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THESIS ABSTRACT

A STUDY OF THE FACTORS INFLUENCING THE COLLAGENASE DIGESTION PHASE OF HUMAN AND PORCINE ISLET ISOLATION

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Human pancreatic islet transplantation offers a potential cure for type 1 diabetes mellitus by restoring normoglycaemia at an early stage of the disease. However, whereas it is possible to routinely reverse diabetes by transplanting islets in rodent models, the results in the human have been more variable. One of the major reasons for this has been the unique difficulty that the mammalian pancreas poses with respect to islet isolation. The crucial stage of the islet isolation process is the collagenase-digestion phase. However, this process is extremely variable and also poorly understood. The aim of this thesis therefore, was to investigate the collagenase-digestion phase of human and porcine islet isolation. The first three chapters consist of reviews of islet transplantation and cell isolation and outline the problems to be addressed. Chapter 4 investigates different aspects of the variability of different batches of crude collagenase. Twelve different batches of crude collagenase were tested on both human and porcine pancreata. Clear differences were noted between collagenase digestions by the different batches and also between the two species. Chapter 5 describes a 'simple in vitro' method that was developed for testing different batches of collagenase or collagenase components on any one mammalian pancreas, thereby controlling for inter-pancreatic variability. Chapter 6 outlines the optimisation of biochemical assays for different components of crude collagenase. Chapter 7 investigates the stability of four different batches of crude collagenase under eight different storage conditions over a six-month period using biochemical assays. Chapter 8 describes a series of experiments investigating the inhibition of crude collagenase by University of Wisconsin solution. Chapter 9 evaluates the influence of different administration pressures on the distribution of collagenase within the porcine pancreas using novel methods with anti-collagenase monoclonal antibodies. Finally, the overall conclusions and suggestions for future research are discussed in Chapter 10.

Statement of Originality

Except where acknowledged, the work described in this thesis is based on my own independent work, undertaken in the Department of Surgery at the University of Leicester.

P.R.V.Johnson

January 2001

Dedication

To Hilary, Thomas, and Tilly - for your patience, support and understanding throughout the production of this thesis.

"If I have seen a little further, it is by standing on the shoulders of giants"

Isaac Newton"

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Presentation of Work Described in this Thesis

Oral Presentations

Johnson PRV, Roberts DL, White SA, London NJM. The influence of different components of crude collagenase on the isolation of islets of Langerhans. Presented at the 8th Leicester-Oxford Islet meeting, Oxford, June 1995.

Johnson PRV, Van Suylichem PTR, Roberts DL, Vos-Scheperkeuter GH, White SA, Van Schilfgaarde R, London NJM. A simple 'in vitro' method for evaluating the efficacy of batches of crude *Clostridium histolyticum* collagenase for islet isolation. Presented at the 5th Congress on Pancreas and Islet Transplantation, Miami, July 1995.

Johnson PRV, Robertson GSM, White SA, Dennison AR, London NJM. First successful case of combined intra-splenic and intra-portal pancreatic islet autotransplantation. Presented at the Congress on Beta-cell, Islet and Pancreas Transplants, Orvieto, Italy, November 1995.

Johnson PRV, Robertson GSM, White SA, Dennison AR, London NJM. First successful case of combined intra-splenic and intra-portal pancreatic islet autotransplantation. Presented at the Transplantation Section of the Royal Society of Medicine, London, November 1995.
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Johnson PRV, Robertson GSM, White SA, Hughes DP, Dennison AR, London NJM. Pancreatic islet autotransplantation combined total pancreatectomy for the treatment of chronic pancreatitis. Presented in the Medawar Medal session at the British Transplantation Society, Oxford, March 1996.

Johnson PRV, Kane C, Shepherd RM, Squires RM, James RFL, Lindley KJ, Aynsley-Green A, Spitz L, Dunne MJ. A new approach to the treatment of nesidioblastosis based upon *in vitro* studies of cell function. Presented at the British Association of Paediatric Surgeons, Jersey, July 1996.
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Johnson PRV. Clinical and metabolic studies of human pancreatic islet autotransplantation. Presented as a Hunterian Professorship Lecture, June 1998

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Collagenase and Islet Isolation. Presented to the Department of Islet Transplantation, Edmonton, Canada, June 1995

Poster Presentations

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Johnson PRV, Chen R, Smith J, Wheatley L, James RFL, London NJM. Influence of different loading pressures on the distribution of collagenase within the porcine pancreas. Presented at the 15th Workshop of the Artificial Insulin Delivery Systems, Pancreas and Islet Transplantation Study Group, Igls, Austria, Jan 1996.

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Johnson PRV, Chen R, Smith J, Contractor HH, Wheatley L, James RFL, London NJM. An initial study of the influence of different loading pressures on the distribution of collagenase within the porcine pancreas. Presented at the International Symposium on Endocrine Cell Transplantation and Genetic Engineering, Giessen, Germany, June 1996.

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Johnson PRV, van Suylichem PTR, Roberts DL, Vos-Scheperkeuter GH, White SA, Van Schilfgaarde R, London NJM. Design of a simple, "in-vitro" method for evaluating the efficiency of crude *Clostridium histolyticum* collagenase and its components for porcine islet isolation. Xenotransplantation 1995; 2:165-168

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List of Abbreviations

ALG	Anti lymphocyte globulin
APC	Antigen presenting cells
ATG	Anti thymocyte globulin
BSA	Bovine serum albumin
CMV	Cytomegalovirus
DCCT	Diabetes control and complications trial
DTZ	Dithizone
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
FALGPA	2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine
HLA	Human leucocyte antigen
HOC	Hyperosmolar citrate
IDDM	Insulin dependent diabetes mellitus
IEQ	Islet equivalents
IITR	International islet transplant register
MEM	Minimal essential medium
MHC	Major histocompatibility complex
NBCS	Newborn-calf serum
NAD	Nicotinamide
PD	Pancreatic duct
PP	Pancreas polypeptide
PV	Portal vein
RPM	Revolutions per minute

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
TNF	Tumour necrosis factor
UW	University of Wisconsin solution

Summary of Thesis

The work in this thesis is divided into 5 main sections. The first section comprises three review chapters which provide a background to the research that I performed. Chapter 1 is a review of the rationale for islet transplantation, together with the results of the human islet autotransplants and allotransplants performed worldwide. It includes an overview of the human islet autotransplants that we performed while I was working in Leicester. Chapter 2 discusses the reasons why the results of islet transplantation have been so disappointing and suggests possible ways to improve these, with particular reference to improving the efficiency of islet isolation. Chapter 3 is a review of the role of collagenase in human islet isolation. It sets the scene for the remainder of the thesis.

The second section investigates the different components of crude collagenase. Chapter 4 compares the digestion profiles of human and porcine pancreata by 9 different batches of crude collagenase. One of its conclusions is that due to the need to control for the considerable variability between different human pancreata, methodology is required that enables the testing of numerous different collagenase components on a single human pancreas. The design of such methods is the subject of Chapter 5. At the start of this project none of the biochemical assays for the components of crude collagenase were established in our department. Chapter 6 therefore, describes the optimisation of these assays together with their modification.

The third section investigates the storage of crude collagenase. Chapter 7 is a study of the stability of 4 different batches of crude collagenase under 8 different storage conditions. It involves the biochemical assays optimised in the previous chapter.

The fourth section investigates the administration of crude collagenase. Chapter 8 investigates the biochemical aspects of collagenase administration with particular reference to the inhibition of collagenase components by University of Wisconsin solution. Chapter 9 studies the biophysical aspects of

collagenase administration with a study of the influence of different administration pressures on the distribution of collagenase within the porcine pancreas.

The final section comprises Chapter 10 which outlines the conclusions of the thesis and suggests future research that is needed to further this work.

SECTION I

Background and Introductory Overviews

CHAPTER 1

Islet Transplantation as a Treatment for Diabetes Mellitus

Chapter 1 Outline

Introduction

- A) Diabetic complications and the influence of glycaemic control
- B) Restoring normoglycaemia in the diabetic patient
 - 1) Physiology of normal insulin synthesis and secretion
 - 2) How can normoglycaemia be restored in the diabetic patient?
 - a) Intensive insulin therapy
 - b) Stimulation of residual beta-cell function
 - c) Gene therapy
 - d) Transplantation of insulin producing tissue
 - 3) Rationale for islet transplantation
- C) Results of clinical islet transplantation
 - 1) Islet autotransplantation
 - 2) Islet allotransplantation

Conclusions

Introduction

Insulin-dependent (Type 1) diabetes mellitus (IDDM) is an autoimmune disorder^{1, 2} of blood glucose homeostasis affecting about 25 million people worldwide³. In the United Kingdom, about 3% of adults over the age of 16 and 20,000 people under the age of 20 are affected^{4, 5}. Until the discovery and isolation of insulin by Banting and Best in 1922 (Figure 1.1)^{6, 7}, the acute clinical presentation of this disease was almost uniformly fatal. This Nobel Prize winning work, performed in the laboratory of Macleod in Toronto, not only forms one of the most fascinating accounts of scientific investigation and intrigue⁸, but more significantly transformed diabetes into a treatable disease. However, whilst the administration of exogenous insulin had a dramatic effect on the short-term complications of diabetes, a number of severe long-term complications of the disorder soon emerged^{9, 10, 11}, shifting diabetes from an acute condition to one of the most common chronic diseases affecting the Western world. This not only had dramatic consequences for type 1 diabetics, but also had profound socio-economic implications for many countries^{12, 13}. Today it is estimated that about 50% of type 1 diabetics will develop one or more of the chronic complications of the disease, with the probability of developing complications increasing with the length of time from initial diagnosis. The most significant long-term complications of diabetes are highlighted in Figure 1.2.

Diabetic *retinopathy* is the commonest cause of blindness in the United Kingdom amongst the 45-65 age-group¹⁴. While it rarely occurs within the first 5 years following initial diagnosis, 90% of type 1 diabetics show evidence of at least background retinopathy after 20 years^{15, 16}. *Nephropathy* develops in 35-45% of patients with IDDM with the subsequent renal failure being the commonest cause of death in those patients who develop diabetes before the age of 30^{17, 18, 19}. It is estimated that up to

Figure 1.1 The first commercially available insulin

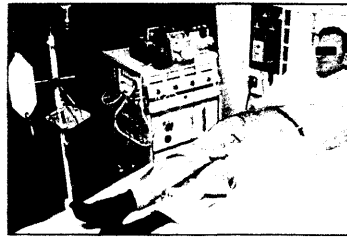


Figure 1.2

The long-term complications of diabetes mellitus occur in about 50% of patients and increase in incidence with the length of time after diagnosis.



Retinopathy



Renal failure



Vascular disease

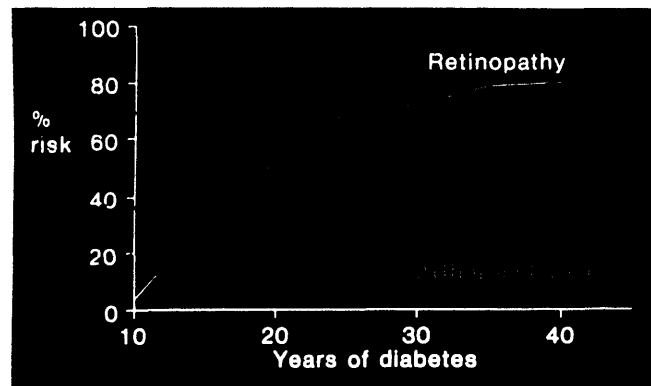
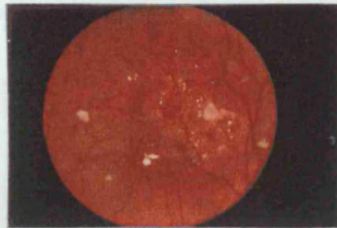


Figure 1.2

The long-term complications of diabetes mellitus occur in about 50% of patients and increase in incidence with the length of time after diagnosis.



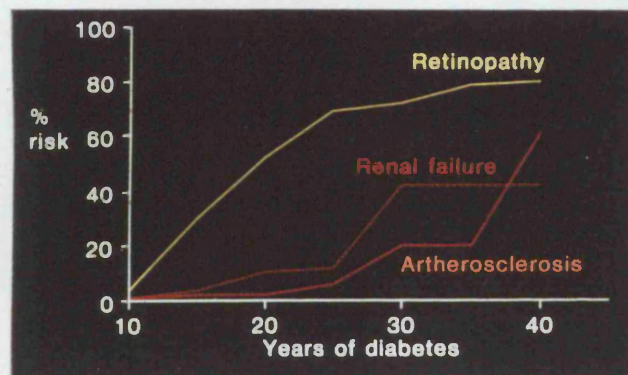
Retinopathy



Renal failure



Vascular disease



60% of insulin-dependent diabetics will develop *neuropathy*, although only about 20% of these will become symptomatic ^{20, 21}. The neuropathy affects both the somatic sensory nerves, resulting in foot ulceration and neuropathic arthropathy, as well as the autonomic nervous system, causing postural hypotension, gastrointestinal disturbance and impotence ²².

Arteriosclerosis is greatly increased in type 1 diabetics, resulting in an overall 2-3 fold increase in cardiovascular mortality in men and a 6-7 fold increase in women compared with age-matched controls ^{20, 23}. The incidence of peripheral vascular disease is increased 4 fold, while the increase in both coronary artery disease and cerebrovascular accidents are doubled ^{14, 24}.

The aim of this introductory chapter is to outline the overall causes of these long-term complications and to discuss different therapeutic options for trying to preventing them. The rationale for islet transplantation is explained and the clinical results of this procedure are reviewed.

A) DIABETIC COMPLICATIONS AND THE INFLUENCE OF GLYCAEMIC CONTROL

Two important points were made in the last section. Firstly, that diabetes is a disorder of glucose homeostasis and secondly, that the risk of developing the long-term term complications of diabetes, increases with the length of time from initial diagnosis. However, does it automatically follow that the long-term complications develop as a result of prolonged fluctuations in blood glucose levels? In other words, are the diabetic complications caused directly by hyperglycaemia itself or are there genetic factors in play that result in 50% of type 1 diabetics developing chronic complications regardless of how tight their glycaemic control. The answer to this question is absolutely key to the design of treatment regimes that aim to prevent the long-term complications of diabetes.

Does the restoration of normoglycaemia prevent / reverse the long term complications of diabetes?

The answer to this question would on the face of it appear straightforward. However, although there have been many studies addressing this issue in small animals, the results of these cannot be directly transposed to the human situation. Indeed as London has pointed out ²⁵, "rodent diabetes is not currently a major problem for the National Health Service!!". Until recently, there had been no conclusive studies on the effects of glycaemic control on the development of diabetic complications in humans. Much of the data available was based on inference from the results of retrospective reviews. For instance, studies involving identical twins in which only one twin was diabetic, had shown that the non-diabetic twin, despite possessing the same genotype, does not develop the diabetic retinopathy encountered in diabetes ^{26, 27}. It had also been observed that the transplantation of kidneys from long-standing diabetics into non-diabetic recipients resulted in the almost complete reversal of both clinical and histological features of established diabetic nephropathy within 7 months ²⁸. The corollary was also observed, that the transplantation of normal kidneys into diabetic recipients resulted in the development of nephropathy within 2 years ^{29, 30}.

However, in 1993, the results of the Diabetes Control and Complications Trial (DCCT) were published and provided the most convincing data yet with regard to the benefit of good glycaemic control ³¹. The DCCT was a multi-centre, prospective, clinical trial performed in U.S.A. A total of 1441 patients with IDDM (726 with no retinopathy - the primary-prevention cohort; 715 with mild retinopathy - the secondary-intervention cohort) were randomised to either receive conventional once or twice-daily insulin therapy or to receive intensive insulin therapy administered either with an external insulin pump (see below) or by three or more daily insulin injections and guided by frequent blood glucose monitoring. The patients were followed for a mean of 6.5 years and the appearance and progression of retinopathy and other

complications were assessed regularly. The results were so conclusive that the trial was stopped earlier than originally planned.

Intensive therapy was found to reduce the risk of developing *retinopathy* in the primary-prevention cohort by 76%. In the secondary-intervention cohort, intensive therapy slowed the progression of retinopathy by 54% and reduced the development of proliferative or severe non-proliferative retinopathy by 47%. In both cohorts, intensive therapy reduced the occurrence of *microalbuminuria* (urinary albumin excretion of >40mg per 24 hours) by 39% and that of albuminuria (urinary albumin excretion of >300mg per 24 hours) by 54%. In addition, clinical *neuropathy* was reduced in both cohorts by 60%. The major side-effect of the intensive therapy was a 2-3 fold increase in severe hypoglycaemia.

The overwhelming message of the DCCT therefore, is that the maintenance of blood glucose concentrations close to the normal range can prevent the chronic complications of diabetes. In the diabetic, normoglycaemia can be restored in several different ways and these are now reviewed in the next section.

B) RESTORING NORMOGLYCAEMIA IN THE DIABETIC PATIENT

1) Physiology of normal insulin synthesis and secretion

Before evaluating different methods of restoring normoglycaemia in the diabetic, it is first helpful to understand the complex process that is responsible for insulin synthesis and secretion in the normal situation.

Insulin is a polypeptide containing 2 chains of amino acids linked by disulphide bridges. The synthesis of insulin begins with transcription of the gene encoding insulin, which in humans is located on chromosome 11. Insulin is synthesised within the endoplasmic reticulum (ER) of the β -cells of islets of

Langerhans as part of a larger pre-prohormone, namely pre-proinsulin. This short-lived precursor is synthesised on the polyribosomes of the endoplasmic reticulum, after which it is transported to the cisternae of the ER, where the 23-amino-acid leader sequence is removed producing proinsulin (Figure 1.3). The remainder of the molecule is then folded and the disulphide bonds are formed. Once the correct tertiary structure has been formed, the pro-insulin is transported to the Golgi apparatus, where proinsulin is cleaved by proteolytic enzymes, such as trypsin-like proteases, carboxypeptidase and kallikrein, to produce insulin and connecting peptide (C-peptide). The latter contains 31 amino-acid residues and has about 10% of the activity of insulin ³². Its significance clinically is that it can be measured by immuno-assay and its level provides an index of β -cell function in patients receiving exogenous insulin. Within the Golgi apparatus the insulin molecule forms hexamers and also complexes with zinc. Insulin and C-peptide are then packaged together in membrane-bound secretion granules and stored within the cytoplasm of the β -cell. The whole process of insulin synthesis, from gene transcription to the formation of secretory granules, takes 1-3 hours ³³.

The secretion of insulin involves the exocytosis of the secretory granules through the plasma membrane. Initially the granules move from the cytoplasmic pool to the cell membrane, via the microtubule / microfilament system of the β -cell cytoskeleton. This is followed by the fusion of the granule with the membrane (a process thought to be regulated by the phosphorylation of membrane proteins) ³³ and the subsequent movement of insulin out of the cell (Figure 1.4).

The stimulus for insulin exocytosis is principally glucose, although other hexoses such as mannose, fructose and galactose, as well as amino acids and 2-keto acids, also have a limited insulinotropic capacity ³⁴. On a sub-cellular level, the stimulus-secretion coupling for insulin centres around the electrophysiology of the K^+ -ATP channel ^{35, 36}. In the resting β -cell, the K^+ -ATP channels are "open" allowing K^+ ions to leave the cell. This

Figure 1.3 The pathway of insulin synthesis

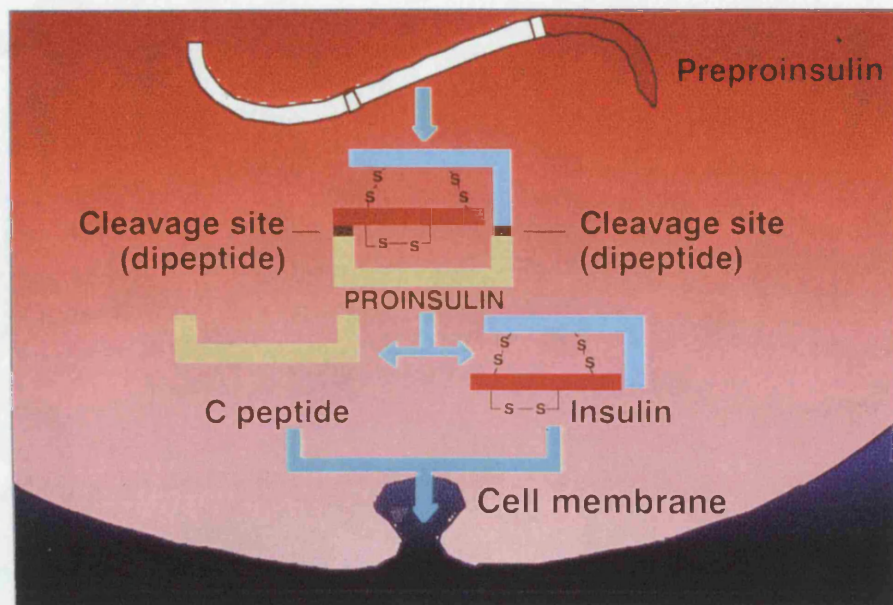
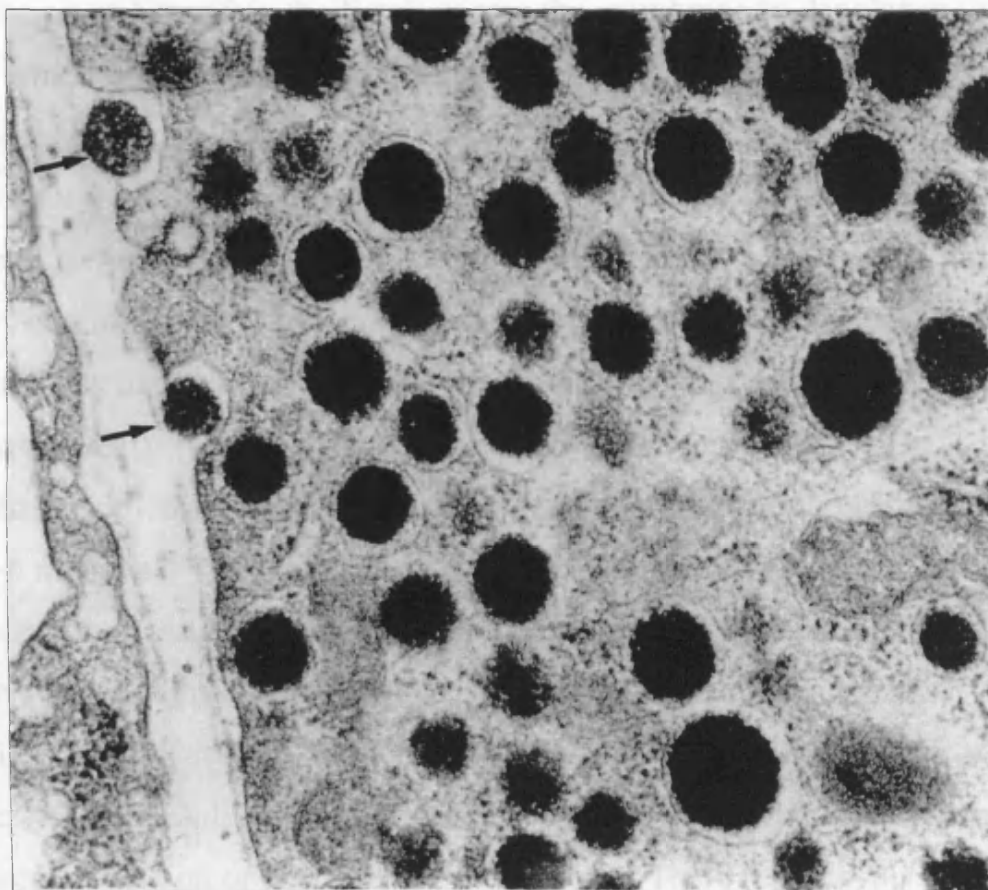


Figure 1.4 Electronmicrograph of insulin secretion



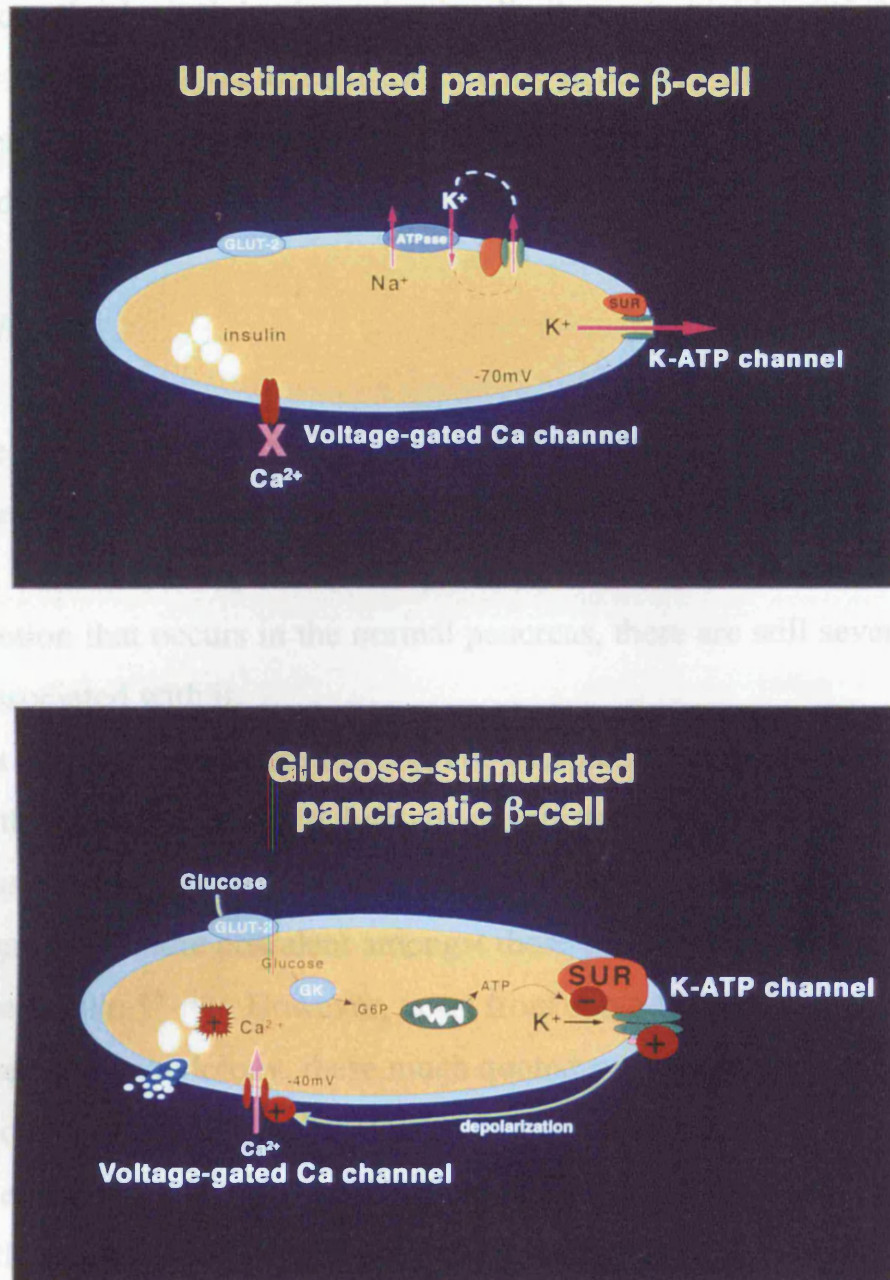
results in a resting membrane potential of -70 mV. When the β -cell is stimulated by glucose, glucose enters the cell via the Glut 2 receptor and is metabolised within the cell resulting in the production of ATP. The direct action of ATP then "closes" the K^+ -ATP channel. The subsequent accumulation of K^+ within the β -cell causes the membrane to depolarise to -40mV, which in turn "opens" the voltage-gated Ca^{2+} channels (Figure 1.5). The resulting calcium influx, together with the mobilisation of calcium from stores within the ER, directly triggers the process of exocytosis from the insulin storage granules. The absence of a functional K^+ -ATP channel has recently been implicated in the pathogenesis of the hyper-insulinaemia that occurs in the congenital condition persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) (previously termed neonatal nesidioblastosis) ³⁷.

In the resting situation, small amounts of insulin are secreted in pulsatile bursts every 5-6 minutes ^{34, 38}. It has been suggested that this pulsatility maintains the sensitivity of insulin receptors ³⁹. It is interesting to note that this pulsatility is maintained, albeit delayed, in isolated islets of Langerhans, suggesting that at least part of the regulatory system of insulin secretion is intrinsic ³⁹. The insulin secretory response to glucose is initiated at a serum glucose concentration of approximately 5 mmol/l. The overall response is sigmoidal, reaching a plateau at about 20 mmol/l ³³. The response to glucose is initially bi-phasic with an initial rapid "spike" followed by a prolonged second phase which continues until the stimulus is removed ³³.

The overall regulation of insulin secretion is a complex process involving metabolic influences, influences from the entero-insular axis(both neural ^{40, 41} and hormonal ^{40, 42}), and influences from the central nervous system (particularly within the hypothalamus ⁴³).

2) How can normoglycaemia be restored in the diabetic patient?

Figure 1.5 The normal electrophysiology of insulin secretion



2) How can normoglycaemia be restored in the diabetic patient?

a) Intensive insulin therapy

The DCCT clearly showed that intensive insulin therapy provides a significant benefit over the conventional once or twice daily injections that is currently the mainstay treatment for most patients with IDDM. Intensive therapy can be administered in two main ways.

Frequent injections

Many of the patients in the intensive therapy group of the DCCT received three or more daily insulin injections, combined with frequent blood glucose monitoring. Whilst this approach attempts to provide a closer imitation of the insulin secretion that occurs in the normal pancreas, there are still several problems associated with it.

First, as has already been stated, this form of treatment is associated with an increase in the incidence of severe hypoglycaemia and hypoglycaemic unawareness ⁴⁴. Prior to the DCCT, several studies had suggested that severe hypoglycaemia was more prevalent amongst those patients using human rather than porcine insulin ^{45, 46}. However, apart from some fundamental problems with the research methodology, these much quoted studies both involved patients who had switched from porcine to human insulin, rather than patients who had been started on human insulin from the time of diagnosis.

Therefore, the most likely interpretation of the findings of these studies, is that it is the change of insulin type rather than the use of human insulin per se that resulted in the increased incidence of hypoglycaemia. Indeed, in his review on human insulin, Gale concluded that the species of administered insulin did not influence the incidence of hypoglycaemia ⁴⁷. This is supported by the fact that hypoglycaemia was not a problem with the conventional therapy group in the DCCT, despite many of these patients taking human insulin.

Second, whilst this approach enables maintenance of glucose levels closer to the normal range (as measured by glycosylated haemoglobin levels), it does not correlate with the minute to minute regulation achieved by normal pancreatic islets ^{38, 48}. Therefore true normoglycaemia is not obtained.

Third, frequent injection is associated with potential complications at the injection sites. These include lipohypertrophy which results from multiple injections into the same site, and lipoatrophy which is more commonly seen with the use of bovine rather than porcine or human insulin ^{49, 50}.

Finally, any treatment aimed at preventing the long-term complications must be applicable to children. It is hard to imagine many pre-adolescent diabetics tolerating injections up to five times a day in addition to frequent blood glucose testing.

Continuous insulin infusion (insulin pumps)

With the limitations of frequent insulin injections in mind, much work has been done to try to produce continuous insulin infusion devices that can either be adjusted by the patient guided by the results of blood glucose measurements (open-loop systems) or in which there is automatic sensing of blood glucose and feedback control of the insulin delivery rate (closed-loop systems or artificial endocrine pancreas).

The first successful clinical use of open-loop continuous infusion devices was described by Slama et al. in 1974 ⁵¹. In this initial series, the insulin was administered intravenously. However, the inherent dangers of a continuous intravenous device led to the development of continuous subcutaneous infusion devices ⁵². Although these open-loop devices have become commercially available, they are associated with several problems. First, several studies including the DCCT, have shown that they are associated with a significant increase in episodes of ketoacidosis and hypoglycaemic coma ^{31, 53, 54}. Second, there is an increase in skin infections at the infusion site ^{54, 55}, especially abscesses ⁵⁶, and systemic infections,

including the toxic shock syndrome ⁵⁷. Third, there are problems with battery failure and administration tubing defects ⁵⁵. Therefore on the whole, their clinical use has been limited to use in patients with "brittle" diabetes. There are two main types of closed-loop devices currently being investigated, namely electromechanical devices and the bioartificial pancreas. Electromechanical devices use microcomputer technology to respond to the constantly monitored blood glucose and either a syringe or peristaltic pump to deliver the insulin. These systems can either be extracorporeal or implanted subcutaneously. Whilst they are an attractive concept, they also are associated with a host of fundamental problems. In addition to all the problems encountered with the open-loop devices, there have been restrictions in both the miniaturisation of the electromechanical components, but more importantly there have been major problems with making the components biocompatible. Therefore, these pumps remain too large for implantation in young children and even with the extracorporeal systems, their long-term use is unlikely for many years ⁵⁸. The bioartificial pancreas is a closed-loop system that instead of using exogenous insulin, contains isolated islets of Langerhans ⁵⁹. These devices can either be situated extra- or intra-vascularly. The extravascular devices consist of islets placed either in microcapsules or hollow fibres (see chapter 2). While this approach is a step closer to the normal physiological situation, the current membranes that are used generate a severe inflammatory reaction in whichever anatomical site the devices are placed. Therefore, the future use of the bioartificial pancreas is dependent on the development of biocompatible materials ⁶⁰.

b) Stimulation of residual beta-cell function

Intensive insulin regimes make the presumption that the endocrine component of the IDDM patient's own pancreas has ceased to function. There are

however, several potential treatment options that aim to stimulate insulin secretion from the residual beta cells within the diabetic pancreas, thereby "reversing" the diabetic process, rather than merely "treating" the established diabetic.

As previously stated, IDDM is thought to be an autoimmune disease ^{1, 2}.

Several groups have therefore attempted to use immunosuppressive agents in order to prevent the autoimmune antibodies from destroying the beta cells of type 1 diabetics ^{61, 62}. Although this treatment can reverse diabetes for up to 2-3 years, the patients all return to insulin independence after the immunosuppressive treatment has been stopped. Apart from the dangers of long-term immunosuppression (see below), it would appear that this treatment provides remission of diabetes, rather than a cure.

Another approach has been to stimulate the growth of the residual β -cells (this is discussed in more detail in Chapter 2). Nicotinamide has been shown to increase the growth of adult β -cells ⁶³ and to protect them by preventing depletion of intra-cellular NAD ⁶¹. Although the use of this agent has been successful in protecting β -cells in animal models ⁶¹, it has not been successful so far in the clinical situation ⁶⁴. However, it is currently the focus of a large multi-centre trial amongst type 1 diabetic children and children of diabetic parents.

c) Gene therapy

Few would disagree that the ultimate "treatment" for diabetes is to "prevent" diabetes developing in the first place. It has been realised for many years that IDDM exhibits a genetic susceptibility. The genetic basis for the disease is thought to be a complex process involving several genes, although it has been estimated that 60-70% of this susceptibility is encoded for in the class II subclass of the human leukocyte antigen (HLA) region of chromosome 6 ⁶⁵, with the DQA1 and DQB1 genes encoding for the DR4 allele being thought to play a crucial role ^{66, 67}. More recently, researchers have localised specific

gene defects that have been implicated in the development of IDDM ⁶⁸. With these findings in mind, it is envisaged that gene therapy could provide the ultimate cure for diabetes. However, whereas such technology has been used in a few diseases which involve single point mutations, it is still many years before gene therapy can be used for diseases such as diabetes, which have a complex aetiology involving several gene defects in addition to environmental factors.

d) Transplantation of insulin producing tissue

Whilst it is possible to maintain steady glucose levels with intensive insulin regimes, the only way of truly restoring normal glucose regulation is by the transplantation of insulin producing tissue. In theory, this can be done by either transplanting the whole pancreas as a vascularised graft or by transplanting isolated islets or β -cells. Whilst the results of vascularised whole pancreas transplants are encouraging (since 1990, 4000 cases were reported to the International Pancreas Transplant Registry with a 1 year graft survival rate of 75% and an actuarial 5-year function rate of 60%) ⁶⁹, this procedure involves the diabetic patient undergoing a major abdominal operation under general anaesthetic with its associated mortality and morbidity, followed as with any whole organ transplant, by the inevitable requirement for long-term immunosuppression. As the risks of side-effects from prolonged use of immunosuppressive agents potentially outweigh the risks of developing the long-term complications of diabetes (as highlighted earlier in this chapter 50% of diabetics never develop long-term complications), it is unlikely that vascularised pancreas transplantation will ever become an option for newly diagnosed diabetic children. As the whole aim of diabetic management is to restore normoglycaemia at an early stage of the disease, the use of whole pancreatic grafts is probably restricted to adult diabetics who already have the chronic complications of diabetes, rather than as a therapy for preventing the complications from developing. A much more promising approach therefore

is the transplantation of isolated islets of Langerhans. Indeed, in a recent review of vascularised pancreas and pancreatic islet transplantation by Remuzzi et al.(1994), it was concluded that "research effort should concentrate on islet transplantation with the aim of improving survival of the insulin-producing cells and protecting them from immune attack" ⁷⁰. The potential merits of islet transplantation are discussed in the next section.

3) Rationale for Islet Transplantation

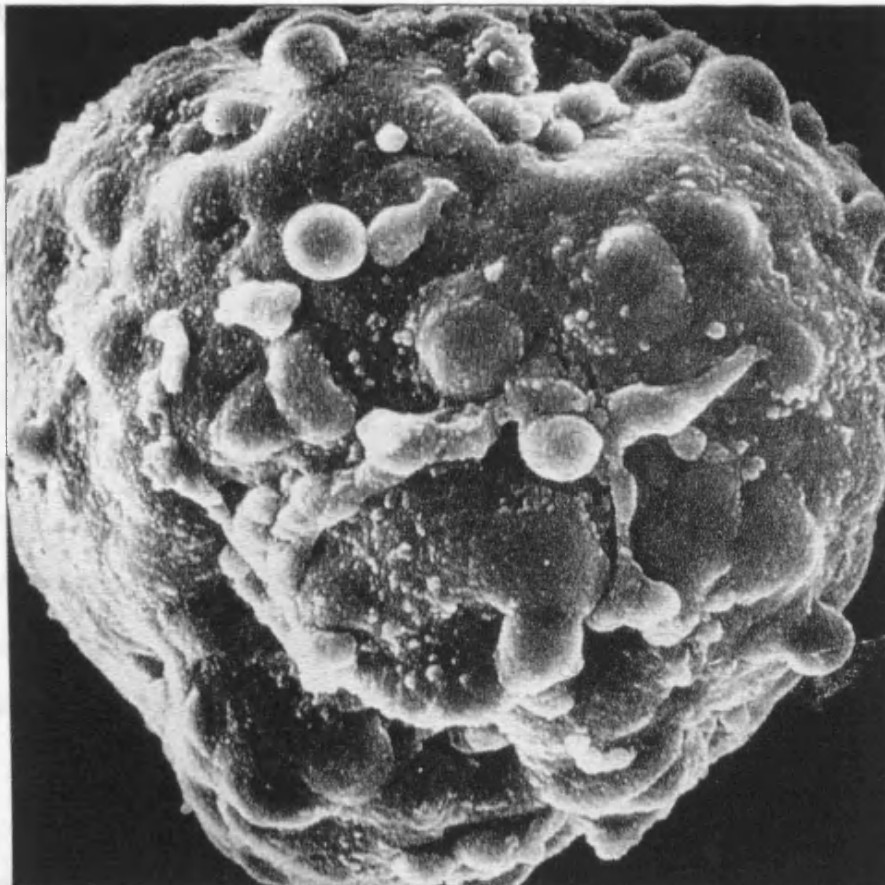
The transplantation of isolated islets offers several theoretical advantages over the methods of restoring normoglycaemia discussed above. First, it is a procedure performed percutaneously under local anaesthetic. Therefore, providing the volume of tissue transplanted is limited, the procedure is associated with minimal mortality and morbidity. Second, because it involves the transplantation of a "cellular" rather than whole organ graft, there is the potential for reducing the immunogenicity of the graft "in vitro" prior to transplantation (see next chapter). This has the potential for obviating the need for prolonged immunosuppression. Both of these factors make islet transplantation an attractive option for use in the young diabetic patient.

The use of β -cells versus islets

Several laboratories have suggested that rather than transplanting whole islets, a better approach is to transplant isolated β -cells. However, such an approach fails to understand the ultra-structure of the islet, and in particular the influences that the different constituent cells have on each other.

Islets of Langerhans make up only 1-2% of the weight of the adult human pancreas ³². Each islet contains between 2 and 5 thousand cells (Figure 1.6), of which the insulin-producing β -cells make up 60-70% by volume ⁷¹. The

Figure 1.6 Electronmicrograph of an islet of Langerhan



principle other cell-types present within the islet are the α -cells which secrete glucagon, the δ -cells which produce somatostatin, and the F-cells which secrete pancreatic polypeptide. The β -cells are generally located in the core of each islet, while the other cell types are distributed around the islet's periphery ³². This intricate arrangement allows the secretion of each hormone to exert a paracrine effect on the other adjacent cell-types ³³ (for more details see Chapter 2, section 2a). It would therefore appear, that although islets are able to function in isolation of the exocrine component of the pancreas, the β -cells require the complete cell complex of the intact islet for optimal insulin secretion and glucose-homeostasis.

In summary, the long term complications of diabetes mellitus can be prevented by the restoration of normoglycaemia at an early stage in the disease. Whilst there are several theoretical ways of achieving this, the most physiological and least invasive method available at present is by the transplantation of isolated islets of Langerhans. The final section of this chapter reviews the results to date of clinical islet transplantation.

C) RESULTS OF CLINICAL ISLET TRANSPLANTATION

1) Islet Autotransplantation

Although this procedure is used for the treatment of surgically-induced diabetes (following total or partial pancreatectomy), rather than for true IDDM, the encouraging results of this procedure mean that potential lessons can be learnt for the islet allotransplant situation. The main differences between the methods used for islet auto- and allotransplants, are highlighted in Table 1.1 and seen in Figure 1.7.

Up to August 1999 (the most recent registry data), a total of 222 autotransplants performed in 24 different institutions had been reported to the

International Islet Transplant Registry (IITR) ⁷². In a more detailed analysis of those autotransplants performed between January 1990 and December 1998, 114 transplants were performed in 12 centres. Of these patients, it is reported that 69% achieved insulin independence for >7 days and 50% were insulin independent for >1 year. However, these figures must be interpreted with caution. In obtaining the 69% figure, only 58 of the 114 were analysed whereas only the data from 50 patients were analysed for the percentage insulin independence >1 year. These data are however, consistent with that of the Minneapolis group who have performed the largest number of islet autografts (54 cases). In an earlier report from their series 58% of 36 patients were still insulin-independent at 2 years ⁷³. The longest insulin-independent follow-up after this procedure is >12 years. This group have reported that the main determinant of success, was the number of islets transplanted ⁷³. Indeed they claim that "insulin-independence after islet autotransplantation following total pancreatectomy can be nearly predicted by the islet mass transplanted" ⁷³. They have found that if >300,000 Islet Equivalents (IEQ) (see next chapter for explanation / calculation of IEQ) were transplanted, the probability of insulin independence for greater than 2 years was 68%. This is also supported by the recent registry data (71% at 1 year). If on the other hand <300,000 IEQ were transplanted the probability of reversing diabetes for longer than 2 years was only 14%. However, there is also a problem in interpreting the results from this particular series as it includes patients who had undergone subtotal (>95%) pancreatectomy, in addition to those who had undergone total pancreatectomy. These two groups of patients have not been analysed separately and so there is no indication of what proportion of the insulin-independent patients had undergone each operation. The number of islets required to obtain insulin-independence in the subtotal pancreatectomy group is clearly influenced by the amount of insulin produced by the remnant pancreas. Many groups use unpurified autograft preparations which may be more accurately termed dispersed pancreas autografts, rather than true islet autografts.

Islet Autografts

no vascular perfusion

minimal pancreatic CIT

usually not purified

no insulin resistance

non-diabetic liver

parenteral nutrition

Islet Allografts

vascular perfusion

long pancreatic CIT

usually purified

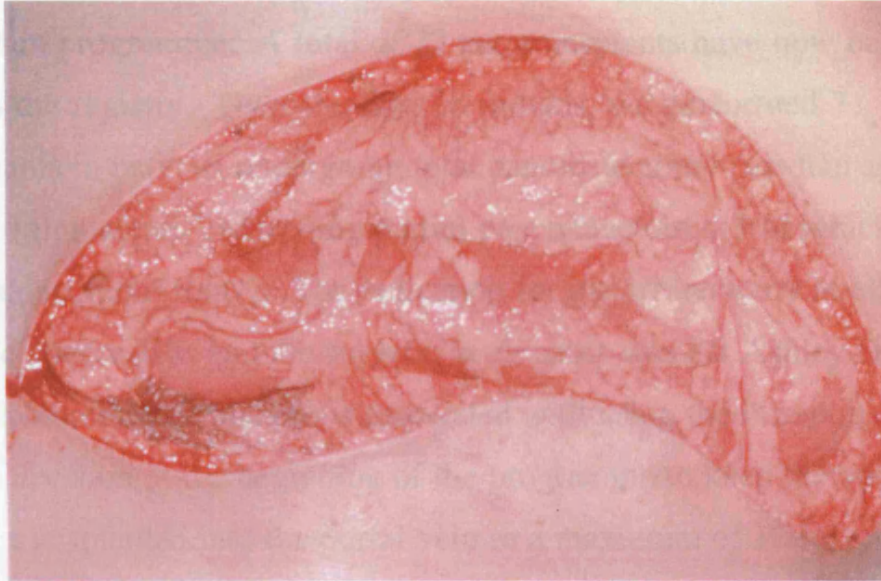
insulin resistance

diabetic liver

enteral nutrition

Table 1.1. A list of the key differences between islet autotransplantation and allotransplantation.

Figure 1.7 Islet transplantation (below) is a minimally invasive procedure compared with whole pancreas transplantation (above).



Following intraportal autotransplantation of impure preparations there have been reports of hepatic infarction ⁷⁴, portal vein thrombosis ^{75, 76}, portal hypertension ^{77, 78, 79, 80}, disseminated intravascular coagulation ^{78, 79}, splenic rupture ⁷⁵ and 3 deaths ^{79, 81, 82}.

During my research post in Leicester, we commenced a clinical islet autotransplant programme. A total of 23 autotransplants have now been reported to the registry. Over the first 18 months, we performed 7 autotransplants in patients undergoing total pancreatectomy (median age 48.5 years) and 3 in patients undergoing partial pancreatectomy (1 of which was a completion pancreatectomy). The indication for pancreatectomy in all patients was chronic pancreatitis and the surgeon in all cases was Mr Ashley Dennison. Due to the potential complications associated with using the intraportal route, we made a decision at the beginning of the programme to keep the volume of islet tissue transplanted into the portal vein to a maximum of 12 ml and to purify the pancreatic digest, thereby ensuring that the majority of the transplanted volume is islet tissue. Even though the volume of digest in the chronic pancreatitic pancreas might be expected to be reduced, the volume obtained in the majority of our cases has exceeded 12 ml. In order to be able to maximise the number of islets transplanted by transplanting all of the isolated islets whilst not exceeding 12 ml of tissue intraportally, an alternative or an additional transplant site was required.

The spleen has been used as a site for islet autografts in both canine ^{83, 84, 85}, and primate ⁸⁶ studies. There is only one reported case of an intrasplenic transplant being performed in the human and this was unsuccessful ⁸⁷.

However, studies comparing the intra-portal and intra-splenic sites for autotransplantation in the dog showed that although the initial results are similar, the intra-splenic site was associated with more prolonged graft survival ^{83, 84, 85}. For this reason, together with the expendability of the spleen as compared with the liver, we decided to combine the intraportal and intrasplenic routes in those preparations which exceeded 12 ml.

Of the total pancreatectomy group, 7 patients received islets embolized into the liver via the portal vein (median transplanted volume= 8.5 ml) and in addition 3 patients had islets embolized into the splenic sinusoids via a short gastric vein (median transplanted volume= 4 ml). One patient died of a stroke 4 weeks postoperatively. Of the 6 surviving patients, all have C-peptides in the normal range (median = 2.94 ng/ml). However, only 1 patient is insulin-independent (14 months post-transplant). In trying to explain the fact that despite normoC-peptidaemia, 5 patients remain insulin-dependent, we have recently found high levels of both intact proinsulin (geometric mean 6.2 pmol/l vs normal 3.8 pmol/l) and 32-33 split proinsulin (geometric mean 22.3 pmol/l vs normal 6.3 pmol/l) in these patients (as measured by a specific two-site immunoradiometric assay). As proinsulin cross-reacts with the commercial C-peptide assay, the measured C-peptide levels are probably falsely elevated. This is confirmed by the fact that endogenous insulin levels were below the normal range. These findings would suggest that C-peptide levels are an unsuitable parameter for assessing the success of the autografts in these patients. More significantly, it has been shown that high levels of split proinsulin are atherogenic and therefore close observation of these patients is mandatory.

Subsequently, we performed a transplant using the splenic route only and this patient achieved insulin-independence for 4 months following the procedure. The main problem faced during this procedure was the technical difficulty of preserving the spleen on the splenic vessels. We now intend to continue with the intrasplenic approach. It will be of interest to measure the intact and split proinsulin levels in these patients to determine whether the splenic site confers an advantage in this regard.

In conclusion, islet autotransplantation has prevented a significant number of patients from becoming insulin-dependent following total pancreatectomy. However, although long-term islet autograft function still requires more complete evaluation, a recent paper by the Minneapolis group has suggested

that islet function deteriorates substantially after 2 years, with a significant number of patients becoming diabetic.

2) Islet Allotransplantation

a) Adult Islet Allotransplantation in type 1 diabetics

Up to August 1999, a total of 407 adult islet allografts, from 33 different institutions, had been reported to the Islet Transplant Registry ⁷². Of those performed before January 1995, insulin-independence was achieved in 28 patients at one month post-transplant, 20 patients at 6 months, 14 patients at one year, 4 patients at two years and 3 patients were still insulin independent at three years ⁸⁸. In an analysis by era in this series, the number of pre-transplant C-peptide negative diabetic recipients showing basal C-peptide levels of >1ng/ml at >1 month post-transplant were 21% between 1985 and 1989 (n=29), 65% between 1990 and 1991 (n=37) and 59% in the 1992-1993 era (n=39). Subsequently, the Islet Registry team at Giessen performed a more detailed analysis on 75 pre-transplant C-peptide negative type 1 diabetics who received adult islet allografts, in order to determine whether it was possible to determine the factors that influenced post-transplant insulin-independence ⁸⁸. Recipient age, gender, duration of diabetes, number of donor pancreata and islet purity did not influence the one-year graft survival rates. However, insulin independence was only achieved if all four of the following criteria were met :1) islets isolated from pancreata with a mean preservation time <8hours; 2) >6000 IEQ / kg body weight transplanted; 3) islets transplanted into the liver via the portal vein; 4) induction immunosuppression with anti-T-cell antibodies. Whilst this study makes an attempt at applying statistical analysis to what had previously been random observations, it is fundamentally flawed in a number of respects. First, the numbers included are too small to make any firm conclusions (the

number of insulin-independent recipients in this group was 11 and 8 at 1 month and 1 year respectively). Second, the statistical analysis has not involved multivariate analysis. Therefore the conclusions made can only be regarded as loose associations rather than true correlations. Third, with 19 different institutions involved, there may well be many different inter-laboratory factors (e.g. differences in organ procurement and islet isolation methods) that have not been included in the analysis, and yet these may have a significant influence on allograft outcome. (Factors influencing allograft success is discussed in detail in the next chapter).

However, in the most recent registry report, 200 islet allografts performed in pre-transplant C-peptide negative recipients between 1990 and 1997 were analysed⁷². Interestingly, for this analysis the definition of graft function had been reduced to a basal C-peptide level of >0.5 ng/ml. This study showed 68% graft function at 1 month with 14% achieving insulin independence for at least 7 days. The graft function at 1 year was 35% with 19 patients still insulin-independent at this time. The 1 year graft survival was 39% for those receiving a simultaneous islet-kidney transplant, compared with 31% for those receiving islets after an established renal graft. The numbers of islets transplanted (>6000 IEQ / kg), a cold ischaemia time of <8 hours, the use of ALG / ATG immunosuppression, and the liver as the site of implantation were all associated with an increased graft survival (83% graft survival at 1 month in those patients in whom all 4 criteria were fulfilled).

In Leicester between August 1990 and June 1992, we performed 3 islet allotransplants^{89, 90}. The first two patients received islet pooled from multiple cadaveric organ donors and both these grafts failed rapidly, almost certainly due to rejection. The third patient received islets from a single well MHC matched donor and his graft continues to function 3 years post-transplant, although he has never achieved insulin-independence.

The first patient was a 40 year old, C-peptide negative, female who received an islet allograft 5 months after receiving a renal transplant. She received a total of 4700 IEQ / kg body weight, pooled from fresh CMV positive islet

preparations. The purity of the pooled preparations was 60% and the total volume of transplanted tissue was 1.1ml. Although her C-peptide level rose to a maximum of 0.9 ng/ml on day 9, by 21 days it had fallen to zero. This correlated with a decrease in insulin requirements, from 40 U insulin daily to 24 U daily by day 12, followed by a gradual increase again. The patient remains well 4¹/₂ years later.

The second patient was a 57 year old diabetic man who had an established renal graft. He received a total of 7600 IEQ / kg body weight, pooled from one fresh and two cryopreserved islet preparations (a total of 565,000 IEQ - 195 000 IEQ fresh; 370,000 IEQ cryopreserved). The purity of the pooled preparation was 80% with a total volume of tissue of 1.7ml. Fasting C-peptide levels reached a maximum of 3ng/ml 18 days post-transplant and then fell to zero by day 35. This was reflected in an initial fall in insulin requirements. Again the patient remains well.

The final patient was a 47 year-old male who became diabetic at the age of 12 years. He received a simultaneous islet-kidney transplant (islets transplanted 4 days post-renal transplant and isolated from the pancreas from the same donor). A total of 2600 IEQ / kg were transplanted. The purity of the final islet preparation was 95%. Stimulated C-peptide levels reached a maximum of 3.2 ng / ml at 3 months. At 18 months the graft was still functioning as determined by a C-peptide level of 1 ng / ml. Although the patient still has measurable C-peptide, it is now less than 1 ng / ml. Pre-operatively the patients insulin requirements were 60 U / day and this has now been reduced to 40 U / day. Post-transplantation, he has had intermittent problems with hypoglycaemia unawareness. Our results would suggest that improved results are obtained using a sufficient number of islets from a single, well MHC matched donor. The problems achieving this ideal are discussed in the next two chapters and form the basis for the work described in this thesis.

Over the past year, islet transplantation has received a welcome boost from the data from the Edmonton group (this will be included in the 2001 analysis of the islet registry). They have recently reported 7 consecutive islet

allografts that achieved insulin independence for a median period of 11.9 months ⁹¹. All the patients were type 1 diabetics with diabetes for more than 5 years. All had pre-transplant C-peptides of <0.48 ng/ml. All patients received an islet graft alone and none had previously received a renal graft. The median age of recipient was 44 years (range 29-54). A further 5 patients at this centre have also subsequently achieved insulin-independence. The main aspects of this series are the glucocorticoid-free immunosuppressive regime (see Chapter 2) and the fact that all recipients received islets from at least 2 donors. The need for islets from more than one donor again outlines that human islet isolation has ongoing problems that must be approached. The total mean number of islets transplanted were 11,547 IEQ/kg (SD +/-1604).

b) Adult Islet Allotransplantation in non type 1 diabetics

The first reported success in this group of patients concerned a patient with haemochromatosis, who was C-peptide negative pre-transplant, and received a combined liver and islet transplant. The patient remained insulin-independent for 49 months post-transplantation.

Another group of non-type 1 diabetic patients received allografts following upper abdominal exenteration for malignancy. These so called "cluster" transplants were performed in Pittsburgh. Of the 11 "cluster" transplants performed at this centre, 6 patients (55%) were insulin-independent until their demise from recurrent malignancy ⁹², with one patient surviving 58 months ⁹² ⁹³. This latter patient is the longest reported case of insulin-independence following an islet allotransplant. Two other islet allografts have been performed in totally pancreatectomised recipients (at Giessen / Wuerzburg and Milan), and once again a period of insulin independence was obtained in both cases.

With the incidence of type 2 diabetes being about 10 times more common than type 1, several have explored whether islet allotransplantation has a place in the treatment of these patients. Thomas et al. have successfully reversed type

2 diabetes in the Zucker rat by islet transplantation ⁹⁴. The team at the Diabetes Research Institute in Miami have transplanted islets into two insulin-resistant cirrhotic patients who were receiving a concurrent liver transplant. Whilst insulin-independence was not obtained in either of these patients, their insulin requirements were significantly reduced ⁹⁵.

c) Foetal Islet Allotransplantation

Whereas it is generally accepted that endocrine cells of the fully mature adult pancreas have limited proliferative capacity ⁹⁶, fetal islets have a marked capacity for growth that is associated with functional maturation ⁹⁷. In addition, fetal islets are thought to be less immunogenic. It is for these reasons that several groups have transplanted foetal rather than adult islets.

It is estimated that more than 2,000 foetal islet allografts have been performed worldwide, with the majority being performed in Russia and China. Data on 187 of these, from 11 institutions, has been reported to the Islet Registry ⁸⁸. Three patients have become insulin-independent, although it is not known whether these patients were C-peptide negative pre-transplant. Eight further patients who were C-peptide negative prior to receiving a foetal islet allograft, have demonstrated post-transplant basal C-peptide levels exceeding 0.5 ng/ml for periods of 2 to >48 months.

Apart from the ethical restrictions of using foetal tissue, the number of foetal pancreata required to isolate sufficient numbers of islets makes this approach impractical.

Conclusions

Islet transplantation clearly offers a potential treatment for children with IDDM. Recent success from the Edmonton group has led to renewed interest in this field and indeed the establishment of a multi-centre trial using the Edmonton Protocol. Nonetheless, clinical islet transplants in adults have so far

resulted in limited success. The reasons for these failures and suggestions for possible improvements, are the subject of the next chapter.

CHAPTER 2

Adult Islet Allotransplantation - Factors Influencing Success

Chapter 2 Outline

Introduction

A) Reasons for islet allograft failure

- 1) Transplantation of insufficient islet numbers
- 2) Failure of the transplanted islets to engraft into the ectopic site
 - a) Revascularisation
 - b) Reinnervation
- 3) Loss of islet function post-implantation
 - a) Antigen-specific T-cell mediated rejection
 - b) Non-specific inflammatory damage
 - c) Recurrence of autoimmunity
 - d) Immunosuppressive drug toxicity
 - e) Metabolic exhaustion of the islets

B) Strategies for improving results of islet allotransplantation

- 1) Improving islet numbers
 - a) Increasing number of donors
 - b) Using alternative sources of islets
 - c) Optimising methods of human islet isolation
- 2) Prevention of immune rejection
 - a) Improved immunosuppressive regimes
 - b) Immunomodulation

c) Immunoisolation

d) Induction of immune tolerance

Conclusions

Introduction

It is clear from the preceding chapter that although a significant number of adult islet allotransplants have now been performed in different centres worldwide, the number of patients achieving prolonged insulin-independence has been limited. On the other hand, relatively good results have been obtained with both vascularised pancreas transplantation and islet autotransplantation. With these observations in mind, this chapter examines some of the reasons for islet allograft failure and outlines possible strategies for preventing them.

A) REASONS FOR ISLET ALLOGRAFT FAILURE

There are a number of different reasons why islet allografts have failed to achieve insulin independence in both the short and long-term. Whilst many of these factors are interrelated, in broad terms there are three main causes. These are: the transplantation of insufficient islet numbers; failure of the transplanted islets to engraft into the ectopic site; and loss of islet function post-implantation. Each of these causes will be reviewed in turn.

1) Transplantation of Insufficient Islet Numbers

One of the major causes of early islet graft failure is that the number of islets initially transplanted was insufficient to supply the insulin requirements of the diabetic recipient. Before discussing in more detail the concept of a "critical number" of islets required to reverse diabetes however, it is important to clarify an important point.

Islets of Langerhans vary considerably in size. Indeed Kaihoh et al. (1986) have shown that the diameters of islets within any one human pancreas range

from 15 to 500 μm ⁹⁸. As islet volume is proportional to the cube of the diameter, the variation in islet volume (i.e. overall size of the islet) can be as great as 30,000 fold. It is therefore important that any calculation of islet numbers takes islet size into account. With this in mind, a workshop was held in Minneapolis in 1990 at the 2nd Congress on Pancreas and Islet Transplantation in order to standardise the way that islet numbers were expressed. It was agreed that a more meaningful way of expressing islet numbers is either by indicating the number of 150 μm islet equivalents (IEQ) or the total islet volume of the final preparation ⁹⁹. The IEQ is a calculation based on the fact that the approximate mean diameter of islets within the human pancreas is 150 μm ⁹⁹ and converts the total islet number into the equivalent number of islets with a diameter of 150 μm (see Table 2.1). It was also uniformly recommended that a rapidly-acting, islet-specific stain, such as dithizone, is used in any sample that is being used for islet quantification ¹⁰⁰ ¹⁰¹, ¹⁰², ¹⁰³. Dithizone binds to the zinc granules within the islet resulting in a characteristic red stain ¹⁰³.

In order to calculate the number of IEQ's that need to be transplanted in order to reverse diabetes in the human, it is necessary to extrapolate data from a number of different sources. Firstly, the results of sub-total pancreatectomy for chronic pancreatitis would suggest that as little as 10-20% of the total islet mass is required to maintain normoglycaemia ¹⁰⁴, ¹⁰⁵. Taking into account that the average human pancreas, weighing 70g, contains between 304,000 and 1.5 million IEQ ¹⁰⁶, ¹⁰⁷, this corresponds to an islet mass of between 30,400 and 300,000 IEQ. However, more recent studies in the dog ¹⁰⁸ and baboon¹⁰⁹ have suggested that as much as 60% of the total islet mass is necessary for normal glucose metabolism. However, in interpreting the results of partial pancreatectomy two points must be remembered. Firstly, glucose-induced insulin secretion from islets within the tail and body of the pancreas is increased compared with islets within the head and neck. Therefore the achievement of normoglycaemia is affected not only by the total number of islets remaining within the remnant pancreas, but also

<u>Islet diameter</u> <u>range</u> (μ)	<u>Mean volume</u> (μ^3)	<u>Conversion into</u> <u>150μ IEQ</u>
50-100	294,525	n / 6.00
100-200	1,145,373	n / 1.50
150-200	2,977,968	n x 1.7
200-250	6,185,010	n x 3.5
250-300	11,159,198	n x 6.3
300-350	18,293,231	n x 10.4
350-400	27,979,808	n x 15.8

Table 2.1. Determination of islet volume for each 50 μ diameter range and relative conversion into equivalent number of islets with a diameter of 150 μ (IEQ).

by the anatomical region of the pancreas that forms that remnant. Secondly, islets within the remnant pancreas have the potential for regeneration.

Therefore, studies of the maintenance of normoglycaemia following partial pancreatectomy tend to underestimate the number of islets that are required in the allotransplant situation.

The next source of extrapolation for determining the required number of islets is the results of human intraportal islet autotransplantation. As has been already discussed in the last chapter, the group in Minneapolis, who have performed the largest series of this procedure, have reported that the main determinant of success was the number of islets transplanted ⁷³. They have found that if >300,000 IEQ were transplanted, the probability of insulin independence for greater than 2 years was 68%, compared with 14% if the recipient received less than this number. As already mentioned, the main problem with interpreting the results from this particular series is that it includes patients who had undergone subtotal (>95%) pancreatectomy, in addition to those who had undergone total pancreatectomy. In a similar way to the partial pancreatectomy data discussed above, the number of islets required to obtain insulin-independence in the subtotal pancreatectomy group is clearly influenced by the amount of insulin produced by the remnant pancreas. It is also pertinent to note that these transplants involved the use of unpurified rather than purified islets (see below). However, the Minneapolis findings are supported by large animal studies. In Cynomolgus monkeys, intraportal autografts of 3000 IEQ per Kg recipient were required to obtain normoglycaemia ^{110, 111}, whereas 3000-5000 IEQ per kg recipient were required to reverse diabetes in the canine model ^{112, 113, 114, 115}. The overall message from autotransplantation data therefore, is that the probability of insulin-independence is significantly improved if the recipient receives 3000-3500 IEQ per kg.

Clearly the data from islet autotransplants however, cannot be directly transposed to the allotransplant situation because of the differences highlighted in the last chapter. Again, in large animal studies, it has been shown that the

IEQ requirements for reversing diabetes in the allotransplantation model are significantly greater than those required for successful autotransplantation. In Cynomologous monkeys the requirements increased from 3000 to 3500 IEQ per kg recipient ¹¹⁶ whilst for allotransplants in the dog, a minimum of 5000 IEQ per kg recipient were required ¹¹⁷. This reflects the data emerging from clinical trials of human adult allotransplantation. Of the 75 adult allografts performed on C-peptide negative IDDM recipients and analysed in the 1995 islet registry, no patients who received <6000 IEQ / kg body weight achieved insulin-independence. This compares with 22% insulin independence at 1 month and 16% insulin independence at 1 year in those recipients receiving >6,000IEQ / kg. Whilst these findings have not been subjected to multivariate analysis, they are certainly consistent with the results of the animal studies. The recent success of the Edmonton group also highlights potential importance of the transplantation of high islet numbers. Indeed any islet preparation was regarded as unsuccessful if <4000IEQ/kg of potential recipient was not obtained ⁹¹.

The discussion so far has concentrated on the number of IEQ required to reverse diabetes in the short-term. However, one of the findings in human autotransplantation and allotransplantation series is that despite initial insulin-independence, in a substantial number of cases islet function seems to diminish with time ^{72, 73, 118}.

Kaufman et al. have established a correlation between the number of islets initially transplanted and the duration of graft function of canine intrasplenic and intraportal autografts ¹¹⁵. This would suggest that although a minimum number of islets required to reverse human diabetes human can be estimated, an additional number of islets may be required for long-term graft function. Indeed, it has been suggested that the optimal number of islets required for long-term allograft survival is 12,300 IEQ / kg recipient and it is interesting to note that the mean transplanted islet number in the recent successful Edmonton series was 11, 547 IEQ/kg (SD +/-1604).

In concluding this discussion on "critical islet mass", a few points of qualification need to be made. First, it should be noted that there is a wide variation in the total number of islets present in different pancreata. As mentioned above, it has been calculated that the average human pancreas contains between 304,000 and 1.5 million IEQ ^{106, 107}. This wide variation in islet number is confirmed by studies on both post mortem pancreata ^{119, 120 121, 122} and pancreata from cadaveric organ donors ¹²³. Therefore any estimation of efficiency of islet isolation should take into account the total number of islets present in that particular pancreas prior to isolation.

Second, even if the number of IEQ's transplanted is above the minimum requirement, not all of the transplanted islets will be viable. It is therefore necessary to qualify the IEQ with an assessment of islet viability. "In vitro" methods available for determining islet viability include light ¹²⁴ and electron microscopic ¹²⁵ morphology, fluorometric membrane integrity assays ^{126 127 128, 129}, colorimetric tests of mitochondrial function ^{130, 131} and glucose-stimulated insulin release studies ^{132, 133}. The most accurate way of assessing islet viability is by transplanting a sample of the prepared islets into a diabetic, immunodeficient rodent ^{134, 135, 136}. However, this method does not give an indication of what percentage of the total number of isolated islets are viable.

Third, although there have been attempts to standardise the procedures used for assessing the outcome of islet isolation, the methods used for both assessing islet numbers and islet viability vary between different islet centres and are subject to both intra- and inter-observer errors. For instance, the sampling technique for assessing islet numbers represents a critical source of potential error as islets settle rapidly in a container and care must be taken to ensure that the preparation is properly suspended before sampling to ensure a representative sample ⁹⁹. Finally, it is important to note that even when a large mass of highly viable islets is transplanted, success is not guaranteed. In three cases reported to the islet registry, the transplantation of >10,000 IEQ / kg failed to reverse the recipient's diabetes even in the short-term ^{137 138 139}.

This implies that other factors are also important for islet transplant success and these are now discussed in more detail

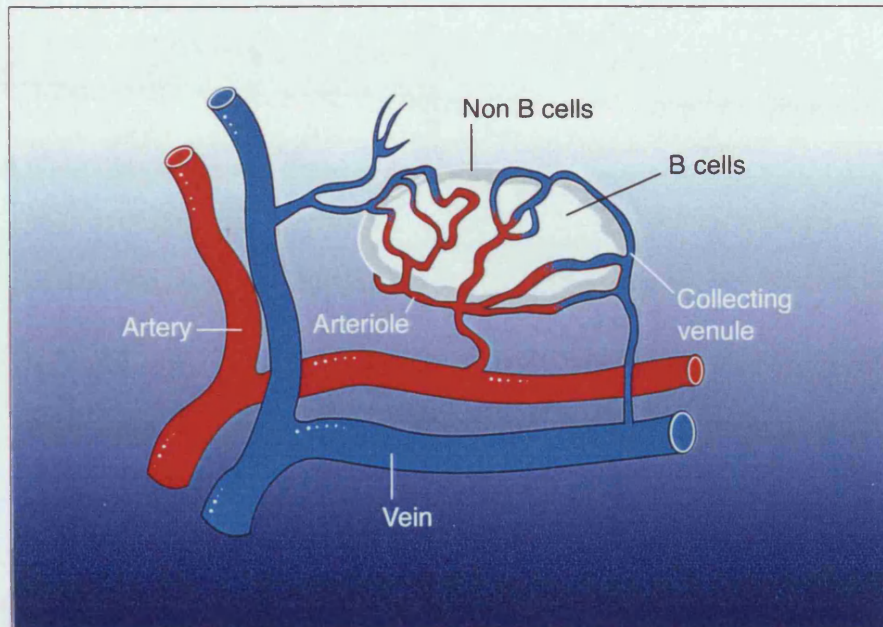
2) Failure of the transplanted islets to engraft into the ectopic site

The engraftment of a transplanted organ or cells into an ectopic site involves two processes, namely revascularisation and reinnervation. Each of these will be considered in turn:

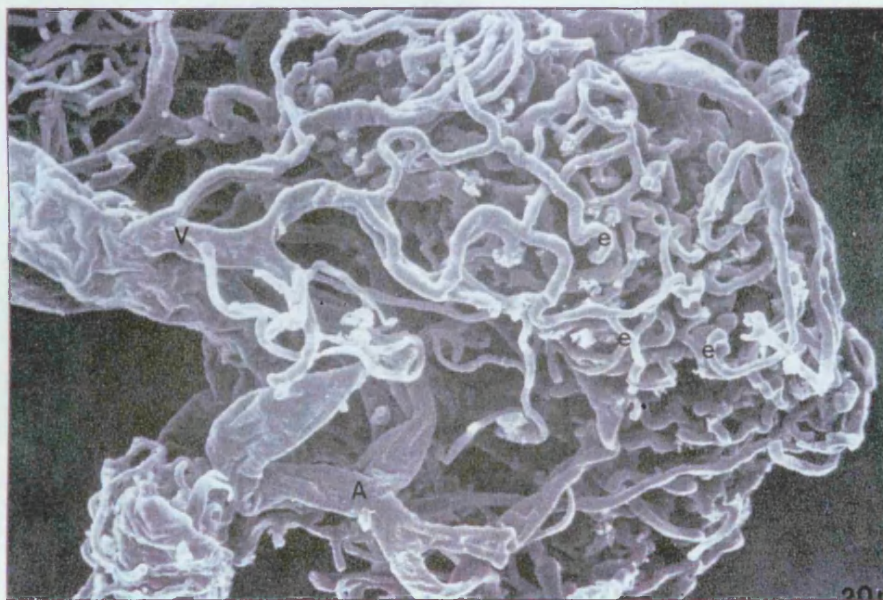
a) Revascularisation

Prior to isolation from the pancreas, islets of Langerhans are highly vascularised structures (see Figure 2.1). Indeed, although the islets represent only 1 to 2% of the total volume of the adult pancreas ^{140, 107}, the islet blood flow is between 10-20% of the total pancreatic blood flow ^{141, 142}. Arterial blood first enters the islets at a gap in the discontinuous mantle of alpha, delta and pancreas polypeptide producing cells (PP, delta 2 or F-cells) and reaches the beta cell core of the islets via small arterioles ^{143, 144, 145}. Within the core of the islets, the arterioles branch into capillaries and follow their route to the non-beta cell mantle. The capillaries coalesce into collecting venules and finally drain into the pancreatic venous system ^{140, 146, 147}. This intra-islet portal system enables insulin to act on the alpha and delta cells, thereby inhibiting the release of glucagon and somatostatin respectively ¹⁴⁸. Islet isolation renders islets avascular ¹⁴⁹. In the rat, revascularisation starts as early as 4 days post-transplantation, is well developed by 14 days and continues for up to 6 months ^{150, 151, 152, 153, 154}. Once the islets are fully revascularised, the vascular micro-anatomy described above is re-established ^{149, 155, 156, 150}.

Figure 2.1



Schematic drawing of islet blood flow



Electronmicrograph of islet blood supply

These findings in animal studies are reflected in the human clinical transplant situation as evidenced by the gradual increase in C-peptide levels over the first 21 days post-transplantation ¹⁵⁷. The revascularisation of islet grafts is potentially influenced by several different factors:

Purity of preparation

It is evident that in order to cure diabetes by islet transplantation, the graft must contain a sufficient amount of endocrine tissue. To what degree the graft should also be free from contaminating exocrine tissue however, has been open to more debate ¹⁵⁸. London et al. (1992) have made the point that "it could be argued that for a transplant to be properly considered an islet transplant (rather than an exocrine transplant) that at least 50% of the final preparation should be islets" ¹⁵⁹. It is clear from both animal studies and results of human islet transplants that although the inclusion of some ductal elements may confer some benefit ¹⁶⁰, the purity of the islet preparation influences the outcome of the islet transplant in several ways.

In theory, exocrine tissue might be expected to impair the engraftment of islet grafts due to the release of pancreatic protease enzymes from pancreatic acinar cells either directly damaging the islets or causing inflammation within the transplant site. These enzymes include trypsin, chymotrypsin, elastase, amylase, kallikrein, lipase, phospholipase A₂, carboxypeptidase A and carboxypeptidase, all of which, apart from amylase, are present within the acinar cells in their inactive forms as zymogens, but can potentially be activated by the islet isolation process ¹⁶¹.

Due to allograft rejection, the influence of islet purity on engraftment can only be studied meaningfully using iso- or auto-graft studies. Surprisingly, few such studies have been performed. In rats, it has been shown that the presence of exocrine tissue in the islet graft impairs the engraftment of islets under the kidney capsule ^{162, 163, 164}. A comparison of purified versus unpurified monkey subcapsular autografts showed that whilst the former

group appeared to undergo successful engraftment, the impure islets produced marked histological evidence of scarring and loss of islet tissue ¹¹¹. There is very little data however, showing impairment of islet engraftment following intraportal islet transplantation. It has been shown in the isogeneic rodent model that transplanting unpurified islets intraportally results in late graft failure ¹⁶⁵. However, this latter finding could also be explained by "islet exhaustion" (see later in this chapter) rather than failure of initial engraftment. Indeed, Gray (1989) has pointed out that the impairment of engraftment observed in the renal sub-capsular site, might not be expected in either the intraportal or intrasplenic sites due to the wider dispersal of tissue and therefore the reduced number of islets engrafting next to acinar tissue ¹⁶⁶. Certainly the experience of the Minneapolis human autotransplant programme described above, would suggest that good graft function can be obtained after the transplantation of unpurified preparations.

Although more detailed research is required, the limited studies to date would suggest that the problems associated with the transplantation of unpurified islet preparations via the intraportal route, appear to lie primarily with the safety of the procedure and possible increased immunogenicity, rather than with impairment of islet engraftment (the influence of impure islets on immunogenicity will be discussed under the section "loss of islet function post-implantation"). The safety of transplanting partially purified or unpurified islets has been brought into question as a result of a significant number of reported complications. As highlighted in the last chapter, following intraportal autotransplantation of impure preparations there have been reports of hepatic infarction ⁷⁴, portal vein thrombosis ^{75, 76} portal hypertension ^{77, 78, 79, 80}, disseminated intravascular coagulation ^{78, 79}, splenic rupture ⁷⁵, and 3 deaths ^{79, 81, 82}.

Although no deaths have been reported, similar complications have been observed following unpurified allotransplants ¹⁶⁷, including a patient in Edmonton who developed a portal vein thrombosis after receiving partially purified islets in a combined liver / islet allograft ¹⁶⁸. Whilst these

complications are probably mainly attributable to the total volume of tissue transplanted and the subsequent disturbance in hepatic blood flow, the intraportal infusion of the pancreatic enzymes mentioned above has also been implicated ^{169, 170}.

Immunosuppressive medication

One of the significant differences between the autotransplant and allotransplant situations, is the requirement for immunosuppressive therapy in the latter group.

Whilst this clearly aids the islet graft in terms of preventing immune rejection, many of the drugs that are used have been shown to either impair islet engraftment or to be islet toxic (the toxicity of immunosuppressive drugs is discussed below). Although there is some conflicting data, it would seem that cyclosporine impairs islet vascularisation. This has been shown following the transplantation of mouse islet isografts under the kidney capsule, the transplantation of rat islet xenografts into hamster dorsal skinfold chambers, and the transplantation of canine islet autografts into the spleen. Prednisolone has been reported to inhibit the revascularisation of Syrian golden hamster isografts ¹⁷¹.

Some of the newer immunosuppressive drugs, such as 15-deoxyspergualin (see later in this chapter), actually appear to improve islet revascularisation ¹⁷².

Other groups are investigating the use of agents that stimulate angiogenesis ^{173 174}.

Hyperglycaemia

Mirkovitch et al. have suggested that in the canine autotransplant model, some degree of hyperglycaemia or an "endocrine deficiency state" is necessary as a stimulus for islet engraftment ¹⁷⁵. However, more recently this work has been repeated by the group in Minneapolis ¹⁷⁶ who found that hyperglycaemia was

not a mandatory stimulus for islet engraftment. Indeed there is increasing evidence from animal studies suggesting that hyperglycaemia actually inhibits the vascularisation of islet grafts 151, 177, 178, 179.

It would appear however, that this inhibition of vascularisation is a result of altered regulation of blood flow through established vessels 177, rather than the impairment of new capillary growth into the islet graft 155, 180. High glucose concentrations have been shown to produce changes within the β -cells at a molecular level causing abnormalities in insulin secretion 181, 182.

Chronic hyperglycaemia has been associated with β -cell loss in rodents, although all these studies involved the deliberate transplantation of an islet numbers insufficient to reverse diabetes 183, 184, 185, 186.

Certainly, it would appear that early graft function is improved, and less islets are required to reverse diabetes, if normoglycaemia is strictly maintained by the administration of exogenous insulin in the immediate post-transplant period 88, 187, 188, 189, although whether this beneficial effect is caused by the prevention of impairment of islet vascularisation, the prevention of the direct effects of hyperglycaemia on the β -cell, or the result of protecting the transplanted islets by metabolically "resting" them is unclear.

Site of islet graft

Many different anatomical sites have been used or proposed for islet allotransplants. Whilst the most important influences on choice of site are ease of access and immune privilege, suitability for islet engraftment is also a key factor. Clearly this is influenced by the vascularity of the chosen ectopic site. Using the renal subcapsular space, 8 human islet transplants (2 autografts and 6 allografts) have been performed, with none of the patients obtaining significant graft function 88, 105, 190. These results could be explained by the relatively poor vascularity of this site. On the other hand both the liver and the spleen provide excellent sites for islet implantation. However, it must be remembered that whereas in the human autotransplant

and most animal allotransplant studies the liver and spleen are normal, the human allotransplant is usually performed in a long-standing diabetic recipient. It is well established that such patients have a microangiopathy ¹⁹¹ and it has been suggested that this in turn might impair islet engraftment ^{192, 193}.

b) Reinnervation

In his description of islets in 1869, Paul Langerhans noticed that "these islands of clear cells" were more richly innervated than the surrounding pancreatic tissue ¹⁹⁴. Since then much research has been undertaken into islet innervation in many species including man and this work has been excellently reviewed by Sundler and Bottcher (1991) ¹⁹⁵.

Islets are innervated by sympathetic, parasympathetic and peptidergic nerves ¹⁹⁶. Sympathetic adrenergic innervation has been studied using both histofluorescence ¹⁹⁷, and immunocytochemical techniques using tyrosine and dopamine hydroxylases ¹⁹⁸. Sympathetic somata have been observed within the pancreas, suggesting that islets are not only innervated by postganglionic nerve fibres originating from the coeliac ganglion ¹⁹⁹, but also fibres originating from intramural ganglia ²⁰⁰. Similarly, cholinergic innervation has been studied extensively ^{200, 201}. Cholinergic nerve fibres appear to be more important in the islets than the exocrine component of the pancreas ²⁰². The density of cholinergic nerve fibres is greatest in the periphery of the islets, resembling the pattern of the muscarinic acetylcholine receptors ²⁰¹. Parasympathetic nerve fibres travel within the vagus nerve as preganglionic fibres which terminate on intrapancreatic ganglia ^{199, 200}. The post-ganglionic nerve fibres are both cholinergic and peptidergic.

As with vascularity, the innervation of the islet is grossly disturbed by the process of islet isolation ²⁰³. However, several groups have shown that islets appear to re-gain some reinnervation after transplantation. Griffith et al. (1977) first showed that intraportal islet isografts in the rat reinnervate

concurrently with revascularisation ¹⁵³. Noda et al. (1992) subsequently demonstrated reinnervation of murine islet isografts transplanted under the kidney capsule ²⁰⁴. Reinnervation has also been shown in intraportal rat allografts ²⁰⁵ and intrasplenic canine autografts ²⁰³. As one might expect, transplanted islets are reinnervated by the types of nerve fibres found within the ectopic site of the transplant ²⁰⁶. Indeed, in a recent comparison of rat islets transplanted into the liver, kidney capsule and spleen, Houwing et al. found that cholinergic innervation of the spleen was limited and that this was reflected by a very limited parasympathetic reinnervation of the intrasplenic islet grafts ²⁰⁷. Therefore the complex pattern of innervation supplying the islets within the pancreas is not necessarily recreated post-transplantation. However, reinnervation may not be essential for the normal glucose control of the islet transplant recipient. This is highlighted by the fact that islets transplanted into the liver of a liver transplant recipient have been able to restore normoglycaemia ^{208, 209}, despite the fact that the liver does not reinnervate after transplantation ²¹⁰.

3) Loss of islet function post-implantation

As has already been stated, islet implantation commences as early as the fourth day post-transplant and is well established by day 14 ^{150 151 152 153 154}.

Therefore, it might be considered a false distinction to separate factors that impair islet engraftment from those that impair islet function after implantation, as many of the processes are occurring concurrently. However, there are several different factors that can cause the partially or fully engrafted islet to cease functioning.

a) Antigen-specific T-cell mediated rejection

In theory, islet transplants should be less susceptible to antibody-mediated

hyperacute rejection because as already discussed, they are revascularised by endothelium from the recipient. However, islet allografts are highly susceptible to cell-mediated rejection ^{211, 212} and this is thought to be a major cause of islet graft failure in the clinical situation ^{157, 213}.

This will now be discussed.

In 1975, Lafferty and Cunningham proposed that T-cells require two signals for activation ²¹⁴. According to this model, signal one (antigen) is provided by occupancy of the antigen-specific T-cell receptor, whereas signal two is provided by a costimulator (CoS) produced by an antigen presenting cell (APC). Cell surface molecules acting as costimulators include CD28 ²¹⁵, and CTLA-4 ²¹⁶ interacting with the B7-1 / BB1 and B7-2 molecules expressed by APC and activated B-cells ²¹⁷. Other molecules expressed by APC such as ICAM-1 ²¹⁸, and LFA-3 ²¹⁹ have also been shown to costimulate T-cells. Two main pathways of graft antigen presentation leading to T-cell stimulation, both of which fulfil the two signal theory, are recognised ^{213, 220}. Firstly, the direct pathway (donor APC-dependent) in which host T-cells recognise graft antigens directly on the surface of donor cells capable of causing costimulatory activity. In this direct pathway, antigen presentation stimulates both CD4 and CD8 T cells specific for donor MHC class II and class I respectively ²²¹. Secondly, the indirect pathway (host APC-dependent) whereby host T-cells indirectly recognise graft antigens which are processed and presented by host APC. The T cells activated by this indirect pathway are restricted to MHC class II molecules. The immune response to islet allografts is dominated by the direct pathway of antigen presentation. This response is marked by the unusually high frequency of precursor T cells which can directly respond to allogeneic antigens expressed by APC. Incidentally, the xenogeneic response would appear to be dominated by the indirect pathway ²²² resulting in the allogeneic / xenogeneic profiles contrasting in an inverse manner (the "Yin / Yang" model) ²²⁰. Islet allograft rejection usually occurs during the first few weeks post-transplantation. However, it is not possible to detect early islet graft failure.

The reason for this is that islet function is monitored by measuring C-peptide levels. As the graft is being suppressed by the administration of exogenous insulin during this time, C-peptides are also suppressed and therefore uninterpretable. Several factors influence islet immunogenicity.

Impure islets are associated with increased immunogenicity, but whether this is a result of the acinar tissue remains unclear. Certainly, the effect is increased if lymph tissue and, to a lesser extent, vascular tissue and ductal elements are present.

The experience of vascularised pancreas transplants would strongly suggest that immune rejection is decreased with good HLA matching ²²³. Data for the period 1986-1990 shows that 1 year graft survival was 54% with two HLA-DR matches, 44% with one DR match and 29% with a complete DR mismatch. In addition, the survival of pancreas grafts is improved by the administration of an anti T-cell agent during induction immunosuppression ²²³. Most human islet transplant groups have incorporated these into their programmes.

b) Non-specific inflammatory damage

In addition to antigen-mediated rejection, the function and survival of allotransplanted pancreatic islets may be impaired by a number of non-specific inflammatory factors. Activated macrophages are known to release numerous cytokines in addition to other toxic metabolites ^{224, 225}. Tumour necrosis factor (TNF), interleukin 1, interleukin 6 and interferon have all been shown to impair glucose-induced insulin production ^{226, 227}, and TNF has also been shown to be directly toxic to human islets in culture ²²⁸. In addition, macrophages produce nitric oxide which is known to cause islet death ²²⁹ as well as to mediate the cytotoxic effect of interleukin 1 on islets ^{230, 231}. There is evidence that β -cells are more susceptible to damage than islet α -cells ²³². Okamoto has suggested that free radical formation is the final common pathway in cytokine β -cell damage, with superoxide production

at the inflammatory site being the source of islet toxicity ^{233, 234}. Certainly the oxygen radical scavenger, superoxide dismutase, can protect murine islet grafts ²³⁵.

The liver, in particular, contains numerous macrophages in the form of Kupffer cells, making intraportal allografts particularly susceptible to this type of damage. Administration of the immunosuppressive drug 15-deoxyspergualin, a macrophage inhibitor, has prolonged intraportal allograft survival in both the rat ²³⁶ and the dog ²³⁷.

Another potential source of non-specific inflammatory damage is the commercially available bacterial collagenase which is routinely used for dispersing the pancreas during islet isolation (see Chapter 3). Although crude collagenase is removed of living bacteria, small amounts of bacterial endotoxin are still present in this product. It has also been shown that this endotoxin is present throughout the different phases of the islet isolation process ^{238, 239} and therefore traces of endotoxin are transferred to the islet transplant recipient. It has been postulated that this bacterial endotoxin, in addition to potential allergic reactions, may cause local inflammation of the recipient site ^{238, 239}. With so little data available, it is impossible to know whether this is a significant problem for human islet grafts or indeed whether any effects are related to impairment of engraftment or direct damage to the islet. Certainly one of the potential advantages of the new collagenase blend *Liberase* ^{240, 241, 242, 243}, is that it is essentially endotoxin free ^{238, 241}.

c) Recurrence of autoimmunity

The development of type 1 diabetes is an immunologically mediated process ^{244, 245}, thought to be a form of autoimmunity ^{246, 247}. Recurrence of autoimmunity might therefore, be expected to be a possible cause of islet graft failure. Autoimmune destruction of β -cells has been observed in segmental pancreatic grafts transplanted between monozygotic twins or HLA-identical siblings ^{248, 249}. However, in all 5 patients immunosuppression had either

been minimal or omitted altogether. Indeed, when the same procedure was performed with full immunosuppression, no such damage was seen. It would therefore appear that current immunosuppressive regimes prevent the recurrence of islet autoimmunity and is therefore, unlikely to have been a cause of failed clinical transplants. However, work by Bartlett et al. (1991) in Baltimore, would suggest that recurrence of autoimmunity may still be a major problem ²⁵⁰. However, in the BB rat model, these effects seem to be prevented by the transplantation of donor T-cells ^{251, 252}.

d) Immunosuppressive drug toxicity

The impairment of islet engraftment by immunosuppressive drugs has already been alluded to. However, many of these agents can also be directly toxic to the islet itself. Cyclosporine has been shown to impair β -cell function ^{253, 254 255, 256}, by interfering with insulin stimulus -secretion coupling ²⁵⁷. It has also been found to be diabetogenic in the clinical situation ^{258, 259, 260, 261, 262, 263}. Prednisolone has a similar effect, impairing insulin secretion and causing insulin resistance ^{264, 265}, as does FK 506 ^{266, 267, 268, 269, 270}, although in the latter case, the effect appears to be less pronounced.

e) Metabolic exhaustion of the islets

One of the phenomena that has been observed in both human and large animal islet transplant series, is the late cessation of function of a previously functioning graft ^{271, 272, 273, 274}. This has been particularly observed after transplantation of a sub-optimal islet mass. The timing of graft failure and the fact that this delayed failure is also seen in islet autotransplant series, means that is unlikely to be explained by immune mechanisms. It has however, been postulated that the islets are subject to metabolic exhaustion ^{113, 275}. This is thought to arise from the chronic stimulation of an initially sub-optimal islet

mass, which causes a progressive metabolic deterioration and loss of β -cells, finally resulting in a reversal of the patient to the diabetic state ¹⁵⁷.

B) STRATEGIES FOR IMPROVING RESULTS OF ISLET ALLOTRANSPLANTATION

It can be seen from the previous discussion that the two overwhelming factors preventing islet allotransplantation from becoming a mainstay treatment of diabetes mellitus are the inability to consistently isolate sufficient islets from the donor pancreas and the problems associated with rejection of the transplanted graft. The second half of this chapter now discusses potential ways of overcoming these difficulties.

1) Improving islet numbers

As has already been discussed, the number of islets transplanted has a critical influence on the outcome of adult islet allotransplantation. Current methods of islet isolation however, are unable to guarantee sufficient yields of islets from a single human pancreas. Several other approaches have therefore been proposed.

a) Increasing number of donors

In an attempt to improve the number of transplanted islets, many groups have opted to "pool" islets from several different donors. Indeed, of the 75 islet transplants performed on C-peptide negative IDDM recipients between 1990 and 1993, 39 were performed using >1 donor, and as stated in Chapter 1, the 12 patients in the recent Edmonton series all received islets from at least 2 donors. However, this approach is potentially problematic for several reasons.

First, it has been shown that the survival of canine islet allografts decreases as the number of donors increases ²⁷⁶. The reason for these findings is thought to be due to the "cytokine storm" caused by the increased antigenic load ^{213, 277}. A possible way of reducing the antigenic diversity of multi-donor islet transplants, is to cryopreserve the islets, thereby enabling closer HLA matching ^{278, 279, 280}. However, in Leicester we have shown that 27 - 51% (median 38%) of the islets are "functionally lost" during this process ²⁸¹. This is partly explained by actual physical islet loss, but in addition several groups have shown that many of the surviving cryopreserved islets have impaired viability ^{124, 282}. This has been confirmed at a sub-cellular level by electrophysiological recordings using cell-patch techniques ²⁸³. However, the second and indeed most compelling reason why multi-donor islet transplantation is not the answer, is the large number of pancreata that would be required. In every country there is already a shortage of donated pancreata. Even if this number was to increase, there will never be enough to make multi-donor islet transplantation a viable option. In addition there is an exponential increase in the number of whole pancreas transplants being performed. As Sutherland has pointed out "it is totally unethical to consider using 4 or 5 pancreata for an islet transplant, when 4 or 5 patients could potentially be cured of their diabetes by a whole organ transplant" ²⁸⁴.

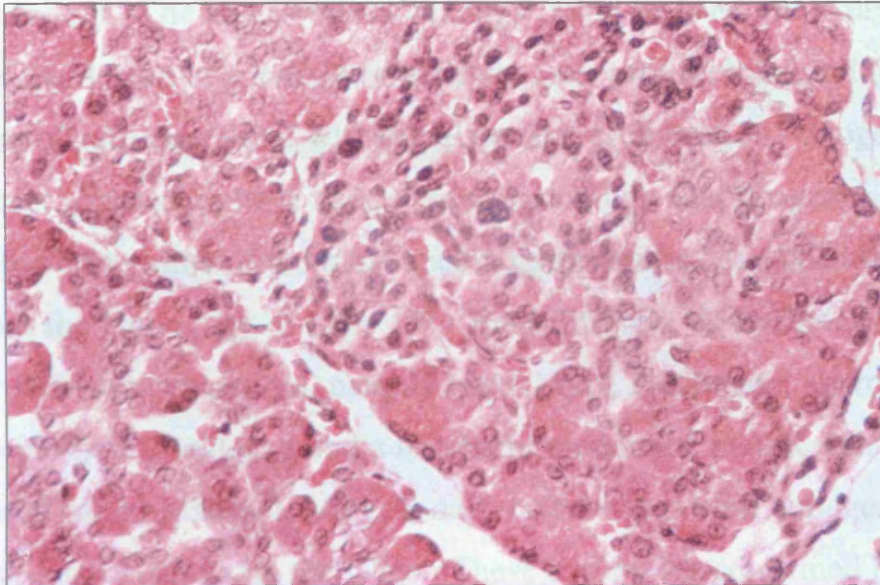
b) Using alternative sources of islets

Even if it was possible to routinely isolate sufficient islets to enable one donor to one recipient transplants to be performed, the number of diabetic patients who would require an islet transplant would clearly still greatly exceed the number of cadaveric pancreata available from organ donation. With this in mind, several alternative sources of islets have been considered.

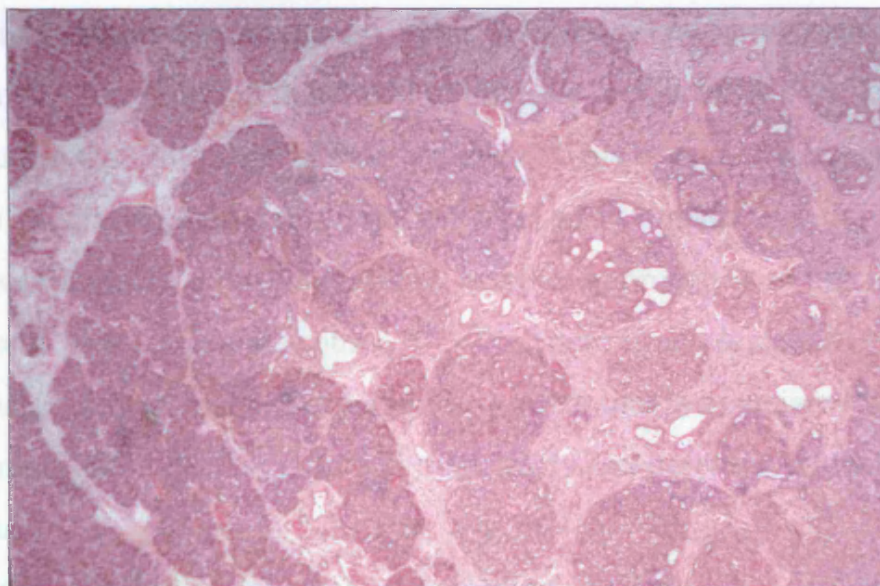
During the early stages of pancreatic embryology, a multi-potential stem cell is formed which may differentiate into cells which possess either an endocrine or exocrine phenotype ^{285, 286}. Endocrine cells develop by budding from embryonic duct-like cells ^{287, 288, 289}, a process termed nesidioblastosis. This term is somewhat confusing due to the fact that it is often used synonymously for the clinical condition of neonatal hyperinsulinism associated with diffuse endocrine cell proliferation. However, a more recent term for this latter condition is persistent, hyperinsulinaemic hypoglycaemia of infancy (PHHI), distinguishing the normal embryology from a rare metabolic disorder (see Figure 2.2).

The classical teaching is that the entire complement of β -cells in an adult is established in the prenatal period and after birth the rate of cell division in β -cells is quite low ²⁹⁰, the fully mature pancreas having essentially no proliferative capacity ⁹⁶. However, several recent studies have questioned this ^{291, 292}. Unequivocal data on the increase of β -cell mass in the developing rat ^{293, 294, 295} and human ²⁹⁶ pancreas indicate that the vast majority of β -cells are added after birth. Finegood et al. (1995) have recently performed a mathematical analysis of the available data in the literature on the β -cell mass, replication and cell size at different ages of Sprague Dawley rats ²⁹⁷. They found that the β -cell mass increases at least eight-fold between 10 days and 10 weeks of age, whilst the individual cell size almost doubles during this period. The rate of replication was greatest during late foetal and neonatal periods, with limited replication occurring beyond 30-40 days of age. In the post-neonatal period, therefore, any further expansion of the pancreatic endocrine cell mass can only be achieved by a limited proliferation of the existing differentiated β -cells, or by a reiteration of embryological development involving the differentiation of precursor cells remaining in the adult pancreatic ducts ^{290, 298, 299}. It is this latter process that is termed neogenesis.

Figure 2.2 Histology of the two main types of nesidioblastosis



Diffuse



Focal

Neogenesis technology has the potential for the induction of islet cell differentiation and proliferation "in-vivo", thereby enabling islets to be "grown" as well as the potential for augmenting islet cell mass "in-vivo". It also offers the possibility of being able to cause in situ β -cell regeneration in the diabetic patient. The key to this technology being realised clinically however, is the design of a suitable matrix for islet proliferation and the identification of substances that will stimulate islet proliferation / neogenesis. Many different approaches have been used to encourage islet neogenesis in vivo ²⁹⁰. However, some of the most encouraging results have been reported recently from the University of Lille ³⁰⁰. Four different three-dimensional protein matrices were compared, namely rat tail type 1 collagen, agarose gel, purified bovine type 1 collagen and a basement membrane matrix rich in growth factors called Matrigel. After 3 days culture in rat-tail collagen, cysts were observed associated with the islets. These cysts were confirmed by the ductal marker carbohydrate antigen 19-9, to be formed from ductal epithelia. Cyst formation was enhanced by matrigel, but was not seen with islets suspended in the other two matrices. After 7-10 days, endocrine cells were observed both intercalated in the ductal epithelium and as structures "budding" from the ductal wall into surrounding collagen. This is one of the first reports of "in-vitro" ductal epithelial proliferation from adult human islet tissue, with initial signs of endocrine differentiation. Another approach is to use foetal or neonatal tissue as the neogenic source ^{97, 301}. There is ample data to suggest that foetal islets have a marked capacity for growth that is associated with functional maturation ^{97, 302}, although this clearly has potential ethical implications.

A whole range of substances have been investigated as potential stimuli of islet proliferation. It is known that glucose can increase mitotic activity in adult islets ^{303, 304}. Various different hormones and gut-peptides have also been implicated. The polypeptide growth factor, hepatocyte growth factor, appears not only to stimulate mitosis in adult human islets, but also to enhance β -cells to form clusters and stimulates proliferation within these clusters ³⁰⁵.

Nicotinamide has been shown to increase the growth of endogenous adult beta cells ⁶³ and there is also data suggesting that it may increase the differentiation rate of human foetal islets ³⁰⁶.

The approach of neogenesis is an exciting one. However, it is still a long way from becoming a clinical reality and clearly requires much more research.

Xenotransplantation

Although this discussion is centred on the islet allograft, another alternative source of islets for transplantation is from the pancreata of other species. Clearly, the use of insulin from other species is not a new concept. Prior to the availability of human recombinant insulin, porcine and bovine insulin were the mainstay treatments for patients with IDDM. Indeed as early as 1893, an islet xenotransplant was performed in Bristol. ⁸⁸. However, whilst this approach clearly addresses the issue of islet availability, it leads to new challenges of xenograft rejection, potential xenograft to host infections, and ethical considerations. This is clearly a very important area of current and future research.

d) Optimising methods of human islet isolation

The alternative approaches described above may be attractive options but are some years away from becoming clinical reality. In the present situation therefore, in addition to ongoing research into these different fields, efforts should be concentrated on optimising the methods for isolating islets from the available human pancreata.

2) Prevention of immune rejection

Knowledge of the allogeneic immune response enables the development of different protocols that attempt to prevent islet rejection, thus enabling long-term graft survival.

a) Improved Immunosuppressive regimes

Many of the previous immunosuppressive regimes used for islet allotransplantation were based on those used for vascularised pancreas transplants. As can be seen from the previous discussion, many of these are less suitable for the islet situation. Over the recent years, several newer drugs have undergone clinical trials and some of these offer potential improvements. One of the potential explanations for the recent success of the Edmonton group is that their immunosuppressive regimen used some of these new agents and was glucocorticoid-free ⁹¹.

Tacrolimus (FK 506)

This macrolide drug blocks the intracellular signal pathway between the T-cell receptor and the nucleus causing interleukin-2 (IL-2) production to be suppressed and therefore IL-2 driven cellular proliferation to be inhibited. Trials have shown reduced rejection following liver transplantation ³⁰⁷ and the drug has been used successfully for transplants of the kidney, heart, lung, pancreas and small bowel ³⁰⁸. It has also been found to be able to rescue grafts with intractable rejection ³⁰⁹. One islet "cluster" patient was treated with tacrolimus and became insulin independent ²⁰⁹. The Edmonton patients received low dose Tacrolimus at a dose of 1mg twice daily which was then adjusted to give a trough concentration at 12 hours of 3-6 ng/ml.

Mycophenolate mofetil (RS-61443)

This drug which is the morpholinoethylester of mycophenolic acid, inhibits the eukaryotic enzyme inosine monophosphate dehydrogenase, thereby blocking the "de novo" but not the "salvage" pathway of purine synthesis. It therefore mainly acts on T- and B-lymphocytes. Randomised, controlled trials of the use of this drug in renal transplantation have shown a reduction in biopsy-proven rejection when compared to cyclosporine, prednisolone and azathioprine ^{310, 311}. Although there is data available for the drugs use in simultaneous kidney-pancreas transplants ³¹², there is little data on the use of this drug for islet transplants. However, it has not been found to be diabetogenic.

Sirolimus (Rapamycin)

This macrolide drug differs from FK 506 in that Sirolimus blocks the signal pathway between the IL-2 receptor and the nucleus, thereby suppressing cellular proliferation. Data would suggest that this drug is synergistic with cyclosporin. In animal studies, this drug has given good results with islet transplantation, although there is no clinical data available. This drug is also reported to have no diabetogenicity. This agent was given to the Edmonton patients immediately before transplantation with a loading dose of 0.2 mg/kg, followed by a dose of 0.1 mg/kg/day. Drug levels were monitored so as to maintain them in the range of 12 - 15 ng/ml for the first 3 months, followed by 7 - 10 ng/ml thereafter.

Gusperimus (Deoxyspergualin)

This drug is a guanine derivative exhibiting potent inhibition of T-and B-lymphocytes, by its antimacrophage activity. A variety of cellular effects have been described, but the mechanisms for these remain poorly understood.

Experimental findings with this drug have aroused interest for using in islet transplant recipients. The drug is highly effective in inhibiting allo- and xeno-antibody production in several animal models ^{236, 313}. There are also encouraging reports that this drug reduces the incidence of early non-function in the human ¹⁶⁷. It also shows no signs of diabetogenicity.

Daclizumab

This is the third immunosuppressive agent used in the Edmonton series. It is a monoclonal against the interleukin-2 receptor. In their patients it was given intravenously at a dose of 1mg / kg every 14 days for a total of 5 doses. If the second transplant occurred more than 10 weeks after the first, the course of daclizumab was repeated.

Whilst these new drugs offer some improvements, they still fall short of one of the main rationales for islet transplantation, namely the potential to modulate the immune response itself, thereby obviating the need for immunosuppression. In theory, this can be done in several ways.

b) Immunomodulation

Islets contain dendritic cells and macrophages, many of which express surface Class II antigens and which may function as antigen-presenting cells (APC) ("passenger leukocytes") in the direct pathway of lymphocyte activation ³¹⁴. One of the potential advantages of cell transplants is to be able to modulate these APC's, thereby preventing allograft rejection.

Many different modalities have been investigated, either singly or in isolation. Low temperature culture (22-24°C) has been used by several different groups with moderate success ^{315, 316, 317}. Cryopreservation has been shown to exert an immunomodulatory effect ^{318, 319, 320}. Irradiation with ultraviolet ³²¹ or gamma irradiation ³²², either singly or in combination has been used. Other

modalities explored include culture at high oxygen tensions ³²³, the treatment of islets with an anti-class II antibody ³²⁴ and anti-dendritic cell antibodies ³²⁵. Whereas these methods have achieved good results in rodents, they have failed to work in large animals or humans without the addition of adjuvant immunosuppression. The main reason for this is that it has been shown that islets devoid of class II antigen are still immunogenic ³²⁶. Different approaches must therefore be considered if this is to be a therapeutic option.

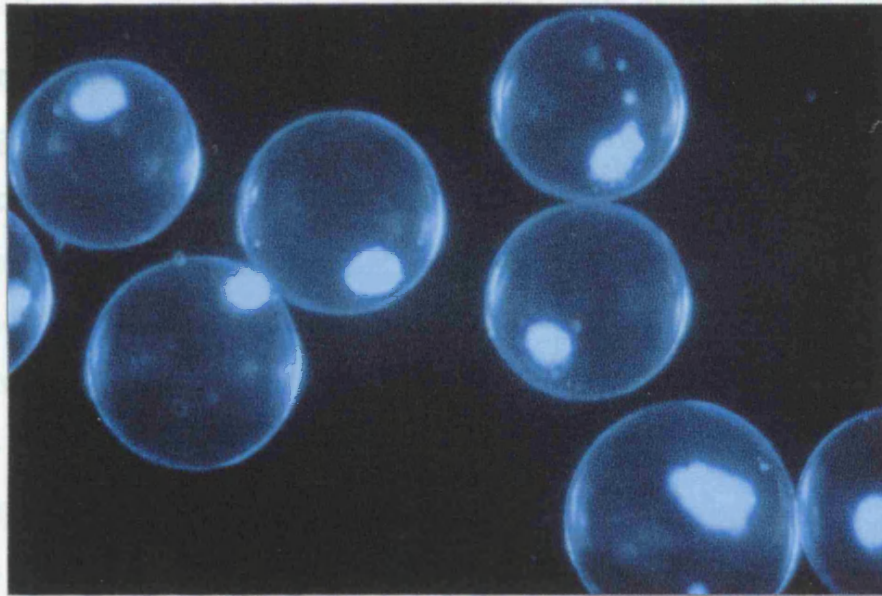
c) Immunoisolation

Instead of modulating the islets in order to render them non-immune to the host defences, another approach is to isolate the islets from the host's immune system altogether (see Figure 2.3). The two main methods that have been attempted to achieve this are the biohybrid artificial pancreas and microencapsulation.

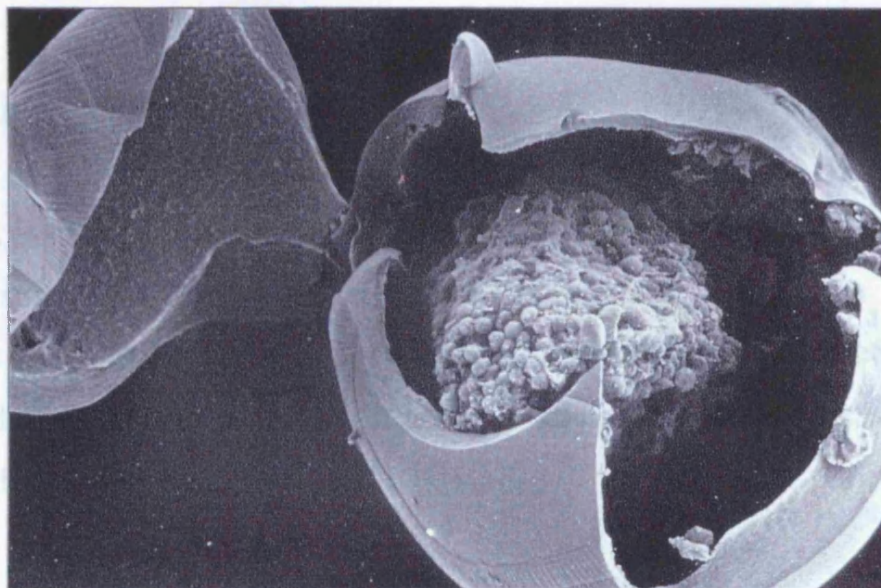
Friedman has defined the hybrid artificial pancreas as "a bionic device constructed of artificial materials that sustain living insulin-secreting cells of pancreatic origin" ⁵⁹. Essentially this device works as a vascular shunt enclosing a compartment containing pancreatic islets. There have been some encouraging results using these systems in animal models ^{327, 328}. However, the complications that are most hindering the application of this approach are thrombus formation ^{327, 329} and the fibrotic reaction that forms around the device ³³⁰.

The initial reports of microencapsulation by Lin and Sun involved the use of sodium alginate as the droplet material ³³¹. This has the advantages that as a hydrogel it is potentially biocompatible and also the sodium salt solidifies in the presence of most divalent ions, enabling outer layers to be coated on the outside in order to construct the final capsule ^{332, 333}. Although Lim and Sun originally used polyethyleneimine for this outer layer, the most commonly used material since has been poly-L-lysine. Numerous different materials have been used to modify the capsule structure ^{334, 335, 336}.

Figure 2.3 Encapsulated islets



Photomicrograph of encapsulated islets



Electronmicrograph of islet within a capsule

d) Induction of immune tolerance

Immune tolerance to allo- or xeno- antigen can be defined as: "the inability of a host, in the absence of concomitant immunosuppression /immunomanipulation, to reject an established primary graft following challenge with a secondary, fresh donor-specific graft or live donor-specific lymphoid cells, e.g. spleen or lymph node cells" ³⁴⁰. The capacity to reject a third party graft remains intact. Tolerance to islet allografts has been achieved in mice by donor-specific bone marrow ³⁴¹. It has also been achieved in rats by inoculating islets intrathymically ³⁴² or into bone marrow ³⁴³. More recently there has been a report of canine islet allograft survival after donor specific vertebral body derived bone marrow cell transplantation ³⁴⁴. The same group have also reported induction of tolerance to islet allografts in man following infusion of perforin deficient bone marrow ³⁴⁵.

Clearly this work is in its relative infancy, but certainly is currently the focus of extensive research.

Conclusions

Although it is clear that there are a number of different factors responsible for the limited success of clinical islet allotransplantation, one of the main obstacles is in the ability to routinely isolate sufficient numbers of islets from a single cadaveric human pancreas. It is this challenge that forms the basis of the remainder of this thesis.

CHAPTER 3

Collagenase and Islet Isolation

Chapter 3 Outline

Introduction

- 1) The rationale for using collagenase
 - a) Non-enzymatic methods
 - b) Enzymatic methods
- 2) Characteristics of collagenase enzymes
- 3) Composition and production of crude collagenase from *Clostridium histolyticum*
- 4) Which components of crude *Clostridium histolyticum* collagenase are required for human islet isolation
- 5) Stability of crude collagenase during storage
- 6) Biophysical factors influencing collagenase administration
 - a) Route of collagenase administration
 - b) Pressure and volume of collagenase administration
 - c) Timing of collagenase administration
 - d) Temperature of collagenase administration
- 7) Biochemical factors influencing collagenase administration
 - a) Which buffer should be used for collagenase administration
 - b) Should calcium be added to the buffer
 - c) pH of collagenase administration
 - d) At what concentration should crude collagenase be administered?
- 8) Pancreatic factors influencing collagenase administration
 - a) Donor factors

- b) Procurement factors
- c) The role of pancreatic enzymes

Conclusions

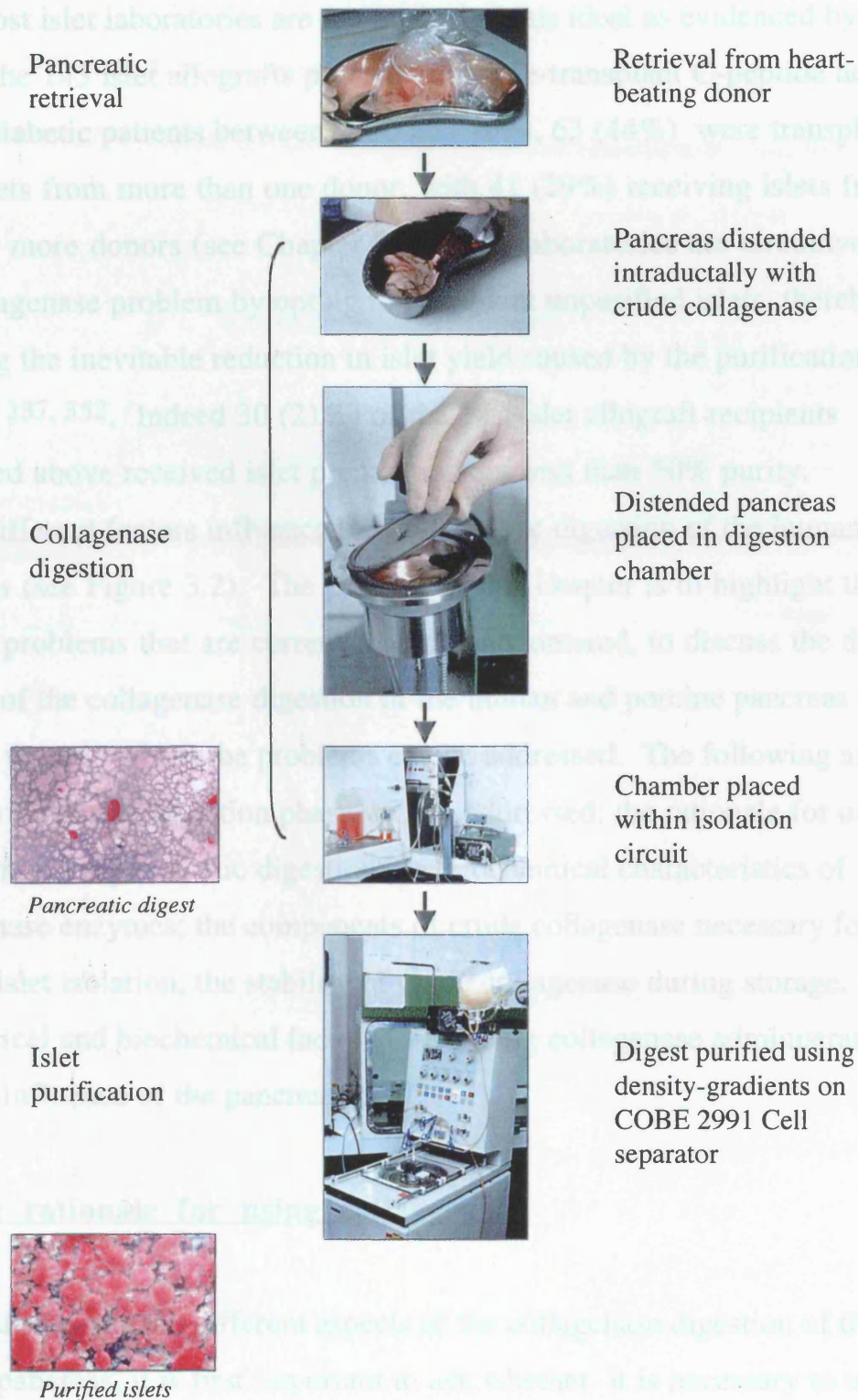
Introduction

The isolation of mammalian islets of Langerhans consists of three main phases (see Figure 3.1). First, the pancreas is excised and transported to the islet isolation laboratory. Second, the pancreas is digested into a suspension of its constitutive cells by a combination of mechanical and enzymatic digestion using commercially available collagenase. Finally, the islets are separated from the other cells by density gradient purification. While the purification of islets has been the subject of extensive research ^{159, 346}, the collagenase digestion phase remains relatively poorly understood and extremely variable. Indeed, the variability of the collagenase digestion phase of human islet isolation is one of the most significant problems hindering clinical pancreatic islet transplantation worldwide ³⁴⁷.

The main reason for this variable collagenase digestion phase is the marked variation between different batches of commercially available crude collagenase ^{157, 348, 349, 350}. This is an inevitable consequence of the non-specific, non-reproducible way in which crude collagenase is currently produced. Even when a good batch is produced, the subsequent deterioration of enzyme activity with storage means that most islet laboratories spend a significant amount of time and resources batch-testing enzymes in order to find the occasional optimal enzyme ³⁵⁰. These problems are not new and Moskalewski wrote in 1964 that "batches of crude collagenase differ in their activity - it is therefore necessary to find the optimal concentration and optimal isolation time for every new batch of the enzyme" ³⁵¹. In this respect, little has changed over the past 30 years!

Few would disagree that if human islet transplantation is going to become a regular therapy for juvenile onset diabetes, it must be possible to routinely transplant purified islets from one well-matched donor pancreas to one diabetic recipient ¹⁵⁷. In order to do this, it is essential that for any one pancreas, the collagenase digestion phase releases large numbers of viable

Figure 3.1 Outline of the principle steps in human pancreatic islet isolation



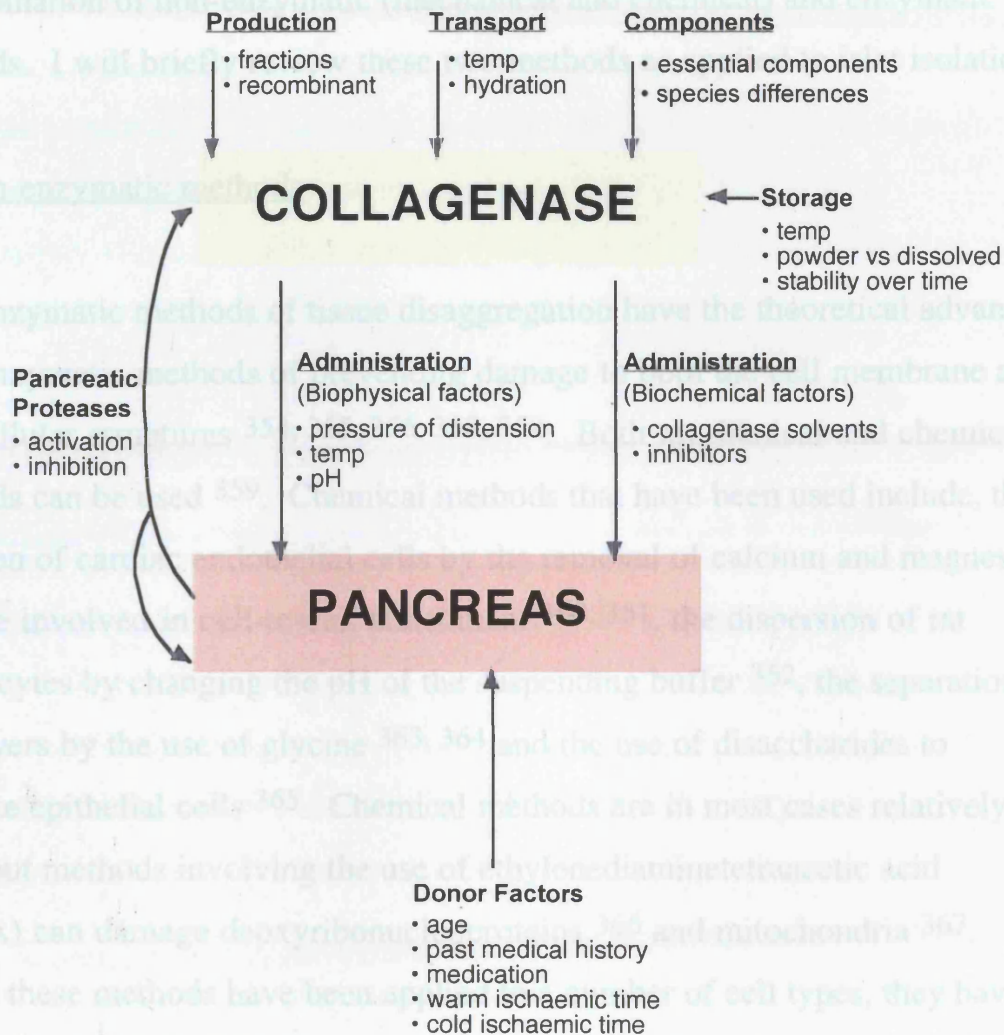
islets that are completely free of exocrine tissue (cleaved) and that are not fragmented (intact) which can then be purified using density gradient centrifugation. Even the smallest amount of exocrine tissue attached to the islet increases its density thereby preventing its separation from acinar tissue³⁴⁶. Most islet laboratories are not achieving this ideal as evidenced by the fact that of the 143 islet allografts performed on pre-transplant C-peptide negative type 1 diabetic patients between 1990 and 1994, 63 (44%) were transplanted with islets from more than one donor, with 41 (29%) receiving islets from three or more donors (see Chapter 1). Other laboratories are circumventing the collagenase problem by opting to transplant unpurified islets, thereby avoiding the inevitable reduction in islet yield caused by the purification process^{237, 352}. Indeed 30 (21%) of the 143 islet allograft recipients described above received islet preparations of less than 50% purity.

Many different factors influence the collagenase digestion of the human pancreas (see Figure 3.2). The purpose of this chapter is to highlight the various problems that are currently being encountered, to discuss the different aspects of the collagenase digestion of the human and porcine pancreas and to suggest ways in which the problems can be addressed. The following aspects of the collagenase digestion phase will be addressed: the rationale for using collagenase for pancreatic digestion; the biochemical characteristics of collagenase enzymes; the components of crude collagenase necessary for human islet isolation, the stability of crude collagenase during storage, the biophysical and biochemical factors influencing collagenase administration, and the influence of the pancreas itself.

1) The rationale for using collagenase

Before discussing the different aspects of the collagenase digestion of the human pancreas, it is first important to ask whether it is necessary to use collagenase at all for human islet isolation. The development of a "successful"

Figure 3.2 Factors influencing the collagenase digestion of mammalian islets of Langerhans



technique for isolating islets took over 20 years and as Gray and Leow have pointed out, "there are probably few other fields of modern science where progress has been so dependent upon empiricism" ³⁵³. The need to separate one intact organ (the islet) from within another organ (the pancreas) while maintaining structural integrity, is a unique requirement of tissue separation techniques ³⁴⁹. In general terms, tissue dissociation and cell harvesting involve a combination of non-enzymatic (mechanical and chemical) and enzymatic methods. I will briefly review these two methods as applied to islet isolation.

a) Non-enzymatic methods

Non-enzymatic methods of tissue disaggregation have the theoretical advantage over enzymatic methods of preventing damage to both the cell membrane and intracellular structures ^{354, 355, 356, 357, 358}. Both mechanical and chemical methods can be used ³⁵⁹. Chemical methods that have been used include, the isolation of cardiac endothelial cells by the removal of calcium and magnesium that are involved in cell-to-cell attachment ^{360, 361}, the dispersion of rat hepatocytes by changing the pH of the suspending buffer ³⁶², the separation of cell layers by the use of glycine ^{363, 364} and the use of disaccharides to separate epithelial cells ³⁶⁵. Chemical methods are in most cases relatively mild, but methods involving the use of ethylenediaminetetraacetic acid (EDTA) can damage deoxyribonucleoproteins ³⁶⁶ and mitochondria ³⁶⁷.

Whilst these methods have been applied to a number of cell types, they have not been used for the large scale isolation of pancreatic islets.

Mechanical methods available include homogenising, sieving, mincing and affinity methods ³⁵⁹. Although some of these methods are traumatic to the tissue, they do have certain applications, in particular for the dispersion of neural tissue ^{368, 369, 370, 371, 372, 373, 374, 375}. The first successful attempts to isolate islets involved microdissection of the mouse, obese-mouse and guinea pig pancreata with a cataract knife under the dissecting microscope ³⁷⁶. This enabled small numbers of islets to be isolated, but was clearly impractical

for large scale islet isolation. Others have managed to isolate rat, rabbit and human islets by the use of a combination of low temperature and mechanical disruption followed by sieving ³⁷⁷. Their explanation for the success of this method is that low temperature selectively destroys the acinar tissue which is sensitive to cold and inhibits the release of pancreatic enzymatic activity, thus protecting the islets which are themselves relatively cold-resistant. The mechanical disruption of the tissue by sieving was essential for successful separation of acinar and islet elements. Whilst these mechanical methods when used alone can enable the isolation of relatively small numbers of islets for experimental purposes, they are much more effective when used in combination with enzymatic methods, enabling the isolation of high islet yields, which are essential for islet transplantation.

b) Enzymatic methods

The choice of enzyme for tissue disaggregation must be made in the knowledge of the connective tissue matrix present within the particular organ from which cells are to be isolated ^{359, 378}. In the case of the pancreas, the principle components of the extracellular matrix are collagen types I, III, pro-III, IV collagen, laminin and fibronectin ^{379, 380, 381, 382, 383}. (The structures of the pancreata of different species are discussed later). The two enzymes most commonly used for cell separation are trypsin and collagenase ³⁸⁴. Trypsin is available as both a crude preparation and as a purified, crystalline form ^{359, 384}. Commercially produced crude trypsin is usually isolated from bovine pancreata and is a mixture of several different enzymes including trypsin, chymotrypsin, elastase, RNase, lipase, collagenase, phosphatase and amylase ^{359, 385, 386, 387, 388}. As a result, as with crude collagenase, "lot to lot" variation is a major problem results in variations in cell yields of up to 250% ³⁸⁵. The first reports of trypsin being used for cell separation involved the detachment of cells from blood clots ³⁸⁹. In 1937, Simms and Stillman used crude trypsin for the isolation of cells from adult chicken aorta ³⁹⁰ and since

then, crude trypsin has been used successfully for a number of cell types including the dispersion of the renal cortex ^{391, 385, 392}. Crystalline trypsin however, is a purified preparation and although it has been used successfully for the isolation of cells from the adrenal gland ^{393, 394} and neuronal cells ^{395, 396}, it has proven unsuccessful in isolating several other cell types, such as rat myocytes, which can readily be isolated by crude trypsin preparations ^{397, 387, 388}. The wider application of crude trypsin when compared with purified trypsin, strongly suggests that it is the other components within the crude preparation, such as collagenase and elastase, that are vital for the tissue dispersion within these particular organs ³⁵⁹. This is highlighted in the case of islet isolation.

Attempts at using purified trypsin for isolation of islets from the rat pancreas have proved unsuccessful ³⁹⁸. However, if purified trypsin is combined with collagenase, good islet yields are obtained ³⁹⁸. In addition, purified trypsin has been shown to inhibit the insulin secretion of islets *in vitro* ³⁹⁹ and also to fragment microdissected mouse islets ⁴⁰⁰. It is for these reasons that trypsin, even in its crude form, is not the ideal choice for islet isolation.

Collagenase is the most widely used enzyme for tissue dispersion ⁴⁰¹ and has been used for the isolation of pneumocytes ⁴⁰², cardiac myocytes ⁴⁰³, osteocytes ⁴⁰⁴, hepatocytes ^{405, 406}, and cells from the thyroid gland ⁴⁰¹. The first successful attempts at using collagenase for islet isolation were reported in 1964 by Moskalewski ³⁵¹. By exposing the chopped guinea-pig pancreas to collagenase, he was able to greatly improve on the islet yields obtained by mechanical methods alone. Whereas this method worked well for the isolation of islets from the guinea pig and rat ⁴⁰⁷ and with some modifications, the mouse ⁴⁰⁸, it was not until the development of the technique of intraductal collagenase administration was developed, that acceptable islet yields were obtained from the dog ^{409, 410} and human pancreas ⁴¹¹. Islet yields were further improved by the development of an automated method ^{412, 413, 414}. This method uses a combination of collagenase digestion and mechanical

dissociation and has become the "gold standard" for human islet isolation. Jingu et al. have reported higher islet yields from the canine pancreas when the collagenase distended pancreas is mechanically chopped into small fragments immediately before being placed into the automated digestion system⁴¹⁵. Their explanation for these findings is that this combined method significantly reduces the digestion time, thereby reducing islet fragmentation. However, other groups have suggested that chopping is too traumatic for the islets^{411, 412}.

Until alternative methods of cell separation are developed, collagenase digestion assisted by mechanical dissociation remains the most effective method of isolating islets of Langerhans.

2) Characteristics of collagenase enzymes

Collagenases are metalloenzymes belonging to the protease group of enzymes^{416 417}. For an enzyme to be defined as a collagenase, it must be capable of causing hydrolytic cleavage of molecules of collagen in their native conformation be capable of degrading the triple-helical region of active collagen under physiological conditions⁴¹⁸. It is this ability to degrade the triple-helical region of the collagen molecule that defines an enzyme as a true collagenase rather than a protease with a limited action on collagen^{418, 419}. There are two main natural sources of collagenase, namely tissue and bacterial⁴¹⁹. (For the purposes of this discussion, mammalian collagenases are included with tissue collagenases). Bacterial collagenases promote more extensive cleavage of collagen than tissue collagenases⁴²⁰, as typified by the fact that the collagenase produced by *Clostridium histolyticum* catalyses approximately two hundred cleavages per collagen α -chain as compared with only one cleavage per α -chain by the tissue collagenase produced by the tadpole tail⁴¹⁹. Bacterial collagenases cleave the X-Glycine bond of collagen and the synthetic peptides at loci which contain the

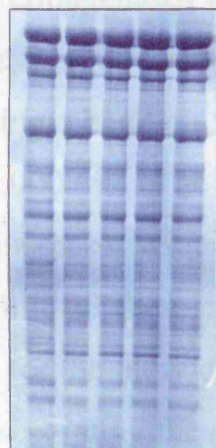
amino-acid sequence Proline-X-Glycine-Proline-where X can be almost any amino acid, provided that the imino terminus is blocked ^{421, 422, 423, 424, 425, 426}. Collagenases require zinc as a catalytic metal as confirmed by the fact that zinc chelators such as 1:10 phenanthroline and cysteine cause reversible inhibition of collagenase ^{419, 425, 427}. Calcium ions are also essential for collagenase activity as evidenced by the fact that the enzymes are inhibited by EDTA and this inhibition is reversed by addition of excess calcium, but not by addition of excess zinc ^{418, 419, 425}.

3) Composition and production of crude collagenase from *Clostridium histolyticum*.

The most prolific producers of bacterial collagenase are the *Clostridial* group of bacteria. In 1923, Henry found that rabbit muscle incubated with the filtrates from *Clostridium welchii*, swelled up, became opaque and underwent changes similar to those occurring during gas gangrene ⁴²⁸. In 1945, Macfarlane and MacLennan conclusively showed that the filtrates of *Clostridium welchii* contained a collagenase and also determined its possible importance in the spread of gas gangrene ⁴²⁹. Since then, the most widely studied and indeed the most efficient known system for the degradation of connective tissue is the crude bacterial collagenase derived from *Clostridium histolyticum* ^{417, 430}. Other bacterial collagenases that have been studied in detail are those derived from *Pseudomonas aeruginosa* ^{431, 432} and *Achromobacter iophagus* ^{433, 434, 435, 436}.

Crude *Clostridium histolyticum* collagenase is produced commercially in large "fermentation vats" using bacteria from a "library" of Clostridial enzymes. Following a simple purification step to remove the bacteria, the enzyme is lyophilised. This production method is both non-specific and non-reproducible and results in extreme batch to batch variation. Crude collagenase is a mixture of at least 12 different components of which collagenases form the major active constituents ⁴³⁷ (see Figure 3.3).

Figure 3.3 Commercially available crude collagenase contains many different components as highlighted in this SDS-PAGE gel



Class I collagenase
Class II collagenase
Neutral protease
Clostripain
Brown pigment
etc.

Indeed a more accurate term for this crude mixture would be Clostridiase!

The main active components of crude collagenase are collagenase, clostripain, trypsin, neutral protease, elastase, β -D-galactosidase, β -N-acetyl-D-glucosaminidase, α -L-fucosidase, phospholipase, neuraminidase, and hyaluronidase ³⁵⁰. In addition there are other less well defined components such as brown pigment. There is evidence to suggest that the enzymes in the crude collagenase mixture, act synergistically to degrade collagen ^{438, 439, 440, 441}. The occurrence of an extracellular collagenase produced by *Clostridium histolyticum* was first suggested by Maschmann in 1938 ⁴⁴². Initially, this bacteria was thought to produce only a single collagenase but in 1959, Grant and Alburn demonstrated the presence of more than one collagenase ⁴⁴³. In 1964, Mandl successfully separated two collagenase fractions ⁴³⁸. These were given the names Clostridiopeptidase A and B or simply collagenase A and B. The collagenases were further subdivided into A- α , B- α , B- β by Kono in 1968 ⁴⁴⁴. It was not until 1984 however, that detailed characterisation of the different collagenases present within crude collagenase was made ^{430, 445, 446}. Six different collagenases were purified to homogeneity ⁴³⁰. These were designated α , β , γ , δ , ϵ , ζ by the order of their purification. Each of the six exhibited a single band on SDS-PAGE gel and had a molecular weight range of 68,000 - 125,000 ⁴⁴⁵. The amount of calcium bound to each subclass of collagenase increases with the molecular weight of the enzyme ⁴⁴⁵. The isoelectric points of these collagenases are in the 5.35 - 6.20 range ^{440, 445}, and many values for the individual collagenases are almost identical explaining the difficulty in separating the subclasses of collagenase by ion-exchange chromatography alone ⁴⁴⁵.

Two distinct subclasses of the α (α_1 and α_2) and γ (γ_1 and γ_2) enzymes were subsequently isolated ⁴⁴⁵. These have the same molecular weights, but different isoelectric points ⁴³⁰. Based on their activities towards native collagen ⁴⁴⁷ and the synthetic peptide 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) ⁴²⁵ the six collagenases were divided into two classes.

Class I collagenases (α , β and γ) have high collagenase activity and moderate FALGPA activity. Class II collagenases (δ , ϵ , ζ) have moderate collagenase activity and high FALGPA activities. Although the two classes have similar physicochemical properties, further studies have confirmed that they have different specificities toward short peptides with class II enzymes having a broader specificity than the class I enzymes ^{445, 446, 430 448, 449, 450}. It has now been suggested that one class evolved from another by gene duplication followed by independent evolution by point mutations to yield enzymes with different substrate specificities ⁴⁴⁶. More recently the amino acid sequences for both class I and class II collagenase have been determined ^{451, 452}.

A wide variety of methods have been reported for the measurement of collagenase activity ranging from qualitative screening assays to those that attempt to quantitate hydrolysis of various substrates ^{430, 453} (see chapter 6).

One of the difficulties in comparing collagenase data between different pharmaceutical companies and indeed different laboratories involved in collagenase research, is the lack of standardisation of these assays ⁴⁴⁵.

It should not be forgotten however, that the ultimate "assay" is the human pancreas itself.

4) Which components of crude *Clostridium histolyticum* collagenase are required for human islet isolation?

The answer to this question is the key to the production of a reproducible, stable collagenase. However, the only detailed studies published to date, have been performed on rodent and canine pancreata ^{454, 455, 456, 457}. These have looked at the roles of class I collagenase, class II collagenase and neutral protease. Studies by Vos-Scheperkeuter et al.(1994) examined the role of different collagenase fractions on rodent islet isolation. After fractionating crude collagenase using a combination of gel-filtration and ion-exchange chromatography on the FPLC and reintroducing the fractions in various combinations, they found that a combination of collagenases was important, but

in particular the presence of high amounts of class II collagenase were favourable ⁴⁵⁶. The same group, performed more detailed experiments and showed that it was possible to dissociate pancreatic tissue with class II collagenase alone, whilst class I collagenase only dissociated the tissue very slowly and incompletely ⁴⁵⁴. When the two classes were combined, a more rapid dissociation resulted. This is consistent with the findings of Van Wart (1985) who suggested a synergistic role for the different collagenase classes ⁴⁴⁸. They concluded that "pancreas dissociation can be manipulated by using a well-defined mixture of collagenases". Studies on the canine pancreas have shown that purified collagenase is ineffective for canine islet isolation, suggesting that components other than collagenases are also required for islet isolation ⁴⁵⁷. The role of protease has been investigated by several different groups ^{454, 455, 458}. McShane et al. (1989) found that enzyme batches low in caseinase activity (i.e. low in neutral protease), were good for islet isolation ⁴⁵⁸. However, in the same study, they found that the addition of Dispase (commercially available neutral protease) significantly improved islet yields. Wolters et al. showed that limited exposure of the pancreas greatly improved islet yields compared to using collagenase fractions alone, but prolonged dissociation in the presence of neutral protease, greatly decreased islet yields, mainly due to islet disintegration ⁴⁵⁴. These studies have shown that limited exposure to protease, in addition to the presence of both classes of collagenase, is essential for efficient islet isolation. This might be predicted from the different specificities of the three components. It can be postulated that the combination of components degrade the extracellular matrix in a sequential manner, with the initial degradation of the matrix proteins by neutral protease. The exposed native collagen is then degraded by class I collagenase, which then enables class II collagenase to fragment the denatured collagen. These collagen fragments can then be further degraded by neutral protease. The results of these studies on rodent and canine pancreata cannot however, be directly transposed to the human situation. The reason for this, is that the substrate for enzymatic digestion i.e. the pancreatic matrix, is different

between different species. As stated earlier, the human pancreas contains collagen types I, III, pro-III, IV collagen, laminin and fibronectin ^{379, 380, 381, 382, 383}. Van Suylichem et al. (1987) have compared the total collagen present in the pancreata of different species ³⁸². They found that the total pancreatic collagen content of the pig, cow, and human, but not the dog or monkey, is significantly larger than that of the rat. The large amounts of collagen measured in the pig, cow and human, is mainly localised to the inter and intralobular septi, a finding confirmed in the human by Uscanga et al. ³⁷⁹. Additional information relating to the structural relationships between the islets and the exocrine tissue for the pancreata of several different species has been provided by van Deijnen et al. (1992) ³⁸¹. They found that in the pig, very little peri-insular capsule is present and the structural integration of the porcine islet within the exocrine tissue, depends almost completely on cell-cell adhesion. In the canine pancreas on the other hand, the islets are almost completely encapsulated with very little islet to exocrine cell-cell contact. The situation in the rat and human is intermediate with a predominance of cell-matrix adhesion. In all four species, the intra-islet adhesion mechanisms, depend largely on cell-cell adhesion. These findings mean that it is unlikely that the same enzyme mixture can be used for all species. Therefore similar studies must be repeated to determine which of the components of collagenase are necessary for the digestion of the human pancreas. Such research however, poses new challenges that were not previously encountered with the studies on the rodent pancreas. First, while it is possible to standardise the inter-pancreatic variables between different rat pancreata, this is not possible for the human. Any study on the role of collagenase components on human islet isolation, must therefore control for these many different donor variables. This can only be done by using methods that enable the testing of numerous different collagenase components and their combinations, on any one human pancreas (see chapter 5). This forms a major emphasis of this thesis. Second, studies in the rodent and dog have been restricted to class 1 collagenase, class II collagenase and neutral protease. As has been mentioned, in addition to

these three components, crude collagenase contains clostripain, trypsin, elastase, β -D-galactosidase, β -N-acetyl-D-glucosaminidase, α -L-fucosidase, phospholipase, neuraminidase, and hyaluronidase³⁵⁰. The role of these components must also be studied both individually and in combination. It can be predicted that several of these factors are not only harmful to islets, but also responsible for the deterioration of crude collagenase during storage.

However, this research requires quantities of collagenase components to be fractionated from crude collagenase that only industry is capable of achieving. Recently, in an attempt to improve the reproducibility of islet isolation, Boehringer Mannheim have produced the 'new' product *Liberase*^{240, 241, 242, 243}. However, this has been developed as a purer 'blend' of components, based on several 'good' Boehringer P collagenase lot numbers, rather than based on systematic, detailed research into the interaction of individual collagenase components and their combinations with the human pancreas.

5) Stability of crude collagenases during storage

As has already been stated, even when a good batch of crude collagenase has been produced, deterioration of enzyme activity during storage remains a major problem. Most pharmaceutical companies state in their product data sheets that the lyophilised crude enzyme mixture is stable at 4°C when stored under dry conditions and that the reconstituted solution is stable at -20°C. Many groups however, store even the lyophilised enzyme at -20°C. Both classes of collagenase are very susceptible to proteolysis⁴⁴⁵. Indeed this proteolysis is thought to have accounted for much confusion over the exact molecular weights of the two classes⁴⁴⁵. Even the smallest amount of hydration, such as that occurring during the freeze thaw cycles between different islet isolations, may in theory cause deterioration of enzyme function. However, surprisingly the detailed study of collagenase activity under different storage conditions that I performed as part of this thesis (see Chapter 7)

showed stability of the key three components namely, collagenase 1, collagenase 2, and neutral protease. The components responsible for the degradation have not therefore been fully elucidated. However, the proteolysis of collagenase samples that occurs during heating is thought to be caused by clostripain in the presence of calcium ⁴⁴⁵.

The deterioration seen with crude collagenase is an inevitable consequence of such a crude mixture of different enzymes. It is to be envisaged that once the components of collagenase required for human islet isolation have been fully determined and simpler, more pure enzyme blends have been produced, that collagenase stability will no longer be such a problem.

6) Biophysical factors influencing collagenase administration

Whilst the correct composition of collagenase is essential, it is also important to deliver the collagenase to the interface between the islets and acinar cells, while maintaining the integrity of the islets themselves. The factors influencing this aspect of collagenase administration are: the route of administration, the pressure and volume of administration, the timing of administration and the temperature of administration.

a) Route of collagenase administration

As has already been discussed, the administration of collagenase via the intraductal route rather than by simple incubation, greatly improved islet yields in all species (see Figure 3.4) ^{409, 410, 411, 459}. Attempts have been made to administer the collagenase via the intra-venous route ^{460, 461, 462}. In a study directly comparing the administration of collagenase via the pancreatic duct (PD) and portal vein (PV) in the rat, Gotoh et al. found that although the PV produced a similar macroscopic appearance following collagenase

Figure 3.4 Human pancreas distended with crude collagenase



administration to that observed with the PD route, the latter was much superior in its ability to digest exocrine tissue and as a result produced significantly higher islet yields ⁴⁶⁰. One of the explanations for this difference put forward by this group is that the mechanical forces generated by PD injection is limited to the exocrine cells, while the PV administration results in high pressure around the exocrine tissue as well as within the islets themselves. Another study by Downing et al. (1979) compared intravenous versus ductal collagenase administration in the dog ⁴⁶¹. In this study however, the intravenous route produced more than three times the post-Ficoll yield of that obtained with intraductal collagenase administration. It is interesting to note however, that when the percentage yield of islets released at each digestion time is compared for the two methods, there is no significant difference apart from the first digestion time, at which significantly more islets were released by the intraductal method. There are no published studies showing an improvement in islet yields by intravenous collagenase administration in the human and therefore the intraductal method remains the most effective way of administering the collagenase to the islet-acinar interface ³⁴⁹.

b) Pressure and Volume of Collagenase administration

There are two main methods currently used for the administration of collagenase into the pancreatic duct. These are the single injection or loading technique ⁴¹¹ and the perfusion method ^{463, 464, 465}. The only study to directly compare these two methods has shown a moderate improvement in islet yield with the perfusion method ⁴⁶³. However, a large controlled trial is required before any firm conclusions can be made and most groups currently continue to use the loading method. Whichever method is used it is essential that the collagenase is distributed uniformly throughout the pancreas because islets are not isolated from areas of undistended pancreas. In addition the collagenase must be prevented from entering the islets themselves. It has been shown that with perfusion pressures even as low as 40 mmHg, collagenase

enters the cells of the islet ⁴⁶⁶. These findings are supported by two other studies that investigated the patho-physiology of acute pancreatitis ^{467, 468}. All these studies used Indian ink which being particulate, may have altered the perfusion characteristics of collagenase. Although the intraductal method of collagenase administration may inevitably cause some collagenase to enter the islets due to the pancreatic anatomy ⁴⁶⁶, it should be possible to determine a pressure that optimises collagenase delivery to the islet / acinar interface, whilst minimising intra-islet administration. These issues are addressed further in Chapter 9.

The volume of collagenase that is usually used for the distension of the pancreas using the loading method is 2 ml / gram of tissue, as volumes greater than this seem to rupture the ductal system, resulting in collagenase leaking from the gland ^{353, 468}.

c) Timing of collagenase administration

Traditionally the pancreas is distended with collagenase once it has been brought back to the islet isolation laboratory. Several groups however, have shown a significant increase in islet yields if collagenase is administered at the time of harvesting ^{469, 470, 471, 472}. Using this method, improved islet yields have been seen in the rat ^{469, 470}, pig ⁴⁷² and human ^{471, 473}. The explanation for these observations is that the collagenase is delivered into the pancreas before the effects of cold ischaemia have caused a loss of pancreatic ductal integrity ⁴⁷⁰. If this method is to be adopted however, the collagenase must be delivered in a suitable cold-storage solution and kept as inactivate as possible until the pancreas arrives in the islet laboratory.

d) Temperature of collagenase administration

All enzymes function optimally within a certain range of temperature. In the case of *Clostridium histolyticum*, the collagenase component has been studied

in some detail ^{418, 419, 437, 474}. However, crude collagenase is a mixture of different enzymes each with different optimal temperature requirements. Gray (1994) has suggested that the crucial factor for the efficiency of collagenase activity is the rapid attainment of working temperature ³⁴⁹. However, Warnock et al. (1988) have suggested that it is preferable to administer the collagenase at 4°C and to slowly warm it to 39°C ⁴⁶³. Collagenase activity rises with increasing temperature in a linear fashion up to a maximum at 43°C before decreasing again ⁴⁵⁸. Clearly a balance has to be struck between a temperature at which collagenase activity is optimal and one that does not cause irreversible damage to the islets. Currently most islet groups administer collagenase to the human pancreas at either 24 °C or 37°C. However, Sutton (1990) has reported a significant increase in human islet yields when collagenase is administered at 41°C as opposed to 39°C with no decrease in islet viability as shown by supravital staining, electron microscopy and insulin response to perfusion ⁴⁷⁵. Other groups have reported a significant increase in islet yields in rat pancreata which had undergone 48 hours of cold-storage when the collagenase was administered at 20°C rather than 37°C ⁴⁷⁶. A possible explanation for this however, is that with a cold ischaemia time of 48 hours, the collagenase activity is greatly influenced by the release of pancreatic proteases. If collagenase is to be administered at the time of organ procurement, it must be administered at a temperature at which collagenase activity is at a minimum.

7) Biochemical factors influencing collagenase administration

a) Which buffer should be used for collagenase administration?

Crude collagenase is manufactured as a lyophilised powder which requires reconstitution before administration. It is essential that the buffer used for administering the collagenase, does not itself inhibit collagenase activity. In

addition, if the collagenase is to be administered at the time of pancreatic harvesting, ("see timing of collagenase" above), it should be administered in a suitable cold-storage solution. One of the most widely used cold-storage solutions worldwide, is University of Wisconsin solution (UW). In an attempt to optimise the cold storage of the pancreas prior to islet isolation, collagenase has been administered in UW solution immediately after pancreas excision. Whilst this has proven effective in the rat ⁴⁷⁷, dog ⁴⁷⁷ and pig ^{478, 479}, in the human it results in very poor islet yields and poor islet viability ^{480, 481}. The inhibition of the collagenase digestion by UW seems therefore to exhibit a species difference (see Chapter 8). It is clear therefore, that it is pointless having an efficient collagenase delivered to the islet-acinar interface, if the cold-storage solution itself inhibits collagenase. Therefore, cold-storage solutions need to be developed that maintain islet viability, but that do not inhibit collagenase digestion and this is studied in more detail in experiments described in Chapter 8.

b) Should calcium be added to the buffer?

Much confusion exists with regard to whether it is necessary to add calcium to the buffer used for dissolving collagenase. As has already been stated, collagenases are calcium dependent requiring calcium ions as cofactors for their hydrolytic activities ⁴¹⁸. In theory therefore, the inhibitory effect of UW described above could be explained in part by the fact that it contains no calcium. However, we found that the addition of calcium to a solution containing the electrolytes of UW was not significantly less inhibitory than the UW electrolytes alone. This is consistent with the findings of Dono et al. (1992) ⁴⁸² who have noted that although rat islets tend to disperse to a small cluster when no calcium is added to the collagenase-UW solution, the exocrine pancreatic tissue was well digested and dispersed. Indeed, the beneficial effect of calcium in their study was not due to an increase in collagenase activity, but by the prevention of islets from becoming dispersed into single cells, as

calcium plays an important role in cell-to-cell and cell-to-extra cellular matrix adhesion ⁴⁸³. The most likely explanation for the minimal effect on collagenase activity is that whilst collagenase requires calcium as a co-factor, it requires it in its "bound" rather than "free" form. We have found that commercially available crude collagenase contains a significant amount of calcium when dissolved in distilled water (unpublished data). Once the collagenase molecule has 'filled' its calcium binding sites, the presence of additional calcium in the cold storage solution does not significantly increase the collagenase activity. However, Burgmann et al. (1992) have shown that UW has a considerable ability to chelate calcium and it may be the chelation of calcium and its removal from the collagenase binding sites, rather than the absence of calcium, that explains the inhibitory properties of UW ⁴⁸⁴. Wolters et al. have shown that the calcium concentration proved to be of little influence on dissociation conditions ^{485, 486}. They found that the highest islet yield was seen using a calcium concentration of at 2.5 mM. Therefore they concluded that no other concentration of calcium was required other than the physiological one ⁴⁸⁶.

c) pH of collagenase administration

The collagenase activity within crude *Clostridium histolyticum* collagenase measured using collagen as a substrate, changed only slightly over a pH range of 6.2 - 8.0 ⁴³⁷. Other investigators have suggested that a pH of the digestion medium of about 8 is favourable for islet isolation ^{487, 488}. Wolters et al. found that for the rat, islet isolation was not dependent on pH over a range of 6.2 and 8.0 ⁴⁸⁶. The only difference that they observed was at a pH of 8, the digestion of the tissue took more time than at the other pH levels. A recent study compared collagenase digestion in a media with a pH of 7.3. with one of 7.8 ⁴⁸⁹. They conclude that islet yield, viability and purity were all significantly improved with a pH of 7.8.

d) At what concentration should crude collagenase be administered?

During any method of cell disaggregation by enzymes, it must be determined whether the cells being isolated are more likely to be damaged by long-term exposure to low concentrations of the enzyme or short-term treatment with higher concentrations ³⁵⁹. In the case of islet isolation by collagenase, most islet laboratories currently administer collagenase at concentrations of 3-6mg/ml. However, because crude collagenase is a blend of different components, altering the overall concentration also alters the concentration of all the individual components in the same proportion. In addition the enzyme activities listed by different companies on their data sheets are not standardised ³⁴⁸. Different concentrations are required when using collagenases from different companies ³⁵³. However, the concentration of collagenase required should become less empirical once the precise role of the different collagenase components is more clearly understood.

8) Pancreatic factors influencing collagenase digestion

No review of the collagenase digestion phase of islet isolation would be complete without a discussion of the influence of the pancreas itself.

a) Donor Factors

As the collagenase problems have affected most islet laboratories, there has been an increased interest in the influence of pancreatic donor factors. It is interesting to note however, that in our own experience, when we had a good batch of crude collagenase a few years ago, we were able to isolate islets from almost any pancreas that we received, suggesting that the principle problem lies with the collagenase itself that we can influence, rather than the donor factors that we cannot. There are however, several groups of donors from

which islet isolation is more difficult whichever batch of crude collagenase is used. Many studies have suggested that pancreata from donors of less than 20 years old, are associated with a significant reduction in islet yields ^{490, 491, 492, 493, 494, 495}. Several of these studies however, have also shown that both the purity and viability were optimal in this young age-group ^{491, 492}. Other groups report no significant differences in islet yields from this donor age-group ^{496, 497}. There has even recently been a suggestion that the transplantation of islets isolated from pancreata of young donors confer advantages on post-transplant islet function ⁴⁹⁸. It has been postulated that the thin, peri-islet rim of non-endocrine tissue surrounding these islets (so called "mantled islets"), could be important for long term islet survival in ectopic sites. However, it is important not to draw firm conclusions from a single case-report. The influence of donor age on collagenase digestion is not surprising when one considers the changes in collagen structure with age ⁴⁹⁹. Indeed Bedossa et al. (1989) have shown a significant difference in the total collagen content between different age-groups ³⁸³. The group from Giessen, have found that increased islet yields are obtained from donors with a body mass index of >24 ^{490, 494}. Watt et al. (1994) have suggested that a past medical history of asthma, hypertension, epilepsy or psychiatric disorders, all resulted in a significant reduction in islet yields ⁴⁹⁵. We would suggest that the explanation for these latter findings however, is more likely to be related to the patients drug history rather than the conditions per se. Indeed it is our own observation that it is very difficult to isolate islets from donors who have been taking long term phenytoin. The same study by Watt et al. also found that the sex of the donor made no significant difference to islet yields ⁴⁹⁵. Other unfavourable donor factors suggested include a prolonged period on the intensive care unit and a blood glucose level of >10 mmol/L ⁴⁹¹. Some groups have even reported a decrease in islet yields from donors that smoke ⁵⁰⁰.

These studies serve as a useful pointer to those human pancreata that are harder to digest using our current batches of crude collagenase. However,

with relatively few human pancreata available, we do not have the luxury of restricting ourselves to pancreata from non-smoking 35 year olds of medium build!! Instead what is required is a more detailed understanding of donor-factors at a histological level, and the formulation of collagenase blends that are capable of digesting the full range of pancreata that we receive.

b) Procurement Factors

Even with a pancreas that fulfils the "ideal donor" criteria, the actual procurement of the pancreas can greatly influence the subsequent islet isolation. The factors occurring during procurement that have the greatest influence are: warm ischaemia, cold ischaemia and the various solutions used both for vascular perfusion and the transport of the pancreas back to the laboratory. Warm ischaemia is extremely damaging to islets ^{501, 502, 503, 504}. In the case of the human, this is kept to a minimum by in-situ vascular perfusion. Although vascular perfusion with UW solution has been successfully used for the cold storage of the human pancreas prior to vascularised transplantation, this approach has proven problematical prior to islet isolation because UW solution appears to inhibit the collagenase digestion phase of the islet isolation process ⁵⁰⁵. This inhibition was not seen when the pancreas was perfused with hyper-osmolar citrate. The use of Euro-Collins has also been shown to be detrimental to rat ⁵⁰⁶ and porcine ⁵⁰⁷ and to a lesser extent in the human ⁵⁰⁶.

The effect of different lengths of cold ischaemia on human islet isolation, is in part dependent on which vascular perfusate has been used. Eurocollins is not an effective perfusate prior to islet isolation ⁴⁶³. UW has been shown to be effective for up 17 hours ⁵⁰⁸. However, up to 6 hours it offers no advantage over hyper-osmolar citrate in terms of islet yields and islet purity ⁵⁰⁵. For pancreata perfused with both hyper-osmolar citrate ⁶⁴ and UW ⁵⁰⁸, a cold ischaemia time of < 2 hours seems to reduce islet yields. Brandhorst et al.

have also suggested that a reduction in islet yield is also seen in pancreata that have been perfused with < 3 litres of perfusate ⁴⁹⁴.

c) The role of pancreatic enzymes

Whereas the factors discussed in the last two sections vary greatly from pancreas to pancreas, there are more basic factors that are intrinsic to all pancreata and that have a major influence on islet isolation. The pancreas itself contains a large number of different enzymes. These include trypsin, chymotrypsin, elastase, amylase, kallikrein, lipase, phospholipase A₂, carboxypeptidase A and carboxypeptidase ¹⁶¹. All these enzymes apart from amylase, are present in their inactive form as zymogens, and require either trypsin or enterokinase for their activation ¹⁶¹. However, when collagenase is introduced into the pancreas, a cascade of both pancreatic and collagenase enzymatic activation and interaction occurs ⁵⁰⁹. Very few studies have looked at this in any depth. Traverso et al. (1978) have shown that canine pancreatic proteases are activated by commercial collagenase and that this activation was attributable to components within crude collagenase other than the collagenase itself ⁵⁰⁹. Indeed this activation was not seen when purified collagenase was added to the pancreas on its own. Wolters et al. have also shown this for the rat pancreas ^{485, 486}. By adding 10 % BSA to the isolation medium, they were able to significantly increase islet yields. When BSA was added in combination with more specific trypsin inhibitors the islet yields were increased even further and a considerable increase in total islet volume was also seen. This is consistent with the findings that trypsin causes islet fragmentation ⁴⁰⁰. Neither of these studies however, involved the use of an automated method of islet isolation. Heiser et al. have investigated the kinetics of different enzymes during the automated porcine islet procedure ^{510, 511}. They found that both trypsin-like protease and neutral protease activity were greatly increased during the isolation procedure and that the collagenase activity decreased. They tested four different protease inhibitors and found that one inhibitor was

able to inhibit the trypsin-like protease activity completely, thereby improving islet isolation. Indeed they conclude that "low trypsin-activity during the isolation procedure guarantees reproducible islet yields" ⁵¹². No detailed study has been published on the effect of trypsin inhibitors in human islet isolation. It is therefore important that detailed research is conducted to study the interaction of collagenase with human pancreatic proteases and to assess if the addition of protease inhibitors can significantly improve islet yields. These inhibitors must selectively inhibit the pancreatic trypsin rather than the collagenase itself.

Conclusions

It can be seen from this chapter that the collagenase digestion phase is a complex process consisting of a multitude of interrelated variables. However, it is also clear that the future of human islet transplantation is in part dependent on understanding the problems highlighted. Very few laboratories have addressed any of the problems with detailed research. In Leicester, our previous research into islet isolation had been aimed at optimising the density-gradient media used during the islet purification phase. However, as has been outlined, this phase is totally dependent on a consistently efficient collagenase digestion phase. Therefore, our human islet transplant programme, together with those of many other centres worldwide, had been severely hindered by the variability of the collagenase digestion phase. It was with this background that I undertook the work described in the remainder of this thesis.

SECTION II

Collagenase Components

CHAPTER 4

A Comparison of the Digestion Profiles of Human and Porcine Pancreata by Different Batches of Crude *Clostridium histolyticum* Collagenase

Introduction and Aims

As has been highlighted in the last chapter, there is substantial variation between different batches of crude collagenase in several respects. First, due to the non-reproducible manner in which the enzymes are manufactured, the efficiency for islet isolation varies enormously between "batches" / "lots". The usual procedure within our department for selecting an efficient crude collagenase is to 'batch' test several new crude enzymes from the most recent industrial 'campaign' when the activity of a previously good crude enzyme has deteriorated or when the supply of such an enzyme has become exhausted. Usually this involves one or two new enzymes each time. Depending on the individual crude enzyme, this batch testing may be required every few months or in the case of an exceptional crude enzyme in plentiful supply, every few years. Having analysed this 'batch' testing data from the five years prior to the start of my project, it was clear that with relatively few enzymes being tested relatively infrequently, an objective comparisons between different enzymes was difficult. In addition, as the research at this time was directed towards the optimisation of the density-gradient phase of islet isolation, the end point measures of collagenase efficiency were not detailed digestion profiles but studies of islet numbers pre and post purification.

The aim of this first series of experiments therefore, was to compare a number of different 'batches' of crude collagenases concurrently and develop a protocol that enabled a detailed profile of pancreatic digestion.

The second way in which the batches of crude collagenase vary is the efficacy in different species. Although it is generally accepted that the results of research on rodent islet isolation cannot be directly transposed to the human situation, at the time that I commenced this research project, many islet laboratories and indeed pharmaceutical companies, believed that the porcine pancreas was ideal for modelling human islet isolation. Whilst we fully accept that research into porcine islet isolation is important in its own right, it was our observation in Leicester that whereas some batches of crude collagenase

were able to efficiently isolate islets from both the human and porcine pancreas, often different batches were required for optimal islet isolation in the two species. This subjective observation had not been formally compared by repeated experiments using a series of different enzymes. The second aim of this initial series of experiments therefore, was to evaluate and compare the digestion of the human and porcine pancreas by a number of different batches of crude *Clostridium histolyticum* collagenase.

The third area in which the collagenase digestion is variable is between different pancreata. Although the 'donor' criteria are more consistent in the abattoir acquired porcine pancreas, variation between islet preparations is observed in both species. The third aim of these initial experiments therefore, was to demonstrate the variation of digestion efficiency of the same crude enzyme when used on different pancreata.

The fourth aim of these experiments was to evaluate potential experimental designs which could then be used in subsequent experiments . In particular, the development of techniques for the testing of several crude enzymes and their components on the same human pancreas in order to control for variation between different pancreata. Although this was the main aim of the studies outlined in Chapter 5, the initial data from these experiments formed the basis of the subsequent studies. In addition, a modified design of the collagenase digestion proforma used in these experiments was used for many of the other collagenase assessments.

Finally, it was envisaged that by defining efficient and poor crude enzymes early in the project, these enzymes could then be used for the research on enzyme assays, collagenase components, and collagenase stability detailed in subsequent chapters.

Materials and Methods

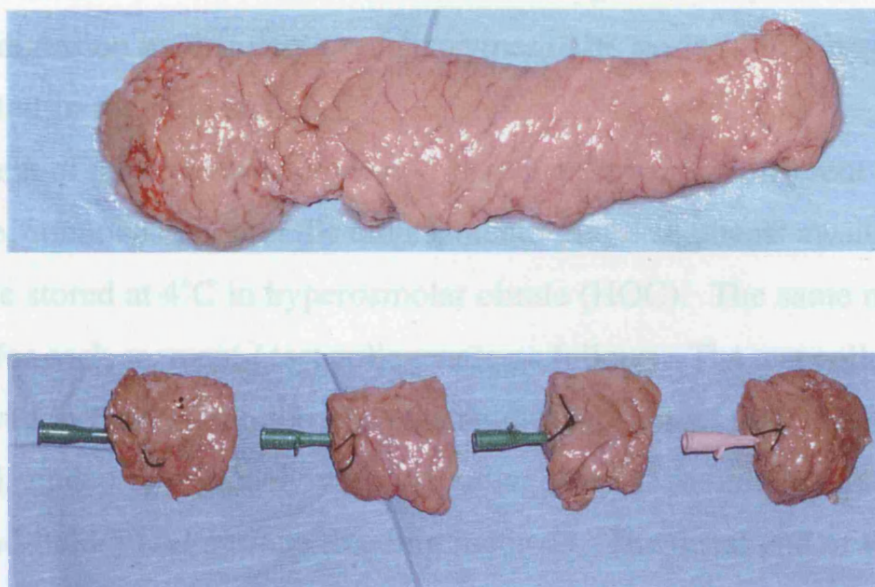
Overall Experimental Design

With the above aims in mind, a total of 12 crude collagenases were tested "blindly" on both human and porcine pancreata. These 12 consisted of 8 different commercially available crude *Clostridium histolyticum* collagenases (Boehringer Mannheim), with 1 enzyme repeated 4 times. The repeated enzyme was used as an internal control. The crude enzymes were coded 1-12 and the code only "broken" after all the tests had been performed. Although the semi-automated method of pancreatic digestion^{412, 414} (see Figure 3.1 in previous chapter) was used for the enzyme testing in both the human and the pig, there were several fundamental differences between the methods used in the two species.

Human Pancreata

Human pancreata were retrieved with appropriate consent from brain-dead, heart-beating donors. Prior to removal, all human pancreata underwent "in-situ" vascular perfusion with 3-6 litres of hyperosmolar citrate (HOC, Travenol, Thetford, UK.), thereby minimising the warm ischaemia time. The pancreata were transported to the islet isolation laboratory in HOC at 4°C. The cold ischaemia time prior to commencing the experiment was 2-4 hours. On return to the islet laboratory, each pancreas was then divided into 4 sections and the pancreatic duct within each section cannulated with either a size 22 or 18 cannula (Abbocath) according to duct size (Figure 4.1). The length of each cannula was trimmed in order not to protrude through the distal end of the segment. Each cannula was secured in place with a 2/0 mersilk suture tied around the cannula whilst avoiding cannula occlusion (Ethicon).

Figure 4.1 Pancreas divided into 4 sections and cannulated for consecutive digestions



I had developed this method of pancreatic division and evaluated it on previous human pancreata as part of a pilot study. This method enables 4 different enzymes to be tested sequentially on any one human pancreas. This is not only important because the number of human pancreata donated for research is very limited, but more significantly allows the immense inter-pancreatic variation encountered in the human to be controlled, enabling true comparisons of the pancreatic digestion by different crude enzymes to be made (this is discussed in more detail in the next chapter).

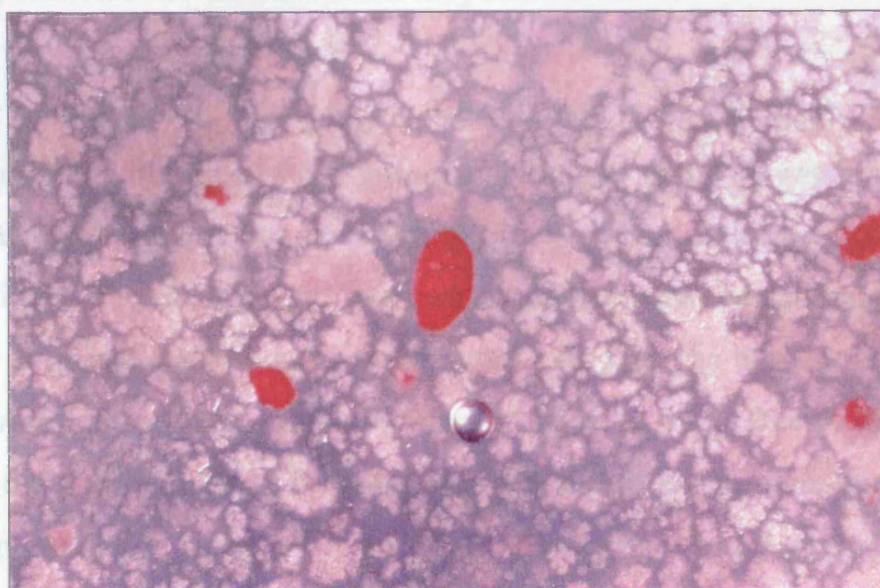
Each segment of pancreas was processed in sequence using a different test collagenase, commencing with the tail segment. Those segments awaiting testing were stored at 4°C in hyperosmolar citrate (HOC). The same methods were used for each segment / test collagenase as follows. The test collagenase was dissolved in Hanks' solution at a concentration of 3mg / ml. The segment was then distended retrogradely with the collagenase at room temperature using a hand-held 50 ml syringe (loading method). The distal end of the pancreatic duct had been clamped with an arterial clamp in order to prevent leakage. A total volume of 2ml / gram of pancreas was used in each case.

Once distended the segment was placed in a 500 ml Ricordi digestion chamber containing a 500 µm mesh (the latter enables the selective passage of tissue from the chamber and prevents undigested tissue from entering the circuit) and 7 metal ball-bearings each measuring about 1cm. The latter enable some physical dissociation of the pancreas during the shaking of the chamber as described below. The digestion chamber was then connected to the isolation circuit. The circuit was primed with MEM. The segment was then digested using the semi-automated method. This comprised two main phases. Firstly, phase 1 consisted of a closed circuit that allows the media and digest to recirculate, driven by a peristaltic pump at a rate of 18 RPM. The temperature of the circulating media and hence within the Ricordi chamber is controlled by the circuit including a metal coil which is immersed in a water bath. The temperature during this phase is maintained between 37 and 38°C. The temperature within the digestion chamber is monitored and recorded

throughout the islet isolation. The chamber is continually shaken and inverted in order to prevent the tissue from blocking the outlet to the chamber and to enhance pancreatic digestion by combining both physical and enzymatic dissociation. Every 3 minutes, 5 ml samples ('biopsies') were taken from a sampling port within the circuit and stained with dithizone (DTZ), a chemical that selectively stains islets red by binding to the zinc granules within the beta-cells (see Figure 4.2). (This is prepared by adding 50mg DTZ to 5ml of dimethylsulfoxide (DMSO), with 1 ml of this solution diluted with 20ml Hanks with 2% foetal calf serum). In order to standardise the amount of tissue within each 'biopsy, the shaking of the chamber was stopped 10 seconds prior to the taking of the sample. Each 5 ml sample was assessed by 2 independent observers.

Phase 1 (closed isolation circuit) was continued until ++ 'cleaved' islets (see 'Parameters analysed for Digestion Profiles' below) had been identified in 2 consecutive "biopsies". At this point the circuit was then 'opened', enabling pancreatic digest to be collected and fresh MEM to be circulated. This second phase is a dilution phase preventing isolated islets from becoming overdigested (fragmented), and during this phase the collagenase activity is significantly reduced both by the dilution of available collagenase itself and reduction of the circuit temperature. The time of conversion from Phase 1 -> Phase 2 of isolation was noted in each case. In this series of experiments the pump rate was increased to 22 RPM during phase 2 and the temperature reduced if fragmentation was observed in the 'biopsies'. If a preparation did not reach the ++ islets criteria, then it remained in Phase 1 throughout the digestion . Each enzyme was assessed for a total of 40 minutes. The reason for choosing such an end-point was based on the observation that efficient enzymes generally enable switching from phase 1 to phase 2 at between 8 and 12 minutes and have usually achieved maximum digestion by about 25 minutes. Any crude collagenase that has not commenced efficient digestion by 20 minutes is by definition a "poor" enzyme for islet isolation (it is to be remembered that islets are being subjected to warm ischaemia for the duration

Figure 4.2 Dithizone stained 'biopsy' showing cleaved and uncleaved islets stained red



that they are within the isolation circuit). Following each digestion, the circuit and chamber were washed out thoroughly and the same procedure repeated using the next segment distended with the next test enzyme.

To enable an objective comparison, a profile of digestion was obtained for each enzyme tested. This was achieved by recording different parameters from each 5 ml 'biopsy'. These parameters and the rationale for their use are discussed below.

Porcine Pancreata

Whilst it is possible to sequentially test up to 4 different enzymes on any one human pancreas, this method is not possible for use on the porcine pancreas for a number of reasons. Firstly, the extensive branching of the porcine pancreatic duct means that cannulation of the more distal segments of the porcine pancreas is seldom possible. Secondly, even when cannulation is technically possible, abattoir-obtained pig pancreata have not undergone vascular perfusion, and therefore the deleterious effects of increasing cold ischaemia mean that sequential "batch-testing" using distended segments of the same pancreas is limited to a maximum of two or at the most three enzymes. In these experiments therefore, a different pancreas was used for each enzyme tested.

The splenic lobe of porcine pancreata were obtained from a local abattoir with a warm ischaemia time of 5-8 minutes. No more than one pancreas was retrieved and tested on any one occasion in order to standardise the cold ischaemia time. The other donor / retrieval variables between test pancreata were as far as possible also kept to a minimum. As with the human, porcine pancreata were transported to the laboratory in HOC at 4°C. Due to the fragility of the porcine islet, it is standard procedure to use a lower enzyme concentration and digestion temperature for porcine digestion than that used for the human. In these experiments the concentration of enzyme used was 1 mg/ml in a volume of 2ml / gram of pancreas and the digestion temperature

during the semi-automated method was maintained between 33-35°C. A 400 ml digestion chamber was used with a mesh size of 280 µm. The remainder of the digestion method was the same as that for the human apart from the fact that physical agitation of the isolation chamber was kept to a minimum, again to minimise the fragmentation of the porcine islets. As with the human, 5 ml 'biopsies' were taken from the isolation chamber at 3 minute intervals and analysed in the same way. A digestion proforma was completed and a digestion profile constructed.

Parameters analysed for Digestion Profiles

One of the concepts that I considered important in the analysis of collagenase digestion was that of an overall profile over time rather than emphasis only on total post isolation islet counts. This is particularly important when comparing different enzymes as the total isolated numbers at the end of the digestion process is dependent not only on an efficient digestion, but on the original number of islets present in a particular pancreas or its segment. By measuring several parameters at each time point an overall assessment of the digestion process can be obtained. The three parameters that I chose to record at each time point therefore, were the number of 'cleaved' islets within each 5ml biopsy (islet number), the percentage of 'cleaved' to embedded islets in that sample (cleavage index), and finally the degree of fragmentation.

The *number of cleaved islets* was defined as the number of intact islets that are completely separated from exocrine tissue (see Figure 4.2). The concept of islet cleavage is important as it is essential for the subsequent isopycnic density gradient purification. An islet that is left with even a small degree of exocrine tissue attached will have a buoyant density approaching the exocrine tissue and therefore cannot be separated with the pure islet fractions. The number of cleaved islets at each time point, although subject to some of the limitations of emphasising islet numbers described above, does give an important measure of

the efficiency of digestion. If only very few cleaved islets appear throughout the whole digestion profile, clearly this is not a good digestion. Also if a large number of cleaved islets appear only in the very late stages of digestion, this delay in the digestion again is sub-optimal. The number of cleaved islets was expressed using a scale from 0 -> +++++ in which 0 = no cleaved islets seen, + = 1-5, ++ = 6-10, +++ = 11-15, ++++ = 16-20 and +++++ = >20 cleaved islets.

The *cleavage index* was defined as the ratio of cleaved to uncleaved islets seen in each sample and was expressed as a percentage in multiples of ten. It is clearly important that this index is interpreted with the number of cleaved islets taken into account. For example, a cleavage index of 100% when only 1 islet is present in the biopsy is much less significant than a 60% cleavage index with 20 islets present.

The *degree of islet fragmentation* was a measure of the degree of destruction of the islets. When present, this can be a measure of a too 'powerful' enzyme or a damaged pancreas. Fragmentation also exhibits a species difference with the porcine islets being more susceptible than human islets. The degree of islet fragmentation was expressed on a scale of 0 -> ++ in which 0 = no fragmentation seen, + = fragmentation present but islets intact, ++ = severe fragmentation present and islets with very irregular surfaces.

In addition to these parameters, an observation of the quality of exocrine digestion was also noted for each profile and graded either poor, moderate, good, very good. In those experiments that reached the criteria for a switch from phase 1 to phase 2, the final collected digest was centrifuged and the packed digest diluted in MEM to 100ml and three 100 µl samples taken. These were also stained with dithizone and an exact count of both cleaved and uncleaved islets was made by me and a second independent observer. A mean cleavage index could then be calculated for the digest of those experiments.

Results

Human Pancreata

The median (range) weight of pancreatic segment used for these experiments was 16.1 (13.1 - 20.1) grams. The median (range) cold ischaemia time for all pancreatic segments was 165 (70 - 260) minutes.

The digestion profiles for the 12 collagenases tested on the human pancreata are given in Figures 4.3 to 4.14. The profile is a composite of the number of cleaved islets, the cleavage index, and the degree of fragmentation for each time point. To enable meaningful comparison, each value is expressed as a percentage of the maximum score possible for that parameter. It must be understood therefore, that a value of 100% fragmentation does not indicate that all the islets in that time point 'biopsy' were fragmented, but rather that the maximum score of ++ was reached. In addition, as is seen in some of the porcine profiles, it is possible to have a time point in which there is still fragmentation but no cleaved islets.

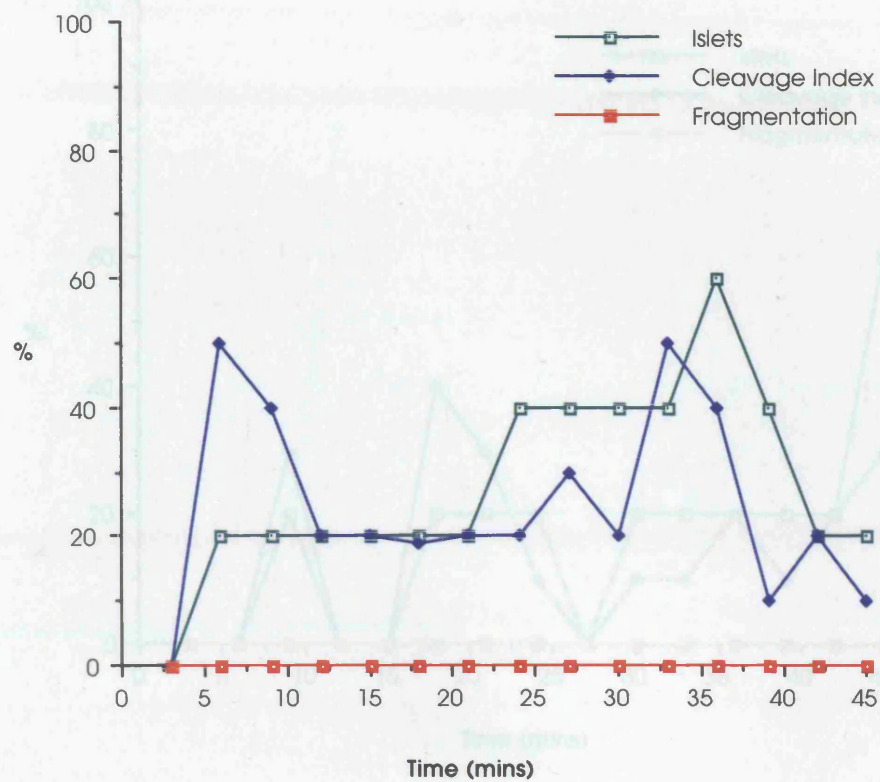
An 'ideal' enzyme is one that produces good yields of well 'cleaved', unfragmented islets from early in the digestion. From these profiles it can be observed that there is considerable variability in digestion between the different enzymes. However, on an overall comparison of the profiles the most efficient digestion profiles were seen with Collagenases 5, 6, 7 and 9. The poorest digestions were with enzymes 1, 2, 8, and 11.

Collagenase 5 produced well cleaved, non fragmented islets quite late in the profile (+++ at 27 mins) but they were of a good quality and when collected had a final digest cleavage index of 40%.

Collagenase 6 was not as efficient as enzyme 5 overall, but produced cleaved islets earlier in the digestion (++ at 21 mins) and still had a final digest cleavage index of 38.6%.

Collagenase 7 was associated with poor exocrine digestion and this is also reflected in the lower cleavage index. However, it produced good quality

Figure 4.3 Profile for Collagenase 1 (Human)

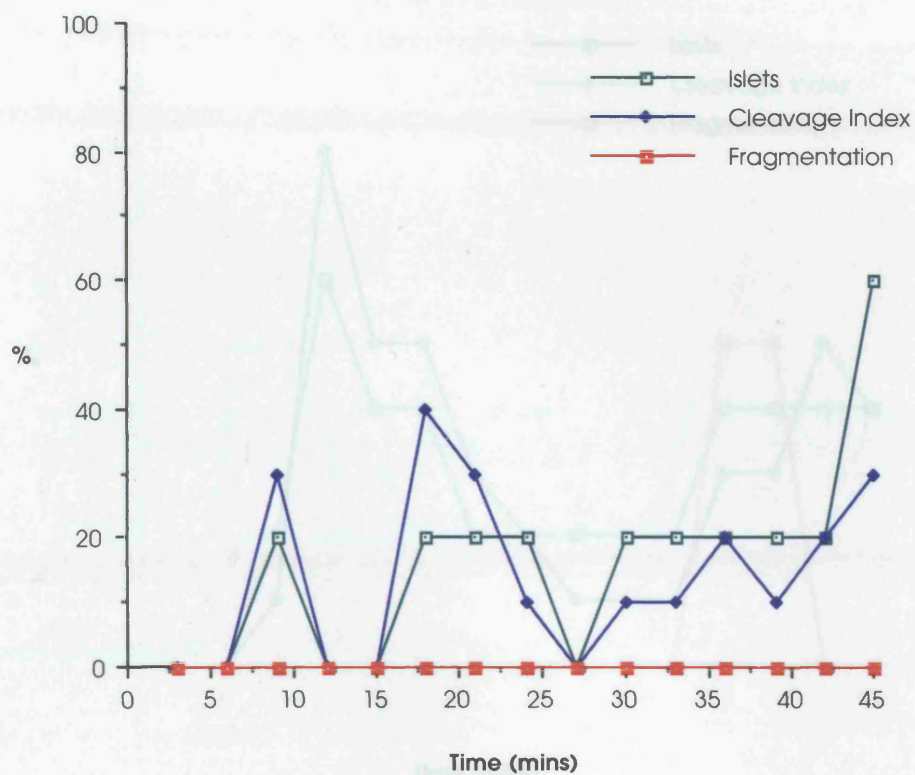


Weight of pancreatic segment = 13.1 g

Cold ischaemia time = 90 mins

Exocrine digestion = moderate

Figure 4.4 Profile for Collagenase 2 (Human)

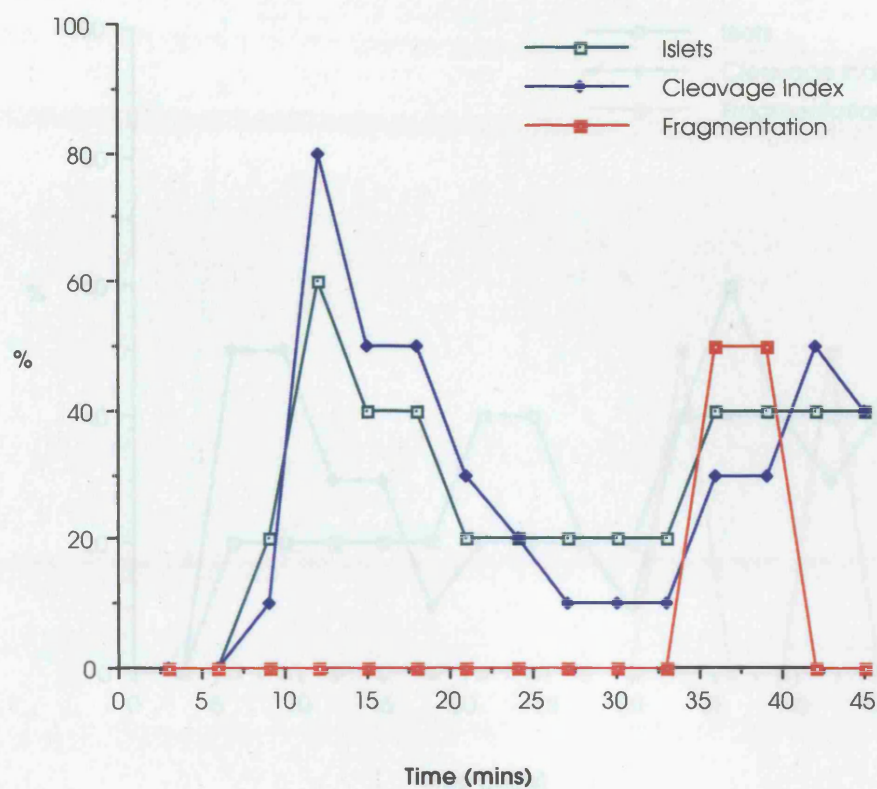


Weight of pancreatic segment = 16.3 g

Cold ischaemia time = 140 mins

Exocrine digestion = poor

Figure 4.5 Profile for Collagenase 3 (Human)

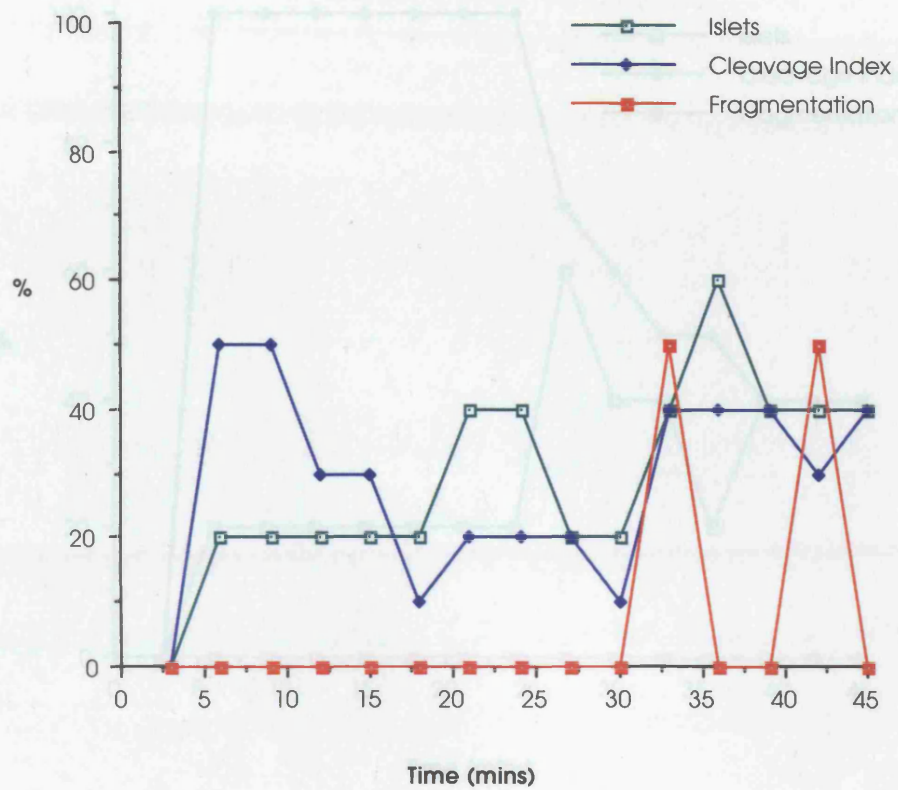


Weight of pancreatic segment = 17.6 g

Cold ischaemia time = 190 mins

Exocrine digestion = moderate

Figure 4.6 Profile for Collagenase 4 (Human)

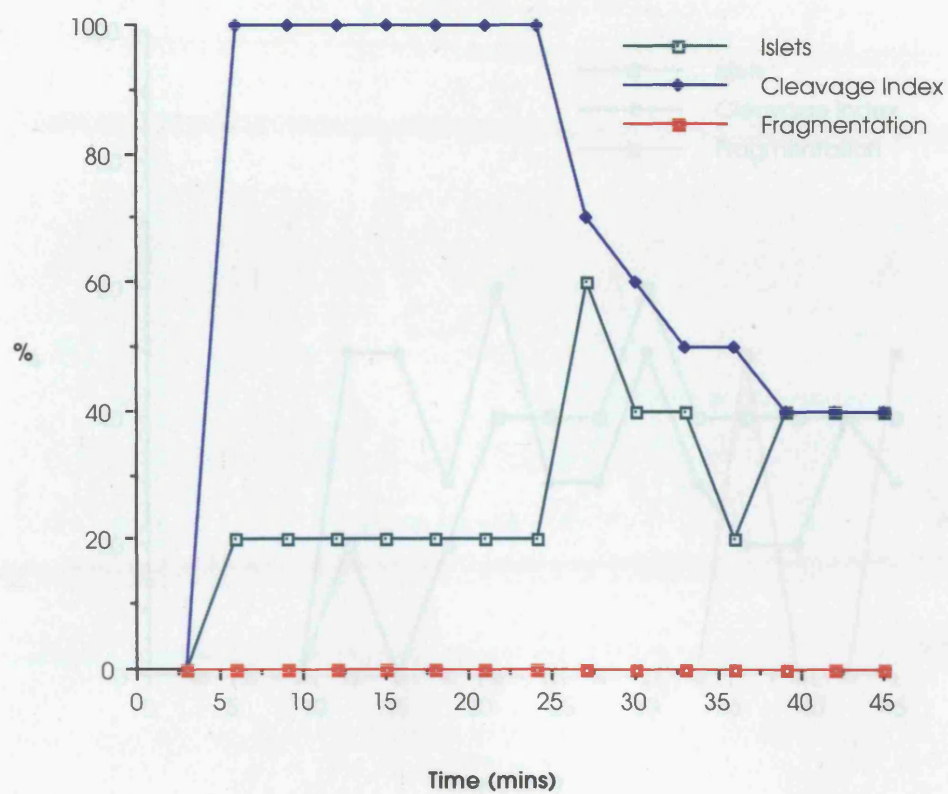


Weight of pancreatic segment = 14.9 g

Cold ischaemia time = 240 mins

Exocrine digestion = moderate

Figure 4.7 Profile for Collagenase 5 (Human)

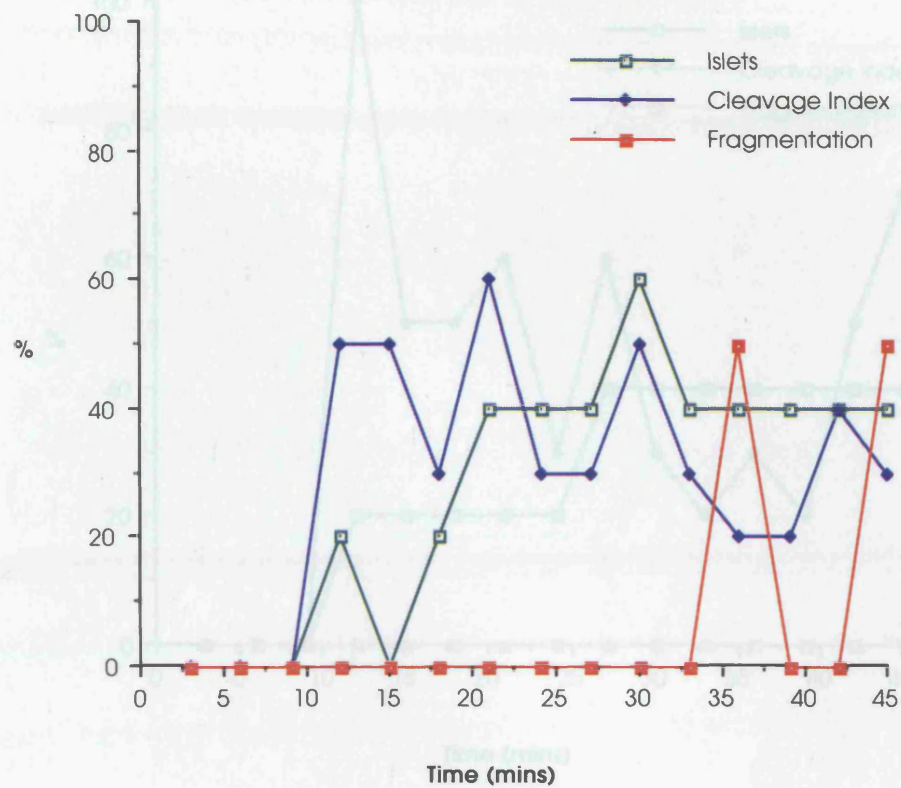


Weight of pancreatic segment = 16.2 g

Cold ischaemia time = 70 mins

Exocrine digestion = good

Figure 4.8 Profile for Collagenase 6 (Human)

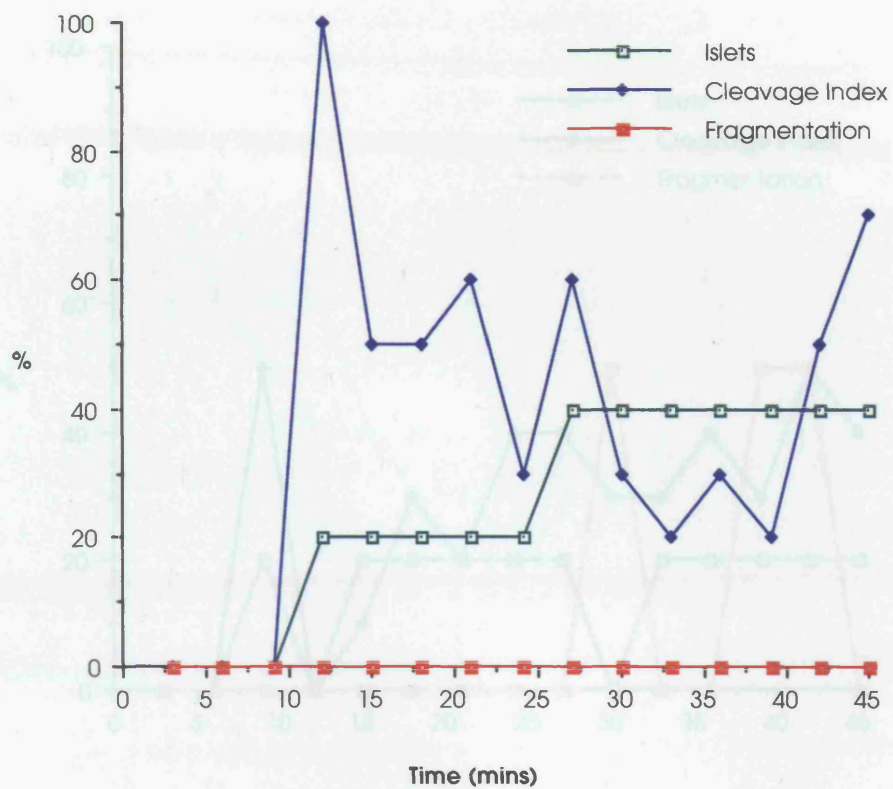


Weight of pancreatic segment = 15.9 g

Cold ischaemia time = 140 mins

Exocrine digestion = good

Figure 4.9 Profile for Collagenase 7 (Human)

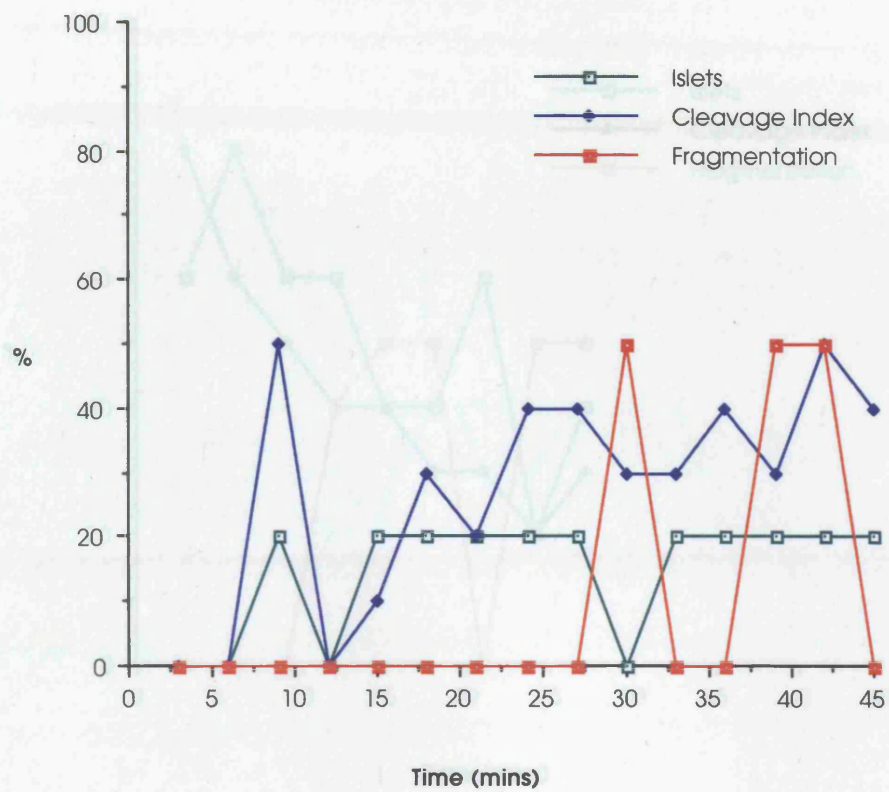


Weight of pancreatic segment = 14.4 g

Cold ischaemia time = 200 mins

Exocrine digestion = poor

Figure 4.10 Profile for Collagenase 8 (Human)

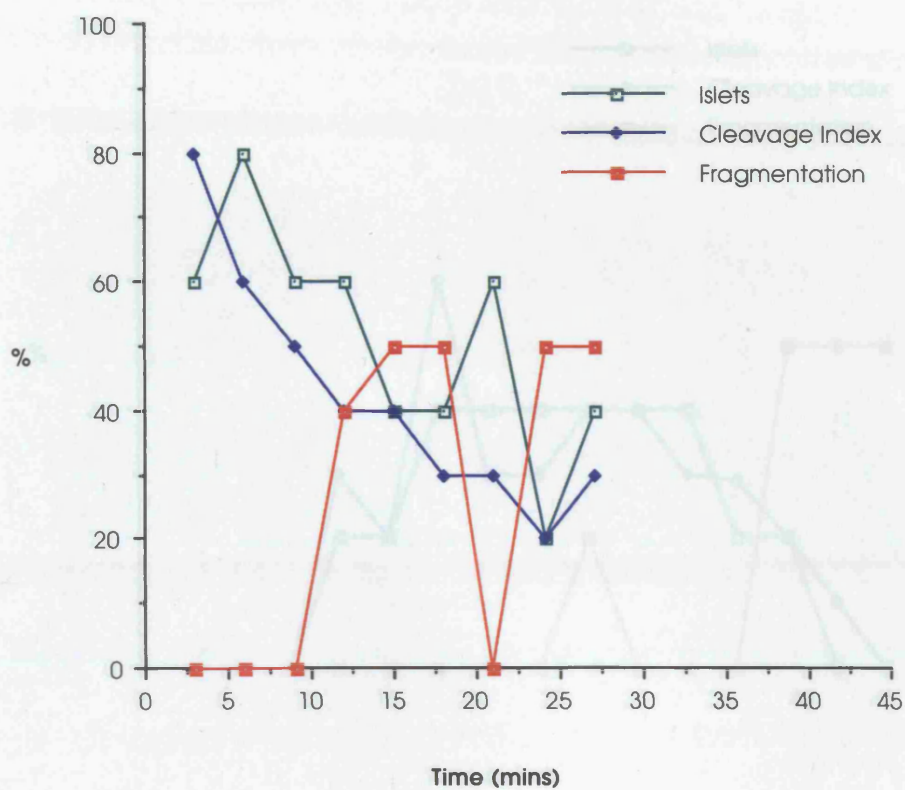


Weight of pancreatic segment = 19.6 g

Cold ischaemia time = 250 mins

Exocrine digestion = poor

Figure 4.11 Profile for Collagenase 9 (Human)

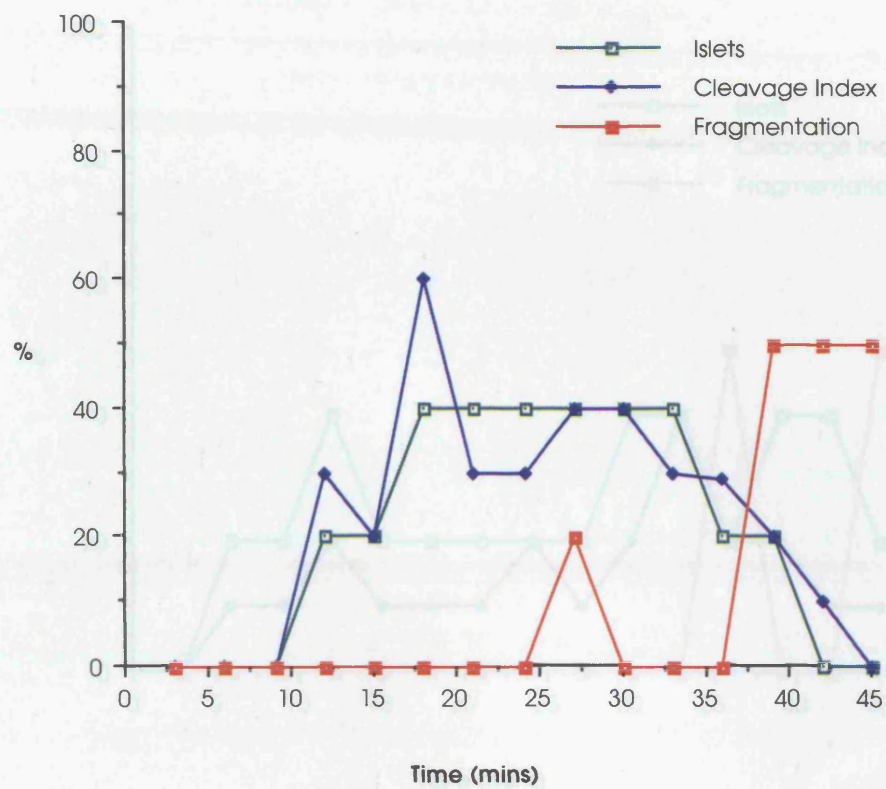


Weight of pancreatic segment = 19.8 g

Cold ischaemia time = 90 mins

Exocrine digestion = very good

Figure 4.12 Profile for Collagenase 10 (Human)

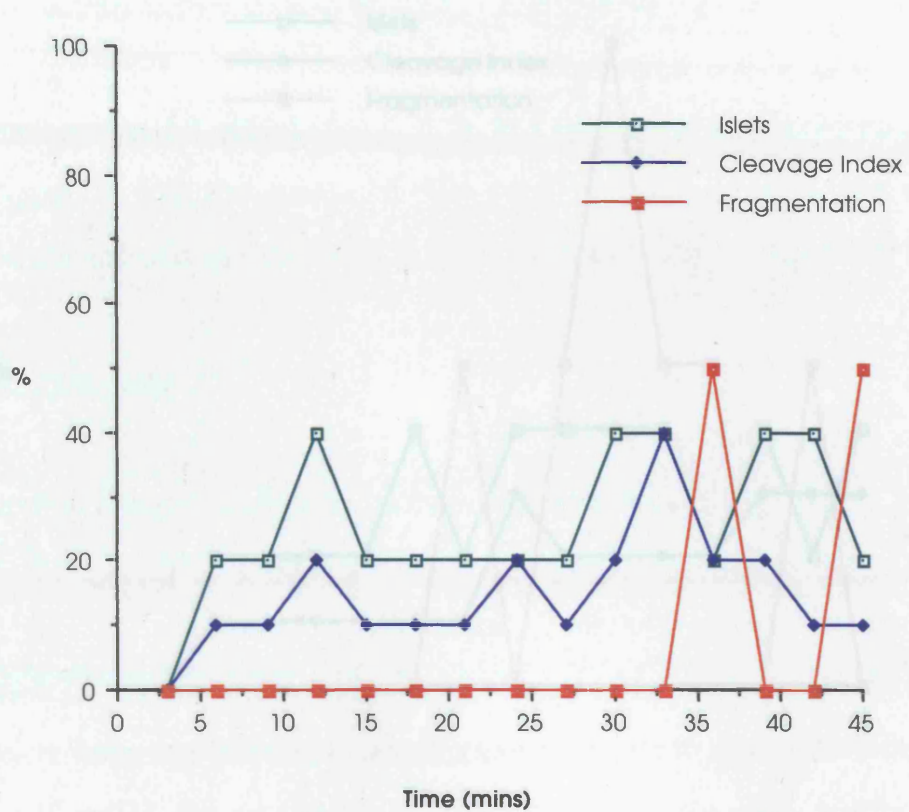


Weight of pancreatic segment = 20.1 g

Cold ischaemia time = 140 mins

Exocrine digestion = moderate

Figure 4.13 Profile for Collagenase 11 (Human)

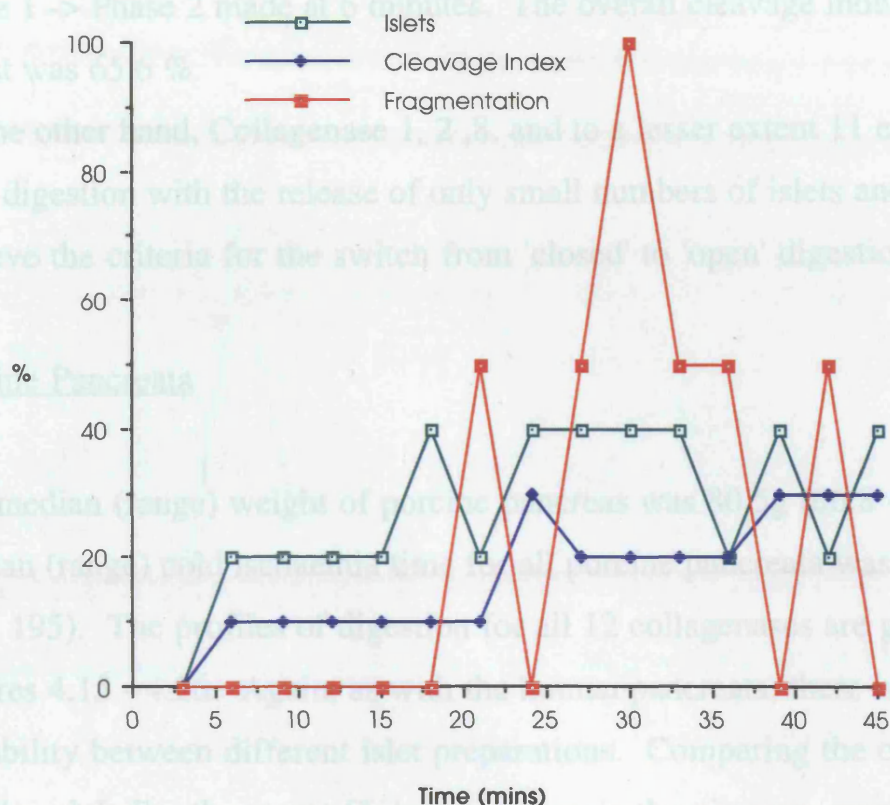


Weight of pancreatic segment = 13.1 g

Cold ischaemia time = 200 mins

Exocrine digestion = moderate

Figure 4.14 Profile for Collagenase 12 (Human)



Weight of pancreatic segment = 15.8 g

Cold ischaemia time = 260 mins

Exocrine digestion = moderate

islets in the second half of digestion, with no fragmentation.

Collagenase 9 was the most powerful enzyme of all, reflected in a very early release of well cleaved islets, but also more fragmentation than the other 'good' enzymes. The digestion was completed at 27 mins and the switch from Phase 1 -> Phase 2 made at 6 minutes. The overall cleavage index of the digest was 65.6 %.

On the other hand, Collagenase 1, 2, 8, and to a lesser extent 11 each had a poor digestion with the release of only small numbers of islets and did not achieve the criteria for the switch from 'closed' to 'open' digestion circuit.

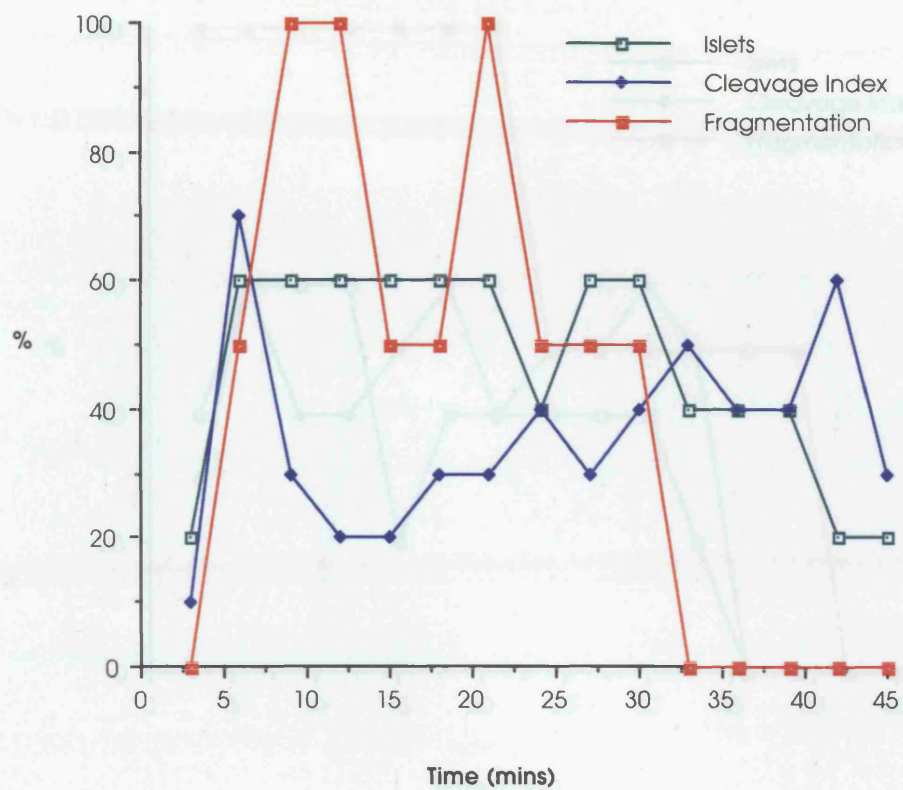
Porcine Pancreata

The median (range) weight of porcine pancreas was 80.5g (66.8 - 120). The median (range) cold ischaemia time for all porcine pancreata was 120 mins (75 - 195). The profiles of digestion for all 12 collagenases are given in Figures 4.15 - 4.26. Again, as with the human pancreata, there is considerable variability between different islet preparations. Comparing the overall profiles globally, the most efficient enzymes in the pig were numbers 1, 2, 6, 7, 8 and 11. The poorest results were obtained using Collagenases 5, 9, and 10.

Comparing this with the human profiles, there are clearly some differences. Collagenase 1, 2 and 11 were all poor in the human, but gave good results when used on the pig. On the other hand Collagenases 5 and 9 were excellent in the human, but disappointing when tested on the porcine pancreas.

Enzymes 6 and 7 worked well in both species. However, it is interesting to note that although the porcine digestion with these two enzymes was associated with very good cleavage indices and numbers of cleaved islets in the pig, this was at the expense of significant fragmentation (even accounting for the fragile nature of porcine islets).

Figure 4.15 Profile for Collagenase 1 (Porcine)

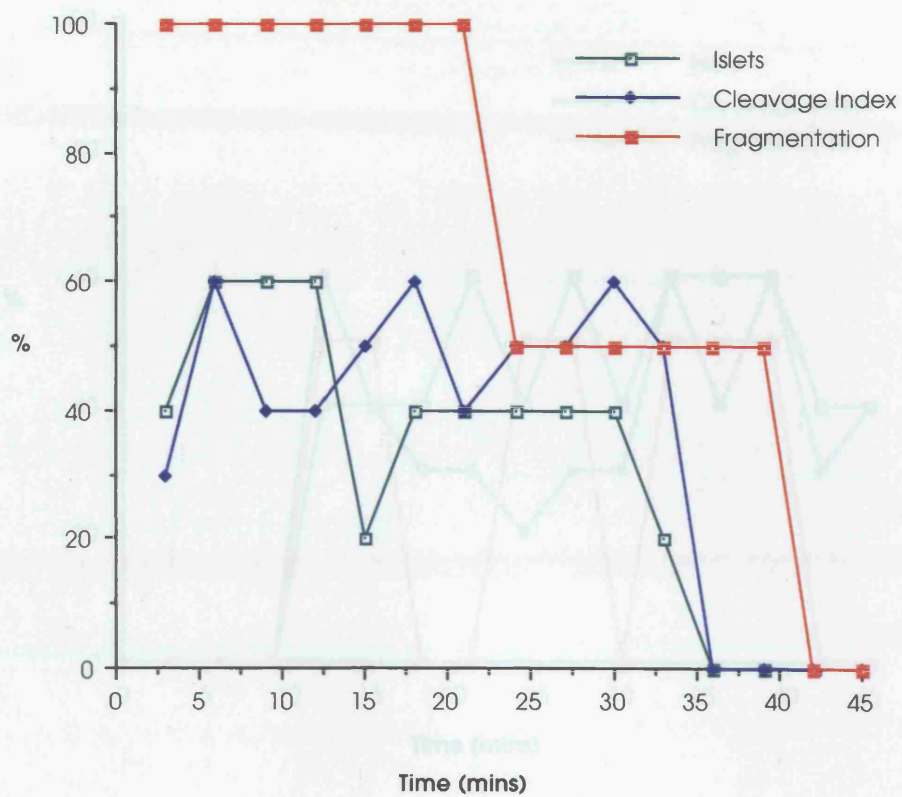


Weight of pancreas = 85.9 g

Cold ischaemia time = 75 mins

Exocrine digestion = good

Figure 4.16 Profile for Collagenase 2 (Porcine)

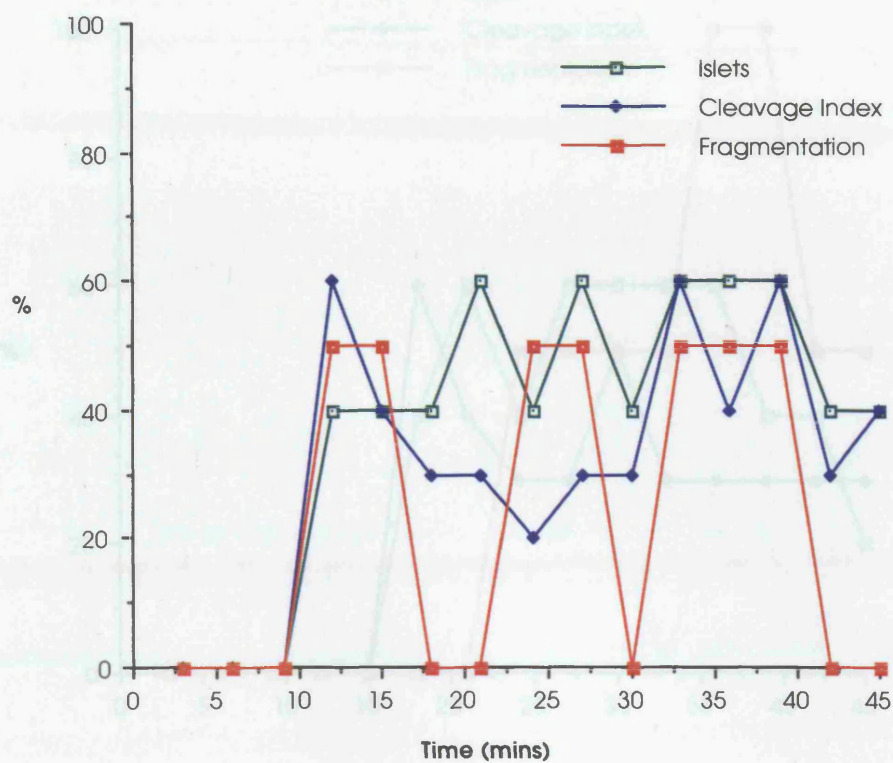


Weight of pancreas = 92 g

Cold ischaemia time = 135 mins

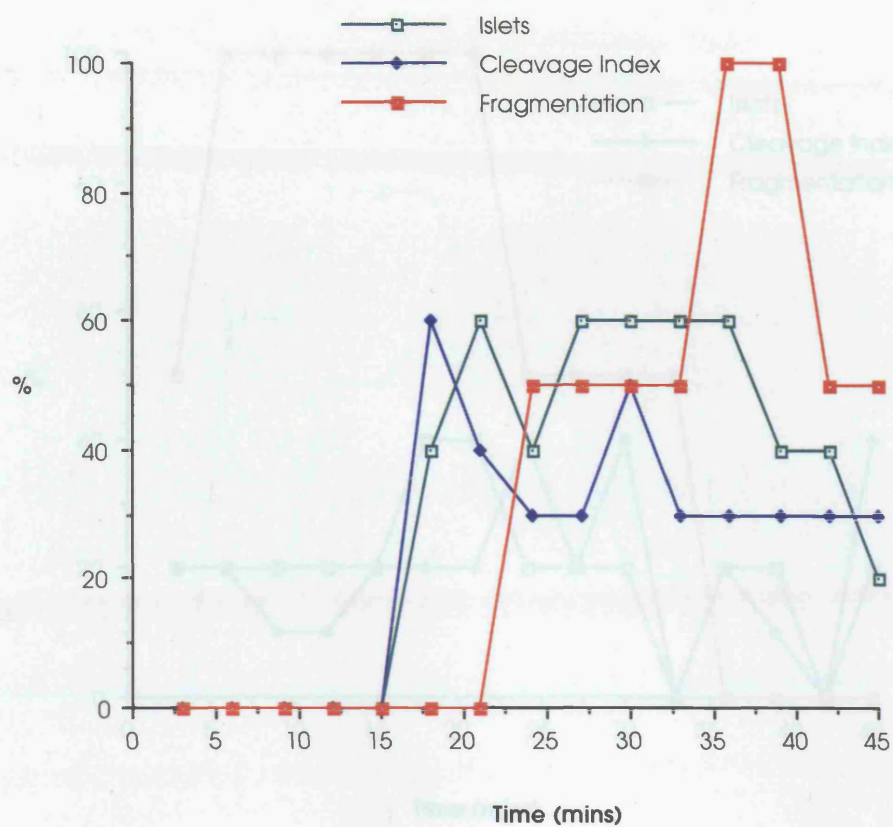
Exocrine digestion = moderate

Figure 4.17 Profile for Collagenase 3 (Porcine)



Weight of pancreas = 80 g
 Cold ischaemia time = 105 mins
 Exocrine digestion = moderate

Figure 4.18 Profile for Collagenase 4 (Porcine)

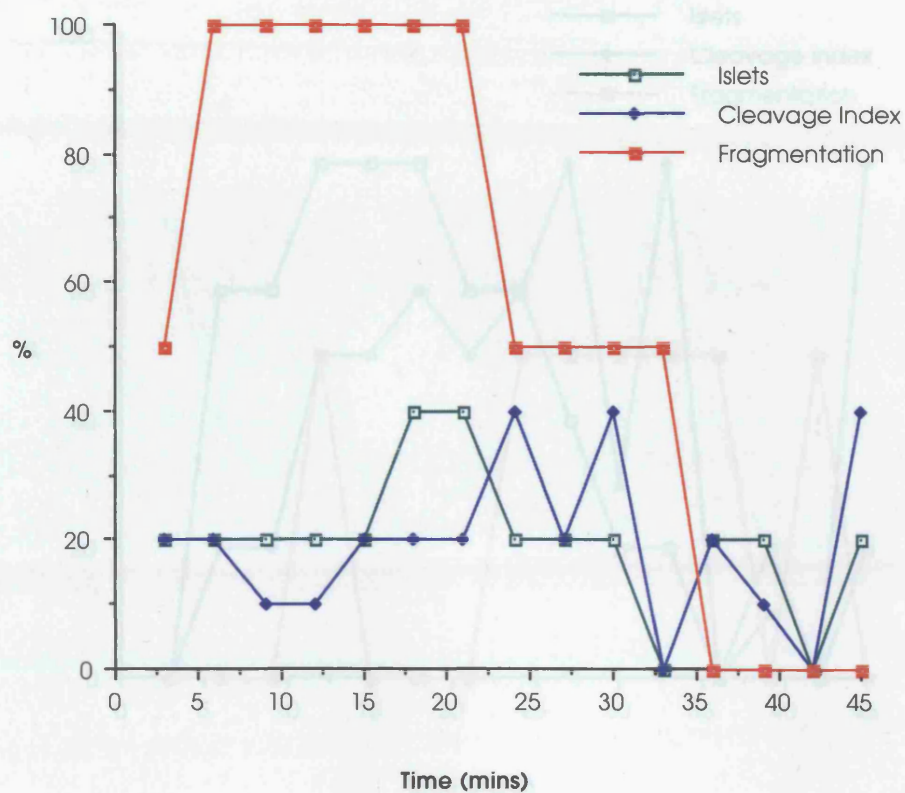


Weight of pancreas = 70 g

Cold ischaemia time = 240 mins

Exocrine digestion = good

Figure 4.19 Profile for Collagenase 5 (Porcine)

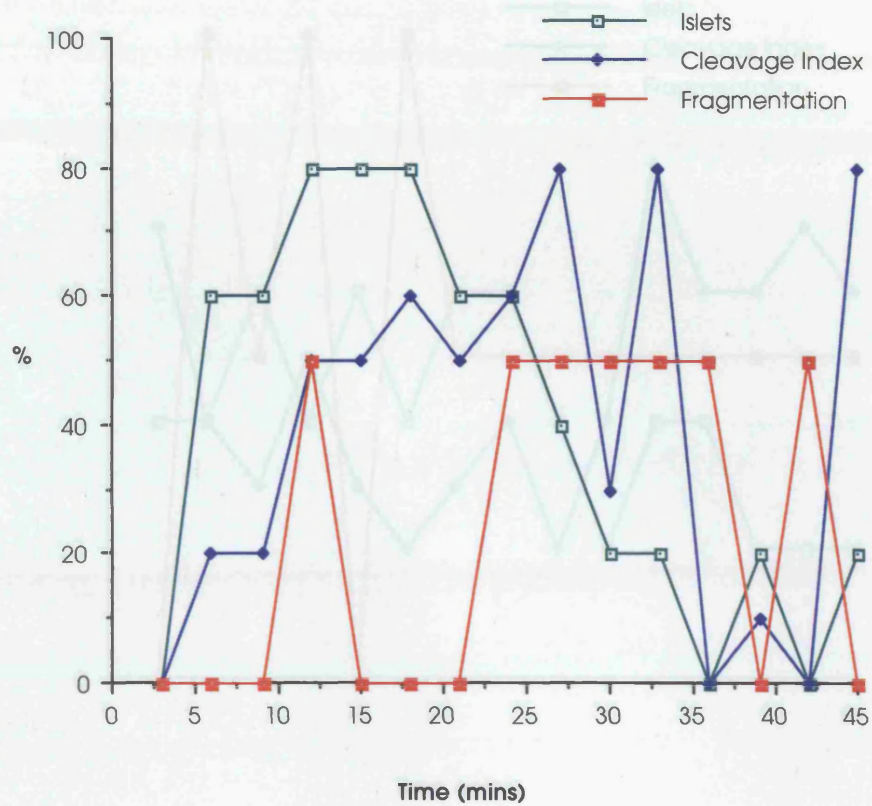


Weight of pancreas = 80 g

Cold ischaemia time = 120 mins

Exocrine digestion = poor

Figure 4.20 Profile for Collagenase 6 (Porcine)

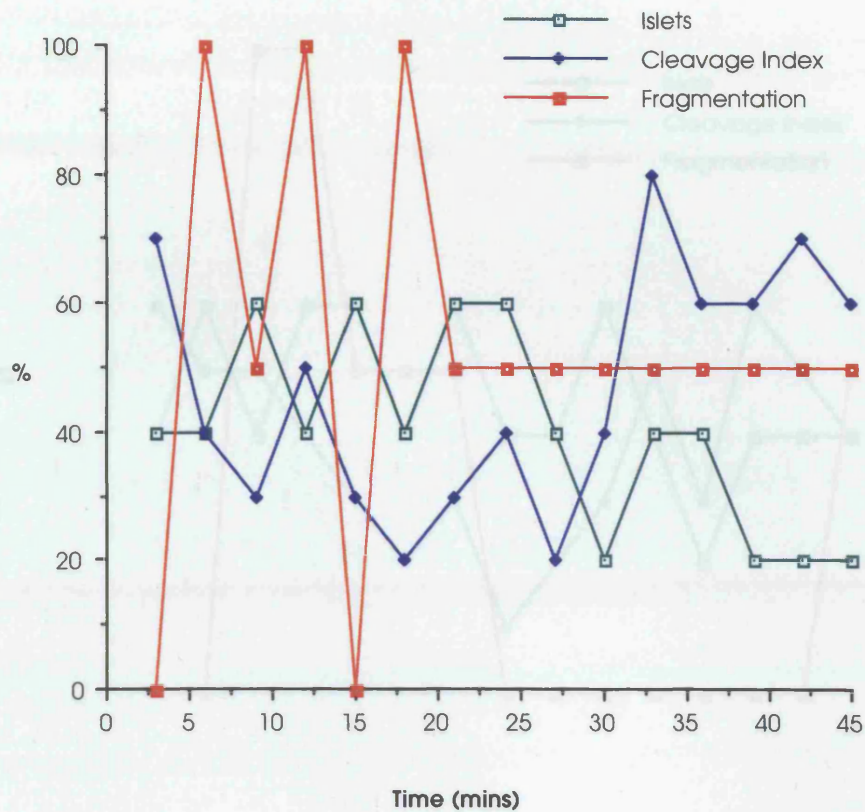


Weight of pancreas = 66.8 g

Cold ischaemia time = 180 mins

Exocrine digestion = very good

Figure 4.21 Profile for Collagenase 7 (Porcine)

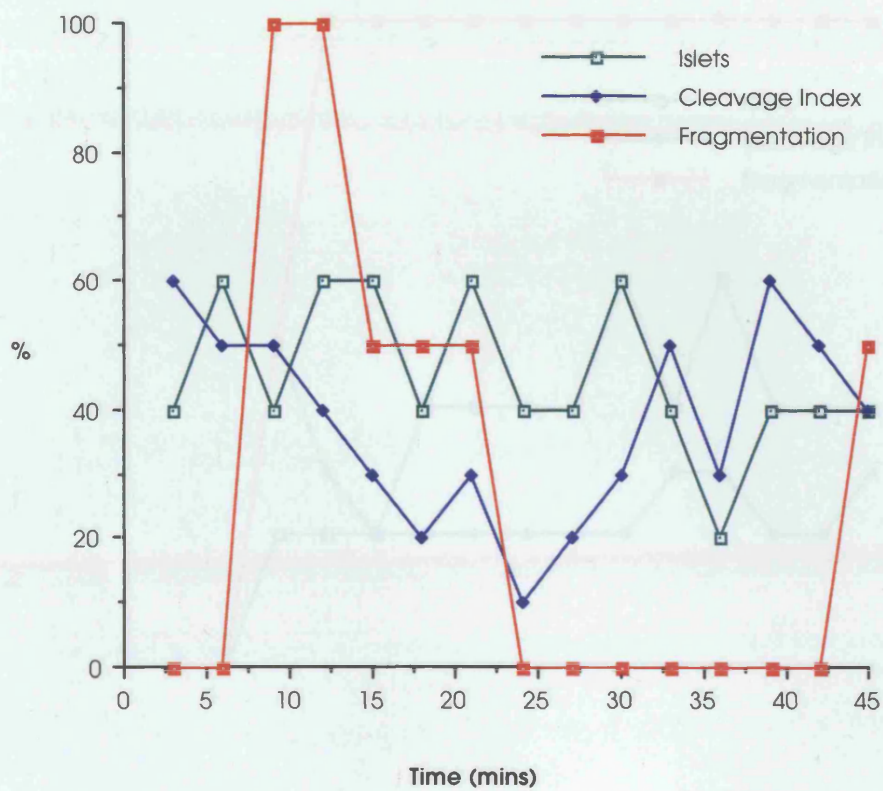


Weight of pancreas = 79.6 g

Cold ischaemia time = 105 mins

Exocrine digestion = moderate

Figure 4.22 Profile for Collagenase 8 (Porcine)

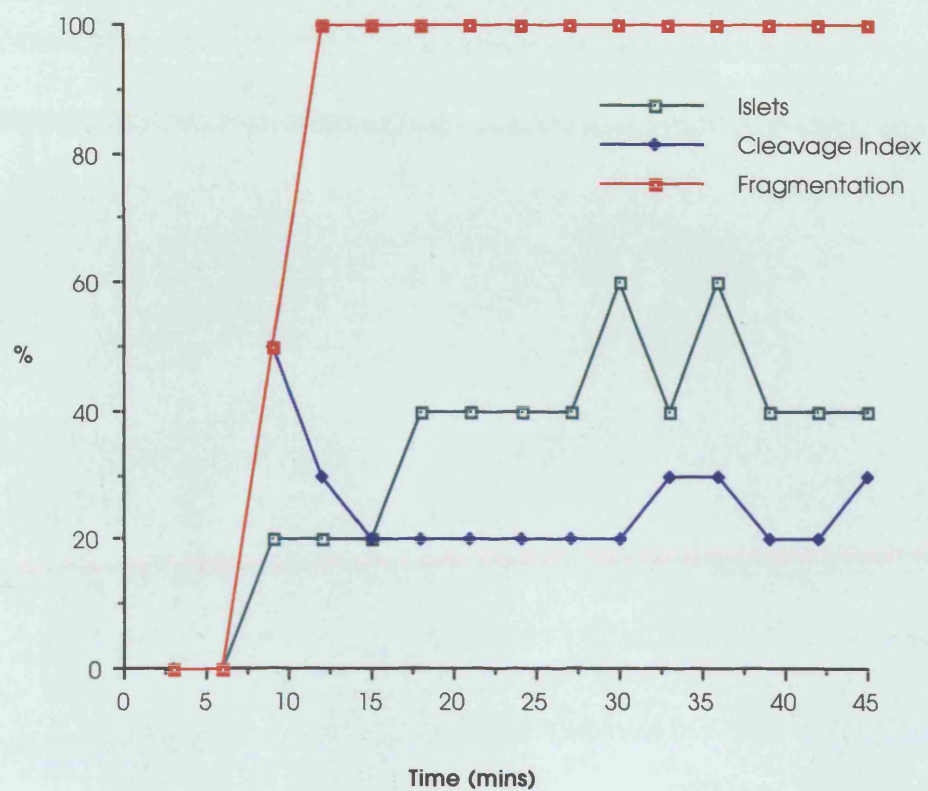


Weight of pancreas = 120 g

Cold ischaemia time = 120 mins

Exocrine digestion = moderate

Figure 4.23 Profile for Collagenase 9 (Porcine)

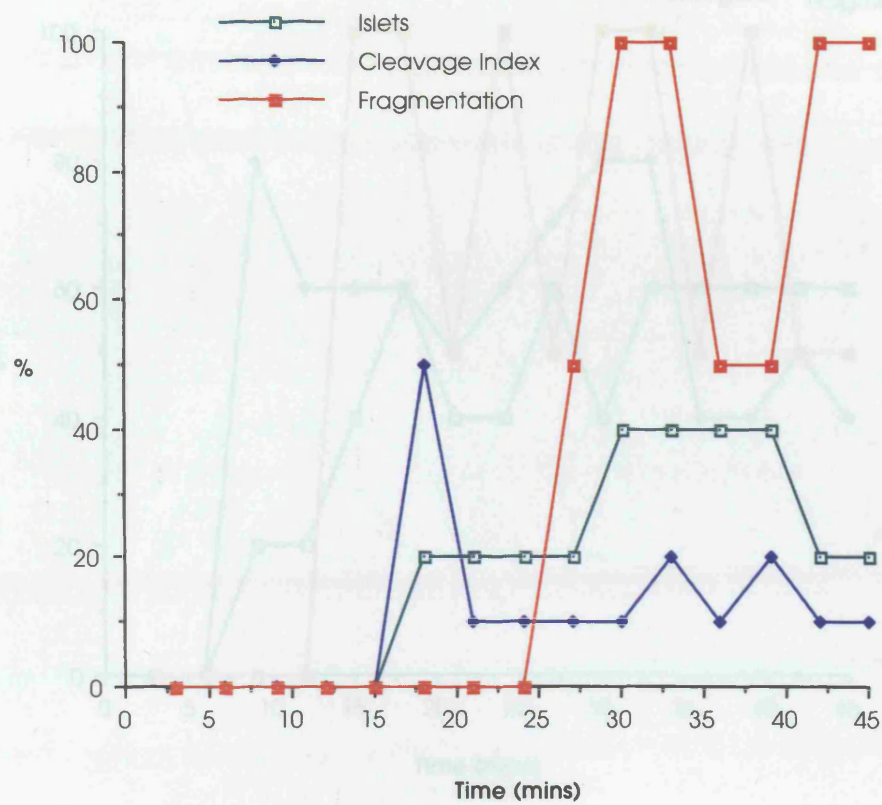


Weight of pancreas = 68 g

Cold ischaemia time = 110 mins

Exocrine digestion = good

Figure 4.24 Profile for Collagenase 10 (Porcine)

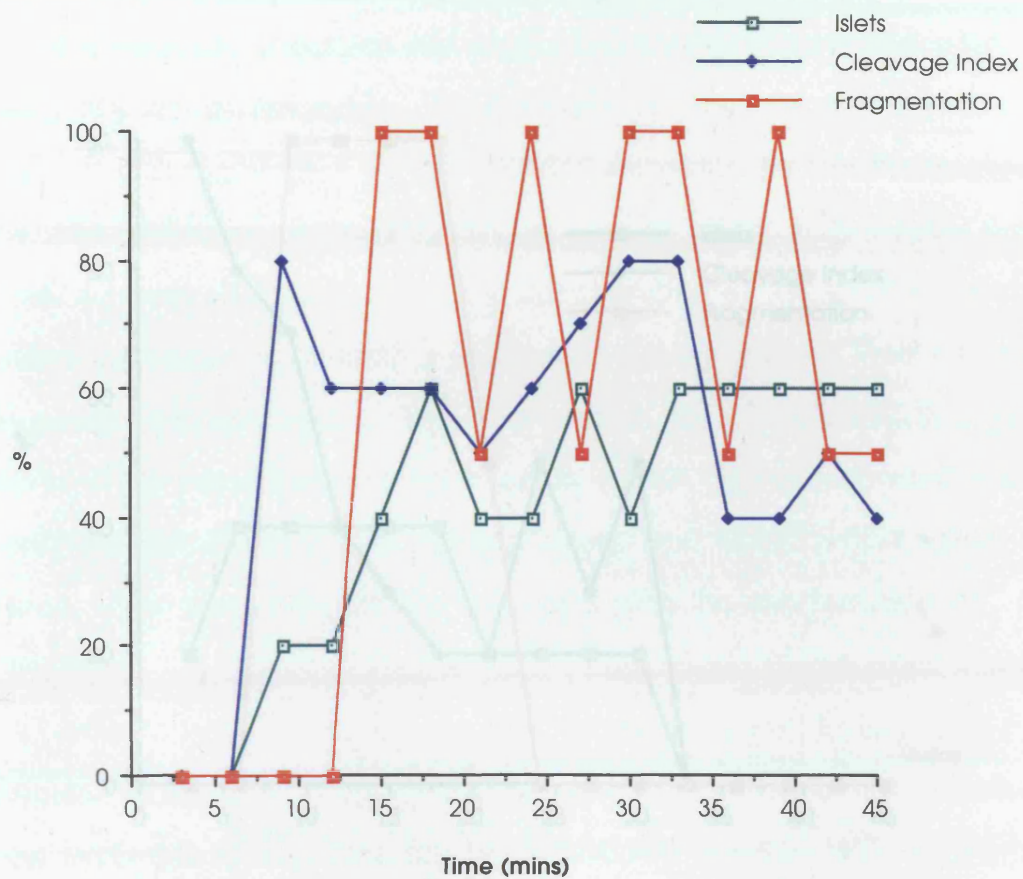


Weight of pancreas = 81 g

Cold ischaemia time = 120 mins

Exocrine digestion = moderate

Figure 4.25 Profile for Collagenase 11 (Porcine)

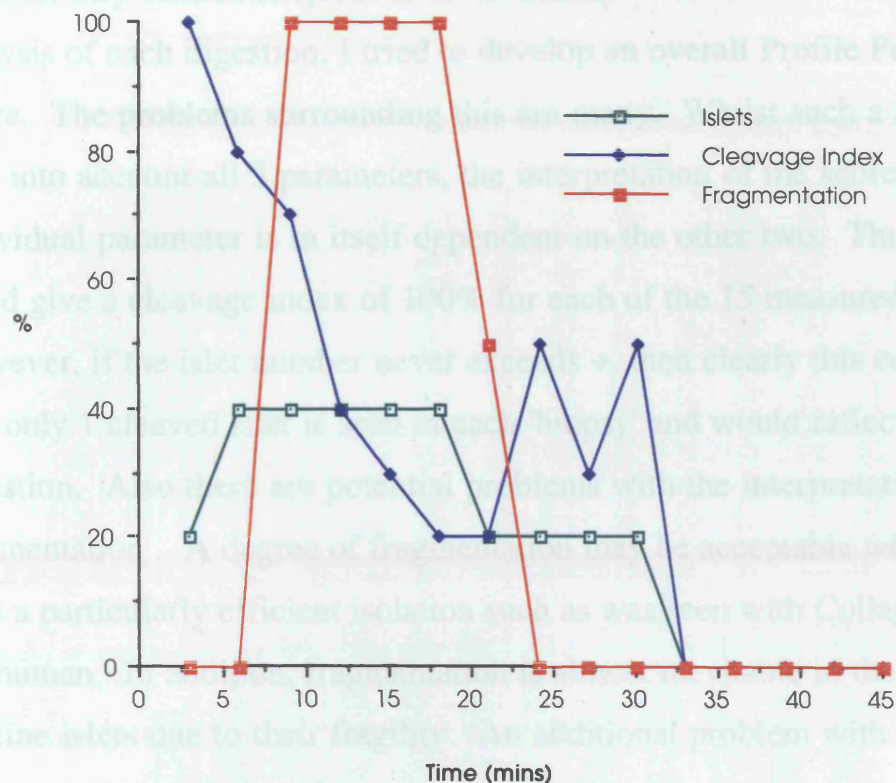


Weight of pancreas = 89 g

Cold ischaemia time = 120 mins

Exocrine digestion = good

Figure 4.26 Profile for Collagenase 12 (Porcine)



Weight of pancreas = 90 g

Cold ischaemia time = 195 mins

Exocrine digestion = poor

Percentage Profile Scores (PPS)

Whilst my concept of the digestion profiles are a useful way of viewing the events of a particular digestion and enable broad comparisons with other profiles, they remain subjective. In an attempt to make a more objective analysis of each digestion, I tried to develop an overall Profile Percentage Score. The problems surrounding this are many. Whilst such a score needs to take into account all 3 parameters, the interpretation of the score for each individual parameter is in itself dependent on the other two. Thus a profile could give a cleavage index of 100% for each of the 15 measured time points. However, if the islet number never exceeds +, then clearly this could mean that only 1 cleaved islet is seen in each 'biopsy' and would reflect a poor digestion. Also there are potential problems with the interpretation of fragmentation. A degree of fragmentation may be acceptable when associated with a particularly efficient isolation such as was seen with Collagenase 5 in the human. In addition, fragmentation is almost inevitable in the isolation of porcine islets due to their fragility. An additional problem with comparing fragmentation with the other parameters is that it is on a narrow scale of 0 -> ++. The percentage leap between + and ++ is therefore 50%, influencing the profile disproportionately more than the other 2 parameters. Finally, the time scale over which the islets appear (rate of islet production) has to be considered.

As outlined previously, islets starting to appear for example after 36 minutes have been subject to considerable warm ischaemia within the isolation chamber and are sub-optimal. In addition, very few islets appeared in any of the profiles within the first 3 minutes. All these points must be taken into account in the development of any mathematical model. This leads to the concept of weighted means.

Firstly, Mean Percentage Scores were calculated for each parameter for each profile. These are outlined graphically for the human and pig in Figures 4.27 and 4.28 respectively. For the reasons stated above, the calculation of each

Figure 4.27 Mean Percentage Scores for Human Profiles

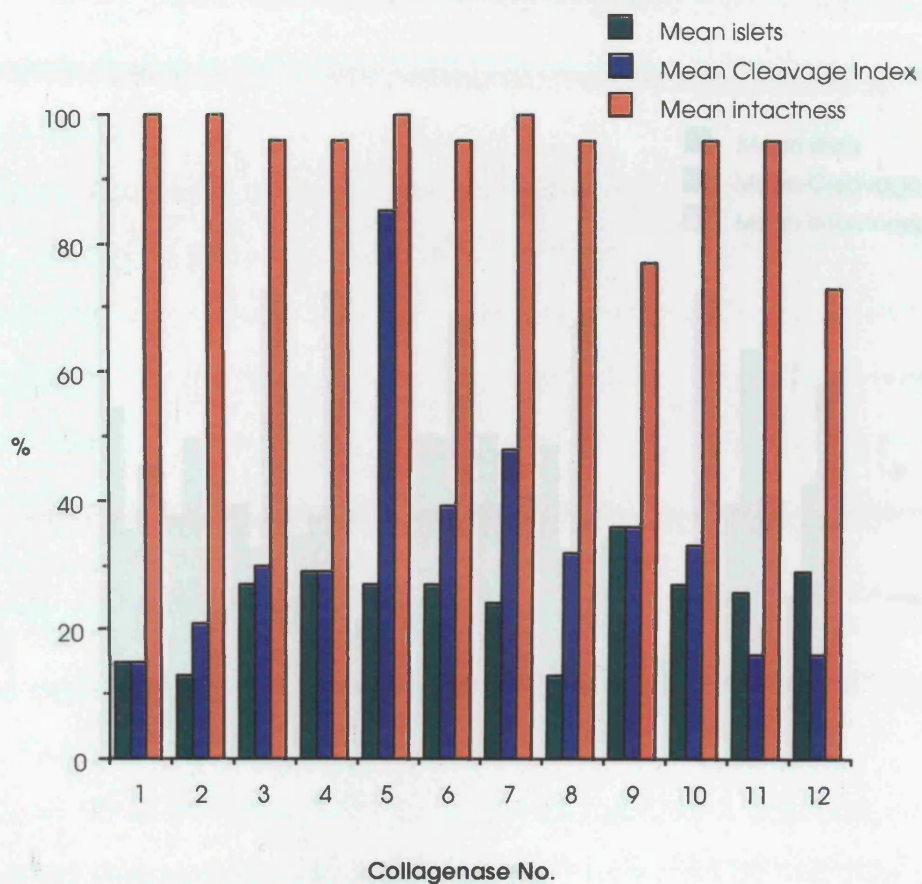
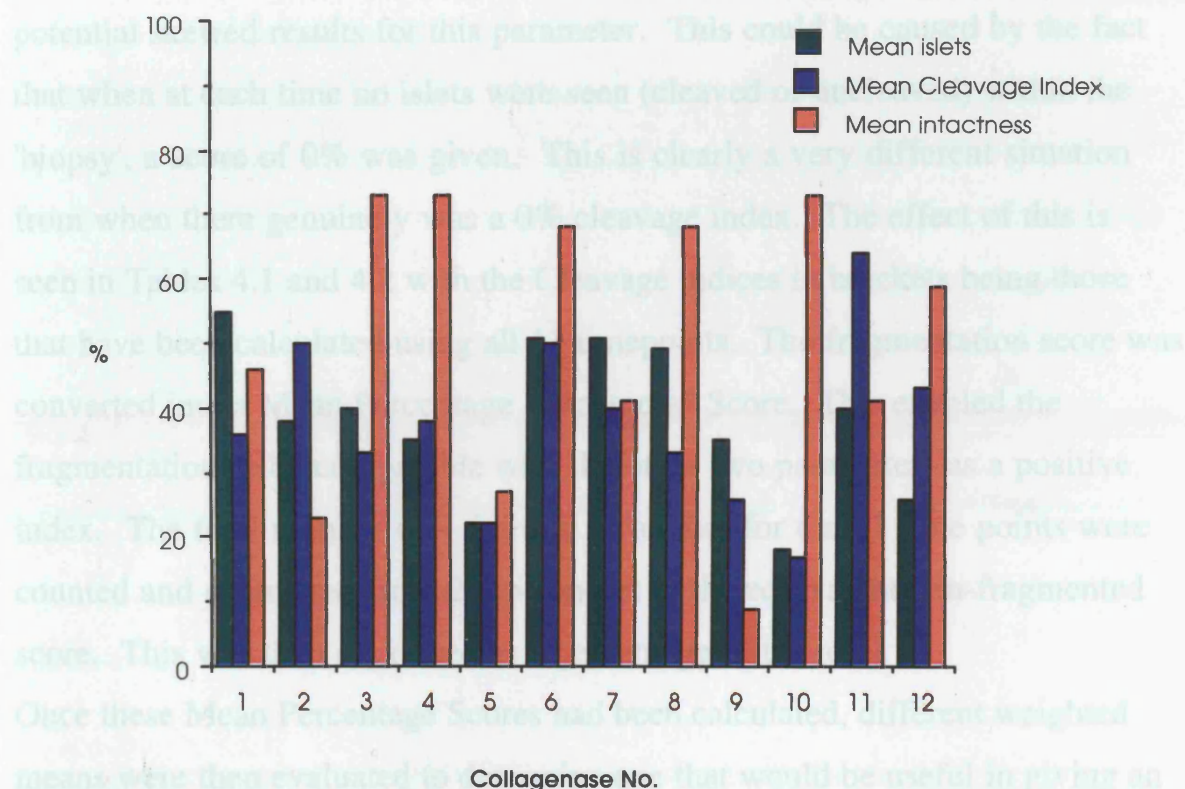


Figure 4.28 Mean Percentage Scores for Porcine Profiles



$$PPS = 10 \times \left(\frac{1}{55} \times 100 \right) + 5 \times \left(\frac{c}{n} \right) + \left(\frac{22-f}{22} \times 100 \right)$$

score was confined to the eleven time points between 6 and 36 minutes which are the time-points of most significance. Each score was simply calculated as an overall percentage of the scores actually achieved during the 11 timepoints compared with the total score possible. For the islet numbers this was simply the total score achieved on the + scale out of 55 expressed as a percentage. For the cleavage index, only those time points at which islets were actually seen were included in the calculation. The rationale for this was to prevent potential skewed results for this parameter. This could be caused by the fact that when at each time no islets were seen (cleaved or uncleaved) within the 'biopsy', a score of 0% was given. This is clearly a very different situation from when there genuinely was a 0% cleavage index. The effect of this is seen in Tables 4.1 and 4.2 with the Cleavage indices in brackets being those that have been calculated using all 11 timepoints. The fragmentation score was converted into a Mean Percentage "intactness" Score. This enabled the fragmentation to be comparable with the other two parameters as a positive index. The total number of + for this parameter for the 11 time points were counted and subtracted from 22 to convert to the equivalent non-fragmented score. This was then expressed as a percentage of the total.

Once these Mean Percentage Scores had been calculated, different weighted means were then evaluated to determine one that would be useful in giving an objective score for digestion profiles. A comparison of weighted means is given in Table 4.1. The weighted mean that gave the most objective score and accounted for the concerns described above was a 10:5:1 score for islets:cleavage:fragmentation in which the cleavage score was calculated only from those time-points in which islets were present rather than the full 11 time-points (see also Table 4 3). To calculate the Profile Percentage Score for each profile therefore, the following equation was used:

$$\text{PPS} = 10 \times \left\{ i / 55 \times 100 \right\} + 5 \times \left\{ c / n \right\} + \left\{ 22 - f / 22 \times 100 \right\}$$

where: i = total number of islet +'s for 11 timepoints
 c = total cleavage index for the n timepoints
 n = number of the 11 time points in which islets were seen
 f = total number of fragmentation +'s for 11 timepoints
 16 = Total weighting of parameters (i.e. 10 + 5 + 1)

The PPS for each of the Collagenases for both human and pig are listed in Tables 4.2..and 4.3 respectively.

In order to confirm the validity of such a score, the original completed porcine proformas were 'blinded' with an A-J system and scored by three independent observers, all of whom were familiar with islet preparations, but unfamiliar with the PPS scoring. Each observer gave each digestion an overall score out of 10, which was then converted to a percentage. The comparison of the Observer Scores and the PPS for the porcine digestions are given in Table 4.4. The final digest Cleavage indices are also included in this table.

'Unblinding' the test Collagenases

Once all the experiments had been completed and all the profiles plotted, the 12 collagenases were 'unblinded'. The Boehringer lot numbers corresponding to each of the test Collagenases are given in Table.4.5. It can be seen that Collagenase numbers 1, 6, 9, and 12 were the same enzyme repeated 4 times and this corresponded to Boehringer lot 52. The good profiles for the human of Collagenases 5, 6, 7 and 9 correspond to B56, B52, B55, and B52 respectively.

Human and Porcine Profiles for B52

The degree of variability of pancreatic digestion is seen clearly when the profiles for the 'control' Collagenase B52 for the two species are reviewed.

Table 4.1 Comparison of Weighted Means for Human Profiles (* = PPS)

<u>Collag No.</u>	<u>1:1:1</u>	<u>2:2:1</u>	<u>3:3:1</u>	<u>5:5:1</u>	<u>5:5:1</u> (cleaved/11)	<u>10:5:1 *</u>	<u>10:5:1</u> (cleaved/11)
1	43.2	31.8	26.9	22.5	20.6	20.0	18.7
2	44.7	33.6	28.9	24.5	21.0	20.9	18.4
3	50.9	42.0	38.2	34.5	35.5	32.4	31.6
4	51.2	43.4	38.6	35.1	35.1	33.3	33.3
5	70.6	64.7	62.2	59.9	59.9	49.7	49.7
6	53.9	45.6	42.0	38.7	34.0	35.2	32.1
7	57.1	48.6	44.9	41.5	37.6	35.9	33.2
8	46.8	37.1	32.9	29.1	26.5	24.0	22.1
9	50.0	44.5	42.2	40.1	35.6	38.9	35.8
10	52.0	43.3	39.6	36.2	33.5	33.4	31.6
11	45.8	35.9	31.6	27.7	27.7	27.0	27.0
12	39.1	32.4	29.5	26.9	26.9	27.6	27.6

Table 4.2 Mean Percentage Scores and Percentage Profile Scores for Human

<u>Collagenase No.</u>	<u>Mean % islets</u>	<u>Mean Cleavage Index</u>	<u>Mean % Non-Fragment.</u>	<u>PPS</u>
1	14.5	15 (10.9)	100	20.0
2	12.7	21.4 (13.6)	100	20.9
3	27.3	30 (27.3)	95.5	32.4
4	29.1	29.1 (29.1)	95.5	33.3
5	27.3	84.5 (84.5)	100	49.7
6	27.3	38.9 (29.1)	95.5	35.2
7	23.6	47.8 (39.1)	100	35.9
8	12.7	32.2 (26.4)	95.5	24.0
9	36.4	36.3 (26.4)	77.3	38.9
10	27.3	33.3 (27.3)	95.5	33.4
11	25.5	16.4 (27.3)	95.5	27.0
12	29.1	15.5 (15.5)	72.7	27.6

Table 4.3 Mean Percentage Scores and Percentage Profile Scores for Pig

<u>Collagenase No.</u>	<u>Mean % islets</u>	<u>Mean Cleavage Index</u>	<u>Mean % Non-Fragment.</u>	<u>PPS</u>
1	54.5	36.4 (36.4)	45.5	48.3
2	38.2	50.0 (45.5)	22.7	40.9
3	40.0	33.3 (30.9)	72.7	40.0
4	34.5	38.0 (24.5)	72.7	38.0
5	21.8	22.0 (20.0)	27.3	22.2
6	50.9	50.0 (45.5)	68.2	51.7
7	50.9	40.0 (40.0)	40.9	46.9
8	49.1	32.7 (32.7)	68.2	45.2
9	34.5	26.0 (23.6)	9.1	30.3
10	18.2	17.1 (10.9)	72.7	21.3
11	40.0	64.0 (58.2)	40.9	47.6
12	25.5	43.3 (35.5)	59.1	33.2

Table 4.4 Comparison of Porcine Observer Scores, Final Digest Cleavage, and PPS

<u>Collag. No.</u>	<u>Observer Scores</u>			<u>Mean Observer %</u>	<u>Digest Cleavage</u>	<u>PPS</u>
	<u>1</u>	<u>2</u>	<u>3</u>			
1	4	7	5	47	23	48.3
2	4	7	5	40	38	40.9
3	4	7	4	40	46	40.0
4	4	5	4	43	39	38.0
5	2	4	2	23	19	22.2
6	7	8	8	77	35	51.7
7	7	7	6	67	34	46.9
8	4	6	5	50	29	45.2
9	2	4	2	23	29	30.3
10	2	2	1	13	21	21.3
11	7	8	4	63	47	47.6
12	3	4	2	30	47	33.2

<u>Test Collagenase No.</u>	<u>Boehringer Lot No.</u>
1	B52
2	B59
3	B53
4	B58
5	B56
6	B52
7	B55
8	B53
9	B52
10	B57
11	B54
12	B52

Table 4.5. The Boehringer 'lot' numbers of the 12 test collagenases

A comparison of the PPS scores for the 4 profiles of B52 (test Collagenases numbers 1, 6, 9, and 12) for both the human and pig are given in Table 4.6.

Discussion

These experiments have demonstrated a number of important points concerning the collagenase digestion of both the human and porcine pancreas. First, it is clear that there is substantial variability in the digestion of the pancreata of both species when different 'lots' of commercially available crude collagenase are used. This is not surprising when one considers what is required in islet isolation, namely the cleaving of an in-tact islet from its surrounding exocrine mantle, compared with the relative crudeness of the way that collagenase is manufactured. Although the criteria for determining a collagenase P is based on fulfilling certain broad biochemical assay criteria, there is substantial variability between different 'lots' of collagenase in terms of efficiency of islet isolation.

Second, these experiments, in particular those involving the repeat collagenase B52, show the enormous variability of the pancreatic substrate itself. Even when the 'lot' of collagenase has been standardised, there are still significant differences in the efficiency of islet isolation between different pancreata and this is seen in both species. It must be pointed out that the collagenase distension of each pancreas / segment of pancreas was similar making it unlikely that these differences can simply be accounted for by differences in collagenase distribution. In addition, although the cold ischaemia times varied, the repeat enzyme B52 was tested on pancreata having a wide range of cold ischaemia and there seemed to be no correlation between a bad digestion profile and a long cold ischaemia time. One of my additional concerns as I planned these experiments was that the increasing cold ischaemic times associated with the method of sequential testing of 4 enzymes on the same

<u>Test Collagenase No</u>	<u>Human PPS</u>	<u>Porcine PPS</u>
1	20.0	48.3
6	35.2	51.7
9	38.9	30.3
12	27.6	33.2

Table 4.6 PPS for the four digestion profiles for B52 in each species

human pancreas could make comparison of an enzyme tested on the 1st segment with another tested on the 4th segment (up to 3 hours longer cold ischaemia) invalid. Although I had already investigated this in initial pilot studies, I further confirmed the validity of the sequential testing method by repeating the testing of some of the enzymes on other human pancreata, but using the inverse anatomical region (and hence the cold ischaemia time) from before. During the second time of testing, no enzyme had a poorer digestion profile when associated with being tested on a more proximal (later) segment. In addition, the digest cleavage index was similar between the two pancreatic digestions for each of the enzymes tested. Of interest is that Collagenase 1 was actually associated with an improved profile when tested on a segment with a longer cold ischaemia time, confirmed by an increased PPS from 20.0 -> 37.5. The interpretation of this is of course difficult, but again supports the suggestion that delayed cold ischaemia times were not deleterious as measured by parameters during the digestion phase. It must of course be noted that all the cold ischaemia times for the human pancreatic segments used in these experiments are significantly shorter than the accepted norms for pancreata used for islet isolation in other islet centres in Europe and North America. The findings also quash another potential criticism of the sequential testing, namely the fact that the islet distribution varies in different regions of the pancreas, being maximal in the pancreatic tail. Not only are these methods not measuring total isolated islet numbers, but also if such a criticism was valid, the poorer preparations would again be expected from the more proximal segments.

The third observation from these experiments is that it is clear that although there is some overlap, human and porcine pancreata have different enzymatic requirements for efficient islet isolation. Again this is to be expected by the different nature of the pancreatic connective tissue matrix and also the islet structure between the two species. This does confirm that the results from research on the components of crude collagenase and porcine islet isolation

cannot be directly applied to the human. Of course due to the shortage of donated human pancreata, there are some aspects of research on islet isolation that can be performed on the pig. In addition, it must be remembered that optimal porcine islet isolation is a prerequisite for porcine xenotransplantation and therefore research on collagenase and porcine islet isolation is required in its own right.

Finally, some comments on methodology. First, while the PPS is a helpful objective score, it must be remembered that it is not an absolute score and must be considered together with the profiles. Second, it could be argued that it would have been better to have a score of 0 - .+++ for the degree of fragmentation. This would have not only have made comparison with the other parameters easier, but also there were several times in which the restriction of a two point scale were realised. Finally, it is clear from these experiments that with all the different variables in the islet isolation process, any studies on the components of collagenase must control for the pancreatic variable. These require methods that enable the testing of numerous enzymes in parallel on the same pancreas.

Conclusions

In summary, the experiments described in this chapter have confirmed first that there is immense variability in the efficiency of both human and porcine islet isolation by different batches of commercially available crude collagenase. Second, these experiments suggest that although there is some overlap, human and porcine pancreata exhibit a species difference in their enzymatic requirements for optimal islet isolation. Third, the influence of the variability of the pancreas itself is clearly demonstrated. This final point means that the development of simple methods of testing a large number of components / crude collagenases on the same pancreas are essential to enable any meaningful

research in this area. This is therefore the subject addressed in the next chapter.

CHAPTER 5

Design of a Simple,"In-vitro" Method for Evaluating the Digestion of the Human and Porcine Pancreas by Crude Collagenase and its Components

Introduction

As highlighted in the last chapter, research into the collagenase digestion of the human or pig pancreas poses novel problems compared to such studies in the rat. Not only does the up scaling of the experiments on collagenase components produce logistical problems, but perhaps more importantly the influence of the pancreatic substrate itself produces significant challenges. Whereas there is little variability between the pancreata of the same strain of laboratory rat, this is not the case for both the human and porcine organs. As discussed in Chapter 3, human pancreata are subjected to a number of different donor variables. These include age, body mass index, past medical history, drug history, length of time on the intensive care unit, blood glucose level prior to death and the social history of the donor. In addition, variables during the procurement / transport of the pancreas such as the type of vascular perfusate used and the length of cold ischaemia, can have a significant influence on the results of islet isolation. Although in the porcine situation it is possible to control the variables more closely, nevertheless inter-pancreatic variation still remains a problem. Due to the financial cost and practical difficulties of keeping pigs in a research institute, most islet laboratories use abattoir-obtained porcine pancreata for islet isolation. Whilst the methods of pancreatic excision and period of cold ischaemia can be kept fairly constant, it is often not possible to standardise for factors such as strain of pig, nutrition, warm and cold ischaemia times etc. In order that true conclusions can be drawn, any studies investigating the role of crude collagenase and its different components on the digestion of the human and porcine pancreas must employ methods that control for this inter-pancreatic variation. In addition, there is a limited supply of human pancreata and there are difficulties in processing consecutive porcine pancreata on a single day because of the fact that abattoir obtained pancreata have not undergone vascular perfusion. It is therefore important that such studies use methods that enable the testing of different batches of collagenase or

collagenase components on any one human or porcine pancreas, thereby providing an internal "control" for each series of experiments. As has already been shown in the last chapter, it is possible in the human to sequentially test up to 4 different crude enzymes or collagenase components on segments of the same pancreas using intraductal collagenase distension and the semi-automated isolation method. However, apart from the fact that this approach is not routinely possible in the pig for the reasons previously described, this method is clearly suboptimal for the detailed individual and combined testing of up to 30 different components of crude collagenase individually and in combination. In addition, in order to eliminate the influence of increasing cold ischaemia, batches or components should be tested concurrently rather than sequentially.

Aims

The overall aim of this study was to design a simple "in-vitro" method using blocks of undistended pancreas, that correlated well with the digestion occurring during the semi-automated method and that would enable many different batches of collagenase or collagenase components to be evaluated simultaneously on a single porcine or human pancreas. Such a method could also potentially be used to reduce the number of pancreata required for the 'batch-testing' of currently available crude collagenases.

Methods

The majority of the studies outlined in this chapter were performed on porcine pancreata for two main reasons. First, they are readily available and these experiments (including the pilot studies) required a significant number of pancreata. Second, once I had designed the methodology and

protocol for these experiments, I established a collaboration with the Department of Surgery in Groningen who had studied the effects of collagenase on the rat pancreas. Groningen do not have access to human pancreata and had recently started porcine islet isolation. In order to establish the reproducibility of these experiments, I performed experiments in their islet isolation laboratory in addition to the laboratory in Leicester (see below). Subsequently, the results of the initial porcine experiments were validated in the human pancreas.

Designing the Experiments

Pilot studies were performed in order to evaluate different approaches to the problem. These included attempting to use a Fogarty embolectomy catheter to catheterise the fine branches of the pancreatic duct and inflating the catheter balloon during the distension with collagenase in order to prevent back flow and effectively section off a region of pancreas enabling different collagenases to be infused into different pancreatic regions. This was tested in both the human and the pig, but in both species the interconnections of the side-branches of the pancreatic duct were so extensive that it was not possible to prevent the mixing of different collagenases.

At that time I was involved in isolating islets for a research project on the ion channel defects in persistent hyperinsulinaemic hypoglycaemic of infancy (nesidioblastosis) ^{37, 516, 517, 518}. The islets were isolated from operative specimens from neonates who had undergone a 95% pancreatectomy. These operations were mainly performed at Great Ormond Street Hospital, London but I also retrieved 2 specimens from Paris and 1 from Munich. Due to the small size of the pancreatic duct it was not possible to cannulate it. I therefore, had to develop methods that enabled islets to be isolated with a combination of pancreatic disruption and incubation in collagenase. These were based on a modification of the techniques described by London et al ¹²³ and involved the following:

Following subtotal pancreatectomy, the pancreas was transported to the Leicester department of surgery in hyperosmolar citrate solution (Marshall's solution) at 4°C, with warm and cold ischaemia times of 4 mins and 1.5 hours respectively. The weighed pancreas was cut into small pieces and placed in a Falcon centrifuge tube containing 30ml of crude *Clostridium histolyticum* collagenase (Boehringer Mannheim type P) dissolved in Hanks' solution in a concentration of 3mg / ml. The tube was placed in a waterbath at 38°C, regularly manually shaken, and incubated for 5 mins. At the end of this time, a 0.5ml sample was taken and stained with diphenylthiocarbazone (Dithizone). The sample was then assessed under the microscope at x40 magnification. Providing 'cleaved' islets were seen, the contents of the tube were then poured into a pre-warmed metallic kidney dish which had been divided into 2 halves by a 1000 µm metallic mesh. By tilting the kidney dish, the solution was passed through this mesh, thereby separating the remaining pancreatic tissue from the digest. The digest was then drawn up into a 50ml syringe using a Universal quill, passed through a 500µm funnel filter, collected into a 500ml glass beaker containing 10% newborn calf serum (NBCS) which both prevents adherence of the islets and also inhibits the collagenase, and then placed on ice. In the meantime, the remaining pancreatic tissue was 'teased' apart using 2 pairs of toothed forceps, replaced in the centrifuge tube in fresh 3mg / ml collagenase solution and again incubated in the waterbath. This process was repeated several times until all the pancreas was digested, with serial dilution of the collagenase to prevent islet fragmentation. If there was significant exocrine contamination, the islets were then purified using a small-scale, Ficoll-based density-gradient system. Once the islets had been isolated, they were placed in CMRL culture medium and placed in a CO₂ incubator at 28°C. Whilst these methods were aimed at maximal yield of islets from the specimens and hence the 'teasing' apart of the pancreatic tissue was an important adjunct, it became clear during these experiments that neonatal islets could be released by simple incubation with collagenase, combined with manual shaking of the incubation tube. It was with this background that I

planned the next series of experiments in order to determine a simple in vitro method for testing collagenases on the porcine pancreas. This involved comparing various different incubation / 'shaking' methods with the digestion occurring within the digestion chamber.

1) COMPARISON OF DIFFERENT 'IN VITRO' METHODS USING PORCINE PANCREATA

Four different "in-vitro" methods were evaluated simultaneously and compared with the semi-automated method of digestion. The 4 methods were as follows:

i) Undistended x 1 method

In this method, undistended blocks of pancreas were incubated in collagenase at the same concentration as that used during the semi-automated method, which for porcine pancreata was 1mg / ml.

ii) Undistended x 4 method

In this method, undistended blocks of pancreas were incubated in a collagenase solution which had a concentration 4 times that usually used during the semi-automated method.

iii) Distended method

In this third method, blocks of pancreas distended with collagenase by intraductal administration, were incubated in Hanks' solution in order to see the influence of collagenase distension of the pancreas.

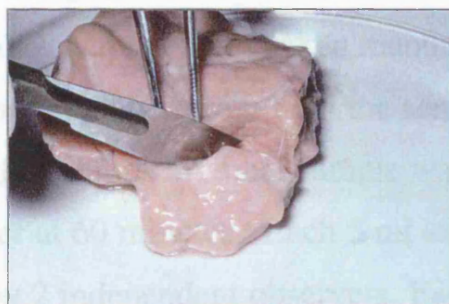
For each of these first 3 methods, a set of 5 tubes, corresponding to one tube for each sampling time point, was incubated simultaneously (the serial method of sampling).

iv) Undistended-repeated method

Finally, in this method, an undistended block of pancreas was incubated in collagenase at 1x concentration and placed in a single centrifuge tube. At each sampling time point, samples were taken from the same tube. After each sample was taken, 5ml of warm Hanks' solution was added to the tube in order to keep the volume constant (the repeated method of sampling). The aim of this method was to evaluate sampling from a closed system in which progressive sampling reflected digestion of the same piece of pancreas.

As stated at the beginning of this Chapter, in order to evaluate these methods in two independent laboratories, I established a collaboration with the Department of Surgery in Groningen, Netherlands. Apart from some minor differences highlighted below, each laboratory used the protocol which I had designed. To ensure conformity the team from Groningen visited our laboratory during the first series of experiments and I went to Groningen for their initial experiments. Porcine pancreata were obtained from a local abattoir with a warm ischaemia time of 5-8 minutes and a cold ischaemia time of 40-50 minutes. These had not undergone 'in-situ' vascular perfusion. The pancreata were transported to the laboratory either in hyperosmolar citrate (Leicester) or Hanks' (Groningen) solutions at 4°C. Blocks of pancreas measuring approximately 1.5 cm³ were taken from the splenic lobe of the pancreas and placed into 50ml centrifuge tubes (see Figure 5.1). Crude Collagenase (Boehringer P in Leicester and Sigma type IX in Groningen) dissolved in Hanks' solution in the test concentration was added to each tube to give a total volume of 20ml. At the same time, the remainder of the splenic lobe of the pancreas was distended with the same batch of collagenase in a

Figure 5.1 Outline of methodology used during 'simple, in vitro method'.



Block of pancreas
taken



Block placed in
universal tube



One experimental
series



Incubation of several
series in waterbath

concentration of 1 mg / ml, distended blocks taken and placed in centrifuge tubes containing 20 ml of Hank's solution. All the tubes were incubated simultaneously in a waterbath at 35°C. The distended pancreas was digested at the same time using the semi-automated method. After 10, 20, 30, and 40 minutes, the tubes were gently shaken manually for 10 seconds and 5ml samples ("biopsies") taken from both the semi-automated circuit and the relevant centrifuge tubes. A final sample was also taken from the tubes, but not the chamber at 60 minutes. Each 5 ml sample was stained with dithizone and assessed by 2 independent observers. Each observer used a scoring system which evaluated the same three parameters as in the previous chapter, but in addition a fourth parameter, namely the quality of the digestion of the exocrine tissue in each 'biopsy' was also scored. The scoring system for each of the 4 parameters was as follows:

First, the *Number of Cleaved Islets*, as with the experiments described in the last chapter was defined as the number of intact islets that were completely separated from exocrine tissue and was expressed using the same scale from 0 -> +++++ in which 0 = no cleaved islets seen, + = 1-5, ++ = 6-10, +++ = 11-15, ++++ = 16-20 and +++++ = >20 cleaved islets.

Second, the *Cleavage index* was again defined as the ratio of cleaved to uncleaved islets seen in each sample and was expressed as a percentage in multiples of ten.

Third, the *Degree of Islet Fragmentation*. In accordance with the comments made in the discussion of the last chapter, the scoring system for this parameter was modified from that used previously. The degree of islet fragmentation was expressed on a scale of 0 -> +++ in which 0 = no fragmentation seen, + = fragmentation present but islets intact, ++ = fragmentation present and a number of islets with irregular edges, and +++ = significant fragmentation with very few intact islets. Again it is to be emphasised that this was expressed as fragmentation during the experiments and in the profiles, but converted to an intactness index (i.e. 1-fragmentation) for the purposes of Profile Percentage Scores.

Fourth, the *Quality of Exocrine digestion*. When comparing the efficiency of pancreatic digestion by different methods, it is useful to assess the exocrine digestion in addition to the islet isolation. The quality of exocrine digestion was expressed on a scale of 0 ->3 in which 0 = no exocrine tissue seen, 1 = fine exocrine tissue seen, typical of that occurring at the beginning and end of pancreatic digestion, 2 = a mixed exocrine digest, and 3 = the presence of large, undigested pieces of exocrine tissue (Figure 5.2).

During the piloting of these experiments, I performed one of these full experiments with the Groningen team present in Leicester in order to ensure that the scoring system was standardised between both centres. A very close correlation of scores was confirmed for all 4 observers (2 from each centre). Using these parameters, for each experiment a profile of digestion over time was established for both the semi-automated and each of the "in-vitro" methods. Each department performed 5 series of experiments.

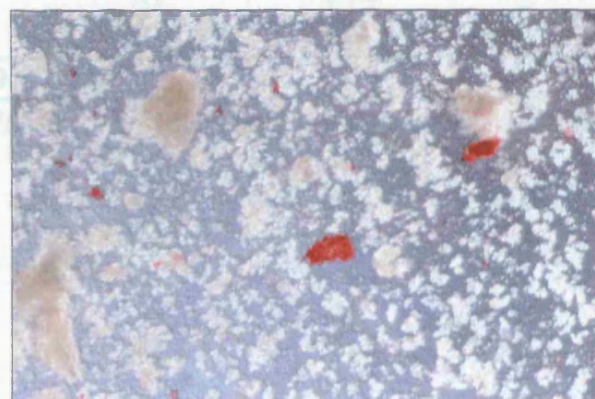
Results

The profiles for the complete set of the experiments performed in Leicester is given in Tables 5.1 to 5.5. These are given in proforma rather than graphic form. Initially, in order to give an objective assessment of whether a particular "in-vitro" method correlated well with the semi-automated method, each of the profiles for the 4 "in-vitro" methods was "blinded" and compared with the profile for the semi-automated method for that particular experiment by 4 independent observers (2 from each laboratory). Each observer gave each "in-vitro" profile a score from 1-10, where 1 meant a very poor correlation and 10 was an exact correlation. It should be reiterated that due to the fact that different methods were being used, the assessment of whether a particular method showed a close correlation or not, could only be made by comparing the whole profile of digestion rather than individual time-points. Table 5.6 shows an example of the results of the "blind" scoring performed by the 4 independent observers for one experiment. It can be seen that the

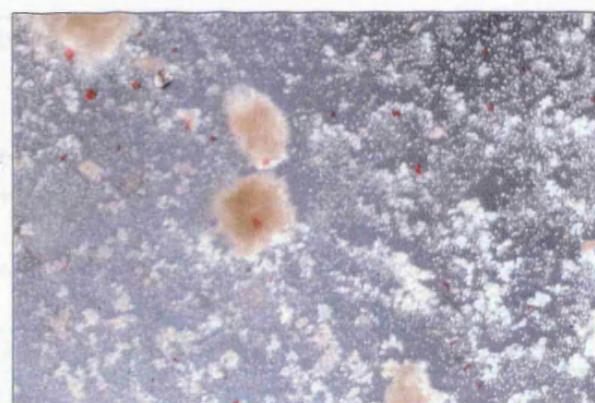
Figure 5.2 Scoring of exocrine tissue



+



++



+++
(large pieces)

Table 5.1 LEICESTER PORCINE PANCREAS 1**CHAMBER**

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	0
10	+++++	80	++	2
20	++++	60	++	2
30	+	30	+++	2
40	++	70	+++	2

UNDISTENDED x1

Time	Islets	Cleavage	Fragment	Exocrine
0	+	100	+	2
10	+++	100	+	2
20	++	80	+++	1
30	++	90	+++	2
40	++	70	+++	2
60	+	90	++	2

UNDISTENDED x 4

Time	Islets	Cleavage	Fragment	Exocrine
0	+	100	+	2
10	+	90	++	2
20	0	-	+++	2
30	+++	70	++	2
40	+	80	+++	2
60	+++	80	++	2

DISTENDED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	+	3
10	+++++	50	++	2
20	++++	40	++	3
30	+++	50	+++	2
40	+++++	80	++	2
60	+++	80	+++	2

UNDISTENDED - REPEATED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	+	2
10	0	-	+	1
20	0	-	+	1
30	0	-	+++	2
40	+	100	+++	2
60	0	-	+++	2

Table 5.2 LEICESTER PORCINE PANCREAS 2**CHAMBER**

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	• 0	0
10	+++	90	+	2
20	+++	80	++	2
30	+++	80	++	2
40	+	80	+++	2

UNDISTENDED x1

Time	Islets	Cleavage	Fragment	Exocrine
0	0	0	• +	1
10	0	0	+	1
20	+	100	+	1
30	+	100	+	1
40	0	-	+	1
60	+	100	+++	2

UNDISTENDED x4

Time	Islets	Cleavage	Fragment	Exocrine
0	0	0	• +	1
10	++	90	0	1
20	++++	70	++	2
30	+++++	80	++	2
40	++++	70	++	2
60	++++	90	+++	2

DISTENDED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	• 0	0
10	0	0	+	1
20	+	100	+	2
30	++	90	++	2
40	+++	90	++	2
60	+	50	+++	2

UNDISTENDED - REPEATED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	• 0	0
10	0	-	+	1
20	+	100	+	1
30	+	100	++	2
40	+	100	+++	2
60	0	-	+++	2

Table 5.3 LEICESTER PORCINE PANCREAS 3**CHAMBER**

Time	Islets	Cleavage	Fragment	Exocrine
0			.	
10	++++	40	0	2
20	+++	30	++	2
30	++	50	+	2
40	+	50	++	2

UNDISTENDED x1

Time	Islets	Cleavage	Fragment	Exocrine
0			.	
10	0	-	+	1
20	+	90	+	1
30	+	90	+	2
40	0	-	+	2
60	++	50	+	2

UNDISTENDED x4

Time	Islets	Cleavage	Fragment	Exocrine
0			.	
10	0	-	+	1
20	0	-	+	1
30	++	80	+	2
40	0	-	+	2
60	0	-	+	2

DISTENDED

Time	Islets	Cleavage	Fragment	Exocrine
0			.	
10	++++	50	+	2
20	++	80	+	1
30	+++	80	++	2
40	+++	80	++	2
60	++	90	+	2

UNDISTENDED - REPEATED

Time	Islets	Cleavage	Fragment	Exocrine
0			.	
10	0	-	+	1
20	0	-	+	2
30	0	-	+	2
40	0	-	+	2
60	+	100	++	2

Table 5.4 LEICESTER PORCINE PANCREAS 4**CHAMBER**

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	1
10	+	100	0	2
20	++++	60	+	2
30	+++++	60	++	2
40	++	40	+	2

UNDISTENDED x1

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	1
10	0	-	0	1
20	0	-	0	1
30	+	50	+	1
40	+	40	++	2
60	++	40	++	2

UNDISTENDED x4

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	1
10	++	70	0	2
20	+++	50	++	2
30	+++++	60	++	3
40	++	40	+++	2
60	++	50	+++	2

DISTENDED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	2
10	++	70	0	2
20	+++	50	++	3
30	++++	50	++	2
40	++	40	++	2
60	++	50	+++	2

UNDISTENDED - REPEATED

Time	Islets	Cleavage	Fragment	Exocrine
0	0	-	0	1
10	0	-	0	1
20	0	-	+	1
30	+	50	++	1
40	+++	60	+	2
60	+++	50	+	3

Table 5.5 LEICESTER PORCINE PANCREAS 5

CHAMBER

Time	Islets	Cleavage	Fragment	Exocrine
			.	
0	0	-	0	+
10	++++	90	+	2
20	+++	80	++	2
30	++	80	+	2
40	++	90	0	2

UNDISTENDED x1

Time	Islets	Cleavage	Fragment	Exocrine
			.	
0	0	-	0	1
10	0	-	0	1
20	++	80	+	2
30	++	90	(+)	2
40	+++	90	+	3
60	+++	90	++	2

UNDISTENDED x4

Time	Islets	Cleavage	Fragment	Exocrine
			.	
0	+	100	0	+
10	+	50	0	2
20	++	100	++	2
30	+++	90	++	2
40	++++	90	+	2
60	+++++	90	+	2

DISTENDED

Time	Islets	Cleavage	Fragment	Exocrine
			.	
0	+	90	+	++
10	+++	90	+	2
20	+++	50	+	3
30	+++++	70	+	2
40	+++++	70	+	2
60	+++++	70	++	2

UNDISTENDED - REPEATED

Time	Islets	Cleavage	Fragment	Exocrine
			.	
0	0	-	+	1
10	+	100	+	2
20	++	50	+	2
30	+++	90	+	2
40	+++	80	++	2
60	+++	60	+++	2

	I	II	III	IV
A	8	8	7	8
B	3	3	3	3
C	6	5	6	5
D	3	3	3	3

Table 5.6. Results of the 'blind" correlation scoring for a single experiment. The 4 different "in-vitro" methods are coded A-D, while I-IV at the top of each column correspond to the 4 independent observers. The closer the score is to 10, the better the correlation with the semi-automated method.

scoring was comparable for each of the 4 observers. A mean score was then calculated for each profile. The mean for each "in-vitro" method from all the experiments (n=10) was then used to calculate a median value for each of the 4 "in-vitro" methods. These median values together with the ranges are shown in Table 5.7.

Although the method using distended blocks of pancreas most closely correlated with the digestion occurring during the semi-automated method (median=7), as highlighted in the introduction to this chapter, this method cannot be used for assessing large numbers of collagenase batches or collagenase components. However, the method using undistended blocks incubated in 4x collagenase concentration also showed a good correlation (median=6). This was judged to be better than the method using undistended blocks incubated in 1x collagenase concentration (median=5). The method using repeated sampling from the same tube showed a poor correlation (median=4).

These results were further analysed using the Mean Percentage Scores for each parameter and also a modified Percentage Profile Score (PPS) calculated for each profile. These results are given in Tables 5.8 and 5.9. The modification of the PPS is due to the reduced timepoints in these experiments (only 10 - 40 mins included in calculations to enable comparison with chamber). It involves the cleavage index being a mean of all time points, rather than just those with islets present. This prevents the bias introduced by having the potential for a single cleaved islet to give the whole profile a mean of 100%. The exocrine digestion is noted in the original profiles, but is not included in the Mean Profile Score or PPS calculations as the scale is not a linear + -> +++, but rather a score where both + and +++ are suboptimal and the ideal digestion is reflected in a ++. It is significant however, that it was possible to obtain the ++ in many of the 'in vitro' profiles.

It can be seen from a comparison of the data, that the PPS scores support the 'blinded' assessments in confirming that the 'in vitro' method involving distended pancreatic blocks was the one most comparable with that occurring

"In-vitro" Method	Range	Median
Undistended x1	2 - 7	5
Undistended x4	2 - 8	6
Distended	6 - 8	7
Repeated	1 - 7	4

Table 5.7. The median values and ranges for the 'blind' scoring of each of the 4 "in-vitro" method (n=10)

	Islets	Cleavage	Fragment.	PPS
Chamber	55	60	33	55.2
Undistended x1	45	85	17	55.8
Undistended x4	25	60	17	35.0
Distended	85	55	25	72.0
Repeated	5	25	33	13.0
Chamber	50	83	33	59.3
Undistended x1	10	50	67	26.0
Undistended x4	75	78	50	74.4
Distended	30	70	50	43.8
Repeated	20	75	42	38.6
Chamber	50	43	58	48.3
Undistended x1	10	45	67	24.5
Undistended x4	10	20	67	17.0
Distended	60	73	50	63.4
Repeated	0	0	67	4.2
Chamber	60	65	67	62.0
Undistended x1	10	45	67	18.1
Undistended x4	60	55	42	57.3
Distended	55	53	50	54.1
Repeated	20	28	67	25.4
Chamber	55	85	67	65.0
Undistended x1	50	65	75	56.0
Undistended x4	50	83	58	61.0
Distended	80	70	67	76.0
Repeated	45	80	58	56.8

Table 5.8. Mean Percentage Scores for each parameter and modified PPS for the 5 Leicester porcine digestion profiles.

		Islets	Cleavage	Fragment.	PPS
Chamber		65	63	50	63.4
Undistended	x1	5	25	83	16.1
Undistended	x4	25	68	58	40.5
Distended		100	48	75	82.2
Repeated		15	60	42	30.8
Chamber		80	63	58	73.3
Undistended	x1	25	75	50	42.2
Undistended	x4	65	85	33	69.3
Distended		100	90	33	92.7
Repeated		25	68	50	40.0
Chamber		55	23	75	46.3
Undistended	x1	5	25	83	16.1
Undistended	x4	0	0	0	0
Distended		95	35	42	72.9
Repeated		5	25	92	16.7
Chamber		70	70	58	69.3
Undistended	x1	5	25	83	16.1
Undistended	x4	10	25	83	25.5
Distended		90	88	50	86.9
Repeated		10	25	83	19.3
Chamber		70	80	75	73.4
Undistended	x1	75	75	75	75.0
Undistended	x4	75	98	75	82.2
Distended		100	95	75	96.9
Repeated		35	75	50	48.4

Table 5.9 . Mean Percentage Scores for each parameter and modified PPS for the 5 Groningen porcine digestion profiles.

within the digestion chamber. Overall the undistended x4 method gave a better correlation than the undistended x1 method, even though one of the experiments showed no digestion in the undistended x4 tube during the course of the whole experiment. The most inconsistent method was the repeated sampling method. It is interesting to note that as one might expect, the profiles were more delayed in the 'in vitro' methods (other than the distended blocks) than in the chamber. Indeed, the correlation with chamber is closer when the 60 minute time-point is taken into account.

2) VALIDATION WITH HUMAN PANCREATA

Materials and Methods

These experiments were performed in Leicester because the laboratory in Groningen did not have access to human pancreata.

After obtaining appropriate consent, human pancreata were obtained from brain-dead, heart-beating donors. Pancreata were only used from donors between the ages of 30 and 55 years. All the pancreata were retrieved by me in order to standardise the procurement procedure. The vascular perfusate and transport solution was HOC in all cases.

Using blocks of human pancreas, 3 of the 'in-vitro' methods as used in the pig (undistended x1, undistended x4, and distended) were compared with the semi-automated method. The repeated method was excluded because of its inconsistent correlation in the previous experiments. This comparison was performed in 3 human pancreata.

Results

These results are outlined in graphic form in Figures 5.3 - 5.5. Again, only the timepoints from 10 to 40 minutes have been included.

Table 5.10 outlines the Mean Percentage Scores and the modified Percentage Profile Scores for all 3 human pancreata. These results in the human confirm the results in the pig, namely that the most comparable non-distended 'in vitro' method was the undistended x4. However, the distinction between the 4x and the 1x concentration was less marked in the human. As with the porcine studies, the distended method was the most comparable overall.

3) EVALUATION OF FURTHER 'IN VITRO' METHODS IN THE PIG

Although there had been moderate correlation with the undistended blocks of tissue, I wanted to evaluate several other methods in order to see if a closer correlation could be obtained. First, in both the human and the pig, the "in-vitro" method that correlated most closely with the "gold standard" of the semi-automated method, involved the use of distended blocks of pancreas. I therefore decided to determine if it was intraductal distension per se that improved pancreatic digestion or whether it had to be distension with collagenase. Second, further work on the PHHI (nesidioblastosis) pancreata had indicated that islet yields were increased when additional collagenase was injected directly into the parenchyma of the pancreas. With this observation in mind, I decided to evaluate whether parenchymal injection could be used as part of an "in-vitro" method. Finally, I needed to validate a negative control by confirming that merely incubating pancreatic blocks in Hank's solution did not cause pancreatic digestion.

Figure 5.3 Human Pancreas 1

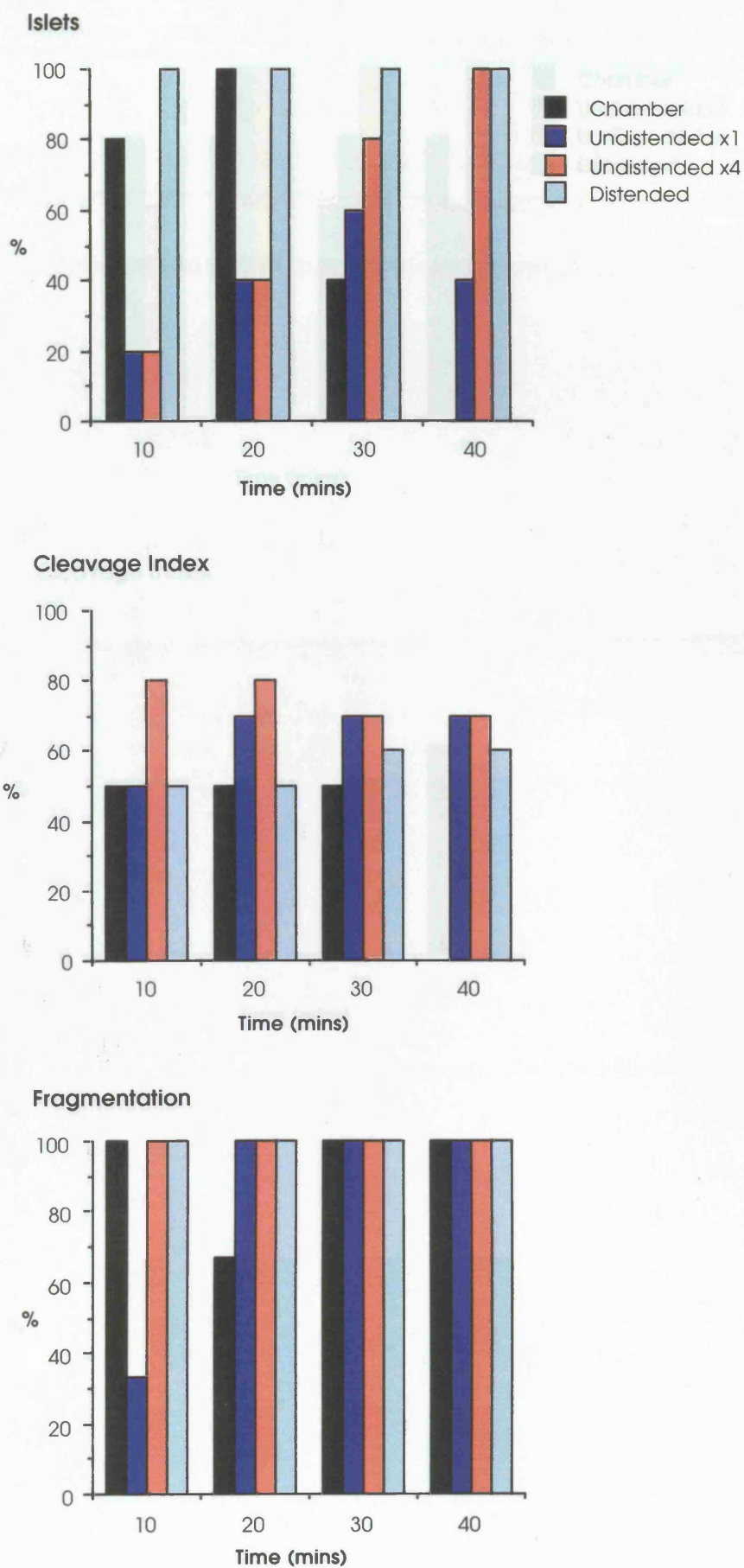


Figure 5.4 **Human Pancreas 2**

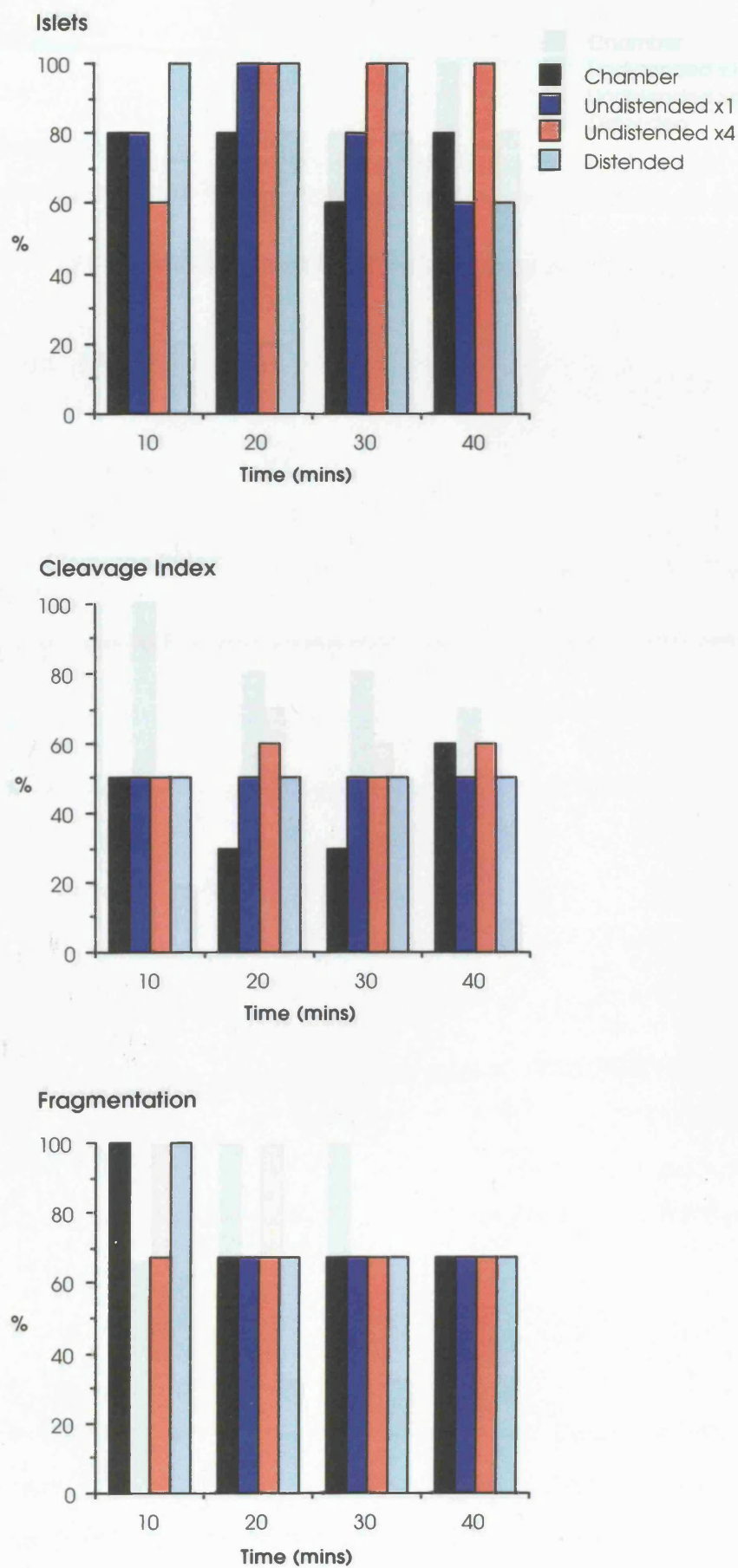
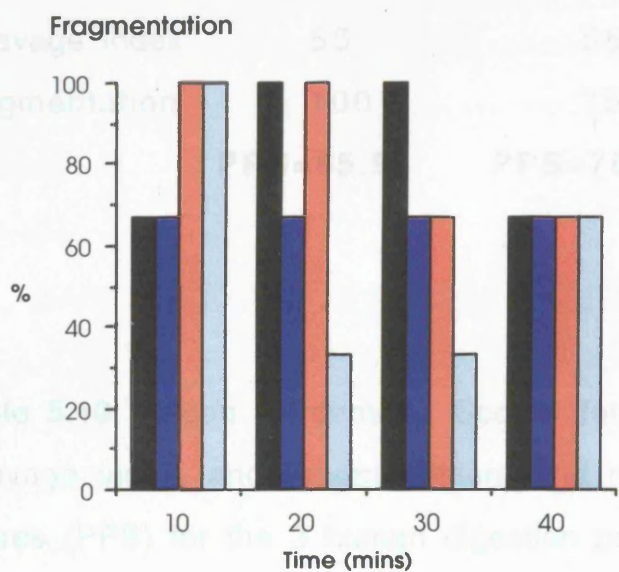
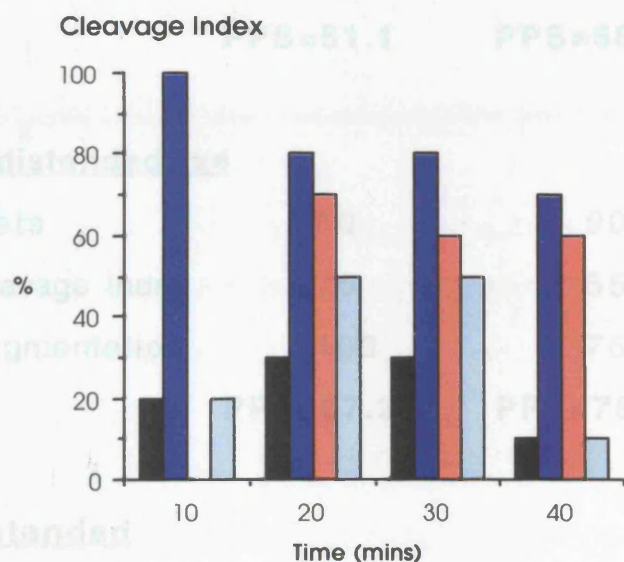
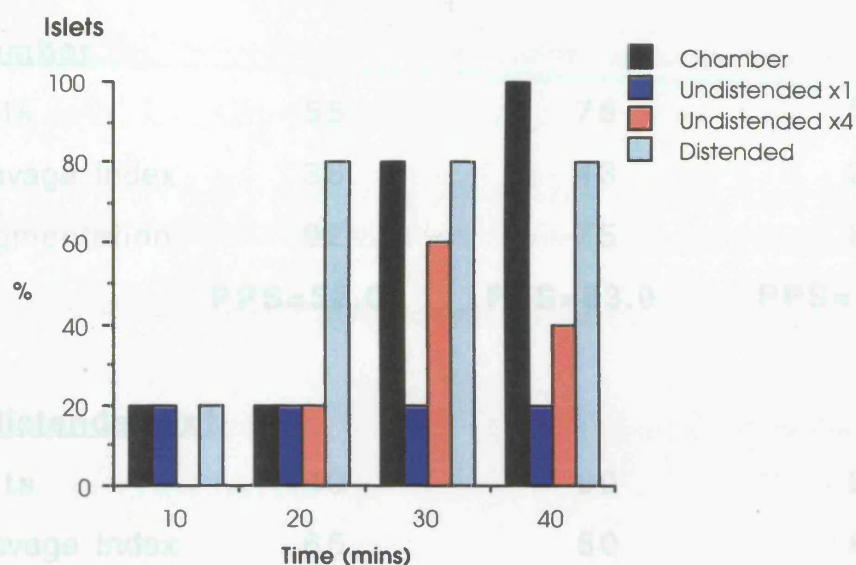


Figure 5.5

Human Pancreas 3



	<u>Pancreas 1</u>	<u>Pancreas 2</u>	<u>Pancreas 3</u>
<u>Chamber</u>			
Islets	55	75	55
Cleavage Index	38	43	23
Fragmentation	92	75	83
	PPS=52.0	PPS=53.0	PPS=46.8
<u>Undistended x1</u>			
Islets	40	80	20
Cleavage Index	65	50	83
Fragmentation	92	50	75
	PPS=51.1	PPS=68.8	PPS=38.4
<u>Undistended x4</u>			
Islets	60	90	30
Cleavage Index	75	55	48
Fragmentation	100	75	83
	PPS=67.2	PPS=78.1	PPS=38.9
<u>Distended</u>			
Islets	100	90	30
Cleavage Index	55	55	33
Fragmentation	100	75	58
	PPS=85.9	PPS=78.1	PPS=54.6

Table 5.10. Mean Percentage Scores for each parameter (islets, cleavage index, and Fragmentation) and modified Percentage Profile Scores (PPS) for the 3 human digestion profiles.

Methods

Using the same methods and scoring system as in the first series of experiments, the following methods were compared with the digestion chamber:

i) Undistended x4

As the method that showed the best correlation of the non-distended methods, this was included as a positive control.

ii) Distended with Hanks / Incubated in Collagenase x4

After undistended blocks of pancreas had been taken from the most proximal part of the pancreas, the next most proximal part of the pancreas was excised and cannulated with occlusion of the distal cut end of the duct. This portion of pancreas was then distended with Hank's solution. Blocks were cut from this distended portion of pancreas and incubated in collagenase in a concentration of 4 mg / ml.

iii) Distended with Hank's / Incubated in Hank's

Blocks of pancreas were taken from the portion of pancreas that had been distended with Hank's (see ii), but were incubated in Hank's only rather than collagenase.

iv) Intraparenchymal injection of collagenase incubated in Hank's

Using a hypodermic needle, blocks of undistended pancreas were injected with collagenase prior to incubation. The concentration of the collagenase was 1mg / ml.

These experiments were performed on 5 porcine pancreata.

Results

The Mean Percentage Scores for all three parameters together with the modified Percentage Profile Score for the 5 different experiments are given in Table 5.11. There are several important findings. Firstly, the pancreatic blocks distended with Hank's and incubated in Collagenase x4 did not show better digestion than the undistended blocks in the same concentration of collagenase. This suggests that distension alone does not improve pancreatic digestion. This was further supported by the second finding, namely that no significant digestion was seen with blocks of pancreas that have been distended in Hank's solution alone and are then incubated in Hank's alone. Finally, it can be seen from the data that the intraparenchymal injection method was very unpredictable and again did not exhibit an advantage over the undistended blocks.

4) TESTING THE 'IN VITRO' METHOD ON CRUDE COLLAGENASE AND COLLAGENASE COMPONENTS

With the data from the previous experiments in mind, the aim of these final series of experiments was to test the 'in vitro' method using both crude and purified collagenase.

Methods

Crude collagenase (Sigma X1 lot 40H-6804) was purified by gel-filtration on Sephacryl S-200 HR in 50 mmol/l Tris-HCL, 5 mmol/l CaCl₂, pH 7.5 in accordance with previously described methods ⁴⁵⁶.

	Islets	Cleavage	Fragment.	PPS
Chamber	65	93	75	74.4
Undistended x4	65	98	75	75.9
Hank's / Collag x4	70	90	58	75.5
Hank's / Hank's	0	0	0	0
Injected Collag.	5	15	92	5.8
Chamber	65	28	58	53.0
Undistended x4	0	0	0	0
Hank's / Collag x4	15	38	58	24.9
Hank's / Hank's	0	0	0	0
Injected Collag.	0	0	0	0
Chamber	40	53	92	47.3
Undistended x4	25	70	50	40.6
Hank's / Collag x4	40	60	25	45.3
Hank's / Hank's	5	25	83	16.1
Injected Collag.	45	55	75	50
Chamber	55	93	75	68.1
Undistended x4	30	90	58	31.8
Hank's / Collag x4	30	90	58	31.8
Hank's / Hank's	5	25	75	12.8
Injected Collag.	30	35	75	34.4
Chamber	80	70	33	73.9
Undistended x4	25	7.5	42	20.6
Hank's / Collag x4	5	25	58	14.6
Hank's / Hank's	0	0	0	0
Injected Collag.	0	0	0	0

Table 5.11. Mean Percentage Scores for each parameter and modified PPS for the second series of porcine experiments.

The purified collagenase was then analysed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by biochemical assays (see Chapter 6) for Class 1 collagenase (collagen orange), Class 11 collagenase (FALGPA), and neutral protease (casein). These confirmed the presence of Class 1 and Class 11 collagenase within the purified enzyme but the removal of neutral protease. The purified collagenase was not further fractionated into its components. It was then freeze-dried and stored at -25°C. The following 3 combinations of enzyme were evaluated:

i) Crude Collagenase

This was tested at a concentration of 2.5 mg / ml. The main reason for choosing this concentration was that as outlined in Chapter 3, the purification of large amounts of enzyme is outside the capabilities of most academic laboratories. In addition, the concentration of crude collagenase needed to have the same class I and class II collagenase activity as the concentration of purified collagenase. The earlier experiments had shown that crude collagenase concentrations as low as 1mg / ml (although suboptimal) could show islet cleavage within our system and therefore a concentration of 2.5 mg / ml was considered to be acceptable.

ii) Purified Collagenase

This was tested at a concentration of 1.25 mg / ml. This was essentially testing the digestion of an enzyme devoid of neutral protease, which as stated above had the same class I and class II collagenase activity as the crude collagenase.

iii) Purified Collagenase with added Neutral Protease

This further tested the role of neutral protease by recombining it with the purified enzyme. Purified collagenase in a concentration of 1.25

mg / ml was combined with commercially available purified neutral protease (Sigma Blend N) in a concentration of 0.5 mg/ml. This concentration of neutral protease showed the same caseinase activity as the crude preparation.

The 3 combinations of enzymes were tested against themselves within the 'in vitro' system rather than comparing them with the digestion within the semi-automated methods. As with the experiments described previously in this chapter, the test volume within each tube was 20 ml and 5 separate tubes were incubated at 35°C for each enzyme combination, enabling serial sampling at 10, 20, 30, 40, and 60 minute time-points.

Results

Examples of the digestion profiles and bar charts for one complete 'in vitro' experiment are shown graphically in Figures 5.6 - 5.9. In addition, the Mean Percentage Scores for all three parameters together with the modified Percentage Profile Score for the 5 different experiments are given in Table 5.12. It should be noted that because the digestion chamber was not used in these experiments, these figures have been calculated from all 5 timepoints from 10 - 60 minutes, enabling the delayed digestion within the tubes to be included in the analysis.

First, it can be seen that using the 'in vitro' method, it was possible to see good numbers of cleaved islets in most of the profiles, together with an evaluation of the other parameters. This included the exocrine digestion which is not included in the PPS calculation, but which achieved a score of 2 (optimal exocrine digestion) during many of the profiles.

Second, from this limited number of tests, it can be seen that the presence or absence of neutral protease did not appear to influence the digestion of the porcine pancreas.

Figure 5.6. Digestion Profile for Crude Collagenase

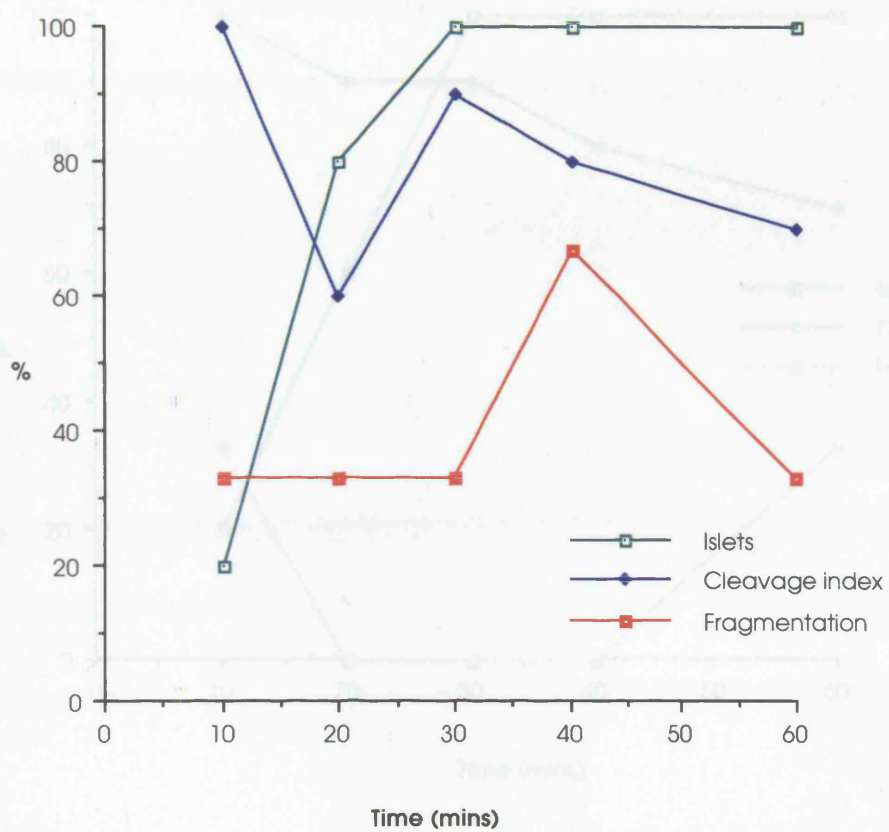


Figure 5.7. Digestion Profile for Purified Collagenase

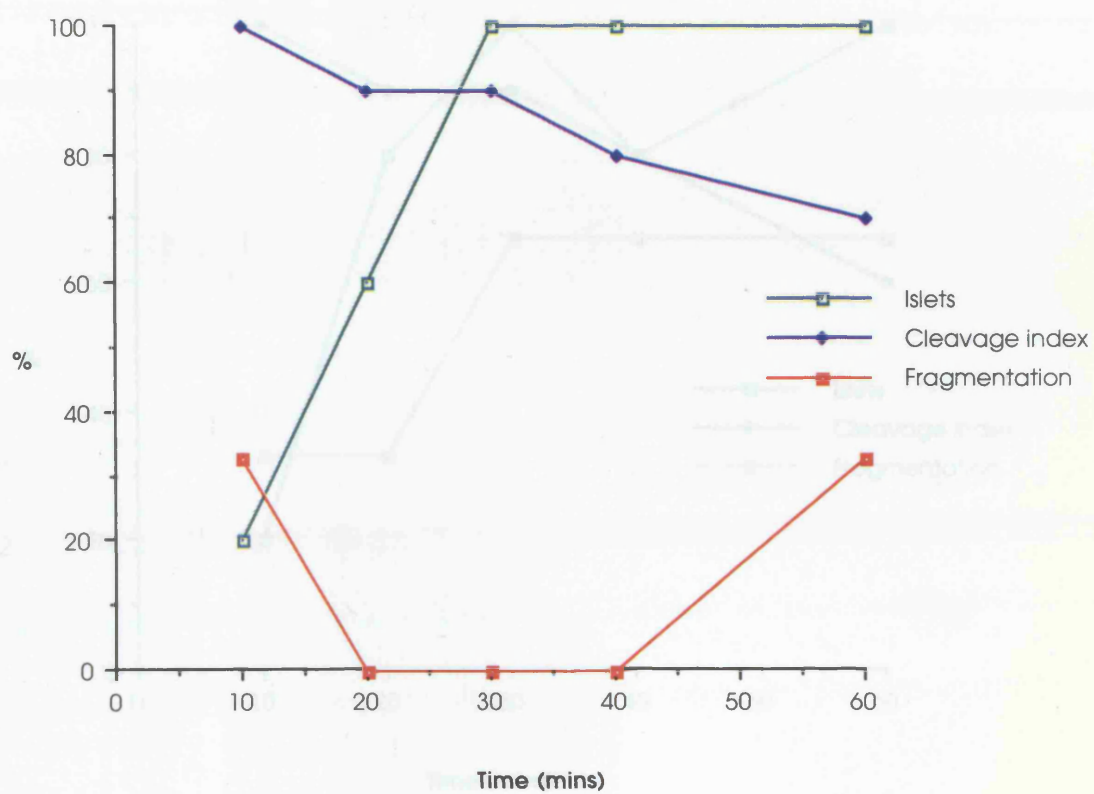


Figure 5.8. Comparison of test outcomes for each parameter

Figure 5.8. Digestion Profile for Purified Collagenase + Neutral Protease

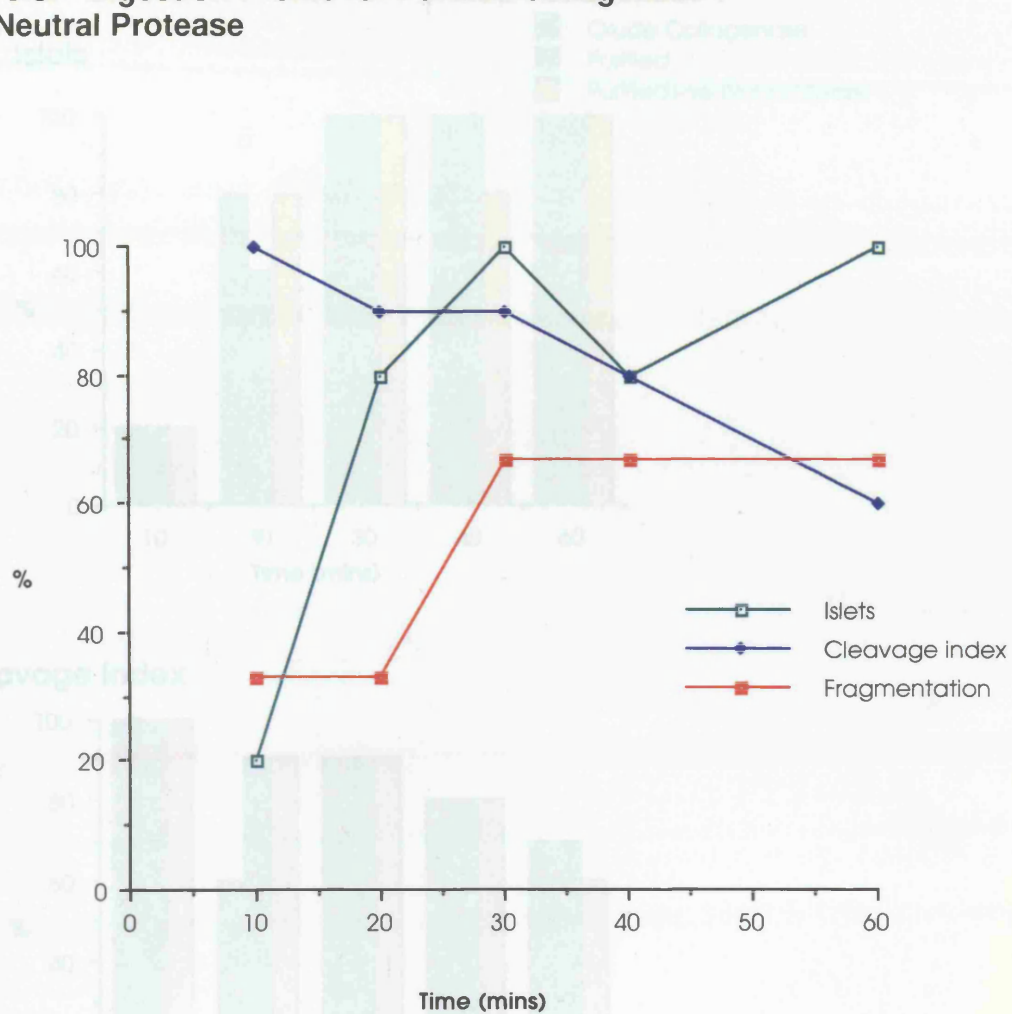
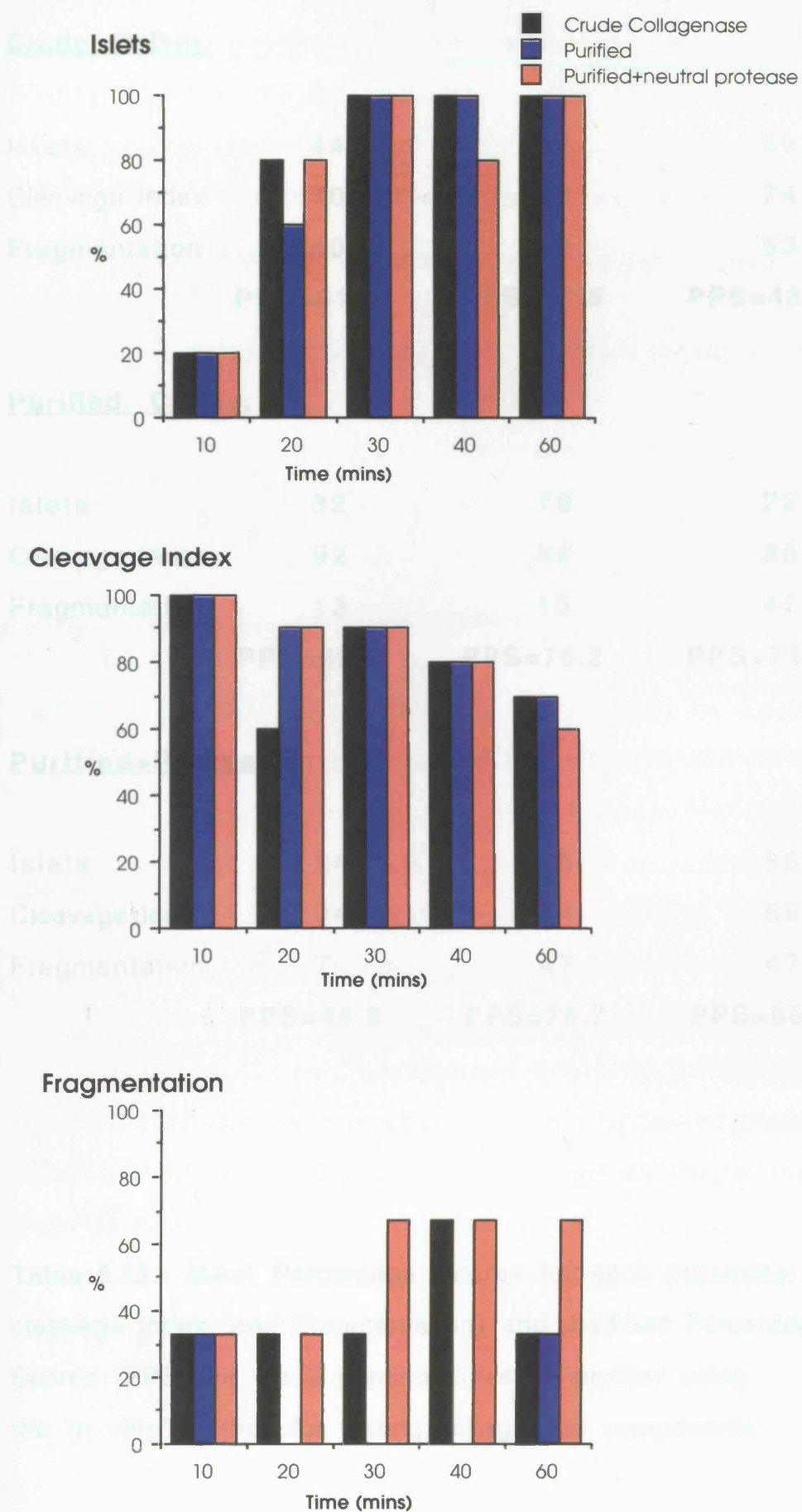


Figure 5.9. Comparison of test enzymes for each parameter



	<u>Expt.1</u>	<u>Expt.2</u>	<u>Expt.3</u>
<u>Crude Collag.</u>			
Islets	44	80	36
Cleavage Index	70	80	74
Fragmentation	40	40	53
	PPS=51.9	PPS=77.5	PPS=48.9
<u>Purified Collag.</u>			
Islets	32	76	72
Cleavage Index	92	86	86
Fragmentation	13	13	47
	PPS=49.6	PPS=75.2	PPS=74.8
<u>Purified+Protease</u>			
Islets	24	76	88
Cleavage Index	94	84	88
Fragmentation	7	47	47
	PPS=44.8	PPS=76.7	PPS=85.4

Table 5.12. Mean Percentage Scores for each parameter (islets, cleavage index, and Fragmentation) and modified Percentage Profile Scores (PPS) for the 3 porcine digestion profiles using the 'in vitro' method for testing collagenase components.

Discussion

These series of experiments were designed to develop a method that could be used for examining the differences between the digestion profiles of different batches of crude collagenase and to investigate the role of collagenase components. To those not involved in islet isolation, they may seem simplistic. However, no pure biochemical model or more complex islet evaluation studies can measure the essential parameters of islet isolation, namely the isolation of intact (non-fragmented) islets devoid of exocrine tissue (cleaved) from the complexity of the human or porcine pancreas.

The first series of experiments enabled a method to be identified that could be used for screening collagenase batches. The fact that the method using undistended blocks of pancreas in a 4x collagenase concentration showed a better correlation than the method using blocks incubated in 1x collagenase concentration implies, as might be expected, that undistended blocks of pancreas require a higher concentration of collagenase for digestion than distended ones. Even so, it is clear that the 4x method still did not correlate exactly with the digestion occurring within the digestion chamber of the semi-automated system. However, the parameters that are important for assessing islet isolation were identified in this method. In addition, these methods were never designed to replace the semi-automated method, but instead to be used for initial screening and testing, before upscaling to the whole porcine or human pancreas. The poor correlation seen with the 'Undistended-Repeated' method was mostly due to islet fragmentation. Again, this might be expected when one considers the fragility of porcine islets and the fact that the repeated method involves repeated sampling and manual shaking of the same tube.

The second series confirmed the findings of the porcine experiments on the human. It is interesting to note that the correlation between the 1x and the 4x collagenase concentrations was closer in the human than in the pig. Again the positive effect of pancreatic distension was observed.

The third series of experiments confirmed that pancreatic distension per se as opposed to distension with collagenase, did not improve islet isolation. This data taken together with the first series would support the data (see Chapter 9) suggesting that the positive effect of collagenase distension is the delivery of collagenase to the islet / extracellular interface. The poor digestion with the Hank' distended / Hank's incubated pancreatic blocks was important as a negative control, confirming that the results could not just be explained by manual shaking and incubation in warm Hank's alone. The intraparenchymal injection method did not show improved digestion. This might be expected from the potentially unpredictable delivery of collagenase that this method gives.

The final experiments confirmed the use of the 'in vitro' method on crude collagenase and three of its main components namely, class I and class II collagenase, and neutral protease. The main purpose of these experiments was a confirmation of the methodology. However, the findings, although based on small numbers, were of interest.

Conclusions

The experiments described in this chapter have suggested a method that can be used for research into the components of collagenase required for optimal islet isolation. It is clear from this chapter however, that methods involving pancreatic distension with collagenase remain the 'gold standard' and a version of the semi-automated method would still need to be used subsequently for assessing the key components in both the pig and the human. The problems with purifying / isolating such large quantities of enzymes have already been highlighted. The importance of efficient delivery of collagenase in an optimal solvent to the islet / extracellular interface has been inferred in this chapter and forms the basis of Chapters 7 and 8. The next chapter describes the

optimisation of biochemical assays for the principal components of crude collagenase.

CHAPTER 6

Optimisation of Biochemical Assays for the Components of Crude Collagenase

Introduction

Before the start of this project, the research in our department had concentrated on optimising the purification stage of islet isolation. There had been no previous work on collagenase and it was therefore necessary to establish new techniques. One of the main areas required investigation was the establishment and optimisation of biochemical assays for class I collagenase, class II collagenase, and neutral protease.

Collagenases are unique in their ability to efficiently hydrolyse the triple-helical region of collagen under physiological conditions ⁴¹⁹.

However, the two classes of collagenase show different but complementary specificities towards substrates ⁴⁴⁸. In general, class I collagenase has a higher specificity for native collagen substrate whereas class II collagenase has more activity against synthetic substrates (see below) ⁴⁴⁵.

The primary structure of collagen has a high content of glycine, proline, and hydroxyproline. Studies on the beta subtype of class I collagenase have indicated specific amino-acid / peptide sequences that are optimal for this collagenase class (see Table 6.1) ^{449, 448}.

In general, the best substrates have glycine in subsite P₃ and P₁', proline or alanine in subsite P₂', and hydroxyproline, arginine, or alanine in subsite P₃' (subsite nomenclature from Schechter and Berger 1967) ⁵¹⁹. These findings are consistent with the frequency of occurrence of these residues in the glycine-X-Y triplets of collagen. However, the work by Steinbrink et al.(1985) have also suggested that the most sensitive substrates for the β -subtype do not have collagen-like amino acid sequences in subsites P₁ or P₂ ⁴⁴⁹. Indeed, a substrate such as cinnamoyl-4-nitrophenylalanine-glycine-proline-alanine is a rapidly hydrolysed substrate for beta-collagenase with a k_{CAT} / K_M value of $4.4 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$. However, this substrate has neither alanine in the P₁ subsite nor proline or alanine in the P₂. Indeed, subsequent studies by the same group have confirmed that both class I and class II collagenases prefer aromatic, hydrophobic amino acids in the P₁ subsite which

$P_2 - P_1 - P_1' - P_2' - P_3'$	$k_{CAT} \text{ (min}^{-1}\text{)}$	$K_M \text{ (mM)}$	k_{CAT}/K_M ($\times 10^{-4} \text{ M}^{-1} \text{ min}^{-1}$)
FA - Leu - Gly - Pro - Ala	660	1.1	59
CNM - Leu - Gly - Pro - Ala	430	0.46	94
CNM - Phe - Gly - Pro - Ala	2100	0.24	870
BOC-Gly- Pro - Nph - Gly - Pro - Ala	26000	2.5	1100

Table 6.1. Kinetic parameters for the hydrolysis of synthetic peptides by the beta-subunit of class I collagenase. (BOC = tert-butyloxycarbonyl). (Data from Van Wart and Steinbrink 1985)

are not found in this position in type 1 collagen ⁴⁴⁸. These studies also demonstrate that substrates with leucine in subsite P₁ are inferior substrates for class I collagenase compared with class II. This finding is thought to explain the basis for the reduced hydrolysis rates for class I collagenase with synthetic substrates. Unlike their previous study however, these subsequent studies did show that all three subtypes of class I collagenase that were tested (beta, gamma, and eta) did have a preference for proline and alanine in subsite P₂.

1) ASSAYS FOR CLASS I COLLAGENASE

The assays available for class 1 collagenase take these findings into account. They are all based on the hydrolysis of denatured collagen. There are numerous assays described with a wide range of methods of measuring the cleavage of the native collagen. These include monitoring the lysis of collagen gels ⁵²⁰, the lysis of collagen films ⁵²¹, monitoring changes in the viscosity or optical rotation of collagen solutions ⁴¹⁹ or measuring the production of soluble hydroxyproline from insoluble collagen ⁵²². The most widely used assays however, involve variations on fluorogenic and radioactive collagenase fibril assays ^{417, 447, 453, 523, 524}. The main problem with these assays is that they have variable specificity and not all are quantitative. Three such assays are discussed with a description of attempts to validate others.

i) Orange-Collagen Assay

This is a rapid, simple, and sensitive assay used commercially by companies such as Sigma and by laboratories such as that in Groningen.

It was first presented by Rashap in 1990 at the X11th FECTS meeting in Bialystok, Poland and subsequently described in the literature ⁵²⁵. The substrate is collagen type 1 coupled with the reactive dye Active Orange (a dye

used in the Soviet textile industry). The assay involves the incubation of collagenase with the insoluble Orange-collagen with the solubilised digestion products being measured spectrophotometrically at 490nm. Non-specific degradation of the substrate can be corrected for by performing parallel incubations of substrate and enzyme in the presence of 5 mM EDTA which inhibits both collagenase and neutral protease.

Assay Protocol

- 1) Weigh 5 mg of Orange-collagen per Eppendorf vial
- 2) Add 970 - 1000 μ m of Tris-acetate buffer (50mM Tris buffer, pH 7.5 containing 5mM $\text{Ca}(\text{CH}_3\text{COO})_2$) to make a final volume of 1.0 ml and either enzyme (max. 20 μ l) for samples; enzyme and 10 μ l of 500mM EDTA for the sample blanks; and nothing added for the blank.
- 3) Incubate for 1 hour at 37°C in a shaking waterbath at 110 rpm with one manual overhead rotation after 30 mins.
- 4) Stop reaction by adding 10 μ l of 500 mM EDTA, mix and place vials on ice.
- 5) Centrifuge for 5 mins at 13000 rpm.
- 6) Remove 800 μ l of the supernatant. homogenise and measure the absorbance at 490 nm against the blank.

Calculating the enzyme activity

The absorbance at 490 nm (A_{490}) of the sample blank (representing proteolytic activity in the absence of collagenase and neutral protease activity) is subtracted from the A_{490} of the sample and the enzyme activity is calculated as follows:

$$U / \text{mg} = \text{nkcat collagen degraded} / \text{mg} = \frac{A_{490} \times V_{\text{tol}}(\text{ml}) \times 1000}{20.2 \times 13.3 \times t(\text{sec}) \times E(\text{mg})}$$

where:

nkcat = nmol / sec

A₄₉₀ = A₄₉₀ (sample) - A₄₉₀ (sample blank)

V_{tol}(ml) = final volume in assay in ml

t(sec) = incubation time in seconds

E(mg) = amount of enzyme in mg (solid/protein)

20.2 = molar extinction coefficient of Active Orange

13.3 = mole of dye coupled to 1 mole of collagen

1000 = converting factor from (μkat to nkat)

Optimising Assay

I learnt this assay within the Department of Surgery in Groningen and it was there that I studied its optimisation. Although this assay is simple and has the advantage of avoiding radioactivity, it does have several limitations.

First, the substrate can be very variable from one batch to another. This means that full evaluation is required on receipt of each new stock.

Second, although the A₄₉₀ increases linearly with enzyme amounts in the A₄₉₀ range of 0.2 - 1.0 (for Sigma type X1 collagenase this corresponds to a maximum of 5 μg enzyme added per 5 mg of substrate), a 2 fold increase in enzyme does not increase the A₄₉₀ two fold outside this range. This means that the assay is optimal when used for comparing enzyme activities from similar A₄₉₀ values obtained within one assay. In other words it is a valid assay in experiments in which comparison rather than absolute values are required.

Third, as might be expected, although it is most sensitive for class I collagenase, it is not completely specific. This is almost certainly due to impurities within the substrate.

ii) Radiolabelled Collagen

There are several reports in the literature that suggest that the most sensitive assay available for class I collagenase is the radioactive acid-soluble collagen assay.

There are several different radiolabelled collagen assays described ⁵²⁶.

However, two of the main methods applicable to bacterial collagenases are based either on the hydrolysis of [¹⁴C]-acetylated collagen or the hydrolysis of [³H]-acetylated collagen.

[¹⁴C]-acetylated collagen

This assay was described by Cawston and Barrett ⁴⁴⁷ and represents a rapid, reproducible assay for detecting class I collagenase. With some modifications, it was this assay that we established in our department.

Commercially available type 1 Rat tail acid soluble collagen was labelled by [¹⁴C]-acetic anhydride (NEN Dupont) in a reductive methylation reaction to yield a [¹⁴C]-collagen. We found that both crude collagenase P and purified class I collagenase had a high affinity for this substrate and activity could be quantified as a specific activity (μmoles / min / μg protein. The assay is performed as follows:

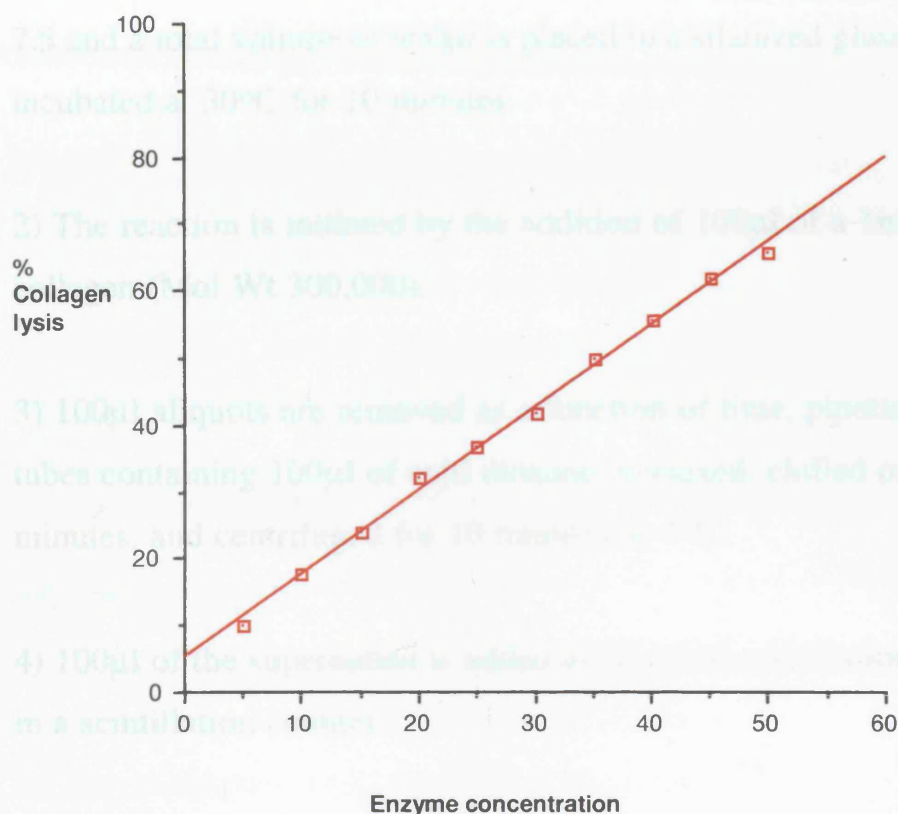
Assay Protocol

It is essential that the enzyme sample is dissolved in a suitable buffer and does not contain any bovine serum albumin (BSA) which is a potent inhibitor.

- 1) 50µl of [¹⁴C]-collagen solution (0.6 mg / ml in 2.5 mM of acetic acid is added to 85µl of 50mM Tris / HCl buffer, pH 7.5).
- 2) The reaction is started by adding a 10µl sample of enzyme and incubation for 1 hour at 37°C.
- 3) The reaction is stopped by adding 5µl of 5% BSA (made up in 2mM of HCl, together with 20µl of 60% Trichloroacetic acid.
- 4) The mixture is then vortexed and kept on ice for 10 minutes. The mixture is then spun in a microfuge for 10 minutes at 13000 rpm. A protein pellet is then visible at the bottom of the tube.
- 5) A 100µl aliquot is then taken and prepared for scintillation counting by adding the aliquot to 400µl of HILOAD scintillation fluid. The mixture is then vigorously vortexed and then counted for radioactivity on a scintillation counter. It is important to note that a substrate blank must be included each time the assay is performed, together with a total count sample (i.e. substrate and HILOAD fluid).

This assay is straightforward and enables the quantification of class I collagenase. In optimising it in our laboratory I found it had a linear relationship with increased calcium concentration and also with sequential increases in substrate and enzyme. However, one of the interesting features of this assay reported by Cawston and Barrett is the fact that the plot of the amount of collagen hydrolysed in response to increasing amount of enzyme does not pass through the origin, but rather intercepts the vertical axis at about 6% collagen lysis (see Figure 6.1). Although the reason for this is unclear, it is likely that the change in the structure of the substrate during the first 5-10 minutes of the assay is responsible for these findings.

Figure 6.1 Effect of enzyme concentration on collagen hydrolysis during [14C]-acetylated collagen



[³H]-acetylated collagen

This method reported by Mallya et al. ⁴⁵³ uses [³H]-acetylated type 1 rat tail tendon collagen. The method is as follows:

- 1) Enzyme is dissolved in 50mM Tricine, 0.4 M NaCl, and 10mM CaCl₂, pH 7.5 and a total volume of 900μl is placed in a silanized glass tube and incubated at 30°C for 10 minutes.
- 2) The reaction is initiated by the addition of 100μl of a 1mg / ml solution of collagen (Mol Wt 300,000).
- 3) 100μl aliquots are removed as a function of time, pipetted into microfuge tubes containing 100μl of cold dioxane, vortexed, chilled on ice for 10 minutes, and centrifuged for 10 minutes at 4°C.
- 4) 100μl of the supernatant is added to 10 ml of scintillation fluid and counted in a scintillation counter.
- 5) The data is then plotted as cpm of [³H] released vs time. Specific activities in units of nanokatals per milligram are calculated by dividing the initial rates by the mass of the enzyme present.

This assay has been used successfully in studies on the specific activities of the subtypes of class 1 collagenase ⁴⁴⁸. While this assay is sensitive and obeys the accepted principles of enzyme kinetics, it does not confer significant advantage over the method using [¹⁴C]-acetylated collagen for the studies that I was performing and therefore we did not establish this assay in our department.

2) ASSAYS FOR CLASS II COLLAGENASE

As has been highlighted above, although class II collagenase has a broader specificity than class I collagenase, the former does appear to be more active toward synthetic substrates than toward native collagen ⁴⁴⁸. There are two main assays most frequently used for class II collagenase. These are the Wunsch assay ⁵²⁷ which involves the hydrolysis of 4-phenyl-azo-benzyl-oxy-carbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine which due to its broad specificity is also used by some companies (Boehringer Mannheim) to assess class I collagenase, and the FALGPA assay ⁴²⁵ which uses the hydrolysis of the peptide 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine. The latter assay is rapid convenient and sensitive and therefore is the preferred assay for class II collagenase. It was therefore this assay that I established in our department and indeed spent some time optimising and revising.

FALGPA Assay

This assay was described by Van Wart and Steinbrink in 1981 and developed for the collagenase of *Clostridium histolyticum* ⁴²⁵. It is a kinetic spectrophotometric assay based on the hydrolysis of the synthetic peptide FALGPA. Class II collagenase cleaves the Leu-Gly bond of FALGPA to give FA-Leu and Gly-Pro-Ala. The hydrolysis of this peptide by collagenase obeys Michaelis-Menten kinetics with $V=1.8 \times 10^5 \mu\text{katal} / \text{kg}$ and $K_m = 0.5 \text{ mM}$ and is hydrolysed more rapidly than any other commonly used substrate ⁴²⁵. It is fairly specific in that it is not cleaved by any of the common proteinases such as trypsin, elastase, or thermolysin. The assay makes use of the blue shift in the near-ultraviolet absorption band of the furanacryloyl peptide when the peptide bond between the first and second residues is hydrolysed. In the original description, a wave length of 324 nm is used. However, during optimisation of this assay, I found that only up to 20 % of the substrate that

was hydrolysed was actually detected at this wavelength. I therefore modified the assay as outlined below.

Optimisation and Evaluation of FALGPA Assay

Collagenase P (Boehringer lot 64) was dissolved in 50 mM Tris-HCl buffer at a pH of 7.5 (Sigma, Poole) to give a final concentration of 1mg / ml.

FALGPA (lots 92HO303 and 51HR719) was dissolved in Dimethyl Sulfoxide (DMSO) to give a final concentration of 10mM. The protein content of the enzyme was determined by a modified method of Bradford ⁵²⁸ using the Bio-Rad protein assay kit. Fraction V bovine albumin (Sigma lot 99FO020 and 123HO811) was used as a standard.

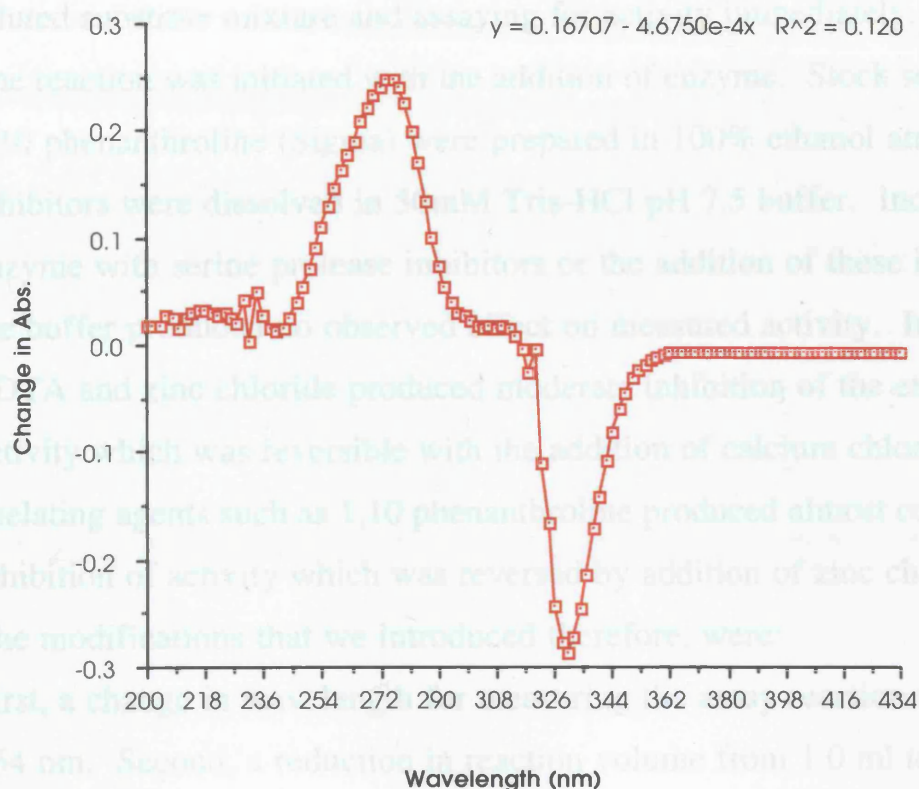
Difference spectra were obtained using a UV-Visible spectrophotometer (Shimadzu uv-1601) with silica micro-cuvettes in both sample and reference positions. A baseline was obtained with 580µl OF 50mM Tris-HCl buffer pH 7.5) and 10µl of 10mM FALGPA in DMSO in both the sample and reference cuvettes. The absorbance spectrum between 190 and 1100 nm was recorded. To start the reaction, 10µl of 1mg / ml collagenase was added to the sample cuvette and immediate consecutive scans within the same range of wavelengths were recorded until no further change in the absorbance spectra was seen.

The difference spectra for these experiments is given in Figure 6.2. It was observed that a wavelength of 254nm rather than 324 nm was associated with a more sensitive assay. At this new wavelength the cleavage of the leucine-glycine peptide bond in the chromophore 'furylacryloyl of the substrate is detected optimally at 254 nm.

The effects of substrate and enzyme concentrations were examined with respect to the rate of hydrolysis of FALGPA by collagenase (crude and purified). It was found that at low concentrations (e.g. 0.03) of FALGPA, the rate of hydrolysis of FALGPA by collagenase at an wavelength of 254 nm decreased exponentially confirming that this assay obeys first order kinetics with respect

to substrate. At high concentrations of FALGPA, the A_{254} decreases linearly

Figure 6.2. Difference Spectra for FALGPA hydrolysis



The redesigned protocol is as follows:

Assay Protocol

As with the assays for class I collagenase, BSA needs to be avoided as this is a potent inhibitor of collagenase activity.

1) 10 μ l of FALGPA in DMSO is added to 500 μ l of 30 mM Tris-HCl buffer at a pH of 7.5.

2) After mixing these well, the mixture is added to a glass microcuvette. This is then used to zero the spectrophotometer at an absorption of 254 nm.

to substrate. At high concentrations of FALGPA, the A_{254} decreases linearly with time suggesting that this reaction obeys zero-order kinetics. At all substrate concentrations studies, the rates of hydrolysis are proportional to the collagenase concentration.

Inhibition studies were performed by adding inhibitor stock solutions to the diluted substrate mixture and assaying for activity immediately.

The reaction was initiated with the addition of enzyme. Stock solutions of 1,10 phenanthroline (Sigma) were prepared in 100% ethanol and all other inhibitors were dissolved in 50mM Tris-HCl pH 7.5 buffer. Incubation of the enzyme with serine protease inhibitors or the addition of these inhibitors to the buffer produced no observed effect on measured activity. Incubation with EDTA and zinc chloride produced moderate inhibition of the enzymatic activity which was reversible with the addition of calcium chloride. Zinc chelating agents such as 1,10 phenanthroline produced almost complete inhibition of activity which was reversed by addition of zinc chloride.

The modifications that we introduced therefore, were:

First, a change in wavelength for measuring the assay reaction from 324 to 254 nm. Second, a reduction in reaction volume from 1.0 ml to 0.6 ml.

Third, the introduction of glass cuvettes rather than plastic ones.

The redesigned protocol is as follows:

Assay Protocol

As with the assays for class I collagenase, BSA needs to be avoided as this is a potent inhibitor of collagenase activity.

1) 10 μ l of FALGPA in DMSO is added to 580 μ l of 50mM Tris / HCl buffer at a pH of 7.5.

2) After mixing these well, the mixture is added to a glass microcuvette. This is then used to zero the spectrophotometer at an absorption of 254 nm.

3) 10µl of the enzyme sample is added to start the reaction and mixed together well for about 10 seconds. Readings are taken every 2 minutes for up to 10 mins for the manual spectrophotometer or an automatic kinetic plot is generated by machines such as the Shimadzu UV-1691 UV-Visible spectrophotometer.

4) The initial rate is then calculated after plotting change in absorbance against time and is expressed as absorbance units per minute. This is then used in the following formula which gives the Specific Activity.

$$\text{Specific Activity} = \frac{\text{Rate (Abs. / min)}}{0.36 \times (\text{protein concentration } (\mu\text{g}) / \text{reaction volume } (\mu\text{l}))}$$

Activities using this calculation are expressed as µMoles per minute per mg of protein. It is also possible to express the activity as U/mg.

Wunsch Assay

Due to its broad specificity, I did not establish this within our department and therefore the assay protocol is not described.

3) ASSAYS FOR NEUTRAL PROTEASE

Biochemical assays which can measure the other major component of collagenase, namely neutral protease, are also readily available. One of the main problems is that this protease is fairly non-specific. However, the two main assays used are either the azocoll assay or the casein assay, the latter may be either radio active (carbon 14) or fluorogenic 529, 530.

i) Azocoll assay

This assay was first reported by Oakley et al. in 1946⁵³¹. Since then it has been widely used for measuring proteinase activity within bacterial extracts^{532, 533, 534}. It is used by several pharmaceutical companies (for example Beohringer Mannheim) to measure the proteinase activity within crude collagenase.

Azocoll is an insoluble ground collagen to which a bright-red protein dye has been attached. It is however, a fairly non-specific assay and one of the features that has led to some concern is that whereas the hydrolysis of azocoll progresses linearly as a function of proteinase concentration, it appears to increase exponentially as a function of time⁵³⁵. Other groups have shown that this nonlinear time course may be due to inhibitory compounds within the commercially available azocoll and that it can be overcome by prewashing the azocoll vigorously with buffer before using it and varying the absorption rate used and the degree of agitation of the assay mixture⁵³⁴. Neither of these studies however, were based specifically on an extract of *Clostridium histolyticum*.

Due to the relative non-specific nature of this assay, I did not establish this assay in our department although I learnt the methodology.

Assay Protocol

1) Azocoll is prepared as a 2.4% solution by adding 1.8g of azocoll (Sigma Chemical Company) to 75 ml of Tris buffer (0.02 mol/l pH 7.7 HCl; +/- 1 mmol/l EDTA. This is incubated at 37°C just before using. It is also important that the solution is shaken before using so as to ensure equal distribution of particles.

2) Control samples (3.0 ml of azocoll) and Active samples (3.0 ml of azocoll and 0.2 ml of enzyme sample) are incubated for 30 minutes at 37°C and again the samples gently shaken several times during incubation.

3) The reaction is stopped by the addition of 2.8 ml of 10% trichloroacetic acid (TCA) in water to each sample.

4) The resulting mixture can then either be applied to filter paper and the absorbance of the filtrate measured at 520 nm or it can be read at the same wavelength in an eppendorf vial after centrifuging the mixture for 20 minutes at 12, 000 rpm. It should be noted that if a measurement is repeated, a fresh 'control' sample must always be prepared as with time the azocoll changes red spontaneously.

5) The change in absorbance (ΔE) is calculated by subtracting the absorption of the Control sample from the Active sample. The activity can then be calculated by the equation:

$$\text{Activity (U/ml sample)} = 1000: (30 \times 0.2) \times \Delta E$$

2) Casein Assay

As has been highlighted above, the casein assay can either involve a radiolabelled or a fluorogenic substrate. In our department we established the radioactive method but I also used the fluorogenic method during research in Groningen.

The radioactive method has the disadvantages intrinsic to the use of radioactivity, but the advantages of being very stable. Indeed, the rate of decomposition is less than 1% for 6 months from date of purification. The radiolabelled casein is prepared by methylating [^{14}C] using formaldehyde and

sodium cyanoborohydride according to methods described by Dottavio-Martin and Ravel ⁵³⁶. It has a Molecular Weight of 23,600 ⁵³⁷. The reported radioactive purity of the product is >98%. Although the substrate is stable, there is 'batch to batch' substrate variation.

Assay Protocol

1) 100µl samples are prepared consisting of 5µl of [¹⁴C]-casein (1mg/ml), 10µl of enzyme (1mg/ml), and 85µl of HEPES buffer (100mM,pH 8.0).

2) These samples, together with a substrate blank and a total count sample of 5µl casein, are then incubated for 60 minutes at 37°C.

3) 20µl of 60% TCA (6g in 10 ml of water) and 5µl of 5% bovine serum albumin (BSA) (5g of bovine albumin in 100ml of 0.1M HCl) are added to each sample. These are then placed on ice for 10 minutes and spun for 10 minutes.

4) 100µl aliquots are taken from each sample and 400µl of HILOAD scintillation fluid added. They are then placed in a scintillation counter for reading. The specific activity is then calculated from the counts, knowing the molecular weight of casein is 23,600.

4) ASSAYS FOR OTHER COMPONENTS OF CRUDE COLLAGENASE

Some of the other components of crude collagenase can also be specifically measured using readily available assays. Trypsin-like proteases cleave peptides preferentially at the carboxyl side of arginine and lysine residues. They can therefore be detected using either the synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEE) ⁵³⁸ or the fluorogenic substrate arginine-AMC ⁵³⁹. The fluorescence change is measured on a fluorometer using excitation and

emission wavelengths of 340 and 415 nm respectively. The other main component clostripain also cleaves peptides preferentially at the carboxyl side of arginine residues. Its activity is measured by its ability to cleave BAEE in the presence of the activator dithiothreitol (DTT) ⁵⁴⁰. Whilst I established these in the department, these assays were not used as part of the experiments included in this thesis.

Discussion

It can be seen from this chapter that there are a variety of assays available for the key components of crude collagenase. It is interesting to note that in spite of the relative non specificity of some of the assays that some pharmaceutical companies manufacturing crude collagenase place emphasis on them. This is well illustrated in the case of Boehringer Mannheim. The results of assays for 10 of the main enzymes that I used in our department during this project are given in Table 6.2. There are a couple of interesting things to note.

First, it is interesting to note the wide variation in enzymatic activity between the compounds and this is seen even between 'lots' that gave good results in terms of islet isolation. For example enzymes 80, 82, and 91 all gave good results during repeated human islet isolation. However, their Wunsch assay activities vary considerably from 0.78 to 1.61 U/ml. The difference in azocoll activity between lot 82 and the other two enzymes is almost double. This highlights not only how variable the assays are, but also how complex the collagenase problem is. The second thing to note is that Boehringer Mannheim normally discard any enzyme that has a Wunsch activity of less than 1.5 U/mg. This is then either used for research or sold as the product collagenase A. However, lot 82 highlights the limitations of such a policy. I found this to be a very efficient enzyme for human and to a lesser extent porcine islet isolation and yet we only obtained this product through a research collaboration with the company. Again this highlights the importance of the pancreas being the only really accurate assay for determining a crude enzyme for islet isolation.

<u>Boehringer Lot No.</u>	<u>Wunsch (U/mg)</u>	<u>FALGPA (U/mg)</u>	<u>Azocoll (U/mg)</u>
62	1.76	0.94	112
63	1.98	1.06	88.4
64	2.17	1.25	78.5
80	0.78	0.63 (1.53)	64.8
82	1.12	0.7 (2.20)	118.2
83	1.67	0.92 (2.95)	138.2
84	1.36	0.73 (2.43)	170.8
85	0.96	00.52 (1.57)	85.5
91	1.61	0.87 (2.91)	63.9
92	1.61	0.74 (2.45)	50.5

Table 6.2. Enzyme activity within the batches of crude collagenase used within our department based on the assays used by Boehringer Mannheim. The FALGPA figure in brackets refers to the optimised assay.

It also outlines the limitations of placing such strong emphasis on arbitrary results of biochemical assays of a product that has not yet been optimised for islet isolation.

Conclusions

This chapter has outlined the main assays available for three of the main components of crude collagenase namely, class I collagenase, class II collagenase, and neutral protease. It has highlighted some of the limitations of such assays and described methods of optimising them.

This work on the biochemical assays was essential as a preparation for the work described in the next two chapters.

SECTION III

Collagenase Storage

CHAPTER 7

A Study of the Stability of Crude Collagenase and its Components under Different Storage Conditions

Introduction

Deterioration of the function of an efficient crude collagenase during storage is a frequently observed problem in islet isolation laboratories. However, this instability is poorly understood in several regards.

First, the components responsible for the degradation have not yet been fully elucidated. It is well known that both classes of collagenase are very susceptible to proteolysis⁴⁴⁵. Indeed this proteolysis is thought to have accounted for much confusion over the exact molecular weights of the two classes⁴⁴⁵. However, the stability of the different individual components of crude collagenase over time has not been well documented.

Second, there is no clarity concerning the effects of different storage conditions. Many pharmaceutical companies state in their product data sheets that the lyophilised crude enzyme mixture is stable at 4°C when stored under dry conditions and that the reconstituted solution is stable at -20°C. However, many islet groups store even the lyophilised enzyme at -20°C and yet still observe deterioration of enzyme function in terms of decreased efficiency of pancreatic digestion. It would also be expected that even the smallest amount of hydration, such as that occurring during the freeze thaw cycles between different islet isolations, would cause deterioration of enzyme function, and yet this is the recommended way of using the crude enzyme.

Third, it is not known whether different batches of crude collagenase are more intrinsically stable than others. Certainly the recent product from Boehringer Mannheim, *Liberase*, is supposed to be a purer, more stable product than the crude collagenase P. Whether the stability of the crude enzymes from different manufacturing companies vary is also not known.

I therefore designed a series of experiments that were performed at different time-points over a 6 month period in order to study the stability of different crude collagenases. I performed these experiments in collaboration with the Department of Surgery in Groningen.

Aims

The aims of this series of experiments was to study the stability of class I collagenase, class II collagenase, and neutral protease within different batches of crude collagenase during a range of different storage conditions.

Materials and Methods

Four different crude collagenases were studied. These were Boehringer lot 82 (the optimal porcine enzyme in Leicester at that time), Boehringer lot 91 (the optimal human enzyme in Leicester at that time), Boehringer Liberase (the purer blend that had recently been released from Boehringer and that was being marketed as a stable product), and Sigma type XI lot 40H68681 (the optimal rat and porcine enzyme in Groningen at that time).

Each enzyme was obtained specially for this experiment and consisted of sealed unopened vials of enzyme that had been transported and recently stored under the recommended, optimal conditions for each manufacturer.

There were three aspects of the investigation of storage conditions. First, four temperatures were studied for each enzyme. These were room temperature, 4°C, -20°C, and -80°C. Second, the effect of freeze-thaw cycles and repeated opening of a vial compared with opening a sealed unit each time collagenase was required, was investigated. Third, the effect of storing crude collagenase in its lyophilised versus its dissolved reconstituted form was studied.

Eight combinations of conditions were therefore studied for each crude enzyme:

i) Room Temperature in covered vial

These samples of lyophilised enzyme were stored in the laboratory with the vials covered to prevent contamination.

ii) 4°C individual samples

Individual samples of lyophilised enzyme were prepared which corresponded to each of the assays at each of the time points. These were stored in the fridge. Prior to each assay, each vial containing the individual samples had therefore only been opened once.

iii) 4°C repeatedly opened vial

The conditions for storage were the same as for ii). However, one large sample of lyophilised enzyme was prepared and stored and the same sample re-opened each time that assays were performed. Although no thawing was required at this temperature, this storage condition included the factor of repeated opening of the same vial.

iv) -20°C individual samples

These were prepared in the same way as for storage condition ii). However, the samples were stored at -20°C. Again, the samples of lyophilised enzyme were only opened once prior to the biochemical assays.

v) -20°C repeatedly opened vial

This sample of lyophilised enzyme was stored at -20°C and opened each time samples were required for the assays. This storage condition therefore included repeated freeze-thaw cycles.

vi) -20°C dissolved in Hank's at 1mg / ml

This storage condition evaluated the effect of dissolving the lyophilised

enzyme prior to storage. Individual samples were prepared for this condition so as not to add the variable of repeated freeze-thaw cycles to this evaluation.

vii) -20°C dissolved in Hank's at 10mg / ml

This condition evaluated the effect on enzyme stability of storing the dissolved enzyme at a higher stock concentration of 10mg / ml. Unlike the other storage conditions, this was only evaluated at selected time points (see below).

viii) -80°C individual samples

This storage condition involved the storage of individual samples of lyophilised enzyme at a temperature of -80°C.

For each of the storage conditions biochemical assays for class I collagenase (collagen orange), class II collagenase (FALGPA), and neutral protease (radiolabelled casein) were performed on Days 0, 1, 3, 7, 14, and then at 1 month, 2 months, 4 months, and 6 months. The exception to this was storage condition i) in which assays were not performed at 6 months because in order to confirm that the results were genuine, these samples were used for rat islet isolation in order to correlate enzymatic activity with pancreatic digestion, albeit that due to the small sample sizes this was conducted in a rodent model. The other exception was storage condition vii) which as stated above was only evaluated at Day 0, 1 month, 2 months and 6 months. For each storage condition, each assay was performed in triplicate. In addition serial sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed over the time course.

Results

With the number of different variables investigated in these experiments (4 crude enzymes, 8 storage conditions, 3 assays performed in triplicate, and 5 time-points) there are several different ways of expressing this data and also a number of different comparisons that can be made. One of the most helpful ways of viewing such data is as a plot of activity over the time course for the 4 enzymes under each individual storage condition. These are shown in Figures 7.1 to 7.24. These plots are comprised of mean enzymatic activity for each assay at each time point.

It can be seen from these plots that over the first 4 months of the study there was no demonstrable deterioration of enzymatic activity for either class I collagenase, class II collagenase, or neutral protease, was evident for any of the 4 enzymes under any of the storage conditions. This demonstrable stability continued up to the final 6 month assays for all the crude enzymes for all the assays with the exception of the FALGPA activity for each storage condition for Liberase. However, this apparent deterioration was not significant when the time plots were tested by rank sign analysis.

In addition, the SDS-PAGE did not show any clear changes over the 6 month time course for any of the enzymes. There were however, several differences in SDS-PAGE between the 4 enzymes themselves (see Figure 7.25).

Discussion

The findings in this series of experiments are unexpected. Investigators in the field of human islet isolation are well used to the observation of deterioration of function of crude collagenase over time. It would therefore, be expected for several of the storage conditions to cause a significant deterioration in measurable enzymatic activity.

Figure 7.1 **Storage at Room Temperature**
- Class I Collagenase

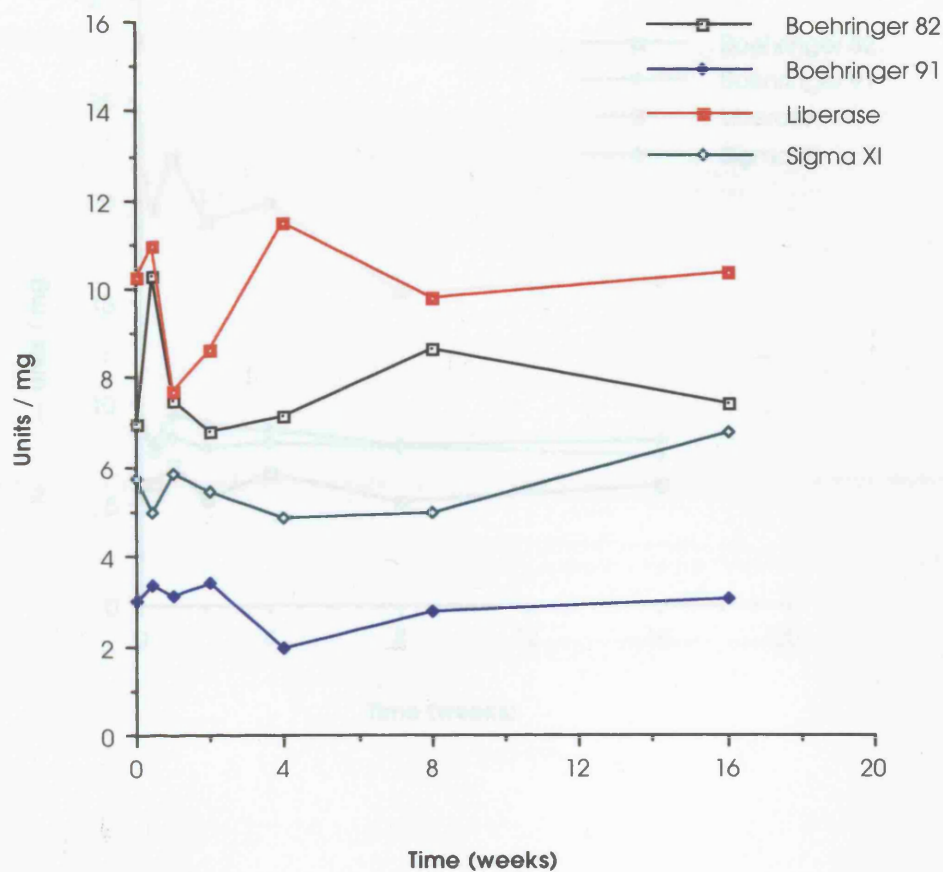


Figure 7.2 **Storage at Room Temperature**
- Class II Collagenase

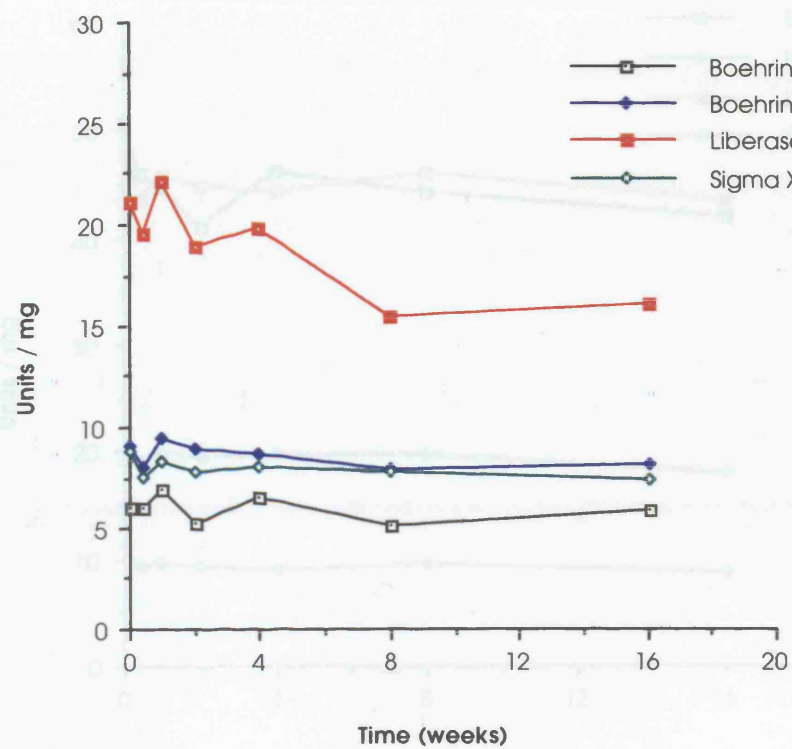


Figure 7.3

Storage at Room Temperature
- Neutral Protease

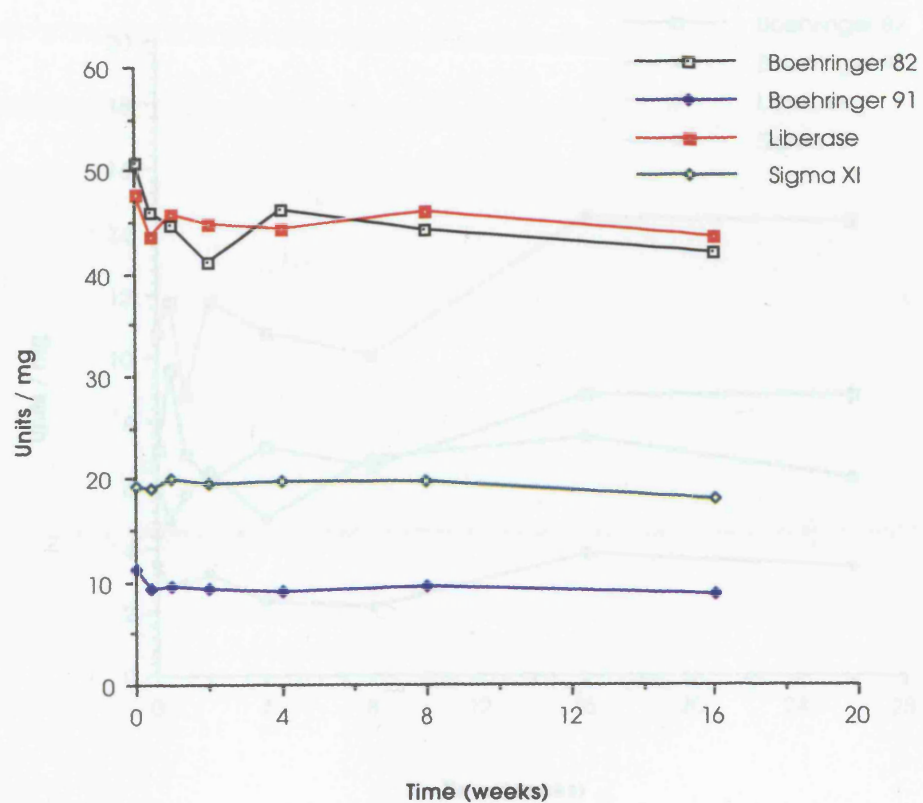


Figure 7.4 **Storage at 4°C (Single) - Class I Collagenase**

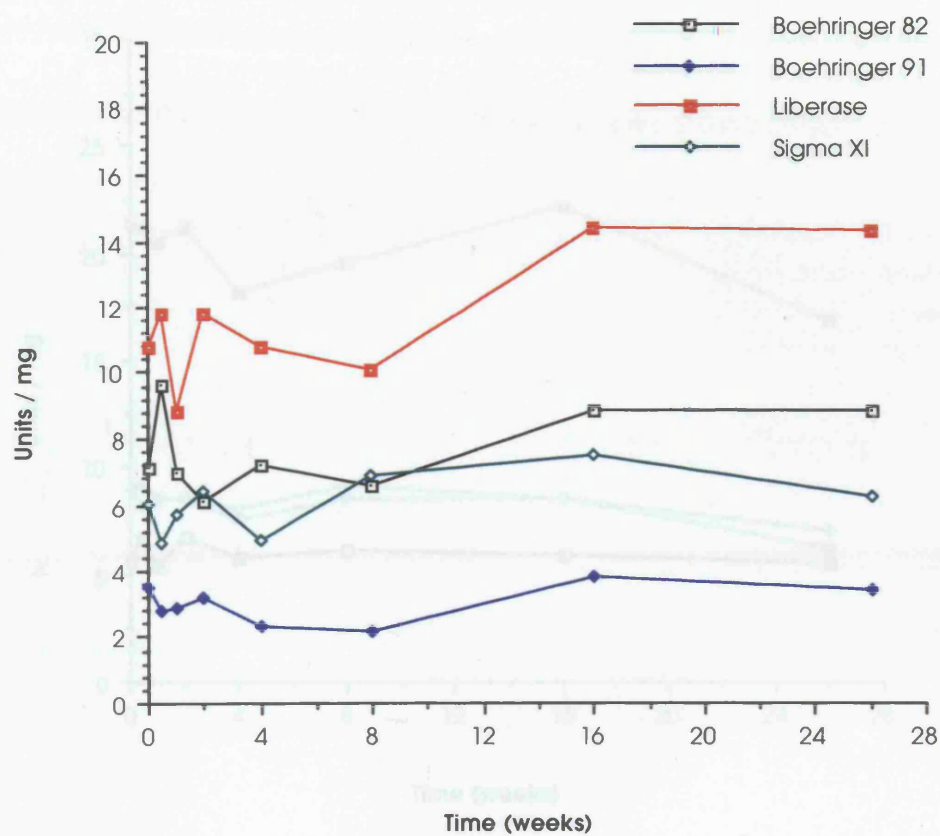


Figure 7.5 **Storage at 4°C (Single) - Class II Collagenase**

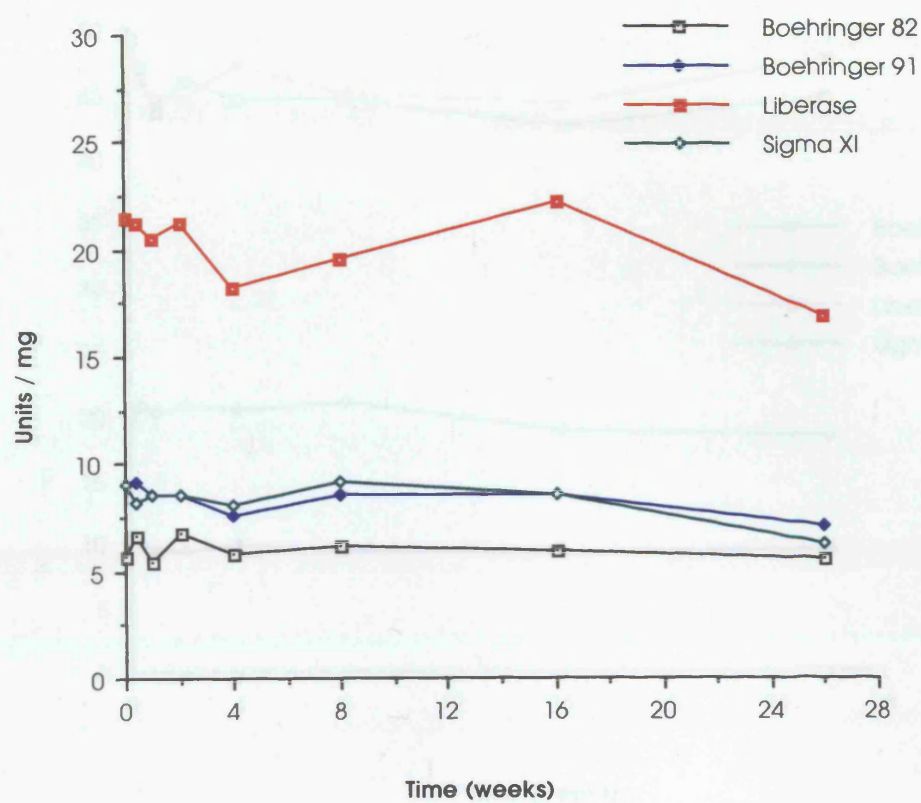


Figure 7.6 Storage at 4°C (Single) - Neutral Protease

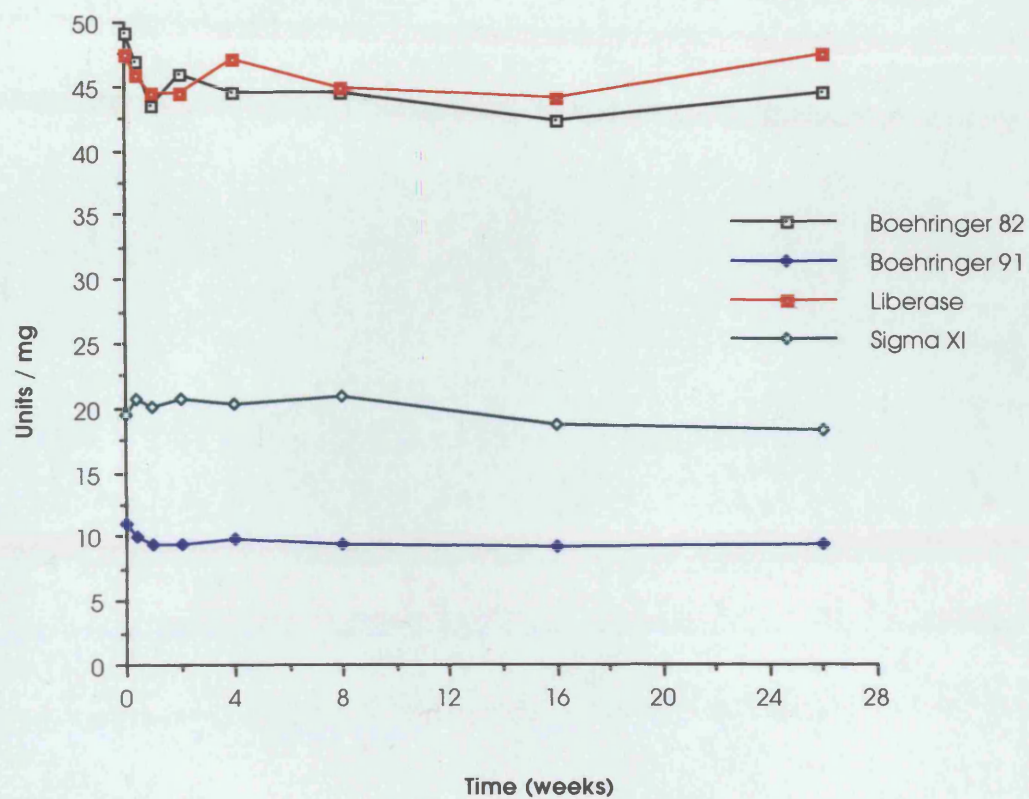


Figure 7.7

Storage at 4°C (Repeated) - Class I Collagenase

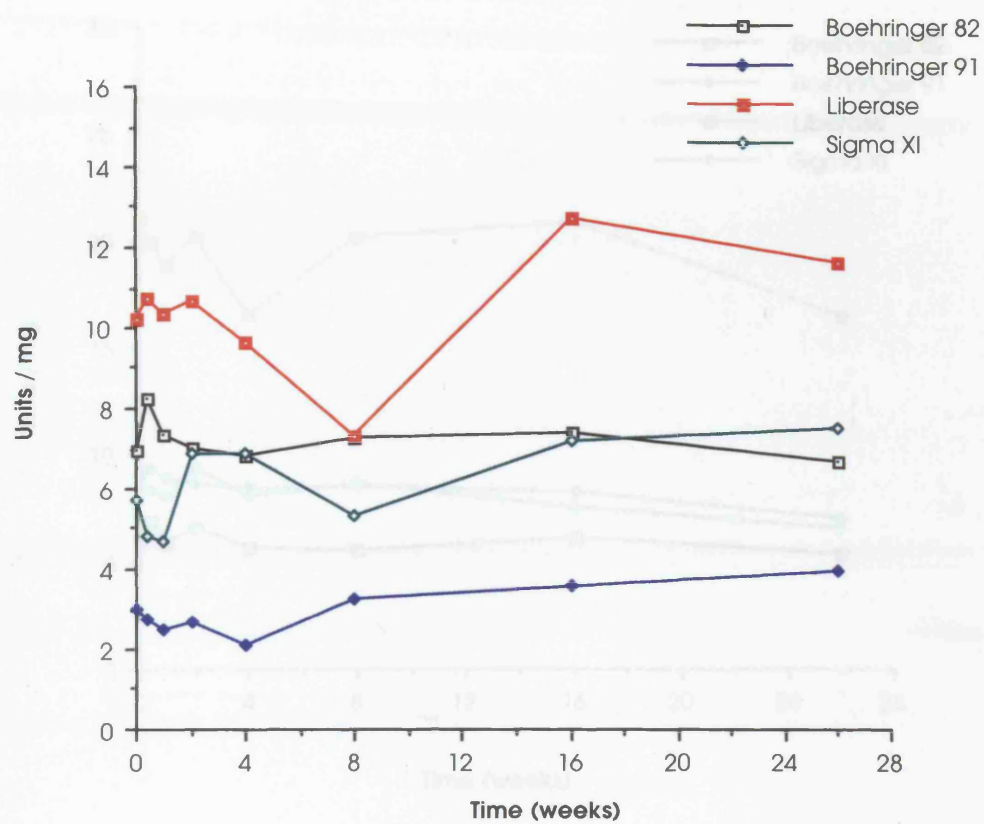


Figure 7.8

Storage at 4°C (Repeated) - Class II Collagenase

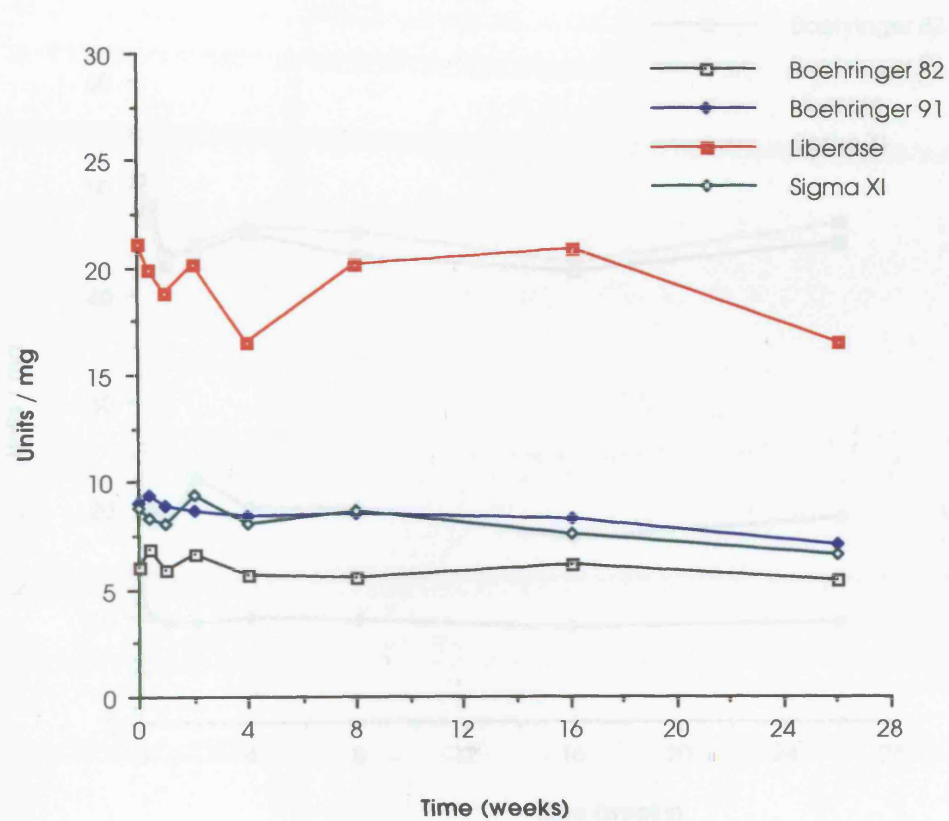


Figure 7.9 Storage at 4°C (Repeated) - Neutral Protease

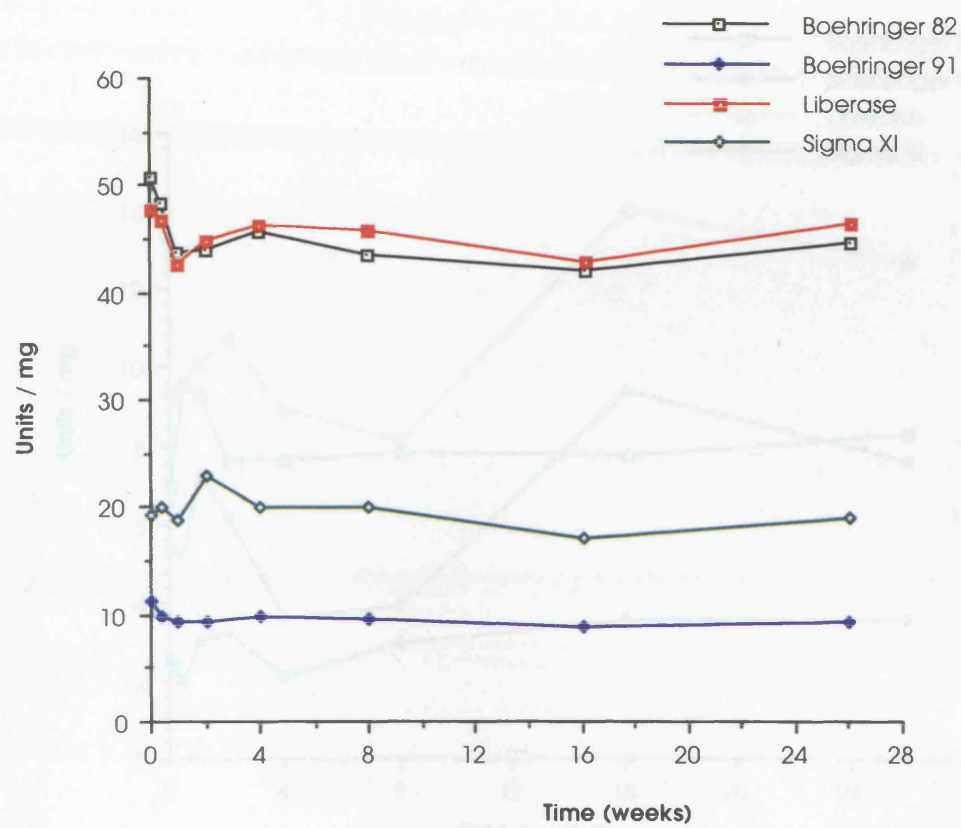


Figure 7.10 **Storage at -20°C (Single) - Class I Collagenase**

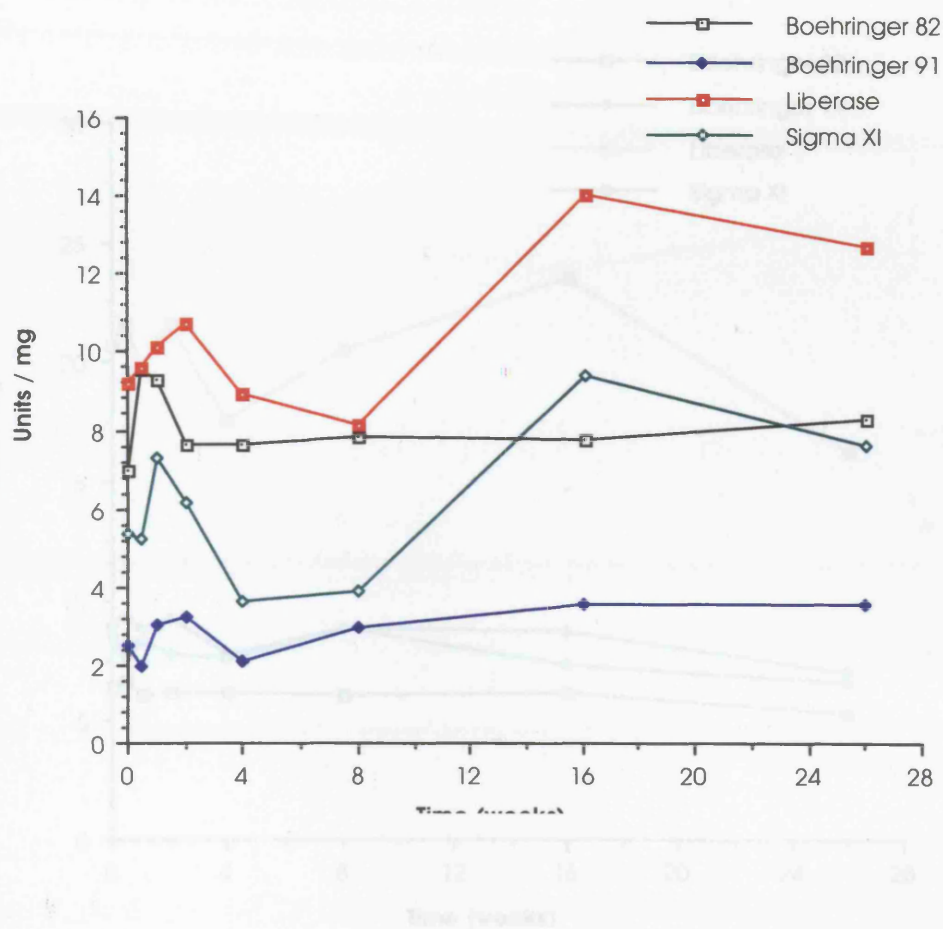


Figure 7.11 **Storage at -20°C (Single) - Class II Collagenase**

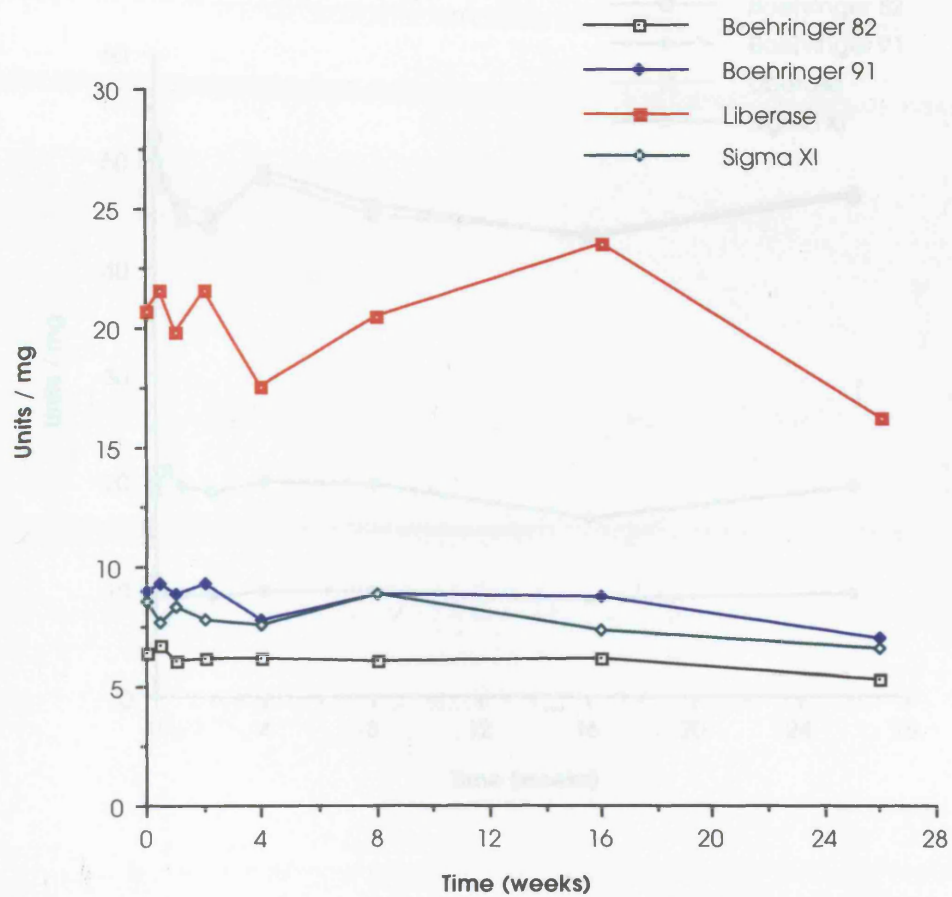


Figure 7.12 Storage at -20°C (Single) - Neutral Protease

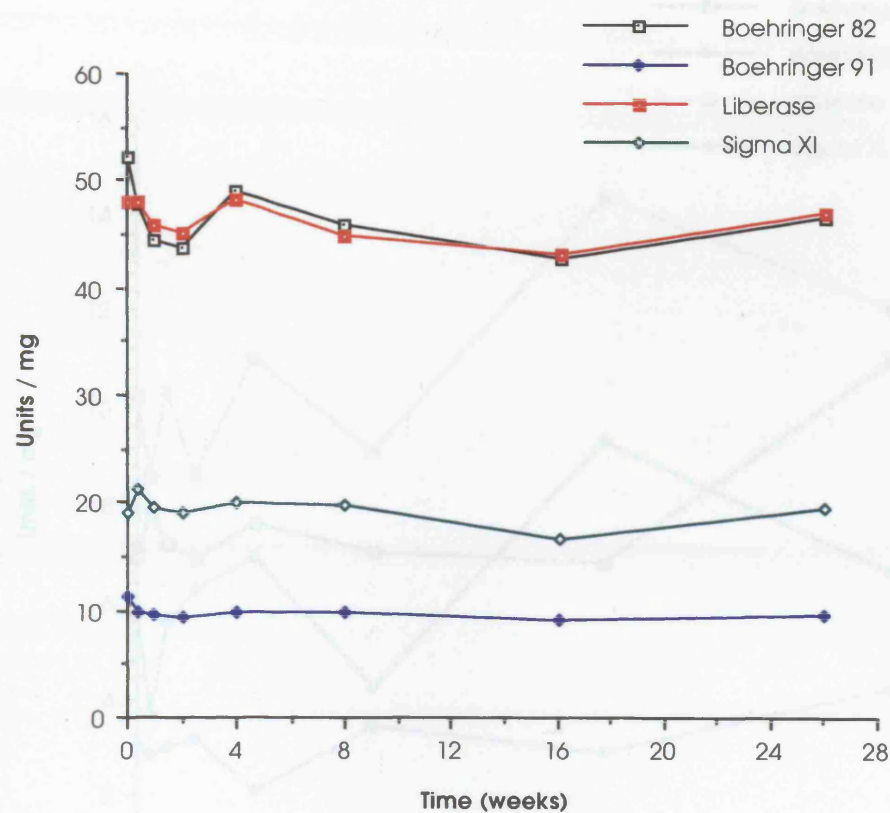


Figure 7.13 **Storage at -20°C (Repeated)**
- Class I Collagenase

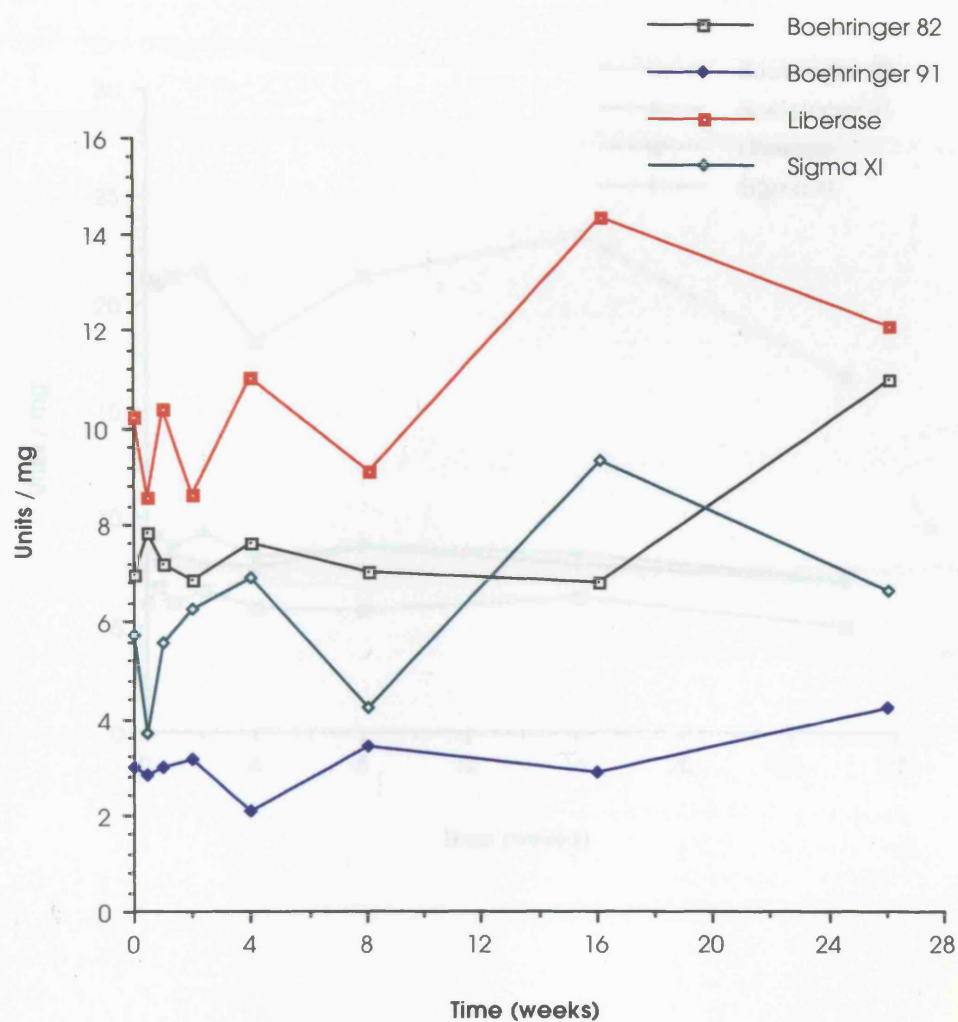


Figure 7.14

Storage at -20°C (Repeated)
- Class II Collagenase

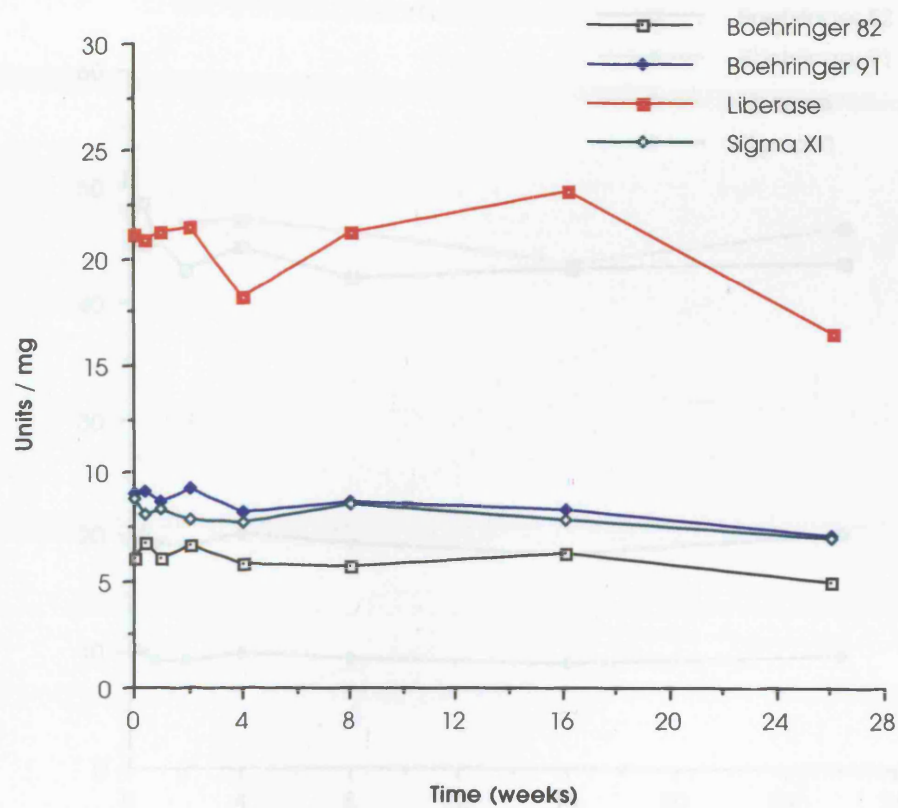


Figure 7.15 Storage at -20°C (Repeated) - Neutral Protease

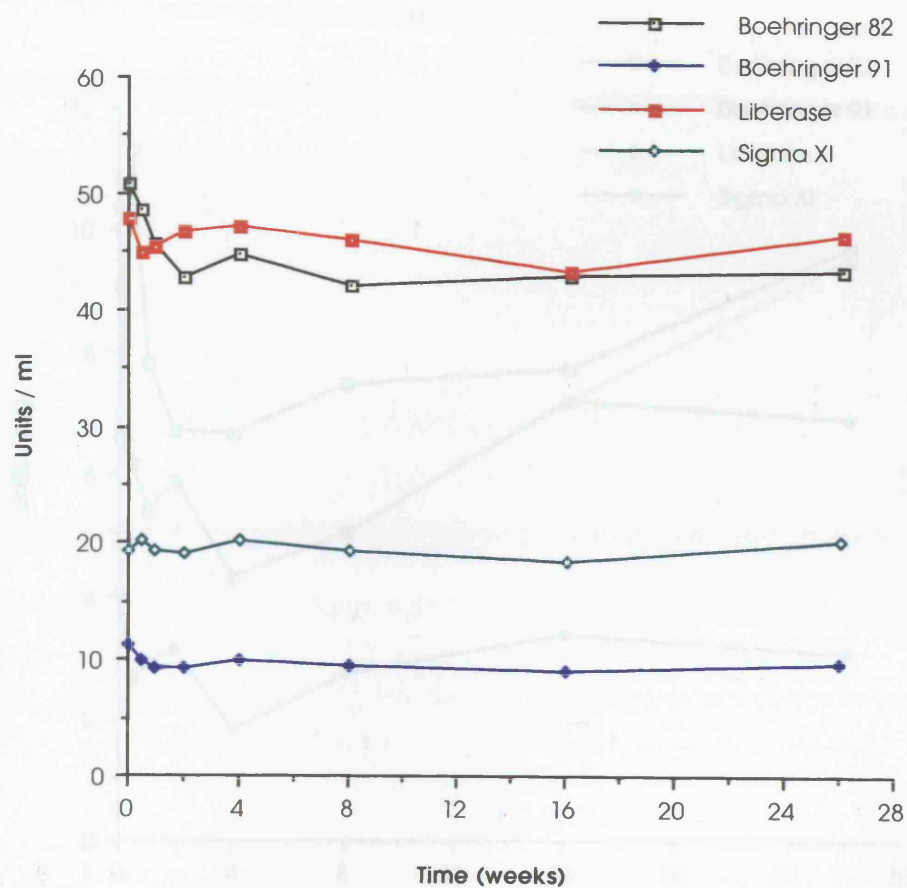


Figure 7.16

Storage at -20°C (Dissolved 1 mg/ml)
- Class I Collagenase

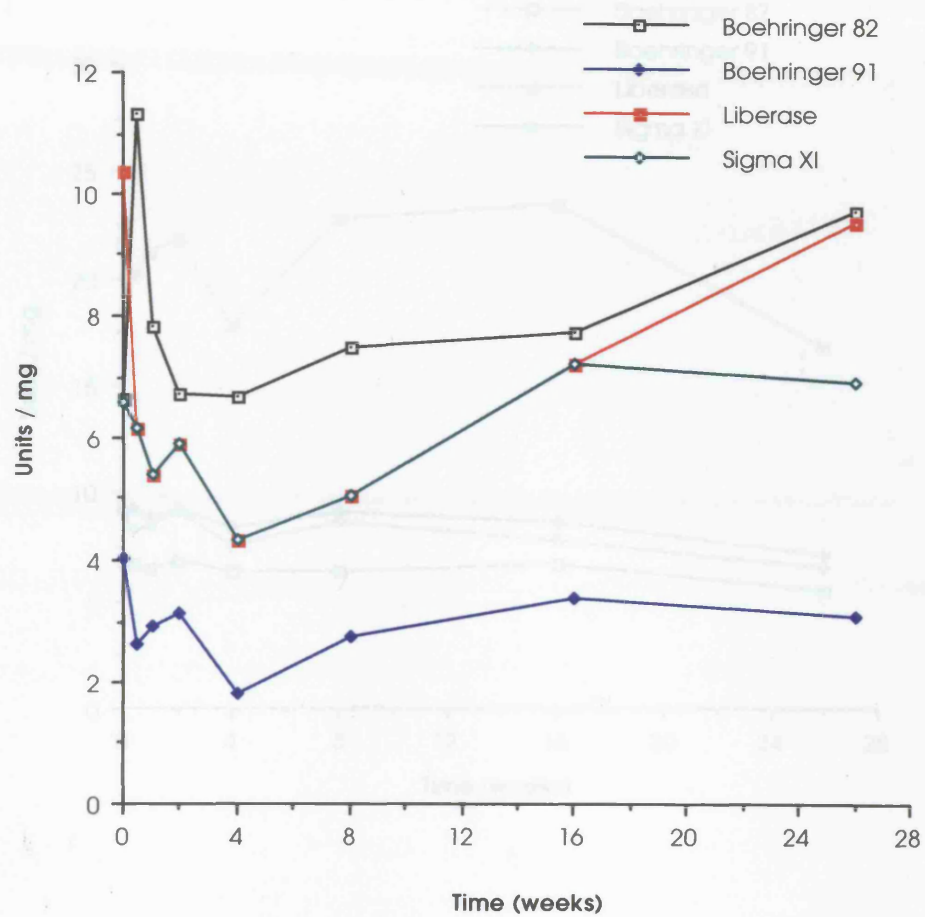


Figure 7.17 **Storage at -20°C (Dissolved 1 mg/ml)**
- Class II Collagenase

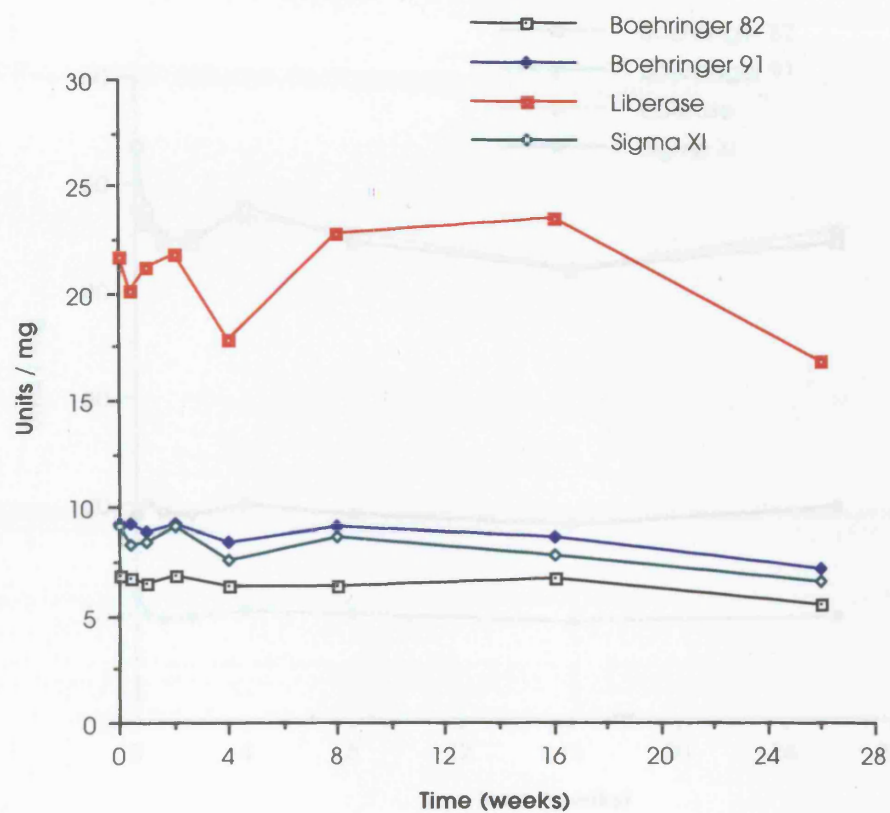


Figure 7.18 **Storage at -20°C (Dissolved 1 mg/ml)**
- Neutral Protease

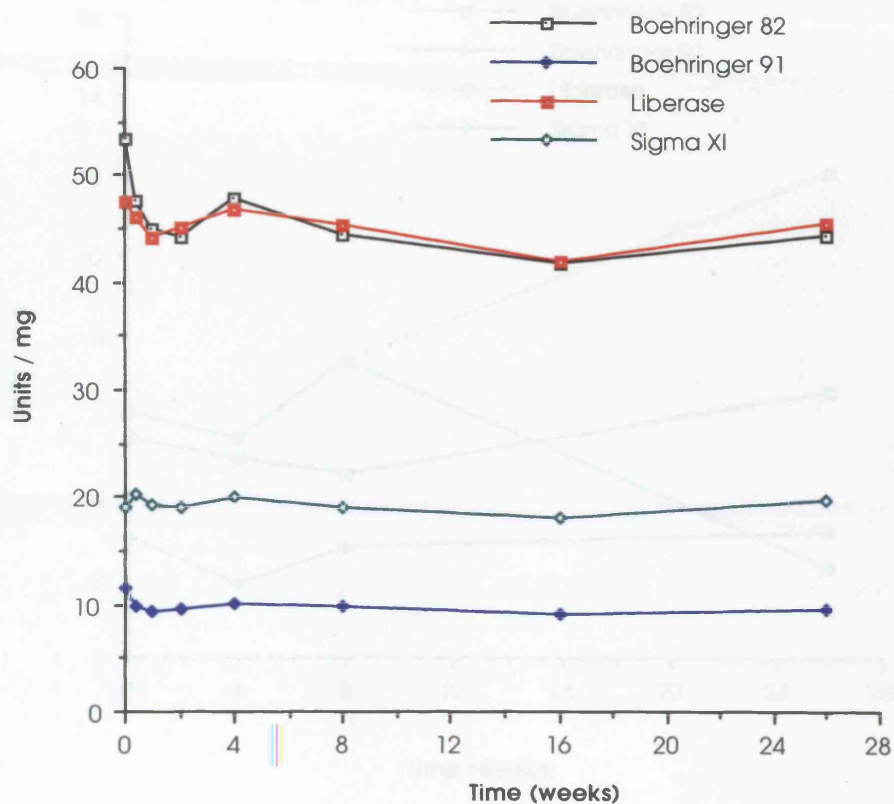


Figure 7.19 **Storage at -20°C (Dissolved 10 mg/ml)**
- Class I Collagenase

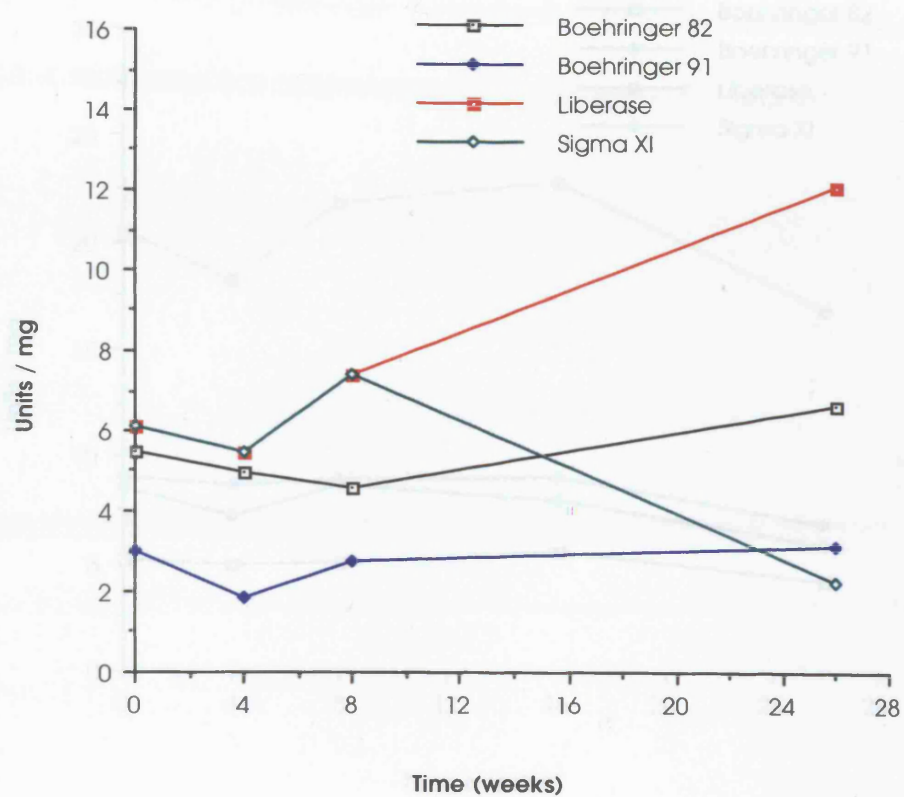


Figure 7.20

Storage at -20°C (Dissolved 10 mg/ml)
- Class II Collagenase

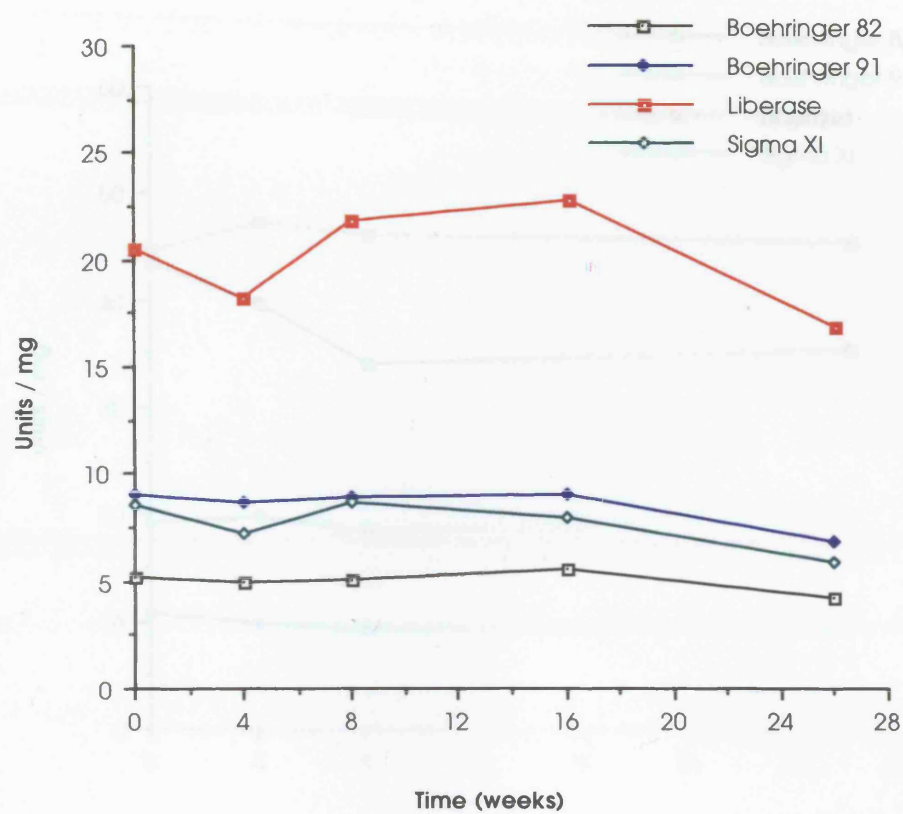


Figure 7.21 **Storage at -20°C (Dissolved 10 mg/ml)**
- Neutral Protease

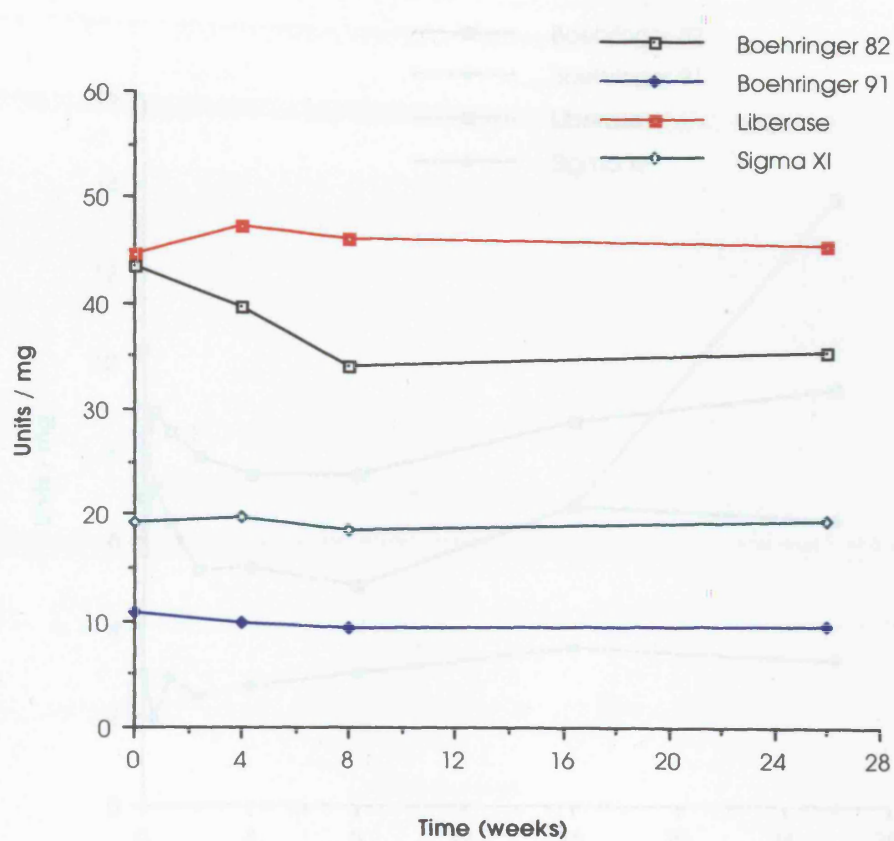


Figure 7.22 **Storage at -80°C (Single) - Class I Collagenase**

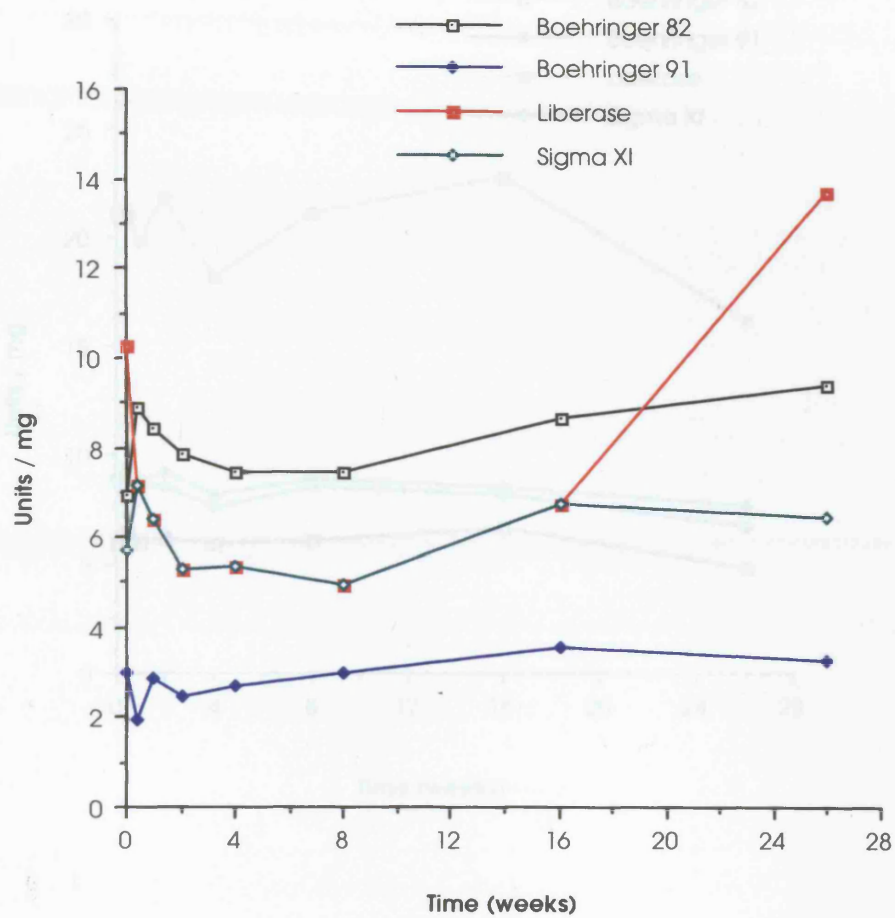


Figure 7.23 **Storage at -80°C (Single) - Class II Collagenase**

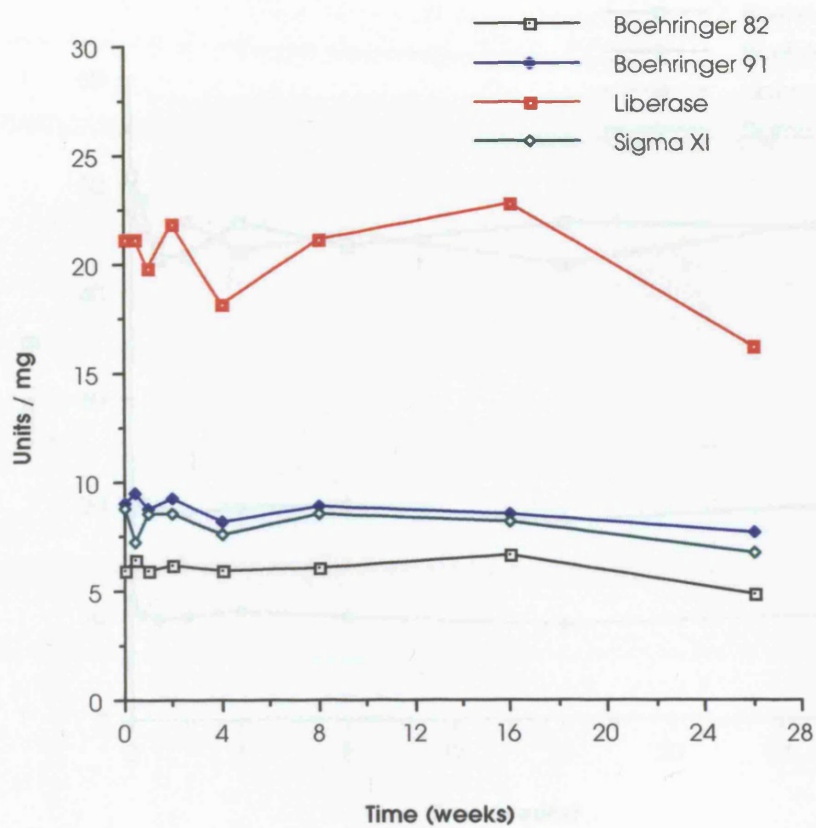


Figure 7.24 Storage at -80°C (Single) - Neutral Protease

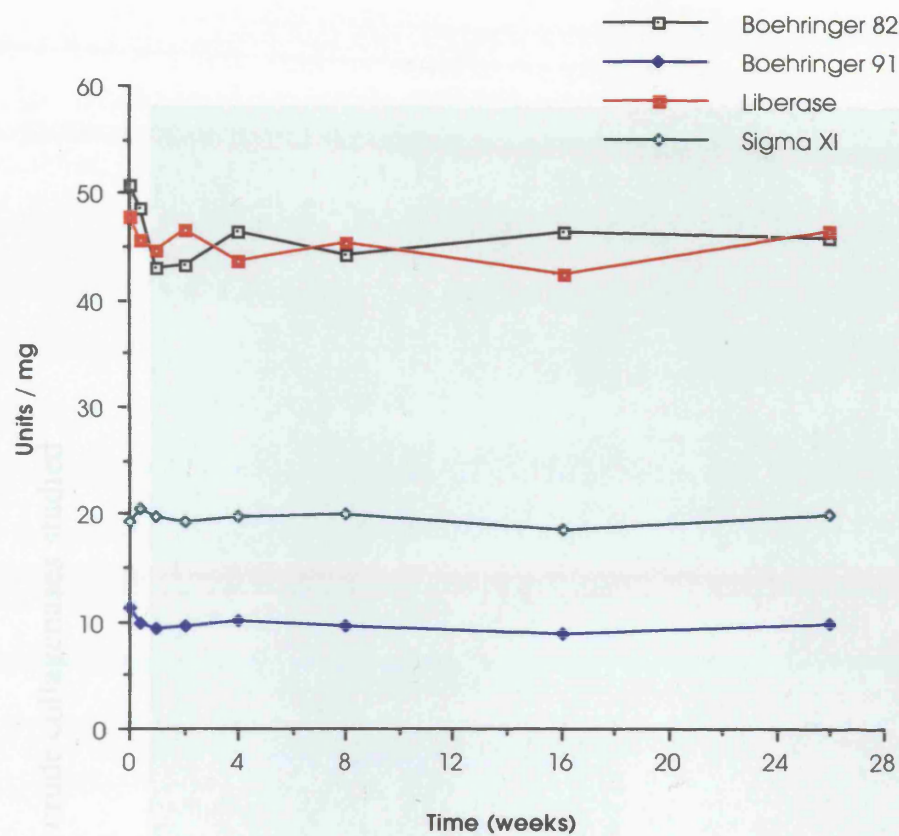
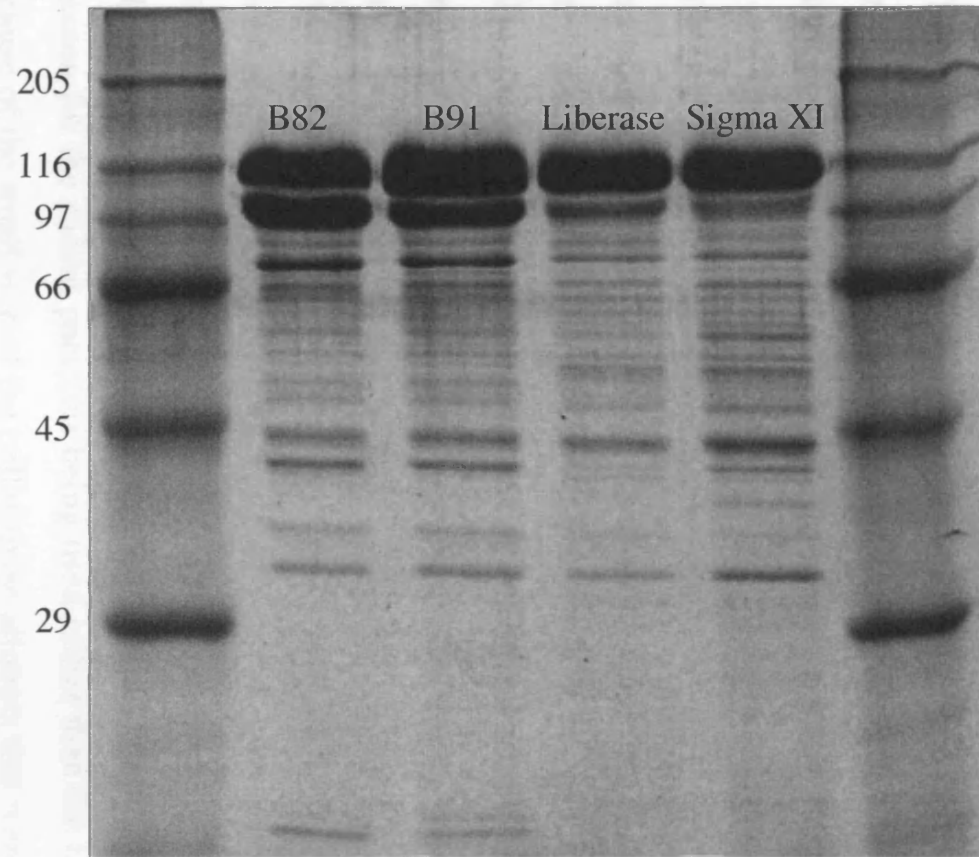


Figure 7.25 SDS-Page of the 4 crude collagenases studied



However, not only did none of the storage conditions confer any stability benefit, but also neither of the 4 different enzymes exhibit any more stability than the others. It is interesting to observe that in these experiments, no product was more stable than the other. Of course it is difficult to make firm conclusions when the exact explanation of the findings is uncertain, but it certainly was envisaged that the simpler, more pure enzyme blends such as *Liberase* would be more stable compounds. Furthermore, the stability recorded for all 4 enzymes stored at room temperature is perhaps the most intriguing. Certainly, each of the experimental samples were kept covered and the laboratory in which the samples stored fairly free of water vapour. Nonetheless it was surprising to find that no significant deterioration in activity was observed for any of the samples stored in this way.

There are three possible explanations for these surprising findings.

First, the results could be artefactual. Clearly this was one of my concerns after no significant deterioration had been observed after the 4 month analysis. However, there are several reasons why this is unlikely. Firstly, all assays were performed in triplicate and the results were consistent. Secondly, several of the samples stored at room temperature were used for islet isolations from rat pancreata after 4 months of storage and all gave good islet yields. This confirmed that the data from the biochemical assays was supported by the ultimate assay of islet isolation, albeit rodent rather than human. The reason for the rodent pancreas being used rather than the human was simply because of the small size of the collagenase aliquots that were being used during the experiments was insufficient for human pancreatic digestion. It is therefore true that these experiments did not evaluate whether the efficiency of human islet isolation had deteriorated during under these storage conditions. Thirdly, it was possible to inactivate the enzymatic activity in all 4 preparations for all 3 assays by simply adding inhibitors to the lyophilised collagenases confirming that the activity recorded during the assays was genuine. Finally, these findings were confirmed by the group in Groningen.

The second explanation for these findings is simply that there was no deterioration for any of these 4 enzymes under any of the 8 variable storage conditions. This may be a valid explanation, but is not a common observation within our laboratory. In addition, it is interesting to note that deterioration of both porcine and human islet isolation was seen in our laboratory during this time period using Boehringer 82 and Boehringer 91 respectively.

Although these aliquots were much larger and this data retrospective, it did suggest that some deterioration in enzymatic activity had occurred for these crude collagenases stored lyophilised at -20 degrees centigrade over this same time period.

The third explanation for these findings is that any deterioration in crude collagenase activity was not reflected in the three enzyme components studied. This is the most likely explanation and does help to postulate what may be important for human islet isolation. In other words, another component / group of components is responsible for crude collagenase instability and may have in fact deteriorated during this time course but was not being measured. In turn, this component may be needed for human islet isolation and not rodent isolation.

Certainly it can be seen from the SDS-PAGE in Figure 7.25 that there are many different factors in crude collagenase that could potentially be implicated in its instability. It can be seen from the last Chapter that the amount of clostripain within crude collagenase varies significantly between batches. It has been suggested that the proteolysis of collagenase samples that occurs during heating is thought to be caused by clostripain in the presence of calcium ⁴⁴⁵. This component in particular warrants further evaluation with regards to collagenase stability.

Conclusions

These detailed studies have shown no significant deterioration in the 3 different enzymatic activities measured for 4 different crude collagenases under 8 different storage conditions over a 6 month period of storage. In particular, none of the storage conditions conferred measurably more stability than the others, and none of the 4 enzymes showed more stability than the others. These experiments would suggest that there may be different components responsible for the deterioration of efficient human islet isolation after periods of storage.

The next section of this thesis addresses some of the issues concerning the optimal administration of crude collagenase into the porcine and human pancreas.

SECTION IV

Collagenase Administration

CHAPTER 8

The Inhibition of Collagenase by University of Wisconsin Solution and its Components

Introduction

As discussed previously, even if a crude collagenase contains the necessary components for optimal islet isolation, the efficient administration of that collagenase into the pancreas is of paramount importance. There are two main aspects to collagenase administration. First, the biochemical nature of the solvent used to dissolve the collagenase, this is the focus of this chapter. Second, the biophysical aspects of administration, including the optimal pressure for delivering the collagenase to the islet - exocrine interface and the temperature / pH of the collagenase during administration. Some aspects of this are investigated in Chapter 9.

University of Wisconsin (UW) solution is one of the most commonly used vascular perfusates during multi-organ donation and is also used extensively as a cold storage solution after organ removal. Although vascular perfusion with UW solution has been successfully used for the cold storage of the human pancreas prior to vascularised transplantation, this approach has proved problematical prior to islet isolation because UW solution appears to inhibit the subsequent collagenase digestion phase of human islet isolation. In addition, attempts to optimise the cold storage of the human pancreas by administering collagenase buffered in UW solution immediately after pancreas excision and prior to transport back to the islet isolation laboratory, have failed. Whilst this approach has proven effective in the rat ⁴⁷⁷, dog ⁴⁷⁷ and pig ^{478, 479}, its application in the human has resulted in very poor islet yields and reduced islet viability ^{480, 481}. The inhibition of collagenase digestion by UW seems therefore to exhibit a species difference.

Aims

The aim of the series of experiments described in this chapter therefore, was firstly to confirm this species difference and then to systematically define the components of UW mediating this effect. This was then followed by more

detailed biochemical experiments to try to determine which components of collagenase were being inhibited by the various components of UW.

1) CONFIRMING THE SPECIES DIFFERENCE IN THE INHIBITION OF COLLAGENASE-DIGESTION BY UW SOLUTION

Materials and Methods

Human pancreata were obtained with appropriate consent from brain-dead, heart-beating donors. All human pancreata underwent vascular perfusion with 3-6 litres of hyperosmolar citrate (HOC, Travenol, Thetford, UK.). The pancreata were transported to the laboratory in HOC at 4°C with a cold-ischaemia time of 2-4 hours. Porcine pancreata did not undergo vascular perfusion and were obtained from a local abattoir with a warm ischaemia time of 5-8 minutes and a cold ischaemia time of 20 - 30 minutes and transported in HOC at 4°C. In this preliminary series of experiments a simplification of the 'in vitro' method described in Chapter 5 was used. Blocks of human or porcine pancreas measuring approximately 0.5 cm³ were placed into 2ml of either Hank's solution (Northumbria Biologicals, Cramlington, UK) or UW (Dupont Pharmaceuticals, Letchworth Garden City, Herts, UK) in a 10 ml test-tube and pre-incubated for 5 minutes at 37°C. Collagenase (Pan Plus 03021C, Serva Feinbiochemica, Heidelberg, Germany), was dissolved in either Hanks or UW solution at a concentration of 8mg/ml and 2 ml of this was added to each tube, giving a final enzyme concentration of 4 mg/ml. The test-tubes were placed into a water-bath at 37°C for the human or 35°C for the pig and every 5 minutes they were manually shaken for 10 seconds and macroscopically examined by two independent observers. The reason that macroscopic evaluation was chosen was that the previous experiments on UW inhibition had shown a dramatic 'all or nothing' effect. Therefore, the more

subtle parameters of islet cleavage, cleavage index, and islet fragmentation were felt to be unnecessary.

The progression of the digestion process was monitored by the macroscopic appearance of fragmented tissue in the supernatant and scored between + and +++ depending upon the amount of tissue seen. The time at which ++ of fragmented tissue appeared was noted for both the UW and Hanks' tubes and this time was used to compare the rate of digestion, and expressed as a median ratio (i.e. time for ++ UW / time for ++ Hanks'). The tubes were observed for a maximum of 100 minutes. Seven human and seven porcine pancreata were studied.

Results

The median (range) Hanks' digestion time for the pig was 36 (25-40) mins and 35 (25-45) mins for the human. The median (range) UW digestion time was 39 (25-50) mins for the pig. All the tubes containing human pancreas in UW did not reach the ++ stage of digestion before 100 minutes.

The median ratio digestion time of UW compared with Hanks' was therefore 1.1 for the pig and >2.7 for the human ($p=0.001$ using paired Wilcoxon Test).

Discussion

The data of these initial experiments confirms that UW markedly inhibits the collagenase digestion of the human but not the porcine pancreas. As crude collagenase derived from *Clostridium histolyticum* is a mixture of many different components⁴⁸⁶, each with different specificities towards different substrates, it can be postulated that UW may inhibit components of the crude collagenase that are important for the isolation of human islets but that are not required for porcine islet isolation. The requirements for different

components may be related to the different composition of the pig and human pancreas. It has been shown that the pancreas of the two species have very different peri-insular extra cellular matrices ³⁸¹. In the pig, very little peri-insular capsule is present, and the structural integration of the porcine islets depend almost exclusively on cell-to-cell adhesion. In the human, however, cell-to-matrix adhesion is predominant. The only other major difference between the pancreata in our experiment was the fact that the human pancreata had undergone vascular perfusion with HOC whereas the porcine pancreata had not. However, the experimental design i.e. using blocks of pancreas rather than whole ones, and bathing the blocks in collagenase rather than using collagenase distension, makes this difference an unlikely explanation for the observed differences in inhibition. The results of these experiments further support the notion that porcine and human collagenase pancreatic digestion are intrinsically different.

2) DEFINING WHICH COMPONENTS OF UW SOLUTION ARE RESPONSIBLE FOR COLLAGENASE INHIBITION.

A series of experiments were then performed in order to define which of the individual components and combinations of components within UW solution are responsible for the inhibition of collagenase digestion of the human pancreas.

Materials and Methods

This series of experiments were confined to human pancreata. The same methods were used as for the initial experiments. As before, the digestion time of the solution being studied was compared with that of Hanks' and UW. The components of UW and Hanks' are listed in Table 8.1 and the

experimental solutions are shown in Table 8.2. The concentrations of the UW components in the experimental solutions were the same as in UW itself. All of the UW components were dissolved in Hanks' except for the solutions containing the UW electrolytes. Hanks solution contains electrolytes with a high sodium and low potassium and in order to study the effect of the electrolytes in UW, a UW electrolyte solution was prepared with a low sodium-potassium ratio. The solution containing calcium was also added to the UW electrolyte solution, in order to see if the addition of calcium to UW affected collagenase digestion. The amount of calcium that could be added was limited by the presence of phosphate in the aqueous UW electrolyte solution resulting in calcium phosphate precipitating out of solution. These experiments were performed on 7 human pancreata.

Results

The results of these experiments are summarised in Table 8.2. The median ratios are given together with the ranges for all the test solutions.

The p-values were calculated using a paired Wilcoxon test and refer to the median ratio digestion compared with Hank's. Whilst all the individual components of UW inhibited collagenase in the human to some extent, the most inhibitory individual components (i.e. median ratio ≥ 1.5) were magnesium, allopurinol, the Na⁺ /K⁺ ratio, hydroxyethyl starch (HES), and adenosine. Allopurinol in combination with either lactobionate or glutathione was markedly inhibitory (i.e. median ratio 1.8 and 1.9 respectively). The most inhibitory solution tested was a combination of the three components raffinose, glutathione and lactobionate (median ratio 2.1). This combination was almost as inhibitory as UW itself (median ratio 2.7).

<u>Component</u>	<u>UW (mM)</u>	<u>Hank's (mM)</u>
Na ⁺	20	141
K ⁺	140	6.6
Lactobionate	100	0
Chloride	0	138
Raffinose	30	0
MgSO ₄	5	0.09
H ₂ PO ₄	25	3.8
Ca ²⁺	0	1.26
Glucose	0	5.4
Glutathione	3	0
Allopurinol	1	0
Hydroxyethyl starch	50g/L	0
Bicarbonate	0	4
Adenosine	5	0

Table 8.1. A comparison of the composition of University of Wisconsin and Hank's solutions.

Solution	Median Ratio	Range	P-value
Hank's	1.0		
Hank's + Raffinose	1.2	1 - 1.4	0.08
Hank's + Lactobionate	1.3	0.9 - 1.6	0.02
Hank's + Glutathione	1.4	1.3 - 1.5	0.02
Hank's + Lactobio. + Raffin.	1.4	1.0 - 2.2	0.02
Water + UW electrolytes + Ca ²⁺	1.4	1.2 - 1.7	0.02
Hank's + Glutathione + Raffinose	1.5	1.1 - 2.2	0.02
Hank's + Allopurinol	1.5	1.3 - 2.2	0.02
Hank's + Magnesium	1.5	1.2 - 1.6	0.02
Water + UW electrolytes	1.6	1.3 - 1.8	0.02
Hank's + Allopurinol + Raffinose	1.6	1.0 - 2.2	0.02
Hank's + Lactobio. + Glutathione	1.6	1.1 - 1.7	0.02
Hank's + Hydroxyethyl starch	1.6	1.4 - 1.8	0.02
Hank's + Adenosine	1.7	1.3 - 2.0	0.02
Hank's + Allopurinol + Lactobio.	1.8	1.3 - 2.2	0.02
Hank's + Allopurinol + Glutathio.	1.9	1.6 - 2.3	0.02
Hank's + Raff. + Lacto. + Glutath.	2.1	1.6 - 2.5	0.02
University of Wisconsin solution	2.7	2.4 - 3.4	0.02

Table 8.2. Results of human inhibition experiments. The p-values were calculated using a paired Wilcoxon test and refer to the median ratio digestion compared with Hank's (n=7).

Discussion

As has been outlined in Chapter 3, as zinc metalloenzymes, the collagenases present within the crude collagenase produced by *Clostridium histolyticum* require calcium ions as cofactors for their hydrolytic activities ⁴¹⁸. In theory therefore, the inhibitory effect of UW in these experiments could be explained in part by the fact that it contains no calcium. However, these experiments also show that the addition of calcium to a solution containing the electrolytes of UW was not significantly less inhibitory (median ratio 1.4) than the UW electrolytes alone (median ratio 1.6). This is consistent with the findings of Dono et al. ⁴⁸² who have noted that although rat islets tend to disperse to a small cluster when no calcium is added to the collagenase-UW solution, the exocrine pancreatic tissue was well digested and dispersed. Indeed, the beneficial effect of calcium in their study was not due to an increase in collagenase activity, but by the prevention of islets from becoming dispersed into single cells, as calcium plays an important role in cell-to-cell and cell-to-extra cellular matrix adhesion ⁴⁸³.

The most likely explanation for the minimal effect on collagenase activity is that whilst collagenase requires calcium as a co-factor, it requires it in its "bound" rather than "free" form. I have found that commercially available crude collagenase contains a significant amount of calcium when dissolved in distilled water (see Table 8.3). Once the collagenase molecule has 'filled' its calcium binding sites, the presence of additional calcium in the cold storage solution does not significantly increase the collagenase activity. However, Burgmann et al.⁴⁸⁴ have shown that UW has a considerable ability to chelate calcium and it may be this chelation, rather than the absence of calcium, that explains the inhibitory properties of UW. Their study found that phosphate and to a lesser extent lactobionate were the two main components responsible. It is also known that reduced glutathione binds divalent metal ions ⁵⁴¹. This is consistent with the findings in this study, that

<u>Test solution</u>	<u>Ca²⁺ (mmol)</u>
Distilled Water	0.18
Boehringer 63	0.89
Boehringer 64	0.69
Boehringer 80	0.98
Boehringer 82	0.89
Boehringer 84	0.96
Boehringer 85	0.98
Serva 16063	0.50
Serva 2025	0.30

Table 8.3. Measured calcium concentrations in different batches of crude collagenase dissolved in distilled water.

lactobionate and glutathione were inhibitory both individually (median ratio 1.3 and 1.4 respectively) and when in combination (median ratio 1.6). These components therefore, could inhibit collagenase, either by removing the 'bound' calcium ions from their binding sites in addition to cheating any 'free' calcium available, or by sterically hindering the interaction of the 'bound' calcium and zinc ions.

The inhibition observed by magnesium could be explained by an exchange between the 'bound' calcium ions and the 'free' magnesium ions. This competitive binding of the magnesium ions would cause a conformational change within the collagenase binding site leading to an inability for it to recognise and bind to collagen within the pancreatic matrix.

HES markedly inhibits the collagenase digestion of the human pancreas (median ratio 1.6). It is a high molecular mass colloid (250 kDa) that could block the active site or alter the configuration of the collagenase molecule, thereby decreasing its activity. The precise chemical basis for the inhibitory effect of the other inhibitory components and the mechanism for the synergistic inhibition by combinations of components e.g. allopurinol added to either lactobionate or glutathione, or raffinose added to both in combination, has not been fully elucidated. However, it is interesting to note, that we have previously shown that the Na^+ / K^+ ratio, adenosine, allopurinol, and glutathione, all of which markedly inhibit collagenase digestion either individually or in combination, are not essential for the cold-storage of pancreatic digest prior to islet purification ⁵⁴². We have also shown in previous studies that although the presence of a colloid is important for optimal islet purity, this does not have to be hydroxyethyl starch ⁵⁴³.

In summary, these experiments suggest that the most inhibitory individual components are magnesium, allopurinol, the Na^+ / K^+ ratio, HES and adenosine. Combinations of components increase the inhibitory effect and the most inhibitory combination is raffinose, lactobionate and glutathione. Whilst the basis of inhibition can be explained for several components, the precise mechanisms for the others remains to be elucidated. Although in these

experiments the collagenase was not administered via the intraductal route, the design of the experiments was a qualitative assessment of the interactions between crude collagenase, UW and its components, and pancreatic tissue, rather than a quantitative assessment of islet isolation. Therefore, these findings are also applicable to the intraductal situation. The results of these experiments taken in conjunction with previous work performed in our laboratory ^{542, 543} are important for the design of effective cold storage solutions that do not inhibit collagenase digestion.

3) THE EFFECT OF UW SOLUTION ON THE CLASS I COLLAGENASE, CLASS II COLLAGENASE, AND NEUTRAL PROTEASE ACTIVITY WITHIN CRUDE COLLAGENASE

The results of the previous experiments led me to further elucidate the biochemical basis for the inhibition of collagenase digestion of the human pancreas and to determine more precisely the components of UW responsible. The aim of the third series of experiments therefore, was to evaluate the influence of UW solution, Tris Buffer, and Hanks solution on the three major components of crude collagenase class I collagenase, class II collagenase, and neutral protease.

Materials and Methods

Samples of crude collagenase (Boehringer P lot 64) were dissolved in a concentration of 1mg / ml in either UW solution, Hanks' solution or the single component buffer Tris / HCL pH 7.4. Protein concentrations for each sample were determined by the method of Bradford using Bio-Rad reagent as described in the last chapter. This involved the creation of a calibration curve using 2 mg / ml crystalline bovine serum albumin (BSA) (Sigma Chemical

Co) dissolved in 50mM Tris/HCl as a protein standard. A calibration constant of 0.0614 was calculated. The protein concentration of the crude collagenase samples was 0.62 mg / ml.

Each of these samples were then assayed for class I collagenase using the radiolabelled collagen assay, class II collagenase activity using the modified 2-furanacryloyl-L-leucyl-glycyl-L-prolyl-L alanine (FALGPA) assay, and neutral protease using the radioactive casein assay. The methods for all these assays are given in Chapter 6. It was confirmed at the beginning of these experiments that none of the three test solutions inhibited the assay substrates in the absence of collagenase.

Results

In these initial experiments, none of the three test solutions showed significant inhibition of either the class I collagenase activity as evaluated by the radioactive collagen assay or the neutral protease activity as measured using the radioactive casein assay (see Table 8.4). However, the class II activity was found to be significantly inhibited in the samples dissolved in UW (see Table 8.5). This inhibition of class II was not seen in the samples dissolved in either Hanks' solution or Tris/HCL. The experiments on class II collagenase were repeated using dialysed enzyme to confirm that the experiments were reproducible using more purified enzyme. Dialysis was performed against 50mM Tris / HCl, pH 7.4. The results for the FALGPA assay with dialysed enzyme confirmed the results of that with undialysed (mean activity = -18.40 μ moles/min/mg protein).

	<u>[¹⁴C] Collagen Assay</u> (mean c.p.m)	<u>Casein Assay</u> (mean c.p.m)
<u>Hank's</u>	3080	13,855
<u>Tris/HCl</u>	2883	12,070
<u>UW</u>	2710	14,808

Table 8.4. The results of the radioactive collagen and radioactive casein assays for dialysed Boehringer 64 enzyme dissolved in Hank's, Tris / HCl, and UW.

<u>Buffering Solution</u>	<u>Mean Specific Activity</u> (μ moles/min/mg protein)
Hank's	16.8
Tris/HCl	3.4
UW	-29.2

Table 8.5. Mean specific activities of class II collagenase within crude collagenase dissolved in different buffering solutions.

Discussion

These results demonstrate that University of Wisconsin solution inhibits class II collagenase, a key component of crude collagenase. This inhibition does not appear to occur for the other two components of collagenase tested, namely class I collagenase or neutral protease. None of the assays demonstrated inhibition when the crude collagenase was dissolved in either Hank's solution or the buffer Tris / HCl pH 7.4.

Putting this data together with the data from the first experiments described in this chapter, it can be postulated that class II collagenase is more essential for human rather than porcine islet isolation. This might be explained by the different structures of the pancreatic extracellular matrices of the two species. Both human and rodent pancreata contain higher levels of collagen and laminin than the porcine pancreas ⁵⁴⁴ Certainly class II collagenase has been shown to be essential for efficient rat islet isolation ⁵⁴⁵ and seems to be responsible degrading the collagen fibrils that have previously been denatured by class I collagenase. Whether this is also the case for human islet isolation remains to be elucidated.

However, great caution must be taken in making such a postulation into a firm conclusion. It must be remembered that there are numerous other components within crude collagenase that have not been investigated. Some of these components may well be more significantly inhibited by UW solution than class II collagenase and it may be the inhibition of these other components that accounts for the inhibition of human pancreatic collagenase digestion by UW. In addition, it may be that the inhibition of class II collagenase upsets the balance of the different components within the crude collagenase rather than is the cause of digestion inhibition itself.

4) INVESTIGATION OF THE COMPONENTS OF UW **INHIBITING CLASS II COLLAGENASE**

The aim of the final series of experiments was to determine which components of UW are responsible for the inhibition of class II collagenase activity as measured by the optimised FALGPA assay.

Materials and Methods

The effects of individual components of UW were then studied in order to elucidate which components were responsible for this inhibition. Molar solutions of the individual components of UW were prepared by adding the solid component to Tris/HCL pH 7.4 in the concentrations in which they are found in UW itself. Incidentally, Tris/HCL was used at a pH of 7.4 throughout this chapter, because it has been shown that this buffer can inhibit collagenase activity at pH's of greater than 7.5 ⁴⁸⁴.

The pH of each test solution was tested throughout the experiments to ensure that this did not change and therefore in itself inhibit collagenase activity. A spectrum of all stock solutions was taken before the assays were performed to determine whether these absorbed at 254 nm (the optimal FALGPA Absorption wavelength). Any absorption could have resulted in inaccurate readings. The only component that did absorb at this wavelength was allopurinol. However, on further evaluation by the addition of collagenase to allopurinol and the Tris / HCl buffer, and recording over 6 minutes, no changes in absorption were observed. Subsequent evaluation confirmed that the absorption of allopurinol was constant and therefore the change in absorption would accurately reflect the degradation of FALGPA.

Results

The effects of the individual components of UW on class II collagenase activity are given in Table 8.6. Those components that inhibited the class II activity by more than 10% were considered to be significantly inhibitory. It can be seen that the components causing significant inhibition were: Na⁺ (-17.4%), H₂PO₄ (-51.5%), adenosine (-474.5%), raffinose (-14.2%) and HES (-11.1%).

Discussion

This study helps to explain the biochemical basis for the inhibition of collagenase digestion of the human pancreas by UW. The inhibition of collagenase by Na⁺, adenosine and HES supports the findings of the initial series of experiments. In addition class II collagenase was significantly inhibited by H₂PO₄ and raffinose. The inhibition of collagenase by H₂PO₄ can be explained by its considerable ability to chelate calcium which is exposed in the class II collagenase molecule and is required as a co-factor for collagenase activity ⁴⁸⁴. The precise chemical basis for the inhibitory effect of raffinose is uncertain. However, it is important to re-emphasise, that the inclusion of neither the UW Na⁺ / K⁺ ratio, adenosine, nor HES, all of which markedly inhibit class II collagenase activity, are essential for the cold-storage of porcine pancreatic digest prior to islet purification ^{542, 543}. The results of this current study further aid the design of effective cold storage solutions that do not inhibit the collagenase digestion of the human pancreas.

<u>UW Components</u>	<u>Mean Activity</u>	<u>Reference Activity</u>	<u>% Difference</u>
Na ⁺	8.9	10.8	-17.4
K ⁺	12.5	10.8	+15.8
H ₂ PO ₄	5.2	10.8	-51.3
MgSO ₄	10.4	10.8	-3.0
Allopurinol	6.6	5.4	+23.3
Adenosine	-25.5	5.4	-474.5
Raffinose	7.1	8.3	-11.1
HES	7.4	8.3	-14.2
Glutathione	8.8	8.9	-1.8

Table 8.6. Changes in activity during FALGPA assay with different components of UW. All activities are expressed as $\mu\text{moles/minute/mg}$ of protein.

Conclusions

The experiments outlined in this chapter have confirmed that UW inhibits the collagenase digestion of human but not porcine pancreata. The most inhibitory components confirmed both in the macroscopic experiments and also the biochemical studies are the Na^+ / K^+ ratio, Adenosine and HES. The biochemical assays have demonstrated that it is the class II collagenase within crude collagenase that is being inhibited and these same experiments confirmed that the same components within University of Wisconsin are responsible. The results of these experiments may help to determine an ideal cold storage solution for the human pancreas that confers cellular preservation during and after pancreatic excision, and yet does not inhibit the subsequent collagenase digestion phase of islet isolation. The next chapter addresses the issue of optimising the delivery of collagenase to the islet-exocrine interface.

CHAPTER 9

The influence of distension pressure during collagenase administration on the distribution of collagenase within the porcine pancreas

Introduction

Even if the composition of crude collagenase and its solvent is optimal, accurate administration of collagenase to the islet-exocrine interface, together with distribution of collagenase to all regions of the pancreas, is vital if efficient human islet isolation is to be achieved. In addition, the collagenase must be prevented from entering the islets in order to prevent digestion of the islets themselves.

As has been previously outlined, there are two main methods currently used for the administration of collagenase into the pancreatic duct. These are the single injection or loading technique ⁴¹¹ and the perfusion method ^{463, 464, 465}. The loading method has been used routinely in our department. Several studies have suggested that even at very low loading pressures, collagenase can enter the cells of the islet ^{466, 467, 468}. However, all these studies used Indian ink in order to study the distribution after administration. One of the main criticisms of such studies is that using Indian ink which is particulate, may produce different loading characteristics from that of collagenase.

Although the intraductal method of collagenase administration may inevitably cause some collagenase to enter the islets due to the pancreatic anatomy ⁴⁶⁶, it should be possible to determine a pressure that optimises collagenase delivery to the islet / acinar interface, whilst minimising intra-islet collagenase administration.

Aims

The aim of the pilot studies described in this chapter therefore, was to investigate the influence of different loading pressures on the distribution of intraductally administered crude collagenase in the porcine pancreas

All the studies described in this chapter involved porcine rather than human pancreata due to the large number of pancreata required for these pilot studies.

1) EVALUATION OF AVAILABLE STAINING METHODS

Before the intrapancreatic distribution of collagenase could be studied, it was important to evaluate and develop staining methods that enabled accurate detection of intraductally administered collagenase within the pancreas and that avoided the limitations of Indian ink described above.

Materials and Methods

i) Testing of conventional staining techniques

The starting point was to evaluate simple tinctorial methods. Blocks of porcine pancreas were taken from the splenic lobe of abattoir obtained pancreata with a warm ischaemia time of 5-8 minutes and a cold ischaemia time of 40-50 minutes. These pancreata had not undergone 'in-situ' vascular perfusion and had been transported back to the laboratory in hyperosmolar citrate at 4°C. The pancreatic blocks were then incubated in crude *Clostridium histolyticum* collagenase (Boehringer Mannheim P lot 64) at a concentration of 3mg / ml for increasing periods of time. Digestion was stopped by placing the blocks in cold formal saline. The pancreatic blocks were then processed and placed in paraffin wax and 4µm sections cut. These sections were then stained using the standard haematoxylin and van Gieson's staining methods as follows: The sections were stained in Weigert's haematoxylin for 15 minutes after which they were rinsed briefly in 1% acid alcohol to remove background staining, followed by rinsing with water. They were then stained in van Gieson solution for 2-5 minutes, after which they were blotted dry and quickly dehydrated, cleared and mounted.

Results

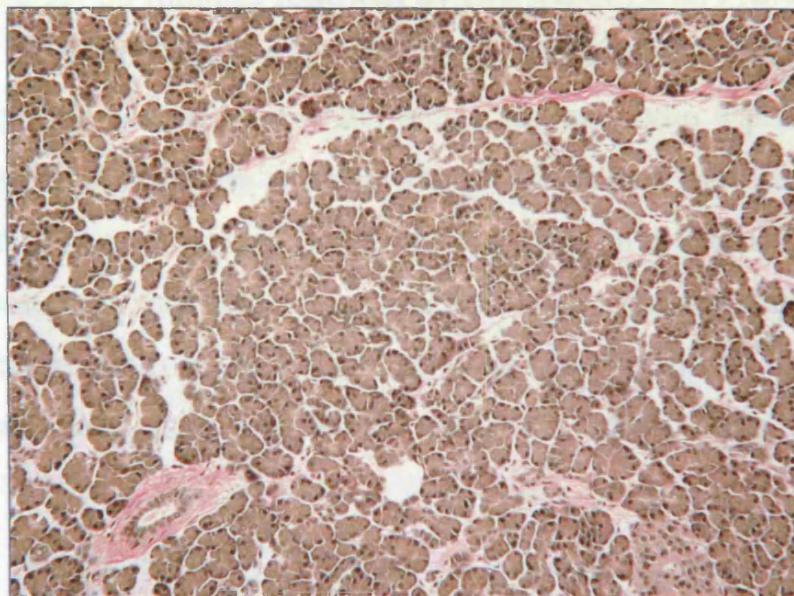
Although the effect of the collagenase activity could be seen after about 1 hour of incubation, the resulting staining was too non-specific to clearly demonstrate the distribution of collagenase effectively. In particular, the collagenase was seen clearly within the ducts of the pancreas but was obscured by the van Gieson stain in the parenchyma of the pancreas (see Figure 9.1).

ii) Testing of FITC conjugated collagenase

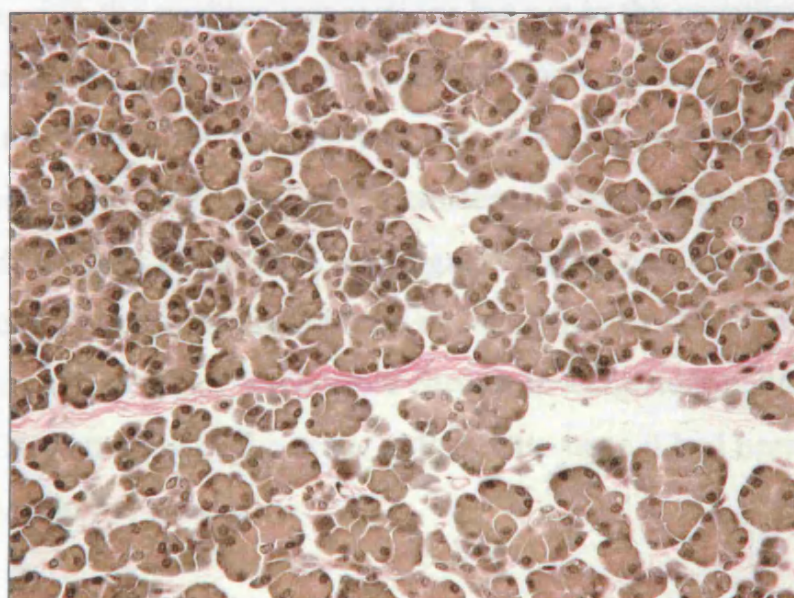
This method made use of the fluorescent stain FITC. Crude collagenase (Boehringer P lot 64) was conjugated to FITC and then injected intraductally into the splenic lobe of porcine pancreata obtained from an abattoir as described above. The blocks were fixed, processed and cut as above. The sections were then treated as follows to determine whether any fluorescence remained within the tissue: The sections were first treated in xylene for 5 minutes. This was then repeated. Following this they were treated twice in 99% alcohol each for 3 minutes. Then they were treated for 3 minutes in 95% alcohol for 3 minutes followed by a further 3 minute wash in water. The sections were then mounted and viewed under the fluorescence microscope.

No fluorescence was seen in any of the sections. The methodology was therefore modified for a light microscope. The same methods were used as for the previous experiments. However, following the washing stage, the sections were trypsinized at 37% for 25 minutes. They were then further washed in water for 2 minutes. This was followed by the addition of

Figure 9.1 Van Gieson Staining
Collagenase is seen clearly within pancreatic ducts but
poorly within parenchyma



X20



X40

hydrogen peroxide for 10 minutes, after which the sections were washed in water for 2 minutes and the buffer P.B.S. for a further 2 minutes. A 1 in 10 solution of normal rabbit serum was then applied for 10 minutes after which the sections were drained rather than washed. A 1 in 50 solution of anti-FITC was then applied for 30 minutes followed by two washes with P.B.S. for 15 minutes each. Diaminobenzidine (DAB) was then added for 5 minutes followed by a 5 minute wash with water before haematoxylin was added for 30 seconds before a final 2 minute wash in water. All washes were carried out at room temperature in approximately 300 ml of buffer on a magnetic stirrer. The sections were then mounted. A second set of experimental slides were also processed but the sections in this second set were not trypsinised. Negative controls were also processed in which the anti FITC antibody was omitted.

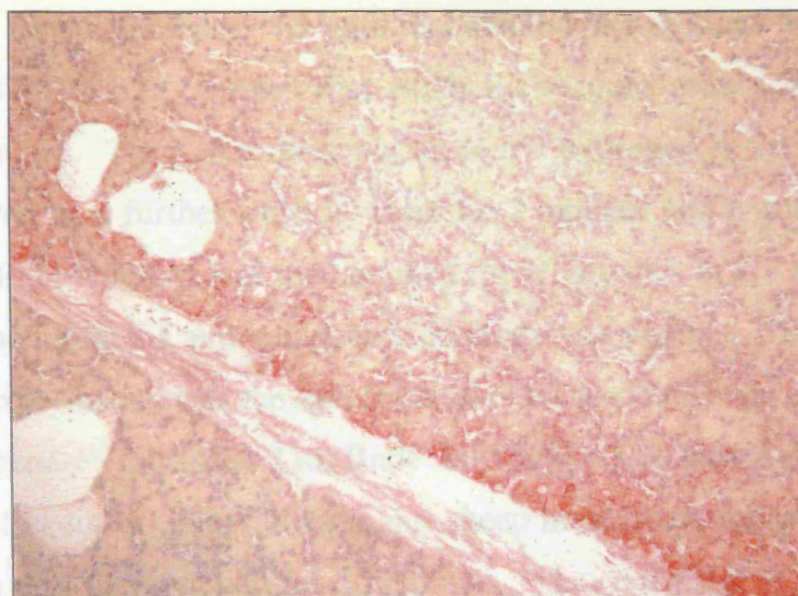
Results

Staining was seen in the positive test sections but not in the negative controls. However, the accurate assessment of intraislet collagenase was difficult with this method. In addition, the staining was not seen uniformly within the parenchyma (see Figure 9.2). I also had concerns about whether the flow / perfusion characteristics of the FITC conjugated collagenase (with its increased viscosity) were truly representative of the unconjugated crude collagenase used during collagenase digestion. I therefore further investigated methods that enabled secondary staining of the crude collagenase once it had been administered within the pancreas.

iii) Testing of a commercially available anti-collagenase antisera

Several commercially available anti bacterial collagenase antisera are available. I therefore evaluated one of these.

Figure 9.2 FITC stained pancreas
Note non-uniform staining of parenchyma



X20

Results

Secondary staining of the crude collagenase was achieved. However, two problems were noted. Firstly, the negative controls stained brown due to cross-reactivity of the antibody. Secondly, the collagenase staining was not intense and therefore it was difficult to distinguish collagenase (see Figure 9.3).

iv) Testing of other commercially available anti-collagenase antisera

Using the same methods as described in iii) several other commercially available anti-collagenase (bacterial) antisera were evaluated. These included anti-collagenase (bacterial) IgG fraction (The Binding site,

Porcine pancreata procured in the same way as described above were distended intraductally with commercially available crude collagenase (Boehringer P lot 64). Blocks of distended pancreas were then taken and fixed, processed and sectioned as above. The sections were then treated using an indirect polyclonal method. This method was almost identical to that for the light microscope in ii) with several key differences. As with that method, the sections were trypsinised at 37°C for 25 minutes after which the sections were washed in water for 3 minutes. They were then placed in 6% hydrogen peroxide for 10 minutes followed by a further wash in water for 2 minutes and a wash in the buffer P.B.S. Rabbit serum was then applied for 10 minutes in a 1 in 20 concentration and then the anticollagenase antibody was applied. The antibody used in these experiments was the IgG fraction of a sheep antibody against bacterial collagenase (Euro-path Ltd, Bude lot x526). This was then washed with P.B.S. buffer for 20 mins. Rabbit anti-sheep immunoglobulins in a 1 in 25 formulation was then applied for 30 minutes. The remainder of the protocol was identical to that in ii).

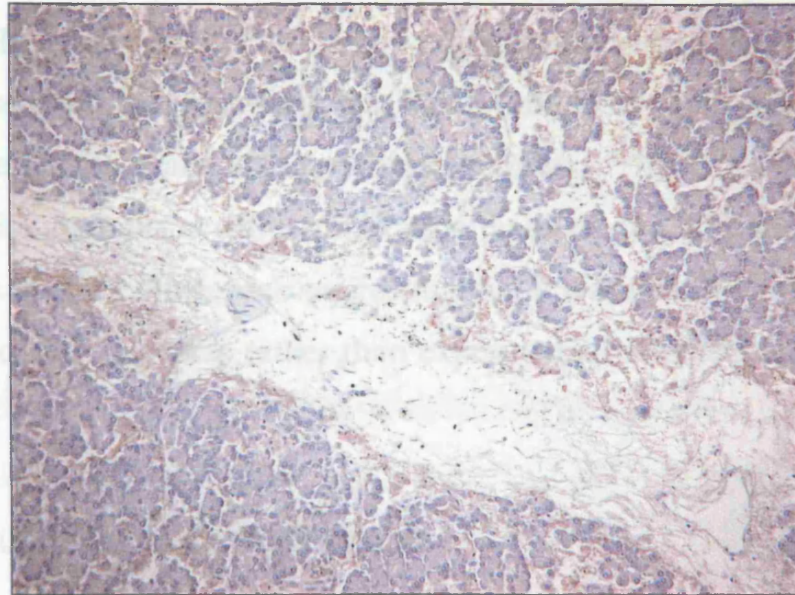
Results

Secondary staining of the crude collagenase was achieved. However, two problems were noted. Firstly, the negative controls stained brown due to cross reactivity of the antibody. Secondly, the collagenase staining was not intense and therefore it was difficult to evaluate intraislet collagenase (see Figure 9.3).

iv) Testing of other commercially available anti-collagenase antisera

Using the same methods as described in iii), several other commercially available anti-collagenase (bacterial) antisera were evaluated. These included anti-collagenase (bacterial) IgG fraction (The Binding site,

Figure 9.3 Commercially available anti-collagenase
The collagenase is not seen as clearly as with the
produced anti-collagenase and cross reactivity with
controls was seen



X20

Birmingham lot AX2658D/1), anti-collagenase (bacterial) whole serum (The Binding site, Birmingham lot X2564C) and anti-collagenase (bacterial) peroxidase conjugate The Binding site, Birmingham lot G4949).

Results

The same results were seen as for the anti collagenase used in iii). The IgG fraction gave the best result but was again limited by cross-reactivity to normal porcine pancreas and also poor islet visibility. This cross reactivity was seen despite attempts at different fixation methods and different concentrations of anti-collagenase suggesting that it was genuine cross reactivity to porcine pancreas rather than experimental artefact.

v) Testing of double -labelling methods

In an attempt to obtain clearer visualisation of the islet in relation to the anti-collagenase labelled collagenase, several double labelling methods were tried. Firstly, the sections were stained for insulin (Life Science International) after the initial staining with the anti-collagenase. Whilst this method achieved some double-staining it was weak. This was not improved by various modifications of the protocols including using a different mountant (insolmount EBIO). Secondary staining with dithizone was attempted. However, even after 8 hours staining was not seen. This was not improved with different amplification systems including Avidin Biotin technology. Following this detailed series of experiments, I then decided that the most sensible way forward was to use an anti-collagenase antisera and anti-collagenase monoclonal antibodies produced in our laboratory and that was specific to the crude *Clostridium histolyticum* collagenases that I was using.

2) PRODUCTION AND TESTING OF AN ANTI-COLLAGENASE ANTISERA AND MONOCLONAL ANTIBODIES

Materials and Methods

i) Production of the collagenase antisera and monoclonal antibodies

A polyclonal antisera against crude collagenase (Boehringer Mannheim lot 50) was produced using the method of Kohler and Millstein. Subsequently monoclonal antibodies were produced. These were produced from fusions between splenocytes from BALB/c mice immunised with crude collagenase (Boehringer Mannheim lot 50) and the mouse myeloma cell line NS-0/Uncl. Supernatants from uncloned hybrids were screened by ELISA and Western blotting.

Results

Seven monoclonal antibodies were produced and these detected a doublet band with molecular weight of 114.5 and 141 kd. Some monoclonal antibodies reacted more strongly with the higher molecular weight band and appeared to have a different specificity. The size of the bands suggests they are precursor forms of Class 1 and 2 collagenase as they didn't bind to purified collagenase or neutral protease. The monoclonal antibodies also showed a different spectrum of binding to 10 different batches of Boehringer collagenase type P.

ii) Testing of the produced antisera on collagenase distended porcine pancreas.

Using the same experimental methods as described in section 2 iii) above, the antisera and monoclonal antibodies produced in our laboratory were tested on sections from collagenase distended porcine pancreata.

Results

The antisera produced in our laboratory produced a significant improvement compared with the commercially available antisera previously tested. Of most importance was that cross reactivity was not seen in the negative controls (see Figure 9.4) The monoclonal antibodies were also evaluated but for the series of experiments described in this chapter did not confer benefit. They were however, further modified by Dr Ruoli Chen and their future use in collagenase experiments discussed in the final chapter of this thesis.

3) EVALUATING THE INFLUENCE OF DIFFERENT LOADING PRESSURES ON INTRAISLET COLLAGENASE ADMINISTRATION

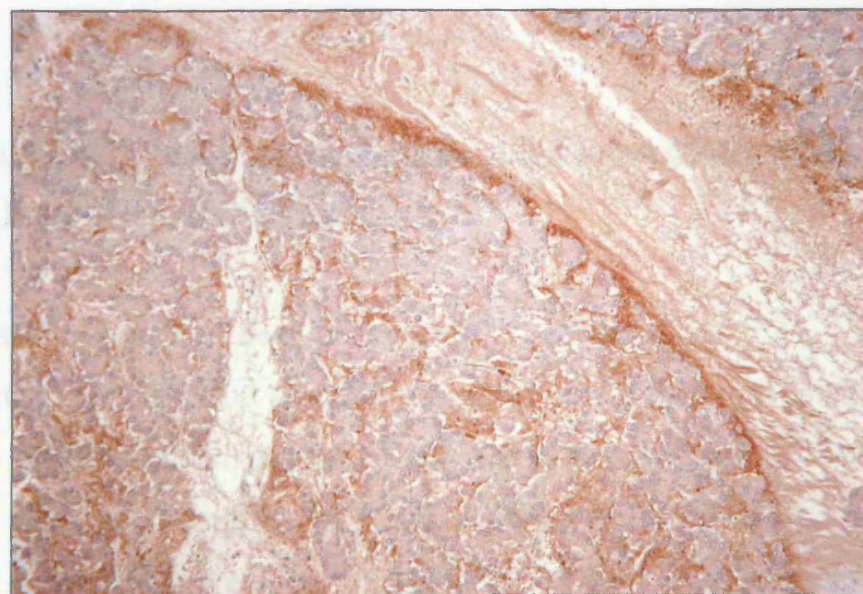
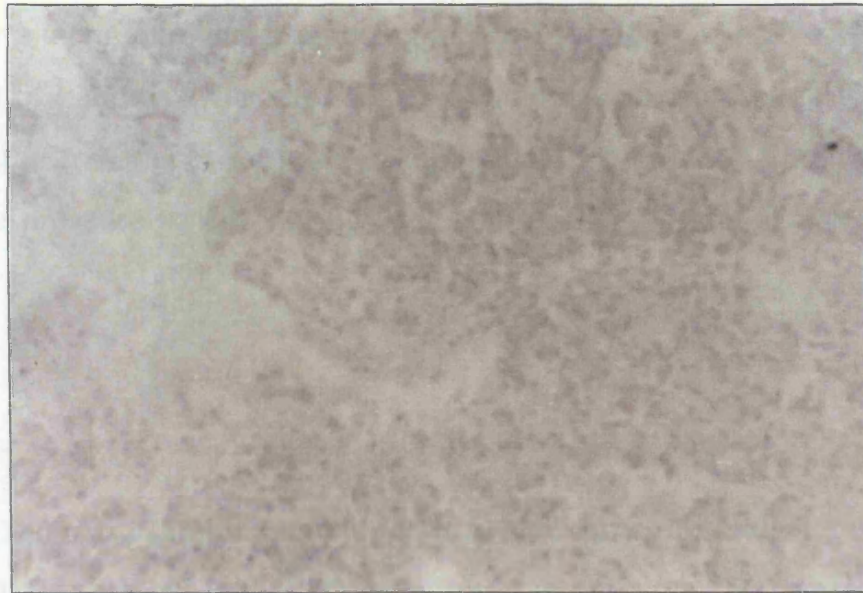
Materials and Methods

i) Evaluating the normal pressure range during collagenase
administration using the manual loading technique

Splenic lobes of porcine pancreata were procured from the abattoir in the manner as described above. The warm and cold ischaemia times were comparable for all pancreata studied.

Figure 9.4

Produced anti-collagenase
Collagenase clearly seen (brown) and no cross-reactivity with control seen



Each pancreas was cannulated with a 22F gauge cannula which was connected via a three-way tap to a pressure transducer (see Figure 9.5) which had been previously primed and calibrated. Each pancreas was distended via the cannula with crude collagenase (Boehringer P lot 50) dissolved in a concentration of 4 mg / ml in Hank's solution at room temperature. The manual loading technique was used using a 50 ml syringe. A total volume of 2ml per gram of pancreas was used for each pancreas. The pressure was measured by the transducer throughout the manual loading but administrator blinded to the pressure profile during each study. Five porcine pancreata were studied.

Results

The pressure range during the manual loading technique of collagenase administration used in our laboratory was found to be 125 - 200 mmHg (see Figure 9.6).

ii) Evaluating the pressure at which crude collagenase enters islets during the manual loading technique

Once the pressure range had been determined, a second series of experiments was performed to study the influence of different loading pressures on collagenase distribution. Using the same experimental set up as in i), the two pressures 50 mmHg and 200 mmHg were studied. The pressure traces for one experiment are seen in Figure 9.7. These pressures were chosen because pressures as low as 50 mmHg were rarely observed during the measurement of the normal loading pressures and therefore collagenase entering islets at pressures as low as 50 mmHg is highly significant. Pressures of 200 mmHg were commonly observed during manual loading.

Figure 9.5 Pressure recording apparatus and perfusion/transducer circuit

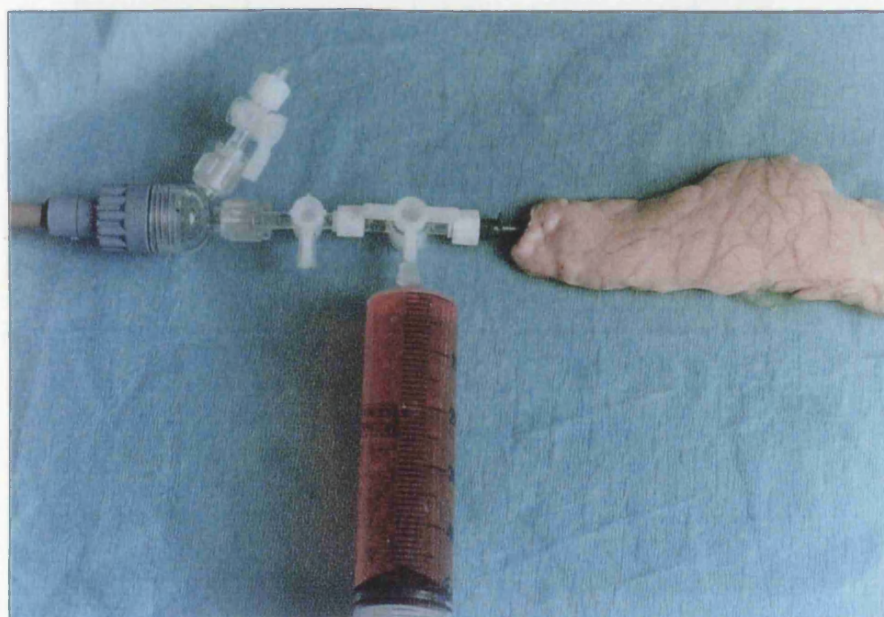
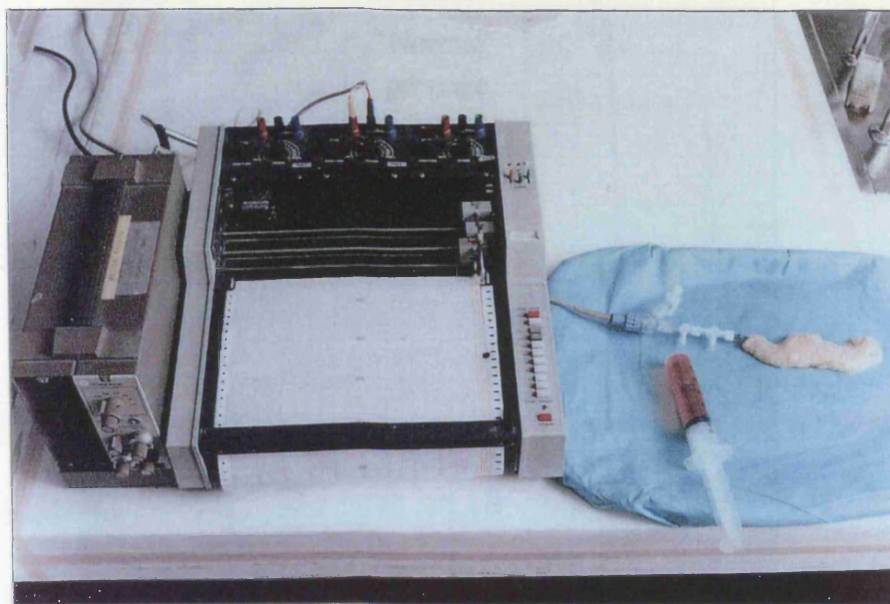


Figure 9.6 Pressure trace during 'normal' collagenase administration

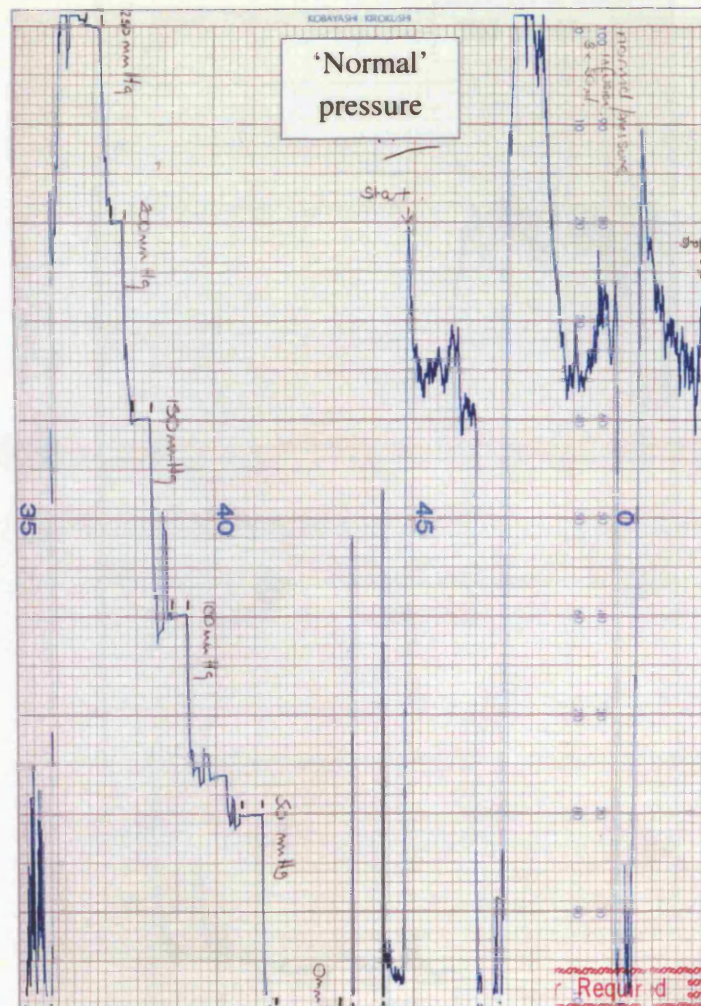
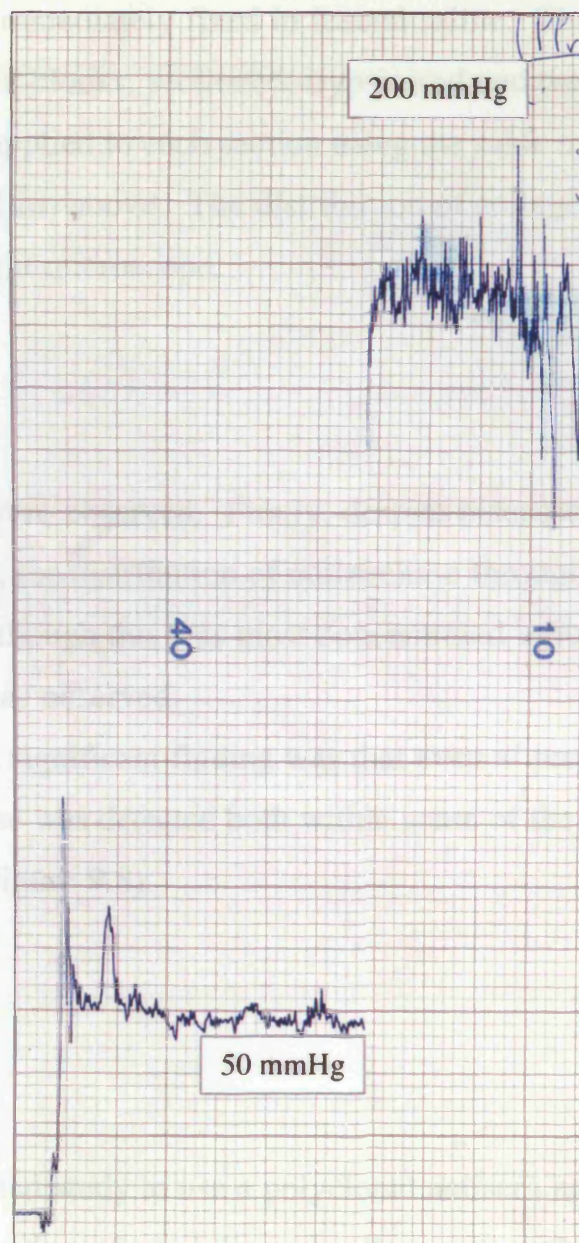


Figure 9.7 Pressure trace during one experiment



Five pancreata were studied at each pressure. In all cases pancreatic distension was macroscopically uniform throughout the different regions of the pancreas. After pancreatic distension, 1-2 cm blocks of tissue were taken from the head, body and tail of each pancreas. Two samples were taken from each region. These were immediately fixed in formal saline. The samples were then placed in paraffin blocks, sectioned, trypsinised and the produced collagenase antisera applied to each section using the same methods as described in section 2 iii) above. The distribution of collagenase was then assessed under the light microscope.

Results

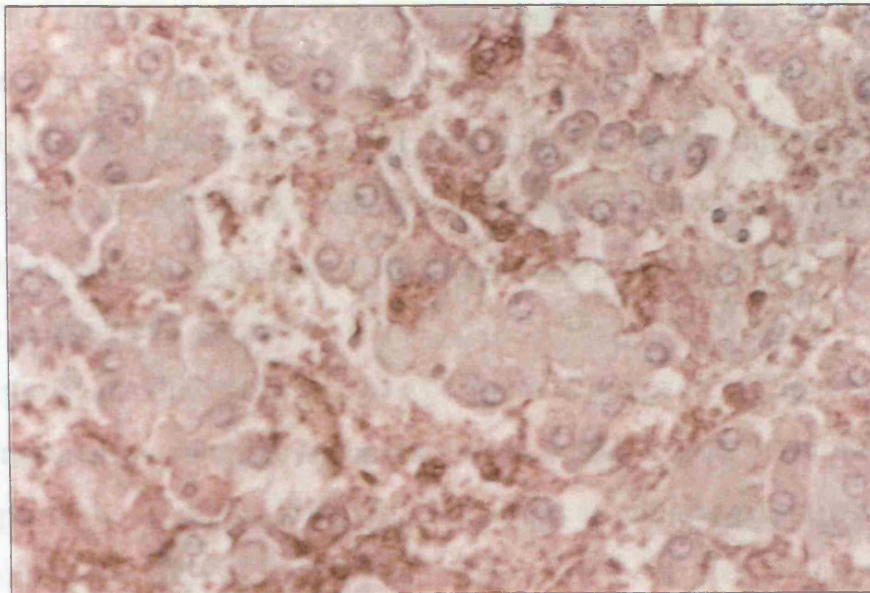
Two main findings were observed. Firstly, despite macroscopically uniform pancreatic distension, the distribution of collagenase throughout the pancreas was not uniform. Although this was most concentrated around the ductules, no discernible pattern was observed.

The second and most significant finding was that even at pressures as low as 50 mmHg, collagenase was detected both within some of the islets and also intracellularly (see Figure 9.8).

Discussion

This is the first reported study to use a polyclonal antisera against collagenase to evaluate the distribution of administered collagenase within the pancreas. The methodology designed and used in these experiments has the advantage over previous studies using Indian ink in two main regards. First, the perfusion characteristics being studied are those of collagenase itself rather than particulate ink. Second, unlike indian ink that tends to provide a crude picture with post administration tissue permeation, this method enables the accurate detection of even small traces of collagenase.

Figure 9.8 Intra islet collagenase seen at 50 mmHg



X60

The development of monoclonal antibodies against different components of crude collagenase is important for future studies of the collagenase digestion phase of islet isolation. These are discussed further in the next chapter.

The finding that collagenase is not distributed uniformly throughout the pancreas is important because islets are not isolated from areas of undistended pancreas. The findings in these experiments could be explained by the fact that in some parts the concentration of collagenase was too low for detection.

However, similar distribution was observed with the earlier fluorescence studies in which the collagenase was labelled prior to administration. It has also been observed with subsequent studies that I performed using the monoclonal antibodies.

The most significant finding in these experiments however, is the observation that collagenase enters islets at distension pressures as low as 50 mmHg. This finding supports the findings of van Suylichem et al. who have shown that indian ink enters the cells of the islet with perfusion pressures even as low as 40 mmHg ⁴⁶⁶.

Although the majority of islet laboratories have traditionally used the loading method for collagenase administration, it is interesting to note that the only study to directly compare these two methods has shown a moderate improvement in islet yield with the perfusion method ⁴⁶³. However, the pressures used during the perfusion method are as high as 200 mmHg ⁴⁶³.

The study described in this chapter would suggest that although the distribution of collagenase may be improved by the perfusion method, constant pressures of 200 mmHg will certainly result in significant intra-islet collagenase administration.

Traditionally the porcine pancreas is distended with collagenase once it has been brought back to the islet isolation laboratory. Several groups however, have shown a significant increase in porcine and human islet yields if collagenase is administered at the time of harvesting ^{471, 472, 473}. An explanation for these observations is that the collagenase is delivered into the pancreas before the effects of cold ischaemia have caused a loss of

pancreatic ductal integrity 440.

Clearly these experiments have all been confined to the porcine pancreas. However, now that the methodology has been optimised, subsequent experiments can be performed using human pancreata using the same collagenase anti-sera.

Conclusions

The methods outlined in this chapter provide an accurate way of assessing the distribution of collagenase within the pancreas following intraductal collagenase administration and enables further studies on the optimal distension pressure to be performed. The results show that some collagenase enters into the islets even at pressures as low as 50 mmHg. This may in part explain why the yields from a single pancreas are not at present routinely sufficient for transplantation, resulting in the need to 'pool' islets from more than one donor.

SECTION V

Conclusions and Future Prospects

CHAPTER 10

Conclusions and Future Research

Introduction

This chapter concludes the thesis with a summary of the main conclusions and suggestions of future research that is needed to further optimise the isolation of human islets of Langerhans. For clarity this chapter is divided into the three different experimental sections of the thesis.

1) COLLAGENASE COMPONENTS

Chapter 4 demonstrated that there is considerable variability both between different batches of crude collagenase and different pancreata. It also suggested that the requirements for optimal islet isolation are different for the human and the pig. Digestion profiles were created for each digestion with the three main parameters islet number, cleavage index, and degree of fragmentation measured at different time points. The concept of Profile Percentage Score was introduced with weighted means. As part of this scoring the mean fragmentation was converted into a measure of islet intactness.

The chapter highlighted the need for methods that enable the testing of numerous components of crude collagenase on any one human pancreas. This need arises both from the importance of controlling for the variability between different human pancreata and also the limited donor supply. Although it was possible to divide and cannulate the pancreas into 4 segments, the progressively increased cold ischaemia times associated with sequential testing of the segments also highlighted the need for new methods to enable consecutive testing.

Chapter 4 investigated such methods. A comparison was made between different 'in-vitro' methods and compared with the gold standard of the semi-automated method of pancreatic digestion. Although methods involving blocks of collagenase distended pancreas gave the closest correlation, these were not technically feasible. However, methods involving the incubation of

collagenase with blocks of undistended pancreas also gave an acceptable correlation and this method was further tested using different combinations of fractionated collagenase components.

These methods clearly need further improvement. More recently I have designed apparatus that enables 5 simultaneous semi-automated digestions to be performed (Figure 10.1). Each has its own mini isolation circuit and has the advantage that five segments of the same pancreas can be tested simultaneously. Although this enables fewer simultaneous digestions to be evaluated than the 'in vitro' method, it has the distinct advantage that distended pancreas can be used. In addition, it may be that by using larger segments of pancreas rather than small blocks, the digestion may be more representative of that would occur in the whole pancreas. This method needs detailed evaluation.

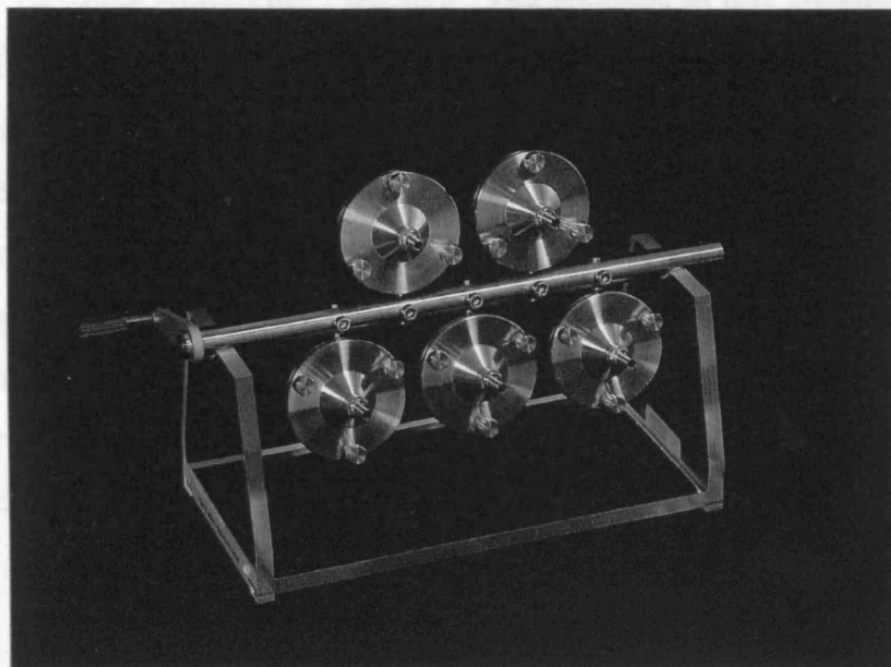
Chapter 6 outlined the different biochemical assays available for the three main components of crude collagenase, namely class I collagenase, class II collagenase, and neutral protease. The assays were optimised in our department and in particular experiments showed that the FALGPA assay for class II collagenase was optimal at a different wavelength from that previously described.

One of the problems however, is the relative non-specificity of the available assays even after optimisation. Specific assays are required for each component of crude collagenase to aid future studies.

One final comment on collagenase components. This work is in vital need of a pharmaceutical company collaborating at a scientific level. Although detailed biochemical studies can be performed within academic departments, the purification of single components from crude collagenase requires large supplies of crude product in addition to large capacity isolation columns.

Although, this work has recently been made easier by improved knowledge of the genetic sequences of the different components, many of the issues could be solved by the scientific collaboration of industry. Now that islet transplantation teams are again active, this may be a possibility.

Figure 10.1 Newly designed experimental apparatus for evaluating 5 simultaneous collagenase digestions



2) COLLAGENASE STORAGE

Chapter 7 demonstrated the stability of four different batches of crude collagenase. These included collagenases from two different companies in addition to the purer compound *Liberase*. The findings in this chapter were surprising but important. The validity of the enzyme stability was confirmed by rodent islet isolation. These data suggest that another component of collagenase is important for the deterioration of crude collagenase. By extrapolation, this component is also important for human islet isolation. I would suggest that a possible candidate is clostripain. Chapter 6 demonstrated that the clostripain levels vary significantly from one batch of Boehringer collagenase to another. Further studies should focus on this component.

3) COLLAGENASE ADMINISTRATION

The biochemical aspects of collagenase administration were investigated in Chapter 8. The principle findings were that collagenase digestion of the human pancreas is inhibited by University of Wisconsin (UW) solution, whereas porcine digestion is not. This led to detailed studies of the different components of UW responsible. Further research then elucidated that the mechanism of inhibition seems to be the inhibition of class II collagenase. This data implies that class II collagenase is necessary for human islet isolation. These findings taken in conjunction with previous studies of cold storage solutions should enable the design of cold storage solutions for different stages of human islet isolation (from organ procurement to storage of pancreatic digest) that do not inhibit collagenase digestion.

Chapter 9 researched the biophysical aspects of collagenase administration. The principle study was to evaluate the distribution of crude collagenase within the porcine pancreas after administration by the loading method. Previous

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