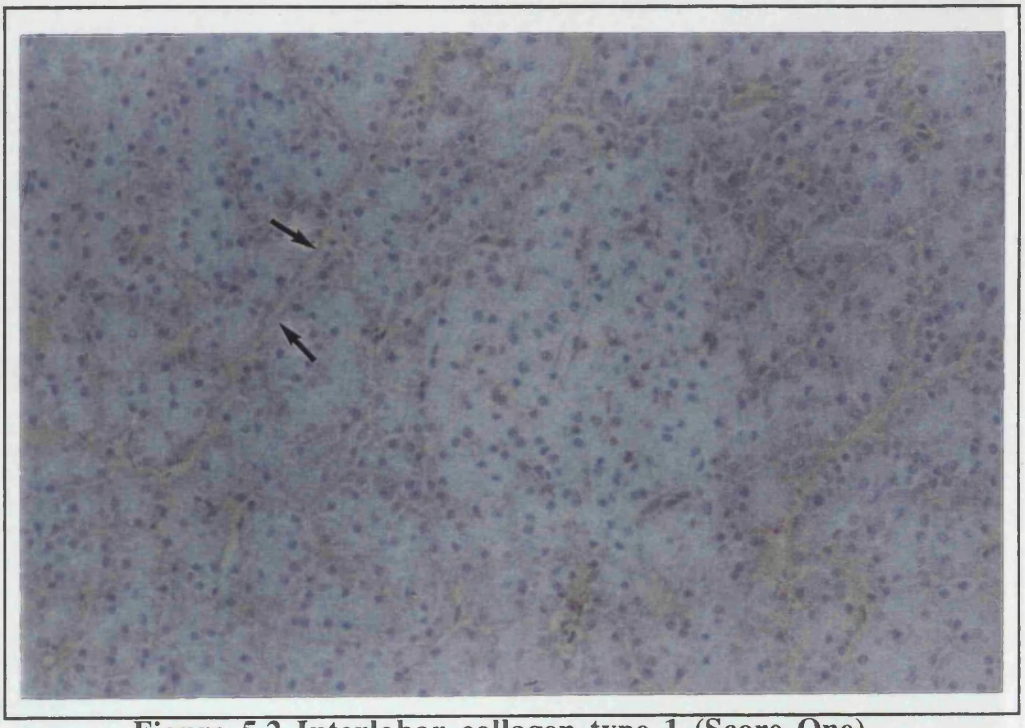


Figure 5.2 Interlobar collagen type 1 (Score One)

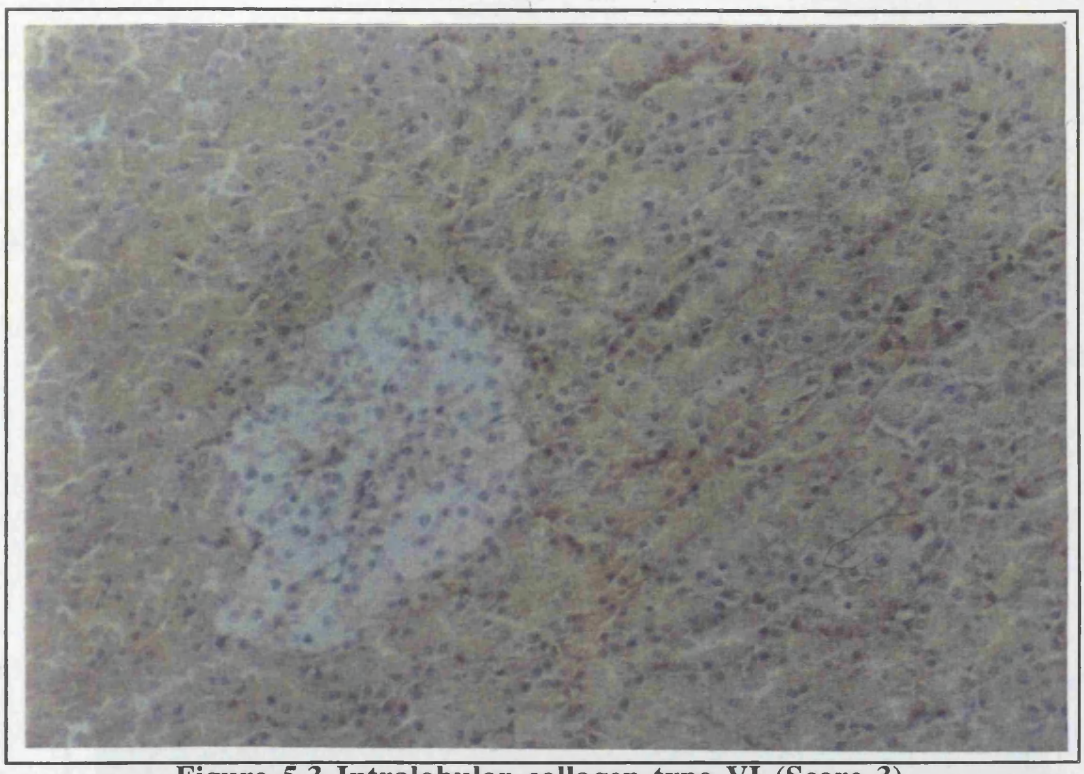
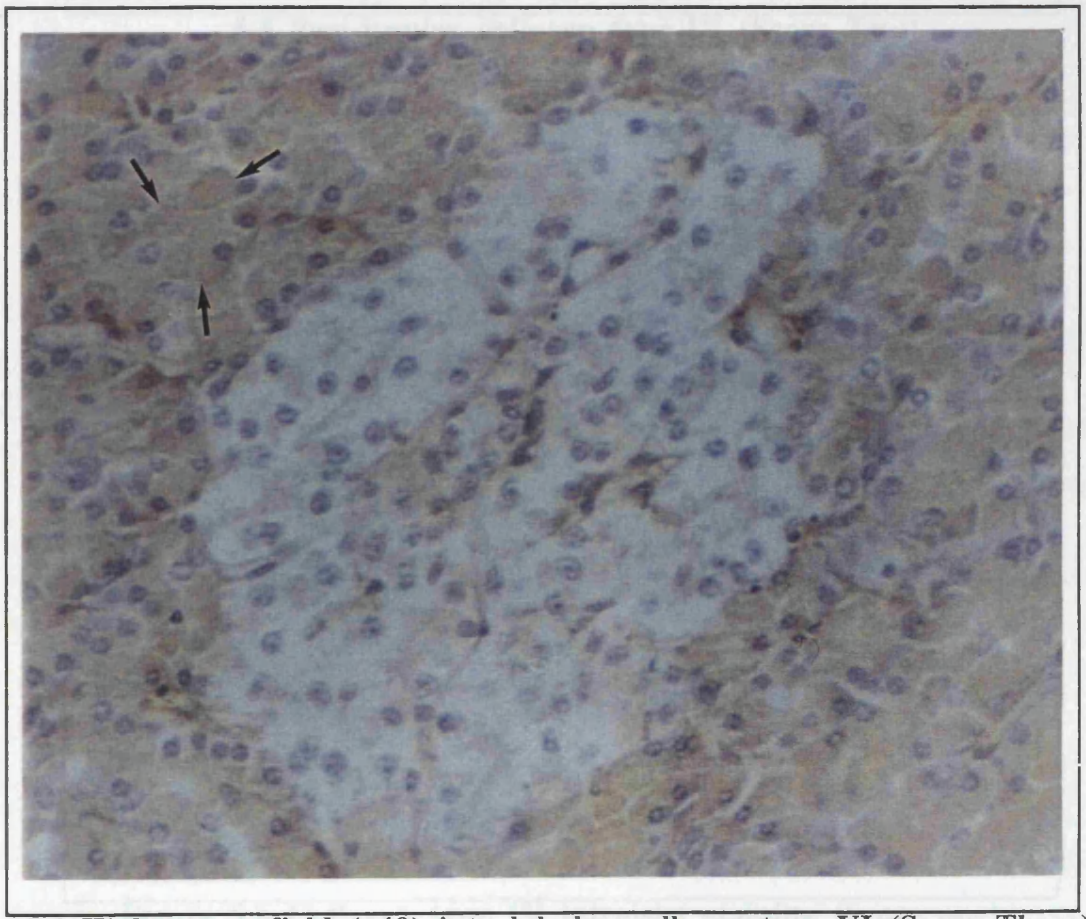
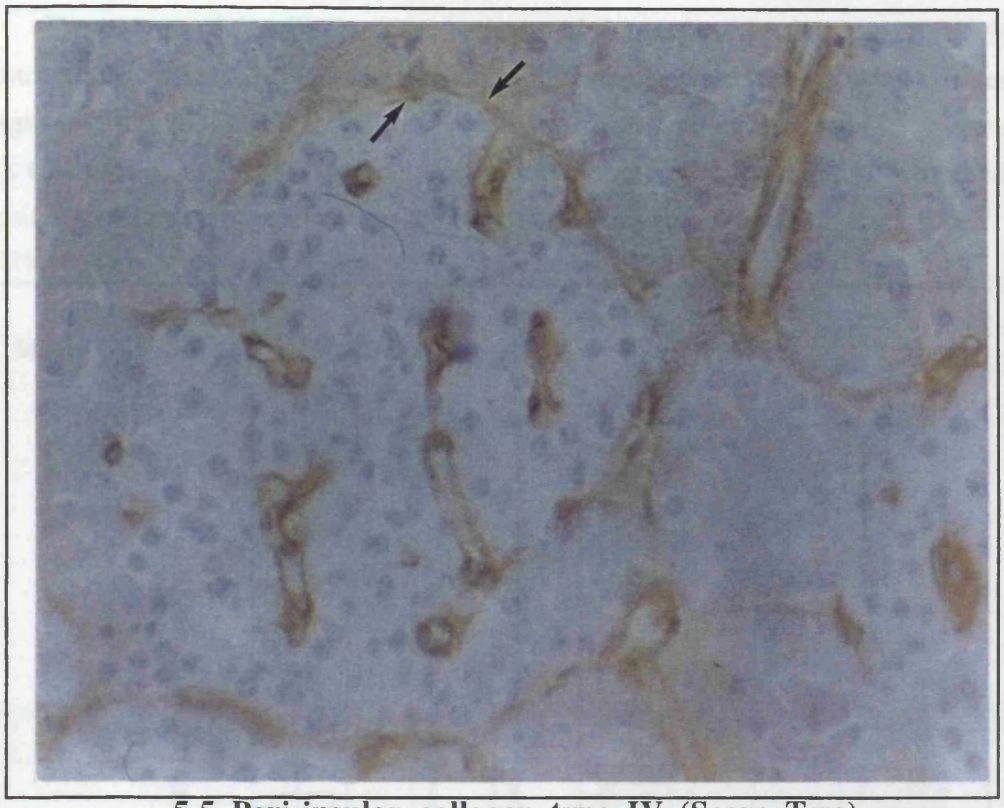


Figure 5.3 Intralobular collagen type VI (Score 3)



5.4 High power field (x40) intralobular collagen type VI (Score Three)



5.5 Peri-insular collagen type IV (Score Two)

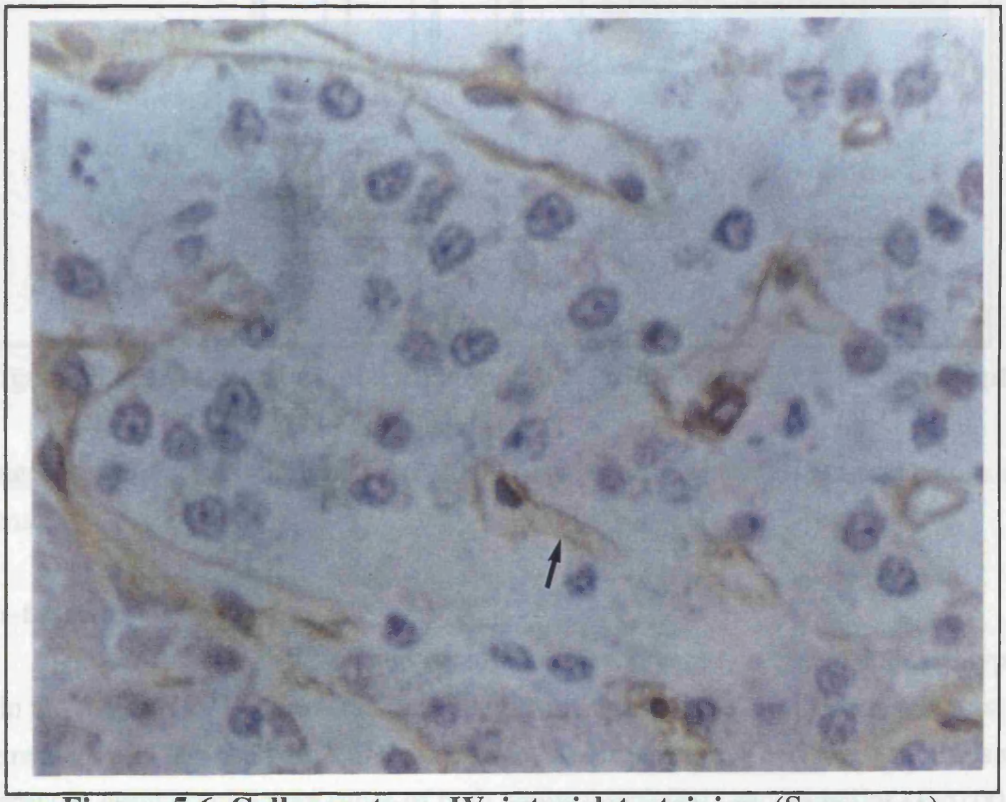


Figure 5.6 Collagen type IV intraislet staining (Score one)

Intralobular septa

The pattern of distribution seen in the interlobar septa was also consistent within the intralobular (acinar) septa where statistical differences were seen between different collagens within adults ($P< 0.001$) and between different juveniles ($P<0.001$; *Figure 5.8*). Collagen type VI was seen completely enveloping all acinar cells with marked intensity, greater to that seen within the interlobar septa and more significant than

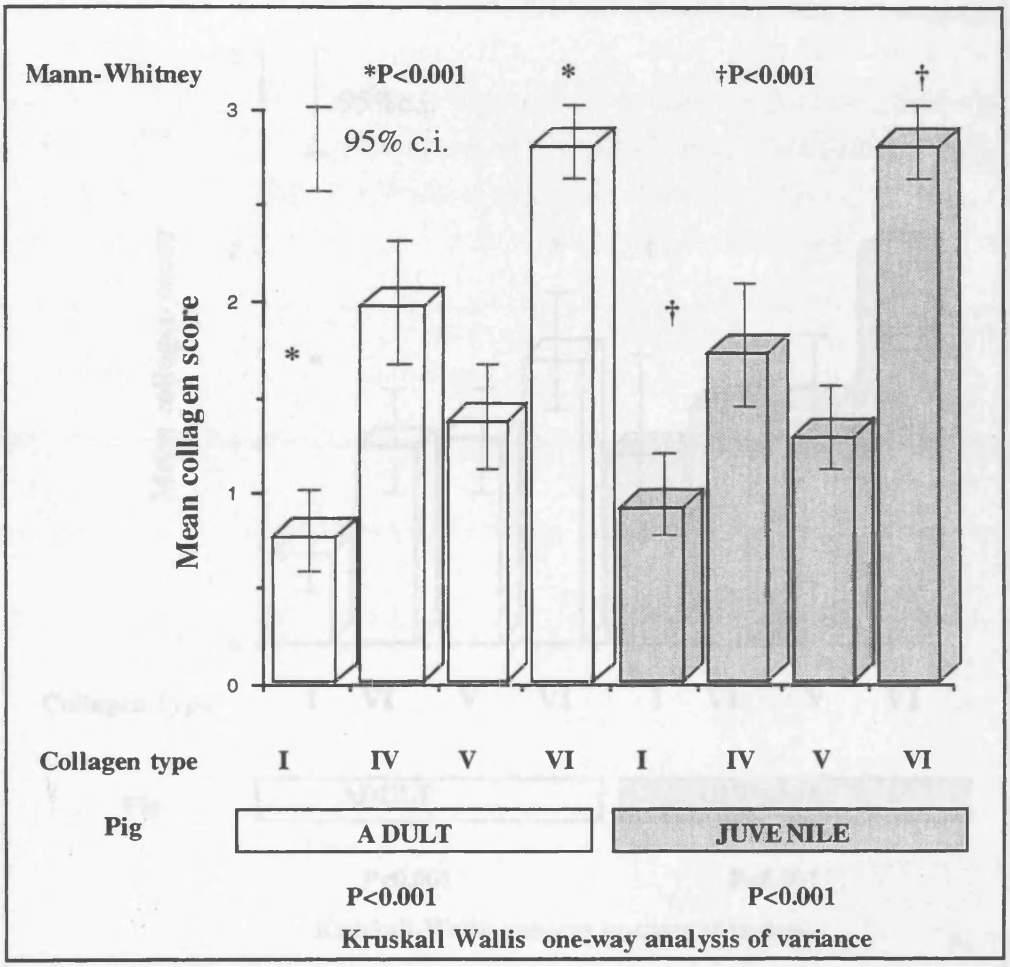


Figure 5.8 Distribution of collagen types within the intralobular septa

collagen type I $P< 0.001$), type IV ($P<0.001$) and type V ($P<0.001$) in both adults and juveniles.

Peri-insular collagen

The peri-insular collagen content also showed diversity between collagen types but in the majority of both juvenile and adult pancreata, the peri-insular capsule was fragmentary and less than 50% of the total islet circumference. There were significant differences between collagen types of both adults ($P<0.001$) and juveniles ($P<0.001$), this mainly being attributable to the quantitative differences of collagen types I (weak)

and type VI (intense) in adults ($P<0.001$), and diversity between collagen type I , IV and V (moderate) compared to type VI (intense) in juveniles ($P<0.001$; *Figure 5.9).* Moreover, the quantitative content of collagen within the islet peri-insular capsule showed an increasing trend in juvenile pancreata compared to that seen adults.

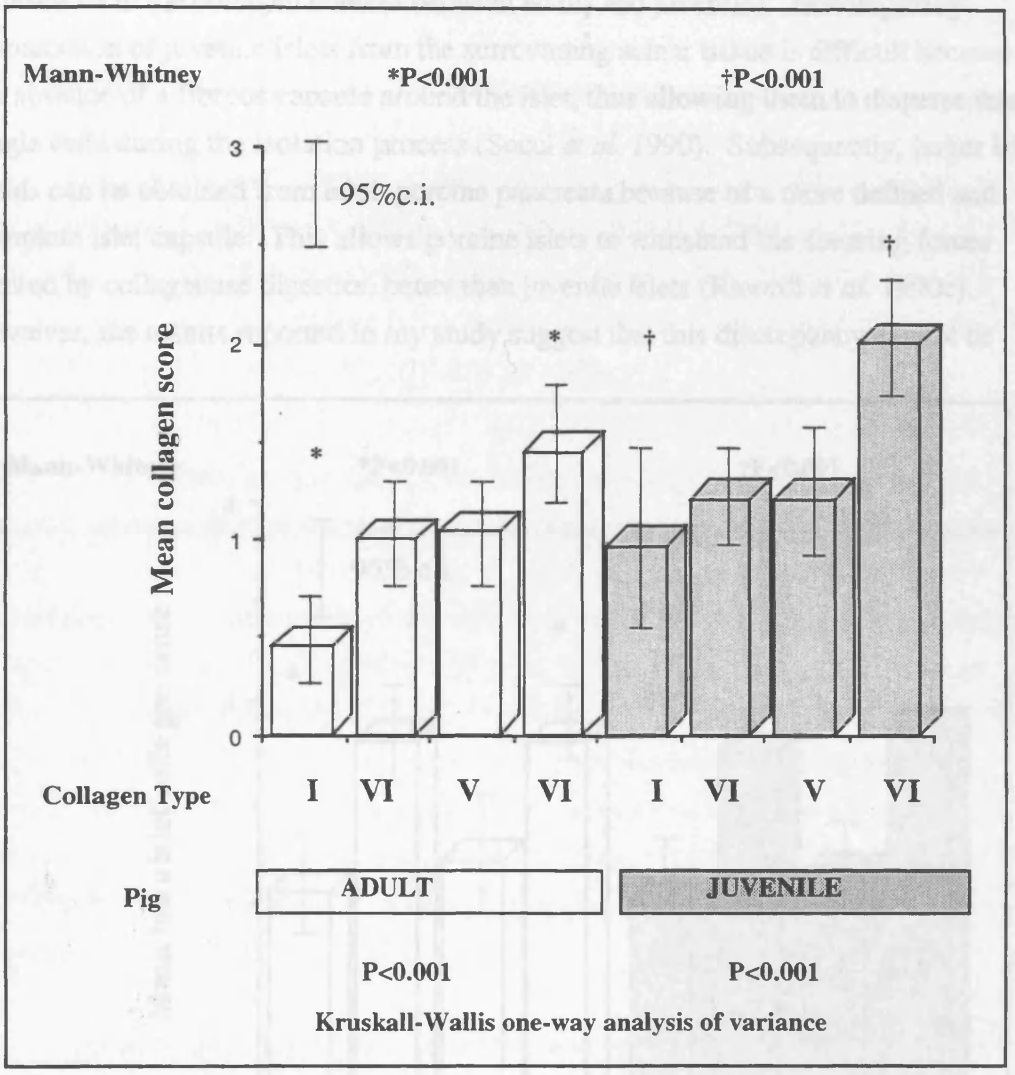


Figure 5.9 Distribution of peri-insular collagen content

Intraislet

The intraislet collagen content also showed significant differences in adults ($P<0.001$) and juveniles $P<0.001$). Collagen type IV was as intense as type VI, with types I and V being less intense *(Figure 5.10 p.159).* All intraislet collagen was identified within islet capillaries.

5.4. Discussion

One of the factors accounting for the fragility of porcine islets during the isolation procedure was thought to be the young age of porcine donors because of differences in the collagen content between adults and juveniles. Histologically, demarcation of juvenile islets from the surrounding acinar tissue is difficult because of the absence of a fibrous capsule around the islet, thus allowing them to disperse into single cells during the isolation process (Socci *et al.* 1990). Subsequently, larger islet yields can be obtained from adult porcine pancreata because of a more defined and complete islet capsule. This allows porcine islets to withstand the shearing forces created by collagenase digestion better than juvenile islets (Ricordi *et al.* 1990c). However, the results reported in my study suggest that this discrepancy cannot be

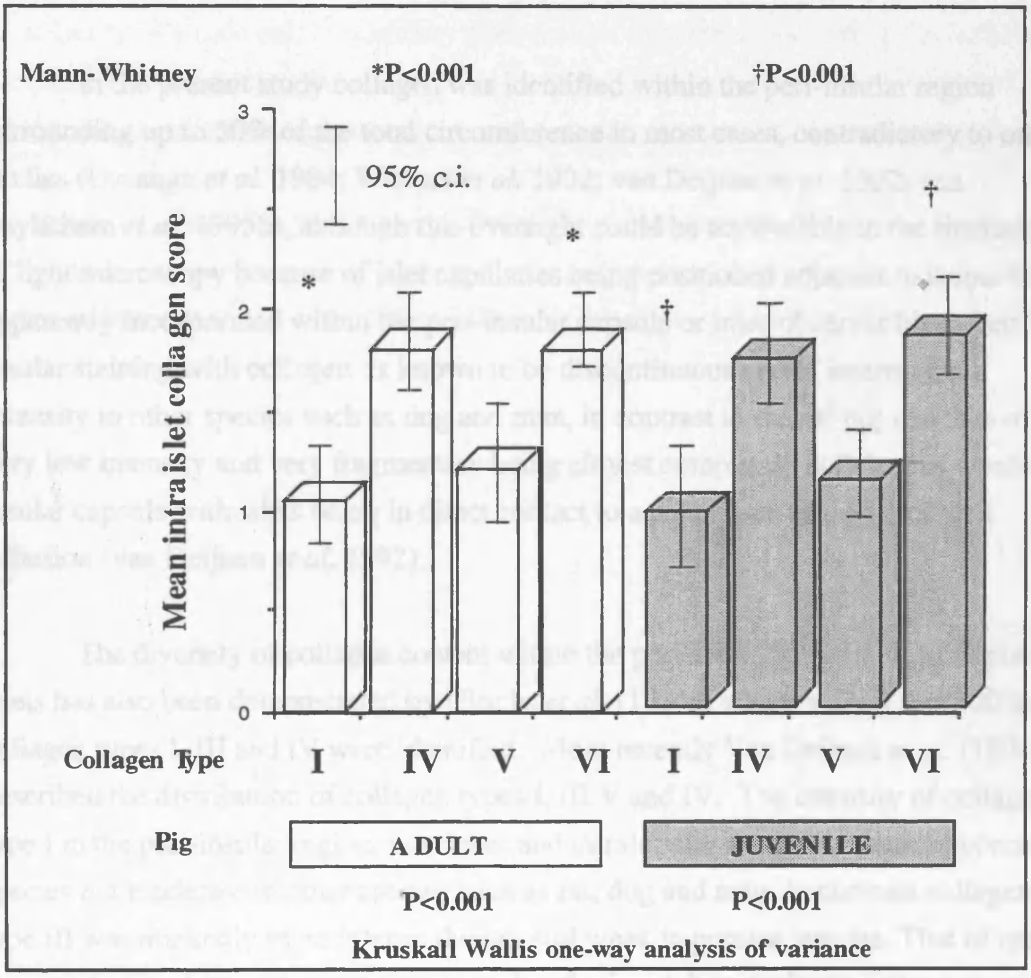


Figure 5.10 Distribution of intraislet collagen

explained in terms of differences in collagen content between adult and juvenile pancreata. No difference in either collagen type (I, IV, V, VI) could be observed in the interlobar or intralobular septa, peri-insular, or intraislet regions between adult and

juvenile porcine pancreata, although there was a general trend towards an increased collagen content of juvenile peri-insular regions, particularly of collagen type VI. Nevertheless, the integrity of adult porcine islets in contrast to juvenile could be explained by age where collagens from older individuals are more resistant to degradation by bacterial collagenase through the formation of stable crosslinking, and the amount of crosslinking cannot be identified from collagen staining (Weiss, 1982).

These results support the findings of van Suylichem *et al.* (1995b), where no difference in the collagen content could be observed between juvenile and adult peri-insular regions. Furthermore, it was also suggested in the above study, using sirus red absorbance, that the amount of collagen was significantly less within the pancreatic septa of adult pancreata compared to juveniles indicating a lower amount of collagen specifically within interlobar and intralobular septa.

In the present study collagen was identified within the peri-insular region surrounding up to 50% of the total circumference in most cases, contradictory to other studies (Uscanga *et al.* 1984; Wolters *et al.* 1992; van Deijnen *et al.* 1992; van Suylichem *et al.* 1995b), although this oversight could be attributable to the limitations of light microscopy because of islet capillaries being positioned adjacent to acinar tissue apparently incorporated within the peri-insular capsule or inter-observer bias. Peri-insular staining with collagen is known to be discontinuous and of intermediate intensity in other species such as dog and man, in contrast to that of pig which is of very low intensity and very fragmentary being almost completely deficient of a peri-insular capsule with islets being in direct contact to acinar tissue through cell-cell adhesion (van Deijnen *et al.* 1992).

The diversity of collagen content within the peri-insular capsule of adult porcine islets has also been demonstrated by Ulrichs *et al.* (1994a) where at least three different collagen types I, III and IV were identified. More recently Van Deijnen *et al.* (1994) described the distribution of collagen types I, III,V and IV. The intensity of collagen type I in the peri-insular region, interlobar and intralobular septa was weak in porcine species but moderate in other species such as rat, dog and man. In contrast collagen type III was markedly more intense though still weak in porcine species. That of type V also reacted weakly in pig, particularly within the interlobar and intralobular acinar pancreatic septa but moderate in rat, dog and man (van Deijnen *et al.* 1994). The results in the present study also complement those findings, but indicate that collagen type VI predominates in both adult and juvenile peri-insular regions.

The function of collagen type VI is not known but its ultrastructure suggests that it is an independent fibrous system perhaps important to the development and maintenance of spatial separation of distinct tissue components from large banded collagen fibers (Burgeson and Nimni, 1992). The relevance of this collagen type still needs to be determined so as to allow its exploitation by a commercial collagenase as it appears to be the most abundant collagen type within porcine pancreata analysed in the presented study.

The assembly of collagens into a complex fibrillar pattern within porcine pancreata is composed of many different types, at least types I, III, IV,V and VI (Ulrichs *et al.* 1994a; van Deijnen *et al.* 1994). This assortment obviously having constraints upon the rate of degradation of native collagen substrates through their different rates of individual degradation by collagenases (Johnson *et al.* 1996b). It can therefore be assumed that to minimise porcine islet fragmentation during the isolation procedure, a collagenase that does not degrade peri-insular collagen typeVI would be ideal but the problem is that collagen type IV is also abundant within the acinar tissue as well. In summary, it can be seen from this study and others (Uscanga *et al.* 1984; Wolters *et al.* 1992; van Deijnen *et al.* 1992; van Suylichem *et al.* 1995b), that the distribution of the different collagen types and the content of the extracellular matrix within pancreata has the potential to detrimentally affect islet isolation but suggests that other factors are also important.

CHAPTER SIX

THE INFLUENCE OF DIFFERENT COLLAGENASE SOLVENTS AND TIMING OF THEIR DELIVERY UPON PORCINE ISLET ISOLATION

6.1 Introduction

One of the problems relating to porcine islet isolation is the need to excise the pancreas from a local abattoir. In this situation the adverse consequences of contamination (see Chapter Eight), warm ischaemia and cold ischaemia are largely uncontrolled due to the nature of the slaughtering process.

One of these problems that needs to be addressed relates to the ischaemic tolerance of porcine pancreata prior to islet isolation, as all organs removed for transplantation are subject to an inevitable period of both warm and cold ischaemia. In an attempt to circumvent the deleterious effects of warm ischaemia upon porcine islet isolation previous investigators have procured the pancreas from a heart beating donor as a prerequisite to successful islet isolation (Ricordi *et al.* 1990b). With regard to minimising the damaging effects of cold ischaemia, a number of different approaches have been used. Application of a cold intravascular perfusate (Calne *et al.* 1963) of either EuroCollins (Collins *et al.* 1969), HOC or UW have proved efficacious in reducing warm ischaemic time and, in combination with hypothermic organ storage, has improved cold ischaemic tolerance of human pancreata, prior to islet isolation (Robertson *et al.* 1993a). However, when applied to the preservation of porcine pancreata, cold *in situ* vascular perfusion has been shown to culminate in very poor islet yields, mainly due to islet disruption (Ricordi *et al.* 1990b).

An alternative and simpler approach to enhance hypothermic pancreas preservation in the absence of *in situ* vascular perfusion, may be to deliver intraductal collagenase immediately after excision of the pancreas. Studies in rats (Ohzato *et al.* 1991) have shown this technique to improve islet yields with administration of collagenase in HBSS, although HBSS is not a cold storage solution (Hanks and Wallace, 1949). An improvement on this approach may be to deliver the collagenase in UW, immediately after excision of the pancreas and before cold pancreas storage. This procedure is effective in both dogs and rats (Munn *et al.* 1989b) but has been shown to be deleterious in the human because of certain components of UW (e.g. glutathione, allopurinol, HES and the reversed sodium ion to potassium ion ratio)

having an inhibitory action upon collagenase (Toomey *et al.* 1991; Casanova *et al.* 1994; Contractor *et al.* 1995a). Furthermore, porcine studies have been anecdotal (Mellert *et al.* 1991; Arbet-Engels *et al.* 1993). The object of this study to determine the influence on porcine islet isolation of intraductal collagenase administration in cold storage solutions.

6.2 Materials and methods

Study design. Islet isolations were randomised using sealed envelopes to: (1) collagenase in HBSS either in the islet laboratory (n=8) after cold pancreas storage, or (2) in the local abattoir immediately after excision of the pancreas (n=8) before cold

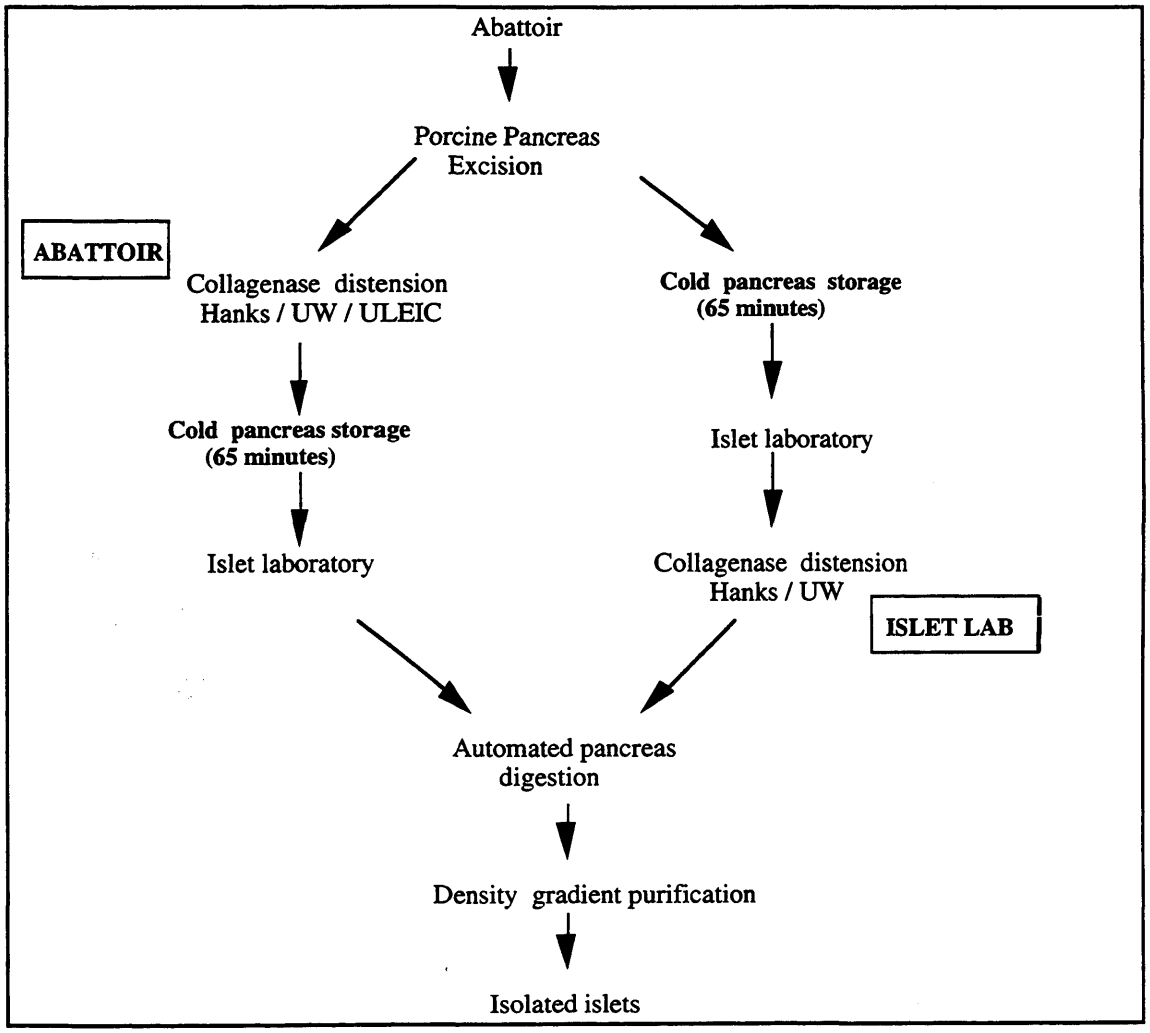


Figure 6.1 Flow diagram of methods

pancreas storage *(Figure 6.1)* ; (3) collagenase in UW in the islet laboratory (n=8) after cold pancreas storage and (4) in the abattoir immediately after excision of the pancreas (n=8) before cold pancreas storage; (5) collagenase in a specially designed

cold storage solution, University of Leicester (ULEIC) solution *(Table 6.1)* administered immediately after excision of the pancreas at the abattoir (n=8).

	Collagenase Solvent		
	HBSS	**UW**	**ULEIC**
Sodium	141.0	20.0	131.0
Potassium	6.0	140.0	3.9
Hydroxyethylstarch g/L		50.0	
Raffinose		30.0	30.0
Dextran-40			0.2
Lactobionate		100.0	100.0
Magnesium	0.8	5.0	5.0
Bicarbonate			
Chloride	137.0	10.0	
Phosphate	0.7	25.0	25.0
Sulphate		5.0	
Glucose	5.6		
Adenosine		5.0	
Allopurinol		1.0	
Glutathione		3.0	
Insulin		100.0	
Dexamethasone		8.0	
Mannitol			
Calcium	1.3		2.4
Citrate			
Osmolarity (mmol/kg)	340.0	320.0	289.0
pH	7.3	7.4	7.0

Table 6.1 Solvent composition (Units mmol/l unless otherwise stated)

Organ donors and procurement technique. Forty porcine pancreata were excised from two to three years old Large White sows obtained from the local abattoir (Dawkins International, Congerstone, UK). Each pancreas was subjected to 10 minutes warm ischaemia before excision. The splenic lobe (65 to 100g) was excised on all occasions *(Figure 6.2 p.166)*. Care was taken not to disrupt the pancreatic capsule or perforate the adjacent duodenum.

Collagenase distension. Each pancreas had intraductal collagenase administration (1mg/ml) in either HBSS (Gibco, Life Technologies, Faisley, Scotland; Hanks and Wallace, 1949), UW (Belzer UW-CSS; Du Pont, Wilmington, Delaware, USA; Wahlberg *et al.* 1986) or ULEIC. Of those distended in the laboratory (16 in total) each underwent approximately 65 minutes cold ischaemia during pancreas transportation in HOC at 4°C (Travenol, Thetford , UK; Ross *et al.* 1976). Those assigned to early collagenase administration in the abattoir (24 in total) were also transported in HOC after distension of the pancreas with collagenase. The same batch

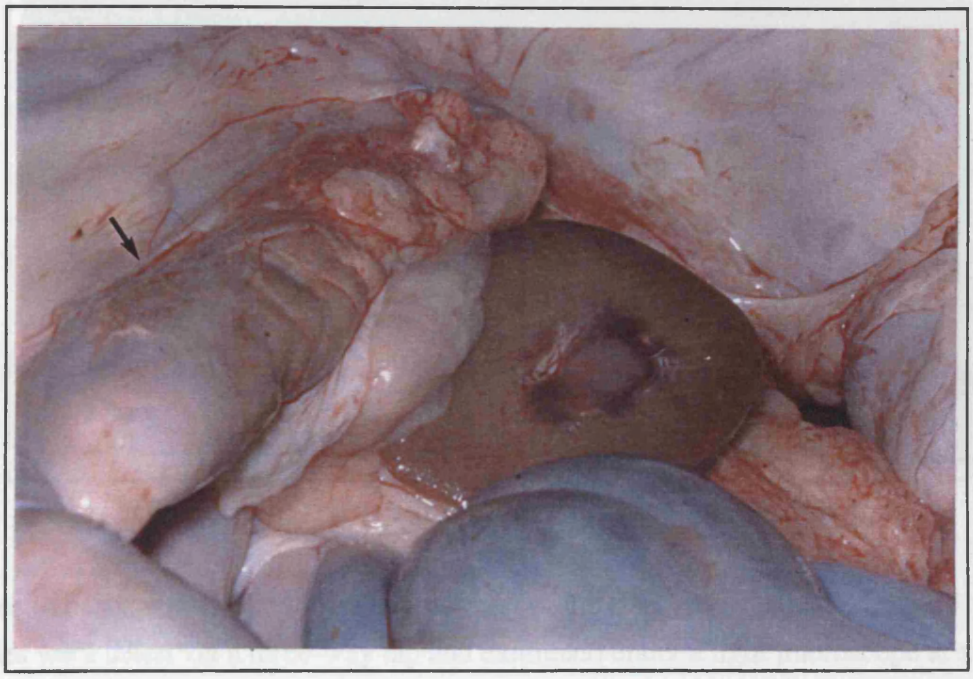


Figure 6.2 Splenic lobe of porcine pancreas

of collagenase (Boehringer Mannheim lot No 14039526-64; Mannheim, Germany) was used throughout the study. Before distension excess fat and lymphoid tissue were removed. The pancreas was weighed (Ohaus Scale Corporation, USA) and the main pancreatic duct was cannulated with an 18-G cannula (Abbocath-T; Abbot Laboratories, Kent, UK) secured by a 2/0 silk tie (Ethicon, Edinburgh, Scotland). Each pancreas was distended by slow injection of collagenase at 4°C using 2ml per g pancreas) *(Figure 6.3 p.167)*. An evenly distended pancreas was obtained by gradual withdrawal of the cannula during infusion, any ductal leaks were immediately clamped with forceps.

Pancreatic digestion. All pancreata (n=40) were digested by the automated method (Ricordi *et al.* 1990c). The pancreas was placed in a stainless steel chamber divided by a 400μm mesh, along with seven $1cm^3$ sized steel spheres to aid pancreatic dispersion *(Figures 6.4 and 6.5 p.168)*. HBSS at 4°C was circulated through a closed circuit of

polyvinyl chloride tubing (COBE Laboratories. Gloucester, UK) by a peristaltic pump (Watson Marlow, Falmouth, UK) set at a rate of 80ml/min into a heat exchanger (COBE Laboratories. Gloucester, UK) intially at 35°C. The chamber was manually agitated between two and three times per minute.

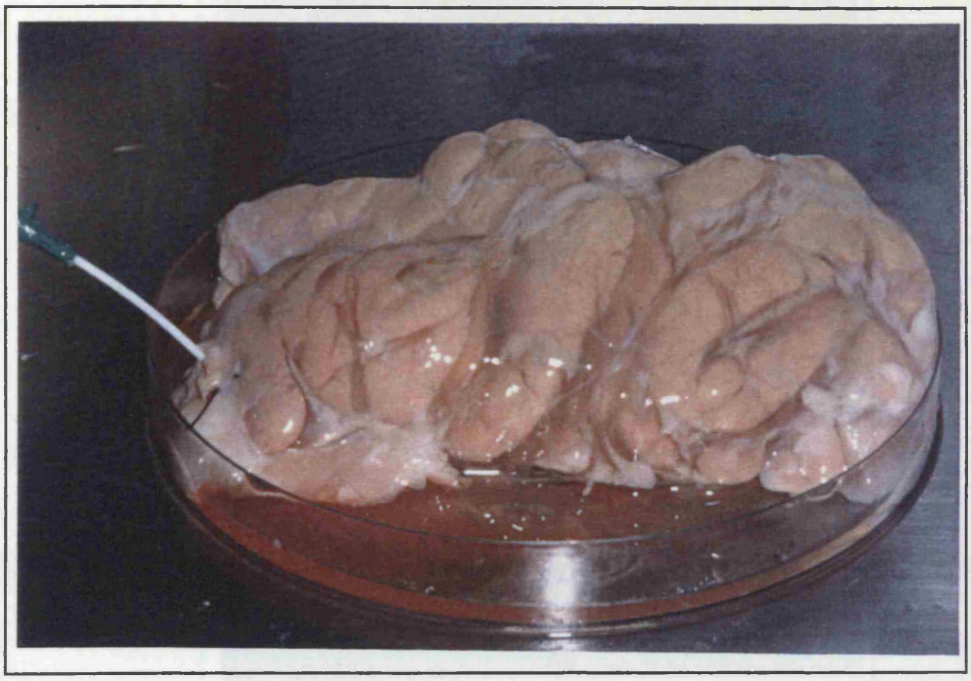


Figure 6.3 Intraductal collagenase distension

Pancreatic tissue was flushed from the exit port of the steel chamber and then 5 ml biopsies were taken via a three-way tap and examined under a light microscope at x 4 magnification (Nikon, Diaphot, Japan) in the presence of dithizone (Sigma Chemicals, Poole, UK; 1.2mmol/l in 5% dimethylsulphoxide and minimal essential medium (MEM) and 1% BSA; Latif *et al.* 1988) for identification of 'cleaved' islet tissue devoid of acinar tissue. At this point the circuit was opened and the flow rate increased to 120 ml/min. The temperature was adjusted according to visualisation of pancreatic tissue within the biopsies so as to avoid fragmentation and overdigestion as islets were liberated (London *et al.* 1990). The digest was collected in 1 litre duran bottles containing 15ml of new born calf serum (Advanced Protein Products, Brierly Hill, UK) to inhibit further action of collagenase. The collected tissue was washed *(Figure 6.6 p.169)* and spun at 600g for two minutes and made up to a volume of 100ml with UW at 4°C. Pancreatic digestion was assumed to be complete when no further islet tissue could be seen within the dithizone stained samples.

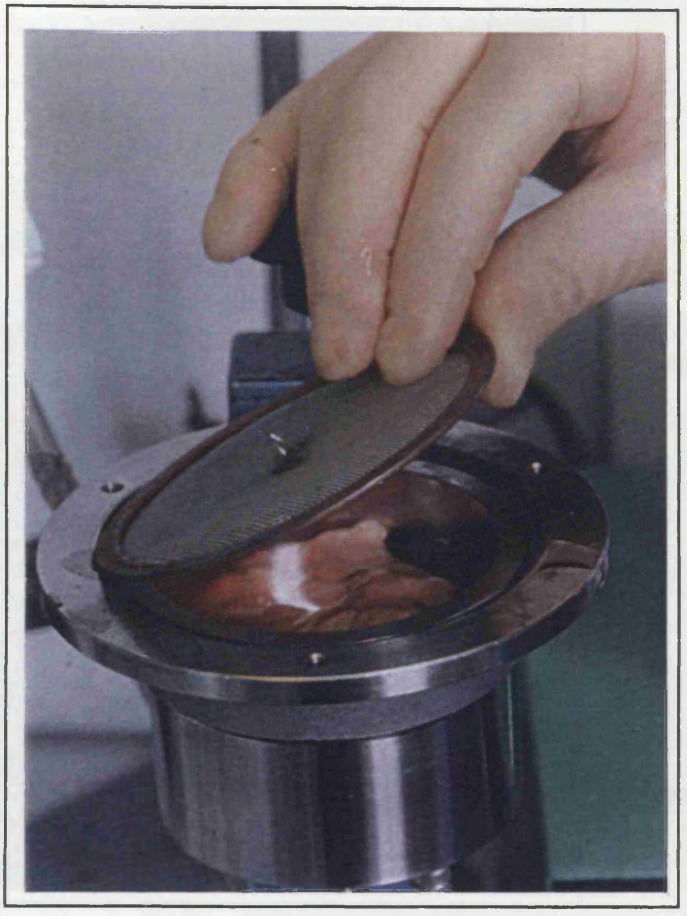


Figure 6.4 Automated chamber

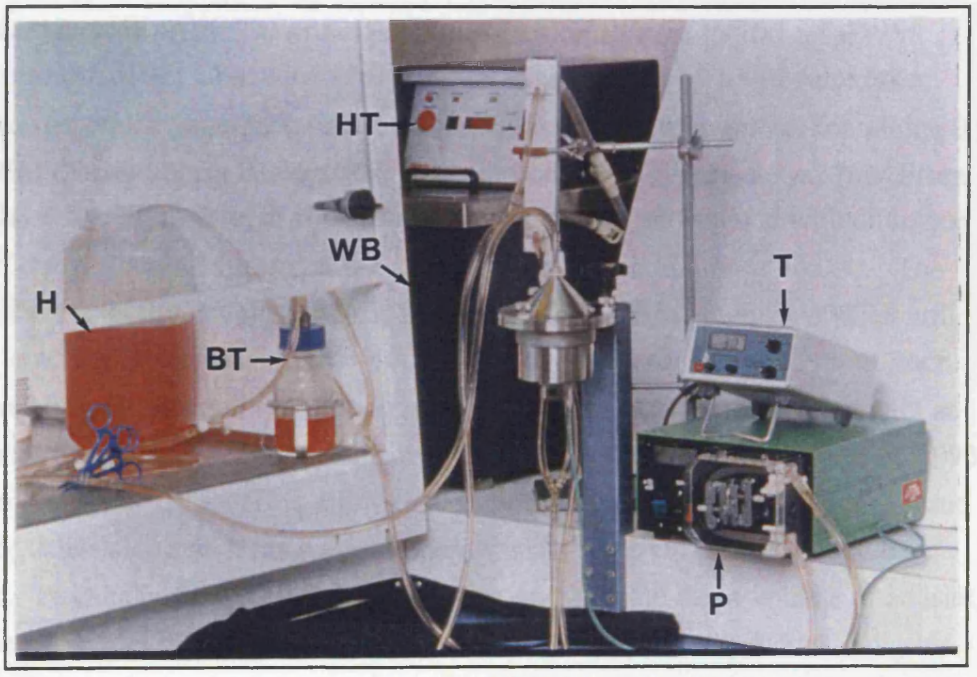


Figure 6.5 Automated circuit (H-HBSS; BT- Bubble trap; P-Pump; HT-Heater; T-Thermometer; WB-Waterbath)

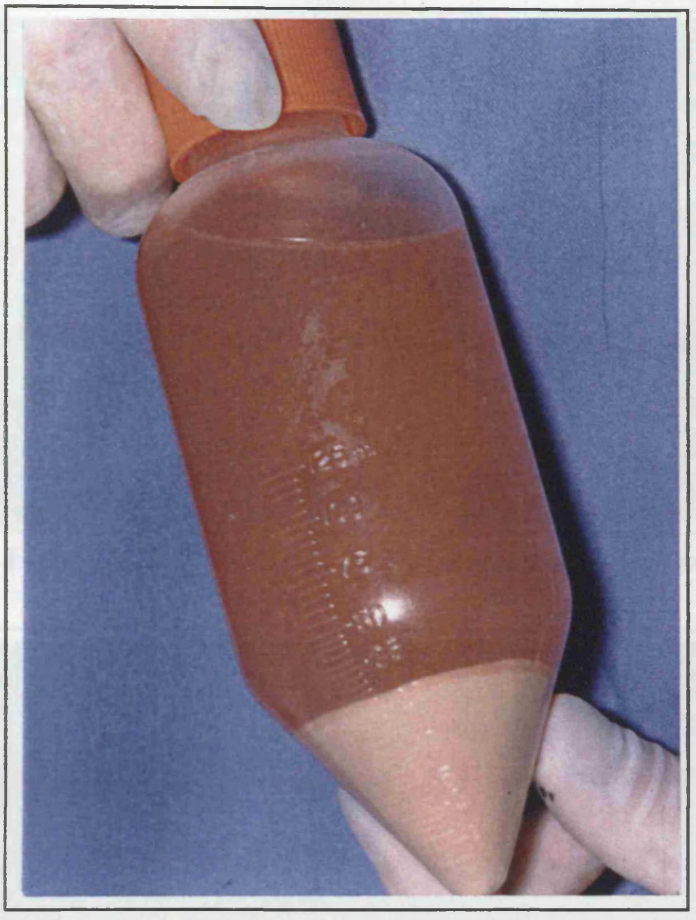


Figure 6.6 Collected pancreatic digest (20ml)

Islet quantification. After 30 minutes prepurification storage in 100 ml UW (Robertson *et al.* 1992; Chadwick *et al.* 1993a), five samples of 100µl were taken from the resuspended pancreatic digest and individually placed in bijoux containing 0.9 ml of MEM (Northumbria Biologicals, Cramlington, UK) *(Figure 6.7 p.170)*. From each of these five bijoux single 100µl strips were aspirated and stained with dithizone *(Figure 6.8 p.170)* in a 140mm petri dish giving a total dilution of 1: 10,000. The number of islets totally devoid of acinar tissue (cleaved) and the number of islets still attached to acinar tissue (uncleaved) with a diameter greater than 50µm, within each strip, were counted under a light microscope (x 100 magnification) calibrated with an optical grid (Cell Finder, Leiden, Holland; Robertson *et al.* 1993a; Toomey *et al.* 1993; *Figures 6.7 to 6.9 p.170*). This allows estimation of the percentage of 'cleaved' and 'uncleaved' islets along with the total number of islets per gram of pancreas to be estimated. To calculate the total volume of islet tissue and the mean volume of an islet, 200 consecutive islet diameters were recorded from each of the 40 pancreatic digests. The total volume of islet tissue isolated was calculated as the product of the mean volume of an islet and the total number of islets (Robertson *et al.* 1993a; Toomey *et al.* 1993).

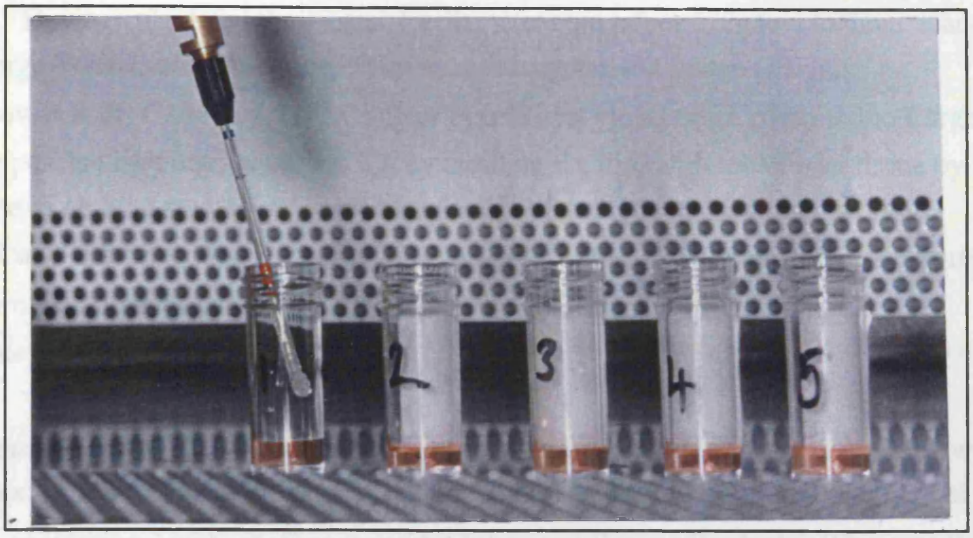


Figure 6.7 Five 100µl samples from resuspended digest

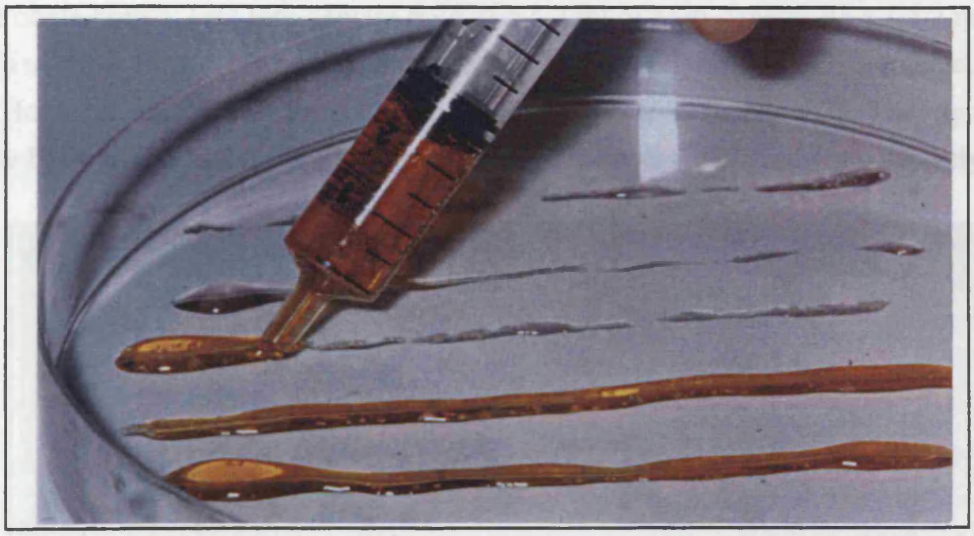


Figure 6.8 Dithizone staining of strips of digest for islet counting

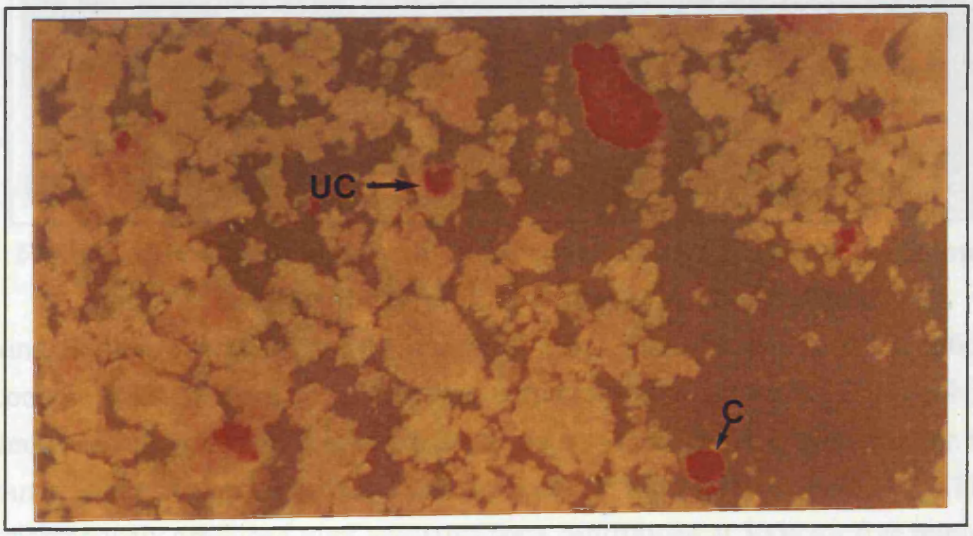


Figure 6.9 Counting of islets (red) 'cleaved'(C) and 'uncleaved' (UC)

Having calculated the mean islet diameter equivalent from two to three years old Large White pancreata to be 80µm using computerised image analysis (Improvision ®, Coventry, UK; Chapter Four), islet yields were corrected to Large White porcine islet equivalents (IEQ) by dividing the total volume of islet tissue by the volume of an 80 µm porcine islet equivalent [0.524 x $(80)^3$], assuming islets are spherical. This correction, therefore, is analagous to the 150 µm equivalents reported for human pancreata but allows a more accurate assessment of the isolation index (Toomey *et al.* 1993; Chapter Four).

Islet purification. A 3 ml sample of the pooled digest was spun at 600g for two minutes. A 65µl aliquot of this packed sample digest was placed in duplicate 10 ml test tubes containing 1ml high-density BSA (Advanced Protein Products). This was then overlaid with a 10 ml, continuous linear gradient of BSA by a two chamber gradient maker containing 5.2 ml low-density BSA and 5.2 ml of high-density BSA (5.2 mls in each to allow for losses within the gradient maker) mixed together with a magnetic flea (Hoefer Scientific Instruments, San Francisco, USA) *(Figure 6.10).* The high-density BSA and low-density BSA were 1.103 g/cm^3 and 1.075 g/cm^3 respectively,

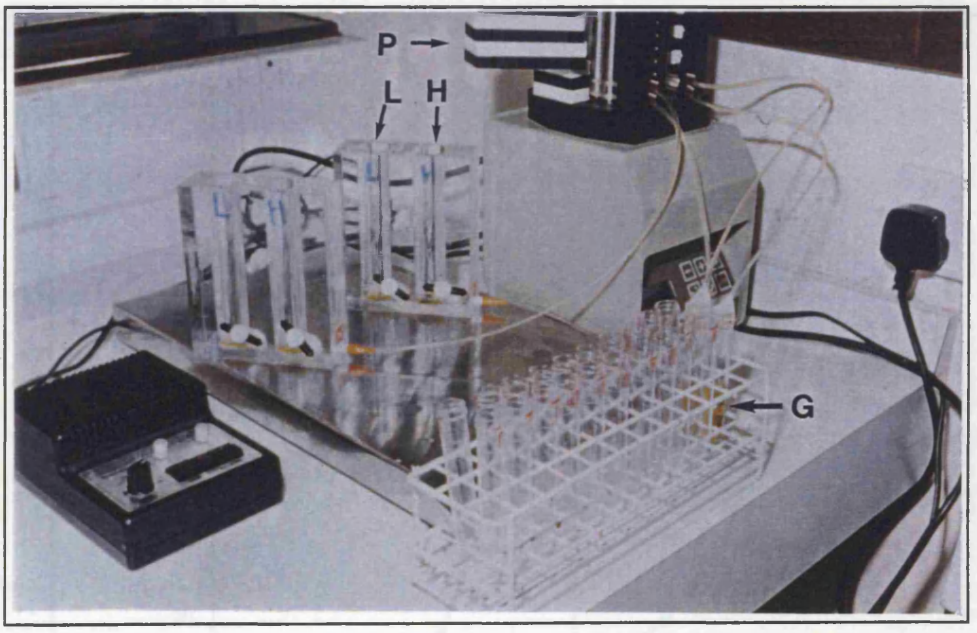


Figure 6.10 Construction of mini continuous gradients (P-Pump; H-High density; L-Low density; G- Gradient)

following dilution with MEM and measurement with a densitometer (Paar Scientific Ltd, London, UK).The final osmolarity was corrected to 400 mOsm/kg H_2O for both high-density and low-density BSA by dilution with sodium chloride upon titration with a vapour pressure osmometer (Chem Lab Scientific Products, Hornchurch, UK).The two synchronously produced mini gradients were centrifuged at 500g for five minutes at 22°C to allow the digest to equilibriate at its isodense point on the gradient

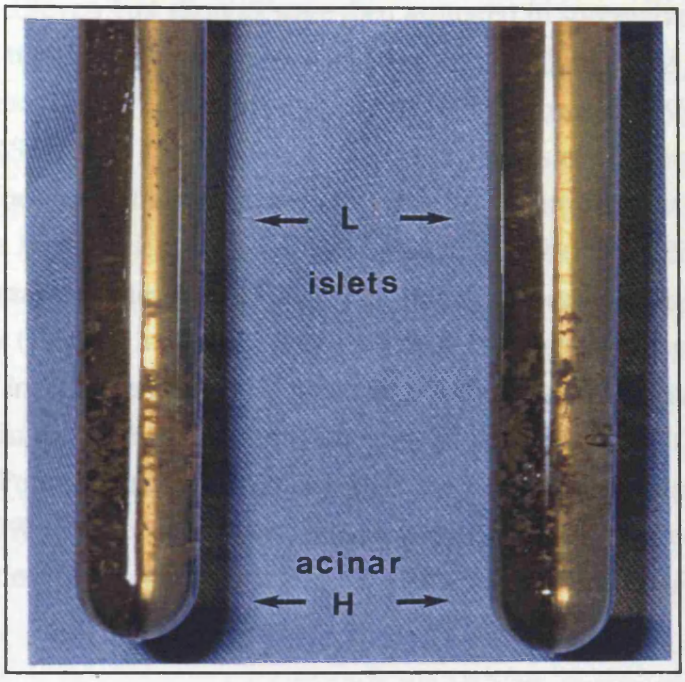


Figure 6.11 BSA mini continuous gradients (L-Low density; H-High density)

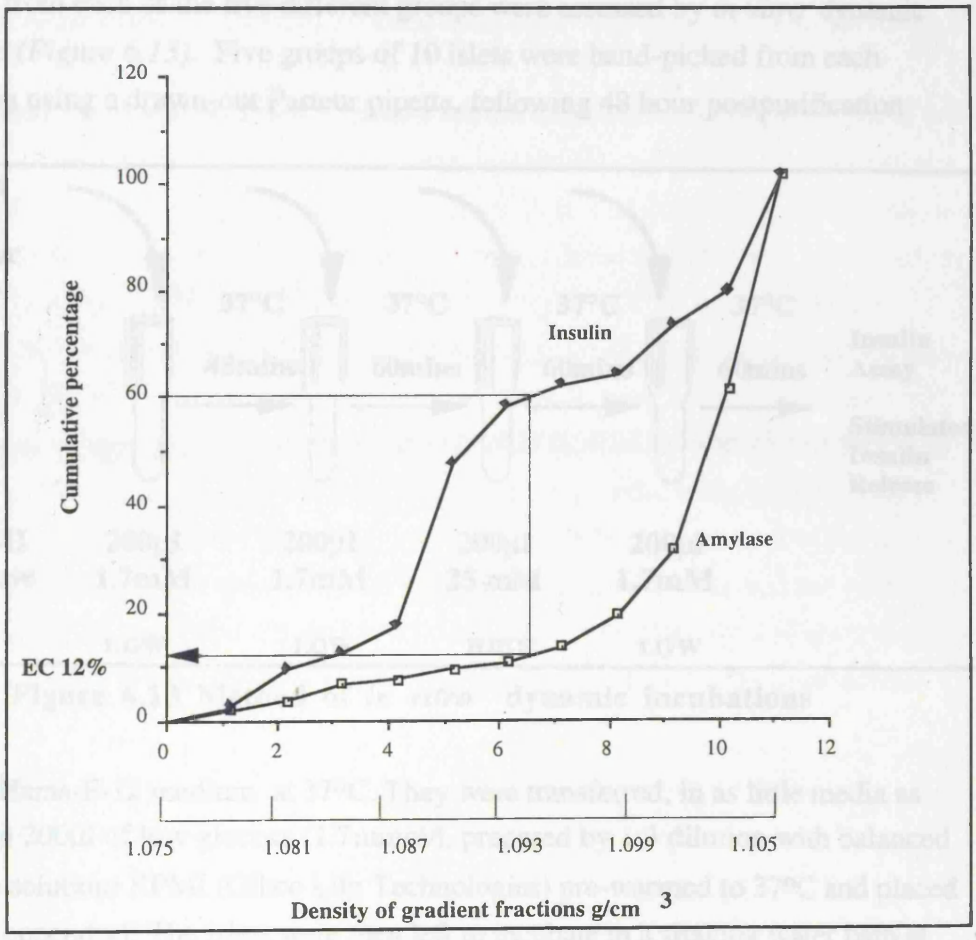


Figure 6.12 Cumulative plots of insulin and amylase content

(Figure 6.11 p.172). Eleven 1ml aliquots were then aspirated in successive order from the meniscus of each tube. Each aliquot was washed twice with 3 ml MEM and finally resuspended in a further 2 mls (Chadwick *et al.* 1993a; Robertson *et al.* 1993b). They were then sonicated (Soniprep 150, MSE, UK) for three periods of 30 seconds on ice; 1ml being aspirated for assay of amylase content (Phadebas ® amylase test kit batch N° 70632; Pharmacia Diagnostics, Uppsala, Sweden) and a further 1ml being mixed with 1 ml of Ziegler reagent (Ziegler *et al.* 1985) for subsequent enzyme linked immunosorbent assay (ELISA; Medgenix-INS-EASIA ® ; Medgenix Diagnostics, Fleurus, Belgium) of insulin content. The cumulative percentages of the insulin and amylase content within the gradient fractions were plotted against density (Cricket Graphs ®, Malvern, Pennsylvania, USA) to ascertain the percentage amylase contamination of a 60% (EC= exocrine contamination) insulin yield, deemed to be a reasonable yield of islets (Robertson *et al.* 1992; Robertson *et al.* 1993b) *(Figure 6.12 p.172).*

Islet viability. Following large scale purification on a COBE 2991 processor (COBE Laboratories; Lake *et al.* 1989a) the viability of islets isolated from five pancreata from each of the five different groups were assessed by *in vitro* dynamic incubation *(Figure 6.13).* Five groups of 10 islets were hand-picked from each preparation using a drawn-out Pasteur pipette, following 48 hour postpurification

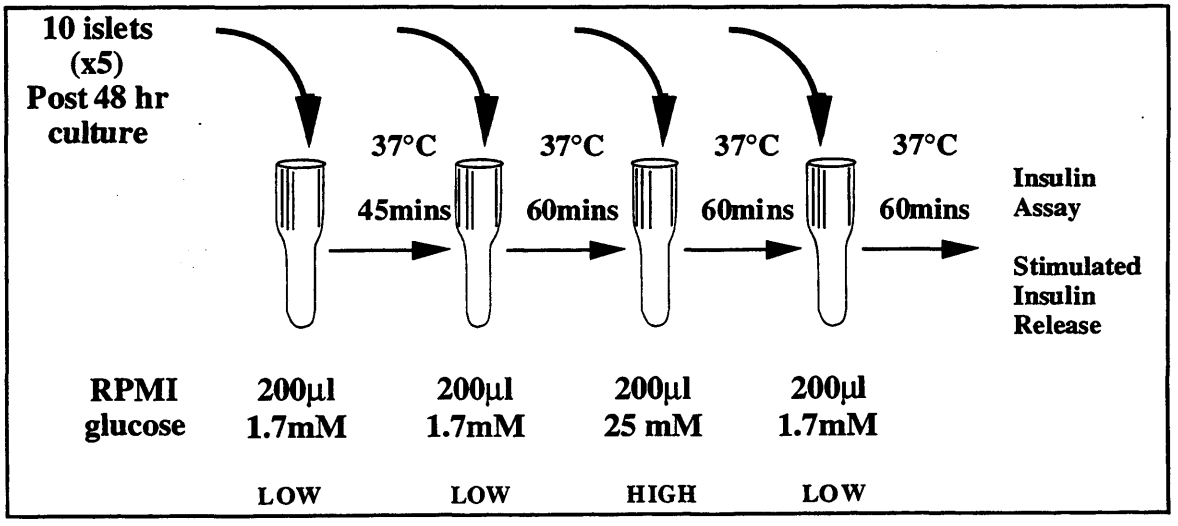


Figure 6.13 Method of *in vitro* dynamic incubations

culture in Hams-F-12 medium at 37°C. They were transferred, in as little media as possible, to 200µl of low glucose (1.7mmol/l, prepared by 1:3 dilution with balanced electrolyte solution) RPMI (Gibco Life Technologies) pre-warmed to 37°C and placed in a 1.5ml eppendorf. The islets were then left to incubate in a shaking water bath at 37°C for 45 minutes. The 200µl supernatant was then removed and replaced by a

further 200µl low glucose RPMI (Gibco Life Technologies) for 1 hour then high glucose (25mmol/l) RPMI for 1 hour and finally with low glucose (1.7mmol/l) RPMI for 1hour as previously described (Ham, 1965; London *et al.* 1990). The insulin content within the supernatant of each of the final two phases was measured by ELISA assay. Stimulated insulin release was expressed in microunits per islet per hour (iuI/per islet/ per hour) calculated as the difference between insulin content in the high-glucose (25mmol/l) response and the final low-glucose (1.7 mmol/l) response.

Statistical analysis. Results are expressed as median (range) values. Differences between each of the five groups were analysed initially by the Kruskall Wallis one-way analysis of variance test. Comparisons between pairs were then made, where valid, using the Mann-Whitney U test with its significance (adjusted for ties) being expressed as a P value. In addition, the 95% confidence intervals (95% c.i.) for differences between the groups were reported where appropriate (Minitab ® release 8.1).

6.3. Results

Pancreas digestion

Results for the five different groups are given in *Table 6.2 p.175*, and *Figures 6.14, 6.15 p.176 and 6.16 p.177.* There were no significant differences between the groups with respect to weight of pancreata (range 59 to 110 grams) and cold ischaemic time (range 48- 99 minutes). The volume of digest differed significantly, with median values ranging from 0.18 mls/g^{-1} to 0.34 mls/g^{-1}, (p=0.002). This was mainly attributable to the increased volume of pancreatic digest following administration of collagenase in UW (median 0.34 mls/g^{-1}) at the abattoir, compared to that after conventional collagenase administration in HBSS at the islet laboratory (median 0.18 mls/g^{-1}; P=0.01; 95% c.i. -0.24, -0.04). This trend also reflects the significantly greater volume of islet tissue (P=0.002) and islet equivalents isolated (P=0.02). Median values ranged between 3.55 mm^3/g^{-1} to 11.02 mm^3/g^{-1} for volume of islet tissue *(Table 6.2)* and 6,924 IEQ/g^{-1} to 21,524 IEQ/g^{-1} for islet yield *(Figure 6.14).* When collagenase was administered in UW at the abattoir, islet yields were trebled

Table 6.2 Summary of results. All median (range) values.

Timing	**Islet Laboratory**		**Abattoir**			**P ***
Collagenase Solvent	Hanks (n=8)	UW (n=8)	Hanks (n=8)	UW (n=8)	ULEIC (n=8)	
Cold ischaemia (mins)	68 (56-88)	63 (52-99)	59 (48-71)	61 (56-67)	69 (61-77)	0.7
Weight of pancreas (g)	80 (71-95)	75 (60-110)	67 (59-92)	79 (62-88)	71 (63-77)	0.5
Pancreatic digest volume mls g^{-1}	0.18 (0.09-0.29)	0.20 (0.03-0.27)	0.21 (0.04-0.24)	0.34 (0.22-0.45)	0.23 (0.16-0.37)	**0.002**
Cleavage index (%)	53 (48-71)	67 (52-75)	58 (28-79)	63 (42-70)	57 (38-72)	0.15
Total volume islet tissue mm^3 g^{-1}	3.55 (0.72-6.6)	4.53 (1.61-9.84)	7.14 (0.40-27.69)	11.02 (3.80-29.85)	10.15 (5.38-16.26)	**0.02**
Mean volume of an islet $x10^{-4}$ mm^3	5.18 (1.44-12.61)	4.10 (1.63-12.60)	7.00 (1.72-26.90)	6.29 (1.68-10.80)	7.74 (4.44-21.02)	0.4

† Porcine islet equivalent (80μm)

* Kruskall Wallis one way analysis of variance

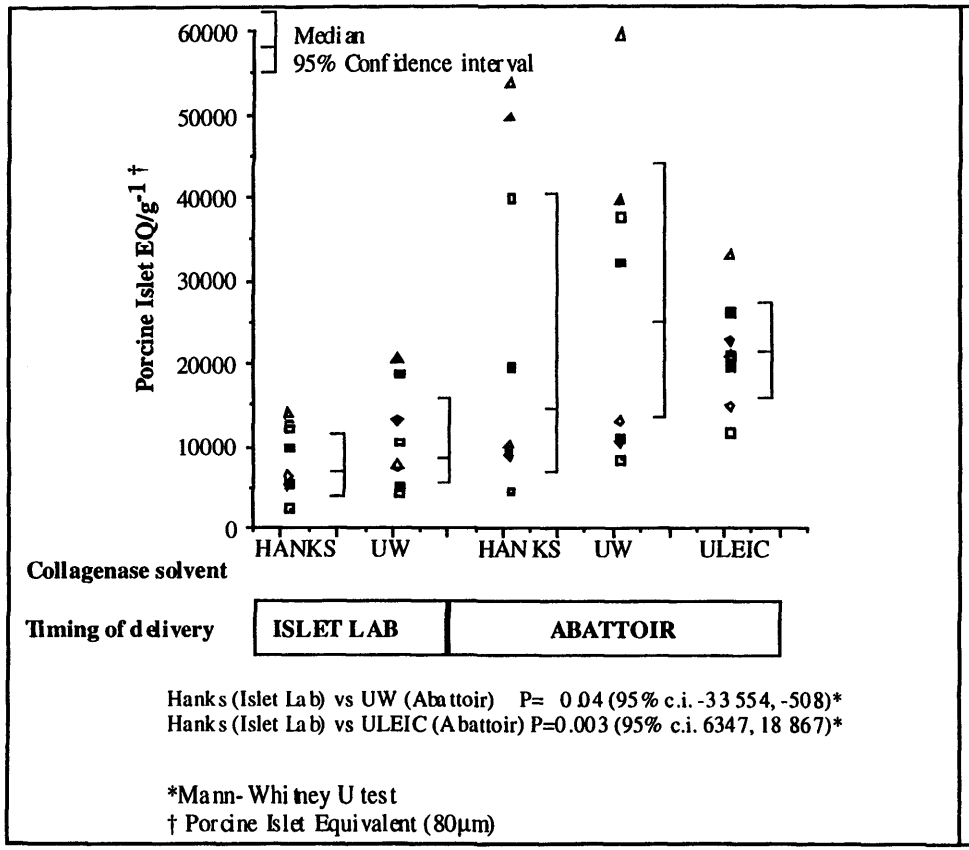
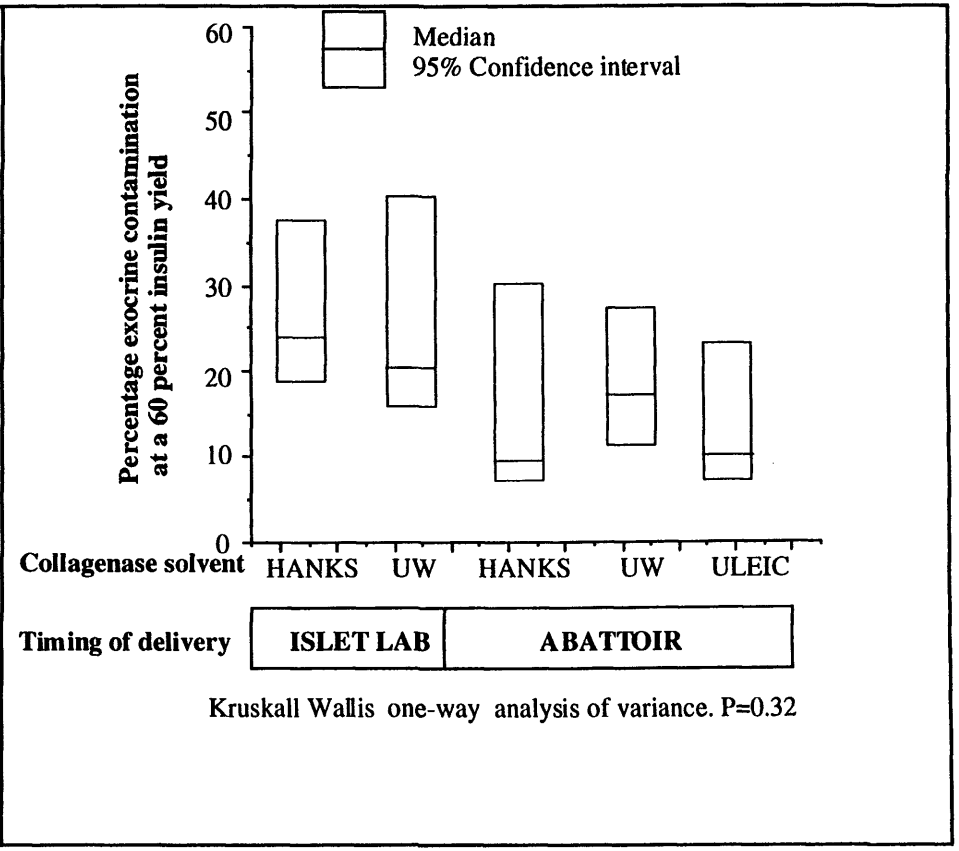


Figure 6.14 Difference in porcine islet yields



6.15 Difference in density gradient purification

compared with those obtained with HBSS at the islet laboratory (median 6,924 IEQ/g^{-1}*versus* 21,524 IEQ/g^{-1}; *P* =0.02; 95% c.i. -33554, -508; *Figure 6.14).* Collagenase administration in ULEIC at the abattoir was also shown to be superior to the conventional method of delivery in HBSS in the islet laboratory (P=0.003; 95% c.i. 6347, 18867). When collagenase was administered in ULEIC, islet yields were similar to those of collagenase administration in UW at the abattoir (median 19,814 IEQ/g^{-1} *versus* 21,524 IEQ/g^{-1}; P =1.0; 95% c.i. -11699, 20762; *Figure 6.14).* Finally, the differences between islet-acinar cleavage index and the mean volume of an islet were not statistically significant *(Table 6.2).*

Density gradient purification and stimulated insulin release

BSA continuous density gradient purification studies demonstrated a range of median values of amylase contamination at a 60% insulin yield between 9% and 24% , none of which differed significantly (P =0.32; *Figure 6.15 p.176).*

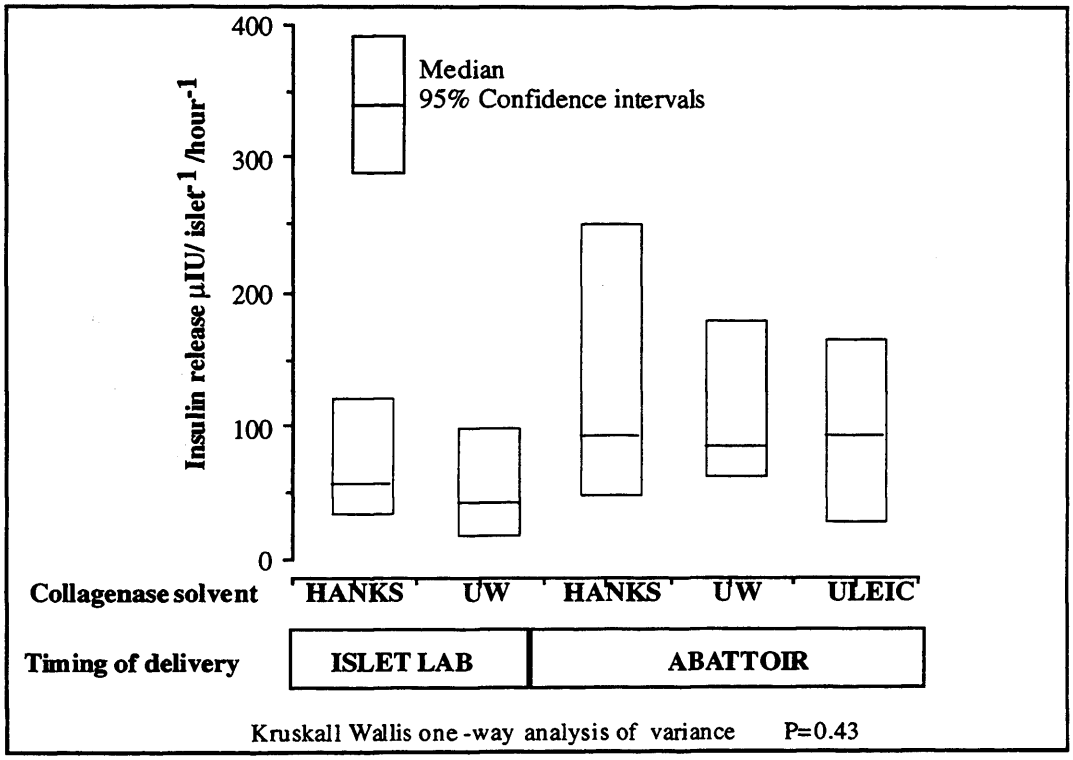


Figure 6.16 Effect upon *in vitro* islet viability

The effect of administering collagenase in either UW or ULEIC, when compared to the appropriate controls, showed no significant difference in the glucose stimulated insulin release (P= 0.43; *Figure 6.16).*

6.4. Discussion

It is well established that short periods of cold pancreas ischaemia before islet isolation are deleterious to both yield (Hesse *et al.* 1987b; Warnock *et al.* 1987b; Lakey *et al.* 1995) and viability (Field *et al.* 1989). Even after as little as three hours of cold pancreas ischaemia, canine islet yields have been shown to be significantly reduced (Hesse *et al.* 1987b). In an attempt to improve cold ischaemic tolerance of rat pancreata Ohzato *et al.* (1991) demonstrated the benefit of early intraductal collagenase administration in HBSS solution at the time of harvesting. Munn *et al.* (1989b) were the first to describe the technique of collagenase administration in a cold storage solution using a modified UW in both rats and dogs. It was shown that preservation with modified UW and collagenase before islet isolation, maintained islet yields for up to 24 hours. The results in this study confirm this hypothesis in the pig, in that regardless of cold storage solution used, islet yields were markedly improved by intraductal collagenase distension immediately after harvesting before any period of cold pancreas ischaemia.

However, in humans a number of reports have suggested an adverse effect of UW (Robertson *et al.* 1993a; Casanova *et al.* 1994; Contractor *et al.* 1995a). Indeed, it has been reported recently that UW is inhibitory to human but not to adult porcine pancreas digestion (Contractor *et al.* 1995a) and could ideally be used, therefore, as a solvent for early intraductal collagenase administration to ameliorate cold pancreas ischaemic damage subsequent to porcine islet isolation.

Notwithstanding the benefits of UW for porcine islet isolation, it is an expensive and complex solution. Some of the additives are thought not to be crucial for its success in preservation of liver (Sumimoto *et al.* 1989), kidney (Wahlberg *et al.* 1989) and pancreas (Morel *et al.* 1992b). Other studies (Chadwick *et al.* 1993a; Chadwick *et al.* 1994; Contractor *et al.* 1995a;) have indicated that adenosine, allopurinol, glutathione and the reversed sodium ion to potassium ion ratio are not essential for cold storage of pancreatic tissue. The key components of UW considered to be of benefit are the impermeants raffinose and lactobionate along with the expensive synthetic colloid HES (Belzer and Southard, 1988; Ploeg *et al.* 1992) used to reduce hypothermic induced cellular swelling. While the role of impermeants is well established, that of HES is less well understood (Morel *et al.* 1992b). For preservation of both canine kidneys and pancreas (Morel *et al.* 1992b; Baatard *et al.* 1993; Candinas *et al.* 1996) results have been as effective when HES is replaced with cheaper additives such as dextran-40. Studies upon porcine islet isolation also support this finding

(Chadwick *et al.* 1994). Moreover, those of human islet isolation have shown some degree of collagenase inhibition by HES perhaps by altering the configuration of the active site of collagenase (Contractor *et al.* 1995a; Burgmann *et al.* 1992), although porcine islet isolation remains unaffected (Contractor *et al.* 1995a). ULEIC was, therefore, specially designed to contain only those components considered beneficial to cold pancreas storage before islet isolation. The main components are lactobionate, raffinose, dextran -40 and a physiological sodium ion to potassium ion ratio.

The findings from this study suggest that collagenase admininstration in either UW or ULEIC have no inhibitory action upon collagenase digestion compared to controls (HBSS). No significant difference could be demonstrated between any of the five groups in terms of islet purification or islet viability *in vitro* ; although the ultimate assessment of long-term *in vivo* islet viability would be by porcine islet autotransplantation studies. However, success has rarely been achieved with that model and *in vitro* function was chosen as an end point (Pattou *et al.* 1995). In summary, therefore, early collagenase delivery at the abattoir in any solution before cold pancreas ischaemia was a prerequisite to markedly greater islet yields. Significantly better islet yields were achieved with collagenase in UW (£116 per litre) or ULEIC (£28 per litre) administered immediately before cold pancreas ischaemia, than with the conventional method of collagenase delivery at the islet laboratory in HBSS following cold pancreas ischaemia. Futhermore, ULEIC solution is the more cost-effective cold storage solution for porcine islet isolation and the benefits of both UW and ULEIC may become more apparent following a longer duration of cold ischaemia.

CHAPTER SEVEN

THE ACTIVATION OF PANCREATIC EXOCRINE ENZYMES DURING PORCINE PANCREAS DIGESTION

7.1 Introduction

Collagenase has the ability to enzymatically separate islet tissue from surrounding acinar tissue. This process then allows islets to be purified from acinar tissue on a density gradient because of the differences in buoyant densities between islets and acinar tissue. However, this selective dissociation of extracellular pancreatic tissue does not always occur and islets can remain enveloped by acinar tissue. The other extreme is that islets can be over digested and become fragmented or dispersed into single beta cells. It is thought that these observations are mainly attributable to batch variation between collagenases (Johnson *et al.* 1996b). Other possible causes of variable yield include donor factors (e.g. age; Benhamou *et al.* 1994b), pancreatic ischaemia (Lakey *et al.* 1995), pancreatic collagen content; van Suylichem *et al.* 1995b) and the technique of pancreas distension (Villani *et al.* 1994; Khalaf *et al.* 1995; Johnson *et al.* 1996a).

One of the least considered areas that could potentially influence the collagenase digestion phase is the release of endogenous pancreatic enzymes (e.g.trypsin, chymotrypsin and elastase etc.) during automated islet isolation (Traverso and Abou-Zamzam, 1978; Heiser *et al.* 1994b). At the time of organ retrieval these enzymes are in their "inactive" proform within the native pancreas but as collagenase degrades pancreatic connective tissue they are released into the automated circuit and become activated into their "active" form (Traverso and Abou-Zamzam, 1978). How these enzymes are activated from the perspective of islet isolation is not entirely known but it has been suggested that it is the inherent impurities within commercial collagenase, particularly trypsin (Traverso and Abou-Zamzam, 1978). Theoretically, activated enzymes have the potential to damage acinar and endocrine cells, particularly those that can digest cell surface components e.g. lipids and proteins.

The purpose of this study, therefore, was to evaluate the activation of the porcine pancreatic protease enzymes trypsinogen, proelastase, chymotrypsinogen, procarboxypeptidase A, procarboxypeptidase B, prolipase and prophospholipase A_2 during porcine islet isolation. Amylase and kallikreininogen were excluded from

analysis because amylase being more relevant to carbohydrate metabolism and kallikrein as it totals less than 0.4% of all pancreatic exocrine secretions (Rinderknecht, 1986).

7.1 Materials and methods

Animals. Four adult porcine pancreata (75 to 100 g) were retrieved from the local abattoir (Dawkins International, Congerstone, UK) and transported to the islet isolation laboratory in HOC (Baxter Health care Ltd, Thetford, UK) at 4°C.

Islet isolation and enzyme collection. Following approximately 60 minutes cold ischaemia each pancreas underwent automated islet isolation (Ricordi *et al.* 1990c) with ULEIC/collagenase digestion as previously described (Chapter Six). The distended pancreas was placed into the automated chamber and brought up to 37°C (approximately two minutes). At this time point (zero minutes) the chamber was shaken and immediately inverted and a 5 ml sample of circulating HBSS (Gibco, Life Technologies, Faisley, Scotland) was aspirated from the 3-way biopsy tap situated immediately below the outlet port of the chamber *(Figure 7.1)* . The biopsies were then filtered and centrifuged at 1500g for five minutes (CR422, Jouan, Herts,UK) to

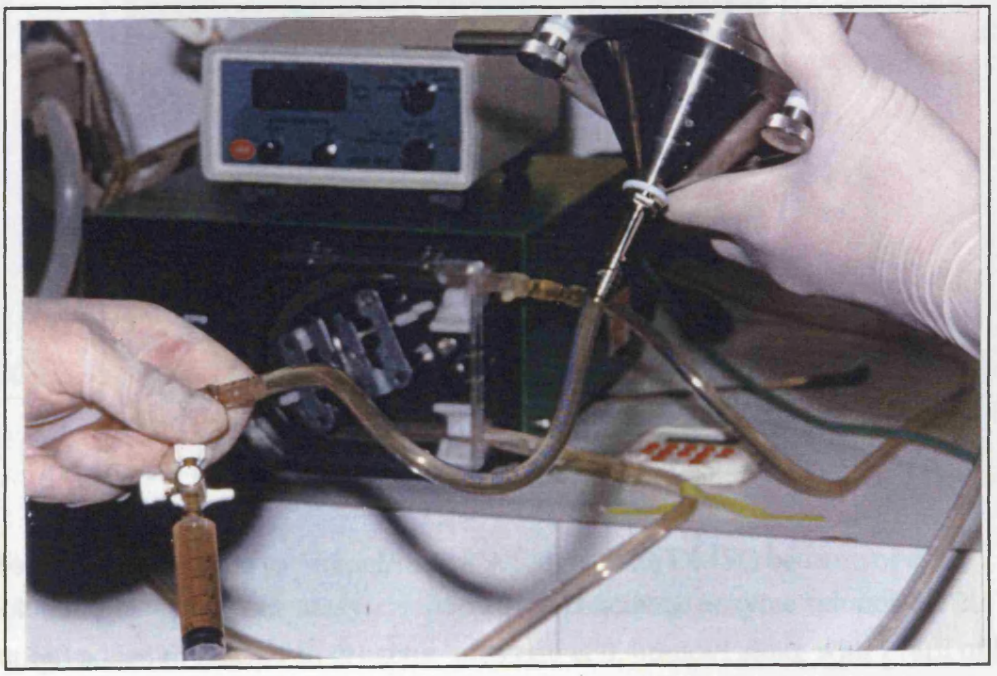


Figure 7.1 Pancreatic enzyme sampling

remove all pancreatic tissue fragments *(Figure 7.2 p.182)* . The supernatant was aspirated and immediately transferred to an Eppendorf for storage at -20°C. Further

biopsies were similarly repeated at five minute intervals up to and including 55 minutes when pancreatic digestion was deemed to be completed

Trypsin, chymotrypsin and elastase Assay. The fluorogenic compounds 7-(N-Benzoyl-DL-argininamido)-4-methylcoumarin for trypsin, N-Acetyl-Ala-Ala-Ala-Pro-Ala-4-methylcoumarin for elastase and Glutaryl-Phe-4-methylcoumarin for chymotrypsin (Sigma Diagnostics,UK) were used as assay substrates. These substrates are highly stable and the cleaved 7-amino-4-methylcoumarin is highly fluorescent. Each substrate is specific for its particular enzyme (Zimmerman *et al.* 1977; *Table 7.1 p.184).*

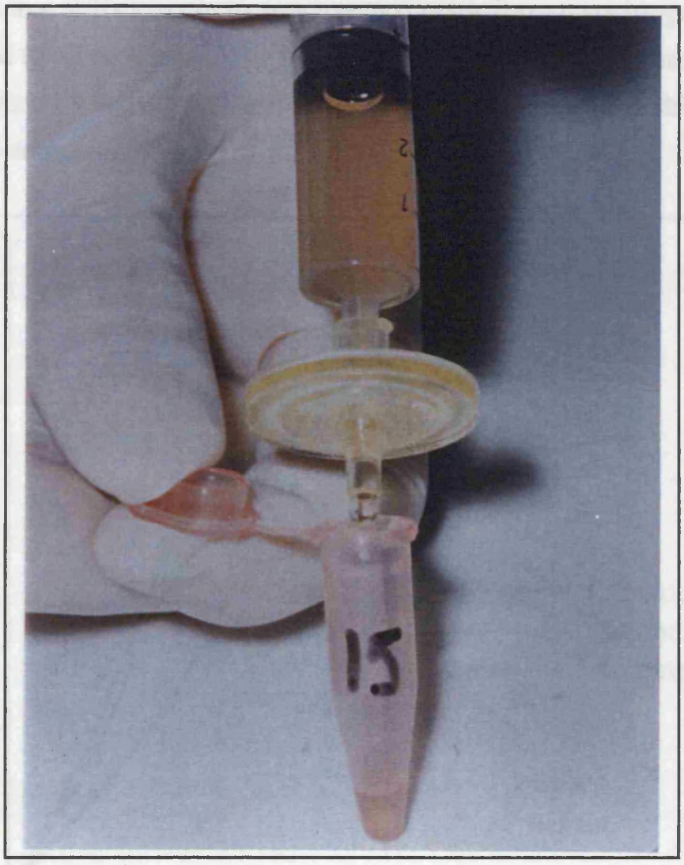


Figure 7.2 Filtering pancreatic enzyme sample (15min)

Substrates were diluted to 5mmol/l stock solutions with DMSO because of their insolubility in water. The assay entailed 2µl of pancreatic enzyme solution (1: 20 dilution) added to 20µl of fluorogenic substrate at 0.4mmol/l along with 178µl of hepes at pH 7.5 (Whatman, Singapore), incubated for 10 mins at 37°C. Appropriate negative controls comprised a further 2µl of hepes in replacement for pancreatic enzyme solution and positive controls utilised purified trypsin, chymotrypsin and elastase. The final volumes were all 1ml. Each individual sample was performed in duplicate. The reaction was halted by the addition of 2ml of distilled water. The cleaved 7-amino-4-

methylcoumarin peptide was measured with a luminescence spectrometer (Perkin-Elmer, UK), activation and emission wavelengths of 380nm and 460nm respectively, with an excitation slit width of 10nm (Zimmerman *et al.* 1977).

The percentage activation was calculated as follows. Within the native pancreas endogenous pancreatic exocrine enzymes exist in their inactive forms. For example, trypsin exists as trypsinogen (inactive precursor) and when activated by enterokinase it becomes trypsin (active form). During automated islet isolation both forms could potentially exist and this experiment was designed to calculate the percentage of activated enzyme relative to the total potentially activatable percentage i.e. the percentage that still exists in its inactive precursor form trypsinogen. This technique was applied to all enzymes. The percentage that existed in its inactive precursor form, was calculated by activating the inactive precursor *in vitro* and this was called the "total percentage activation".

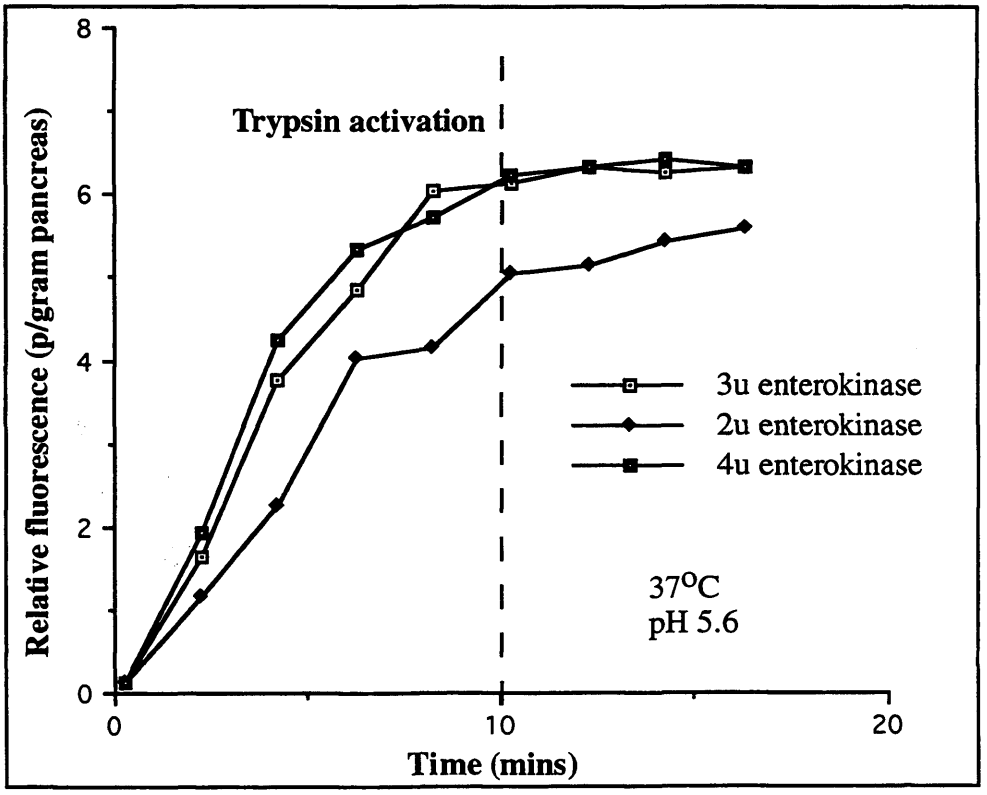


Figure 7.3 Validation of trypsin activation with 3u enterokinase

The *in vitro* enzyme activation of trypsinogen with porcine enterokinase was performed as described by Haverback *et al.* (1960). A provisional validation study *(Figure 7.3)* was carried out to determine the saturation rate of trypsinogen activation with enterokinase. For this study enterokinase was diluted with buffer at pH 5.6 and incubated at 37°C with trypsinogen. Samples were incubated for up to 15 minutes to

estimate the time point at which trypsinogen to trypsin activation reached saturation. This end point was chosen because of the problems of trypsinogen undergoing degradation when allowed to remain at room temperature for long periods at 37°C for greater than 45 minutes. Trypsin was then assayed using the nitroanilide chromogenic substrate N-Benzoyl-DL-argininamido-4-methylcoumarin method *(vide supra).* Increasing concentrations of enterokinase ranging from two to four units were assessed. The percentage of trypsinogen to trypsin activation relative to time was plotted graphically at different concentrations of enterokinase. Saturation was reached at 10 minutes incubation and no difference in the total activated trypsin could be observed when greater than three units of enterokinase were used. Therefore for the purpose of this study incubation of all samples requiring activation with porcine enterokinase (Sigma Diagnostics, UK) was with three units incubated for ten minutes. Similar experiments were performed with "trypsin dependent" activation of pro-elastase, chymotrypsinogen, pro-phospholipase A_2, pro-carboxypeptidases A and B and lipase. For these enzymes enterokinase was replaced with trypsin and saturation was reached at a concentration of at 3µg/ml in 10mmol/lTris/HCl buffer. Each series of activated and unactivated assays were performed on the same day so as to allow for standardisation.

ENZYME	SUBSTRATE
TRYPSIN	7-(N-Benzoyl-DL-argininamido)-4-methylcoumarin
ELASTASE	N-Acetyl-Ala-Ala-Ala-Pro-Ala-4-methylcoumarin
CHYMOTRYPSIN	Glutaryl-Phe-4-methylcoumarin
LIPASE	1,2 diglycerides (Lipase PS ®)
CARBOXYPEPTIDASE A	2-Furanacryloyl-L-phenanalanyl-L-phenanalanine
CARBOXYPEPTIDASE B	(2-Furanacryloyl-L-alanyl-L-lycine)
PHOSPHOLIPASE A_2	Egg yolk suspension (sodium deoxycholate + 1M Ca Cl_2)

Table 7.1 Summary of substrates used for assay

Carboxypeptidase Assays. The most convenient assay for C-A is based on hippuryl-L-phenylalanine or the chromogenic N-carbo-B-naphthoxy-L-phenylalanine substrate because of cleavage at the phenylalanine site (Petra, 1970). Those for C-B use the hippuryl-L-arginine substrate (Folk *et al.* 1960) based on cleavage at the arginine peptide bond. The assay procedures used in this study were a modification of the FALGPA assay described by Van Wart and Steinbrink, (1981). The FAFF (2-Furanacryloyl-L-phenanalanyl-L-phenanalanine) substrate was used for C-A, cleaving through the phe-peptide bond and FAAL (2-Furanacryloyl-L-alanyl-L-lycine) for C-B, cleaving through the lycine residue *(Table 7.1).* These two substrates take advantage

of the chromophore 'furylacryloyl', which upon cleavage leads to increased absorbance when measured at 254nm spectrophotometrically. The two substrates were specific for carboxypetidase activity and showed linear kinetics and have been used for many other specific proteinases (Van Wart and Steinbrink, 1981; *Table 7.1*).

Both substrates were provided in dessicated form and dissolved with DMSO. FAFF was stored at 4°C but FAAL was stored at room temperature because of its propensity for dropping out of solution. The assay procedure consisted of 5µl of 10mmol/l substrate to which 10µl of pancreatic enzyme solution was added, all performed at room temperature. The assay was performed in a final volume of 600µl, the remaining 585µl was made up with Tris/HCl buffer (50mmol/l filtered). The spectrophotometer (Shimadzu Corporation, Japan; *Figure 7.4)* was set to zero and the reaction solutions placed in a silica microcuvette. The pancreatic enzyme solution was added (10µl) and gently mixed with stirring rods and the timer started. The first reading (t=0 minutes) was taken immediately after the readings stabilized. Further readings were taken at two minute intervals up to and including ten minutes. Each sample was assayed in duplicate and the mean of the two recorded. Finally, trypsin activated enzyme proportions were performed following incubation of the pancreas enzyme samples as described for elastase and chymotrypsin *(vide supra).*

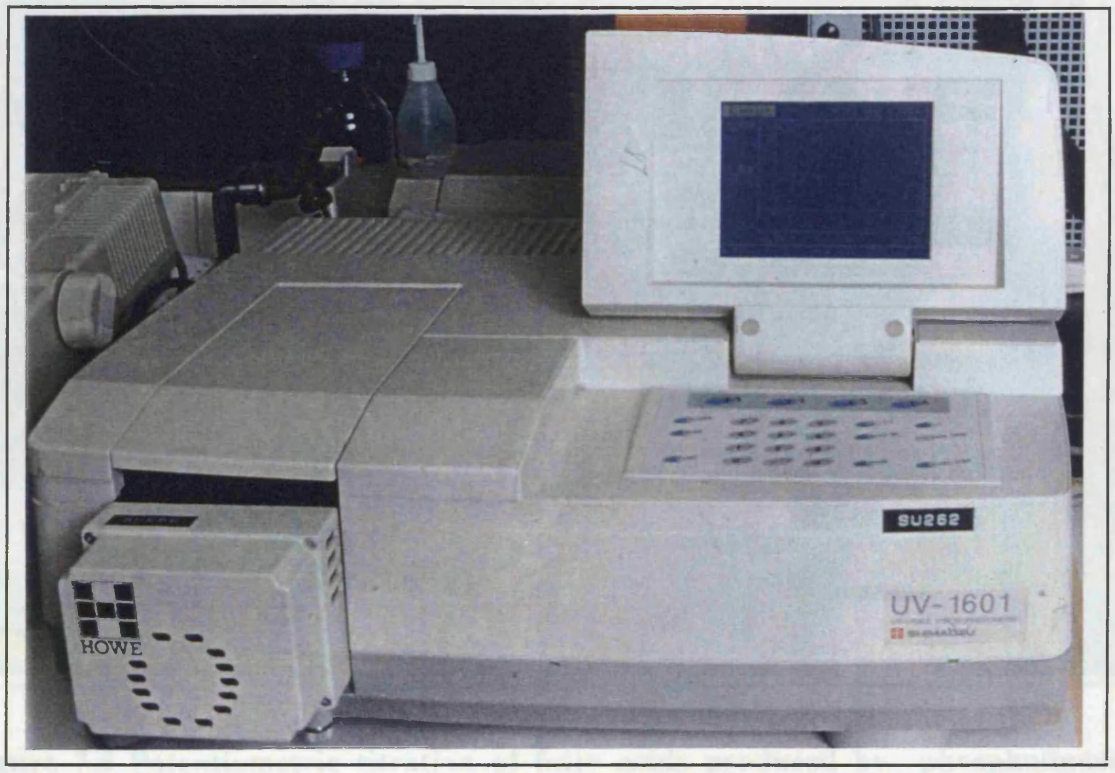


Figure 7.4 UV-spectrophotometer used for carboxypeptidase (A and B) assay

The relative proportion of enzyme was calculated by subtracting the absorbance at t=0 from all the readings taken at two minute intervals and the change in absorbance per minute calculated (Δ Abs per minute).

Phospholipase A_2 assay. This was performed by potentiometric titration of fatty acids produced by its action on an egg yolk emulsion (De Haas *et al.* 1968; Nieuwenhuizen *et al.* 1974). A more accurate method of radioimmunoassay has been developed but substrates were not commercially available in the UK (Nishijima *et al.* 1983).

The substrate for phospholipase A_2 is egg yolk lipoprotein. This substrate consists of a mixture of proteins but is specific for phospholipase A_2 as previous studies have shown no activity with contaminating pancreatic lipase, trypsin, chymotrypsin, and carboxypeptidases. The assay is based on recording the consumption of alkali needed to keep a constant pH when phospholipase A_2 liberates fatty acids (Nieuwenhuizen *et al.* 1974; *Figure 7.5).*

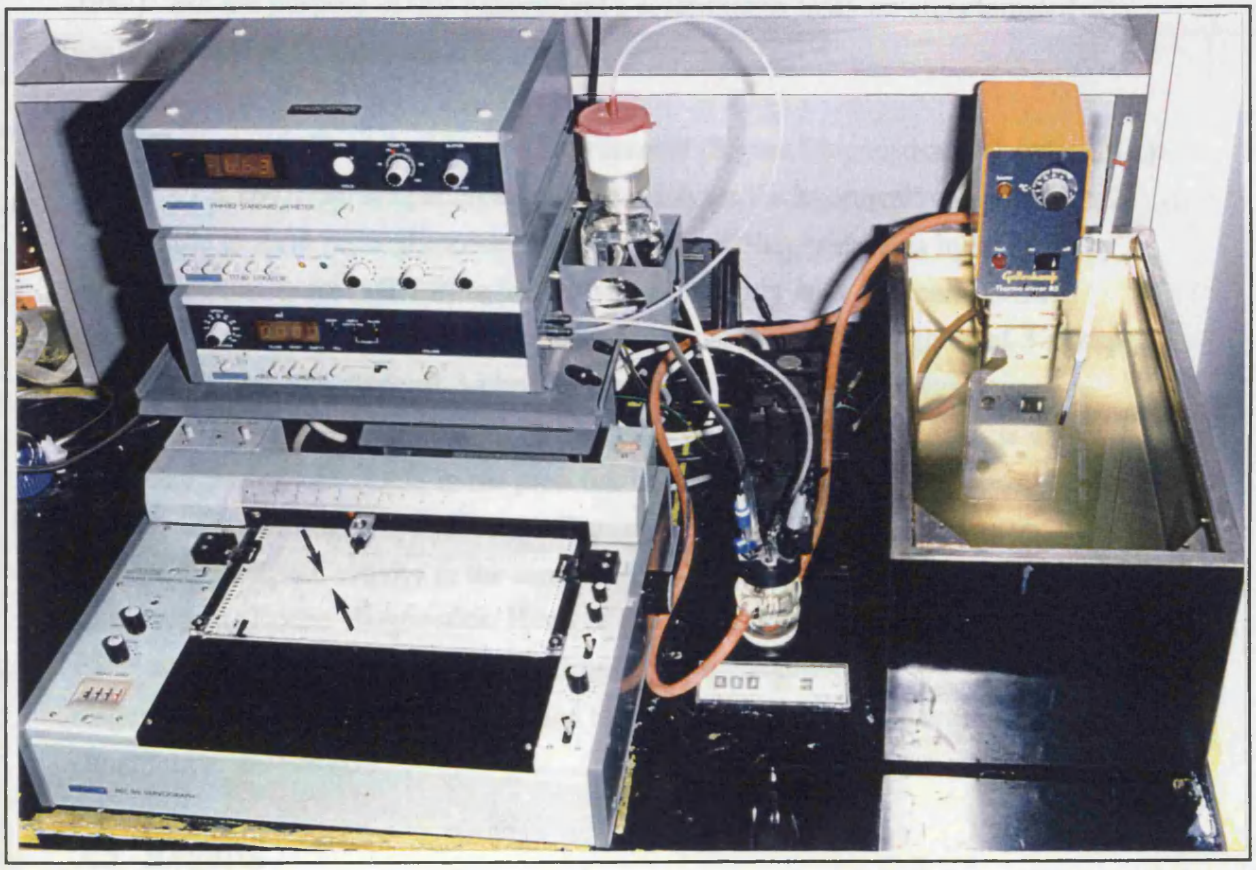


Figure 7.5 Potentiometric titration of fatty acids produced by phospholipase A_2 using a pH stat (see arrows)

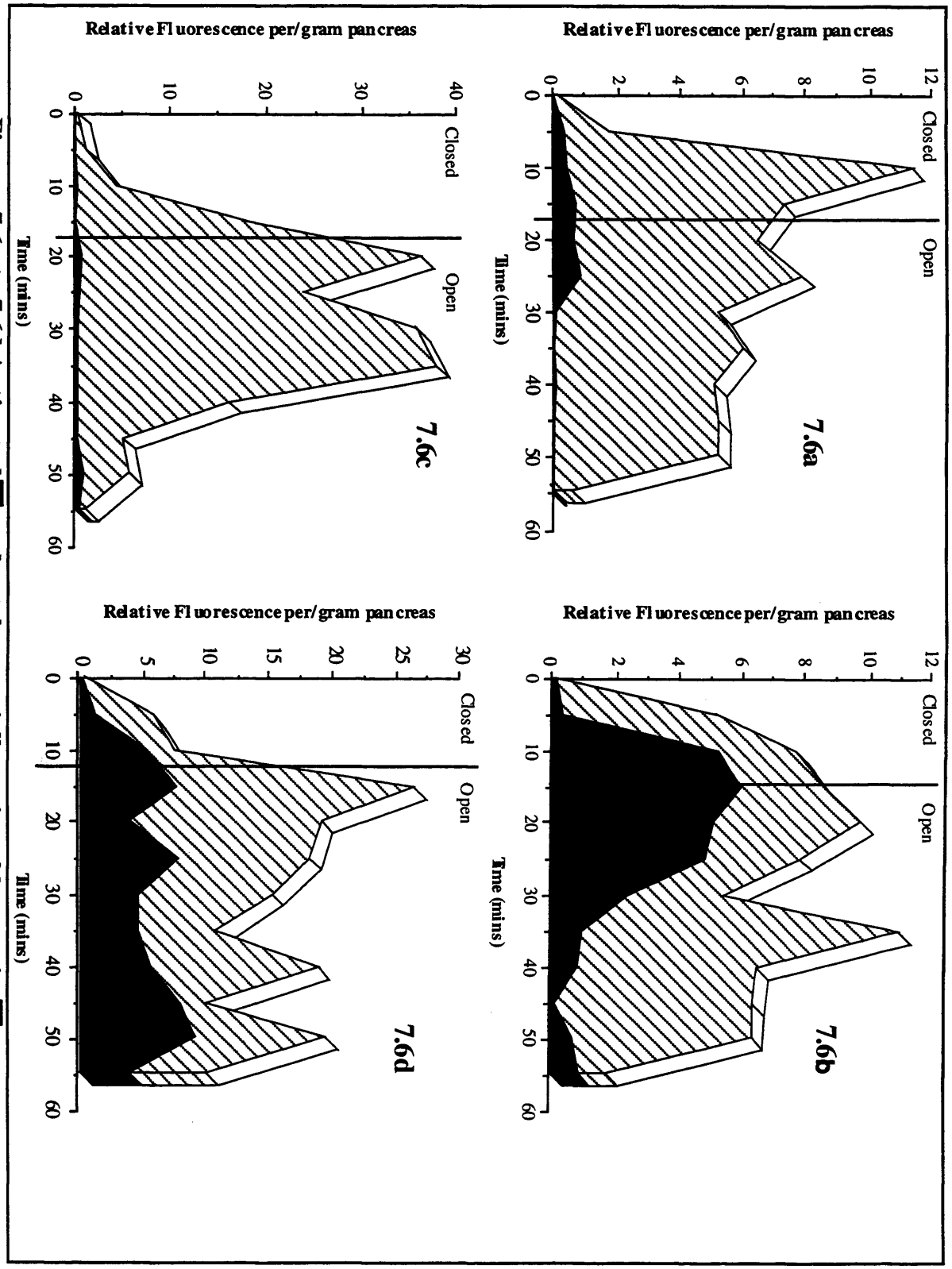
The substrate consists of one egg yolk homogenized in 100 ml of distilled water and stirred for ten minutes *(Table 7.1 p.184)*. To this 4ml of 1M Ca Cl_2 is added. The emulsion is then freed by filtration and 10 ml of suspension is added to 20 ml of 6mmol/l sodium deoxycholate. To perform the assay 2 ml of substrate (egg yolk suspension) was placed into the reaction vessel into which a glass electrode was submerged. The pH was set to 8 by titrating with Na OH (20mmol/l), and a baseline activity recorded for 1 minute. Then 50µl of phospholipase A_2 solution was added. The hydrolysis of substrate liberating fatty acids was recorded automatically by a pH stat. By extrapolating the difference between the baseline gradient and the gradient representing alkali consumption the amount of enzyme within each sample could be calculated (Nieuwenhuizen *et al.* 1974).

Lipase assay. Many different methods of lipase assay have been described including titrimetric (Desnuelle *et al.* 1955; DeCaro *et al.* 1977), turbimetric (Shihabi and Bishop, 1971), fluorometric (Rietz and Guilbault, 1975) and immunological (Grenner *et al.* 1982). For the purpose of this experiment a colorimetric assay on an automated analyser was used (Imamura *et al.*1989).

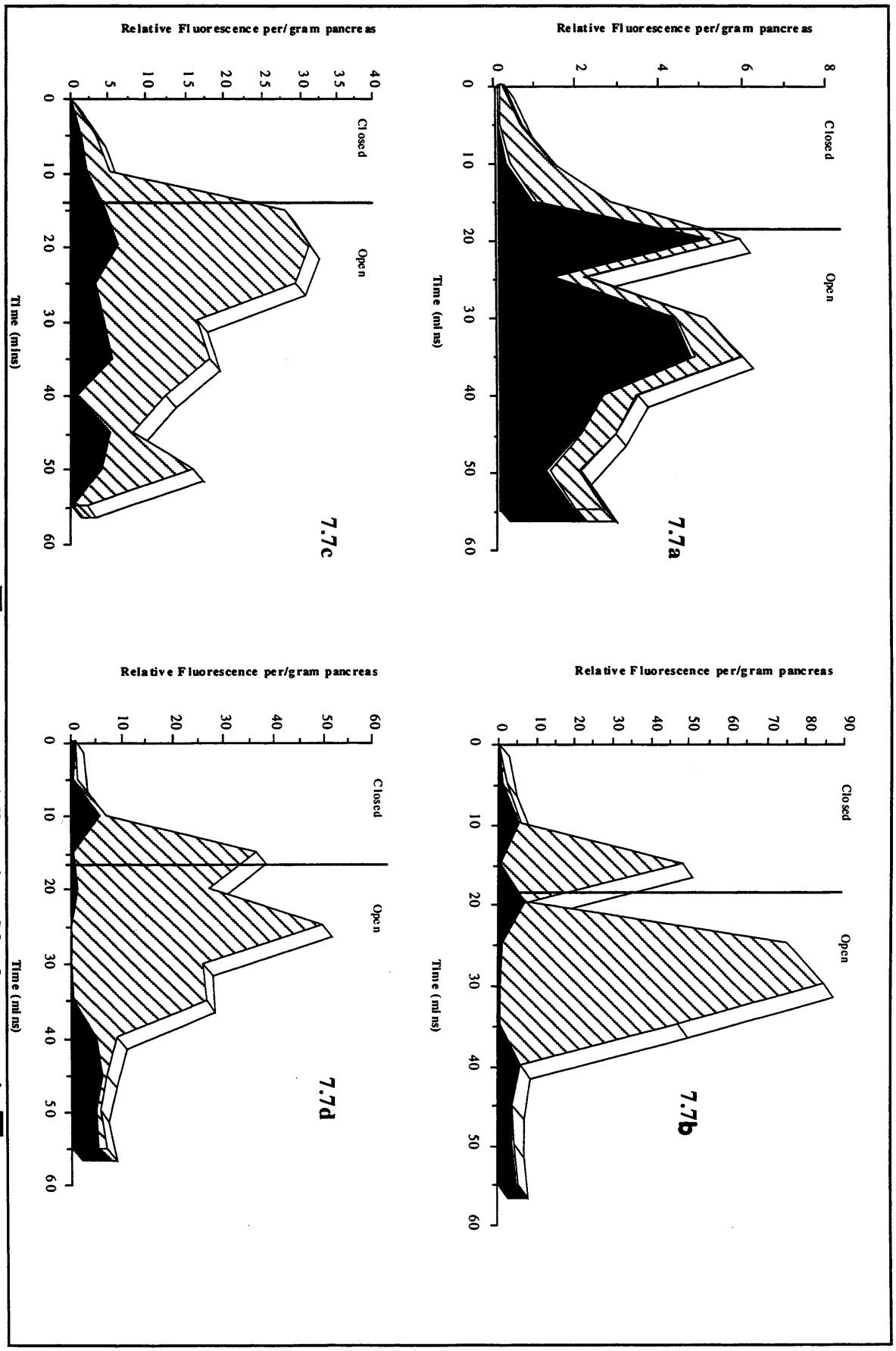
The assay utilised the Lipase-PS ® reagent (Sigma Diagnostics, St Louis, USA) *(Table 7.1 p.184)*. Serum pancreatic lipase catalyses the hydrolysis of a natural 1,2-diglyceride to form monoglyceride and fatty acid. Monoglyceride is hydrolyzed by monoglyceride lipase (MGLP) to form glycerol and fatty acid. Glycerol is then phosphorylated by glycerol kinase in the presence of ATP to form glycerol-3-phosphate which is oxidised by glycerol-3-phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. Subsequently hydrogen peroxide reacts with 4-aminoanti-pyrine and TOOS in the presence of peroxidase to form a quinone diimine dye. The dye absorbs light at 550nm. The rate of absorbance is directly proportional to the pancreatic lipase activity in the sample. This method was applied to a Cobas Bio autoanalyser (Roche diagnostics, Herts ,UK). To perform the assay 120µl of Lipase-PS ™ substrate was mixed with 2µl of lipase enzyme solution and to 40 µl of start reagent. The increase in absorbance due to the quinonediimine dye was measured (Imamura *et al.* 1989).

7.3 Results

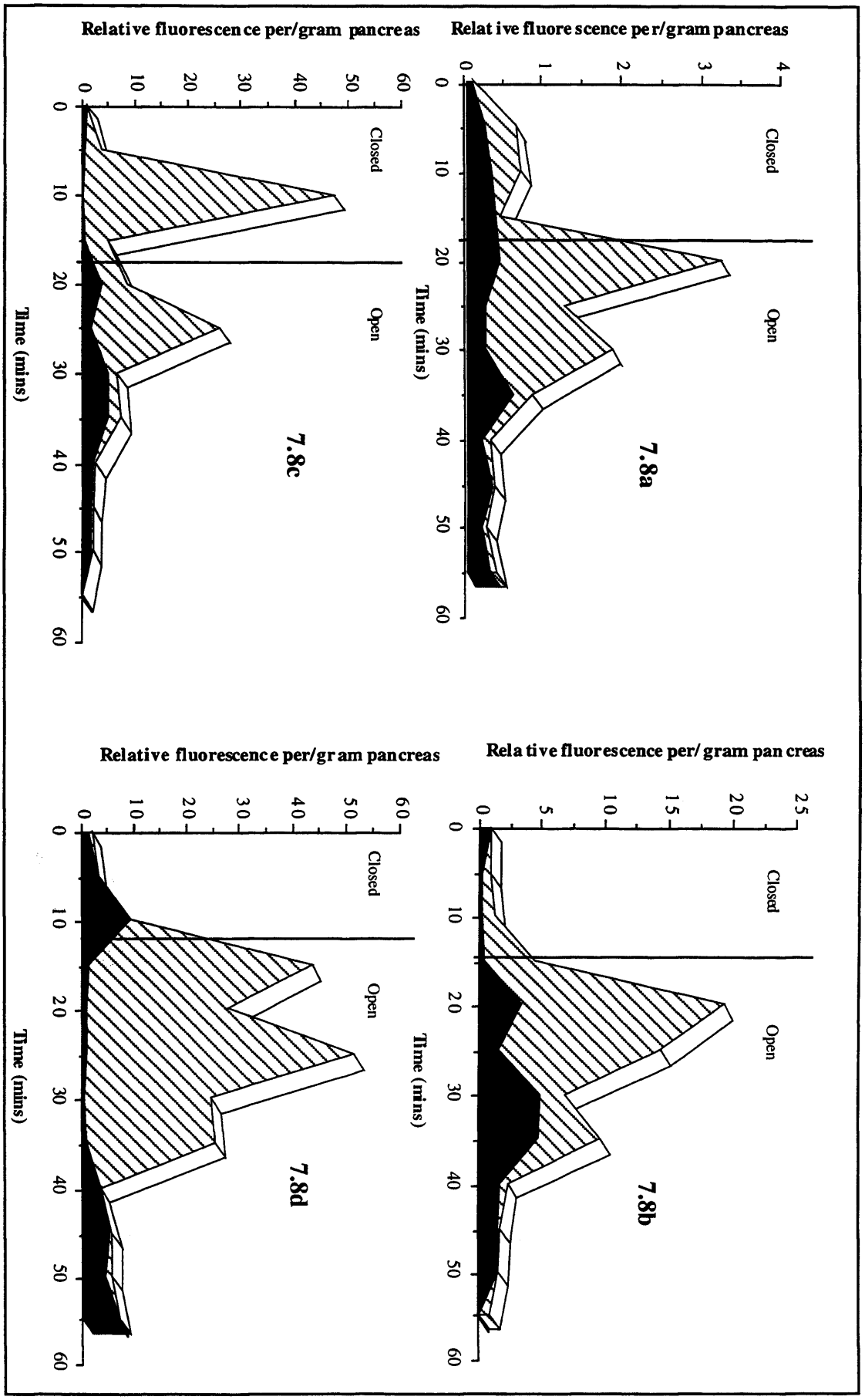
Seven endogenous pancreatic enzymes were studied. The profiles of both activated and the total (potentially activatable) enzyme release were plotted graphically for each individual enzyme during the closed and open circuits. Graphs were plotted in terms of enzyme release per gram of pancreas against duration of automated porcine



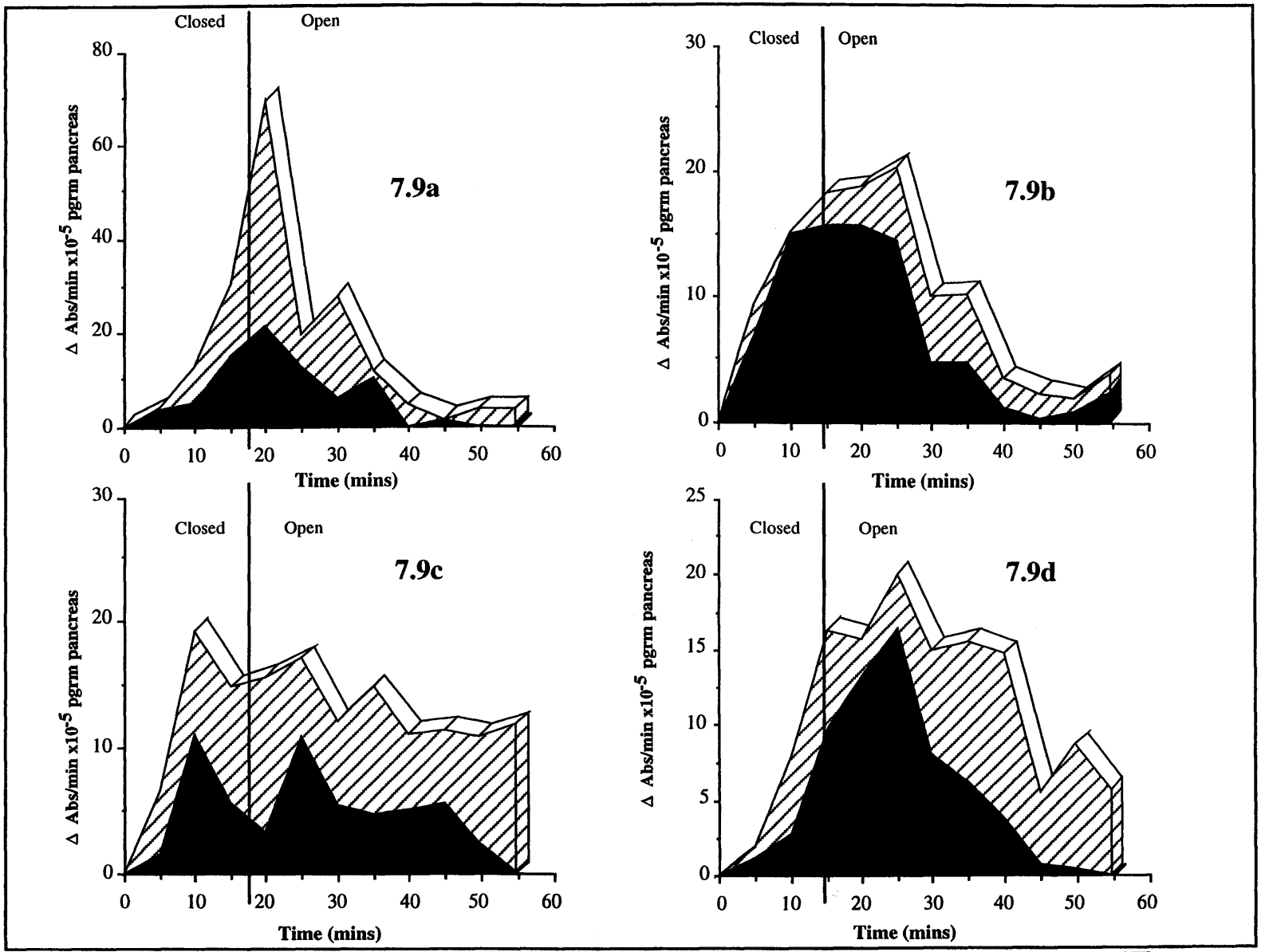
Figures 7.6a to 7.6d Activated ■ and total potentially activatable trypsin ▨



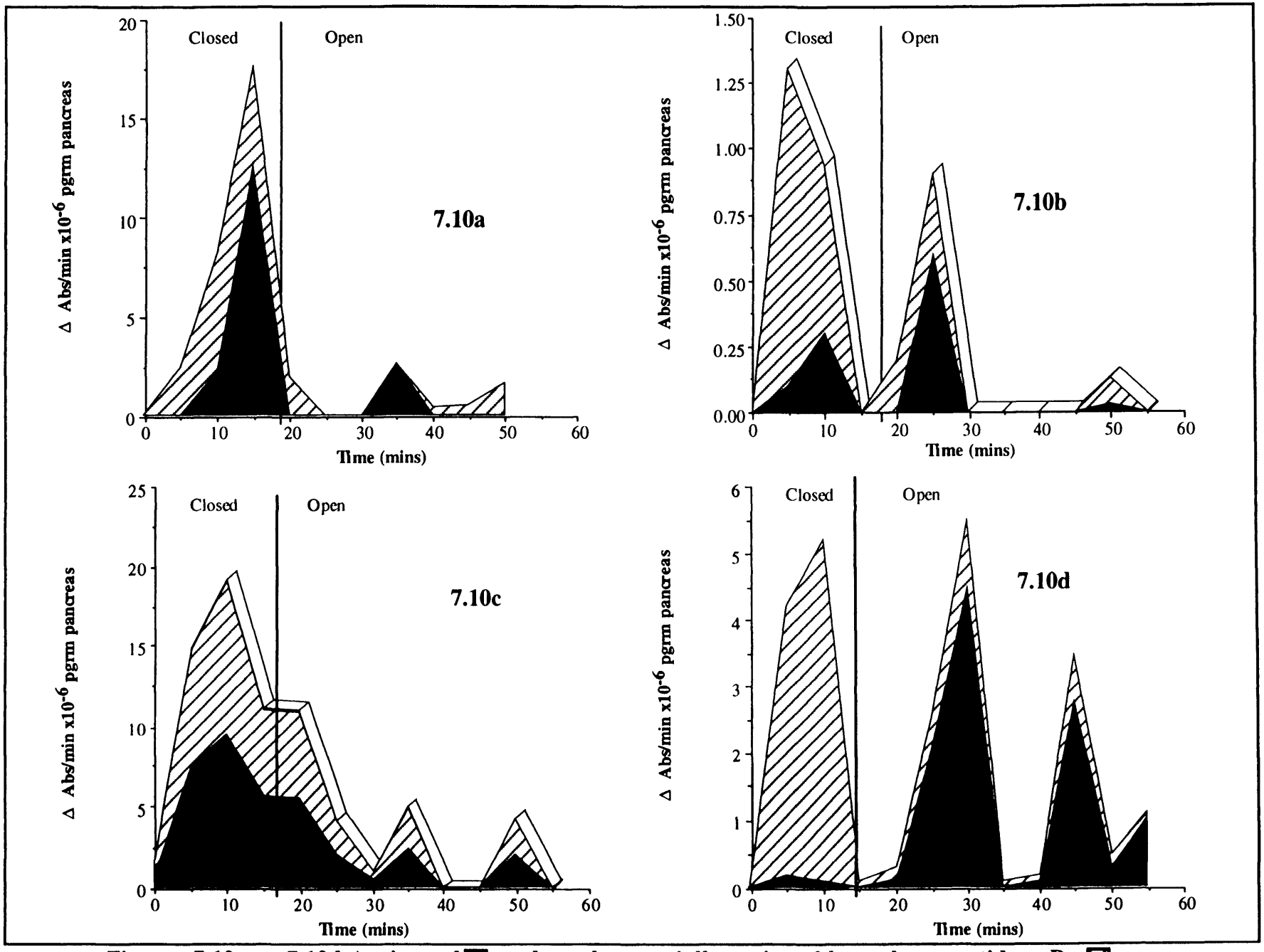
Figures 7.7a to 7.7d Activated ■ and total potentially activatable chymotrypsin ▨



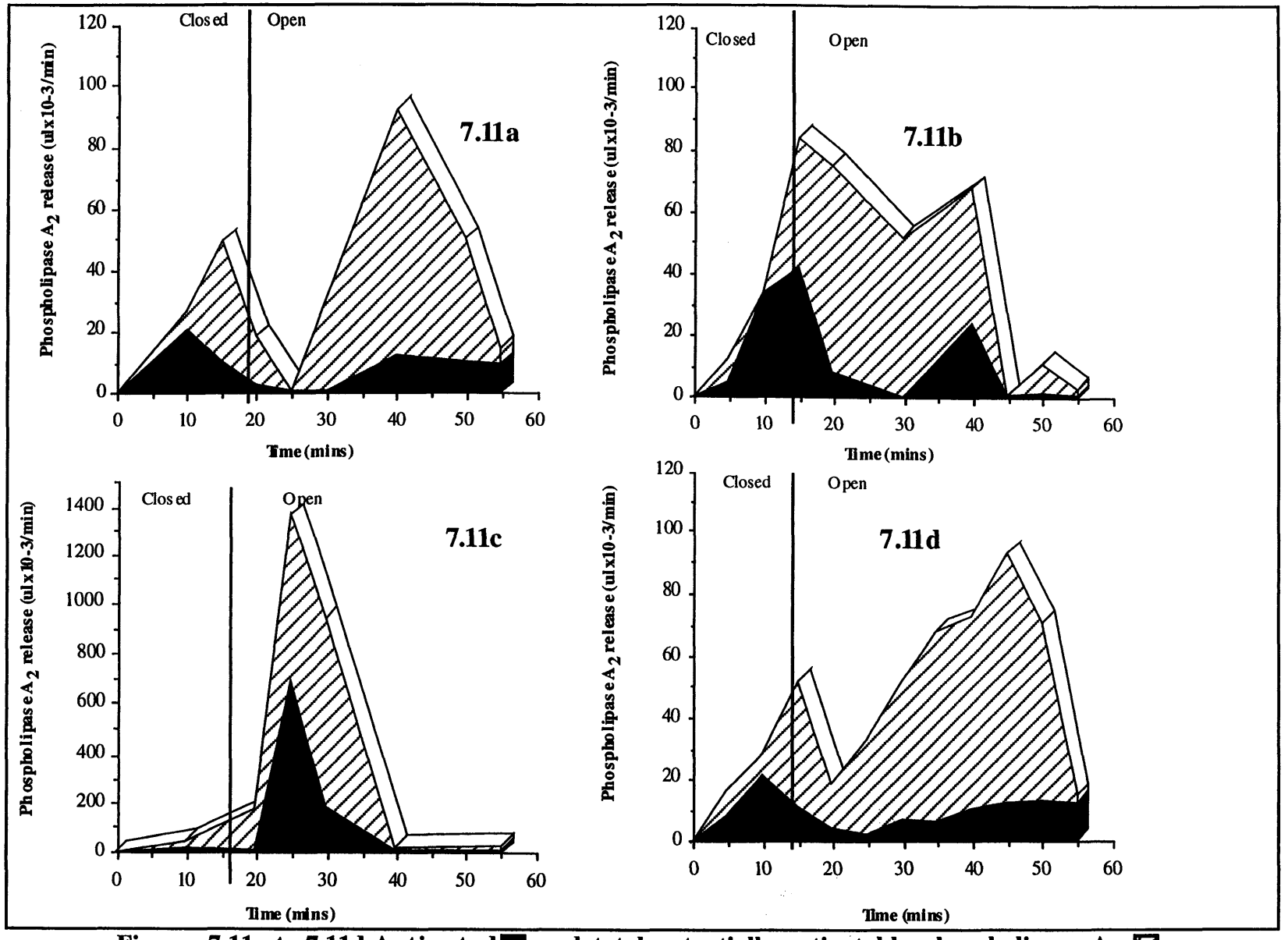
Figures 7.8a to 7.8d Activated ■ and total potentially activatable elastase ▨



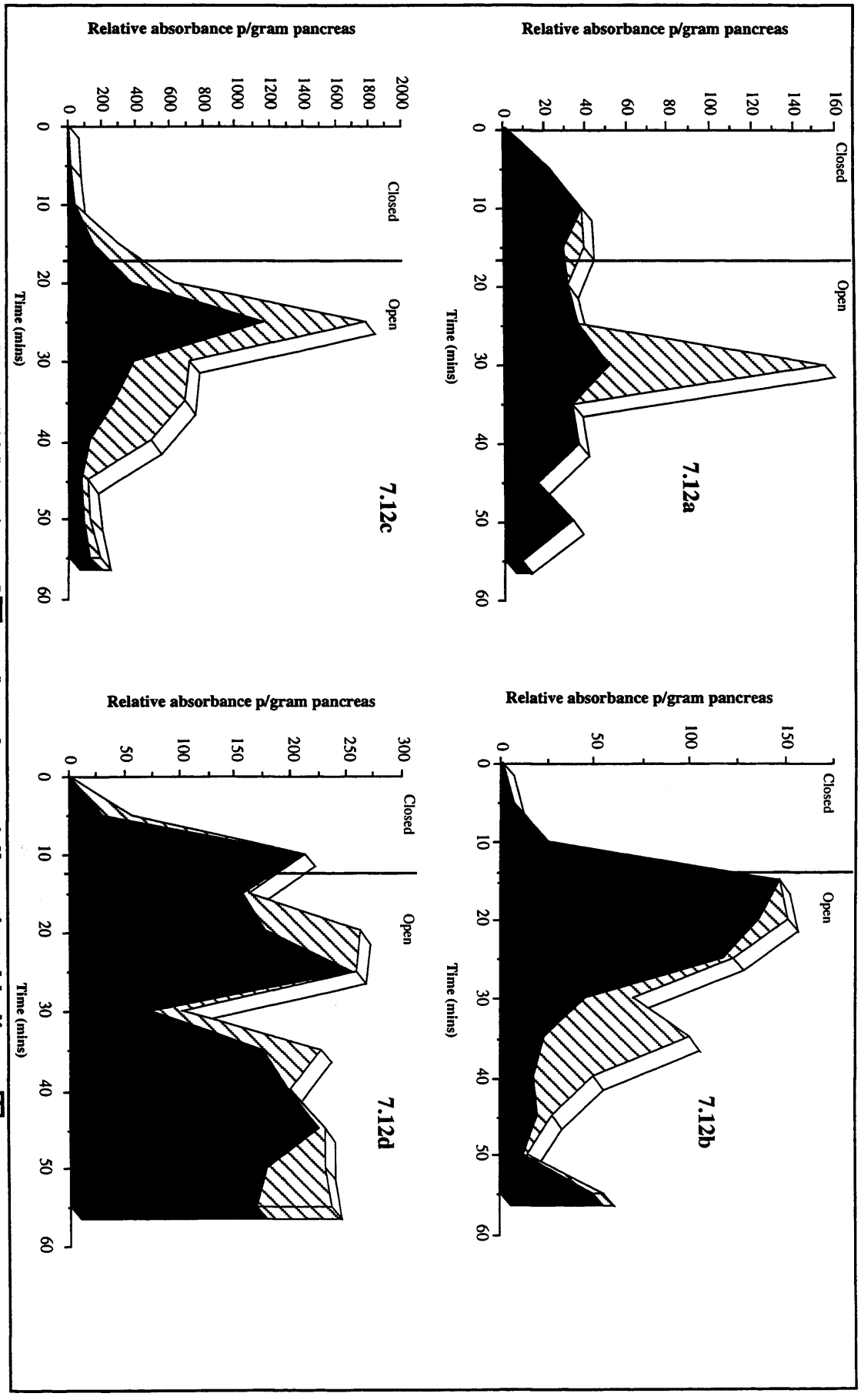
Figures 7.9a to 7.9a Activated ■ and total potentially activatable carboxypeptidase A ▨



Figures 7.10a to 7.10d Activated■ and total potentially activatable carboxypeptidase B ▨



Figures 7.11a to 7.11d Activated■ and total potentially activatable phospholipase A $_2$☒



Figures 7.12a to 7.12d Activated ■ and total potentially activatable lipase ▨

Table 7.2 Summary of activated and total (potentially activatable) pancreatic exocrine enzyme release during automated porcine islet isolation

Enzyme (units of activity)*	Time (min) Peak Activity		Peak Activity g/pancreas		Percentage Activation
	Activated	Total	Activated	Total	
Trypsin (Relative Fluorescence)	23	38	3.3 (0.66-9.03)	19.91 (10.91-37.56)	19.15 (1.74-53.87)
Chymotrypsin (Relative Fluorescence)	20	23	6.27 (5.00-7.12)	40.91 (5.73-84.37)	51.98 (12.55-87.99)
Elastase (Relative Fluorescence)	30	20	2.72 (0.56-9.04)	31.78 (3.22-51.37)	13.80 (4.11-18.51)
Carboxypeptidase A (Δ Abs $x10^{-5}$/min)	15	18	13.8 (11.1-16.4)	21.2 (19.2-69.0)	53.68 (30.4-80.0)
Carboxypeptidase B (Δ Abs$x10^{-6}$/min)	20	13	8.1 (0.67-12.)	10.9 (1.2-17.5)	70.77 (55.83-82.01)
Phospholipase A2 (FFA release/µl/min/$x10^{-3}$)	13	33	31.8 (21.2-42.6)	92.2 (81.1-1320.1)	51.99 (42.86-53.26)
Lipase (Relative absorbance)	25	23	199.8 (51.4-1168.1)	202.8 (146.3-1777.4)	80.65 (35.13-99.0)

† Median (Range) values at time of maximal activation (n=4); * units per gram of pancreas.

pancreas digestion. The profiles from four islet preparations were compared for each individual enzyme. The time of the peak of maximal active enzyme release during the digestions are summarized in *Table 7.2 (p.195).*

Generally, it can be seen that during the closed (recirculating) circuit phase of pancreas digestion there were both gradual and rapid increases in the levels of enzymes released. This to a certain extent reflecting the variable rates of pancreatic dispersion and the activation cascade of endogenous pancreatic enzymes released within the automated chamber. In general the trends of enzyme activation were extremely variable.

Trypsin, *(Figures 7.6a to 7.6d p.188)* activation demonstrated variability between all isolations. The median time of maximal activation occurred at 23 minutes (n=4) but potentially higher levels could have occurred if the trypsinogen was totally activated. The median time of maximal total (potentially activatable) trypsinogen occurred much later at 38 minutes. During two preparations trypsin *(Figures 7.6a to 7.6d)* was mostly in its trypsinogen form, although moderate levels were released in the other two. There was also at least a 10-fold difference between the peaks of maximum release (0.66 *versus* 9.03; Fluorescence/g pancreas) through the four preparations. Interestingly, in isolation four *(Figure 7.6d)* trypsin release showed an increasing trend towards the end of the open digestion phase.

The profiles of chymotrypsin release showed a similar pattern of release *(Figures 7.7a to 7.7d p.189).* As with trypsin release chymotrypsin gradually increased during the closed circuit reaching maximal release during the open circuit phase. The median time of peak activated chymotrypsin was at 20 minutes similar to the peak of the potentially activatable chymotrypsinogen. The vast majority of chymotrypsin existed in its inactive chymotrypsinogen form, with the exception of one isolation where most was completely activated *(Figure 7.7a).* There was little variation between the maximum peak release of activated chymotrypsin or total potentially activatable chymotrypsinogen release (5.00 *versus* 7.12; Fluorescence/g pancreas). There was a 15-fold (5.75 *versus* 84.37; Fluorescence/g/pancreas) difference between isolations for total potentially activatable chymotrypsinogen.

The profiles of elastase release *(Figures 7.8a to 7.8d p.190)* demonstrate that it is mostly in its inactive proelastase state, although there was still a 15 fold difference between the peaks of release for total potentially activatable proelastase (3.22 *versus* 51.37; Fluorescence/g pancreas) values *(Table 7.2 p.195)* . Peak activated elastase release occured during the middle of the digestion phase (30mins) but still showed a 20 fold difference between the 4 islet preparations (0.56 to 9.04; Fluorescence/g pancreas).

The release and activation of both carboxypeptidase A *(Figures 7.9a to 7.9d p.191)* and carboxypeptidase B *(Figures 7.10a to 7.10d p.192)* also demonstrated variability between preparations. Carboxypeptidase A was generally in its active form with more consistent peak values ranging from 11.1 to 16.4 Δ Abs $x10^{-5}$/min/g pancreas. In the case of activated carboxypeptidase B multiple peaks occurred throughout and there was also an 18-fold difference (0.67 to 12 Δ Abs $x10^{-6}$/min/g pancreas) in the median peak value range between islet preparations. The total potentially activatable procarboxypeptidase B median peak was similar to that of the activated peak indicating that the majority existed in its active state during porcine islet isolation, although values were very small (10^{-6}) similar to the low levels for carboxypeptidase A (10^{-5}) .

The median peak of total potentially activatable phospholipase A_2 *(Figures 7.11a to 7.11d p.193)* release occurred at a much later stage during the open phase of pancreas digestion compared to activated phospholipase A_2 (13 mins *versus* 33 mins). Once again this enzyme showed a capacity for further potential activation. During three preparations the levels of activated phospholipase A_2 were low compared to the total potentially activatable enzyme (median peak values 31.8 *versus* 92.2 FFA release/µl/min/$x10^{-3}$ g pancreas; *Table 7.2).* In one preparation there was a 30-fold increase *(Figure 7.11a)* in the value of activated phospholipase A_2 in comparison to the other isolations.

Finally, lipase was similar to the carboxypeptidase enzymes in that it was generally in its active form in all four isolations (median peak values 199.8 *versus* 202.8. absorbance/g pancreas). There was also a 20-fold difference in the range of peak values (51.4 to 1168.1 relative absorbance/g pancreas). Each isolation demonstrated a gradual increase during the closed phase of islet isolation with both the activated and total potentially activatable enzyme peaking at similar times (25 *versus* 23 minutes; *Figures 7.12a to 7.12b p.194).*

7.4 Discussion

The collagenase digestion phase of islet isolation has been previously documented to cause morphological disruption of both acinar and islet endocrine cells (Schwartz and Traverso, 1984). Acinar cells show vacuolation due to disorganisation of the endoplasmic reticulum along with discontinuity of their plasma membrane yet these changes are much less pronounced in islet cells (Schwartz and Traverso, 1984). With respect to islet cells, high intraductal collagenase perfusion pressures (Johnson *et al.* 1996a) along with prolonged pancreas digestion, can cause islet fragmentation and

dispersion into single beta cells that are unsuitable for transplantation. Most investigators refer to collagenase as being the most likely candidate to induce these findings (London *et al.* 1994) though it is conceivable that endogenous pancreatic proteases that are released into the islet isolation medium could also play a role.

Previous studies allude to endogenous pancreatic proteases having both advantages (Henriksson *et al.* 1977) and disadvantages (Wolters *et al.* 1990; Heiser *et al.* 1994b) to islet isolation (Grotting *et al.* 1978; Traverso *et al.* 1981). It is still not known what precipitates the endogenous pancreatic protease activation cascade during islet isolation. Physiologically it is enterokinase that is released from gut enterocytes. In the situation of automated islet isolation, it is perhaps most likely to be the mixture of crude impurities (e.g. neutral protease, trypsin, clostripain etc.) within commercial collagenase rather than the *collagenase* itself. Traverso and Abou-Zamzam (1978), have investigated this hypothesis in a canine model, demonstrating that pancreatic proteolytic activity is potentiated in the presence of a crude commercial collagenase, in contrast to minimal activation with a purified collagenase. Indeed, the combination of crude collagenase and enhanced activation of pancreatic proteases was a prerequisite to rapid pancreatic digestion, although improvements in islet yield were anecdotal. It can, therefore, be assumed, from the results reported by Traverso and Abou-Zamzam, (1978), that perhaps crude commercial collagenases and endogenous pancreatic enzymes work synergistically in degrading pancreatic tissue but the optimum components from either crude commercial collagenase or endogenous pancreatic enzymes still needs further clarification.

Most studies relating to discharging pancreatic proteases during islet isolation have concentrated on the damaging effects of trypsin. Hitherto reports of trypsin inhibitors being efficacious to both, rat (Wolters *et al.* 1990) and porcine islet yields (Heiser *et al.* 1994b) one might believe that trypsin is the most detrimental of all pancreatic exocrine enzymes. Following automated porcine islet isolation, low islet yields (507 islets/g) are associated with high trypsin activity but trypsin inhibition with Pefabloc culminates in significantly better islet yields (6,795 islets/g). This is perhaps not so surprising given that trypsin is known to cause islet fragmentation (Lernmark, 1974). However, it is theoretical that trypsin inhibitors have their effect by inhibiting other endogenous pancreatic enzymes (e.g. elastase, chymotrypsin, phospholipase A_2 etc.). To summise trypsin may not necessarily exert direct effects on the pancreas but merely indirect ones via activation of other pancreatic proenzymes. Evidence also in favour of this hypothesis has to a certain extent been demonstrated in animal models with isolated acinar cells after autoactivation during acute episodes of pancreatitis (Letko *et al.* 1989; Niederau *et al.* 1995)

Consistent with the study of Heiser *et al.* (1994b) *(vide supra)* the findings from the present study show the existence of active trypsin during automated porcine islet isolation. There is, however, considerable differences between the rates of activation of both trypsin and other proenzymes (up to 10-fold), together having the potential to perhaps partly explain some of the extreme differences seen with porcine islet yields (up to 25 fold; Chapter Six). More interestingly they are not totally activated. Trypsin which is most frequently documented to be influential to islet isolation was only 20% activated. Many factors could account for the low levels of trypsin activation including donor factors (e.g. hyperlipidaemia; Niederau *et al.* 1995), contaminating bile, levels of endogenous protease inhibitors (Nagai *et al.* 1989) but perhaps most important is the duration of pancreatic ischaemia (Jönsson *et al.* 1995) and the batch of collagenase (Traverso and Abou-Zamzam, 1978), particularly the latter as crude collagenase is known to possess trypsin activity and this could be the mediator of the pancreatic enzyme activation cascade. Furthermore this property could also vary between batches (Johnson *et al.* 1996b).

Trypsin activation may be the most important enzyme to be activated in terms of activation of other pancreatic proteases but it may not be the most important with respect to damaging islet tissue. The reasons for this come from detailed studies with isolated pancreatic acinar cells that have yet to be transposed to isolated islets. For example, it has been previously shown that isolated rat acinar cells incubated with trypsin (for four hours) in activities comparable to *in vivo* endogenous levels do not appear to influence acinar cell survival (Letko *et al.* 1989). Only after a 10-fold increase in trypsin activity were the cells slightly damaged (blebbing) but a more than 100-fold excess was necessary to kill individual cells. A much stronger correlation has been observed with chymotrypsin where damage prevailed at one tenth the concentration of trypsin. Chymotrypsin activation was 52 % in this study, greater than that of trypsin, but there was marked variability between isolations. Tentatively, this enzyme could be potentially more damaging to isolated islet tissue, although this has not been previously investigated.

Nagai *et al.* (1989) have likewise demonstrated that acinar cells individually incubated with physiological concentrations of trypsin, lipase or phospholipase A_2, have preserved cellular integrity based on morphological analysis and release of 35^S methionine labelled proteins. Nonetheless, incubation times in this study were only 30 minutes, pancreas digestion in contrast can take up to 70 minutes. A difference in the intensity of cellular destruction was only seen when lipase in combination with triglycerides was added to the incubation medium. The release of triglyceride degradation products (e.g. diglycerides and fatty acids) resulted in marked cellular

toxicity and structural disintegration of acinar cells. Interestingly some of these changes were inhibited by albumin but more specifically by the lipase inhibitor tetrahydrolipstatin. In addition the toxic potential of phospholipase A_2 is also significantly enhanced in the presence of its substrate, releasing toxic byproducts such as lysophospholipids that can be further converted into potent pharmacological mediators such as prostaglandins (Flower and Blackwell, 1976) and thromboxane (Hamberg *et al.* 1975). The present results demonstrate lipase can be almost 100% activated during islet isolation, particularly during the middle of the digestion phase (23 to 25 minutes). Even at this stage the presence of contaminating retroperitoneal fat remaining attached to the pancreas could further augment cellular damage. In comparison, phospholipase A_2 was only 52% activated, but in one isolation large amounts of inactive prophospholipase A_2 were released with the potential for a 16-fold increase in further activation. In these situations the presence of large amounts of activated phospholipase A_2 and lipase porcine islets could be particularly vulnerable. This is because compared to other mammalian islets porcine islets lack a complete protective peri-insular capsule (Ulrichs *et al.* 1995; Chapter Five) and would be subjected to greater destruction as lipase and phospholipase A_2 could exert their effects directly on the exposed phospholipid domains of the porcine beta cell plasma membrane, together partly explaining some of the increased dispersion of porcine islets.

More recently a more thorough analysis of damage to acinar cells by pancreatic proteases has been performed by Niederau *et al.* (1995). Rat acinar cells were significantly damaged by elastase at nanomolar concentrations after only 45 minutes incubation whereas lipase, phospholipase A_2 and chymotrypsin caused equivalent injury at micromolar concentrations. Trypsin in contrast failed to cause significant injury even at millimolar concentrations. The noxious potential of pancreatic proteases, in decreasing order was elastase, lipase, chymotrypsin, phospholipase A_2 and trypsin. The results in the present study indicate that elastase activation was only 14%. This relatively low rate of activation may represent inefficient elastase activation during the automated procedure or it may be because of the slight elastase activity within the crude collagenase (Johnson *et al.* 1996b). The inefficient activation could be attributable to the short duration of pancreas digestion in this study (55 minutes) as Wolters *et al.* (1992) suggest that in the presence of collagenase elastin is not degraded until at least 120 minutes of pancreas digestion. In support of this elastase activation is known to occur late in the activation cascade (Niederau *et al.* 1995). Finally, further to the low rate of elastase activation in my study, Niederau *et al.* (1995) suggest that nanomolar concentrations can be damaging to acinar cells, but one can only speculate as to whether the amounts released during islet isolation would be sufficient to produce similar effects upon islet cells.

Lastly, the carboxypeptidase enzymes A and B confirmed a greater degree of activation compared to trypsin and elastase. They are highly specific enzymes and they make up a small proportion of the total active protease content (Rinderknecht, 1986) within a digesting pancreas and are unlikely to be very influential to islet isolation but a further study is needed to confirm this.

In summary, it is unlikely that poor islet yields and islet fragmentation are solely explained by variations between collagenase batches and it may be that activated pancreatic endogenous enzymes are also influential in this process. Current evidence relating to isolated acinar cells suggests the most noxious effects are perhaps orchestrated by elastase, chymotrypsin, lipase and phospholipase A_2 but these studies need to be transposed to isolated islet cells. It is not surprising that the use of trypsin inhibitors has not solved the problem of poor islet yields as they only cause moderate inactivation of chymotrypsin (Niederau *et al.* 1995) and perhaps no inactivation of phospholipase A_2 or elastase. From the presented results, it can be seen that activation of pancreatic enzymes occurs during islet isolation but their activation is extremely unpredictable, thus partly explaining some of the variability seen in porcine islet yields following collagenase pancreas digestion.

CHAPTER EIGHT

DIFFERENT TREATMENT REGIMENS TO REDUCE MICROBIAL CONTAMINATION OF PURIFIED PORCINE ISLETS OF LANGERHANS

8.1 Introduction

One of the potential risks of xenotransplantation is the possibility of introducing microbial pathogens from another species into the human population. Many reagents used for islet isolation (e.g. density gradient purification media and culture media) provide an ideal environment for the proliferation of microbial pathogens. Even after pancreatic digestion and purification, up to 30% of all islet preparations used for human transplantation can still be contaminated with micro-organisms (Lloveras *et al.* 1994), leading to postoperative infective complications (e.g. septicaemia and subphrenic abscess formation) after islet transplantation (Socci *et al.* 1991). Following these reports microbiological quality control has been recommended as a mandatory screening procedure for clinical islet transplant programs (Carroll *et al.* 1992).

In the event of islet xenotransplantation becoming a clinical entity it is likely that porcine organ donors will be the donors of choice and reared in a gnotobiotic environment so as to reduce microbial contamination (Dufty, 1976). Unfortunately, in the majority of transplant centres gnotobiotic rearing is not routinuely available and therefore investigators are left to excise porcine organs from local abattoirs. Previous studies relating to contamination of purified porcine islets have been anecdotal. Microbial contamination of both foetal (Bjoersdorff *et al.* 1995) and adult (Marchetti *et al.* 1992b) porcine pancreata excised in local abattoirs have been reported, but neither of these studies were designed to allow prospective randomization of different treatment regimens. The essence of this study was to firstly determine what microbial organisms contaminate purified porcine islets isolated from pancreata excised from an abattoir and, secondly, to assess different regimens for minimising microbial contamination during post-isolation culture.

8.2 Materials and methods

Animals. Twenty six consecutive adult porcine pancreata were initially assessed (approximate weight 100 g) for microbial contamination. The slaughtering process entailed cerebral death by exsanguination whilst suspended from their hind limbs,

complying with The Ministry of Agriculture for Fisheries and Food Regulations (MAFF). The entire gut was excised and deposited on a conveyor belt. The splenic lobe of the pancreas was excised using sterile surgical gloves and surgical instruments. Each were individually transported in 500 ml of sterile HOC at 4°C (Travenol, Thetford, UK) to the islet laboratory for processing.

Islet isolation and purification. Each pancreas was distended in a sterile laminar air hood (Laminar Flow Class II Hood, Walker Safety Cabinets Glossop, Derbyshire) by intraductal injection of 1mg/ml collagenase (Boehringer Mannheim lot No 14039526-64; Germany) dissolved in sterile HBSS (Gibco, Life Technologies, Faisley, Scotland). The pancreas was placed within a stainless steel automated chamber (Ricordi *et al.* 1989) divided by a 400µm mesh with sterile HBSS at 37°C circulating through the closed automated circuit. Pancreatic tissue was flushed from the exit port of the steel chamber and collected in sterile new born calf serum (Advanced Protein Products, Brierly Hill, UK). Porcine pancreatic digest was then purified on a continuous density gradient of sterile Ficoll amidotrizoate gradient medium placed on a COBE 2991 processor (Lake *et al.* 1989a; London *et al.* 1995). Purified porcine islets were then collected from the gradient fractions and washed three times with sterile MEM (Northumbria Biologicals, Cramlington, Northumberland, UK).

Islet culture. Purified porcine islets isolated from the first series of porcine pancreata (n=26) were divided into two groups (A and B) containing approximately 100,000 islets. Both groups were cultured in sterile Hams F-12 (Gibco, Life Technologies, Faisley, Scotland) placed in a 5% carbon dioxide and air incubator at 37°C for 48 hours. The first group (A) being devoid of antibiotics (control) with the second group (B) being cultured in Hams F-12 with the addition of penicillin (100U/ml) and streptomycin (100µg/ml) antibiotics (P/S). Following 48 hour incubation, 5 ml aliquots of the culture medium were aspirated from both culture dishes and centrifuged to remove any cellular debris and were taken for microbial analysis. Corresponding 5 ml aliquots from the transport medium (TM1) were similarly examined *(Figure 8.1 p.204).*

Analysis of micro-organisms. Using a sterile (flamed) loop, samples from each of the three groups were evenly streaked across the surface of four different agar plates containing either chocolate agar (incubated in CO_2), horse blood agar (incubated anaerobically), Sabouraud agar and finally MacConkey agar. All were incubated for

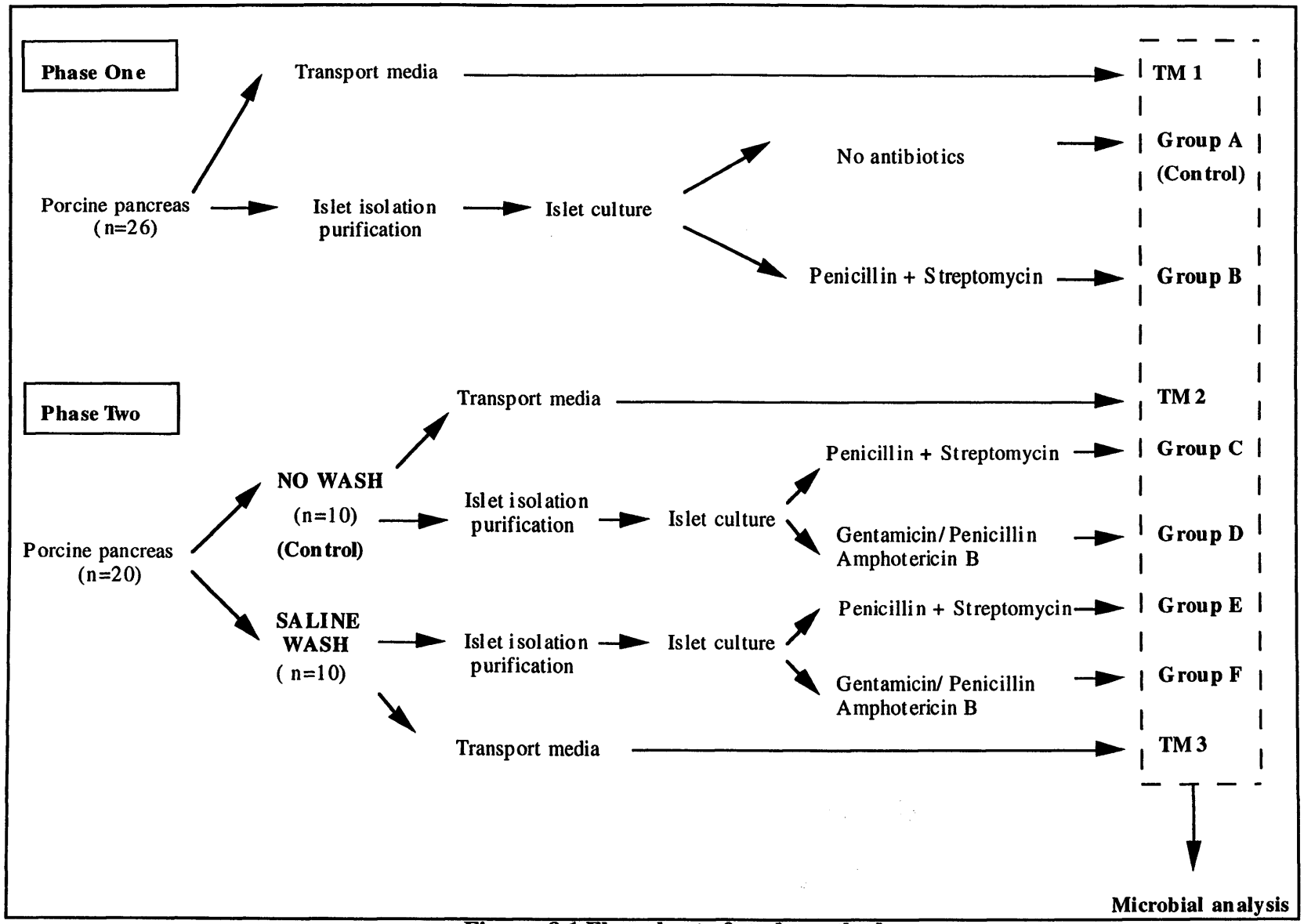


Figure 8.1 Flow chart of study method

five days. To increase the sensitivity of culture and to dilute the inhibitory effect of antibiotic carryover liquid enrichment cultures were undertaken. Oxoid signal blood culture broth bottles were also inoculated with each of the three different specimens (Unipath Ltd, Basingstoke, UK) and incubated for five days *(Figures 8.2 and 8.3)*. If growth was not apparent after initial incubation, a terminal subculture of the broth was made onto solid media and subsequently re-incubated overnight. Mycobacterial analysis

Figure 8.2 Oxoid culture bottles, sterile (S) and contaminated (C)

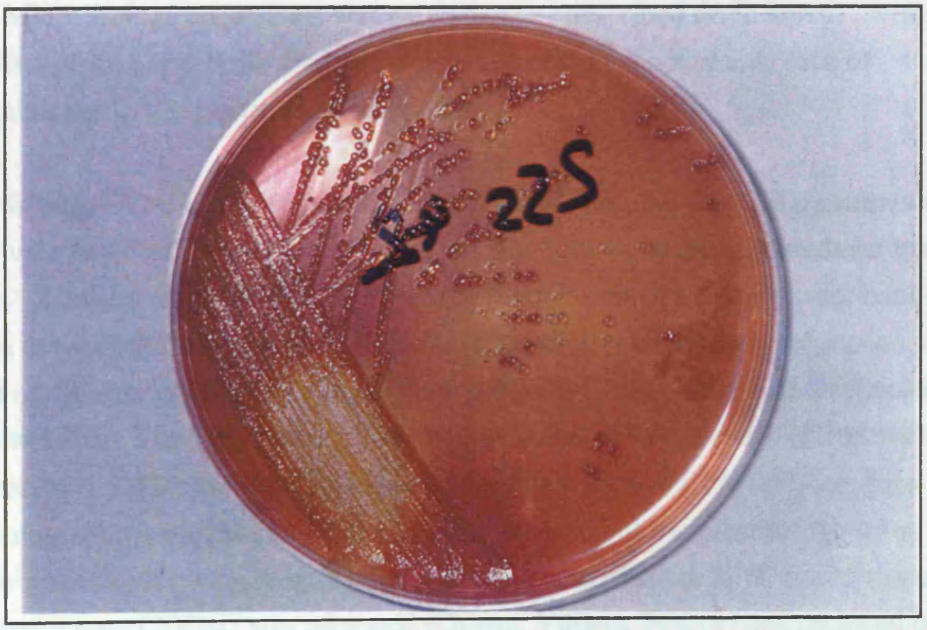


Figure 8.3 Microbial growth (mixed colony) on a blood agar plate

was also performed by Ziehl-Neelsen acid fast staining on specimens of the first 26 transport media only.

The identification of micro-organisms was based on colonial appearance and conventional identification to the genus level. Antibiotic susceptibilities to penicillin, gentamicin and streptomycin were performed by the comparative disc diffusion method.

Eradication of micro-organisms. In the second phase of the study a further 20 pancreata were randomised to receive two different treatments. The first treatment group (n=10), were treated in the standard way (no pancreas washing-'controls') and transported to the islet laboratory immediately after excision. A 5 ml aliquot of transport medium was taken for assessment (TM 2). Purified islets were then divided into two further groups (C and D) and cultured for 48 hours as before, in either Hams F-12 (Gibco Life Technologies) with the addition of either penicillin (100 U/ml) and streptomycin (100µg/ml; P/S; Group C) or, alternatively, with gentamicin (50µg/ml), penicillin (100 U/ml) and amphotericin B (1µg/ml; G/P/A; Group D). The second treatment group of 10 pancreata were cleaned and dissected free of connective tissue and washed with sterile normal saline. They were then transported and digested as previously described with a 5ml aliquot of transport medium analysed for contamination (TM 3). The isolated islets were then cultured in Hams F-12 with the addition of either of the two different antibiotic regimens (P/S- Group E: G/P/A- group F; *Figure 8.1 p 204*). Microbiological analysis was carried out as described *(vide supra)* , with the exception of mycobacterial analysis which was excluded because of lack of contamination in the previous 26 pancreata.

Islet viability. The viability of the purified islets from the final 20 pancreata (Phase Two) were assessed by *in vitro* dynamic incubations by assay of stimulated insulin release (London *et al.* 1990; Chapter Six). Five groups of ten islets were hand-picked using a drawn out Pasteur pipette, from each purified porcine islet preparation after a period of 48 hour postpurification culture in Hams-F-12 (Gibco, Life Technologies), media at 37°C. The islets were transferred to 200µl of low glucose (1.7mmol/l, prepared by 1:3 dilution with balanced electrolyte solution) RPMI (Gibco, Life Technologies) prewarmed to 37°C and placed in a 1.5ml microcentrifuge tube. The sample was then left to preincubate in a shaking water bath at 37°C for 45 minutes. The supernatant (200µl) was aspirated (Gilson, Villiers, France) and replaced by a further 200µl of low glucose RPMI (Gibco, Life Technologies) incubated for one hour then high (25mmol/l) glucose incubated for one hour and finally with low (1.7mmol/l) glucose RPMI (Gibco, Life Technologies) incubated for one hour as previously described (Chapter Six). The insulin content within the supernatant of each of the final

two phases was measured by ELISA assay. The stimulated insulin release was expressed in microunits per islet per hour (μIU/per islet/ per hour), calculated as the difference between insulin content in the high (25mmol/l) glucose response and the final low (1.7 mmol/l) glucose response (Holmes *et al.* 1995; Chapter Six).

Statistical analysis. Results are expressed as median (range) values. Differences between discontinuous variables were compared using the Chi-Squared analysis (X^2), the level of significance being reported as a P value. Comparison between continuous variables were assessed by the Kruskall Wallis one-way analysis of variance Test. Differences were considered significant at $P < 0.05$ where appropriate (Minitab ® release 8.1; State College, Pennsylvania, USA).

8.3 Results.

Microbial contamination

The results from series one (n=26) showed a 100% contamination rate of transport medium (TM1). Following islet isolation and purification 70% of cultured islets (no antibiotics-Group A) were contaminated. The addition of P/S (Group B) to the final culture medium reduced contamination of purified islets to 42% of preparations, in comparison to those of Group A (70%), P=0.05 *(Figure 8.4 p.208).* The most common contaminants seen in those preparations cultured with no antibiotics were coliforms (54%) and faecal streps (15%), whereas those islets cultured in P/S (Group B) were contaminated with coliforms (15 %), Candida spp. (9%) and faecal streptococci (8%) *(Figure 8.5 p.209).*

In series two with pancreatic washing prior to islet isolation, contamination rates were further reduced. The transport media (TM 3) from washed pancreata showed a lower rate of contamination (80%) compared to those unwashed (100 %; TM 2) but was an insignificant finding (P> 0.05). In the case of pancreas washing and the use of P/S antibiotics (Group E), purified islets were contaminated in 30% of cases but washing together with the combination of G/P/A (Group F) eradicated all contamination. The addition of pancreas washing to either combination of antibiotic regimens improved islet contamination rates, it was not a statistically significant finding, for P/S (group C) 53% *versus* (group E) 30%, for G/P/A group D 30% *versus* group F 0% *(Figure 8.4).*

Islet viability

Neither antibiotic regimen had a significant effect upon *in vitro* islet viability. *(Figure 8.6 p.209)* between either of the four groups tested.

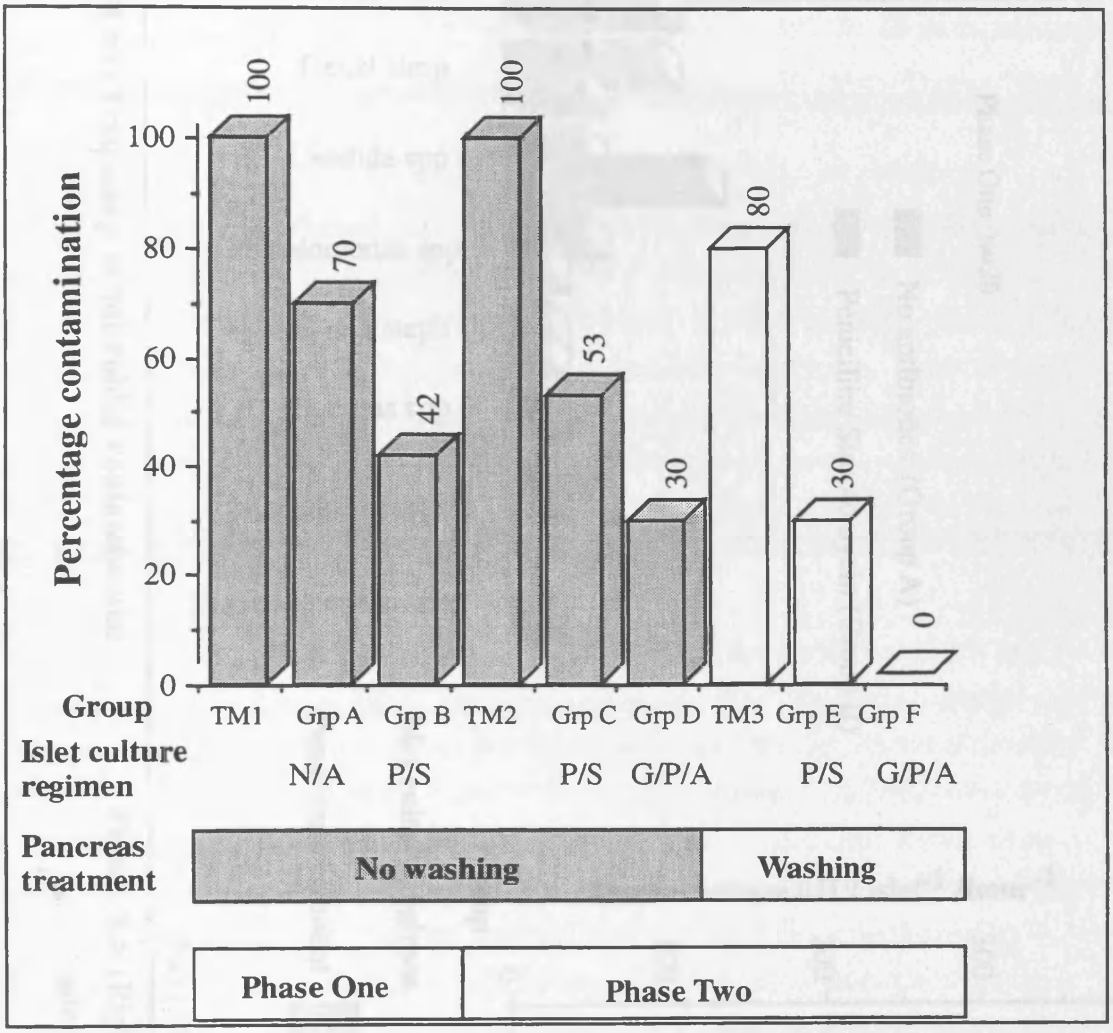


Figure 8.4 **Summary of results. TM1 to TM3, transport medias 1 to 3; P/S, penicillin and streptomycin; G/P/A, gentamicin, penicillin and amphotericin B; Grp A no washing and no antibiotics; Grp B, no washing with penicillin and streptomycin; Grp C, no washing with penicillin and streptomycin; Grp D, no washing with gentamicin, penicillin and amphotericin B; Grp E, washing with penicillin and streptomycin; Grp F, washing with gentamicin, penicillin and amphotericin B.**

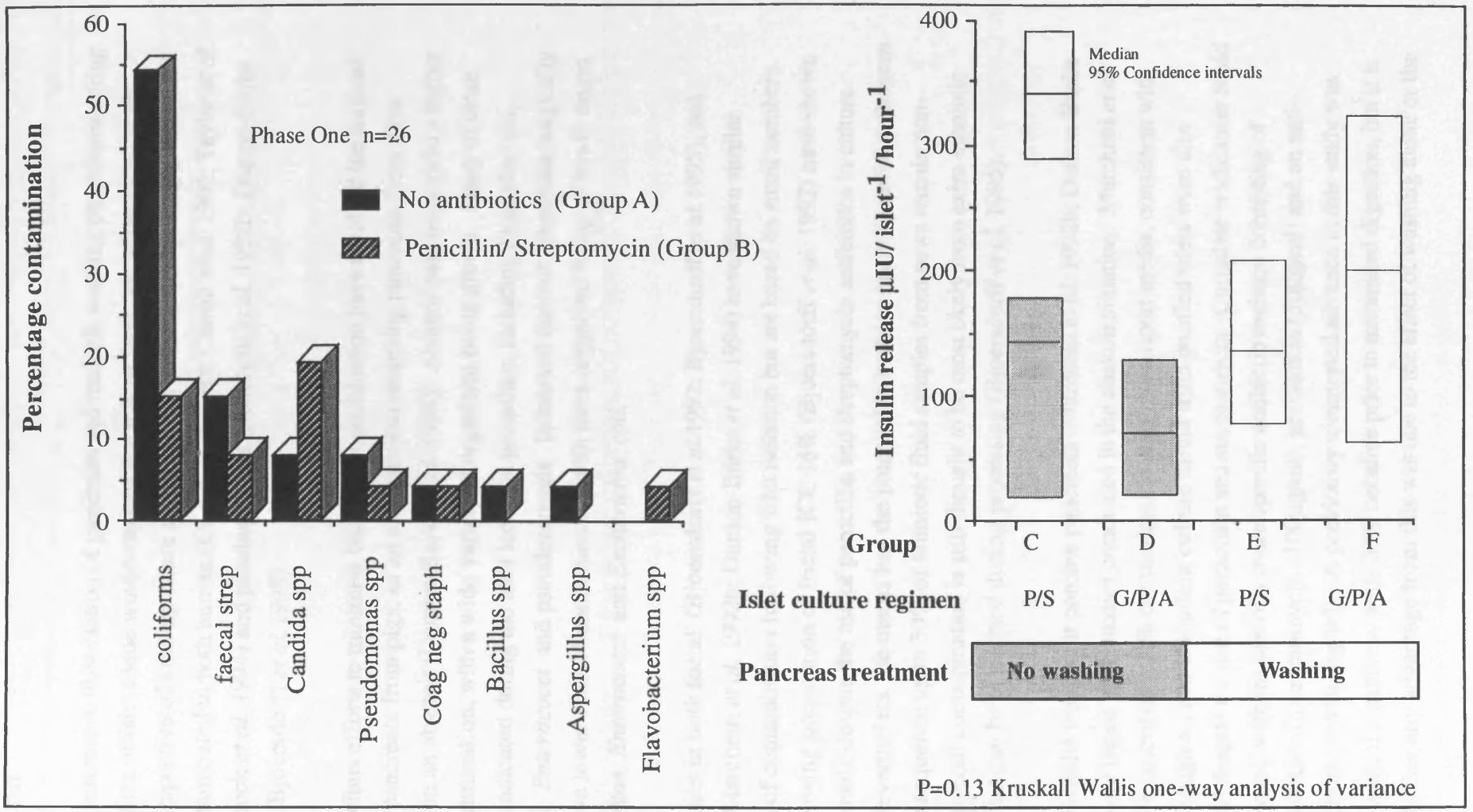


Figure 8.5 (Left) Frequency of microbial contaminants;

Figure 8.6 (Right) Effect of treatment regimens on islet viability

Discussion

The extensive dissociation of pancreata and multiple washing procedures during the preparation of islet tissue would suggest that it is perhaps one of the safest grafts from the perspective of limiting donor transmitted infection, but surprisingly many still remain contaminated in both human (Corlett *et al.* 1988; Carroll *et al.* 1992; Taylor *et al.* 1994; Warnock *et al.* 1994) and porcine studies (Marchetti *et al.* 1992b; Damon-Burke *et al.* 1994; Bjoersdorff *et al.*1995)

Previous efforts to minimise bacterial contamination have involved the surgical removal of pancreata from pigs, as an alternative to retrieving pancreata from those slaughtered in an abattoir (Damon-Burke *et al.* 1994). Abattoir pancreata yield a higher rate of contamination, with a wide variety of organisms being grown. Many of these cannot be eradicated during the islet isolation procedure, including *Staphylococci, Lactobacilli, Enterococci* and pseudomonads. In marked contrast, pancreata surgically removed give lower rates of contamination with most organisms being of bowel origin e.g. *Klebsiellae, Enterococci* and *Escherichia coli.*

Studies in both foetal (Bjoersdorff *et al.*1992; Bjoersdorff *et al.*1995), and adult pigs (Marchetti *et al.* 1992b; Damon-Burke *et al.* 1994) have shown similar frequencies of contamination following islet isolation but are based on small numbers. Firstly, following preparation of foetal ICC 18% (Bjoersdorff *et al.* 1992) have shown bacterial growth, despite the use of penicillin and streptomycin antibiotics in culture medium. Nevertheless, the uterus has the potential to harbour many bacterial pathogens and as a consequence up to 30% of amniotic fluid samples from sows remain non-sterile, but overall contamination is still thought to be most likely from extra-amniotic pathogens that can be eradicated during processing (Bjoersdorff *et al.* 1995).

The results from adult porcine pancreata (Marchetti *et al.* 1992b; Damon-Burke *et al.* 1994) are based upon studies conducted in the abattoir situation. Marchetti *et al.* (1992b) have described 100% contamination rates in transport media, consistent with our study, though all corresponding culture media from purified islets were also contaminated despite the use of penicillin and streptomycin. Complete eradication could only be achieved with the use of a more specific antibiotic regimen consisting of penicillin (100U/ml), streptomycin (100µg/ml), gentamicin (50µg/ml) and an anti-fungal, amphotericin B(1µg/ml). A previously unreported advance in this study was the need to wash the pancreas with 20% betadine prior to automated digestion, but it is not clear whether any advantage from this was due to the effect of washing alone or the

betadine solution. Commonly encountered pathogens in this study were Staphylococci, *Corynebacteria*, and pseudomonads. Candida spp. were likewise frequently reported. In conjunction with cases of candida sepsis and other systemic mycoses, anti-fungal agents have now become a necessary addition to islet culture media (Socci *et al.* 1991). Amphotericin B is currently the drug of choice having no impairment on islet viability when used at a concentrations of up to 2.5µg/ml (Arias-Diaz *et al.* 1994c).

The results from this study compare well with those of Marchetti *et al.* (1992b) and those reported by Damon-Burke *et al.* (1994) in that the majority of our contaminating organisms were coliforms and faecal streptococci, reflecting the high rate of bowel perforation during the slaughtering procedure. Interestingly, these organisms also showed a considerable amount of antibiotic resistance in medium supplemented with penicillin and streptomycin.

Particularly resistant micro-organisms have emerged in most species of *Steptromyces*, severely limiting the usefulness of streptomycin, the first aminoglycoside to be discovered in 1947. Most bacterial resistance to streptomycin is perhaps attributable to the widespread use of prophylactic antibiotics in animal feeds, leading to the selection of resistant organisms (Fishman, 1995) and its unnecessary use in combination with penicillin. As a consequence of these findings gentamicin, a similar aminoglycoside was utilised, it is the most widely used aminoglycoside and is active against most aerobic gram negative rods, including *Pseudomonas spp., Proteus spp. and Staphylococci.* Nevertheless, most streptococci are resistant because gentamicin cannot penetrate the cell wall. However, penicillin and aminoglycosides have a synergistic effect (Jawetz, 1987).

In conclusion, it can be seen that the preparation of porcine islets of Langerhans can be conducted without detectable microbial contamination. This is particularly the case when the pancreata are surgically removed in preference to pancreata removed from a slaughtered abattoir pig. It is also apparent that the historical use of penicillin (Hu *et al.* 1953) and streptomycin (Keilova, 1948) for over 40 years is ineffective in avoiding contamination of cultured islet tissue from porcine species. It is now, therefore, necessary to apply more specific regimens such as those described, thus reducing unnecessary antibiotic use and the risk of selecting resistant organisms. With the addition of pancreas washing in simple saline and the use of pencillin, gentamicin and amphotericin B, microbial contamination can be completely eradicated without compromising islet viability. Whilst these findings relate to the bacterial and fungal contamination of porcine islets, the relevance of virological and protozoal transmission following xenotransplantation still warrants further investigation.

CHAPTER NINE

CONCLUSION OF THESIS AND FUTURE RESEARCH

One can seen that there is a major discrepancy between the number of Type 1 diabetic patients and the number of human organ donors available for human islet allotransplantation and if Type 1 diabetes is to be treated by a transplant in young, newly diagnosed diabetic patients then the only choice is to pursue clinical xenotransplantation. As has already been discussed, the porcine species is the donor of choice but one of the major obstacles, other than graft rejection, is porcine islet isolation because of the fragility of porcine islets leading to islet fragmentation and poor yields after collagenase digestion. The object of this thesis was to systematically review the current literature of porcine islet isolation and to identify some of the more important factors that may influence successful porcine islet isolation for xenotransplantation.

The first phase of this thesis looked at the size and number of islets between adult and juvenile pancreata from the same porcine strain. The volume density of islets cannot be calculated accurately by conventional techniques and an assessment of the CSSA of islets was made using computerised image analysis. There were no differences between CSSA of either adult or juvenile islets of Langerhans. However, there were significant differences between the percentage CSSA density of islets between donors of similar age and strain. Again, this difference was only slight and observed in one donor and taken together this difference would not explain the 20-fold differences seen in porcine islet yields. The challenge now would be to develop a simple technique that would allow investigators to identify pancreata that would yield a large number of islets prior to starting the isolation procedure. Secondly, investigators need to rear an in bred porcine strain specifically for islet isolation but this is not as straightforward as it seems, as it may not necessarily be a strain that has a high islet CSSA density as this does not correlate with high porcine islet yields. If a strain could be identified, it is likely to be a genetically engineered humanised version so as to avoid the complications of xenograft rejection.

A second factor that was considered to be important was the collagen content within the pancreatic extracellular matrix. The crux of islet isolation relies on the cleavage of islets from the surrounding acinar tissue. There are many different types of collagen within the porcine pancreas extracellular matrix but a complete analysis is currently impossible because of the limitations of anti-collagen antibodies available.

There were no obvious differences between donors of similar age or strain. Nevertheless, there were significant differences between the different types of collagen. A previously unreported finding was the intensity of collagen type VI compared to other types (I, IV, V) although the function and influence of collagen type VI upon porcine islet isolation still needs further clarification. Indeed, given that 13 different collagen types have been described, it is likely that other investigators will demonstrate important relationships between other collagen types once other collagen monoclonal antibodies have been developed.

With the advances in genetic engineering, efforts should be made to try and modulate the maturation and cross linking of collagens immediately surrounding the islet. The question is would it be possible to develop a collagenase that would be specific for cleavage of peri-insular collagen ? The current problem is that *Clostridium histolyticum* collagenase is non-specific and full of impurities and no one knows what is the best combination of components and what their optimum substrates are within the pancreas. To an extent this difficult problem is particularly highlighted in my study where collagen type VI predominates in the peri-insular matrix but also in the rest of the pancreas as well. Until the relationships of other collagen types have been identified, a specific collagenase cannot be designed. Even with the introduction of Liberase® for islet isolation, variability is still seen and therefore perhaps the only answer is a genetically engineered collagenase when the correct combination of components have been demonstrated. This will be a costly and time consuming endeavour as advances in this area are slow but urgently needed.

Chapters Six and Seven concentrated on some of the issues pertinent to the collagenase digestion phase. The early administration of collagenase in a cold storage solution was shown to be a major advance to previously described methods. This was most likely because of the improved distribution of collagenase prior to the damaging effects of cold ischaemia. It was also shown that UW was not inhibitory to porcine islet isolation and similar yields could be obtained with a simple and cheaper solution (ULEIC). This technique will be invaluable to investigators who wish to transport pancreata over long distances and further study is needed to assess the effect of longer cold ischaemic upon porcine islet yields. It is likely that other technical refinements to cold storage solutions will be made perhaps involving phosphodiesterase inhibitors, nitric oxide synthetase inhibitors and newer impermeable colloids. Lastly, having developed a method to minimise the damaging effects of cold ischaemia the problems of warm ischaemia also need to be addressed. Most centres retrieve pancreata from abattoirs and ultimately for the purposes of xenotransplantation it is likely that pancreata

will be removed surgically with cold organ perfusion, this area still needs further refinements in techniques with islet isolation in mind.

The study of the effects of endogenous pancreatic enzymes upon porcine islet isolation is still in its preliminary stages. Experiments involving trypsin inhibitors have not conclusively shown any major advance in islet yields, they have a non-specific mode of action and most probably inhibit other more damaging endogenous enzymes (e.g elastase, chymotrypsin, lipase etc). Chapter Seven demonstrated the extreme variability (20-fold) of endogenous pancreatic enzyme activation, thus partly explaining some of the differences seen in porcine islet yields. Further studies are still needed to assess which enzymes are detrimental to porcine islets, similar to those already reported with isolated acinar cells. Also, further work is also needed to determine how these enzymes are activated *in vitro* i.e. is it the collagenase itself, the crude impurities or a self perpetuating cascade. Once this has been carried out investigators could then develop a more specific protease inhibitor that could minimise their damaging affect upon porcine islets.

Finally, Chapter Eight outlined one of the current topical issues concerning xenotransplantation. It is likely that porcine organ donors will have to be bred in a gnotobiotic environment to reduce the risk of microbial contamination. However, it is possible to isolate porcine islets in an aseptic manner and with the correct combination of antibiotics microbial contamination can be kept to a minimum even in the abattoir situation.

Where do we go to now ? Clinical xenotransplantation has evolved very rapidly over the last 10 years mainly in parallel with advances in molecular biology. It is a particularly exciting prospect that xenografts can be manipulated in such a way so as to reduce the need for toxic immunosuppressives and their associated risks, one of the major advantages over allografts. Nevertheless, there are still many drawbacks which limit the widespread application of xenotransplantation including ethical issues, transmission of donor pathogens, emergence of recombinant viral strains of unknown pathogenicity, physiological incompatibilities, particularly different rates of ageing between the xenograft and recipient and lastly severe immunological xenograft rejection. The threat of transmissible disease is perhaps the most discussed as outlined in the recent Nuffield Council on Bioethics (Nuffield Council, 1996) and by the UK Department of Health (1997). These concerns have been particularly reinforced by reports of co-culture of porcine and human cell lines that allow endogenous porcine retroviruses to begin replication (Patience *et al.* 1997). However, one solution to this problem may be to breed virus free pigs by genetic engineering but a recent report

suggests that there are a number of proviruses present (Le Tissier *et al.* 1997) making the feasibility of this venture highly unlikely to succeed. In conclusion, there will always remain a need for the careful assessment of the potential risks of xenotransplantation but it will be difficult to address every concern until clinical trials of xenotransplantation are allowed to begin.

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