STUDIES OF PHYSICAL
AND IMMUNOLOGICAL
CELL SEPARATION METHODS
IN ISLET PURIFICATION.

by
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Statement of originality

Unless otherwise acknowledged, the work described in this dissertation is my own independent work undertaken in the Department of Surgery at The University of Leicester.

G. Robertson

G.Robertson

March 1993
Dedication

This thesis is dedicated to my mother and father
whose lifelong support made my medical career possible,
and to my wife Claire
for her unflagging encouragement throughout this work

Learn as if you were to live for ever,
Live as if you would die tomorrow.

Isidore, Archbishop of Sevilla (570-636)
I would like to thank the following people and organisations without whose help this work could not have been completed.

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References
LIST OF ABBREVIATIONS

a1 & a2 Appendices 1 & 2.
A.D. Anno Domini.
Anti-IgM-AP Anti-mouse IgM (μ-chain specific) alkaline phosphatase conjugated antibody.
B.C. Before Christ.
BSA Bovine Serum Albumin supplied at 300, 400 & 500mOsm (Applied Protein Products, Brierly Hill, UK).
dd H2O Double distilled water.
DMEM Dulbecco’s Modification of Eagles Medium (Northumbria Biologicals Ltd, Cramlington, UK).
DMSO Dimethyl sulphoxide.
EC Kidney perfusion Euro-Collins-Solution, (Fresenius AG, Hamburg, Germany).
ELISA Enzyme linked immunosorbent assay.
FACS Fluorescence activated cell sorting.
FCS Fetal Calf Serum, (S-0001a, SeraLab, Sussex, UK.).
GSH Reduced form of glutathione.
GSSG Oxidation product of glutathione.
HAT Hypoxanthine-aminopterin-thymidine supplemented medium.
HBSS Hanks’ Balanced Salts Solution with 0.35g/L sodium bicarbonate, Northumbria Biologicals, Cramlington, UK. M8122.
HEPES 1M buffer solution pH7.2-7.4. (Northumbria Biologicals Ltd, Cramlington, UK. M932).
HES Hydroxyethyl Starch pentafractron (Dupont Critical Care, Wilmington, USA)
HGMS High gradient magnetic separation.
HGPRT Hypoxanthine-guanine-phosphoribosyl transferase.
HLA Human leucocyte antigen.
HCO (Hyperosmolar citrate) Soltran hypertonic kidney perfusion solution, (Baxter Healthcare, Thetford, UK. B4708).
IDDM Type 1 insulin-dependent diabetes mellitus.
IEq Islet equivalent (conversion calculated on the basis of the volume of a standard 150μm diameter spherical islet).
ip Intra-peritoneal.
LDSno Identity code for Monoclonal antibodies produced in Leicester Department of Surgery.
LCDGs Linear Continuous Density Gradients.
M Molar.
MACS Magnetic cell sorting system (built by Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).
MEM  Minimal Essential Medium with Hank's salts (Northumbria Biologicals Ltd, Cramlington, UK).
MEM+Imp  MEM with lactobionate(100mmol), raffinose (30mmol) and glutathione (3mmol).
MEM+HES  MEM with 50g/l hydroxyethyl starch.
MHC  Major histocompatibility complex.
mmol  MilliMole.
moS  MilliOsmole/Kg of water.
95% CI  Wilcoxon signed-rank based 95% confidence interval.
NMF  Non-magnetic fraction (from magnetic separations).
PBS  Phosphate buffered saline.
PEG  Polyethylene glycol 1500, 50% w/v HEPES.
RPMI  Roswell Park Memorial Institute -1640 tissue culture medium, (Northumbria Biologicals Ltd, Cramlington, UK).
SDS/PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
SRBC  Sheep red blood cell.
TBS  Tris buffered saline.
UK.  United Kingdom.
UV  ultra-violet.
WAG  Wistar Albino Glaxo/Leicester, inbred rat strain.
The prospect of reversing diabetes by the transplantation of isolated pancreatic islets thereby avoiding the major surgery and associated morbidity of whole pancreas transplants, led to the first reports of transplantation of dispersed human islets both purified and unpurified in the late 1970's. Reports of complications and even deaths following intraportal infusion of unpurified islets, led to intensified research into methods for purifying islets from the collagenase dispersed human pancreas.

Separation on the basis of physical differences in the density of islets and exocrine tissue has proven to be the most effective method, and studies in Leicester pioneered the use of density gradients on the COBE 2991 cell processor for large scale islet purification. The first half of the work described in this thesis examines methods for maximising the differences in islet and exocrine densities using cold storage solutions, both at the time of organ retrieval and during pancreas processing. The improvement in human islet purification using University of Wisconsin solution and the components responsible for this are outlined. Simultaneously the mechanical process of density gradient purification was optimised by the introduction of continuous rather than discontinuous density gradients on the COBE.

Despite this work, purified islet yields from a single donor pancreas remain insufficient for successful transplantation and the need to use more than one donor/transplant reduces both the number of transplants and the HLA matching possible. The second part of this work therefore examined the use of alternative immunomagnetic techniques to purify islets in a rat model. By coupling 4.5μm diameter magnetic beads to the exocrine fragments via very specific monoclonal antibody linkages, reliable and effective removal of 90% of the exocrine fragments was possible with islet yields of 60%. Immunomagnetic methods would therefore provide an alternative or adjunct to existing density dependent methods.
INTRODUCTORY OVERVIEW

The first introductory chapter outlines the increasing success of human islet transplantation during the first three years of this decade. This has not only shown it to be a real alternative to whole pancreas transplantation, but has also clarified the problems still to be overcome. Increased yields of purified islets, and islet rejection. With successful transplantation by intraportal infusion of islets into immunosuppressed type 1 diabetic patients requiring 8-10,000 IEq/recipient Kg, current yields of purified islets of around 200,000/donor pancreas are usually inadequate to allow transplantation on a one to one basis. The necessity to use islets from more than one donor means that fewer transplants can be performed, HLA matching of donors to recipients are likely to involve more mismatches, and simultaneous transplants of a kidney and islets from the same donor (so successful in whole pancreas transplantation) are impossible.

Since collagenase digestion of the human pancreas often produces a digest containing in excess of 1 million islets, the problems appear to occur at the purification stage, during which the islets which constitute only 1-2% of the total digest volume are separated from the contaminating exocrine tissue. These problems have led to a number of groups performing both auto- and allo-transplants by infusing the whole unpurified dispersed pancreas into the recipients portal vein. While this overcomes the problem of islet yield it has also been reported to cause significant morbidity and mortality. With the only well documented benefits of pancreas transplantation being an amelioration of certain diabetic complications and an improvement in quality of life, many feel that such risks are unacceptable, and the work described in this thesis aimed to examine a variety of methods to improve purified islet yields, with the hope of allowing sufficient numbers of islets to be purified from a single donor pancreas for successful one to one transplantation.
Islet purification currently relies on the difference in density between islets and the denser exocrine tissue allowing their separation on density gradients. The results of this process are extremely variable, with large numbers of very pure islets being obtained from one pancreas but not from the next, for reasons which remain unclear, but must be related to donor factors and differences in the treatment of the pancreas before density dependent purification. One of the major variables in this respect is the period of cold ischaemia to which the pancreas is subjected before processing. Although the ability to store organs by cooling for periods of several hours between their removal from the donor and their transplantation, is central to the success of whole organ transplantation, hypothermic inactivation of processes such as the cell membrane pumps has been shown to result in cell swelling, which has a direct relationship to the cell’s buoyant density, and this phase could therefore have a profound effect on the results of islet purification.

Chapter 3 describes the standard methods of human and rat islet purification used in the Department, together with methods used in the production and screening of monoclonal antibodies. It also validates the use of mini-continuous density gradients as an investigative tool and these are subsequently used extensively to measure changes in islet and exocrine tissue densities and their effect on islet purity and yield.

The work described in Chapter 4 examines the effect on human islet purification of using different cold storage solutions (University of Wisconsin Solution and hyperosmolar citrate) for in situ organ perfusion during the donor operation. This work was then extended in Chapter 5 to examine the effect of storing the pancreatic digest at 4°C in different solutions prior to purification on density gradients, and Chapter 6 examines the effect of gradient temperature on islet purification. While these Chapters investigate the differential effect of manipulating cell metabolism and swelling on the density of islet and exocrine tissue, Chapter 7 approaches the problem of density
gradient purification from a different perspective. *Discontinuous* step gradients have always been used for islet purification due to their apparent simplicity. They have several problems associated with their use however which limit the scale of purification and the resulting yield of islets. Chapter 7 examines the possibility of purifying digest from whole human pancreases on a single *continuous* density gradient using the COBE 2991 cell processor.

Despite the improvements in islet purification achieved by optimisation of the density dependent process, the results remain variable and the differences in tissue densities often inadequate to produce the degree of purity and numbers of islets required. Chapter 2 therefore reviews the alternatives to isopycnic centrifugation, and in particular the application of immunomagnetic cell separation techniques to islet purification. Chapter 8 describes the production and purification of a number of monoclonal antibodies specific to the non-islet components of both the rat and human pancreas, one of which is then used in work described in Chapter 9 to purify rat islets by adapting immunomagnetic techniques normally used for single cell suspensions.

The results from this thesis are summarised in Chapter 10 which also suggests further work required in the field of immunomagnetic islet purification before its application on a large-scale could be fully exploited for human islet isolation.
CHAPTER 1
HUMAN ISLET TRANSPLANTATION IN TYPE 1 DIABETES MELLITUS

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1.5 SUMMARY
1.1 INTRODUCTION

i) DIABETES MELLITUS

a) History and aetiology

As a disease, diabetes dates back to antiquity with the first reference to a treatment for polyuria occurring in the ancient Ebers Papyrus (1550 B.C.) \(^1\). Although comments on polyuria by Celsius (30 B.C.-50 A.D.) alluded to the disease, considering that patients excreted more fluid in the urine than they took in and advising them to eat as little as possible, Aretaeus (30-90 A.D.) practising in Cappadocia (now part of Turkey) is credited with the first clinical description of diabetes and with coining the term diabetes from the Greek meaning 'to run through a syphon' \(^2\). The sweet taste of the urine in addition to its large quantities were noted in fifth century Sanskrit texts from India in which it was described as ‘honey urine’ \(^4\), and more detailed studies of the disease by Arabian physicians including Avicenna (980-1027 A.D.) \(^5\) led to descriptions of the increased appetite, gangrene and loss of sexual function. There are few European references to diabetes before Thomas Willis (1621-1675) who realised that glycosuria was a constant feature of the disease and distinguished it from diabetes insipidus \(^6\), leading to the addition of the qualifying adjective ‘mellitus’ by William Cullan (1710-1750).

The pancreas as an organ had also been described as long ago as 300 B.C. by Herophilus \(^7\), with a more detailed description being included by Rufus of Ephesus 200 years later in his work ‘On the names of various parts of the body’ in which he coined the term pancreas (“all flesh”). The link between this organ and diabetes however was not established until 1889 when von Mering and Minkowski in repeating the earlier pancreatectomy experiments performed in 1683 by Brunner \(^8,9\), established that total pancreatectomy in dogs caused diabetes \(^10\). Initially it was believed that the exocrine pancreatic secretion played a role, but in 1893 Hédon observed that diabetes did not occur in partially pancreatectomised dogs despite the diversion of the exocrine secretions onto the skin as long as the pancreas remained vascularised \(^11\), and in the same year Laguesse suggested that the clusters of cells first described by Langerhans in 1869 \(^12\) were responsible for the postulated internal secretory function \(^13\).

At the turn of the century Opie \(^14\) among other authors described changes in the islets of Langerhans occurring in diabetes, and over the next 30 years a basic understanding of the morphology and function of the islet cells and their role in diabetes was achieved. At the turn of the century Opie \(^14\) among other authors described changes in the islets of Langerhans occurring in diabetes, and over the next 30 years a basic understanding of the morphology and function of the islet cells and their role in diabetes was achieved.

Attention then began to turn to the aetiology of the disease. While a complete understanding of the aetiology of Type 1 diabetes continues to elude researchers, it has become obvious that its development can be broken down into several stages beginning with genetic susceptibility and ending with complete beta-cell destruction. Population studies have shown that genes within the major histocompatibility complex contribute to the development of diabetes, with 95% of whites with type 1 diabetes expressing histocompatibility alleles DR3 or DR4, compared with 40% of the general population \(^15\). Some genetically susceptible individuals (less than half of identical twins) will then undergo a triggering event. This
remains poorly defined, but a number of viruses are known to be diabetogenic in animals and several studies have suggested that a virus may trigger a potential diabetic state in man 16-19, other environmental factors such as early nutrition 20, and cow's milk 21,22 have also been suggested more recently. Whatever the trigger, a stage of active immunity ensues with the production of autoantibodies to islet cells 23,24 and islet cell proteins including insulin 25,26 and a 64 kDa protein 27,28 and activation of T lymphocytes expressing the HLA-DR antigen 29. Although it was assumed initially that these immunological changes rapidly led to the immune destruction of most of the insulin secreting cells and finally to insulin dependent diabetes, it is now known that this can take many years and may never occur 30. However a substantial proportion of those with high titres of islet cell antibodies will go on to show a progressive loss of glucose stimulated insulin secretion and finally overt diabetes 31.

b) Insulin treatment and the complications

The Nobel prize winning discovery of insulin by Banting and Best 32,33 began a new era in diabetes treatment, in which the risk of death from the short term metabolic complications receded. It took only a few years however for an increasing number of reports of long-term complications of the disease to appear 34-36 and as a result, the importance of diabetes in socio-economic terms 37,38 has continued to increase along with its incidence 39-43. The most important of the chronic complications are the retinopathy, nephropathy, neuropathy and vascular disease. Retinopathy is the commonest complication of IDDM increasing with time, so that while background retinopathy is rare in the first 5 years of the disease it is almost ubiquitous after 20 years and by 40 years 62% of patients will have the more severe proliferative stage of the condition and 10% will have severe visual loss 44,45. While only 40-45% of diabetics develop nephropathy after 40 years, by the time it does develop, other long term complications are already present and the life expectancy of such patients is much reduced, with a combination of deaths from uraemia and cardiovascular causes, reducing median survival in this group to 7-10 years 46,47. Estimates for the development of neuropathy vary widely according to the method of assessment, but probably 40% or more 48,49 of long-term diabetics show evidence of either a distal sensory neuropathy, which contributes to foot ulceration and neuropathic arthropathy, or an autonomic neuropathy producing a range of symptoms relating to postural hypotension, gastric stasis, diarrhoea and impotence 50. The accelerated development of atherosclerosis identical to that developed by non-diabetics may be due to the association of diabetes with risk factors such as hyperlipidaemia, hypertension, elevated fibrinogen levels and hyperinsulinaemia 51,52. As a result, overall cardiovascular mortality is increased 2-3 fold in men and 6-7 fold in women with IDDM compared with age-matched controls 46,53, and similar risks have been observed for the development of peripheral vascular disease and its complications.

Despite some dissent 54-56, the aetiology of these complications is believed to be related to the degree of glucose control, and much time and effort has been expended in
attempts to achieve near normal glucose control using multiple insulin injections, artificial pancreases 57,58 and continuous subcutaneous insulin infusions 59, in the hope of reversing the complications of the disease or at least preventing their progression 60-62. This has met with varying success 63, and it seems unlikely that the delivery of exogenous systemic insulin can ever match the minute to minute regulation of a range of metabolic functions exerted by normal pancreatic islets 64, where the response to changes in portal glucose concentration occurs within minutes 65,66. This realisation has led to an increasing interest in the transplantation of pancreatic islets, either as part of a whole pancreas graft, or in the form of dispersed pancreatic tissue, with or without purification of the islets.

ii) WHOLE PANCREAS TRANSPLANTATION
Since the first report of insulin independence following whole pancreas transplantation 67, the numbers of transplants performed each year have risen almost exponentially to a high of 606 in 1990. This increase has been accompanied by dramatic improvements in the results of whole pancreas transplantation over the last decade (meticulously documented by the International Pancreas Transplant Registry 68), in which patient survival rates have increased from 71-91%, and 1 year functional graft survival from 21-70%.

There is no doubt that the increasing success rate of whole pancreas transplants means an improved quality of life for the recipients 69,70, and can restore normal phasic β-cell function 71-74. The effect on the long-term complications can only be studied in terms of secondary intervention rather than primary prevention and comparison with suitable controls is virtually impossible, nevertheless the experience of the Minnesota group 75 has allowed the progression of diabetic nephropathy 76, neuropathy 77 and retinopathy 78 following whole pancreas transplantation to be examined, documenting the sometimes minimal benefits, and leading to suggestions that pancreas transplantation should perhaps be made available to diabetics who have fewer of the chronic complications of diabetes mellitus 79. However, whole pancreas transplantation constitutes a major surgical procedure with a technical failure rate of 26.8% in the best of hands 75 and a high relaparotomy rate 80,81. Unlike kidney, liver and heart transplants, pancreas transplantation is not life saving, but still involves the risks of major surgery and requires life-long immunosuppression. These considerations have limited its place mainly to diabetics who receive a simultaneous kidney transplant (1,592 transplants in the USA from 8/1987-11/92, 76% 1 year graft survival), with small numbers of transplants being performed in diabetics who already have a functioning kidney transplant (122 US transplants 1987-92, 51% 1 yr graft survival) or who receive a pancreas alone (91 US transplants 1987-92, 48% 1 yr graft survival), and have led many to question its role in the treatment of diabetes at all 82,83, let alone broadening the indications.

As a result, the number of suitable patients appears to be in decline, and only 422 transplants were performed in the first 10 months of 1992. It seems unlikely that whole pancreas transplantation will ever be relevant to the treatment of young diabetics before the
development of the long term complications of their disease, who might see the greatest
benefits in terms of prevention of disease progression. Islet transplantation may just be the
answer!

### RATIONALE BEHIND ISLET TRANSPLANTATION

As long ago as 1892 when Thirloix injected lamp-black and oil into the pancreatic
ducts in an attempt to suppress exocrine secretion, attempts have been made to avoid the
unnecessary transplantation of functioning exocrine tissue, and such techniques for duct
ligation or obliteration continue to be used in whole pancreas transplants particularly in
Europe. An extension of this, has been to remove the pancreatic tissue from the ducts
altogether, producing a “digest” of acinar fragments and intact islets, which can then either be
transplanted as a dispersed mixture, or purified to remove the exocrine fragments, reducing
the digest to the 1-2% constituted by the islets. The transplantation of either the dispersed
pancreas or the purified islets then becomes a much more minor procedure, with the islets
able to engraft themselves if transplanted to a site of suitable vascularity, many of which are
accessible by percutaneous injection.

While islet transplants today require immunosuppression, they offer the potential of
being transplanted in an immunomodulated form without this. The combination of a low
morbidity minor transplant with no immunosuppression would open the procedure to exactly
the group of patients likely to benefit most from it, young diabetics without complications.

At present many problems remain, and this review will concentrate on these and the
current results of clinical islet transplant programs of both auto- and allotransplants. The
transplantation of islet rich fetal and neonatal tissues will not be included. Where insufficient
data is available for conclusions to be drawn from human islet transplants, it is inferred from
that of whole pancreas transplantation or animal studies if appropriate.

### 1.2 PANCREAS RETRIEVAL AND ISLET ISOLATION

#### i) DONOR FACTORS IN ISLET TRANSPLANTATION

The effort to maximise organ function despite the multiple insults involved, is a major
consideration in organ transplantation. In islet transplantation not only can tissue viability
suffer, with decreasing metabolic reserve impairing the islets ability to survive the ensuing
purification, but the ability to purify islets from the fragmented pancreatic tissue on the basis
of density can also be affected. While pancreatic acinar cells and islets have been shown to
differ in density, exocrine discharge is likely to alter this balance, and may be responsible
for the dramatic differences in islet yield and purity from one donor to the next.

Numerous hormonal interactions have been shown to be involved in the control of
pancreatic exocrine secretion, the effect on these of the proven changes which occur in
the haemodynamic and metabolic function of the brain-dead organ donor are unknown
but likely to be considerable. In addition drugs such as opioids, inotropes and
atropine, electrolytes including calcium and potassium, previous surgery,
fasting 101 and both parenteral 102 and enteral 103 feeding have all been shown to have measurable effects on pancreatic secretion. It has already been shown that prolonged care on ITU adversely affects the results of hepatic transplantation 104-106, and it seems highly likely that a period of donor support on ICU will result in a wide variation in the secretory status of the exocrine pancreas contributing to problems in subsequent islet purification.

ii) PANCREAS PROCUREMENT
There are several interrelated elements to this, all of which may have an important impact on the results of islet purification and viability.

a) Warm ischaemia
Despite some experimental work suggesting that pancreatic islets can withstand quite prolonged periods of warm ischaemia 107-110, it is generally accepted that warm ischaemia results in tissue damage and cell death 111 with deterioration in transplanted organ function 112. This has led to the development of techniques for multi-organ retrieval from brain-dead heart-beating organ donors which virtually eliminate it. However, with the shortage of organ donors generally and particularly in countries that do not use heart-beating donors, the criteria for use of pancreases which have suffered a period of warm ischaemia remains important but undetermined. Although for whole pancreases it may well be longer than for the kidney 113, subsequent islet isolation appears to have an additive damaging effect 114-116, which may reduce the tolerance of the islets to warm ischaemia still further, and this period clearly needs to be minimised if good islet viability is to be maintained.

b) Cold ischaemia and storage solutions
Following the pioneering work by Collins 117 and then Belzer 118 in the late 60’s on hypothermic solutions and extracorporeal perfusion for kidney preservation, much of the work on prolonging storage in the multi-organ donor era has been done at the University of Wisconsin looking at the inclusion of cellular impermeant molecules 119-121 and leading to the development of their cold storage solution (UW). This and other similar solutions aim to prevent cell swelling during the period of storage when the Na/K ATPase dependent pump is inactivated by hypothermia. Initially it was felt that a high “intracellular” concentration of potassium ions was important, but it has become obvious that it is the inhibition of cellular swelling which results in benefit 122 and reversal of this high K/Na ratio has been shown to be equally effective 123,124 125 as long as cellular impermeants relevant to the organ are present 126.

Solutions based on this premise such as UW and hyperosmolar citrate 127 have been shown to improve the results of liver 128-130, pancreas 131-134 and kidney 135 transplantation and have become widely adopted as the solutions of choice for in-situ flush during multi-organ donations, allowing storage of the pancreas and liver for longer than 12
hours and permitting transfer of these organs between centres and more convenient scheduling of recipient surgery.

In human islet isolation, in-situ flush with UW has been shown to improve both islet yield and function for periods of up to 18 hours compared with Euro-Collins and other solutions. This benefit may only begin to be obvious after 6-8 hours however, as before this the benefits may be outweighed by an adverse effect of components of the UW on collagenase digestion.

In general, despite some studies showing preservation of function for up to 92 hours, most have shown that islet yields and viability deteriorate rapidly with storage times beyond 6 hours, and no patient has been rendered insulin independent after more than 12 hours cold ischaemia (Figure 1.1). The shortest possible period of cold storage prior to the further insults of the digestion and purification process appears likely to give the best results.

![Figure 1.1 Showing the Effect of Cold Ischaemia on Subsequent Graft Function 1990-6/1992](image)

Includes only the 67 islet transplants in the last 30 months whose pre-transplant C-peptide levels were 0 ng/ml excluding two in 1991

The proven deterioration of the duct system (used for the delivery of collagenase prior to digestion of the pancreas) during warm and cold ischaemia, may contribute to the decline in islet yield, and has led to studies examining the use of a ductal flush using organ preservation solutions with collagenase prior to cold storage. The logistics of this and hypothermic pulsatile perfusion make both difficult to apply in the human situation outside a very limited retrieval area.
Overall these studies suggest that the period of cold ischaemia before islet isolation should be kept to a minimum, and that while differences between the methods of preservation appear small over periods of less than 6 hours, for periods longer than this UW, probably with early ductal distension may significantly improve islets yields.

iii) DIGESTION / FRAGMENTATION OF THE PANCREAS

The isolation of islets from the exocrine components of the pancreas remains one of the limiting factors in human islet transplantation despite years of research, and the fragmentation of the pancreas without simultaneous destruction of the islets constitutes a continuing problem.

a) Collagenase

Although non-enzymatic mechanical methods of disrupting the pancreas were the first to be described 151 and have been used occasionally in the human 152,153, the use of collagenase to disrupt cellular aggregations first described in 1916 154 and adapted for pancreatic islets by Moskalowski 155, has become the most widely used method following Lacy’s description of techniques for acinar disruption by ductal distension followed by exposure of the chopped rodent pancreas to collagenase 156,157.

The application of collagenase in this way to the more fibrous human pancreas however met with only limited success 158, until the description by Gray et al 159 who by adapting the technique first described in the dog 160,161 for the intraductal distension of the pancreas with collagenase, considerably improved on previous results, and established the current method of collagenase delivery, which may be either by constant ductal perfusion 143,162 or by the more straightforward single intraductal injection.

The extreme variability in the process of collagenase digestion remains one of the most frustrating aspects of islet isolation however. Not only is there tremendous variation between batches of collagenase 163-170 with their activity bearing no relationship to the manufacturers activity ratings and depending on the presence of undefined impurities, but there is even variation within a batch from pancreas to pancreas 171 perhaps due to variations in pancreas distension 172, donor diet 173 and in collagen make up 174-176 and islet fragility 177 with age. The enzyme activity is also known to be affected by temperature 143,178,179, storage conditions 180, and a variable 181 inhibition by chemicals such as glutathione 180,182 included in UW and histidine 181 among others 183.

The production of collagenase in batches compounds the problem, since the supply of an effective enzyme can be rapidly exhausted or deteriorate over a period of time. This necessitates an intermittent testing of new batches for activity in digestion of the human pancreas, a time consuming and often frustrating process.
b) Mechanical disruption

In addition to the use of intraductal collagenase the process of digestion also requires a mechanical process to shake or tease the pancreas apart, and some method of removing digested islets from the continuing action of the collagenase. The first methods described used a variety of procedures for teasing the pancreas apart, monitoring this either visually 159 or microscopically 184 and stopping the action of collagenase on digested tissue at an appropriate stage by cooling, dilution and the addition of serum 185. The description in 1988 of an “automated method” in which the pancreas, distended with collagenase, is shaken apart in a sealed chamber from which the digested islets are continuously flushed through a 280μm mesh has with few exceptions 187 replaced the older methods 188.

The rapid removal of the islets from the active collagenase is vital for several reasons, firstly the islets themselves begin to fall apart as their collagen matrix is digested 172, secondly the enzymes are toxic to cells in a dose dependent way 189-191 and although islets appear to be more resistant than acinar cells to this 147, the fact that disruption of the acinar tissue may in theory lead to a self-sustained process of autodigestion makes minimal disruption of both an important aim 185.

The process of digestion may cause islet damage in other ways, and minimisation of mechanical trauma is felt to be important 183,186. The necessity to activate the collagenase enzyme by warming the pancreas to body temperature or above is also likely to initiate a reperfusion type injury 192,193 and the addition of antioxidant compounds requires examination 116,194.

c) Storage of Digest

By applying this combination of enzymatic and mechanical dissociation to the pancreas, as much as 60 ml of digest is produced, of which only 1-2% is islets. It has now been convincingly shown that the same principles of cold storage at 4°C apply to this digest as to the whole pancreas, and that the results of subsequent density dependent purification can not surprisingly be improved, by solutions which prevent cell swelling 141,195 and maintain tissue densities 196,197.

iv) ISLET PURIFICATION

The aim of the purification process is to separate the islets (which can number up to 1 million) from the dispersed exocrine pancreas. The process of hand picking islets 155 is clearly impractical on this scale, and various differences between the physical and immunological characteristics of islets and acinar tissue have been exploited to achieve large scale separation with varying success (Chapter 2).

The apparent robustness of islets in comparison to acinar tissue has formed the basis of many often adjuvant methods of purification, such as warm ischaemia 110, high dose radiation 198, short-term tissue culture 199 and freezing to -30°C 200.
Other methods depending on differences in the cell surfaces are often more applicable to single cell suspensions, however such cell separation methods can be adapted to whole islet purification and the use of anti-exocrine lectins or monoclonal antibodies bound to magnetic microspheres, or fluorescence activated cell sorting, have been used with some success.

The known physical difference in the density of islets and acinar tissue has formed the basis for methods such as sedimentation under gravity in cold Hank's solution or Percoll, and centrifugal elutriation, but the most effective method, remains that of isopycnic (equal density) gradient centrifugation. In this, the digest is centrifuged in a density gradient allowing the islets and acinar tissue to float to positions in the gradient where the density of the gradient medium is identical to that of the tissue fragment. In view of the proven effects of osmolarity, pH, and various ions on cell density it is perhaps not surprising that numerous different gradient media have been tried with varying results. The use of sucrose was the first to be described, followed by the use of its polymer Ficoll. These caused a decrease in islet viability, which improved with dialysis of the Ficoll to remove the impurities, and more recently by the suspension of Ficoll in Euro-Collins instead of Hank's. More iso-osmotic gradient media have also been tried, including Hypaque-Ficoll, Metrizamide, Ficoll-Conray, Ficoll-Nycomed, Percoll, Dextran, and bovine serum albumin. The ideal gradient media, which prevents hypothermic cell swelling and can therefore be run at 4°C minimising further warm ischaemic damage, remains to be defined, the widely used hypertonic Euro-Ficoll is probably the closest.

1.3 HUMAN ISLET TRANSPLANTATION

i) AUTOTRANSPLANTATION OF UNPURIFIED ISLETS

The transplantation of only the endocrine portion of the pancreas avoiding the problems caused by the release of digestive enzymes from the exocrine element, has always seemed an intellectually attractive concept. However the process of islet purification using density gradients in the human will always be imperfect, as there appears to be considerable overlap in the density of acinar tissue and islets, so that the purification process results in a yield of islets which falls with increasing purity. This reduction in islet numbers made reversal of diabetes using islets from one pancreas difficult in large animal models, and led many to question the assumptions underlying the purification step.

In 1977 Mirkovich and Campiche described the prevention of diabetes in 20 of 25 totally pancreatectomised dogs, by autotransplantation of unpurified collagenase dispersed pancreas into the spleen, findings which were subsequently confirmed by others. Animal studies looking at the intraportal transplantation of such grafts, were however much less successful and illustrated some of the potential problems, such as portal hypertension and liver cell damage.
Nevertheless, such studies encouraged the initiation of clinical trials beginning in early 1977, in patients undergoing total pancreatectomy for chronic pancreatitis in whom the spleen had been removed, necessitating the use of the intraportal route. The first reports came from the Minneapolis group, and while they, and a few other groups and have continued achieve some success with this approach (see Table 1.1), it has been obvious from an early stage that "the surgeon and the patient must still be willing to accept diabetes for relief of pain when performing this operation." Not only do many of the grafts not function initially but many will fail in the ensuing years presumably due to graft 'exhaustion'.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>number</th>
<th>Outcome (duration of insulin independence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sutherland et al 234,237,238</td>
<td>25</td>
<td>1m, &gt;1m, 8-18m(2) &gt;16m, 18m, &gt;20m,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;29m, &gt;60m, 6y, &gt;6y1m, &gt;7y2m.</td>
</tr>
<tr>
<td>Cameron et al 235 Baltimore</td>
<td>8</td>
<td>3-8m(3), &gt;9m, &gt;18m, &gt;22m.</td>
</tr>
<tr>
<td>Valente et al 239,240 Genoa</td>
<td>12</td>
<td>&gt;2w to &gt;13m (9).</td>
</tr>
<tr>
<td>Toledo-Pereyra et al 241 Detroit</td>
<td>7</td>
<td>&gt;20m, &gt;25m.</td>
</tr>
<tr>
<td>Grodsinsky et al 242 Detroit</td>
<td>3</td>
<td>&gt;14, &gt;9, &gt;4.</td>
</tr>
<tr>
<td>Hinshaw et al 243 Loma Linda</td>
<td>5</td>
<td>2m, 20m, &gt;4m, &gt;14m.</td>
</tr>
<tr>
<td>Altman et al 244 Paris</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Dobroschke et al 245 Giessen</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dafoe et al 246 Philadelphia</td>
<td>2</td>
<td>&gt;22m</td>
</tr>
<tr>
<td>Traverso et al 247 Los Angeles</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Lorenz et al 248,249 Berlin</td>
<td>12</td>
<td>4m, 8m, 30m, 36m, &gt;48m, &gt;48, &gt;24</td>
</tr>
<tr>
<td>Ricordi et al 250 Pittsburgh</td>
<td>2</td>
<td>&gt;1m, &gt;10m</td>
</tr>
<tr>
<td>TOTAL</td>
<td>84</td>
<td>46</td>
</tr>
</tbody>
</table>

Table 1.1 Published cases of intraportal islet autografts (adapted from Farney and Sutherland 238)

More worrying still, several reports have illustrated the potential complications of embolising large volumes of tissue into the portal vein, with reports of hepatic infarction from Michigan, portal hypertension from Baltimore, disseminated intravascular coagulation, systemic hypotension and death. The cause of these may be related not only to the disturbance in hepatic blood flow, but also to pancreatic enzymes, collagenase and thromboplastin, and may be ameliorated by the addition of anticoagulants and enzyme inhibitors. Attempts to avoid such complications by the use of alternative, extravascular sites such as the kidney capsule, intraperitoneal and intramuscular sites, have met with little success.
In addition to these immediate complications, the gross exocrine contamination may impair the engraftment of the islets both beneath the kidney capsule, and intraportally, by their proximity to necrotic exocrine tissue \(^ {262}\) or perigraft fibrosis \(^ {263}\), and even in the spleen, 'a non-confined site' significantly more islets are required in unpurified preparations to reverse diabetes \(^ {264,265}\). Exocrine contamination has been also been shown to significantly increase the immunogenicity of islet grafts in allotransplantation and increase the rejection rates \(^ {266-270}\) with the acinar and ductal tissues but not the islets expressing MHC class II antigens during the rejection response \(^ {271}\). This may go some way to explaining why the results of allotransplants using the same techniques are less successful \(^ {272,273}\), although success has been achieved in the last year \(^ {274}\). Transplantation of the dispersed pancreas therefore seems peculiarly suited to autotransplantation of patients with chronic pancreatitis, where the number of islets present preoperatively may already be marginal \(^ {275}\) and where rejection is not a problem. In this group transplantation of only 265,000 islets can result in appropriate release of insulin and prolonged periods of insulin independence.\(^ {237}\)

**ii) Human Islet AlloTransplants**

The first report of amelioration of diabetes by transplantation of isolated islets was made in 1970 \(^ {276}\) and it rapidly became obvious that, at least in rodents, purified islets could successfully reverse diabetes when transplanted intraperitoneally \(^ {157}\), intraportally \(^ {277}\), into the spleen \(^ {278,279}\) and beneath the renal capsule \(^ {280}\).

The first report of purified human islet transplantation came in 1977 in a report on 10 transplants into varying sites in recipients who had previously received a renal allograft for end-stage diabetic nephropathy \(^ {281}\). Although no patient became insulin independent, 7 patients temporarily reduced their insulin requirements.

In 1980 \(^ {282}\) Kolb and Largiader described a type 1 diabetic patient who became insulin independent 9.5 months after simultaneous transplantation of two kidneys and intra-splenic injection of dispersed pancreatic tissue containing 200,000 islets from a 2.5 year old donor. No pre-transplant insulin levels were reported, and remarkably the islets appeared to continue to function even after rejection of both kidneys and reduction of immunosuppression, until the patient’s death 20 months post transplant \(^ {283}\).

It was not until 1990 that the first report of insulin independence following purified islet transplantation was published \(^ {284}\), and during the years 1990-92 the number of clinical islet allografts performed has approached that of the previous 20 years, with varying but increasing success (Figure 2). The 11 institutions involved over the last three years, have used methods that varied in nearly every respect however making analysis of the results almost impossible.
<table>
<thead>
<tr>
<th>GROUP</th>
<th>No</th>
<th>Period</th>
<th>Purified</th>
<th>Site</th>
<th>C peptide</th>
<th>1-independence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minneapolis 272-274,281</td>
<td>33(5) (1)</td>
<td>1974-1992</td>
<td>p(3),n(2) p</td>
<td></td>
<td>&gt;5m</td>
<td>2-4d, 13-17d, 90-&gt;120d,123-&gt;268d</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td></td>
<td>n(4),d(33) p</td>
<td>ip</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zurich 283,285</td>
<td>8 (4) (3)</td>
<td>1977-1988</td>
<td>d</td>
<td>pv</td>
<td></td>
<td>9.5-20m</td>
</tr>
<tr>
<td>Genova 286</td>
<td>13(1) (12)</td>
<td>1978-1979</td>
<td>d</td>
<td>dc,i p</td>
<td></td>
<td>3-10m, 3-19m</td>
</tr>
<tr>
<td>Detroit 287,288</td>
<td>7 (5) (2)</td>
<td>1980-1985</td>
<td>d</td>
<td>Kc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Berlin 289</td>
<td>8</td>
<td>1982-1987</td>
<td>d</td>
<td>pv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>St Louis 287,290,291</td>
<td>23(6) (3)</td>
<td>1985-1992</td>
<td>d</td>
<td>S p</td>
<td>&lt;3m(3)</td>
<td>10-25, 33-341</td>
</tr>
<tr>
<td>Paris 295,296</td>
<td>7 (4)</td>
<td>1988-1991</td>
<td>p</td>
<td>eL</td>
<td>-6m, -23m, -23m,</td>
<td>7-&gt;34m, -7d</td>
</tr>
<tr>
<td>Perugia</td>
<td>3</td>
<td>1989</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Berlin 297</td>
<td>1</td>
<td>1989</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edmonton 298,299</td>
<td>5</td>
<td>1989-1992</td>
<td>p</td>
<td>pv</td>
<td>1d-8m(2), 1d-&gt;6m.</td>
<td>69-645</td>
</tr>
<tr>
<td>Milan 300,301</td>
<td>8</td>
<td>1989-1991</td>
<td>p</td>
<td>pv</td>
<td>8-12d, 19d-&gt;1y, 10d-&gt;1y, 2m-&gt;1y, 1m-&gt;1y</td>
<td>110-300d, 213-340d.</td>
</tr>
<tr>
<td>Pittsburgh 250,302,303</td>
<td>23(4) (10)</td>
<td>1990-1992</td>
<td>pv L p Cl</td>
<td>&gt;16, 6m,</td>
<td></td>
<td>24, 13, &gt;6, 9, &gt;13, 10</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td></td>
<td></td>
<td>pv Cl</td>
<td>&lt;1m, &lt;1m, &lt;1m, &gt;1m, &gt;1m-&gt;1m.</td>
<td></td>
</tr>
<tr>
<td>Leicester 304</td>
<td>3</td>
<td>1991-1992</td>
<td>p</td>
<td>pv</td>
<td>&lt;1m, 1m, &gt;6m</td>
<td></td>
</tr>
<tr>
<td>Charlestown</td>
<td>1</td>
<td>1991</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Los Angeles</td>
<td>1</td>
<td>1992</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
No: number of transplants (not patients).

Purified: d=dispersed, p=purified, n=neonatal (unpurified islet rich)

Site: e=epiploic flap, pv=portal vein, ip=intraperitoneal, Kc=kidney capsule, S=intrasplenic, sc=subcutaneously,

C=part of a cluster transplant, L=with a liver transplant, IO=islet only (the rest are associated with kidney transplants), dc=diffusion chamber.

C peptide: duration of islet function in recipients where pre-transplant levels were documented as 0, and who did not become insulin independent.

**Table 1.2** Summary of human islet allotransplants from cadaver donors 1970-1992

In 1980 the International Pancreas and Islet Registry was founded and organised initially at Minneapolis, until the islet component was transferred to Giessen in 1989. This review makes use of up to date information from the registry together with reports in the literature in an attempt to define criteria for successful (insulin independent) islet allotransplants.

**Figure 1.2 Showing the Number and Success of Adult Islet Allografts**

Shows the 166 islet transplants performed since 1916, including two in 1991 whose pre-transplant C-peptide levels were >0 ng/ml

The success of human islet transplants both long- and short-term, is known to depend on many factors, including the viability of the islets, the number transplanted, the type of
recipient and degree of pre-existing insulin resistance, the site used and rate of engraftment, and rejection.

iii) FACTORS AFFECTING SUCCESS

a) The effect of pre-transplant storage

The major difficulties involved in producing enough purified islets from a single pancreas to reverse diabetes can be approached in two ways, either by improving the results of purification until enough islets can regularly be produced from one donor, or by pooling islets from several donors. This latter approach necessitates storage of the islets until sufficient numbers are available to transplant, a facility which could also allow improved HLA matching of donor to recipient.

Culture of human islets

Although adult human islets have been maintained for up to 2 years in culture at 37°C, there is general agreement that the risk of infection, and the gradual loss of islet number and viability and make this an inappropriate form of islet storage, particularly for impure islets, despite the fact that it may improve purity. Low temperature culture (24°C) of human islets has also been shown to preserve function over 7-14 days, and may have an additional immunomodulating effect. While islets cultured in this way revascularise normally, and have been successfully used to achieve insulin independence, most groups have tried to minimise the period of culture and the resulting losses, infusing the islets immediately, or within 24 to 48 hours.

Cryopreservation

Using methods described by Rajotte et al, there is no doubt that in combination with fresh islets, cryopreserved islets can help to render diabetics insulin independent, allowing transplantation from as many as 11 donors and producing long-term normoglycaemia. The process of cryopreservation may have several other advantages; not only does it allow >10,000 islets/recipient kg to be transplanted, but, as with culture, cryopreservation may improve purity and decrease immunogenicity by depleting passenger leucocytes, while transplantation from multiple donors may also prolong allograft survival.

However, while cryopreservation does not affect revascularisation, the process causes numerical losses of at least 20% and morphological changes with further functional losses, so that no patient has been rendered insulin independent using only cryopreserved islets. (Figure 1.3)
Figure 1.3 Showing the Effect of Islet Preservation on Transplant Success 1990-6/1992

- Number of Adult Islet Allografts
- Number with Basal C-peptide >1ng/ml 1 Month Post-transplant
- Number off Insulin for >1 week

Includes only those 67 transplants in the last 30 months with pre-transplant C-peptide levels <0 ng/ml

Figure 1.4 Showing the Effect of Donor:Recipient ratio on graft function 1990-6/1992

- Number of Adult Islet Allografts
- Number With Basal C-peptide >1ng/ml 1 Month Post-transplant
- Number off Insulin for >1 week

Including only the 67 patients from the last 30 months.
As the success rate of whole pancreas transplantation increases, the transplantation of islets from more than one donor is increasingly seen as being wasteful of a scarce resource, with unnecessary exposure of the recipient to an increased number of foreign antigens. These obvious drawbacks have initiated a trend towards using fresh islets in one to one transplantation (Figures 1.3 & 4).

At present if one to one transplants are to be achieved with the already marginal yields, the losses from cryopreservation are unacceptable. However, with improved yields it seems likely that cryopreservation would allow the long-term storage of islets perhaps as part of comprehensive tissue banks, until suitably well matched recipients were available.

b) Transplantation site
This has important implications not only for the survival of the graft, but also for its ability to normalise metabolism and prevent long-term diabetic complications.

The effect on graft survival
While in rodents an amazing variety of sites have been used for successful islet implantation, including potentially immune privileged sites such as the thymus brain and testicle, in man with one exception, success has only been achieved with intravascular sites such as the liver, spleen, or epiploic flap.

Islet autograft studies in large animal models have shown good results with intraportal infusion, renal subcapsular transplantation and intra-splenic grafting by a variety of techniques, such as direct puncture, arterial, or retrograde venous infusion. Although similar islet numbers achieve successful reversal of diabetes using either the spleen or liver, the renal subcapsular space and epiploic flap have been less successful in comparison. The long-term function of such grafts is also important and intraportal grafts in particular have been shown to fail after several months and their survival may be inferior to intra-splenic grafts. While the injection of unpurified pancreatic tissue into both the spleen and liver, can cause problems, the use of purified islets appears to be uncomplicated. In clinical trials, the overwhelming majority of transplants have been intraportal (Figure 1.5), encouraged by the low morbidity, the success of intraportal autotransplants, the ease of access percutaneously, and the histologically proven survival of islets. Although the numbers transplanted intra-splenically and into epiploic flaps have been too low to allow useful comparison, particularly in view of differences in recipients, it is interesting that in the last 3 years, only patients receiving intraportal transplants have successfully achieved insulin independence.
Normalisation of metabolism

Hyperinsulinaemia secondary to insulin resistance is increasingly acknowledged as a contributing factor to the increase in macroangiopathy \(^5\) and cardiovascular mortality \(^3\) seen in the both type 1 and type 2 diabetics \(^3\). In view of the major role of the liver in the clearance of insulin and glucose \(^6\), the site of insulin secretion in relation to the portal circulation may well be important in the normalisation of metabolic control and the prevention of diabetic complications. The delivery of insulin into the systemic rather than portal circulation following whole pancreas transplantation has been shown to increase hyperinsulinaemia \(^7\), as has the administration of immunosuppressive steroids \(^2\). In addition, inferior metabolic control by systemically drained transplants has been demonstrated in several rodent studies \(^3\).

It seems likely that transplantation of islets into the portal circulation where they can respond appropriately to enterically absorbed glucose offers the best chance of normalising glucose metabolism \(^2\) on a long term basis \(^2\).

c) Number of islets transplanted

A study of normal human pancreases, has estimated them to contain 3.6-14.8 million islets, with a mean radius of 157+/−35μm diameter, occupying 0.77-3.78% of the pancreas volume \(^3\), although this number of islets is high and other reports have put the number at around 1 million \(^3\), the figures otherwise agree other studies \(^3\). While surgical studies
looking at the effect of partial pancreatectomy on glucose metabolism have suggested that only 10% of the pancreas may be needed for normal glucose homeostasis \(^{355}\), such conclusions ignore the effect of the concomitant reduction in glucagon secretion \(^{356,357}\), and nutrient absorption resulting from excision of the exocrine pancreas and insulin requirements can actually be higher in patients following partial than total pancreatectomy \(^{357}\). The careful follow-up studies of living related donors after hemi-pancreatectomy have also cast doubt on the estimated requirement of 10% of the pancreas, showing a significant increase in fasting glucose and decrease in insulin secretion one year post-operatively \(^{358,359}\) even resulting in diabetes, and suggesting a much greater requirement.

While insulin independence following autotransplantation has been achieved using only 110,000 islets and normal fasting plasma glucose concentrations using 265,000 \(^{237}\), it seems likely that in allograft situations where the recipient, already insulin resistant as a result of their diabetes and immunosuppression, is rendered hyperglucagonaemic by the islet transplant \(^{360,361}\), much higher numbers might be required. The exact number, probably varies for any individual, depending on recipient variables such as insulin resistance and rejection and transplant variables such as islet viability, engraftment and immunogenicity.

In non-diabetic islet recipients such as patients rendered apancreatic by upper-abdominal exenteration insulin independence has been achieved following allotransplantation of 285,000 150\(\mu\)m equivalent islet numbers (IEq)\(^{303}\), while another patient, diabetic secondary to haemochromatosis, became insulin independent following a liver-islet co-transplant of 105,000 islets \(^{362}\). The co-transplantation of a liver appears to have a beneficial effect on islet survival (Fig. 1.6), reducing the numbers required to those of autotransplants.

![Figure 1.6 Showing Recipient Category and Success Rate 1990-6/1992](image)

Including only the 67 transplants from the last 30 months
In the absence of this effect, although as few as 200,000 islets have been reported as successfully reversing type 1 diabetes, all other reported successes have required at least 592,000 IEq. The fact that groups have required between 10,800 and 18,699 IEq/recipient Kg has led to the conclusion that 'over 10,000 IEq/Kg body weight are needed in the immunosuppressed recipient' and in theory the requirements may be even higher. With islet isolation yielding a mean of 250,000 islets per pancreas, all but 2 successes have utilised more than one donor.

The spontaneous recurrence of hyperglycaemia following successful transplantation also appears to be at least partly dependent on the number of islets transplanted, and long-term follow-up of large animal autografts have suggested that chronic stimulation of an initially marginal β-cell mass might be responsible, with animals receiving most islets remaining normoglycaemic longest. This hypothesis is supported by the finding that prolonged hyperglycaemia can induce diabetes in partially pancreatectomised dogs.

While whole organ grafts are revascularised at the time of transplantation, purified islet grafts require a process of angiogenesis and revascularisation to establish the microcirculation necessary for a nutritional blood supply. In the native pancreas the islets are supplied by a capillary glomerulus with the opportunity for intra-islet regulation of hormonal secretion, and an insulo-acinar portal system, that is capable of responding to changes in glucose concentration. Examination of intraportal islet grafts has shown the survival of morphologically intact islets scattered though out the liver, and demonstrated the ingrowth of capillaries connecting both the portal vein and hepatic artery beginning from day 4, and completed between day 8-11, with a microvascular morphology very similar to that of pancreatic islets in situ. While the islets are clearly bathed in blood from the time of their intraportal infusion, the laws of gas diffusion suggest that the centre of large islets or clumps of tissue will be vulnerable to anoxia as their capillary bed collapses. The death of β cells particularly those in the centre of large islets which have been shown to be the most responsive to glucose, may be responsible for the number of islets required for transplantation, and factors affecting the rate of engraftment would have a significant effect on transplant survival.

The effect of diabetes and hyperglycaemia

One of the factors that has been extensively examined is the effect of the diabetic state and in particular hyperglycaemia. The persistent stimulus generated by elevated glucose levels results in degranulation of islets, with loss of cells and a reduced response to stimulation, while a period of insulin therapy following transplantation has been shown to improve graft function and decrease the number of islets needed to successfully reverse diabetes. Whether this is due to factors such as diabetic microangiopathy
affecting engraftment 381,382 which has been questioned 383, or a direct toxic effect of hyperglycaemia on the islet 384 has been difficult to determine. Extrapolating from this rodent work, all groups performing clinical islet transplants have adopted a policy of maintaining normoglycaemia by intravenous insulin infusion in the peri-transplant period, to maximise islet engraftment 291,293,298,300,303,304.

**Immunosuppressive therapy and engraftment**

The effect of various immunosuppressives on engraftment has also been examined. Prednisolone has a detrimental effect on islet graft function 385 particularly when given at the time of transplantation 386, appearing to suppress angiogenesis and leading to a reduction in the capillary network 387, in addition to its effect in increasing insulin resistance. Cyclosporin has also been shown to have a deleterious effect on islet function by inhibiting revascularisation 388,389, and in kidney transplant recipients 390 and established whole pancreas grafts 391, it has also has been shown to reversibly inhibit insulin release 392,393 and may potentiate the insulin resistance produced by prednisolone 394,395. FK506 has similarly been shown to produce both insulin resistance 396, and a reduction in insulin secretion 397,399, which may exceed that of cyclosporin 400, as has azathioprine 401.

Despite these potential problems, successful islet grafts have been achieved using a combination of prednisolone and either FK506 402, or cyclosporin 291,294,298,300, and it appears that provided sufficient islets are transplanted, the deleterious effects can be overcome. Nevertheless if islet transplantation is to succeed it is imperative that the majority of islets survive in a competent functional condition. Some newer immunosuppressives such as 15-deoxyspergualin have no known adverse effect on islet function 273 and appear to improve revascularisation 403, and the use of this by the Minnesota group in their recent successful allotransplant 274 may have contributed to its success. Other ways of actually stimulating endothelial cell growth and revascularisation are now also being examined 373.

Reinnervation of transplanted islets with the in growth of non-myelinated nerve axons into the islet has also been noted both in the spleen 371,404 and kidney capsule 405. Their significance in terms of control of insulin release and graft blood flow is unknown 406, but their presence might significantly affect long-term metabolic control.

**1.4 FACTORS AFFECTING REJECTION**

Hopes that free cellular grafts might be less immunogenic than vascularised whole organ grafts, and that purified islets like other endocrine tissues 407, with their lack of HLA class II DR antigen expression 408-411, might be particularly privileged 267,412 have proven false. Pancreas grafts are highly antigenic and islet grafts appear even more so 413-415. The reasons for this remain unclear, but the size of the graft is probably relevant, as is the up-regulation of MHC antigen expression that occurs during the islet isolation process.
i) IMMUNOMODULATION OF THE GRAFT

The gene products of the major histocompatibility complex can be divided into two functional groups. Class I antigens (HLA-A, -B & -C in man) are believed to be present on most nucleated cells and are the target of cytotoxic T lymphocytes in transplantation reactions producing the barrier of MHC antigens proposed by Medawar 416,417. In contrast, class II antigens (HLA-DR, -DQ & -DP in man), are expressed on a limited number of cells, such as lymphocytes, and directly initiate the helper T lymphocyte response in transplant rejection, representing the passenger leucocytes or antigen presenting cells suggested by Snell 418,419. Modification of both of these processes have been shown to improve islet graft survival.

a) Removal of passenger leucocytes particularly dendritic cells

Studies on islets have shown that they contain passenger leucocytes 420 and vascular endothelium 421,422 expressing class II antigen. Unlike whole organ grafts, in cellular grafts it is practical to consider removing all these cells, allowing allografts of pure endocrine cells, known to express only class I MHC antigens, to survive even in non-immunosuppressed recipients. Various methods have been used to rid islet grafts of passenger leucocytes, and the use of anti-la (class II) antibody with complement 423-425, UV 426 and γ-irradiation 270,427, in vitro culture 428,429, cryopreservation 315,430 and hypothermic culture 310 have frequently achieved long term graft survival.

b) Level of Class I MHC antigen expression

Although depletion of antigen presenting cells can significantly improve graft survival by preventing the direct pathway for alloantigen recognition the islets remain immunogenic 431. There is evidence that class I antigens on their own can activate recipient T helper cells 432 via the indirect pathway using recipient antigen presenting cells 433, and the level of expression of class I antigens by the non-lymphoid cells of the islet graft has been shown by most 434-438, although not all 439,440, to be a decisive factor in the survival of islet allografts, particularly in large animal models 441.

The importance of class I expression has received support from demonstrable downgrading of class I expression in islets by culture at 24°C 442 which may be one factor in the resulting improved allograft survival. While the masking of class I antigens by F(ab)2 antibody fragments allows long-term function of xenografted human islets in mice without immunosuppression 438,443.

c) Expression of class II MHC expression on islets

Although islets have been shown not to express class II MHC antigens constitutively, they have the potential to do so, and various factors such as collagenase 444 and γ-interferon with 445,446 or without 446,447 tumour necrosis factor or lymphotoxin, have been shown to
stimulate this response, which may be implicated in the development of autoimmune diabetes. While the expression of class II is not sufficient to endow islets with complete antigen presenting cell function, it does confer a susceptibility to conventionally initiated class II-specific immune attack. However class II antigens have been shown to be either absent during rejection, or of dubious significance, and the implications for allograft survival may be small, or even protective.

Although both cryopreservation and hypothermic culture have been used in clinical trials, there is little evidence that either provide sufficient immunomodulation to allow long term function without additional immunosuppression. Until such methods are available, the above evidence suggests that matching of both class I and class II HLA antigens is important in preventing human islet allograft rejection.

### ii) IMMUNOSUPPRESSION OF THE RECIPIENT

#### a) Maintenance immunosuppression

The success of clinical transplantation has relied heavily on the long-term prevention of rejection using immunosuppressive agents that non-specifically suppress the recipient’s immune response. Current strategies in islet transplantation have been extrapolated from experience gained in renal and whole pancreas transplantation modified in the light of large animal work.

The efficacy of drugs such as prednisolone which reduces circulating T and B lymphocytes and azathioprine which inhibits the primary antibody response has been significantly augmented by the addition of drugs such as cyclosporin A and FK506 whose major effect is the inhibition of T-cell activation. Following its initial use in whole pancreas transplantation, cyclosporin was soon shown to significantly improve graft survival. In animal studies its use improved the results of transplantation of both microfragments and purified islets. The adverse effects of both cyclosporin and FK506 in engraftment outlined above (see engraftment Section 1.3 iii d), may make it important to minimise their use in the first few days following islet transplantation, and the manipulation of induction immunosuppression by some groups has attempted this. FK506 has only been used by one group and only patients receiving a simultaneous liver transplant became insulin independent.

The effect of long term immunosuppression on islet graft survival while unavoidable may be deleterious and constitutes another obstacle to achieving insulin independence.

#### a) Induction immunosuppression

The use of polyclonal antilymphocyte serum, has been shown to deplete recipient helper T lymphocytes and induce suppressor cells. This improves islet graft survival on its own, and in combination with immunomodulation by γ-irradiation, or in vitro culture, other immunosuppressives or donor specific blood transfusion. The
effect often requires only a single injection although the timing of this appears critical\cite{467}, perhaps immunomodulating the islet graft itself.

Specific monoclonal antibodies directed against lymphocyte subsets such as the OKT3 a murine monoclonal antibody\cite{468} which blocks the antigen binding of human T cells\cite{469} has also been shown to significantly improve the results of acute rejection therapy compared with conventional high dose steroids\cite{470,471}. The use of equivalent antibodies in rodents has suggested that transient removal of the helper-inducer T cell subset can lead to prolonged\cite{425,472} or even indefinite\cite{473,474} islet allograft survival.

Both agents have been shown to be effective when given in the first few days after renal transplantation\cite{475,476}, and the increasing use of both ALG\cite{272,291,298,300} and OKT3\cite{294} as induction immunosuppression has been accompanied by an improvement in the results of islet graft function in clinical trials\cite{Figure 1.7}.

![Figure 1.7](image)

Comparing the results of “State of the art” transplants in which >8,000 IEq/recipient Kg, with a purity >50%, were transplanted intraportally with induction immunosuppression using ALG, ATG or OKT3.

(only transplants in type 1 diabetics are shown)

c) Diagnosis of rejection

The ability to diagnose and treat rejection episodes at an early stage has undoubtedly improved renal allograft survival, in whole pancreas and islet transplantation however no effective markers of early endocrine rejection exist, and more than 90% $\beta$-cell destruction occurs before the return of hyperglycaemia or falling C-peptide levels, both of which are
therefore late indicators of usually irreversible damage. The lack of any early marker of rejection prevents effective treatment and makes the first rejection episode likely to be the last, contributing significantly to rejection rates.

In whole pancreas transplantation imaging of the graft using isotope scans, ultrasound and duplex-doppler can rarely diagnose rejection reliably. However since the exocrine portion of the pancreas appears to be more susceptible to rejection, utilisation of ductal drainage techniques permitting analysis of exocrine secretions (e.g. pancreatico-cystostomy) allows early diagnosis and prevention of endocrine damage, improving rejection reversal rates from 31-91%. Cytology, falls in urinary amylase levels and increasing neopterin levels have all been shown to be varying successful predictors of pancreas rejection. In addition fine needle aspiration cytology and percutaneous cystoscopically directed biopsies are possible.

More applicable to islet transplants, is the fact that in simultaneous kidney islet transplants from a single donor, kidney rejection serves as an early marker of simultaneous islet rejection, the resulting early treatment, has contributed significantly to the higher 1 year graft survival rates (up to 86%) in simultaneous pancreas kidney grafts. Analysis of serum markers of rejection such as soluble interleukin 2 receptor may also be helpful.

The use of both immunomodulation and immunosuppression appear to act synergistically to improve islet allograft survival, probably by decreasing both the indirect and direct stimulation of rejection. The fact that antilymphocyte immunosuppression at the time of transplantation appears so beneficial may be because it does both.

The need for immunosuppression with all its side effects, has limited the place of islet transplantation like that of whole pancreas transplants, to diabetics who already require immunosuppression for other grafts. However, immunomodulation of isolated islets, with inhibition of T-cell activation (capable of inducing a metastable state in which the islets are rejected when the recipient is challenged with lymphoreticular cells of donor origin), combined with donor-specific manipulation of the recipient immune system causing either anergy or active suppression of donor-reactive T-cells, can produce a state of graft tolerance and might allow islet transplantation of those likely to benefit the most, diabetics without the disease’s long-term complications.

**iii) INDUCTION OF TOLERANCE**

Tolerance is defined as the inability of a recipient in the absence of concomitant immunosuppression or immunomodulation to reject an established allo- or xenograft following challenge with fresh donor specific lymphoid cells, while the capacity to reject a third party graft remains intact.

The way in which antigen is presented to the immune system appears to determine whether immune rejection or tolerance is induced. Several different ways of inducing
tolerance to histoincompatible islet grafts have been demonstrated, with the method employed to prevent primary graft rejection and the nature of the histoincompatibility barrier being important background factors.

**a) Administration of alloantigen**

In 1964 Halasz et al showed that the administration of 2ml of donor blood prolonged the survival of canine renal transplants, although it was not until a decade later that it was shown that transfusion of allogeneic blood, particularly containing lymphocytes, improved renal transplant survival.

More recent studies have suggested that the systematic administration of non-immunogenic donor alloantigen can induce tolerance, and pre-immunisation with donor blood treated with either 1a antibody and complement, or UV irradiation has been shown to prolong islet allograft survival, as has the use of UV irradiated dendritic cells post-transplant, and even the oral administration of pancreatic antigen may prolong graft survival.

**b) Bone marrow chimerism**

It has been recognised for some time that transplantation of MHC mismatched bone marrow stem cells into neonatal recipients induced specific tolerance to the donor antigens with immunocompetence against third party grafts. With conditioning of either the donor or recipient bone marrow or both, this process can be extended to adult recipients to create mixed allogeneic and even xenogeneic chimerism with retained immunocompetence and donor specific transplantation tolerance allowing donor specific islet xenografting.

**c) Other grafts producing chimerism and tolerance**

The intrathymic inoculation of donor antigen has similarly been shown to produce tolerance by intrathymic chimerism and the interaction of maturing thymocytes with foreign alloantigens.

Liver transplants have been shown not only to enjoy prolonged survival despite HLA incompatibility which would cause rejection of a kidney or pancreas, but to produce a chimeric state, with tolerance to other grafts such as kidney, skin and islets from the same donor, and a similar state may be produced following small bowel transplants.

Such studies suggest that migration of dendritic and lymphoid cells is associated with graft acceptance rather than rejection, and has led to suggestions that rather than deleting dendritic cells from grafts, improved strategies might include peri-transplant infusions of donor bone marrow or other immunocompetent cells.
Clinically the improved survival of islets by liver and islet co-transplantation from the same 303,362 or even a third party donor 303 supports the possibility that they can benefit from a state of tolerance (Figure 1.6). A similar explanation may account for the protection apparently conferred on the pancreas during simultaneous kidney pancreas transplants 492,493.

iv) IMMUNOMECHANICAL BARRIERS

Although a combination of immunomodulation and immunosuppression may allow xenotransplantation 534, and fetal porcine islets may have been shown to survive in clinical trials in humans 535, immunoisolation preventing interaction between graft antigens and host immune system by a mechanical barrier, offers one of the most promising approaches to overcoming the formidable obstacles to xenotransplantation and providing a potentially unrestricted source of islets.

a) Microencapsulation

Since the first report in 1980 536 of the microencapsulation and transplantation of individual pancreatic islets, the prospect of being able to immunoisolate islets while maintaining their physiological function, thereby avoiding rejection of even xenografts, has engendered considerable interest, and seen the development of microcapsules in a variety of materials 537-540, together with larger diffusion chambers 541-543 and hollow fibres 544,545 capable of containing greater numbers of islets. Nutrients from the surrounding extracellular fluid diffuse across the membrane to nourish the islets, and insulin secreted in response to glucose levels passes back into the extracellular fluid and eventually into the circulation.

There is no doubt that islets encapsulated using a three layered alginate - poly-l-lysine - alginate membrane 537, release insulin appropriately in-vitro, 536,546,547 and are capable of reversing streptozotocin-induced diabetes in rodents when placed intra-peritoneally in large numbers as isografts 536,548, allografts 549 or xenografts 550,551. However attempts to use them in spontaneously autoimmune diabetic models (the non-obese diabetic mouse or BioBreeding rat) have nearly all, with rare exceptions 552,553, resulted in failure due to peri-capsular fibrosis 554. This inflammatory reaction may be due to bioincompatibility of the membrane 555,556, although in many cases only capsules containing islets appeared to cause it 557, and the permeability of the capsule to cytokines 558 may result in an inflammatory response, depriving the islet of nutrients and causing direct damage 559-561. Although immunomodulation 562 and immunosuppression 554,557,563 reduce this reaction, they also acknowledge defeat of the purpose of the technique.

Other problems with encapsulation include, capsule fragility, and the size of the encapsulated islets (0.25-0.65 mm) which limits their transplantation to less effective sites, such as the peritoneal cavity 564. The size of the capsule, and the diffusion distances involved, also adversely affects the pattern of insulin response to glucose 551,565 and
millipore diffusion chambers (13mm), increase this still further. The development of smaller capsules may help to reduce these problems.

b) Vascular biohybrid artificial pancreas

The incorporation of islets into a vascular shunt with blood (or ultrafiltrate) circulating through the centre of the device, separated by a membrane from a compartment containing islets, is an alternative to individually encapsulated islets, although the two may be combined. The major advantages of such a device compared with the use of capsules are that fibrin deposition or necroendothelialisation rather than fibrosis occurs on the immunoprotective membrane, and insulin, glucose, nutrients and oxygen pass directly to and from the blood reducing response times and islet hypoxia. Such devices are however susceptible not only to some of the problems with diffusion distances and fibrosis seen with encapsulation, but also to thrombosis, bleeding from anticoagulation, membrane rupture, calcification and infection. Despite this they are capable of functioning as allografts or xenografts, eliminating the need for exogenous insulin in pancreatectomised dogs.

1.5 SUMMARY

Two major problems persist in islet transplantation: achieving adequate yields of islets from one pancreas to reverse diabetes, and preventing rejection of allogeneic islet transplants. The solution to both problems is interrelated and multi-factorial depending upon the optimisation of many of the factors outlined above. In many ways, current clinical trials involving transplantation of long standing type 1 diabetics with their insulin resistance, hyperglycaemia and need for immunosuppression provides perhaps the ultimate challenge, and while the progress made in the last 20 years has been slow, the recent successful islet transplants provide hope that the goal of transplantation of young newly diagnosed diabetics without the need for immunosuppression is an achievable one.
CHAPTER 2
REVIEW OF CELL SEPARATION TECHNIQUES AND THE
POTENTIAL OF IMMUNOMAGNETIC METHODS FOR ISLET
PURIFICATION

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      Electrophoresis

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d) Immunomagnetic islet purification

2.4 SUMMARY AND SCOPE OF PRESENT STUDY
2.1 INTRODUCTION

The development of tissue fractionation from an art to a science, has allowed the analytical study of individual cell types and organelles producing many of the advances in our understanding of cell biology at a cellular and molecular level. The technology for the separation of discrete types of cells from complex cell populations is relatively recent, and methods for fractionating and purifying subcellular particles, macromolecules and enzymes were devised long before cells could be reliably separated from each other. The ability to clone and grow homogeneous populations of cells and micro-organisms bypassed this need, and whole tissues were homogenized and treated as though their cellular constituents were identical. The main impetus for devising cell separation procedures has been the increasing interest in animal cell differentiation, in particular in the development of blood elements from haemopoietic stem cells, and in the behaviour of lymphoid cells during the immune response. The desire to produce pure samples of certain cells has led to the development of separation methods utilising every known difference in the physical and biological characteristics of cells, many of which have been adapted with varying success for the purification of pancreatic islets.

The evolution of the diverse range of cell separation methods is reviewed briefly, and their relative merits and application to islet purification discussed. The development and potential advantages of immunomagnetic cell separation methods are then expanded upon.

2.2 METHODS OF CELL SEPARATION

i) CELL SEPARATION USING PHYSICAL DIFFERENCES

Among the first and still most widely used methods of cell separation were those which made use of the physical differences between cells such as size, density and cell surface charge.

a) Cell size

Although simple sieves can achieve some degree of subcellular and cellular purification on the basis of size and deformability, the most widely used method for separating cells on the basis of size difference is that of sedimentation rate or velocity sedimentation which has been well reviewed. A relatively large spherical particle, such as a cell moving through a uniform medium under gravitational or centrifugal force rapidly reaches a constant velocity dependant on the resistance created to movement predominantly by its size, and described by adaptation of Stokes law as:-

\[
V = \frac{dr}{dt} = \frac{r^2(\delta c - \delta m) \omega^2 D}{k \eta}
\]

where \(D\) is the distance of the cell from the centre of revolution, \(t\) is the time, \(r\) is the radius of the cell, \(\delta c\) is the cell density, \(\delta m\) the medium density, \(\omega\) the angular velocity (speed of centrifugation), \(\eta\) is the medium viscosity at the cells location and \(k\) is a constant.
Isokinetic sedimentation is a particular type of velocity sedimentation in which a gradient rather than a uniform medium is used so that the cells sediment at constant velocities with the viscosity ($\eta$) of the medium increasing as a function of the distance (D) from the centre of revolution.

b) Cell density

Density dependent cell separation or isopycnic sedimentation, separates cells on the basis of differences in their buoyant density by equilibration on density gradients. Although the size and shape of the particles may determine the time they take to equilibrate to their isodense point on the gradient, these parameters no longer affect the separation achieved. This has become the method of choice for islet purification based on the fact that pancreatic exocrine cells are denser than most other mammalian cells, including islets. As discussed in Chapters 1&3 however the variable and often considerable overlap in the densities of exocrine fragments and islets limits the yield of purified islets, so that the purification process remains one of the limiting factors in human islet transplantation.

c) Physical differences in the cell surface

Since surface composition is usually a marker of cell type, surface-dependent methods of cell purification are often able to purify large quantities of cells having a specific function and a number of methods have been described, some of which have been adapted for islet cell separation.

Phase extraction

Countercurrent distribution or two-phase aqueous polymer extraction, depends upon the observed phenomenon that concentrated solutions of unlike polymers when shaken together, separate into different phases after standing for a while. The lower phase will be enriched in one polymer and the upper phase in the other polymer. Both phases are rich in water and can be made isotonic to preserve cell integrity. When cells are included in these mixtures, their distribution between the two phases at equilibrium is described by the equation:

$$\frac{C_1}{C_2} = \frac{\lambda A}{e^{\kappa T}}$$

Where $C_1$ is the concentration of cells in one phase, $C_2$ the concentration in the other phase, $A$ describes the surface area of the cell type under consideration, $\lambda$ is a constant other than area of the cell type under consideration, $\kappa$ = the Boltzmann constant, and T the absolute temperature.

The phase system can be both a mild and powerful separation method and by using differences such as surface charge and fatty acid composition it can complement other methods of physical cell separation.
Electrophoresis

The electrophoresis of cells and micro-organisms is relatively ancient among methods for cell separation and has been used since the beginning of the century. It separates cells on the basis of differences in their surface charge, which in turn depends upon the electrically charged groups carried by many of the components of the cell surface. The cells can either be electrophoresed in a medium which is stationary, or using continuous free-flow electrophoresis, in which the cells are introduced into a medium which flows from vertically down through a perpendicular electric field. Cells with differing electrophoretic mobility move along different paths and can be collected continuously at the bottom end of the chamber. The electrophoretic mobility of cells can be manipulated by alteration of functional cell-surface groups by the action of proteolytic enzymes, pH, cell maturation, virus infection and many others, and electrophoretic separation has been used extensively in the study of cells from lymphoid and haemopoietic tissues.

Although it has been used for the effective purification of viable canine pancreatic islet cells from a single cell suspension, the influence of sedimentation rate on the process makes it unsuitable for the purification of intact pancreatic islets.

ii) CELL SEPARATION USING FUNCTIONAL/BIOLOGICAL DIFFERENCES

a) Selective destruction by physical damage

Selective destruction of cells by physical damage of the more sensitive cell types is an alternative method of enriching the other components. Adrenal cortical hormones, ionising radiation, controlled rate freezing, hyperthermia and tissue culture, are among those methods used to enrich lymphocyte cell populations, and some of these such as warm ischaemia, high dose radiation, short-term tissue culture, and cryopreservation, have been used, often in conjunction with more specific methods, to purify islets with limited success.

b) Non-specific adherence

Functional differences between cells such as non-specific leucocyte adhesion, have also been exploited in order to separate them. In 1938 Wildy and Ridley reported the purification of peripheral blood leucocytes by allowing them to adhere to glass bottles. The adherent properties of macrophages, Leydig cells and others have since been used. The use of columns containing a variety of materials such as glass wool, nylon wool and glass beads combined the use of adherence with filtration.

c) Phagocytosis

The fact that neutrophils actively phagocyte iron particles in vitro, has been used to aid their separation from non-phagocytosing lymphocytes. Allowing them to
ingest iron particles, increases their density and allows them to be separated by centrifugation.

While adaptation of these methods for cell separation have been successful in purifying sufficient islets for human transplantation, with successful transplantation of immunosuppressed Type-1 diabetics requiring at least 10,000 IEq/recipient Kg \(^{291,294,298}\), yields averaging 200,000 islets per donor pancreas \(^{300}\) remain inadequate to allow one donor to one recipient transplantation. Although the diverse physical and biological nature of tissues is demonstrated by the ability to separate them in these ways, such methods are unable to distinguish discrete populations and sub-populations of cells and it seems unlikely that the physical and biological differences between islets and exocrine tissue are sufficiently great to allow the degree of separation required, even with optimal methodology.

Recently considerable progress has been made in our understanding of cells by using antigen/antibody interactions to separate cells expressing specific receptors (e.g., immunoglobulins), or antigens (e.g. histocompatibility antigens) on their surfaces.

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iii) IMMUNOLOGICAL METHODS OF CELL SEPARATION

In 1975 Kohler and Milstein described a method for producing immortal clones of cells producing a single antibody of preselected specificity (hybridomas) \(^{641}\). The ability to produce large quantities of monospecific antibody probes directed to specific cellular antigens such as membrane proteins has led to a rapid expansion in immune-mediated techniques for cell separation or destruction.

They can be divided into three broad categories:

i) Antigen or antibody specific for cell populations, is linked to a solid phase to which the relevant cells then adhere during incubation, removing them from the cell mixture.

ii) Elimination of specific cell types by antibody mediated cytotoxicity.

iii) Markers are attached by specific antigen/antibody interaction to specific cell populations, changing their physical characteristics, and allowing their removal by procedures such as flow cytometry, density centrifugation or exposure to a magnetic field. Labelling and physical segregation of cells are thus two separate processes.

a) Specific absorption of cells on a solid phase.

The linkage of antigens, haptons or antibodies to a solid phase support either by chemical covalent linkage or by adherence, allows the subsequent absorption and removal of cells expressing the relevant antibody or antigen. If required, they can be subsequently recovered to a variable extent from the solid phase, by disruption of the antibody-antigen complex.

A variety of materials have been used as solid phase immunoabsorbents in an attempt to optimise the balance of stability, specificity, inertness and ease of elution. They include cellulose \(^{642}\), glass beads \(^{643,644}\), polymethacrylate plastic beads \(^{645,646}\), polystyrene \(^{647}\),
polyacrylamide 648, polyacrolein 649, polyurethane foam 650, agarose 651,652, collagen 653,654, gelatin 655, sepharose, sephadex 659,655,657, nylon 658-660 and rayon. After proper treatment the non-specific retention of cells in comparison to the selective binding is minimised, nevertheless the optimal balance between stability, specificity and inertness has been difficult to establish in any system, and neither the problem of non-specific interactions between cells and matrix nor that of elution of the specifically bound cells, have been adequately resolved.

They are used in a variety of configurations including test tubes 661, fibres 658-660 and digestible 653,654 or meltable 662 matrices, but the two most widely used, are either in the form of affinity chromatography columns where the nonadherent cells are moving downward by gravity, or as flat immunoabsorbents (e.g. petri dishes) where the nonadherent cells are carefully removed by decantation and washing (panning).

**Affinity Chromatography columns**

The principles of affinity chromatography were initially applied to protein purification 663, with the purification of antibodies using immobilised antigen and subsequent specific elution using hapten, first being described by Campbell in 1951 642. They were first used for the depletion of specific antigen binding cells in 1969 643,644 and since then have been adapted to allow separation or depletion of a variety of lymphocyte subpopulations on the basis of antigen and antibody expression, including B lymphocytes 639,656,657,664 T cells 665, complement-receptor bearing cells 666, Fc-receptor bearing cells 667, lectin receptors, specific antigen or hapten-binding lymphocytes 654 and cytotoxic T lymphocytes.

**Panning**

A variety of antigen and antibody coatings have been used to separate specific cell populations by adherence to flat surfaces. Cells forming adherent monolayers in culture such as macrophages 668 and fibroblasts 669,670, have been used as target cell monolayers to remove sensitised lymphocyte populations, while the use of poly-L-lysine to attach cell monolayers to plastic tissue culture plates 671 has allowed the use of tumour cells 670,672 spleen cells 673,674 and other lymphoid and non-lymphoid cells as immunoabsorbent antigens.

Hapten-conjugated gelatin in plastic petri dishes was described for the separation and recovery of specific antigen binding cells 675,676. While the gelatin-hapten conjugates form insoluble matrices at 4°C to which the cells can be bound, the cells can be recovered by incubation of the dishes at 37°C at which temperature the gelatin melts, and the method has remained popular for positive cell selection 653,677,678.

The observation that antibody molecules adsorb onto polysterene surfaces and bind antigen 579 led to the use of polysterene tissue culture dishes coated with adsorbed antibody specific for mouse Ig, to separate Ig+ mouse spleen cells which became bound to the plastic, from Ig- cells which could be decanted off 647,680. Panning can handle up to 2x10^5 cells per 100mm diameter petri dish and be used for the preparation of both major and minor
populations of cells in a mixture, with purities of 95% or better being reported using polyclonal antisera. The size of islets and exocrine fragments make immunoabsorption procedures less satisfactory for islet purification. Passage through affinity columns would result in trapping of both exocrine and islet populations, while in panning disruption of the antibody bound fraction during removal of the nonadherent element occurs, and there are no reports of their use.

b) Cytotoxicity

The induced killing of cell populations can be achieved biologically by exploiting processes such as complement mediated lysis or chemically with poisons. Complement mediated cell lysis

The complement system is composed of a set of interacting proteins which react sequentially in a cascade when activated, resulting in the assembly of membrane attack complexes which puncture the cell membrane and result in cell lysis. Rabbit or guinea pig serum is usually used as the source of complement, since few mouse monoclonal antibodies effectively bind human complement and rigorous quality control and careful storage is needed to ensure sufficient activity to cause strong reproducible reactions with minimal nonspecific toxicity and to prevent damage to the thermolabile complement. Other problems include antigenic modulation, complement inhibition and loss of other cells trapped by clumping of lysed cells. Despite all the pitfalls complement mediated lysis remains a widely used technique for obtaining subpopulations of lymphocytes being quick and easy to perform.

Elimination using specifically labelled cell toxins

Immunotoxins consisting of antibody covalently bound to a toxic component usually of plant or bacterial origin are an alternative method of deleting specific cell populations. Ricin which binds to galactose residues in the cell membrane, is internalised and inhibits protein synthesis resulting in eventual cell death is the best described. Other options include the linking of radioactive isotopes such as to specific antigens or antibodies for the depletion of cell colonies, or the use of chemosensitizing agents (directed by antibody coupling to specific tissues) which are subsequently activated by exposure to light at a specific wavelength, resulting in destruction of the labelled cells.

Although complement-dependent cytotoxicity and cytotoxic methods using monoclonal directed laser photodynamic therapy, or light activated lysis have been used for the specific destruction of acinar cells, the risk of damage to the islets by the release of exocrine enzymes, is likely to discourage the use of cytotoxic methods for large scale islet isolation, and other groups have not reported the method to be successful.
c) Labelling of specific cell populations

Fluorescence activated cell sorting

Optical/electronic devices which measure cell size and detect the presence of cell-bound fluorochrome labelled antibodies or antigens have become widely used over the last 15 years as both counters and separators of cell populations. A single cell suspension is injected into the flow cytometer and focused into a line of cells. This stream of cells passes through a laser beam in which the cells physically interact with the light scattering it in all directions. Light scattered forward is related to the size of the cell, and that at 90° to the degree of refraction from its internal structure. At the same time excitation of the fluorochromes occurs. The scattered and reflected light and that emitted by the excited fluorochromes is detected by a suitable arrangement of lenses, mirrors, filters and photoelectric devices, allowing the generation of analogue electrical signals for each particle, which are then computer analysed. If cell sorting is required, the properties of the cells to be selected in terms of size and fluorescence are programmed into the computer, and once the cell containing droplets leave the detection point, either a positive or negative charge is applied to the stream so that for instance droplets containing brightly fluorescent cells can be given a positive charge and those containing dull cells a negative one. The droplets then pass through an electric field which deflects them according to their charge.

Separation of single cell suspensions from isolated islets into β and α/δ cell populations has been performed by light scatter flow cytometry of unstained cells while purification of β cells can also be done using fluorescence-activated cell sorting (FACS). The process of FACS usually requires a single cell suspension, but with appropriate modifications of piezoelectric valve based light-activated cell sorters, particles of 300-500μm diameter can be sorted according to their size and/or staining with neutral red, allowing the purification of intact islets. However, while FACS is capable of producing islets of 70-95% purity with only 15% losses, the hardware is expensive to purchase and maintain, requiring trained personnel, and is slow, with maximal sorting speeds of 100,000 islets per hour from a starting purity of 50%. Faster speeds create problems because of the shear forces generated and the limits of instrumental response times.

Rosetting

This technique involves the interaction of a single lymphocyte with several erythrocytes (>3-4) forming structures known as rosettes which can be removed from the mixture on the basis of the high density of the erythrocytes and the large size of the rosette structure using either velocity or density gradient centrifugation. The spontaneous formation of rosettes between unsensitised sheep erythrocytes (SRBC) and non-immune human lymphocytes was initially noted, and shown to be a specific marker for human T lymphocytes. Since then, the coating SRBCs with artificially coupled antigens, has widened the application of the technique to allow the purification of antigen specific B lymphocytes, human lymphocyte subpopulations bearing Fc receptors, and cells bearing complement receptors, while the ability to bind
immunoglobulins to SRBC via CrCl\textsubscript{3} \textsuperscript{730,731} allows the removal of any cell to which a specific antibody can be raised \textsuperscript{732}.

**Immunomicrospheres**

Over the last 15 years functional polymeric microspheres with diameters of less than 5\textmu m have been developed for use in immunological systems \textsuperscript{733,734}. These reagents were initially used as visual markers to identify specific cell types and analyse the distribution of surface antigens by microscopy \textsuperscript{735-737}, or the use of a variety of labels including fluorescence \textsuperscript{738}, radiolabels \textsuperscript{739}, and others \textsuperscript{740}.

![Diagram](image)

**Figure 2.1** The direct and indirect methods for labelling and separation of cells using immunomicrospheres

More recently, on similar lines to rosetting and related to affinity chromatography, polymeric microspheres of various materials (see Table 2.1) coated in antigen or antibody, have been specifically attached either directly or indirectly (Figure 2.1) to cells altering their characteristics and allowing their separation on the basis of the properties conferred by the microsphere such as size, density, electrophoretic mobility, fluorescent label or magnetic moment.
Polyacrylamide spheres with a density of 1.12g/cm³ non-covalently bound to anti-human lymphocyte globulin, have been used to increase the density of specific lymphocyte populations and allow their separation by low speed centrifugation on Ficoll-Isopaque 741, while OKT3 coated colloidal gold has been used to separate T cells from bone marrow, and floating beads have been used to purge bone marrow 742. The density change caused by microsphere attachment has also been used to fractionate red cells 743 fat cells 651 and lymphocyte 744 and platelet 745 membranes.

<table>
<thead>
<tr>
<th>Type of Microsphere</th>
<th>Composition</th>
<th>Size</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>phospholipid</td>
<td>4μm</td>
<td>cell separation-centrifugation 746</td>
</tr>
<tr>
<td>Polysterene latex</td>
<td>S</td>
<td>0.23μm</td>
<td>Phagosome separation - centrifugation 747</td>
</tr>
<tr>
<td>Polymeric microspheres</td>
<td>S</td>
<td>0.783μm</td>
<td>Cell separation - FACS 748,749</td>
</tr>
<tr>
<td>Copolymeric methacrylate</td>
<td>HEMA, MMA, EGDMA</td>
<td>30-340nm</td>
<td>Cell labelling - SEM 735,738</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Membrane separation - centrifugation 743</td>
</tr>
<tr>
<td>Hydroxyethyl methacrylate</td>
<td>HEMA, AM, BAM</td>
<td>0.3-3μm</td>
<td>cell labelling 750</td>
</tr>
<tr>
<td>Polyvinylpyridine</td>
<td>VP, AM, BAM</td>
<td>0.15μm</td>
<td>Cell separation - electrophoresis 751</td>
</tr>
<tr>
<td>Polyglutaraldehyde</td>
<td>G</td>
<td>0.2μm</td>
<td>Cell separation - electrophoresis 751</td>
</tr>
<tr>
<td>Polyacrylamide</td>
<td>AM, BAM</td>
<td>1-5μm</td>
<td>Cell separation - centrifugation 741</td>
</tr>
<tr>
<td>Polyacrolein</td>
<td>AC, MAA</td>
<td>150-350nm</td>
<td>Cell separation - electrophoresis 752</td>
</tr>
</tbody>
</table>

*SEM = scanning electron microscopy, S = Styrene, MMA = Methyl methacrylate, HEMA = 2-hydroxyethyl methacrylate, MAA = methacrylic acid, AM = acrylamide, BAM = N,N'-methylene bisacrylamide, VP = 4-vinylpyridine, G = glutaraldehyde, EGDMA = ethylene glycol dimethacrylate, AC = acrolein*

Table 2.1 Documents the polymer microspheres used for cell separation by centrifugation, electrophoresis and FACS 753,754.

Alteration of the electrophoretic mobility of cells using polymeric microspheres has also been reported, reducing the mobility and allowing the separation of specific erythrocyte 751,752 and lymphocyte 755 populations as well as other cells 613.

The separation of rare cells with a low density of a given marker remains a problem using FACS technology, and the use of fluorescent labelled polymer microbeads may help to
overcome this problem by amplification of the signal. They can be attached to the outside
748, 756, 757 or inside 749 of the cell, or phagocytosed 758-761.

The use of macro-microspheres developed in Wick's laboratory for the identification
of cells by 'fluoro-immuno-cyto-adherence' 762 is an adapted methodology. Spheres much
larger than the cells (26µm diameter) are coated in specific tracer molecules, cells possessing
the relevant surface antigens or receptors adhere to the spheres to form "inverted rosettes".
By coupling several ligands of differing specificities to each bead, such rosettes can allow the
removal using FACS or density centrifugation of several cell types at once 763.

iv) SUMMARY

<table>
<thead>
<tr>
<th>METHOD</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>The separation produced by physical and biological methods of cell separation relies upon the presence of existing differences in cell properties and the specificity of these.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation</td>
<td>Rapid, high capacity, relatively cheap</td>
<td>Variable resolution, traumatic</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>non-traumatic</td>
<td>Variable resolution, limited capacity</td>
</tr>
<tr>
<td>Biological</td>
<td></td>
<td>Variable resolution can be traumatic</td>
</tr>
</tbody>
</table>

All the immunological methods depend upon the use of specific target/receptor interactions.
They therefore require the isolation of either target or receptor molecules, and their resolution will vary with the affinity and specificity of this for the cell.

<table>
<thead>
<tr>
<th>METHOD</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACS</td>
<td>Very high resolution</td>
<td>expensive, slow, complex, limited capacity</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>High resolution, relatively cheap, simple, rapid</td>
<td>Negative selection only, potential damage of other cells, cell losses during washing, batch to batch variation in complement</td>
</tr>
<tr>
<td>Solid phase affinity</td>
<td>High resolution, high capacity, simple</td>
<td>Dislodgement of selected cells by shear forces</td>
</tr>
<tr>
<td>immunomicrospheres</td>
<td>High resolution, rapid</td>
<td>Relies on separation method selected</td>
</tr>
<tr>
<td>Immunomagnetic cell separation</td>
<td>High resolution, rapid, gentle, simple, high capacity</td>
<td>Trapping of unselected cells in magnetic fraction</td>
</tr>
</tbody>
</table>

Table 2.2 Summarising the advantages and disadvantages of the categories of cell separation
Any process of cell separation which is to be adapted for islet purification should be
gentle and rapid minimising cell damage to both the islet and exocrine components, in order
to maintain the functional and structural integrity of the islets. It needs to have a high
capacity, allowing separation of large amounts of digest, and it should produce good
resolution of the islets and exocrine tissue with minimal non-specific retention, allowing high
islet yields and purity. Other factors which need to be considered are the accessibility and
cost of the required instrumentation and reagents.

It can be seen that no single method is ideal, and the use of more than one method in
sequence, combining the advantages and minimising the disadvantages of each, may
maximise the yield of pure islets obtained from a single pancreas.

The well established process of density dependent isopycnic centrifugation seems a
logical first step in any islet purification process, rapidly separating the bulk of exocrine
tissue from the least dense islets. However, the considerable overlap in the density of the
denser islets and the less dense exocrine fragments, prevents the purification of the large
numbers of islets which remain excessively contaminated in the denser areas gradient
medium. A secondary method designed to retrieve islets from this poorly purified digest
would therefore significantly improve on current yields.

Any method chosen has to be density independent, highly specific, capable of
processing moderately large amounts of digest, and perhaps most importantly, separate the
islets rapidly and gently, without subjecting the already traumatised islets to any further
damage. Only the immunologically based methods fulfil these criteria, with immunomagnetic
cell separation methods offering the least traumatic option.

2.3 IMMUNOMAGNETIC CELL SEPARATION

i) THE DISCOVERY OF MAGNETISM

Few of the materials and natural phenomena recognised and used today are new, and
magnetism is no exception 764,765. There is good reason to believe that the attractive
powers of permanently magnetised lodestones composed of magnetite (Fe₃O₄), for iron
artefacts (which have been found dating back to 4000 B.C. in Egypt), had been observed
3000 years before the Christian Era, while the application of the earth's magnetic influence
dates back to B.C.2637 when it is related that the Chinese emperor Huang-ti constructed a
chariot upon which stood an erect female figure whose outstretched arm always pointed to
the south. By B.C. 1110, the Chinese had combined the two observations by discovering that
steel needles could be permanently magnetised by the lodestone and used as compasses
indicating both north and south. Although the Greeks provided the word magnet from the
area Magnesia in which lodestones were found, very little new knowledge was contributed
until after the middle ages.

In 1269 Petrus Peregrinus, in his "Epistola Petri Peregrini de Maricourt ad Sygerum
de Foucaucourt militem de magnete" described a series of experiments with magnets and the
development of the floating and pivoted compass, the invention or rediscovery of which
resulted in a greatly increased interest in the study of geomagnetism. This culminated in the series of experiments on electricity and magnetism by William Gilbert (1544-1603) described in his great work "De Magnete, Magneticisque Corporibus, et de Magno Magnete Tellure: Physiologia nova, Pluribus et Argumentus et Experimentis Demonstrata", to which he had dedicated 17 years of his life and in which he described the earth as a huge magnet and presented the first published work on electricity.

ii) CURRENT CONCEPTS OF MAGNETISM

a) Introduction to magnetic fields and forces

One of the most fundamental concepts in magnetism is that of the magnetic field. When a non-uniform field is generated in a volume of space, it means that an energy gradient is produced, resulting in a force which can be detected by the acceleration of an electric charge moving in the field, the force on a current carrying conductor, the torque on a magnetic dipole such as a bar magnet or even by the reorientation of spins on electrons within certain atoms.

The magnetic field produced by a permanent magnet is the result of the orbital motions and spins of electrons (the so called "amperian currents") within the permanent magnetic material, which produce a magnetic field of strength \( H \) (amps/meter) capable of exerting a force on both current carrying conductors and permanent magnets. This generates a magnetic flux \( \Phi \) (Webers) in the surrounding space or medium, and small magnetic particles such as iron filings align themselves along the direction of this. The flux density generated by the magnetic field in any medium in Webers/metre\(^2\) is known as the magnetic induction of the medium \( B \) (Teslas). While in free space this is linearly related to \( H \) by the formula \( B = \mu_0 H \) where \( \mu_0 \) is the permeability of free space (a constant, \( 1.257 \times 10^{-6} \mathrm{H/m} \)), in other media such as ferromagnets \( \mu \) varies with \( H \), so that the relationship is no longer linear but becomes one of a hysteresis loop (Figure 2.2, p50).

The flux density generated is also dependent on the magnetisation \( \mathbf{M} \) of the material generated by the spin and orbital angular motion of electrons within a permanent magnet where \( B = \mu_0 M \). \( B \) can be related under all circumstances to both the magnetic induction in free space \( \mu_0 H \) and the magnetisation \( M \) of the material by the equation \( B = \mu_0 (H + M) \).

A further property of materials in regard to magnetism is their magnetic susceptibility \( \chi \) which can be defined as \( B/\mathbf{H} \). This is closely related to the relative permeability of the material \( \mu = \mu_0 \chi \), the relative permeability of free space is therefore 1 and \( \mu = \chi + 1 \)

Using these parameters, materials can be divided according to their magnetic permeability \( \mu \) and susceptibility \( \chi \) into three broad categories.

i) Diamagnets such as copper, silver, gold, bismuth and beryllium in which \( \chi \) is small and negative \( \approx -10^{-5} \), so that their response opposes the applied magnetic field

ii) Paramagnets such as platinum, aluminium and manganese, for which \( \chi \) is small and positive \( \approx 10^{-3} \) to \( 10^{-5} \), their magnetisation is weak but aligned parallel with the direction of the magnetic field.
iii) Ferromagnets are the most widely recognised magnetic materials including iron, cobalt, nickel and several rare earth metals and their alloys. Their magnetic susceptibility is much greater, \( \chi = 50 \) to 10,000. One of the most widely known properties of this group is their ability to retain their magnetisation even when the field generating it is removed. This distinguishes them from paramagnets, which although they acquire a magnetic moment in an applied field cannot retain the magnetisation after the field is removed.

b) Atomic theories of magnetism

On the macroscopic scale, magnetisation (\( M \)) is clearly field induced, and dependent on some property of the material being magnetised. Weber suggested that, in the demagnetised state, rather than the magnetic moment everywhere being zero, the material could already have small atomic magnetic moments which are randomly aligned summing to zero, but which become aligned under the influence of a magnetic field \(^{767}\). Ampère had already suggested that these atomic moments were due to 'electrical currents continually circulating within the atom' \(^{768}\). Some years later one of the most important advances in the understanding of magnetism was made by Weiss who suggested the existence of magnetic domains in ferromagnets, consisting typically of \( 10^{12} \) to \( 10^{15} \) atomic moments aligned in parallel, with these domains being randomly aligned in the demagnetised state \(^{769}\). The magnetisation process consisted of orientating the domains so that the volume of domains aligned with the field exceeded those aligned against it. Visual confirmation of the effect of domains gave support to this theory soon afterwards \(^{770},771\).

On this basis:

Diamagnets are solids with no permanent magnetic moment per atom and their diamagnetic susceptibility arises from the realignment of electron orbitals under the influence of a magnetic field.

Paramagnets have a net magnetic moment per atom due to unpaired electron spins in each atom, but these are not ordered into domains.

Ferromagnets contain ordered domains, the behaviour of which explain many of their unique properties, including the hysteresis loop obtained when the change in flux density \( B \) in a ferromagnetic material is plotted against the strength of the applied magnetic field \( H \) (Figure 2.2).
c) **Important properties of ferromagnets**

The changes which arise during magnetisation of a ferromagnet can be either reversible or irreversible, dependent on the domain processes involved. More often both reversible and irreversible changes occur, so that the on removal of the magnetic field the magnetisation decreases but does not return to its initial value. Both the ability of a ferromagnetic material to retain its magnetisation, termed its *remanence* $H_r$ (T), and the strength of the field required to reduce the magnetic induction to zero, its *coercivity* $H_c$ (A/m) are determined by the shape of its hysteresis loop (Figure 2.2), and determine the suitability of a ferromagnetic material for various applications. The *hysteresis loss* is the area enclosed by the hysteresis loop and represents the energy expended in one cycle of the loop. This increases with the maximum field strength reached and is closely related to the coercivity of the material, so that reducing the coercivity reduces the hysteresis loss.

*Permeability* which is the most important parameter of soft magnetic materials (those with a low coercivity) such as iron, indicates how much magnetic induction is generated by a given field, as shown in Figure 2.2 materials with a low coercivity necessarily have a high permeability and vice versa. Ferromagnetic materials used in magnetic microspheres need to have high permeability so that they rapidly become magnetised on entering a magnetic field, with a low remanence and coercivity so that magnetisation can be easily reduced to zero, minimising spontaneous clumping. Permanent magnets on the other hand need a high remanence and coercivity in order to retain their magnetisation as much as possible. The important properties of some hard and soft ferromagnetic materials are shown in *Table 2.3*. 

![Figure 2.2 Hysteresis loop showing the variation in flux density with changes in the strength of the applied magnetic field](image)
Table 2.3 Showing the important properties of some ferromagnetic materials in order of increasing coercivity or magnetic hardness. Those with coercivities below 1kA/m are regarded as soft.

<table>
<thead>
<tr>
<th>Material</th>
<th>Composition</th>
<th>Remanence Br (T)</th>
<th>Coercivity Hc (kA/m)</th>
<th>Relative permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permalloy</td>
<td>78%Ni, 22%Fe</td>
<td>0.5</td>
<td>4 x 10⁻³</td>
<td>8000</td>
</tr>
<tr>
<td>Iron</td>
<td>100%Fe</td>
<td>0.8</td>
<td>80 x 10⁻³</td>
<td>5000</td>
</tr>
<tr>
<td>Steel</td>
<td>99%Fe, 1%C</td>
<td>0.9</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Permalloy</td>
<td>72%Co, 21%Fe, 17%Mo</td>
<td>1.6</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>Adraste</td>
<td>78%Al, 24%Ni, 1%Co</td>
<td>1.7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ho Ferrite</td>
<td>HoO₆Fe₂O₁₄</td>
<td>0.39</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Samarium</td>
<td>Sm₂Cu₂O₅</td>
<td>0.3</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Garnet</td>
<td>Gd₃Fe₂O₁₂</td>
<td>0.5</td>
<td>3.9</td>
<td></td>
</tr>
</tbody>
</table>

Relative permeability is measured at a magnetic induction of 2 tesla.

**d) Permanent magnetic materials**

A permanent magnet is a passive device for generating a magnetic field without requiring an electric current. The energy needed to maintain the magnetic field has been stored when the permanent magnet was ‘charged’, i.e. magnetised initially to a high field strength and then to remanence when the applied field was removed.

It is essential that they are not easily demagnetised by working conditions in which they are often subject not only to their own demagnetising field, but to various demagnetising effects from other magnetic materials or magnets in their vicinity. This requires a hard magnetic material with a high coercivity. In addition a permanent magnet is only of use if it has a relatively high remanence when removed from the applied magnetic field which generated it and this inevitably means an initially high saturation magnetisation.

A wide variety of naturally occurring and man made materials have been used as permanent magnets. Lodestone or magnetite (Fe₃O₄) a naturally occurring iron oxide was the first ‘permanent magnet’ to be recognised. Today it is not even considered a hard magnetic material, and as ‘ferrofluid’ has been incorporated as the magnetic component of many magnetic microspheres.

The addition of carbon to iron has long been known to increase coercivity. The first commercially produced permanent magnets were high-carbon steels containing about 1% carbon. These were also mechanically hard while low carbon steels were mechanically soft, leading to the labels ‘hard’ and ‘soft’ which later transferred their attachment to the
coercivity of the material. The addition of tungsten, chromium and cobalt further improved the coercivity of steel (see Table 2.3) \(^{772}\).

The development of Alnico alloys of iron, cobalt, nickel and aluminium with small amounts of copper in the 1930s \(^{773}\), ceramic magnets of barium or strontium ferrite in the 1950s \(^{774}\), samarium-cobalt in the late 60s \(^{775}\) and most recently neodymium-iron-boron in the 1980s \(^{776,777}\) has led to a progressive improvement in the properties of permanent magnets \(^{778}\) which has proved vital to their use for immunomagnetic cell separation, producing permanent magnets capable of generating the flux density required to attract magnetic microspheres without resorting to complex electron magnets.

Permanent neodymium-iron-boron magnets, which are relatively inexpensive and readily available, are used throughout the studies on immunomagnetic islet purification detailed later, in order to generate the magnetic fields required. The MPC-6 magnet produced by Dynal for instance has a remanence of 1.080T (10800 gauss) and a coercivity of 870kA/m.

### iii) SEPARATION BY MAGNETIC FIELDS

#### a) Industrial

Magnetism has been used as a practical tool for the separation of materials on the basis of differences in their magnetic properties since the days of Thomas A Edison \(^{779}\), who patented 10 distinct types of magnetic ore separator, the first in 1880 (Figure 2.3). Relatively simple structures such as this allowed the processing of materials rich in iron which could be concentrated from relatively unsophisticated mineral processing streams.

Since then the use of magnetism as a separation tool has progressed ingeniously in a number of areas employing both wet and dry applications. The development of increasingly intense magnetic fields and an appreciation of the importance of non-uniform or high gradient magnetic fields (HGMS), with their ability to polarise weakly paramagnetic materials, has led to wider application of magnetic separation techniques to separate even

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**Figure 2.3** Diagram illustrating the principles of Edison's magnetic ore separator
micron size particles 780,781. One of the first applications was the cleaning of kaolin clay by removing feebly magnetic <1 μm coloured particles 782.

b) Phagocytosis

Cell separation using magnetic fields were first introduced by Rous and Beard 783 for the removal of kupffer cells that phagocytosed iron particles, and the technique has since been used to separate polymorphs capable of phagocytosis from whole blood 637,784-787. The use of magnetite particles, coated with sensitising agents such as polylysine, polybrene, polyarginine or other basic polypeptides, further improve their uptake by phagocytic cells 788. However no selective uptake by specific cell types has been demonstrated.

c) Intrinsically magnetic cells

It is also possible to separate cells which already contain iron using magnetic fields. For example, certain flagellate bacteria found in salt marshes have chains of iron particles inside them which orientate the cells when a uniform magnetic field is applied producing magnetotactic migration 789.

One of the obvious iron containing cells is the red blood cell with the magnetic properties of haemoglobin being well established 790,791. While oxyhaemoglobin is diamagnetic, with all the electron spins in the iron and oxygen molecules paired, deoxyhaemoglobin has a paramagnetic moment so that red blood cells containing it, when placed in a magnetic field B (Tesla) with a field gradient dB/dz, may be expected to experience a magnetic force F (Newtons) in the direction z given by:

\[ F = \frac{\chi V}{\mu_0} B \left( \frac{dB}{dz} \right) \]

where \( \chi \) is the (SI) susceptibility of the red blood cell, V (m^3) its volume and \( \mu_0 \) the permeability of free space.

As long ago as 1946 792 attempts were made to separate blood cells containing malarial parasites using their magnetic properties, but the low field gradient available (0.1Tm^-1) meant that the force experienced by the red cells was only a few percent of the gravitational force, frustrating this and other early attempts 637. The description of a new type of high gradient magnetic separator which enables the generation of magnetic field gradients of 10^4-10^5Tm^-1 793 led to a re-evaluation of magnetic red cell separation in the mid 1970's 794. The technique involved placing fine steel wool mesh in an electromagnet. When the magnet was activated a field was induced in the individual strands of the steel wool. This field was greatest at the strand surface and reduced to the background field approximately one strand radius from the surface. Since the strands in the filter are only a few microns thick the magnetic field gradient obtained was very high.

Using this technology, it is possible to extract erythrocytes from a flowing cell suspension on the basis of the cell's contents of paramagnetic deoxyhaemoglobin 795,796, methaemoglobin 797-799 or oxidised haem products 800. It has also made possible the
separation of cells capable of forming rosettes either spontaneously \textsuperscript{801,802} or with antigen labelled red cells \textsuperscript{803}, traditionally separated using density or velocity sedimentation.

**iv) DEVELOPMENT OF MAGNETIC MICROSPHERES**

Along similar lines using hetero-antisera or more recently monoclonal antibodies as targeting agents a variety of particles have been used to coat and render cells magnetic. Magnetic microspheres used in cell separations require certain properties many of which are common to all polymer microspheres used for cell labelling and separation.

1) They should be stable and durable in the media or isotonic buffers used in cell separation.
2) They should not spontaneously aggregate in physiological media, remaining monodisperse.
3) They should bind immunoglobulin molecules or appropriate ligands strongly, by either adsorption or the formation of covalent bonds under appropriate reaction conditions.
4) They should not bind cells non-specifically, so that the binding to cells is determined exclusively by the antigen-antibody reaction. This requires the presence of a hydrophilic surface of low negative charge.
5) They and any substances adsorbed by them during their preparation should be biocompatible, not affecting cell function in any way.
6) Their specific density should be similar to that of the cells for optimal cell/microsphere interaction (1.05-1.15g/cm\textsuperscript{3}).
7) They should allow satisfactory physical separation in magnetic fields, of unlabelled cells from those labelled with particles, this requires a uniformity of size and magnetic properties, so that they behave predictably in a magnetic field.
8) Since antibody coating requires repeated exposure to a magnetic field and remaining magnetism would severely reduce the ease of redispersion, their remanent magnetisation should be small enough to prevent this.

Such microspheres consist of two components, a matrix often composed of a polymer to which the ligands are bound allowing cell attachment, and which also acts as a carrier for the second component, the magnetic core.

a) **The matrix**

In an effort to attain the optimal combination of these properties numerous materials have been used for the construction of the matrix of magnetic microspheres (*Table 2.4*) many based on those developed as non-magnetic immunomicrospheres (*Table 2.1,p55*).

**Development**

The first description of magnetic affinity beads conjugated with antibody and used for cell separation was by Molday in 1977 \textsuperscript{738}. However the method of bead production was complex, the time period required for the magnetic separation step was long at 2 hours, and the microspheres tended to form aggregates.
Table 2.4 Outlining the properties of magnetic micropsheres which have been used for cell separation starting in 1977.
(Compiled and updated from 740,753,754,804-805).

<table>
<thead>
<tr>
<th>Microsphere</th>
<th>chemical composition</th>
<th>polymerisation method</th>
<th>size</th>
<th>activation</th>
<th>applications</th>
<th>Magnetic separation system</th>
</tr>
</thead>
<tbody>
<tr>
<td>methacrylate 735,738,750</td>
<td>MMA, HEMA, MAA, EGDMA, Mag</td>
<td>60Co gamma irradiation</td>
<td>30-50nm</td>
<td>Coupling to carboxyl groups via carbodiimide</td>
<td>mouse T and B cells</td>
<td>Permanet magnet</td>
</tr>
<tr>
<td>polyglutaraldehyde 807,808</td>
<td>G, Mag</td>
<td>Chemical at high pH</td>
<td>0.1-0.5μm</td>
<td>Direct coupling to aldehyde groups</td>
<td>Separation of RBCs from different species</td>
<td>Permanet magnet</td>
</tr>
<tr>
<td>polycrolein grafted polyesterene 809-811</td>
<td>PS, A, C, Mag</td>
<td>60Co gamma irradiation</td>
<td>3-10μm</td>
<td>Direct coupling to aldehyde groups</td>
<td>Separation of RBCs from different species</td>
<td>Permanet magnet</td>
</tr>
<tr>
<td>polycrolein 736,812,813</td>
<td>AC, Mag</td>
<td>irradiation or anionic polymerisation at pH 10.5</td>
<td>4μm variable</td>
<td>Direct coupling to aldehyde groups</td>
<td>Separation of RBCs from different species</td>
<td>Permanent magnet</td>
</tr>
<tr>
<td>Hydrogel 814-816</td>
<td>HEMA, BAM, MAA, Mag</td>
<td>redox polymerisation</td>
<td>&gt;50nm</td>
<td>Coupling to carboxyl groups via carbodiimide</td>
<td>Neuroblastoma cells</td>
<td>Electromagnet</td>
</tr>
<tr>
<td>Material</td>
<td>Type</td>
<td>Chemical</td>
<td>Size</td>
<td>Coupling Method</td>
<td>Cells and Tissues</td>
<td>Method</td>
</tr>
<tr>
<td>-------------------</td>
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</tr>
<tr>
<td>Magnogel 817-819</td>
<td>AM, Ag, Mag</td>
<td>Chemical polymerisation</td>
<td>50-160μm</td>
<td>Direct coupling to aldehyde groups</td>
<td>Mouse and rat T and B lymphocytes, oligodendrocytes</td>
<td>Permanent magnet</td>
</tr>
<tr>
<td>Albumin 820-824</td>
<td>A, pA, Mag</td>
<td>Emulsion polymerisation</td>
<td>0.2-1.5μm</td>
<td>Indirect adsorption via protein A, glutaraldehyde activation</td>
<td>RBCs from various species, Rat T and B lymphocytes, Human T cells, Islet purification</td>
<td>Permanent magnet</td>
</tr>
<tr>
<td>Iron-dextran 826-828</td>
<td>D, Mag</td>
<td>Precipitation</td>
<td>30-40nm</td>
<td>Indirect adsorption via protein A</td>
<td>Separation of mouse RBCs from myeloma cells</td>
<td>Permanent magnet and HGMS</td>
</tr>
<tr>
<td>Cobalt immunocolloid 829-832</td>
<td>A, Co, Mag</td>
<td>Precipitation/reduction</td>
<td>150nm or 30-60nm</td>
<td>Avidin-biotin linkage</td>
<td>Elimination of leukaemic cell clones from blood</td>
<td>Electron magnet or HGMS</td>
</tr>
<tr>
<td>Protein magnetite</td>
<td>A, Mag</td>
<td>Precipitation</td>
<td>30-40nm</td>
<td>Coupling to amino groups via N-hydroxysuccinimide</td>
<td>RBCs from different species, Mouse T and B cells</td>
<td>HGMS</td>
</tr>
<tr>
<td>Polymer</td>
<td>SB, Mag</td>
<td>Chemical</td>
<td>Diameter (μm)</td>
<td>Treatment</td>
<td>Magnetic Method</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>---------------------------------------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Polystyrene monosized particles 835-839</td>
<td>SB, Mag</td>
<td>Chemical</td>
<td>3-4.5μm</td>
<td>Physical adsorption Bone marrow purging of tumour cells &amp; T lymphocytes, Separation of T and B lymphocytes, islet purification</td>
<td>Permanent magnets</td>
<td></td>
</tr>
<tr>
<td>Tosylated polystyrene particles 840-842</td>
<td>SB, Mag</td>
<td>Chemical</td>
<td>4.5μm</td>
<td>Direct coupling to OH groups via sulfonyl chloride Bone marrow purging, separation of T and B cells.</td>
<td>Permanent magnets</td>
<td></td>
</tr>
<tr>
<td>MACS 843-846</td>
<td>D, Mag</td>
<td>precipitation</td>
<td>30 or 65 μm</td>
<td>Coupling via biotin avidin linkage Separation of B and T cells, bone marrow purging</td>
<td>HGMS</td>
<td></td>
</tr>
<tr>
<td>Biomag 697,847-852</td>
<td>Mag, Silane</td>
<td>not recorded</td>
<td>1μm</td>
<td>coupled to amino groups via glutaraldehyde + others Bone marrow purging, T cell separation, islet purification</td>
<td>HGMS and permanent magnets</td>
<td></td>
</tr>
</tbody>
</table>

MMA = Methyl methacrylate, HEMA = 2-hydroxymethacrylate, MAA = Methacrylic acid, EGDMA = Ethylene glycol dimethacrylate, Mag = Magnetite, G = glutaraldehyde, PS = polystyrene, AC = Acrolein, BAM = N,N'-methylene bisacrylamide, AM = Acrylamide, A = Albumin, Ag = agarose, pA = Protein A, D = Dextran, Co = Cobalt, SB = Styrene divinyl-benzene. HGMS = High gradient magnetic separation.
Guesdon and Avrameas described the preparation of relatively large and heterogeneous magnetic particles whose size ranged from 50-160nm (Magnogel 44®) 817. They were composed of hydrophilic agarose-polyacrylamide containing free amino-groups and allowing attachment of antibody to the surface by simple glutaraldehyde coupling, and being used for the fractionation of lymphoid cells 818 and other more specific cell populations 819.

Microspheres of starch 853, dextran 826-828,844, cellulose 854,855 and latex 856 containing Fe₃O₄ have been produced, usually for specific purposes and without general applicability. Biodegradable and non-toxic albumin microspheres in a number of sizes 820-822,833 linked to protein A, have proved more useful, allowing separation of mixtures of RBCs 821,833, subpopulations of lymphocytes 823,824,834, and islet purification, by means of anti-acinar lectins 857-860.

In a further attempt to optimise the magnetic and surface properties of magnetic microspheres a variety of iron containing polymer microspheres have been produced by polymerisation of hydrophobic monomers such as styrene 835,836,838,861, and hydrophilic monomers of glutaraldehyde 808, acrolein 813 and acrylates 738,750,814,815 or combinations of the two 809-811, using processes such as Coy irradiation 738,750,813, redox potential systems 814,815, and anionic polymerisation 813.

Numerous approaches to the coupling of antibodies to solid microspheres have been published (Table 2.A), most of them initially described for the preparation of column matrices in affinity chromatography.

b) Antibody binding

While hydrophilic immunomicrospheres have advantages in terms of biocompatibility 862 and avoiding non-specific interactions with cells which are generally hydrophobic, hydrophobic particles, such as those made of polystyrene 863 (M-450 Dynabeads), polyhexylcyanocrylate 757, and colloidal metals 830,864, can be easily coated with immunoglobulins by physical adsorption, which renders them more hydrophilic 835,836,838,861, and reduces non-specific cell interactions. At their isoelectric point immunoglobulins bind to the surface of such particles by van der Waals-London forces, tending to do so via their Fc portion, leaving the Fab immunoreactive site free to interact with antigen.

Specific Adsorption

Antibody can also be specifically adsorbed onto the surface of microspheres by means of a ligand which interacts specifically with the intact or modified antibody.

Protein A. The cell wall of Staphylococcus aureus contains a protein (mol wt 42,000) which has a high capacity to bind the Fc portion of the majority of IgG subclasses 865. This has linkage been exploited by either coupling Protein A covalently to the surface of dextran...
Microspheres or incorporating it into the polymerisation mixture during the manufacture of micropsheres based on albumin, allowing antibodies to be coupled to the microspheres after brief incubations at 37°C, without the use of chemical coupling agents (see below) which might reduce antigen binding activity.

**Biotin-avidin.** Avidin is a 68,000 mol wt glycoprotein with four high affinity binding sites for biotin (Figure 3.7). When biotin is conjugated to proteins, avidin will form essentially irreversible complexes with these biotinylated proteins. This highly specific interaction has been used either by coating cobalt polymethacrylate microspheres with avidin, or dextran microspheres with biotin, and coupling them either directly or via an avidin bridge, to biotinylated antibodies.

**Direct coupling**

Covalent coupling of antibodies to functional groups such as aldehydes can occur directly, while other groups such as carboxyl, hydroxyl or amino groups have to be linked to antibodies employing a coupling reagent or modified to give reactive aldehyde groups. The relative simplicity of direct coupling to aldehyde groups led to the development of microspheres specifically designed to maximise such sites using polyglutaraldehyde and polyacrolein which antibodies could be directly linked, with the repeated units of conjugated aldehyde groups forming stable bonds with the primary amino groups in the protein (Equation 2.1).

\[ \text{CHO} \rightarrow \text{NH}_2-\text{Antibody} \]

**Coupling via a reagent**

Often the microsphere of choice will not carry a group which can be directly linked to monoclonal antibodies, and a variety of different reagents such as carboiimide, cyanogen bromide and glutaraldehyde have been used to allow covalent coupling to these. **Carboiimide Method**. A water soluble carboiimide derivative such as 1-ethyl-3-(3-dimethylaminopropyl) carboiimide, can be used to activate carboxyl groups present on the surface of methacrylate and silane microspheres, coupling them to amino groups on the protein molecule via an amide linkage (Equation 2.2).

The coupling procedure is simple to perform and occurs under very mild conditions, with the antibody being conjugated directly to the microsphere without the introduction of additional groups. However since proteins contain both carboxyl and amino groups, intra- and inter-molecular cross linkage can occur in the antibody molecules.
Cyanogen Bromide method. In this method, hydroxyl groups are first activated with cyanogen bromide at alkaline pH and then coupled at pH values between 7 and 10 to amino groups on the antibody (Equation 2.3).

Glutaraldehyde method. This reagent can be used to link amino groups present on the microsphere to amino groups on the antibody molecule. One problem with this method is that amino groups are not common on the surface of microspheres, but their number can be increased by a preliminary step in which amino groups in the form of diamino compounds are derivatised onto the surface of methacrylate microspheres using the carboiimide and cyanogen bromide methods, before the amino groups are linked using glutaraldehyde (Equation 2.4).
In comparison with the previous two methods, the glutaraldehyde method was found by Molday et al to be the most effective method of binding antibody to microspheres, and other groups have found it to be superior to the use of protein A with albumin based microspheres and optimal for silane coated iron oxide and even liposomes. This method converts hydroxyl groups present on the beads surfaces to corresponding esters (tosylates) which then form stable bonds with the amino terminal of antibodies (Equation 2.5).

\[
\begin{align*}
\text{CH}_2\text{-OH} + \text{ClSO}_2\text{-CH}_3 & \rightarrow \text{CH}_2\text{-O-SO}_2\text{-CH}_3 \\
+\text{H}_2\text{N-antibody} & \rightarrow \text{CH}_2\text{-NH-antibody} + \text{HOSO}_2\text{-CH}_3
\end{align*}
\]

Equation 2.5

It has been used effectively for the activation of agarose, methacrylate and polysterene M-450 Dynabeads. \(N\)-Hydroxysuccinimide 3-(2-pyridyldithio) propionate (SPDP) reacts with molecules containing primary amino groups on both the beads and antibodies, introducing dithiopyridine groups (DTP) via amide bonds.

\[
\begin{align*}
\text{NH}_2 + \text{N-CH}_2\text{-CH}_2\text{-S-S-CH}_2\text{-CONH-antibody} & \rightarrow \text{NHOC-CH}_2\text{-CH}_2\text{-S-S-CH}_2\text{-CONH-antibody} \\
\text{NH}_2\text{-Antibody} + \text{Antibody-NHOC-CH}_2\text{-CH}_2\text{-SH} & \rightarrow \text{NHOC-CH}_2\text{-CH}_2\text{-S-S-CH}_2\text{-CONH-antibody}
\end{align*}
\]

Equation 2.6
By reduction of the antibody bound DTP with dithiothreitol the antibody can then be covalently bound to the DTP groups on the bead (Equation 2.6).

This reaction has been used to couple both antibodies and protein A to albumin microspheres.

*p*-benzoquinone. In excess and at neutral pH this molecule reacts with amino and sulfhydryl groups via only one of its two active sites. After removal of the excess *p*-benzoquinone the activated bead will then react with added antibody at a slightly alkaline pH (Equation 2.7).

\[
\begin{align*}
&\text{Antibody} \\
\rightarrow &\text{activated bead} + \text{antibody}
\end{align*}
\]

This method of coupling has been exploited for the activation of albumin coated cobalt colloid.

Several other reagents such as *N*-ethyl-5-phenylisoxazolium 3'-sulfonate (Woodward's reagent K), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinilone, *p*,*p'*-difluoro-m,m'-dinitrophenyl sulfone, toluene disocyanate and bis-diazotized benzidine have been described for the covalent coupling of antibody to ferritin and other molecules for specific cell labelling, but they have yet to be exploited in cell separation procedures.

c) **The magnetic core**

As outlined above the ideal characteristics of a magnetic immunomicrosphere are a high permeability resulting in intense magnetisation of the sphere on entering the magnetic field, with a low remanence and coercivity so that once the field is removed they no longer retain their magnetism, minimising clumping before and after cell separation. This depends not only on the properties of ferromagnetic material used (*B*<sub>r</sub>, *H*<sub>c</sub>, *μ<sub>r</sub>*), but also the size of the ferromagnetic particles incorporated in relation to the size of a domain.

Particles the size of single domains cannot become magnetised. If they are suspended in a gel, although they will rotate within a magnetic field, brownian motion will randomly orientate them again once it is removed. This means that such a gel will have the properties of a ferromagnet at its Curie point (the temperature at which its properties become...
diamagnetic), and the gel is termed 'superparamagnetic' having a high permeability and saturation induction, without a remanent magnetisation. All magnetic microspheres have made use of this principle, incorporating many magnetic subdomain or domain sized particles allowing them to experience much greater forces in saturating magnetic fields than a particle with a single particle magnetic core.

Production of such particles requires the incorporation of a colloidal suspension of magnetic material either onto the pre-formed polymer matrix or more effectively, during the process of polymerisation, to produce a more homogeneous distribution. Nearly all the magnetic microspheres have incorporated iron based magnetic materials such as magnetite, the only exception being the use of colloidal cobalt particles.

Nearly all the magnetic microspheres have incorporated iron based magnetic materials such as magnetite, the only exception being the use of colloidal cobalt particles.

d) **Selection of a microsphere for immunomagnetic islet purification**

There were three criteria for selecting a microsphere for use in this study.

1) The magnetic microsphere should be commercially available, allowing procedures developed to be simply and rapidly used by others, and avoiding the problems of developing magnetic microspheres.

2) It should be easy to couple IgM monoclonal antibodies (produced in the Department of Surgery) to the beads.

3) When coupled to relatively massive fragments of exocrine tissue, the beads should have sufficient magnetic moment to allow separation in fields generated by permanent magnets, without the need for the latticework of iron wire involved in HGMS, which would trap particles the size of pancreatic digest.

This last requirement was crucial, whatever system was developed for islet purification, it seemed likely that unlike single cells, the digest would sediment rapidly remaining in suspension only for a limited period of time, during which the magnetically labelled exocrine tissue would have to be removed. If we were to use the most magnetically 'massive' particles available (M-450 Dynabeads®) the following calculation illustrates the potential problem.

A permanent magnet creates a magnetic field or flux gradient, pulling the superparamagnetically labelled particles towards the magnet according to the formula

$$F_x = V \cdot M \frac{dH_x}{dx}$$

Where $V$ = the volume of the spherical paramagnetic bead ($4/3 \pi r^3$), where $r$ = the radius), $M$ is the magnetic moment of the sphere (in gauss = $10^{-4}$ tesla) and $dH_x/dx$ is the flux gradient (gauss/cm) $815$.

Resisting this is the viscous force on the sphere and any attached particle moving through fluid described by Stokes Law as:-

$$F_V = 6\pi \eta R v$$
Where $R = \text{the radius of the exocrine particle with attached beads}$, $\eta$ is the viscosity of the medium and $v$ the velocity of the sphere.

In the case of a steady state motion, the magnetic force is just balanced by the viscous force, so that $F_x = F_v$. This means that:

$$v = \frac{V_{M} M_{x}}{6 \eta R v}$$

The magnetic field gradient $dE_x/dx$ generated by the MPC®-6 neodymium-iron-boron permanent magnet produced by Dynal can be approximated by assuming the field generated to be spherical and at a distance $x$ of 1.2 cm from the centre of the magnet = 1500 gauss/cm (personal communication, A.T.Skjeltorp, Institutt for energeteknikk, N-2007 Kjeller, Norway).

For a particle of exocrine tissue of radius 75 μm, labelled with 50 M-450 Dynabeads (each with a magnetic moment of 10 gauss) with a volume each of $4.6 \times 10^{-11}$ cm$^3$, suspended in medium with a viscosity $\eta = 0.001$ Pa s (that of water at 20°C), the velocity with which it will move under the influence of the magnetic gradient:

$$v = \frac{2.3 \times 10^{-9} \times 50 \times 10}{6 \eta \cdot 0.001 \cdot 0.075} \times 1.500 \times 1.22 \text{ cm/sec}$$

The other commercially available immunomagnetic microspheres are much smaller and the effect of this decrease in size greatly decreases the speed of movement in field gradients of the order of 1000 gauss/cm produced by commercial permanent magnets. Beads of 1 μm in diameter such as the ferritin-silane beads used in the BioMag system can be removed using flat permanent magnets, but such separations require exposure to magnetic fields for periods of up to 7 minutes (manufacturers details). This is feasible with suspensions of single cells but not with rapidly sedimenting pancreatic digest. Smaller beads such as the 100nm microspheres used in the MACS system cannot be removed without HGMS of the order of 10,000 to 100,000 gauss/cm.

The decision to use Dynabeads had another major advantage as the beads are individually visible under the light microscope. This allowed their binding to tissue particles to be viewed directly, rather than inferred from the behaviour of the labelled particles in a magnetic field, making the establishment of procedures for coating the beads with antibody, and attaching them to exocrine particles considerably simpler.

e) Development and production of M-450 Dynabeads

These monosized and monodisperse magnetic microspheres produced by the polymerisation of styrene divinylbenzene in a two stage swelling process, were developed by John Ugelstad (Figure 2A). Iron was later incorporated into these beads by allowing the uptake of iron salts into the porous bead structure where it became oxidised and precipitated as iron oxides and hydroxides which on heating formed $\gamma$-Fe$_2$O$_3$, maghemite, which has the same spinel lattice as magnetite. Magnetisation curves show that because the maghemite crystals (5-10nm in size) behave as single domains with no hysteresis
minor thermal effects are strong enough to spontaneously demagnetise the microspheres, so that both the remanence and coercivity at room temperature are zero.

Figure 2.4 Electron-micrograph showing the remarkable uniformity of the M-450 Dynabeads®
(reproduced courtesy of Dynal AS, Oslo, Norway).

These beads were the original 3μm size beads with a very large surface area ~100m²/g. While they were used for effective cell separation, the large surface area necessitated the use of large amounts of antibody, the majority of which became buried in the pores and rendered sterically inactive, and they exhibited a degree of nonspecific cell binding. To reduce the surface area, the surface pores were therefore filled after the process of magnetisation, with polymeric material reducing their surface area to 3-5 m²/g and increasing their size to 4.5μm (the M-450 Dynabead®). The hydrophobic nature of these particles allow high levels of relatively strong nonspecific adsorption of both IgM and IgG antibody to the surface, with adsorption studies showing that by applying 10mg of antibody per gram of beads during incubation, about 3mg of antibody can be bound/gram of beads.

Alternatively the presence of free hydroxyl groups on the surface allow covalent binding of proteins by the tosyl chloride activation method of Nilsson and Mosbach (Equation 9.1, p195). Ready tosyl-activated M-450 beads have been produced, and used for T cell depletion and B-lymphoma cell purging of bone marrow. A smaller version,
the M-280 bead has also been produced with some advantages for single cell separations but not for islet purification 898.

V) APPLICATIONS OF M-450 DYNABEADS®

The need for large scale manipulation of specific cell populations in order to allow transplantation of haemopoietic stem cells, has provided much of the impetus behind the development of cell separation methods, including immunomagnetic ones. Bone marrow transplantation has been used for decades as a means of curing haematological and non-haematological malignancies. The patient's bone marrow is ablated and allogeneic bone marrow from HLA matched donors transplanted. Alternatively, autologous bone marrow harvested prior to ablation can be used to reconstitute patients lacking a histocompatible donor or for whom allogeneic transplantation is inadvisable. The use of bone marrow cells whatever their source, involves ex vivo manipulation of the collected marrow 899,900, with preliminary filtration to remove cell aggregates, fat globules and bone particles, followed by red cell removal. More specific manipulations may then be required in order to eliminate malignant cells harvested with the bone marrow in autologous transplants, or the mature T-cells responsible for graft versus host disease in allogeneic transplantation.

Figure 2.5 Electron micrograph of Dynabeads bound to a Burkitt's Lymphoma cell.

(reproduced courtesy of, Norwegian Radium Hospital, Oslo, Norway)
Although the use of monoclonal antibodies and complement, SRBC rosettes, lectin agglutination, immunotoxins, cytotoxic drugs, and physical separation by centrifugation have all been used for selective removal of cells from marrow, immunomagnetic cell separation offers advantages already discussed, and in addition to their use for bone marrow manipulation, immunomagnetic methods have also been used extensively to purify cell subsets from peripheral blood (see below), spleen cells and hybridoma mixtures, and for the immuno-isolation of non-haematological cells and subcellular organelles.

The immunomagnetic beads make it easy to accomplish either positive or negative selection of cell populations. Isolation of cell subsets especially for functional studies has usually been carried out by negative selection procedures, which ensure that the cells under study are unaffected by either the monoclonal antibody or the separation procedure itself. However for certain purposes even the most thorough depletion of cells with immunomagnetic beads cannot provide cell populations of sufficient homogeneity, while in other situations the monoclonal antibody developed determines that positive selection of the required cell population is used.

For both positive and negative selection the immunomagnetic separation may be accomplished in two different ways. The direct method in which the beads are coated in the monoclonal antibody and bound directly to the cells, and the indirect method in which the cells are first incubated with the mouse monoclonal antibody, washed, and subsequently rosetted with anti-mouse IgG-coated beads. A third alternative is to first coat the beads with a secondary polyclonal antibody and then with the appropriate monoclonal antibody, with the secondary polyclonal antibody acting as a spacer and ensuring a favourable orientation of the monoclonal antibody.

The type of monoclonal available, determines which is most suitable, with the large pentameric IgM molecule apparently providing the necessary space between beads and cells for effective direct coupling, while IgG usually results in suboptimal binding when used in this way. The avidity of the antibody and the density of its corresponding epitope on the target cell also influence the decision, with the indirect method proving more effective for low affinity antibodies and low target antigen densities, optimising the number of interaction possibilities between beads and cells.

A further variable to be considered is the number of cycles used, with two or more cycles of separation with monoclonal antibodies and magnetic beads often proving more effective than just one.

a) **Removal of tumour cells in autologous bone marrow transplantation**

Although it is well known that most tumours exhibit a dose-related response to chemotherapy and radiation, and that administration of high levels could eradicate all of certain tumour cells from the body, the use of such toxic levels are prohibited by the
subsequent failure of the haemopoietic system and death of the patient. This can be prevented by removing bone marrow from the patient before treatment and later reinfusing it to achieve haemopoietic reconstitution. However, most tumours metastasise at an early stage to the bone marrow and reinfusion of even small numbers of tumour cells dramatically increases the likelihood of relapse, the removal of such cells (purging) has therefore become a prerequisite.

Using a panel of monoclonal antibodies to neuroblastoma cells and coupled indirectly (see Figure 2.1, p54) to 3µm diameter beads, Kemshead and co-workers were the first to purge tumour cells from bone marrow using Dynabeads (the use of colloidal cobalt for the immunomagnetic purging of lymphoma and leukemic cells from bone marrow had been described the year before in 1983). Although the porous 3 µm beads demonstrated non-specific binding to other cells reducing yields of re-infused stem cells to 65%, they allowed successful re-engraftment after purging of neuroblastoma cells and both B-cell lymphoma and acute lymphoblastic leukemic cells. The subsequent development of the M-450 particle with its reduced nonspecific cell binding has made it the standard particle for immunomagnetic bone marrow purging of neuroblasts, lymphoma, leukemia, myeloma and other cancer cells.

The reasons for some of the divergent results are unclear, but are most likely to be related to variations in the quality of antibody reagents used. The secondary antibody should be of high titre, and both affinity purified and species specific, with the reactivity against various mouse Ig classes and subclasses optimised. The quality of the monoclonal antibody is also critical. In general high avidity antibodies will perform better than low avidity ones, although in positive cell selection low avidity antibodies can be an advantage allowing more efficient detachment of beads from the cells).

b) Depletion of T cells in allogeneic bone marrow transplantation

For the treatment of conditions such as severe combined immunodeficiency, some leukemias and aplastic anemia, allogeneic bone marrow transplantation has been suggested to be the treatment of choice. When harvesting bone marrow from healthy donors however mature, immunocompetent T lymphocytes from peripheral blood will inevitably contaminate the suspension, and by reacting to foreign tissue antigens in the recipient, can lead to graft versus host disease. Removal of most of the T-cells from the donor marrow should prevent this, but may reduce graft versus leukemia reaction and successful engraftment, increasing relapse and rejection rates.
Vartdal in 1987 was the first to show that immunomagnetic depletion using Dynabeads coated in anti-mouse-Ig antibody and then T-cell-specific monoclonal antibodies, could produce a 3 log decrease in the % of contaminating T lymphocytes from 20-30% to below 0.025% \[950\]. Similar results were obtained by the indirect method \[842,951-953\] using a variety of antibodies to T-cell surface antigens, capable of selective depletion of T-cell subsets \[954\].

c) The purification of specific cell populations from peripheral blood

The quantification and isolation of cell subpopulations in the peripheral blood is an important part of both routine and experimental clinical immunology. **Negative selection** (using a large panel of monoclonal antibodies to specifically remove all the unwanted populations of cells in human peripheral blood) has been used for the isolation of functionally intact T \[955,956\] and B lymphocytes \[957\], monocytes \[958\], eosinophils \[959\] and basophils \[960\], either directly from peripheral blood or from mononuclear cell suspensions. Logically however **positive selection** is likely to result in greater homogeneity of the selected cell population and despite the problem of contamination of the selected cells by attached beads, this approach has been extensively used, and in addition to the selection of whole classes of cells such as B lymphocytes \[957\], has been used to select cell subgroups as discrete as antigen-specific B cells \[961\], CD8+ T cells \[962,963\] and other functional lymphocyte subsets with varying phenotypic markers \[964,964-966\], CD34+ haemopoietic stem cells \[967,968\] and reticulocytes \[969\], and megakaryocytes \[970,972\].

Although their presence has been shown not to affect cell function \[963,973,974\], the beads can usually be detached by a period in culture \[957,965,975\], or the use of chymopapain \[968\] or goat anti-mouse-F'ab antiserum \[976\] to break the hinge region of Ig and then removed by a magnet if necessary. The alteration of cell function and activation that occasionally occurs following the procedure appears to be related to the antibody used and the site of antigen binding rather than the Dynabeads \[973,975\].

As well as their use as research tools, immunomagnetic cell separation procedures have also been used to improve routine laboratory work, such as HLA typing \[913,977-979\] and lymphocyte quantification \[961,974,980,981\].

Unlike the process of bone marrow purging both direct \[957,962,982\] and indirect \[967,973,975\] methods of bead attachment appear to provide similar results \[970\], and the preferred method will depend on the system under study and the functional capabilities of the antibodies. The easiest method is undoubtedly the direct method, but some antibodies still necessitate an indirect technique with prior sensitisation of the cells with the monoclonal antibody.
d) **Immunomagnetic islet purification**

The use of immunomagnetic procedures for islet purification was first described in 1987 by Muller-Ruchholtz et al \(^{859}\) who used 0.2-1.5 μm albumin based magnetic spheres coupled to lectins (sugar-specific, cell-agglutinating proteins) which specifically bound to the surface of rat, large mammal and human exocrine tissue \(^{857}\). Using an electromagnetic field and 3 cycles of purification, islet yields (assessed visually) varying from 20-70% were possible with a purity of 90-95% \(^{858}\).

The first and only description of the use of antibody coupled Dynabeads for rat islet purification came from Soon-Shiong and Fujioka in 1989 \(^{983,984}\). They coupled blood group-reactive monoclonal antibodies which cross reacted with pancreatic acinar cells but not islets \(^{985}\) to Dynabeads. Although they described purities of 84% with only 27% islet losses using an electromagnet and three cycles, it required islets from ‘a minimum of three donors’ to reverse diabetes by isografts in streptozotocin treated recipients \(^{983}\). The procedure used for the magnetic separation in both cases involved collecting the islets from the bottom of a 50 ml flask exposed to an electromagnetic field \(^{858,983}\), and no attempt to optimise the separation procedure was described, although devices involving flow through a series of coiled tubes and magnetic fields were already in use for single cell separation \(^{805}\).

2.4 **SUMMARY AND SCOPE OF PRESENT STUDY**

Chapter 1 outlines the current status of human islet transplantation and the need to increase islet yields in order to reliably achieve one donor to one recipient transplantation. Optimisation of the existing density dependent methods is obviously important and can be approached in one of two ways. Work described in Chapters 4-6 examined the effect of storing both the whole pancreas and the pancreatic digest in storage solutions known to influence hypothermic induced cell swelling. The importance of specifically designed cold storage solutions and their components, in maximising the existing differences in islet and exocrine densities is discussed. Secondly Chapter 7 details investigations improving the mechanical process of density gradient separation using continuous rather than discontinuous gradients on the COBE 2991 cell processor. Despite using these methods to maximise islet yields and purity, there will always be considerable overlap in the density of some of the islets and exocrine tissue fragments, preventing satisfactory purification of these islets using density dependent methods.

This chapter has outlined some of the alternative methods already in use for cell separation, which could be used either in isolation or in conjunction with density dependent processes to improve islet isolation. The recent development of an magnetic immunological based method offers the advantages of rapid, gentle, highly specific and potentially large scale purification, and has been rapidly applied to many areas of cell separation. Its application and adaptation to islet purification however has received scant attention.
Chapter 8 describes the production and purification of a panel of mouse monoclonal antibodies specifically reactive to the exocrine components but not the islets of the rat and human pancreas. Using a monoclonal to rat exocrine tissue preliminary work exploring ways of optimising the binding of beads to exocrine tissue and subsequent immunomagnetic islet purification are then recorded in Chapter 9.
CHAPTER 3
METHODS

3.1 GENERAL INTRODUCTION

3.2 THE ISOLATION OF HUMAN ISLETS AND METHODS OF EVALUATION
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iv) Visual and assay assessment of digest distribution
v) Reproducibility of measurements
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3.1 GENERAL INTRODUCTION

This chapter outlines methods already established within the Department from the outset of this work in January 1991, for:
1) The purification of human pancreatic islets and methods of evaluating these.
2) The purification of rat pancreatic islets
3) The production of monoclonal antibodies

The development and assessment of a mini continuous-gradient system for the measurement of tissue densities, which was subsequently used in much of the work on improving human islet purification is then described in section 3.5.

The experimental work which led to modifications of the existing methods for human islet purification, and the methods used in the development of immunomagnetic islet purification are covered in the relevant Chapters which follow.

3.2 THE ISOLATION OF HUMAN ISLETS AND METHODS OF EVALUATION

i) Donor operation

Human pancreata were removed with appropriate consent from brain-dead heart-beating organ donors. In each case the kidneys were also removed for transplantation, and in most cases the procedure was part of a multi-organ donation involving retrieval of the liver, heart and lungs. After initial dissection of the organs to minimise the period of cold ischaemia, the systemic circulation was perfused in each case with HOC. Where the liver was involved the portal system was also perfused 986,987, but with UW. At this point ventilation and the heart were stopped, the pancreas was estimated to undergo a minimal period of warm ischaemia of ~ 2 minutes before being cooled by the in-situ vascular perfusion to 4°C. The period of cold ischaemia was regarded as commencing at this point.

After removal of the liver and kidneys, the entire pancreas was removed with care being taken not to breach the capsule. The head of the pancreas was dissected from the duodenum with the duct (s) being ligated and divided at its point of entry into the duodenum. After removal of all fat and blood vessels the pancreas was transported in HOC at 4°C to the laboratory.

ii) Collagenase distension and automated digestion

The pancreas was processed immediately to minimise the period of cold ischaemia. A sample of HOC transport fluid was removed for microbiological assessment, and all procedures were carried out within a Class II Microbiological Safety Cabinet (Medical Air Technology Ltd., Manchester). The pancreas was transected across the neck of the gland, and the head and tail of the pancreas were weighed. The ducts in both the head and the tail were cannulated using an Abbocath ®-T (Abbott Ireland Ltd., Sligo, Republic of Ireland) of
appropriate gauge (14 - 20G). The cannulae being secured in place with a suture tightened around a minimal amount of pancreatic tissue including the duct.

Figure 3.1 Intraductal distension of the pancreas with collagenase

The pancreas was then distended by gentle intraductal injection of 2ml/g of collagenase at a temperature of 22°C (Figure 3.1). A single batch (03091C) of collagenase (Serva Feinbiochemica, Heidelberg, Germany) was used throughout this work. It was dissolved by sonication in HBSS at a pre-determined concentration of 3mg/ml for donors over the age of 20, and 2.5mg/ml for younger donors, before being filter sterilised using a 0.22µm filter. During the process of distension the cannula was slowly withdrawn so that the whole pancreas was effectively distended, before the cannula was removed.

The automated method of digestion first described by Ricordi with minor modifications, was then used for the sterile digestion of the pancreas. The distended head and tail of the pancreas were placed in a chamber containing 10 stainless steel marbles 1 cm in diameter, with a 500µm mesh across the outlet (Figure 3.2).

Phase 1 MEM a1 at 37-38°C was recirculated through the chamber using a peristaltic pump (503S, Watson-Marlow Ltd., Falmouth, UK) at 96 ml/min, maintaining the pancreas at the optimal temperature for enzymatic digestion of the collagen framework, and at the same time flushing islets and exocrine fragments out of the chamber, through the 500µm mesh, as they were liberated. Gentle shaking of the chamber mechanically aided the digestion, and occasional inversion of the chamber, ensured that free digest was not retained within it by
gravity. The digestion process was monitored by sampling the outflow from the chamber, with dithizone staining used to allow clear and rapid identification of the islets.

**Figure 3.2** The stainless steel digestion chamber containing the pancreas and covered by a 500μm mesh

**Phase 2** Once islets devoid of any adherent exocrine tissue (cleaved islets) were seen, the circuit was opened, so that fresh MEM still at 37-38°C was pumped in at 130ml/min, and the outflow from the chamber instead of re-circulating, was collected into 1 litre sterile bottles containing 100ml NCS (10%). This phase was designed to continue the digestion process of the pancreas within the chamber, while rapidly removing cleaved islets and exocrine fragments from the injurious action of collagenase. Sampling of the chamber outflow was continued during this phase.

**Phase 3** If evidence of fragmentation of the collagen matrix of the islets themselves was seen at any stage, the temperature of the in-flowing MEM was lowered to 22°C to prevent overdigestion. This phase was rarely needed however.

The MEM containing digest was centrifuged at 200g for 2 minutes to pellet all the digest, and the pellet was then resuspended in cold MEM and stored at 4°C until purification.
iii) Large-scale density gradient purification using the COBE 2991 cell processor

The COBE 2991 cell processor (Figure 7.2, p153) was used to separate islets from the exocrine fragments on the basis of differences in their densities, with up to 20 ml of digest being purified by centrifugation on a single 550ml discontinuous density gradient. The gradient medium used was 500mOsm/KgH2O BSA 591.

The digest was suspended in 200ml of high density BSA, (1.106g/cm^3) which was loaded under gravity into the 2991 bag as the 'pellet'. Any air in the bag was then vented by centrifugation at 1000rpm and using the 'superout' facility. The density of the 150 ml, 'isolation' layer of BSA was adjusted by the addition of appropriate amounts of 500mOsm MEM to vary from 1.089-1.092g/cm^3, the precise density being chosen on the basis of a mini-continuous gradient (see section 3.5). This variation in density was designed to optimise the islet yield from any individual pancreas, allowing maximum numbers of islets to rise through it while retaining most of the exocrine fragments below it. This layer and then 50ml of 'capping' BSA (1.065g/cm^3) and 150ml of MEM were pumped onto the 2991 while continuing to spin at 1000rpm, producing a discontinuous density gradient (Figure 3.3).

After 5 minutes of centrifugation at 2200rpm the top 160 ml of the gradient containing MEM and 10 mls of the capping BSA was allowed to flow off the top of the gradient while continuing to spin. The next 115 mls was collected as the 'islet' layer using the 'superout' facility, and the bottom 'exocrine' interface was collected in the next 160 ml, with the residual 115ml of BSA containing exocrine tissue being left in the 2991 bag.

iv) Islet quantification

The number of islets in both the pooled digest and following purification was routinely estimated 592. The digest was suspended in 200ml of MEM and 4x50μl samples were taken and streaked in lines on a petri dish. After dilution with 200μl MEM and staining with 100μl dithizone, all the islets >50μm in diameter along each line were counted microscopically at 150x magnification. Multiplication of the total by 1000 gave the total number of islets in the digest.

Using a graticule at the same magnification the diameters of 200 consecutive islets were then measured and any uncleaved islets were recorded during this, to give a cleavage percentage. The diameters were entered into a computer statistical package and used to calculate the mean volume of an islet using the formula 4/3πr^3 or 0.524d^3 = the volume of a sphere. Multiplying this figure by the total number of islets gave the total volume of islet tissue.

The number of 150μm equivalent islets 593,594 was then calculated by dividing the total islet volume by the volume of a 150μm equivalent islet (0.00177ml).

The same process was used to calculate these variables for purified islet yields using 5x100μl samples from a total of 200ml 593, while an estimate of % purity was made on each sample by two observers.
v) **In vitro islet viability**

The viability of islets produced was regularly assessed using either static incubation or perifusion.

**Static incubation of islets**

Purified islets were cultured in RPMI 1640 for 24-48 hours. After confirmation of purity using dithizone, 5 groups of 10 islets were handpicked in minimal amounts of medium, into Eppendorf tubes containing 200μl of low glucose MEM (1.7mM glucose) in a waterbath at 37°C and incubated for 1 hour. After this period, the medium was aspirated off and replaced with a further 250μl low glucose MEM for 1 hour at 37°C, this medium was then aspirated and the insulin content subsequently assayed (L1). The islets were then incubated for 1 hour in MEM containing 25mM glucose (H) and finally 1.7mM glucose for a further hour (L2). The three samples (L1, H and L2) were stored at -20°C until determination of the insulin release during the three phases using a radioimmunoassay. This allowed a stimulation index to be calculated by dividing the value for H by the mean of L1+L2.

**Perifusion of islets**

After culturing for 24-48 hours in RPMI 1640, 4 groups of 50 islets were handpicked and trapped in a chamber containing glass wool through which low glucose (1.7mM) Gey and Gey buffer was running at 1ml/hour at 37°C. After 1 hour during which the insulin release settled to a basal level, samples of the buffer as it left the chamber were collected for 1 minute at the following times:

- 0, 10, 19, low
- 21, 23, 25, 27, 29, 40, 50, 60, 70, 79, high
- 90, 100, 110, 120, 130 and 140 minutes, low

At the end of the 19th minute the perfusion was switched to a high glucose (25mM) buffer for 1 hour, before being switched back after the 79th minute to the low glucose perifusion for the last 1 hour. After insulin assay, the profile of insulin release over time was plotted, to show the biphasic nature of the insulin release (Figure 4.4, p.113). The response time could then be determined as the time taken for the islets to respond to the increase in glucose by a 2-fold increase in insulin release (allowing for the 4 minute lag time of the perifusion system). Several methods have been used for calculating an index of islet viability from perifusion results, including the rate of stimulated insulin secretion in μU/islet/minute, division of the peak insulin release by the first basal level, and more complex but meaningful methods involving the comparison of areas under the insulin release profile with time. For these studies a stimulation index was calculated from the ratio between the insulin level during both basal periods of insulin release, and that over the period of stimulated insulin release.
3.3 ISOLATION OF ISLETS FROM WAG (Leic) RATS

Under halothane anaesthesia, the common bile duct was exposed, and clipped as it entered the duodenum. The pancreas was then distended by injection of 5 mls of ice cold collagenase solution into the duct at the point where the two hepatic ducts joined, avoiding any intrahepatic flow (Figure 3.4). The distended pancreas was carefully resected and incubated in MEM at 37°C for 18 minutes, after which the digestion process was stopped by cooling the pancreas to 4°C in MEM. Mechanical shaking was used to dissociate the pancreas, before the digest was filtered through a 500μm mesh and washed with MEM + 0.5% BSA.

Figure 3.4 the intraductal distension of the rat pancreas with collagenase (4°C)
Discontinuous density gradients of BSA were used to purify the islets. The digest was suspended in high density BSA (1.085 g/cm³) 6 ml pancreas, and 3 ml of this suspension was layered at the bottom of a 12 ml tube, and overlaid with a discontinuous gradient of BSA of densities 1.069 and 1.056 g/cm³ (Figure 3.5). The gradient was centrifuged at 800 g for 25 minutes with no brake, and the islets were removed from the upper interface using a pasteur pipette, washed and placed in RPMI 1640.

3.4 PRODUCTION OF MONOCLONAL ANTIBODIES

i) Immunisation of mice.

Rat and human exocrine pancreatic digest was collected following islet purification as detailed above, with pig exocrine digest being produced by similar methods to the human islet purification process. The exocrine fragments were divided into 100 µl aliquots and suspended in 0.5 ml of MEM before storage at -20 °C. BALB/c mice > 6 weeks of age were immunised by intraperitoneal (ip) injection of aliquots of exocrine tissue antigen at 2-3 week intervals. After a minimum of 3 injections the antibody response of the mice was checked by immunohistology of their serum on pancreatic sections (see section 3.4 iii), if this was positive the mice were boosted by a further ip injection of antigen 4 days prior to removal of the spleen for hybridoma production.

ii) Hybridoma formation.

On the day of the fusion the immunised mouse was anaesthetised under halothane anaesthesia and killed by cardiac puncture to obtain serum and inject a lethal dose of barbiturate. Five ml of 50:50 medium was then injected intraperitoneally and aspirated to obtain a suspension of peritoneal macrophages which were irradiated and used as feeder cells. The spleen was then removed.

Working in a Class II hood the spleen was reduced to a single cell suspension by passage through a 125 μm mesh. Viable cells in a 1:10⁵ sample, were counted in an Improved Neubauer Haemocytometer (Weber Scientific International Ltd, Lancing, UK) using trypan blue, after lysis of the red cells with 0.75% tris ammonium chloride (pH 7.65).
NS-0 mouse myeloma cells 1005,1006 (a kind gift from Geoffrey Butcher, Agricultural Research Centre, Cambridge, UK) were then added to the spleen cells in a ratio of 1:4.

Figure 3.6 Illustrating the production of hybridomas by fusion of spleen cells with NS-Os

Fusion of the 8-azaguanine-resistant non-secreting mouse myeloma cell line NS-Os and the spleen cells was accomplished at 37°C, by the addition of 800μl of polyethylene glycol (PEG 1500, 50%w/v HEPES) (Boehringer Mannheim) 1007-1009, first described for the fusion of plant protoplasts in 1974 1010,1011, over a period of 1 minute. After a further minute, the PEG was diluted by the addition of 3 ml of 50:50 medium over 9 minutes, and the PEG was then washed out and the cells plated out into 3x24-well tissue culture plates (Nunc, Denmark), in 50:50 medium with 15% FCS at 2 million spleen cells/well (Figure 3.6).

After 24 hours, the 50:50 medium was changed for hypoxanthine-aminopterin-thymidine (HAT) supplemented medium 1012, which was then changed every 3rd day. In this, although the aminopterin blocks purine and pyrimidine synthesis, the spleen cells are only able to survive as usual for 1-2 weeks utilising the intermediary substrates hypoxanthine and thymidine. However the NS-0 cells which are usually immortal, are deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT-) necessary to use this alternative pathway, and consequently also die. Only the hybrids which have inherited the complementary HGPRT gene of the spleen cells, and the immortality of the myeloma cells are able to survive indefinitely and become selected by 2 weeks growth.
iii) **Screening of hybridomas using immunohistology**

Once the hybridoma cells were almost confluent within the wells the supernatant from each well was screened for pancreatic exocrine antibodies, by immunohistology on frozen sections of rat, human or pig pancreas.

Fresh pancreas for use as histological sections was cut into small oblong blocks and mounted in OTC cryoprotectant gel on a cork block, before being snap frozen in isopentane cooled in liquid nitrogen. The block was then stored at -196°C in a liquid nitrogen bank.

Using a cryostat (2800, Frigocut E, Reichert-Jung GmbH, Heidelberg) 5μm thick frozen sections were cut from these blocks, and dried onto multispot slides (Hendley-Essex, Loughton, Essex) which had been pre-gelatinised. The slides were stored in batches of 25 slides of human (O-negative donor tissue), rat and pig pancreas sections at -20°C. Before use the sections were thawed over a period of 1 hour, and fixed by immersion in acetone for 10 minutes.

100μl of the supernatant from each hybridoma was placed on the pancreas sections, existing anti-exocrine monoclonals within the Department were used as positive controls, and 50:50 medium with 15% FCS as the negative control. After incubation at 4°C for 40 minutes, the supernatant was washed off with TBS.

![Diagram](image_url)

*Figure 3.7* showing the indirect method using the biotin-avidin complex for further amplification of the alkaline phosphatase enzyme signal.

In order to amplify the signal identifying IgM antibody from the supernatants bound to the sections, an indirect staining method using the avidin-biotin bridge system was used. 100μl of a goat μ-chain specific anti-mouse IgM antibody tagged with biotin...
(B9265, Sigma) was added to each section. After incubation for 30 minutes at 22°C and washing with TBS, this was in turn labelled (Figure 3.7) by incubation for 30 minutes with extravidin® conjugated to alkaline phosphatase (E2636, Sigma) 1016.

The addition of 100μl of chromogenic substrate was used to develop the red colour reaction, and the rest of the section was counter-stained blue with acid haematoxylin solution (Sigma 285-2). Once dry, the slide was sealed using glycerol gelatin (Sigma GG-1) and a cover slip.

Light microscopic examination of each section under 150x magnification, allowed hybridomas producing antibodies to various components of the exocrine pancreas to be identified, and these were then cloned.

iv) Cloning and freezing of hybridomas

The hybridomas from each positive well, were resuspended in 1ml of RPMI + 10% FCS. Cell numbers were estimated by counting 10μl, and a proportion of the cells were then plated out in a 96 well tissue culture plate at an estimated 1 cell/well (Figure 3.8). The rest of the cells were maintained in culture as a back up.

Figure 3.8 Cloning of hybridomas producing anti-exocrine antibodies

Wells containing colonies of cells were re-screened about 1 week after cloning and positive wells were re-cloned by dilution for a second time to ensure monoclonality. Only if >95% of these second clones were positive for antibodies to the same antigen, was one of
these clones accepted as monoclonal, if <95% were positive the cloning procedure was repeated. Clones of cells from positive wells were harvested for storage in liquid nitrogen. 2 million cells were placed in freezing vials in 50:40:10 RPMI:FCS:DMSO (Dimethyl sulphoxide), and gradually cooled, 4°C for 1 hour, -20°C for 1 hour, -70°C overnight, before storage at -196°C in a liquid nitrogen bank.

v) Production of ascites

High concentrations of the required antibody were produced by growing the cells in mice to produce an ascites. Cells producing the desired monoclonal antibody were thawed rapidly to 37°C and washed in RPMI before being grown up in a tissue culture flask. BALB/c mice were primed by injection of 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane, T7640, Sigma) ip 1 week prior to ip injection of 2-5x10⁶ viable hybridoma cells. The mice were then checked daily for ascites. Once 2-5 ml of ascites had been produced the mouse was culled, and the ascites stored at -20°C.

3.5 VALIDATION OF MINI CONTINUOUS GRADIENTS FOR ASSESSING ISLET AND EXOCRINE DENSITIES

i) Introduction

Improvements in the density-dependent purification of human pancreatic islets depend on maximising the existing difference in density between the islets and the contaminating exocrine tissue. Much of the work which follows aimed to improve both the purity and yield of human pancreatic islets. This required the ability to measure the effect of various manipulations on the density of both islets and exocrine tissue, and to produce from this data, a measurement of both islet yield and purity which would allow comparison, and was meaningful in terms of large-scale human islet purification.

While the use of discontinuous density gradients in which the cells concentrate in the steep areas of the gradient at each interface, and neutral density separation, where cells are separated simply on the basis of whether they float or sink in a particular suspension, have both been used for isopycnic islet purification, neither method is suitable for quantitative investigation of the density distribution of the islets and exocrine fragments within the digest. As de Duve has pointed out, “the discontinuous gradient is essentially a device for generating artificial bands. This may be a convenient way of compressing together for preparative purposes certain segments of the distributions observed in continuous gradients. But it is also a very dangerous procedure in that it creates the illusion of clear-cut separation.” It has also been pointed out that performing “isopycnic centrifugation over a neutral density column or in a discontinuous gradient” involves an internal contradiction in terms.
Manually generated continuous gradients had been generated before 1018,1019, but Leif and Vinograd in 1964 first described the production of a linear isotonic continuous density gradient of BSA, and used it to measure accurately and reproducibly the density distribution of human erythrocytes 1020. These linear continuous density gradients were mixed in a single chamber, from which the gradient was removed at twice the rate that the dense BSA was being added, and these have since been developed into two chamber gradient makers which are simpler to use 596,1021. Since then, a general consensus has been reached among cell separatists that only continuous gradients provide consistent and reliable results 578,591,595 lending themselves "to an entirely objective assessment of the frequency distribution curves of certain physical properties, such as density”. Discontinuous gradients on the other hand produce an artificial banding of tissue which impedes cell distribution in the gradient, and enhances aggregation of different cell types into heteroagglutinates with a different buoyant density to the two parent cells. Continuous density gradients produced using a similar method to Leif’s, have been used to visually assess rat islet and exocrine banding in different density gradient media 1022.

We aimed to examine the formation of mini-continuous density gradients using a simple two chamber gradient maker, and validate their use for the measurement of the density distribution of exocrine and islet tissue, and assessment of islet yield and purity, following collagenase digestion of the human pancreas.

Bovine serum albumin (BSA) was used as the gradient medium since it produces gradients whose osmolality remains unchanged with increasing density 222, unlike gradients using Ficoll which have been shown to produce a marked non-linear increase in osmolality which prevents the 'true' density of cells being measured on them 210,602,1023. Also unlike Ficoll, the viscosity of BSA changes very little over the density range required for these studies 602,1022, making it ideal for analytical studies of cell density.

Production of a 10 ml linear continuous density gradient

Using a two chamber continuous gradient maker (SG100, Hoefer Scientific Instruments, San Francisco) (Figure 7.7, p160), a series of 10 mini continuous gradients were produced, 500mOsm BSA was used throughout, with the density adjusted by the addition of appropriate amounts of 500mOsm MEM to stock BSA whose initial density was 1.106g/cm³. The densities were all absolute densities measured on a densitometer (DMA 35, Parr Scientific Ltd, London) calibrated to read 0.998 using distilled water at 21°C and 1.000 at 40°C.

Each 10 ml mini continuous gradient was produced by pipetting 5.2 ml of low density BSA (1.066g/cm³) into the low density chamber, and 5.2 ml of high density BSA (1.098g/cm³) into the other, (the use of 5.2 ml rather than 5.0 ml was found to produce a 10 ml gradient, due to BSA losses in the pipette and gradient maker). The tap between the two chambers was opened, and a magnetic flea used to achieve mixing of high and low density gradient media as the media was pumped from the high density chamber at 2ml/min. The
gradient was formed in a test tube (144AS, Sterilin) to which 1 ml of high density BSA had already been added.

Once formed, the gradient was centrifuged (CR422, Jouan, Tring, Herts) at 800g for 5 minutes, with no brake being used during deceleration to avoid vortexing. A series of 11x1ml aliquots down the gradients were removed using a Gilson pipette (Gilson, Villiers France), and after pooling matching fractions from all 10 gradients, the density of each pooled fraction was determined using the densitometer (Figure 3.9).

![Graph Showing the Mean Density of Each Fraction from Ten Gradients]

**Figure 3.9** Showing the linear increase in density down the gradient

This established the linearity of the mini continuous gradients, with a correlation, even when the 1ml high density pellet, (designed to hold the pancreatic digest in future experiments), was included of r=0.997, p<0.001 between fraction and density.

The pH of the BSA remained unchanged down the gradient at pH7.0, and the osmolality of each fraction was shown to be unchanged at 500mOsm/KgH₂O using a vapour pressure osmometer (5500, Chem Lab Scientific Products Ltd, Hornchurch, UK).

iii) **Determination of islet yield and purity**

The same technique was then used, but 100µl of human pancreatic digest was suspended in the 1ml of high density BSA placed at the bottom of the mini continuous gradient. The use of bottom loading rather than top loading visibly decreased wall effects during centrifugation, reducing the possibility of indiscriminate sedimentation of the resulting aggregates. The exocrine fragments and islets rose through the gradient during 5 minutes centrifugation, until they reached their iso-dense point, so called isopycnic or “equal density” cell separation. The gradient was then divided into 11x1 ml aliquots, each
Graph Showing the Cumulative Distribution of Insulin and Amylase Down the gradient

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**Figure 3.10** Graph showing the method of calculating the ‘median’ islet (I den) and exocrine (E den) densities and the % exocrine contamination of a 60% islet yield (% EC)

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**Figure 3.11** Diagram showing the balance necessary between islet yield and purity during density gradient separation

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was washed twice in MEM, and the tissue contained in each fraction was sonicated in 2ml of MEM. This allowed the insulin and amylase content of each fraction to be determined by radioimmunoassay and Phadebas® amylase assay (Pharmacia Diagnostics, AB Uppsala, Sweden) respectively.

The percentage of insulin and amylase containing tissue in each fraction was calculated and plotted graphically (**Figure 3.10**).

From these graphs, the median density (g/cm³) of amylase (E den) and insulin (I den) containing tissue was determined as the density of the 50th percentile of the tissue, 1.0846 for insulin and 1.0963 for amylase in this example. Islet purification using density gradients involves a balance between islet yield and purity with greater yields always obtainable at the expense of lower purity, it was felt that a realistic yield of purified human islets was 60%, and the % of exocrine tissue contaminating a 60% yield of islets was used (% Ec) as a standardised measure of islet purity.
standardised measure of islet purity (7% in this case), effectively controlling one of the variables in the yield/purity equation (Figure 3.11).

These figures allowed the density of tissue from one human pancreas to be compared with another, and examination of the effect of various manipulations not only on these densities, but also on the purity of a standard 60% islet yield.

The reproducible nature of these measurements was examined by comparison in two ways, firstly by comparing the visual assessment of gradient purification with that obtained by assay of sonicated tissue, and secondly by comparing the results of assaying two identical gradients.

iv) Visual and assay assessment of digest distribution

For 14 human pancreases, two gradients were run synchronously, each gradient was divided into 11x1ml aliquots and then washed twice in MEM. The distribution of insulin and amylase containing tissue in one gradient was then determined by assay, and in the other was assessed visually after dithizone staining of each fraction. This involved scoring the amount of exocrine tissue and islets in each fraction on a scale from -,-/+,-/+ etc. to +++++++. The correlation between the visual and assay assessment of % islet and exocrine distribution, was determined using Pearson's product-moment correlation coefficient (r).

The exocrine tissue distribution down each pair of 14 gradients showed a strong correlation in the assessment of the two methods (with a median value of r=0.8055, p<0.001, range 0.4380-0.9860) (Figure 3.12).

**Figure 3.12** Showing the correlation between visual and assay assessments of tissue distribution on four gradients
The correlation in islet distribution was not as great, although still highly significant (median $r=0.6565$, $p<0.022$, range $0.3130-0.9440$). The reason for this seemed to be that the visual scoring system underscored the % of islets contained in the highly contaminated lower fractions of the gradients.

The assessment of median tissue densities was correlated for both exocrine ($r=0.762$, $p<0.001$) and islets ($r=0.670$, $p=0.009$) and there was also a significant correlation between the visual and assay estimates of the % exocrine contamination of a 60% yield of islets for each of the 14 pancreata ($r=0.538$, $p<0.05$).

In view of the problems encountered estimating the % of islets in the lower gradient fractions visually due to the presence of large numbers of exocrine fragments, the more objective assays were used in all reported results.

v) **Reproducibility of measurements**

Having decided to use assay assessment of tissue distribution on the gradient as the more accurate method, the reproducibility of this methodology was itself examined by comparing the results obtained from two identical gradients run synchronously using digest from the same pancreas.

28 pairs of gradients using different pancreases were used to assess the repeatability of the method over a wide range of values, with the median exocrine density varying from 1.0910 - 1.0974, the variation in median islet density from 1.0820 - 1.0955 and the % of exocrine contamination of a 60% islet yield ranging from 0-55%, mean 11.6%. The results from the two gradients showed a very high correlation between median exocrine densities ($r=0.995$, $p<0.001$), median islet densities ($r=0.762$, $p<0.001$) and % exocrine contamination ($r=0.956$, $p<0.001$).

However, use of correlation coefficients to assess the repeatability of a single method has recently been justifiably criticised since if one measurement is always twice that of the other the correlation will be 1.0 although absolute value of the two measurements is very different this can be seen from Figure 3.12. The repeatability coefficient adopted by the British Standards Institution (the standard deviation of the differences) which takes into account the difference between each value (Figure 3.13), was therefore calculated, this showed that we could be 95% confident that the value for the median exocrine density would lie between +/-0.0013g/cm³ of the value given by a single gradient, for islet density the figure was +/-0.0045g/cm³, and for exocrine contamination +/-8.95%. These limitations have to be borne in mind when discussing changes in density and purity detected using the gradients.
Figure 3.13 showing the difference in median exocrine densities between the two matched gradients

vi) Discussion

The use of continuous gradients has become accepted as the most reproducible way to assess the density of cells in single cell suspensions, and has been described as producing visible separation of the multi-cellular tissue fragments in rat pancreatic digest. This work shows that the results obtained using easily produced linear isotonic continuous density gradients of BSA, can be not only used to reproducibly assess the densities of the components of human pancreatic digest, but also to provide a standardised measurement for comparing islet purity in terms of the % of exocrine contamination of a 60% yield of islets.

The purification of human pancreatic islets is a very variable process with large numbers of pure islets obtained from one pancreas, and minimal numbers from the next. While some of this variability is due to differences in the digestion process, the majority of it results from variations in the relative overlap of islet and exocrine densities and the effect that this has on isopycnic density separation (Figure 3.11).

Many factors are known to affect density dependent cell separation, with the effect of osmolality 210,1020, pH 211 and anionic composition 212 of the gradient medium changing cell density by alterations in cellular volume 1026. The period of cold storage of organs during transplantation is also known to have a profound effect of cellular swelling 121, and although the effect of this on subsequent islet purification has not been investigated it is likely to be as important as the composition of the density gradient medium. In each case cellular swelling results from alterations in the balance of solutes across the semi-permeable membrane of the cell described by the double-Donnan hypothesis in which the Na+/K+ ATPase dependent pump maintains a balance of osmotic activity 1026. Since the
maintenance of cellular volume in this way is clearly dependent on cellular metabolism, many of the disease processes which contribute to brain death in potential organ donors are also likely to have an effect on cellular swelling contributed to by the maintenance of fluid balance, haemodynamic stability and nutrition during a variable period on ITU. Identification and perhaps manipulation of such factors, is vital to improving isopycnic islet purification.

Using a series of 6 SG100 gradient makers and an 8-channel peristaltic pump (Gilson, Villiers, France), the mini continuous gradient technique will be extensively used during this study to examine the effect of gradient temperature, cold storage medium, and digest storage on tissue densities and subsequent human islet purification. Although the changes noted predominantly reflect cellular swelling, the use of the continuous gradient technique allows their effect on islet purification (the clinically important end-point) to be measured directly.
CHAPTER 4
A PROSPECTIVE RANDOMISED COMPARISON OF PANCREATIC VASCULAR PERFUSION WITH HYPEROSMOLAR CITRATE OR UNIVERSITY OF WISCONSIN SOLUTION AND ITS EFFECT ON HUMAN ISLET ISOLATION.

4.1 INTRODUCTION
i) Principles of anaerobic cold storage
ii) Development of cold storage solutions and their effect on organ preservation
iii) Effect on islet purification
   a) Islet viability
   b) Collagenase digestion
   c) Density dependent purification
iv) Aims of study

4.2 METHODS

4.3 RESULTS
i) Pancreas digestion
ii) Islet purification
iii) Islet viability

4.4 DISCUSSION
i) Effect of donor age on digestion
ii) Effect of UW on digestion
iii) Comparison of the theoretical and practical advantages of HOC with UW
iv) Comparison of cost
4.1 INTRODUCTION

i) Principles of anaerobic cold storage

Hypothermia is the key to successful organ preservation, decreasing the rate at which intracellular enzymes degrade the essential cellular components necessary for organ viability. Between the temperature range 0-35°C, the rate of chemical reactions doubles for every 10°C increase in temperature, expressed by van’t Hoff’s rule where:

\[
\text{The coefficient for a 10°C temperature change} = \frac{\text{rate of reaction at (x+10)°C}}{\text{rate of reaction at x°C}} = 2.
\]

Outside the range 0-35°C the rate of reaction processes continue to decrease slowly down to -6°C, so that hypothermia does not stop metabolism, it simply slows down reaction rates and postpones cell death, until ultimately the organ ceases to function. Above 40°C there is a rapid decrease in the reaction rate due to denaturation of enzyme proteins, with cell death ensuing rapidly above 45°C.

As suggested by Van’t Hoff’s rule, cooling of the whole body 1028,1029, or isolated organs 1030, has been shown to suppress the metabolic rate by 12-13 fold, and preservation times for organs such as the kidney 113,1031, pancreas 1032 and liver 1032 which can withstand 30-60 minutes of warm ischaemia, have been extended to 12-13 hours in the dog simply by surface cooling 1033-1036, before cellular damage and deterioration in function begin to be evident.

Organ survival remains limited during hypothermia however by the development of cellular swelling due to inactivation of the Na+/K+ ATPase dependent pump 1037, and intracellular acidosis due to the switch to anaerobic metabolism. During reperfusion, organ viability deteriorates still further due to cell injury from the generation of oxygen-free radicals, compounded by a lack of high-energy phosphate compounds. The use of solutions adapted to minimise these problems has resulted in extended cold storage times.

The mechanism behind cellular swelling has been extensively investigated. At normal body temperatures the Na+/K+ ATPase dependent pump effectively renders Na⁺ (and with it Cl⁻) an extracellular impermeant, counteracting the osmotic effect of the

\[
\begin{align*}
\text{4mM} & \quad \text{Cl⁻103mM} \\
10mM & \quad \text{Na⁺142mM} \\
140mM & \quad \text{K⁺4mM} \\
\end{align*}
\]

**Figure 4.1** Diagram showing the concept of active sodium transport out of the cell balancing the intracellular colloid osmotic pressure in the normal metabolising cell.

The mechanism behind cellular swelling has been extensively investigated. At normal body temperatures the Na⁺/K⁺ ATPase dependent pump effectively renders Na⁺ (and with it Cl⁻) an extracellular impermeant, counteracting the osmotic effect of the
Figure 4.2 illustrating the swelling that occurs with hypothermic inactivation of the Na\(^+\)/K\(^+\) ATPase dependent pump intracellular proteins and impermeant anions (110-140mOsm) and maintaining the osmotic balance across the cell membrane (Figure 4.1)\(^{1026}\), the so called double-Donnan equilibrium.

Anaerobic-hypothermic preservation suppresses the activity of the Na\(^+\) pump \(^{1037,1038}\), and Na\(^+\) and Cl\(^-\) enter the cell down a concentration gradient accompanied by water, causing the cell to swell as a result of an isosmotic increase in cellular hydration \(^{1039,1040}\) (Figure 4.2), a process analogous to the cloudy swelling and hydropic degeneration which occur during the later stages of cell death \(^{1041}\).

Figure 4.2

The use of solutions which contain molecules impermeant to the cell, balances the colloid osmotic pressure across the cell membrane when the Na\(^+\) pump has been inactivated (Figure 4.3), and have been shown to prevent normothermic \(^{120}\) and hypothermic cell swelling of both the kidney \(^{119}\) and pancreas \(^{121,126}\).

Figure 4.3 Diagram illustrating the prevention of cell swelling using impermeant molecules during cold storage
ii) Development of cold storage solutions and their effect on organ preservation

The use of appropriately adapted flush-out solutions increase cold storage times well beyond the 12-13 hours possible with just hypothermia. In 1969 Collins showed that the use of solutions simulating intracellular fluid, rather than the previously used plasma based solutions prolonged kidney preservation up to 30 hours, equalling that achieved by continuous perfusion circuits. While the solution was rapidly assimilated into clinical practice making kidney transplantation a non-emergency procedure, it was not particularly effective for the preservation of other organs such as the liver, pancreas and heart, which continued to have safe ischaemic times of only 6-10 hours.

Following the description by Sachs in 1973 of a hyperosmolar solution based on mannitol, Ross and Marshall in 1976 described a hypertonic solution (Table 4.1) based on a large carbohydrate based anion, citrate (mol wt 189, 55mmol), and a relatively impermeant sugar, mannitol (mol wt 182, 34mmol) showing it to be superior to Euro-Collins solution for renal preservation. With studies proving its efficacy for kidney, liver and pancreas preservation, this has become the most widely used vascular perfusate in the UK.

<table>
<thead>
<tr>
<th></th>
<th>EC</th>
<th>HOC</th>
<th>UW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobionate(mmol)</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Citrate (mmol)</td>
<td>0</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Raffinose (mmol)</td>
<td>0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Glucose (mmol)</td>
<td>195</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HE Starch (g/l)</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Mannitol (mmol)</td>
<td>0</td>
<td>185</td>
<td>0</td>
</tr>
<tr>
<td>Na+ (mmol)</td>
<td>10</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>K+ (mmol)</td>
<td>115</td>
<td>80</td>
<td>140</td>
</tr>
<tr>
<td>Cl- (mmol)</td>
<td>15</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Glutathione(mmol)</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>mOsmol/Kg</td>
<td>375</td>
<td>400</td>
<td>320</td>
</tr>
<tr>
<td>pH</td>
<td>7.25</td>
<td>7.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 4.1 Showing the important differences in the constituents of Euro-Collins (EC), Hypertonic citrate (HOC) and UW cold storage solutions.
The realisation that it was the effective cellular impermeants in the solution rather than its intracellular nature that were beneficial, and that the glucose in the Collins' solution was not an effective impermeant for the liver and pancreas, led to the development of other solutions containing impermeant anions such as lactobionate (mol wt 358, 100mmol), an impermeant saccharide, raffinose (mol wt 504, 30mmol) and a colloid (hydroxyethyl starch, 0.2mmol). The resulting UW solution (Table 4.1) was also shown to improve the results of renal transplantation and effectively prolong storage times for the pancreas and liver in comparison with Euro-Collins, although storage times greater than 12 hours remain a risk factor for graft function.

While successful preservation of the whole pancreas has now been achieved for up to 72 hours and preservation times of at least 24 hours shown to have no effect on the outcome of whole pancreas transplants, the requirements for cold storage prior to islet transplantation may be very different and appear particularly demanding for several reasons.

Firstly, the islets must not only be viable at the end of the period of cold storage, but must have sufficient metabolic reserve to survive the subsequent injurious effects of the isolation process. Secondly, the integrity of the ductal system used for collagenase delivery appears to deteriorate rapidly during cold storage preventing effective pancreas distension and impairing the digestion process. Thirdly, with islet purification relying on density dependent separation of islets from exocrine tissue, hypothermic cell swelling decreases the density of cells towards that of water, and has a deleterious effect on islet purification.

Despite some recent encouraging reports, periods of cold storage have been shown to result in islet degranulation, and a rapid loss of islet viability. Although insulin release in vitro and in vivo is better preserved in islets from UW stored pancreases than with Euro-Collins, even those stored in UW respond abnormally.

Cold storage of islets or digest following isolation and before transplantation has also been shown to cause a deterioration in function, preserving islet viability for only 24 hours using tissue culture medium, 48 hours with cold storage solutions based on colloid and mannitol, and slightly longer using UW.

The use of UW as the cold storage solution for in situ flush has been shown to increase islet yields compared with Euro-Collins over 24 hours cold storage, but, regardless of the solution used, cold storage of the pancreas for times as short as 3 hours in rodents, large animal models and humans has been shown to adversely affect pancreatic digestion and reduce islet yields.

The likely explanation for this is the proven deterioration in the pancreatic ductal system during cold storage, the effect of which appears to be minimised by early ductal...
distension. Immediate intraductal distension of the pancreas prior to the period of cold storage with collagenase dissolved in UW, returned islet yields towards those from immediately processed controls in all three studies. However, as the use of UW both for intraductal distension and for short term storage has been shown to decrease islet yields, the ideal solution for intraductal distension remains undetermined. The adverse effect seen with UW may be the result of direct toxicity to the islets themselves, with exposure to high potassium concentrations during digestion at 37°C, or an effect of UW's components or lack of them on collagenase digestion, with the addition of ionic calcium increasing islet yields to control values.

c) Density dependent purification

Density dependent purification is also adversely affected by cold storage, even in UW, and deteriorates even further with the use of Euro-Collins. Storage of the pancreatic digest at 4°C in UW compared with tissue culture medium has been shown to increase the density of islets and acinar tissue, resulting in a marked improvement in the yield of islets following purification.

iv) Aims of study

Comparisons between Marshall's citrate based solutions and UW are uncommon, but they have been shown to give similar results in terms of microvascular injury and the outcome of both liver and kidney transplantation. The effect of in situ vascular perfusion with HOC on islet purification has never been examined, indeed there has never been a randomised trial examining the effect of vascular perfusate on islet isolation in the human at all. In view of the proven benefits of UW in many aspects of the process, the continued use of HOC rather than UW needed to be justified.

This Chapter describes a study examining the effect of systemic vascular perfusion with either UW or HOC during organ donation, the effect of no vascular perfusion, was impossible to study, as the pancreases were usually retrieved at the end of a multi-organ donation. Seven pancreases were randomised to perfusion with HOC and 7 with UW. After uniformly short periods of cold ischaemia (≤5 hours), the effect on collagenase digestion and subsequent islet purification and viability was examined. The effect of longer periods of cold ischaemia, while they might have exaggerated differences, are invariably associated with lower yields and are avoided in practice by all groups. Since they are never needed in our unit, studying their effect was felt to be less relevant.

4.2 METHODS

With appropriate consent, pancreases were removed from 14 brain-dead heart-beating organ donors. During the process of organ donation the solution used to perfuse the systemic circulation was randomised by opening sealed envelopes, to UW in 7 cases and HOC in the other 7, these were consecutive apart from 4 where vascular perfusion had been completed by another transplant team prior to randomisation, which were therefore excluded from the
study. In 12 of the 14 cases the pancreas was removed as part of a multi-organ donation, in these cases the liver was perfused with UW via the portal vein, but the cannula was tied beyond the splenic vein which was vented during perfusion preventing reflux of UW and venous hypertension affecting the pancreas.

After a cold ischaemic time of less than 6 hours, each pancreas was weighed and then distended intraductally with collagenase, the same batch being used throughout (Number 03092C, Serva, Feinbiochemica Gmbh & Co, D-6900 Heidelberg 1, Federal Republic of Germany). The whole pancreas was transected and in each case both the head and tail were distended with 2 ml of collagenase dissolved in HBSS/g of pancreas (Figure 3.1, p84). The entire pancreas was digested using an automated method with minor modifications (see Chapter 3.2 ii, p83), and the time taken during phase 1 of the digestion process was recorded, as an indicator of the rate of digestion. The digest was collected at 22°C into MEM, pooled, and then suspended in 200 ml of UW at 4°C. From this, four 50µl samples were taken, diluted and stained with dithizone for assessment (Chapter 3.2 iv, p87) of the total number of islets in the digest, the cleavage percentage, the mean volume of an islet, the total volume of islet tissue and the number of 150µm equivalent islets/g of pancreas. These figures were used to accurately compare the results of digestion.

Initially the concentration of the collagenase used for the digestion was 3 mg/ml, however preliminary studies on 2 pancreata perfused with UW suggested the digestion to be less effective, taking 1.5 times as long as in the usual HOC perfused organs.

Further studies were performed to study the effect of UW and its components on collagenase digestion of the human pancreas. Pieces (0.5 cm³) of 7 HOC perfused pancreases were placed in 4 mg/ml of collagenase dissolved in UW, HBSS or HBSS containing one of the individual UW components, lactobionate (100 mmol), glutathione (3 mmol) or raffinose (30 mmol). The test tube was then shaken every 5 minutes, and the time taken for visible digestion to occur was recorded.

The digestion time for the whole pancreas returned to normal when the concentration of collagenase in the HBSS was increased from 3 to 4 mg/ml for UW perfused organs, and the higher concentration was therefore used for the UW perfused organs.

100 µl of the digest was centrifuged on mini-continuous density gradients of 500 mOsm/KgH₂O BSA, varying linearly in absolute density from 1.076-1.106 g/cm³. Sequential 1 ml fractions of these gradients were then assayed after sonication, for insulin and amylase content, and the results used to determine the median exocrine and islet densities, and the percentage of exocrine tissue contaminating a 60% yield of islets (Chapter 3.5 iii, p96).

The in vitro viability of islets from five pancreata in each group was examined following purification, using perfusions (Chapter 3.2 v, p88).

Differences in the effect of collagenase within the same pancreas were examined using the Wilcoxon matched-pairs signed-rank test, reported as Wilcoxon statistic T, with p value. The effects of HOC or UW perfusion on all the examined parameters were compared using the Mann-Whitney U test. Reported as a W score with its significance (adjusted for
ties) as a p value, where appropriate the 95% confidence intervals for the difference between HOC and UW are also reported (95%CI). Possible links between factors were examined using Pearson’s product-moment correlation coefficient (r).

### 4.3 RESULTS

The two groups were well matched (Table 4.2), with no significant differences in donor age, the length of cold ischaemia which varied from 0.5 to 6 hours (median 2.7), or the weight of the pancreases which ranged from 50-133g (median 88g).

Looking at all 14 pancreases as one group, a positive correlation existed between increasing donor age and both islet cleavage (r=0.753, p=0.002) and the duration of phase 1 (r=0.595, p=0.025). There was also a significant positive correlation between increasing organ weight and the time taken for digestion (r=0.748, p=0.002).

<table>
<thead>
<tr>
<th></th>
<th>HOC PERFUSED</th>
<th>UW PERFUSED</th>
<th>SIGNIFICANCE OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor age</strong> (years)</td>
<td>16,18,30,37,</td>
<td>13,24,24,37,</td>
<td>W=53.5</td>
</tr>
<tr>
<td></td>
<td>38,46,56.</td>
<td>45,45,50.</td>
<td>p=0.95</td>
</tr>
<tr>
<td><strong>Period of cold ischaemia (hrs)</strong></td>
<td>0.75,2.0,2.0,2.5,</td>
<td>0.5,0.75,1.5,3.5,</td>
<td>W=57.5</td>
</tr>
<tr>
<td></td>
<td>3.0,5.0,6.0.</td>
<td>3.0,3.75,4.0.</td>
<td>p=0.56</td>
</tr>
<tr>
<td><strong>Weight of pancreas (g)</strong></td>
<td>50,63,84,92,</td>
<td>68,70,75,83,</td>
<td>W=54.5</td>
</tr>
<tr>
<td></td>
<td>100,121,122.</td>
<td>92,94,133.</td>
<td>p=0.85</td>
</tr>
</tbody>
</table>

*Table 4.2* Comparing donor age, cold ischaemic time and pancreas weight in pancreases randomised to the two groups (showing individual values and highlighting the median)

i) **Effect on pancreas digestion**

The addition of glutathione or lactobionate to HBSS resulted in significantly prolonged periods of digestion with a median time taken of 55 minutes compared to the median of 40 minutes taken for visible pancreatic digestion to occur in the collagenase dissolved in HBSS alone (T=28.0, p=0.022 for glutathione and T=26.5, p=0.043 for lactobionate), the addition of raffinose had no significant effect (T=15.0, p=0.06). Dissolving collagenase in UW completely inhibited the action of collagenase which failed to produce any digestion after 100 minutes, and this was significantly different (T=28.0, p=0.022) from any of the other groups.
The time taken to digest pancreases perfused with UW at a collagenase concentration of 3 mg/ml increased to 26.5 minutes, compared to a normal time of 17.5 minutes for HOC perfused glands. Since this represented a period of warm ischaemia for the islets, such an increase would have prevented comparison of the two groups and was avoided by increasing the collagenase concentration for UW perfused pancreases to 4mg/ml.

The time taken for digestion of the pancreases using either 3mg/ml collagenase for HOC perfused pancreata or 4 mg/ml for UW perfused organs, ranged from 13 to 22 minutes (that for HOC perfused organs being slightly but non-significantly longer, see Table 4.3).

Following digestion, the numbers of 150μm islets in the digest expressed both as total numbers and per gram of original pancreas, are shown in Table 4.3, for comparison between the two groups, while Table 4.4 shows the results for each pancreas. The mean volume of an islet is also shown, with the percentage of islets totally cleaved from exocrine tissue in the two groups. There were no significant differences between the groups with respect to any of these variables.

<table>
<thead>
<tr>
<th></th>
<th>HOC PERFUSED</th>
<th>UW PERFUSED</th>
<th>SIGNIFICANCE OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion time (mins)</td>
<td>15,15,18,21,</td>
<td>13,14,15,18,</td>
<td>W=61.0</td>
</tr>
<tr>
<td></td>
<td>22,22,22.</td>
<td>18,19,22.</td>
<td>p=0.30</td>
</tr>
<tr>
<td>Total number of 150μm islets in the</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>digestive process</td>
<td>73805,266338,</td>
<td>163478,253770,</td>
<td>W=58.0</td>
</tr>
<tr>
<td></td>
<td>478618,544876,</td>
<td>315723,364475,</td>
<td>p=0.53</td>
</tr>
<tr>
<td></td>
<td>640910,700177,</td>
<td>475389,569485,</td>
<td>95%CI -401,584 to +384,449</td>
</tr>
<tr>
<td></td>
<td>718652.</td>
<td>1268467.</td>
<td></td>
</tr>
<tr>
<td>Number of 150μm islets/g of pancreas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>604,4545,5786,</td>
<td>2335,2373,2738,</td>
<td>W=59.0</td>
</tr>
<tr>
<td></td>
<td>4791,7208,</td>
<td>3969,5057,</td>
<td>p=0.44</td>
</tr>
<tr>
<td></td>
<td>7629,9572.</td>
<td>7933,1863.</td>
<td>95%CI -4453 to +4834</td>
</tr>
<tr>
<td>Mean volume of an islet mm³x10⁻³</td>
<td>0.70,0.81,0.81,</td>
<td>0.93,1.14,1.40,</td>
<td>W=50.0</td>
</tr>
<tr>
<td></td>
<td>1.53,1.70,2.70,</td>
<td>1.53,1.70,1.94,</td>
<td>p=0.80</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>2.09</td>
<td>95%CI -0.89 to +1.17</td>
</tr>
<tr>
<td>Percentage of totally cleaved islets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52,74,80,84,</td>
<td>33,63,74,87,</td>
<td>W=48.5</td>
</tr>
<tr>
<td></td>
<td>84,86,90.</td>
<td>89,93,96.</td>
<td>p=0.65</td>
</tr>
<tr>
<td></td>
<td>95%CI -15 to +21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 Comparing the results of digestion on pancreases in both groups.
<table>
<thead>
<tr>
<th>Pancreas No</th>
<th>perfusate</th>
<th>liver removed</th>
<th>weight</th>
<th>Donor age</th>
<th>cold ischaemia</th>
<th>Durat of phase 1</th>
<th>Collag-erase Conc</th>
<th>% Cleavage</th>
<th>Total no 150um(eq islet/g pancreas)</th>
<th>150um eq islet/g pancreas</th>
<th>Mean volume of islet density</th>
<th>Median exocrine density</th>
<th>% exocrine contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>190</td>
<td>HOC</td>
<td>no</td>
<td>84.0 g</td>
<td>37 yrs</td>
<td>2.0 hrs</td>
<td>21.0</td>
<td>3.0</td>
<td>74.0</td>
<td>640910</td>
<td>7620.9</td>
<td>0.030810</td>
<td>1.0880</td>
<td>1.0952</td>
</tr>
<tr>
<td>191</td>
<td>UW</td>
<td>yes</td>
<td>92.0</td>
<td>24</td>
<td>1.50</td>
<td>18.0</td>
<td>4.0</td>
<td>63.0</td>
<td>253770</td>
<td>2758.4</td>
<td>0.031140</td>
<td>1.0974</td>
<td>1.0975</td>
</tr>
<tr>
<td>192</td>
<td>HOC</td>
<td>yes</td>
<td>50.0</td>
<td>18</td>
<td>3.00</td>
<td>18.0</td>
<td>2.5</td>
<td>82.5</td>
<td>478618</td>
<td>9572.4</td>
<td>0.001530</td>
<td>1.0974</td>
<td>1.0975</td>
</tr>
<tr>
<td>194</td>
<td>UW</td>
<td>yes</td>
<td>67.7</td>
<td>37</td>
<td>2.50</td>
<td>14.0</td>
<td>4.0</td>
<td>93.0</td>
<td>1208467</td>
<td>18633.9</td>
<td>0.002050</td>
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<td>38</td>
<td>6.00</td>
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<td>3.0</td>
<td>90.0</td>
<td>700177</td>
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<td>0.003597</td>
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<td>75.0</td>
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<td>87.0</td>
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<td>63.0</td>
<td>30</td>
<td>2.50</td>
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<td>84.0</td>
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<td>200</td>
<td>HOC</td>
<td>no</td>
<td>99.7</td>
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<td>0.75</td>
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<td>86.0</td>
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<td>5057.3</td>
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*Table 4.4* Showing the details for each pancreas consecutively
ii) **Effect on islet purification**

Analysis of the results of continuous gradient centrifugation of this digest, was used to assess the effect of vascular perfusate on islet purification. The median densities of neither the islets nor exocrine tissue was significantly different between the two groups; not surprisingly therefore the percentage of exocrine tissue contaminating a 60% islet yield was also similar (Table 4.5).

<table>
<thead>
<tr>
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<th>UW PERFUSED</th>
<th>SIGNIFICANCE OF DIFFERENCE</th>
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<tr>
<td>Median islet densities g/cm³</td>
<td>1.0890, 1.0904,</td>
<td>1.0878, 1.0890,</td>
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<td>1.0925, 1.0941,</td>
<td>1.0893, 1.0904,</td>
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<tr>
<td></td>
<td>1.0947, 1.0974,</td>
<td>1.0912, 1.0965,</td>
<td>95% CI -0.0027 to +0.0069</td>
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<tr>
<td></td>
<td>1.0977</td>
<td>1.0974</td>
<td></td>
</tr>
<tr>
<td>Median exocrine densities g/cm³</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>1.0975, 1.0986,</td>
<td>1.0975, 1.0989,</td>
<td>95% CI -0.0023 to</td>
</tr>
<tr>
<td></td>
<td>1.1017</td>
<td>1.0999</td>
<td>+0.0035</td>
</tr>
<tr>
<td>% Exocrine contamination of 60% islet yield</td>
<td>7, 24, 24, 26, 28, 32, 47, 46, 60</td>
<td>1, 2, 3, 22, 41, 46, 60</td>
<td>W=56.5</td>
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<td></td>
<td>2, 3, 22, 41,</td>
<td>46, 60</td>
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<td></td>
<td>28, 32, 47</td>
<td></td>
<td>95% CI -23.00 to +25.01</td>
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</table>

*Table 4.5* Comparing the results of islet isolation in the two groups.

iii) **Effect on islet viability**

In-vitro assessment of islet viability in the two groups showed no significant differences either, with islets from all the pancreases demonstrating good biphasic insulin release in response to a glucose challenge (*Figure 4.4*). The median (range) stimulation index of islets in the HOC group was 2.9 (2.1-3.8), compared with 2.6 (1.9-4.3) in the UW perfused pancreases (W= 27.0, p=1.000).
Comparison of Biphasic Insulin Release From an HOC and UW Perfused Pancreas During Perifusions

![Graph showing comparison of insulin release from HOC and UW perfused pancreas.](image)

**Figure 4.4** Showing the phasic pattern of insulin release from islets in both groups during perifusion.

4.4 DISCUSSION

i) Effect of donor age on digestion

The significant effect of increasing age in prolonging the time taken to free islets from the pancreas and improving islet cleavage during digestion although not previously documented is not unexpected. Compared with the rodent, the effectiveness of collagenase in catalysing the hydrolytic cleavage of undenatured collagen and disrupting the collagen framework of the pancreas to release clean islets, has proven particularly critical in large animals such as the pig, cow and human, due to the increased percentage of collagen tissue, mainly localised in the interlobular and intralobular septa of the pancreas. Age is known to be an additional factor, with the overall amount and cross-linking of collagen in the normal human pancreas increasing with age and the fragility of islets from paediatric donors decreasing, as the framework within the islets becomes more defined. The fact that this effect continues beyond the paediatric age range (13-56 years in this study), presumably represents a continuity in change in the collagen matrix over this time.

ii) Effect of UW on digestion

UW appeared to contain one or more inhibitors of collagenase, and its use as the perfusate increased by 33% the amount of collagenase enzyme needed to maintain pancreatic digestion. Overall a median cleavage index of 84% was achieved. The similarity between the two groups in this respect, together with the similar time taken for digestion, showed that the
inhibition of collagenase by UW was not absolute and could be overcome by increasing the collagenase concentration.

The adverse effect of UW on the action of collagenase has been hinted at in previous studies 1060,1061 but no explanation has been attempted. One possibility is the inclusion of the reduced form of glutathione (GSH), which is known to be a potent inhibitor of collagenase 180-182. This is included in UW in an attempt to protect cold-injured cells from oxidative stress during reperfusion, and maintain the integrity of sulphydryl intracellular enzymes and membrane proteins, and it has been shown to be of benefit in prolonged periods (>20 hours) of cold storage 1073. GSH exists in its reduced form for a maximum of only 3 weeks, before conversion to its major oxidation product (GSSG) 1074. This is considerably shorter than the shelf life of UW and by the time UW is used, most of the GSH will have been oxidised. While GSSG may be as effective as GSH in improving cells tolerance to injury 1075, the inhibition of collagenase relies on reduced -SH groups, and would therefore be expected to decrease with time if the GSH was solely responsible. This fact, coupled with the demonstration in this study that the addition of GSH to HBSS produces significantly less inhibition of collagenase than UW, suggests that other components are also important.

Other possibilities are the presence of magnesium, which in concentrations as low as 0.01mM have been shown to have an inhibitory effect on collagenase as great as that of glutathione 180, and the absence of calcium ions. Calcium has been shown to be necessary for the catalytic activity of collagenase 181 and its essential nature is noted by both Serva and Boehringer Mannheim in their Manufacturer's Information sheets. Although the presence of Ca^{2+} has not always proven necessary 142,185, the addition of Ca^{2+} ions to UW has been shown to reverse its inhibitory effect on collagenase 1062 and appears to have an optimal concentration, beyond which its effect becomes inhibitory 168. This may be due to the role of calcium in maintaining the intercellular matrix 183,1076 and could account for the conflicting reports on its potentiation of collagenase. It is also possible that the inhibition of collagenase by UW, like the action of many collagenases, is species dependent and seen to a significant degree only in the human, which would also contribute to conflicting reports using various animal models. The fact that HOC is very similar, in containing Mg^{2+} and not Ca^{2+}, make the contribution of these ions like that of glutathione and lactobionate, unlikely to be the entire answer, but a synergistic effect of all of these factors could explain the effect of UW on collagenase dependent digestion.

Whatever the cause, the need for a greater concentration of collagenase to digest the UW perfused pancreas may reverse any potential benefit of UW over short periods of cold ischaemia by increasing tissue trauma during the digestion phase, and adds weight to the arguments in favour of the continued use of HOC.
Comparison of the theoretical and practical advantages of HOC with UW

Based largely on comparisons with Euro-Collins, over the last 5 years UW has become one of the most widely used vascular perfusates in multi-organ donations worldwide. There is no good evidence supporting its use when compared with HOC however, which in many respects appears very similar.

Anions

Both UW and HOC contain anions of carbohydrate based carboxylic acids (citrate or lactobionate). The inclusion of a large and effectively impermeant cation has been repeatedly shown to be a crucial element of cold storage solutions. Studies comparing lactobionate (mol wt 358) with more permeable agents such as gluconate (mol wt 195), or chloride (mol wt 35), have shown it to reduce cell swelling in the pancreas \(^{121}\) and improve the results of rat liver transplants \(^{1077, 1078}\). It seems likely that it would also be more effective than citrate (mol wt 189).

Cations

Early work suggested that changes in the \(\text{Na}^+:\text{K}^+\) ratio, which in UW is 12:88 compared with 50:50 in HOC might have an effect on organ preservation \(^{1038, 1079}\). A substantial proportion of cellular energy production was known to be utilised by the \(\text{Na}^+\) membrane pump, and decreasing extracellular \(\text{Na}^+\) produced a proportional decrease in cellular respiration at 30°C \(^{119}\). Moreover in hypothermic conditions the cells were shown to lose \(\text{K}^+\) ions \(^{119, 1079, 1080}\) while gaining \(\text{Na}^+\) and \(\text{Cl}^-\) \(^{1040, 1081, 1082}\). Although the significance of these findings for organ preservation were not known, they formed the basis for the development of the \(\text{K}^+\) rich Collins solution \(^{123}\).

Since then Southard and Belzer have shown that varying \(\text{Na}^+:\text{K}^+\) ratios had no effect on hypothermic swelling of kidney slices \(^{119}\), a finding confirmed in the pancreas by Wahlberg \(^{121}\), and in studies on erythrocytes, which demonstrated that their density remained unchanged with differing ratios \(^{212}\). Studies examining the effect of reversing the cation ratio on kidney, liver and pancreas preservation in the rat \(^{1083, 1084}\), rabbit \(^{1078}\), dog \(^{125}\) and man \(^{1085-1087}\) have shown that a high sodium solution results in either unchanged or improved performance.

Carbohydrate and colloidal impermeants

Glucose, the main component in Euro-Collins, acts as an impermeant in the kidney but not in the liver, which is freely permeable to small carbohydrates such as glucose and mannnitol, with only larger sugars such as maltose, sucrose and raffinose being restricted to the extracellular fluid. Both solutions include sugar molecules, but when used at 30mmol raffinose has been shown to be more effective at reducing hypothermic cell swelling in the pancreas than mannitol \(^{121}\), although increasing the concentration of mannitol to 195mmol more than counteracts this in the kidney \(^{119}\). The effect of an impermeant sugar molecule on liver and pancreas preservation may have been over-estimated in the presence of an
effectively impermeant anion however 1077,1084, and the raffinose in UW may actually cause shrinkage of cells and impair organ function 1088.

The inclusion of the colloid HES in UW has been one of its most contentious components. Its inclusion was based on its effectiveness in perfusion solutions 1089 on which UW was modelled, and designed to prevent leakage of fluid under hydrostatic pressure into the interstitial space during in situ perfusion, preventing collapse of the vascular network and improving washout of blood cells. This expansion of extracellular fluid has been suggested to occur during storage as well, and to be prevented by colloids in solutions which allow hypothermic cell swelling 1090 and also in UW 132. However earlier work had suggested that the extracellular water remains a constant fraction of the weight of tissue slices, whether hydration is normal or increased 1040,1091, and it seems likely that the colloid contributes not only to the reduction of extracellular fluid but also to the reduction in the isosmotic intracellular expansion. In solutions which already effectively prevent this, their effect would therefore be minimal. This is supported by studies showing that its removal from UW simplifies the solution with no effect on liver or pancreas transplantation in the rat 1066,1083,1084,1092, rabbit 1078, pig 1093 or dog 1048.

Taking these known parameters of a successful cold storage solution into account, it had seemed likely that HOC, which uses citrate rather than lactobionate, mannitol rather than raffinose and contains no colloid, was likely to be less effective as a preservation solution over prolonged periods of cold storage than UW. The hypertonicity of HOC which might be expected to counteract cell swelling, may actually increase it, if all the molecules involved are actually permeable to the hypothermic cell, since the isosmotic point will be reached at a higher concentration of intracellular ions accompanied by more water and swelling. Since most of the studies examining the effect of UW on transplantation demonstrate benefits after periods of 24 hours or more, it is perhaps not surprising that UW had no discernible benefit on islet viability over the short periods of cold ischaemia of <6 hours involved in this study, and it seems likely that most of the potential benefits in this respect would be seen over periods in excess of 12 hours, which were not examined.

It is more surprising however that no effect on islet and exocrine density, or islet purification was seen. Hypothermic cell swelling has been shown to occur very rapidly 1040,1082, and even brief exposure of potentially swollen pancreatic digest to UW has been shown by van der Burg 195,1064 to produce a beneficial increase in tissue density. It may be that the brief exposure of the digest in this study to UW following digestion and before density gradient analysis, eliminated any existing differences. If this was the case it does not detract from the use of HOC since any effect must be rapidly and fully reversible.
iv) **Comparison of cost**

While the use of UW has been shown to be cost effective in renal transplantation by decreasing the rate of primary non-function \(^{1094}\), it increases the cost of islet purification considerably (*Table 4.6*).

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<th>HOC PERFUSION</th>
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<td>COST OF 4 LITRE PERFUSION</td>
<td>£40</td>
<td>£464</td>
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<tr>
<td>COST OF COLLAGENASE</td>
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<td>TOTAL COST OF ISLET PURIFICATION</td>
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*Table 4.6* showing the effect of using UW for the in-situ vascular flush on the overall cost of islet purification.

To justify its use, a considerable benefit would have to be shown in terms of islet yield, purity or viability. Although statistically there was no demonstrable difference between the two groups, the 95% CIs clearly include a range of possibly important differences, suggesting the possibility of a type II statistical error. However, in each case these differences range equally about 0, and in view of the accepted variability between pancreases, it seems unlikely that greater numbers would have produced a more categorical advantage one way or the other.

While in theory over periods of cold ischaemia greater than 12 hours UW might be expected to improve islet viability, in practice, cold ischaemic times of this magnitude have been found to prevent the reliable isolation of large numbers of islets, even with storage in UW, and are therefore avoided. Since perfusion with UW in situ is expensive, does not improve islet purification and has an inhibitory effect on the digestion process, the continued use of HOC seems logical until islet isolation following prolonged storage times is improved by other means.
CHAPTER 5
THE EFFECT ON ISLET PURIFICATION OF STORING HUMAN PANCREATIC DIGEST IN COLD STORAGE SOLUTIONS BASED ON THE UNIVERSITY OF WISCONSIN SOLUTION

5.1 INTRODUCTION

5.2 AIMS

5.3 METHODS
i) Comparison of UW and MEM
ii) Examination of the effective components in UW
iii) Statistical analysis

5.4 RESULTS
i) Comparison of UW and MEM
   a) Effect on changes in density of exocrine tissue
   b) Effect on changes in density of islets
   c) Effect on islet purity
   d) Comparison of HOC and UW perfused pancreases
   e) Summary
ii) Examination of the effective components in UW
   a) Effect on changes in density of exocrine tissue
   b) Effect on changes in density of islets
   c) Effect on islet purity
   d) Summary

5.5 DISCUSSION
i) Density as a measurement of cellular swelling
ii) Comparison of cold storage in UW or MEM
iii) The effect of adding cellular impermeants and colloid to MEM

5.6 CONCLUSION
119

INTRODUCTION

The preceding chapter reviewed the rationale behind hypothermic preservation of organs during transplantation and the development of solutions specifically designed for this purpose. In islet transplantation not only is the whole pancreas stored at 4°C preceding collagenase digestion, but the digest produced by digestion at 37°C, has routinely been cooled again to 4°C and stored for brief periods before islet isolation.

Cell storage may be subtly different from whole organ storage due to the lack of a vascular network, but the same principles governing cell swelling, should apply to this period of cold storage. Very little attention however has been paid to this potentially crucial stage of the isolation procedure, and most groups, when they have described this phase at all, have stored their digest in tissue culture medium 184,272,298,300,302, ignoring the fact that such media have been documented to cause immediate cell swelling in whole organs 122,1038, tissue slices 119,121,122,1039,1040,1081,1082,1095,1096 and single cells 1020, due to the isosmotic influx of saline during the hypothermic inactivation of the Na⁺/K⁺ ATPase dependent pump.

Although it has never been scientifically documented, the resulting changes in cell density have been thought to have a deleterious effect on islet purification, and separation of islets from the digest has therefore always been performed as rapidly as possible, in order to minimise this. In 1989 in studies in the dog, van der Burg reported the increase in tissue density which occurred if the digest was placed in UW rather than RPMI before density dependent purification 1064, and the dramatic improvement in purification which resulted from this 195. This study suggested that the principles of cold storage of whole organs, could be applied to the storage of pancreatic digest at 4°C, with benefit to subsequent density dependent islet purification.

While cold storage solutions were designed to prevent cell swelling, it has been suggested that UW goes further, and results in shrinkage of tissues 1088,1097. A period of cold storage in UW may therefore not only maintain the intrinsic difference in density between islets and exocrine fragments, but actually produce an increase in density of the tissues per se. The concept that this increase in density might result in an overall improvement in purification, rather than just preventing a deterioration has not been examined at all, but a differential response of the islets and exocrine fragments to cold storage is implied in the results of van der Burg’s studies.

AIMS

This study aimed to compare the effect on islet and exocrine densities and resulting purification, of washing human pancreatic digest at 4°C in either tissue culture medium (MEM) or UW. It also examined whether the immediate effect of this brief period of cold storage could be maintained, or even increased over a further period of 1 hour’s cold storage, and whether the perfusate (UW or HOC) used for the in situ perfusion and storage of the whole organ prior to digestion had any effect.
The second part of the study aimed to determine the components in UW responsible for the results produced in the first part, by adding various components of UW to MEM. Three solutions were studied in addition to MEM and UW which were included as 'controls'. These solutions were designed to examine the effect of increasing the proportion of impermeant molecules (such as lactobionate and raffinose) in the MEM, (MEM+Imp). The composition of MEM+Imp was designed to produce a measurable effect on tissue swelling (based on work from The University of Wisconsin 119,121,126), but not to inhibit swelling completely, so that the importance of adding a colloid to the solution could also be examined (MEM+HES and MEM+Imp+HES).

While the pH of these solutions was controlled, the osmolality varied, allowing the effect of hyperosmolar solutions to be examined.

### 5.3 METHODS

#### 1) Comparison of UW and MEM

A total of 23 pancreases, removed with appropriate consent from brain-dead heart-beating organ donors were studied, with 7 being randomly allocated to in situ vascular perfusion with UW and the rest to HOC.

Following collagenase digestion of the pancreas at 37°C in a circuit containing MEM (Chapter 3.2 ii), the resulting digest was collected into MEM containing 10% NCS to inhibit the further action of collagenase. Five hundred μl of digest was then washed twice at 4°C in cold MEM (by centrifugation at 200g for 2 minutes), and another 500 μl was washed twice in cold UW. Following the second centrifugation, 100 μl of tissue from each solution, was immediately suspended in 1 ml of 1.105g/cm³ high density BSA (500mOsm/kgH₂O), in the bottom of a test tube and overlaid with a continuous gradient of BSA from a gradient maker containing 500mOsm BSA with a density of 1.075g/cm³ in one chamber and 1.105g/cm³ in the other (Chapter 3.5). The two synchronously produced linear continuous density gradients one for MEM and one for UW washed digest, were centrifuged at 22°C at 800g for 5 minutes, before analysis of the density distribution of islets and exocrine tissue and the % of exocrine tissue contaminating a 60% islet yield (Chapter 3.5 iii & iv).

The remaining 400μl of digest was resuspended following the second wash in 10ml of the relevant media (either MEM or UW) and stored for an hour at 4°C before 2 further gradients were run synchronously to examine any change in tissue density and islet purity which had occurred.

In 10 pancreases the effect of storage for 2 hours in UW was examined in the same way.

The effect on digest volume during storage in UW was examined by storing the whole of the pancreatic digest (mean volume 40.6 ml) in UW in a 200ml graduated flask. After centrifugation for 2 minutes at 4°C at 200g, the volume at the initiation of storage was measured. The digest was resuspended in the UW and before purification, after a mean
storage time of 30 minutes, the volume was again measured after centrifugation in the same conditions.

### ii) Examination of the effective components in UW

<table>
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<tr>
<th>Characteristic</th>
<th>MEM</th>
<th>MEM+Imp</th>
<th>MEM+Imp</th>
<th>MEM+Imp</th>
<th>UW</th>
</tr>
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<tr>
<td>No (of 23) in each group</td>
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<td>9</td>
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Table 5.1 Showing the differences in components of the 5 solutions studied.
The effect of adding impermeant ions, or a colloid, or both to MEM in the same concentrations used in UW, was examined in pancreases all perfused in situ with HOC. Using a series of five gradient markers and a multi-channel pump, the effect on pancreatic digest of washing and 1 hour's storage in five different solutions was analysed, using synchronously produced gradients, in the same way that the effect of UW and MEM had been compared.

The five solutions were MEM, MEM with the addition of raffinose, lactobionate and glutathione (MEM+Imp), MEM with the addition of hydroxyethyl starch (MEM+HES), MEM with both the impermeants and HES added (MEM+Imp+HES) and UW (Table 5.1).

The solutions were produced by the addition to MEM of 35.83g/l of lactobionic acid (D7924, Aldrich Chemie Steinheim, Germany, form wt 358.3), 17.83g/l of raffinose pentahydrate (R7630, Sigma, form wt 594.5), 0.922g/l of glutathione (G6013, Sigma, anhydrous wt 307.3) and 50g/l of HES (HES pentafraction, Dupont Critical Care, mol wt 250,000). As in UW, the pH at 4°C was corrected to between 7.35-7.45 by the addition of 10M NaOH using a pH electrode (PHM110-010X, Gallenkamp). The osmolalities of the resulting solutions were determined using a vapour pressure osmometer.

The gradients from each pancreas acted as their own controls. The changes in the median islet and exocrine densities which occurred with storage in either UW or MEM, or in the second series of experiments in the modified MEM solutions, were compared using the Wilcoxon matched-pairs Signed-rank test (reported as Wilcoxon statistics (T) with p values), with the Wilcoxon signed-rank based 95% confidence interval for the differences (95% CI) being determined. Differences in the resulting purity were examined using the same test, and differences between UW perfused and HOC perfused pancreases were determined using the Mann-Whitney U test (M-W U), reported as a W score with its significance (adjusted for ties) as a p value.

5.4 RESULTS
i) Comparison of UW and MEM
a) Effect on changes in density of exocrine tissue

Over the course of 1 hour's storage in UW (n=23), the density of exocrine tissue increased significantly (T=2.0, p<0.001), from a median of 1.098 (range 1.093-1.102)g/cm³ to a median of 1.099 (1.094-1.104)g/cm³. The 95% CI for this change showed an increase of between 0.0009 and 0.0025 g/cm³ (Figure 5.1). In the pancreases in which the digest was stored for a second hour in UW (n=10), this increase in density was maintained and in some pancreases increased even further, reaching a median of 1.101 (1.094-1.104), although this increase was not significant (T=4.0, p=0.208).
In MEM (n=21) on the other hand the density of exocrine tissue significantly decreased (T=153, p=0.021) from 1.096 (1.087-1.105) to 1.095 (1.079-1.100). The 95%CI for the change, being a negative one from -0.0002 to -0.0031 (Figure 5.1).

Comparing the density of exocrine tissue after just the brief exposure to either UW or MEM during the period of 5 minutes washing, it could be seen that these changes had already begun to occur, with the exocrine tissue washed in UW significantly denser than that washed in the MEM, (T=177.5, p<0.007, 95%CI = 0.001 to 0.004). After 1 hour, as would be expected from the differential effects of MEM and UW on exocrine densities, this difference had become even more significant (T=187, p<0.001, 95%CI = 0.003 to 0.007), indicating an ongoing process.

**b) Effect on changes in density of islets**

The effect on islet densities was very similar to that on exocrine tissue. Storage in UW for 1 hour significantly increased the median islet densities (T=40, p=0.005, 95%CI = 0.001 to 0.003), from 1.093 (1.088-1.100) to 1.095 (1.088-1.102)g/cm³ (Figure 5.2). Storage for a second hour did not result in a further increase (T=34, p=0.541).

Storage in MEM again had the opposite effect, causing a significant decrease (T=155.5, p=0.016, 95%CI = -0.000 to -0.003) in densities from a median of 1.091 (1.085-1.099) to 1.091 (1.085-1.097)g/cm³ (Figure 5.2).
Figure 5.2 Illustrating the 95% CIs for the change in islet densities which occurred with storage in either UW or MEM (including comparison between UW and HOC perfused pancreases - see below)

Again this effect was seen to occur rapidly during the washing, with islets washed in UW becoming significantly denser than those washed in MEM ($T=194, p=0.007, 95\%CI = 0.001$ to $0.004$), and the difference then increased further during the hour's storage period ($T=228, p<0.001, 95\%CI = 0.003$ to $0.006$).

c) Effect on islet purity

The percentages of exocrine tissue contaminating a 60% islet yield before and after storage, as a result of these changes in density, were examined. With storage in UW, as both the exocrine tissue and islets became denser, the purity did not change significantly ($T=144.5, p=0.322, 95\%CI +3.0$ to $-12.0$), but there was a tendency, reflected in the 95%CI for the % exocrine contamination to decrease (Figure 5.3).

With storage in MEM similarly there was no significant effect on islet purity ($T=65, p=0.140, 95\%CI = +17.5$ to $-3.0$), however in MEM the tendency was towards an increase in exocrine contamination (Figure 5.3).
Figure 5.3 Illustrating the 95% CIs for the changes which occurred with storage in either MEM or UW in the % of exocrine tissue contaminating a 60% islet yield.

There was no significant difference in purity as a result of just washing the digest in UW compared with MEM (T=88, p=0.794, 95% CI = 6.5 to -5.0). When the islet purity was compared after 1 hour’s storage however, the % of exocrine contamination was significantly greater after storage in MEM than after storage in UW (T=48, p=0.035, 95% CI = 19.5 to 0.5).

d) Comparison of HOC and UW perfused pancreases

When the pancreases which had been perfused in situ with UW were analysed as a separate group from those perfused with HOC, there were no significant differences between the two groups in the changes in density undergone by the exocrine tissue during washing and storage. On the whole however, they changed less if the pancreas had been perfused with UW, particularly during storage in UW (Figure 5.1), in which the exocrine densities only changed from a median of 1.098 (1.093-1.100) to 1.098 (1.094-1.103) g/cm³ (T=2.0, p=0.178), considerably less than the significant change (p=0.002) noted in exocrine densities in HOC perfused pancreases (W=164.5, p=0.070).

Similarly the changes in islet density during storage in MEM were insignificant in UW perfused pancreases (Figure 5.2). However, islets from UW perfused pancreases were noted after 5 minutes washing in UW, to be significantly less dense than those perfused with HOC (W=258, p=0.004). During storage in UW they were then noted to increase in density very significantly (T=0.0, p=0.022), unlike their exocrine counterparts.
In terms of purity (Figure 5.3), the fact that islets from UW perfused pancreases became denser during storage in UW while the exocrine tissue did not, meant that storage in UW had no beneficial effect on islet purity compared with storage in MEM in this group. However, when this group was removed from the analysis, the improvement in purification seen in pancreases perfused with HOC during storage in UW, compared with storage in MEM, became more significant (T=10.5, p=0.016), and purification after 1 hour’s storage in UW appeared better than before it (T=93.0, p=0.065).

e) Summary

The densities of both exocrine tissue and islets increased significantly during 1 hour’s storage in UW, while in MEM they became significantly less dense. This difference started to occur during the 5 minutes wash period, after which tissue washed in UW was significantly denser than that washed in MEM. These changes meant that islet purification was significantly better after storage in UW than in MEM (p=0.035).

If the pancreas was perfused with UW during the donor operation, the changes in exocrine density, particularly during storage in UW were considerably less than if HOC was used. The islets behaved differently and continued to become denser. When this group of pancreases was removed from the analysis, and only HOC perfused pancreases were compared, a period of storage in UW was not only significantly better than storage in MEM (p=0.016) preventing any deterioration, but actually appeared to produce better purification than processing the digest immediately (p=0.065)

ii) Examination of the effective components in UW

The effect of washing and 1 hour storage in either MEM, MEM+Imp, MEM+HES, MEM+Imp+HES or UW in all the 23 pancreases studied are shown for reference in Table 5.2.

a) Effect on changes in density of exocrine tissue

Over the period of 1 hour’s storage, exocrine tissue stored in MEM+Imp, like that stored in MEM alone, became less dense although this change just failed to reach significance (T=64, p=0.055, 95%CI = 0.0000 to -0.0026). Storage in MEM+HES prevented any change in density at all (T=21, p=0.726, 95%CI = +0.0012 to -0.0013), while storage in MEM+Imp+HES produced an increase in density similar, but not as significant, as that seen in UW (T=4, p=0.108, 95%CI = +0.0022 to -0.0002). The 95% CIs for the changes in exocrine density which occurred with storage are shown in Figure 5.4, p131.
Table 5.2 Giving the values calculated graphically for the exocrine and islet densities and the % of exocrine
tissue contaminating a 60% islet yield from continuous each gradient

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Figure 5.4  Showing the 95% CIs for the change in density of exocrine tissue stored for 1 hour in each of the 5 solutions

As with washing in UW and MEM these differences had already started to occur during the wash, and before the period of storage began, so that comparison of the densities of exocrine tissue after the combination of washing and storage (rather than just the changes during the hours storage shown in Figure 5.4) revealed even more significant differences between the groups (Table 5.3).

<table>
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<th>Density after 1 hour storage in</th>
<th>compared with density in</th>
<th>T</th>
<th>p</th>
<th>95%CI</th>
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<td>UW</td>
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<td>0.002</td>
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<td>0.009</td>
<td>-0.0024 to -0.0073</td>
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<td>0.014</td>
<td>-0.0026 to -0.0065</td>
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<td>-0.0024 to 0.0015</td>
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<td>0.014</td>
<td>0.0060 to 0.0015</td>
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<td>0.0</td>
<td>0.022</td>
<td>0.0065 to 0.0013</td>
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</table>

Table 5.3  The densities of exocrine tissue after washing and storage in the adapted MEMs compared with those from the same pancreas in UW or MEM
The addition of the impermeant molecules lactobionate, raffinose and glutathione present in UW to the MEM, resulted in a solution which produced changes similar to MEM alone, and significantly different from those occurring in UW. The addition of the colloid HES however, prevented any significant change in density during storage, unlike both the increase seen in UW or the decrease in MEM. Combining the impermeant molecules and HES together, began to produce the increase in density seen during storage in UW but the magnitude of this was significantly less.

b) Effect on changes in density of islets

The effect of the solutions on the densities of islets was very similar, with storage in the modified MEMs producing changes which were closer to those seen in MEM than UW (Figure 5.5). Storage in MEM+Imp produced a significant decrease in density as had been seen in MEM (T= 72, p=0.011, 95%CI = -0.0006 to -0.0023). In MEM+HES no significant change in islet density occurred (T=33.5, p=0.214, 95%CI = 0.0003 to -0.0021) although the trend was still towards a decrease. In MEM+Imp+HES no change in density occurred (T=19, p=0.944, 95%CI = 0.0014 to -0.0018).

**Figure 5.5** Showing the 95% CIs for the change in density of islets stored for 1 hour in each of the 5 solutions

The combination of washing and storage in the modified MEMs resulted in islets which were significantly less dense than those washed and stored in UW, but tended to be denser than those washed and stored in MEM alone (Table 5.4).
Table 5.4 The densities of islets after washing and storage in the adapted MEMs compared with those from the same pancreas in UW or MEM.

c) Effect on islet purity

In all the solutions containing HES, the increase in density of the exocrine tissue was more significant than that of islets, and this meant that these solutions tended to improve purity, while solutions which contained impermeant molecules only, without a colloid, tended to increase the exocrine contamination (Figure 5.6).

Graph Showing the Change in Exocrine Contamination With Storage

Figure 5.6 Showing the 95% CIs for the changes in % exocrine contamination of a 60% islet yield which occurred during storage in the 5 solutions.
Once again the fact that these changes started to occur during the washing period, meant that analysis of the changes during the 1 hour storage only, tended to minimise the overall effects, but Figure 5.6 demonstrates convincingly that the improvement in purification which occurs during storage in UW, appears to be the result of inclusion of a colloid and not the impermeant molecules.

Comparison of the differences in purity after both washing and storage in the various solutions are shown in Table 5.5. The more positive the confidence interval, the better the purity produced by the UW or MEM storage.

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<th>p =</th>
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<td>0.142</td>
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Table 5.5 The % exocrine contamination of a 60% islet yield after washing and storage in the adapted MEMs compared with those from the same pancreas in UW or MEM.

**d) Summary**

The addition of the impermeant molecules in UW to MEM, produced effects during digest storage which were virtually unchanged from those of MEM alone. However the inclusion of the colloid HES in MEM effectively prevented the decrease in tissue densities seen during storage in MEM, particularly for the exocrine component of the digest. This resulted in a similar beneficial effect on islet purification to that seen with storage in UW, although it was produced without significant changes in tissue densities.

**5.5 DISCUSSION**

**d) Density as a measurement of cellular swelling**

There are two possible explanations for the changes in the density of pancreatic digest seen to occur during storage, one possibility is the hypothermia-induced release of dense
cellular enzymes, and the other, is changes in the cellular water content producing changes in cellular volume.

**Hypothermia-induced enzyme release**

There is a well documented link between periods of hypothermia and pancreatitis while cold storage of the whole pancreas prior to transplantation has been shown to produce an elevation of serum amylase in the recipients, which can be minimised by storage in UW. Both phenomena are presumably a reflection of the sensitivity of exocrine tissue to hypothermia and the resulting discharge of dense exocrine enzymes, with demonstrable vacuolation at the ultrastructural level. This in turn, has been suggested to result in a corresponding decrease in tissue density as seen in mast cells. However, there are several reasons why this is unlikely to produce the effect on density seen in this study. The fact that the exocrine fragments in the digest both decrease in density during storage in MEM, and increase in density during storage in UW, demonstrates that the process responsible for the changes is a reversible one. The release of enzymes from tissue in which the metabolic processes have been effectively arrested, is not.

In addition, the parallel changes in density of islets, suggests that the process responsible affects both exocrine and endocrine tissue similarly. As islets have been shown to be more resistant to both ischaemic and cold induced damage and are only capable of releasing 10% of their insulin content physiologically, with cellular impermeant molecules such as sucrose inhibiting even this insulin release, it seems doubtful that hypothermic induced insulin release from islets would match that of exocrine enzyme release from acinar tissue.

**Hypothermic cell swelling**

The second explanation, that the changes in density of the digest, reflect changes in hypothermic induced cell swelling during storage in the various media, seems much more likely. Leaf and Vinograd in 1964 analysed the effect of changes in cellular volume on the density of cells. They showed that the equation previously derived by Ponder for the relationship between osmolality and cellular volume could be adapted to express the effect of changes in cellular volume in terms of their buoyant density, showing that the two were directly related, according to the equation,

\[
\rho = \frac{\left(\frac{1}{T} - 1\right)\rho_{\text{osm}} + \left(\frac{V}{V_0}\right)\rho_{\text{iso}}}{\left(\frac{1}{T} + 1\right) + \left(\frac{V}{V_0}\right)\rho_{\text{iso}}}
\]

Where \( \rho \) is the buoyant density of a cell at tonicity \( T \) and \( \rho_{\text{osm}} \) and \( (V/V_0)\rho_{\text{iso}} \) are the buoyant density and the ratio of the total volume to the volume of water in a cell at unit tonicity.

During the course of our study the volume of the pooled pancreatic digest from each of 10 pancreases was measured, by centrifugation for 2 minutes at 4°C at 200g in a graduated
flask, before and after storage in UW. The results showed that the volume of digest decreased significantly during the period of cold storage in UW, from a mean of 40.6 ml pre-storage, to 33.6 ml post-storage (T=55, p=0.006, 95%CI -3.5 to -10.00ml). It can therefore be assumed that the changes in tissue densities noted using the mini continuous gradients, reflect the same hypothermic induced changes recorded in terms of cellular swelling by other groups. In addition to their direct relevance to the process of islet purification, they should therefore have the same implications in terms of organ viability, with denser tissue (less swollen) reflecting improved viability.

ii) Comparison of cold storage in UW or MEM

The results of the initial part of the study, comparing the effect of cold storage in UW or MEM, on tissue density and islet purity, are therefore not surprising, and are easily explicable in terms of the known reduction of cellular swelling by UW. The fact that UW was noted to increase density rather than just prevent the decrease seen with storage in MEM, may reflect either a reversal of cell swelling which had occurred during the period of whole organ storage and subsequent collagenase digestion, or a shrinkage of tissue which has been noted by others. The rapidity with which it occurred suggests that it is likely to be the result of movement of water rather than ions.

The effect on islet isolation following the period of storage represents a difference in the sensitivity of islets and exocrine tissue to hypothermic cell swelling, which has previously been noted in response to osmolality. The significance of these changes can be readily appreciated when it is remembered that the islets constitute only 2% of the volume of the pancreas. This means that if all the islets are isolated (100% yield) with a contamination by only 2% of the exocrine tissue, the result will be islets which are only 50% 'pure'. If the exocrine contamination increases by as little as a further 2%, the purity of the same islets falls dramatically to 33%. Although only the difference in exocrine contamination following storage in UW or MEM was statistically significant, the 95%CI for the change in exocrine contamination with storage in UW shows a tendency towards an improvement which in practice is highly significant, allowing islets to be purified from digest which, had it not been stored, or been stored in MEM, would have been heavily contaminated and untransplantable.

The results of this study, therefore confirmed both the impression that storage of digest in tissue culture medium resulted in a deterioration in the results of islet purification with time, and the findings of van der Burg in the dog, that storage of digest in UW rather than tissue culture medium resulted in a significant improvement in islet purification. It also demonstrated that this was the result of UW's effect in preventing hypothermic induced cell swelling and probably reversing swelling that had already occurred during the preceding stages of islet purification. Although this was an immediate effect, storage in UW for a period of 1-2 hours potentiated it and resulted in improvement in islet purification over time, rather than just preventing deterioration.
Since hypothermic induced cell swelling results in a demonstrable deterioration in the results of whole organ transplantation, it would be expected that storage in UW by preventing this, would minimise any loss of viability, a finding supported by autotransplantation in the dog. In vitro viability tests do not provide qualitative data on the degree of viability and the influence that this might have on long term graft function, but showed that islets stored in MEM and UW, both demonstrated comparable insulin release.

c) The effect of adding cellular impermeants and colloid to MEM

It seems clear that the principles that apply to whole organ storage, apply equally to the storage of tissue fragments, with short periods of cold storage in tissue culture medium producing an adverse effect on purification due to cell swelling, followed over longer periods by a deterioration in tissue viability.

The principles which govern hypothermic cell swelling were established in the red cell model. When cell metabolism is inhibited at 4°C the cell reverts from a state in which the double-Donnan equilibrium taking into account the activity of the Na⁺ pump at 37°C applies, to one in which only the Gibbs-Donnan equilibrium is effective, and the cell then behaves as a perfect ion osmometer, surrounded by a semi-permeable membrane, permeable to water and small molecules but not to macromolecules. As a result the cell initially reflects increases in extracellular salt concentration, by shrinkage and increased density. The cell swelling produced by cooling the cell to 4°C is then explainable as Na⁺ stops being effectively impermeant, resulting in an influx of Cl⁻ and Na⁺ ions accompanied by water. (Figure 4.2, p104).

Cellular impermeants

With this in mind, it was expected that adding cellular impermeants to the MEM would prevent cell swelling and mimic the effect of UW, since the concentration of impermeants added was designed to balance the osmotic force of 110-140 mOsm/KgH₂O exerted by the impermeable intracellular proteins and anions, responsible for the end result of isosmotic hypothermic cell swelling (Chapter 4.1 i, p103).

Although 30mmol raffinose on its own does not completely suppress tissue swelling in either the pancreas or the kidney, alterations in the ratio of permeable chloride ions to impermeable lactobionate have an additive effect, and a lactobionate-raffinose solution has been shown to decrease pancreas weight during 24 hour storage. The ratio of chloride:lactobionate ions in such solutions has been shown to be crucial, particularly in solutions high in Na⁺ rather than K⁺ ions. In our study the ratio was altered from 100% chloride to 60% chloride + 40% lactobionate. Although this would not be expected to prevent cell swelling completely, coupled with the addition of 30mmol raffinose, it should produce a measurable decrease compared with MEM alone, and was selected to allow any further improvement due to the addition of HES to be detected.
The lack of any detectable effect on cellular swelling at first appears difficult to explain, since the concentration of impermeable ions outside the cell, should have balanced the effect of the impermeable ions inside the cell, and prevented the isosmotic entry of water which is the crucial factor in generating cell swelling. However this study demonstrates that it is not so much the presence of impermeable anions, as the absence of permeable anions outside the cell, which is responsible for the effect of UW. The fact that the concentration of chloride ions remains constant in all the MEMs and is completely absent in UW, means that the same influx of Cl⁻ down a concentration gradient occurs, accompanied by Na⁺ down the electrical charge gradient created. Despite the presence of impermeable ions outside the cell this movement will still result in an influx of water and cell swelling.

Although the study of the changes in density of pancreatic fragments seems likely to have a direct bearing on the results of whole organ preservation as shown by the clear differences in the effect of MEM and UW, there are several important differences. The mechanical forces thought by some to be important in controlling cellular swelling by maintaining an intracellular hydrostatic pressure ("the mechanochemical hypothesis), are obviously very different in tissue fragments, compared with whole organs with an intact collagen framework, and renal tubular cells treated with collagenase have been shown to swell significantly more than in intact tubules 1119. In addition the permeability of the cell membrane may be altered by the processes such as collagenase digestion which have preceded the storage period. Rinaldini has suggested that "the permeability (of the cell) to ions or to nonelectrolytes may be substantially changed" by the action of collagenase 183, and Schwartz noted that following collagenase digestion, "many of the isolated acinar cells had discontinuous plasma membranes 147. If the cell was to become permeable to larger molecules such as lactobionate or raffinose, it would make this model less applicable to illustrating the effect extracellular molecules would have in whole organ preservation. The results of this study have not demonstrated such an effect however, since the changes in density demonstrated must reflect an intact semi-permeable membrane.

Colloid

Although the inclusion of a colloid has been shown to decrease tissue water content 132 and prevent increases in organ weight during storage 1088,1090,1097, the inclusion of HES in UW was primarily designed to improve its functional capacity for in situ flushing of vascularised whole organs 1097,1120 and isolated normothermic organ perfusion 1089, by preventing accumulation of extracellular fluid under hydrostatic pressure 1121, and on its own has been shown to allow significant increases in tissue water 126. It was not expected that its addition to MEM would be beneficial in the reduction of cell swelling in tissue fragments, in the absence of either hydrostatic pressure or a vascular endothelial barrier across which to act.

The conclusion of this study that the addition of HES is more important than the addition of impermeant molecules in preventing the decrease in tissue densities seen during
storage in MEM and prevented by UW, is therefore difficult to explain using accepted theories of whole organ cold storage, but has been documented before \cite{1114,1122,1123}. It is obvious that while the colloid will contribute to counteracting the osmotic effect of intracellular colloids the inclusion of 50g/l of HES (0.2mmol) is unlikely to be more effective in this role than the addition of 133mmol of impermeable molecules.

One explanation for the unexpectedly greater effect seen, is that the addition of HES actually increased the osmolality of the MEM+HES solution by 20mmol (see Table 5.1). Analysis of a solution of 50g/l of HES in dd H$_2$O using inductively coupled optical emission spectrometry (Fisons Scientific Equipment Division using an ARL 3580 ICP OES) showed that this was not due to the presence of contaminating molecules, with a concentration of only Na$^+$ = 0.03mmol, K$^+$ = 5x10$^{-5}$mmol, Ca$^{2+}$ = 2x10$^{-4}$mmol, Mg$^{2+}$ = 4x10$^{-4}$mmol /g of HES. The increase in osmolality is therefore likely to be the result of binding of water by the HES (which has been calculated at 20ml/g) resulting in a much greater colloid osmotic effect $^{1124}$.

An additional factor which may further increase the effectiveness of HES in preventing cell swelling in this particular situation, is the presence of large amounts of amylase and other pancreatic enzymes free in the solution of pancreatic digest. HES like any other starch based compound is hydrolysed to progressively smaller polymers of glucose $^{1125}$ by amylase, and even at 4°C this would be expected to produce large numbers of cellular impermeants of large molecular weight.

Lastly, the effect seen in this study may be peculiar to pancreatic tissue, since the presence of a colloid in cold storage solutions has been shown to be particularly important in the pancreas $^{132,1126}$, while having little effect on liver preservation $^{1078,1083,1092}$. However this has been presumed to be due to prevention of over-perfusion of the fragile pancreas, rather than an effect on cellular swelling $^{1097}$, and it seems unlikely that the cells of the pancreas would be intrinsically different to those of other organs in their response to extracellular colloid.

5.6 CONCLUSION

Overall, while changes in the intracellular hydrostatic pressure as a result of the dissolution of the collagen matrix, together with a possible alteration in the permeability of the cellular membrane produced by the enzymes involved in the collagenase digestion process may occur, the effects seen in this study correspond with those seen by Belzer’s group in developing their UW solution, suggesting that they too reflect cell swelling.

The results therefore confirm the efficacy of UW in preventing cell swelling, demonstrating a direct benefit in terms of islet purification. While it has always been suggested that it was the ratio of permeable:impermeable anions in the extracellular fluid which was important, this study demonstrates that it is actually the low concentration of permeable anions which is crucial in reducing cell swelling. It also suggests that in this
model at least, the presence of HES as a colloid, is important in producing the improvement in islet purification seen with storage in UW.

These results, while particularly applicable to the cold storage of pancreatic digest, are likely to have wider implications to hypothermic organ storage in general, since the changes seen in tissue density accurately reflect changes in cell volume which have a proven relationship to organ viability.
CHAPTER 6

THE EFFECT OF TEMPERATURE ON HUMAN ISLET PURIFICATION USING DENSITY GRADIENTS OF BOVINE SERUM ALBUMIN.

6.1 INTRODUCTION

i) Use of BSA for density dependent cell separation

ii) Use of density dependent methods and BSA gradients for islet purification

iii) Temperature of density gradients

iv) Aims of the study

6.2 METHODS

6.3 RESULTS

6.4 DISCUSSION
6.1 INTRODUCTION

i) Use of BSA for density dependent cell separation

The density dependent separation of cells, was initially developed for the separation of blood components, and is most commonly performed in density media consisting of an aqueous solution containing carefully adjusted concentrations of macromolecules a number of which have been examined. The three most successful have been albumin (bovine serum albumin, BSA) 212, a polysucrose (Ficoll) 1127,1128, and colloidal silica (Ludox) 1129 with dextran (Percoll) 1130.

BSA was first described for the neutral density separation of cells invaded by malarial parasites, by Ferrebee and Geiman 1131, who also touched on the possibility of red cell fractionation using discontinuous BSA gradients. Its use in continuous gradients was initially described by Spriggs and Alexander in 1960 1019, and perfected by Leif and Vinograd 4 years later, when they described the production of linear isotonic continuous density gradients, and used these for analysis of erythrocyte buoyant density 1020.

Its proponents, have highlighted several advantages in the use of BSA, the main one undoubtedly being its lack of cell toxicity, and its use in the culture of human cells 1133 demonstrates this protective effect 1132, and its ability to reduce cellular aggregation. Cells separated in BSA therefore do not need to be washed completely free of the medium, as simple dilution reduces the concentration to ‘normal’. Lastly, although viscous, it can still be filter sterilised though a 0.2μm filter.

ii) Use of density dependent methods and BSA gradients for islet purification

The various techniques examined over the years for the purification of pancreatic islets from the pancreatic digest, have often had their origins in methods established predominantly for the separation of single cell blood components. It is not surprising therefore, to find that the density gradient media described for density dependent islet purification also owe much to this technology. Sucrose which had been extensively used for the separation of sub-cellular organelles 1134 was the first medium to be described 156. The increase in tonicity of the gradient medium was known to produce irreversible cell damage however 1135, and was shown to reduce islet viability. While Percoll has been among the many other gradient media examined with varying success 219, Ficoll 157 was the next to be studied, and with subsequent improvement by dialysis 214,1135 and then suspension in Euro-Collins 215 to produce “Euro-Ficoll”, it has remained popular.

The first description of BSA’s use in islet purification came from this Unit in 1987 222, and was rapidly transferred on a large scale from the rat model to the human, using the COBE 2991 cell processor 990. Its adoption by other groups 1136, has meant that most human islet purification world-wide is now performed using either Euro-Ficoll or BSA density gradients.
iii) Temperature of density gradients

Logically 4°C would be the ideal temperature at which to perform the anaerobic process of density dependent purification, as a further period of warm ischaemia following the collagenase digestion, would be expected to adversely affect tissue viability. However, while the dissolution of Ficoll in Euro-Collins produces a gradient medium which can justifiably be used at 4°C [1137], since its ionic composition is based on that of a cold storage solution (Table 6.1), BSA on the other hand is a 35% solution of BSA in predominantly NaCl. The effect of this, over even brief periods of hypothermia, would produce cell swelling, and for this reason BSA gradients have usually been run as a compromise at 22°C (room temperature).

<table>
<thead>
<tr>
<th>Euro-Ficoll</th>
<th>BSA (500mOsm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/cm³)</td>
<td>1.115</td>
</tr>
<tr>
<td>pH</td>
<td>7.21</td>
</tr>
<tr>
<td>Osmolality (mOsm)</td>
<td>450</td>
</tr>
<tr>
<td>Na⁺ (mmol)</td>
<td>8.2</td>
</tr>
<tr>
<td>K⁺ (mmol)</td>
<td>91.2</td>
</tr>
<tr>
<td>Cl⁻ (mmol)</td>
<td>1.5</td>
</tr>
<tr>
<td>Mg²⁺ (mmol)</td>
<td>0.02</td>
</tr>
<tr>
<td>Ca²⁺ (mmol)</td>
<td>0.06</td>
</tr>
<tr>
<td>Zn²⁺ (mmol)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 6.1 showing the difference in the ionic composition of 500mOsm BSA and Euro-Ficoll, measured on an inductively coupled optical emission spectrometer (ARL 3580 ICP OES by Fisons Scientific Equipment Division, Loughborough, UK.) or calculated from the composition of the diluent⁸.

However the use of hyperosmolar BSA (500mOsm) at 22°C has been shown to increase the density of both islets and exocrine tissue, improving the purification of islets [991]. This effect is due to the higher permeability of the cell membrane to water than to inorganic ions, which results in an initial immediate movement of water out of the cells when placed in a hypertonic medium, producing cell shrinkage and an increase in density [1114]. This would be followed, after the first hour, by a much slower movement of extracellular ions and water into the cell producing subsequent cell swelling over the next 8 hours, an effect which has been demonstrated in hyperosmolar solutions at 4°C [1026,1114]. The behaviour of cells suspended in a hypertonic sodium chloride solution such as 500mOsm
BSA, depends therefore on the total osmolality of the solution, the activity of the Na\(^+\) pump, the ionic permeability of the cell membrane, and the time spent by the cell within the solution, and is complicated by the fact that different cells appear to respond to these effects at different rates, accounting for the improvement in purification of some cells with alteration in osmolality. The behaviour of human pancreatic digest exposed to these counteracting forces over the brief 15 minute period during isopycnic centrifugation, is therefore impossible to predict theoretically.

The ability to refrigerate the COBE 2991 cell processor to 4°C, with the potential improvement in islet viability that might result, made it important to establish the result that these conflicting forces might have on islet purification. While the high potassium concentration in Euro-Ficoll makes it unsuitable for use at temperatures above 4°C, hyperosmolar BSA could be effective over a range of temperatures, and was therefore used in this study.

iv) Aims of the study

This study aimed to examine the effect of temperature (4°C or 22°C) on the isopycnic purification of human pancreatic islets on BSA continuous gradients, by comparing the effect on islet and exocrine tissue densities and consequent islet purity.

This would answer the important question of whether brief exposure to hypertonic gradient media at 4°C results in a deleterious effect on islet purification by producing cell swelling, or whether the initial movement of water out of the cell as a result of the hypertonicity would be effective in counteracting the effect of turning the Na\(^+\) pump off.

6.2 METHODS

Eight pancreases were retrieved with appropriate consent from brain-dead heart-beating organ donors, with a median age of 45 (range 14-65) years. In each case HOC was used for the in situ vascular perfusion. After a median cold ischaemic time of 3.2 (range 3-8) hours, the pancreas was distended with 2 mls/g of collagenase solution (3mg/ml in HBSS), and shaken apart in the digestion system (Chapter 3.2 ii).

Following collection at 22°C in MEM + 10% NCS, 2 mini continuous gradients of 500mOsm BSA were run synchronously one at 4°C and one at 22°C. Two gradient makers were used, allowing one to be kept at 4 and one at 22°C. The absolute density of the BSA solutions used to produce the gradients were adjusted at the relevant temperature, to 1.098g/cm\(^3\) (high density) and 1.069g/cm\(^3\) (low density), since lowering the temperature increases the absolute density of solutions. After suspending 100μl of digest in 1 ml of high density BSA at either 4 or 22°C, these were overlaid with linear isotonic continuous density gradients using the gradient makers (Chapter 3.5). The two gradients were centrifuged at either 4 or 22°C for 5 minutes at 800g, allowing the tissue to equilibrate to its iso-dense point on the gradient, before being fractionated for analysis.
Pearson's product-moment correlation coefficient ($r$, with significance level $p$) was used to assess the correlation between the results from gradients at 4 and 22°C. Since the two gradients from each pancreas acted as their own controls, the median density of islets and exocrine tissue and the % of exocrine tissue contaminating a 60% islet yield at the two temperatures were compared using the Wilcoxon matched-pairs signed-rank test (reported as a T value with significance level, $p$). The Wilcoxon signed-rank based 95% confidence interval 95%CI was calculated for the differences which occurred with temperature, in islet and exocrine densities and the results in terms of purity.

6.3 RESULTS

The results for each pancreas are summarised in Table 6.2.

\[
\begin{array}{cccccc}
\text{Pancreas no} & \text{Median exocrine density} & \text{Median exocrine density} & \text{Median islet density} & \text{Median islet density} & \% \text{ exocrine contamination} & \% \text{ exocrine contamination} \\
& 22°C & 4°C & 22°C & 4°C & 22°C & 4°C \\
177 & 1.0913 & 1.0920 & 1.0856 & 1.0852 & 19 & 12 \\
178 & 1.0933 & 1.0905 & 1.0858 & 1.0846 & 22 & 27 \\
180 & 1.0908 & 1.0912 & 1.0863 & 1.0858 & 32 & 14 \\
182 & 1.0972 & 1.0940 & 1.0856 & 1.0877 & 4 & 14 \\
185 & 1.0916 & 1.0939 & 1.0893 & 1.0925 & 44 & 44 \\
186 & 1.0887 & 1.0885 & 1.0846 & 1.0841 & 32 & 21 \\
187 & 1.0963 & 1.0961 & 1.0846 & 1.0863 & 7 & 5 \\
189 & 1.0929 & 1.0932 & 1.0892 & 1.0911 & 36 & 45 \\
\end{array}
\]

Table 6.2 showing the median densities (g/cm$^2$) of islets and exocrine tissue and the % of exocrine tissue contaminating a 60% islet yield from gradients at 4 and 22°C.

Analysis of the results, shows that tissue from each pancreas gave very similar results regardless of the gradient temperature. There was a significant positive correlation between the density of exocrine tissue ($r=0.769$, $p=0.026$), islets ($r=0.902$, $p=0.002$) and % exocrine contamination ($r=0.770$, $p=0.025$) at the two temperatures.

There was no significant difference in the density of either exocrine tissue ($T=18.0$, $p=1.000$) or islets ($T=10.0$, $p=0.294$) at the two temperatures, with the positive correlation between the two, showing exocrine tissue or islets from a pancreas which were dense at 22°C were likely to also be dense at 4°C. Not surprisingly, the lack of any significant changes in
densities was reflected in an absence of any change in islet purity, expressed as % exocrine contamination \((T=17.0, p=0.673)\). The 95% CIs for the differences \((4^\circ C-22^\circ C)\) between these values are shown in \textit{Figure 6.1}. The more positive the value, the greater the density or the % contamination at the lower temperature.

\textbf{Figure 6.1} Showing the 95% CIs for the differences between the results at 4\(^\circ\)C and those at 22\(^\circ\)C.

\textbf{6.4 DISCUSSION}

The fact that 15 minutes exposure to a hypertonic gradient medium produces the same effect on tissue densities, whether it occurs at 4 or 22\(^\circ\)C suggests that the movements of water into or out of the cell over this period of time are not significantly temperature dependent. Since the movement of water is considerably more rapid than that of ions, this result is not unexpected as both metabolising cells and non-metabolising cells would be expected to shrink at similar rates when initially placed in hypertonic solutions. The effect of turning off the \(\text{Na}^+\) pump, would subsequently result in an influx \(\text{Na}^+\) and \(\text{Cl}^-\) ions accompanied by water to produce hypothermic cell swelling. As long as the digest is not left in the gradient medium for prolonged time periods, the predominant factor determining the changes in cellular volume is therefore the hypertonicity of the gradient medium rather than the inactivation of the \(\text{Na}^+\) pump.

While this study suggests that density dependent purification of human pancreatic islets can be performed at 4\(^\circ\)C in hypertonic BSA without affecting islet yields and purity, it would seem more logical to use an aqueous medium specifically designed for cold storage containing one of the several available dense macromolecules. For the brief periods
necessary for isopycnic density separation, this medium should be hypertonic since this increases the difference in densities between islets and exocrine tissue improving purification.
CHAPTER 7
THE OPTIMISATION OF DENSITY GRADIENTS FOR LARGE SCALE ISLET ISOLATION ON THE COBE 2991 CELL PROCESSOR

7.1 INTRODUCTION
i) Density dependent cell separation methods and their application to islet purification
   a) Separation by velocity sedimentation
   b) Separation by centrifugal elutriation
   c) Isopycnic separation
   d) Artefacts during centrifugation

ii) The development and usage of the COBE 2991 cell processor
   a) Historical development
   b) Use for blood component separation
   c) Advantages for islet purification

7.2 AIMS

7.3 METHODS & RESULTS
i) Optimisation of discontinuous gradient separation
ii) Production of 300ml linear continuous BSA gradients
iii) Linearity of BSA gradients on the 2991
iv) Human islet purification using linear continuous gradients on the 2991

7.4 DISCUSSION
i) Advantages of the 2991
ii) Advantages of continuous gradients
iii) Advantages of ‘top’ loading
iv) Conclusion
7.1 INTRODUCTION

i) Density dependent cell separation methods and their application to islet purification

There are three different methods, for cell separation which rely at least in part on differences in the densities of the cells being separated, and the use of each of these has been examined for islet purification.

a) Separation by velocity sedimentation

In its simplest form this involves the sedimentation of cells at unit gravity through a column of medium. A relatively large spherical particle, such as an animal cell, moving through a uniform stable medium rapidly attains a constant velocity which depends on a balance between the earth's gravitational force and the resistance to movement through the fluid. Equating the two forces, the velocity \( v \) is described by adaptation of the Svedberg equation as:

\[
v = \frac{2(\delta c - \delta m) g r^2}{9\eta}
\]

where \( \delta c \) is cell density, \( \delta m \) is medium density, \( r \) is the cell radius, \( \eta \) is the viscosity of the medium, and \( g \) is the earth's gravitational force.

Since most mammalian cells have a density in the range 1.040-1.090g/cm\(^3\) [130], the difference in the \( (\delta c-\delta m) \) element of the equation is usually much less significant than the difference in the radii which is squared. The degree of separation produced by sedimentation rates is therefore primarily determined by differences in cell size. While sedimentation at unit gravity through either HBSS [156,206] or Percoll [207], has been claimed to purify islets, it is interesting that during sedimentation at unit gravity through HBSS, one group retrieved their islets from the sedimented pellet [156], and the other from the supernatant HBSS [206]. This illustrates the fact that any method which relies predominantly on the relative size of the islets and exocrine fragments in order to separate them, is unlikely to produce reliable results, due to the considerable and variable overlap in size between the two.

The same problem applies to sedimentation of cells in a centrifugal field described by the equation:

\[
v = \frac{d r}{d t} = \frac{r^2 (\delta c - \delta m) \omega^2 D}{k\eta}
\]

where \( D \) is the distance of the cell from the centre of revolution, \( t \) is the time, \( r \) is the radius of the cell, \( \delta c \) is the cell density, \( \delta m \) the medium density, \( \omega \) the angular velocity (speed of centrifugation), \( \eta \) is the medium viscosity at the cells location and \( k \) is a constant.
Centrifugal elutriation was first described by Lindahl in 1948 as counterstreaming centrifugation. It is based on the principle that cells are subjected to a centrifugal force while suspended in a medium which flows continuously in a centripetal direction (Figure 7.1). The sedimentation of a spherical cell subjected to these forces is given by the equation:

\[ v = \frac{2}{9} \alpha^2 D_c^2 \frac{8\mu \delta m}{b \cdot O(D)} F \]

Where \( F \) is the flow rate and \( O(D) \) is the cross sectional area of the sedimentation chamber as a function of \( D \).

As a method, despite its relative complexity, it is capable of separating large numbers of cells over a relatively short period of time, and in a wide variety of media. As with velocity sedimentation however, although it has been used for islet purification, the separation achieved, is predominantly the result of differences in particle size, and the counteracting forces make islet fragmentation a major problem.

Figure 7.1 Illustrating the principle of centrifugal elutriation

Isopycnic separation

Isopycnic or buoyant density sedimentation refers to the sedimentation of cells in density gradients, with sufficient force and for a sufficient period of time for them to reach the point on the gradient whose density equals their own. No further sedimentation then occurs and the cells will be separated according to their respective densities, the cell size which influences the rate of sedimentation becomes unimportant. Although isopycnic sedimentation has very rarely been shown to be more effective than velocity sedimentation and the greater forces involved can be damaging to cells, the lack of any reliable size difference and the documented densities difference between acinar tissue and islets, have made it the most popular and successful method of islet purification, with numerous gradient media being examined.
The distribution of particles on isopycnic gradients is determined by the shape of the density gradient, with the major choice being between discontinuous (step) gradients, in which the tissue aggregates in "artificial bands" at the interfaces, and continuous gradients in which the cells will be distributed over a wider range, equilibrating to their actual density, rather than in bands above or below medium either more or less dense than themselves. Discontinuous gradients have been used for islet purification because of their simplicity, but they limit the amount of tissue which can be separated before the accumulation of cells at the interfaces begins to block the movement of other cells through the gradient, produces cell aggregation impairing separation, and, beyond a critical point renders the system unstable due to density inversion between the cell band and the gradient below it, ruining the separation. For these reasons only continuous gradients are capable of giving consistent and reliable results.

The introduction of excessive numbers of cells or tissue particles can result in concentrations in certain areas of the gradient which result in the density of that part of the gradient exceeding that of the more centrifugally located gradient. This zone then broadens, and sediments to a denser part of the gradient capable of supporting it as a stable zone. The use of continuous rather than discontinuous gradients increases the gradient capacity considerably.

**Artefacts during centrifugation**

**Gradient capacity**

The introduction of excessive numbers of cells or tissue particles can result in concentrations in certain areas of the gradient which result in the density of that part of the gradient exceeding that of the more centrifugally located gradient. This zone then broadens, and sediments to a denser part of the gradient capable of supporting it as a stable zone. The use of continuous rather than discontinuous gradients increases the gradient capacity considerably.

**Cell streaming**

This is a small scale version of the problems produced by gradient overloading, and occurs when a local point in the gradient becomes overloaded, often when the tissue or cells are first layered onto the gradient and before centrifugation occurs. The particles form droplets, which stream through the sample-gradient interface and settle down the gradient. Since the droplets take a few minutes to form, it can be prevented by carrying out centrifugation rapidly after the cells have been layered onto the gradient.

**Cell aggregation**

Most viable cells exhibit a variable but appreciable tendency to aggregate, and this also applies to pancreatic digest. Since cell aggregates will not be separated from each other, islets involved in such aggregates will be lost from the purified fraction. The problem can be minimised by working at 4°C, reducing the pH of the medium, suspending the dispersed cells in solutions containing BSA, and reducing the time before purification.

**Swirling**

This artefact results from the "rotational movements of the fluid" around the tube axis, which occur as a result of the changes in angular velocity during acceleration and deceleration of the centrifuge, complicated by the changing axis of the tube as it swings from vertical to horizontal and back again. The effect can be reduced by the use of
subdividing lamellae within the centrifuge tubes and by low rates of acceleration and deceleration. Alternatively if the gradient is never exposed to acceleration or deceleration forces, the disruption produced by this artefact is minimised.

**Wall effects**

These result from the fact that the axis of sedimentation in parallel walled tubes is not parallel to the walls of the tube, but instead follows the direction of the force vectors directed radially out from the centre of revolution during centrifugation. The cells therefore become relatively more concentrated at the periphery of the tubes and can be assumed to collide with the walls, becoming lost from the separation. The effect can be minimised by placing the cells towards the bottom of the gradient, and by locating the tubes as far as possible from the centre of revolution. The use of gradient containers in which the walls are aligned with the effective centrifugal force also reduces wall effects.

**Thermal convection**

The effect of even small changes in temperature is greatly magnified as the gravitational field increases. In a field of 1000g for example, a change of 0.1°C will effectively change the density of water by 6mg/cm³, so that differences in temperature along the gradient will produce disruption of the gradient during centrifugation. These effects can be minimised by careful control of temperature.

ii) **The development and usage of the COBE 2991 cell processor**

a) **Historical development**

The development of centrifuges specifically designed for density dependent cell separation began in the 1950's, and resulted in the development of "zonal rotors", which not only minimised wall effects, but allowed the density gradients to be produced under the stabilising influence of a centrifugal field while the centrifuge was running, and to be emptied during rotation. This coincided with the development of continuous-flow centrifuges, based on De Laval's cream separator and designed specifically for the rapid fractionation of blood components, which resulted in the development of the Cohn ADL centrifuge, which allowed the glycerolisation and deglycerolisation of red cells for long-term storage, and the preparation of platelet concentrates and cell-free plasma. The Latham bowl was a simplification of this, producing an intermittent flow device for the rapid and atraumatic separation of red cells, white cells and platelets, and this formed the basis of a number of machines devised by IBM including the 2991 cell processor (Figure 7.2).

b) **Use for blood component separation**

The 2991 cell processor reduced many of the artefacts produced during density dependent centrifugation. The walls of the centrifuge chamber are aligned with the force vectors during centrifugation, reducing wall effects. It allows the loading and unloading of
samples and gradients while spinning reducing swirling, and it can be used for large scale separations in a sterile environment over short periods of time.

Figure 7.2 The COBE 2991 cell processor

It was initially designed and used for the automated deglycerolisation of stored red blood cells 1155. This process not only removed the cryoprotectant, but also helped to remove potentially injurious anticoagulants, and plasma proteins. Moreover, slight modification of the use of the machine, was found to produce leucocyte depletion of whole blood 1156-1161, and platelets 1162, reducing non-haemolytic transfusion reactions common in multi-transfused patients 1163-1165. Similar methods were used to concentrate granulocytes 1166 for use in patients with severe granulocytopenia.

Fractionated collection of the red cells themselves was also possible, since red cells increase in density with age, young red cells with an increased life span could be
concentrated by collecting the more superficial layers of the red cell concentrate following centrifugation 1167-1169.

The use of the 2991 for the concentration of mononuclear cells from bone marrow, for use in allogeneic bone marrow transplantation 1170, pioneered the use of a density gradient medium within the 2991 centrifuge chamber, rather than just centrifugation of blood 1171, and also involved manual control of the off-loading procedure bypassing the red cell detector. The process involved two stages, with initial red cell depletion of the buffy coat layer of the marrow, followed by purification of the mononuclear cells by layering them onto gradient media such as Ficoll-metrizoate 1171, Ficoll-diatrozoate 1172,1173 and Ficoll-hypaque 1174,1175. These combined the stability and inertness of the Ficoll with the high density range of the iodinated compounds originally developed as X-ray contrast media 602.

c) Advantages for Islet Purification

The use of the 2991 was first described for human islet purification on discontinuous BSA gradients in 1989 990, and its efficacy for islet purification with both BSA 1135 and Euro-Ficoll 1176 gradient media has since been confirmed by other groups. The use of large volume discontinuous density gradients (up to 640 ml), allowed the purification of as much as 20 ml of pancreatic digest at 4°C, under sterile conditions.

Although the 2991 improves the purification of large volumes of digest compared with centrifugation in numerous tubes, one of its major limitations remains the capacity of the gradient. Since digestion of most human pancreases produces between 30 and 60 ml of digest the fact that only 20 ml can be purified at any one time on the 2991 means that unless several of these expensive machines are available, the digest is stored for periods of up to one hour during the purification process. As has been shown in Chapter 5, this can dramatically affect the density of the digest components, and on discontinuous gradients will alter the optimal density of the layers of medium.

7.2 AIMS

This study had two main aims, firstly to maximise the amount of digest which could be loaded onto discontinuous BSA gradients before they became overloaded, by maximising the area of the interfaces at which the digest accumulated.

Secondly, we examined the ability to produce 300 ml linear continuous gradients within the 2991 at 4°C. The volume of human pancreatic digest suspended in UW, that could be loaded onto the top of these without causing gradient disruption was determined.

The implications for islet purification of combining of the advantages of continuous gradients, with those of the 2991, are discussed.
7.3 METHODS & RESULTS

The detailed instructions for the use of the COBE 2991 cell processor are contained in the manufacturers manual, and their specific adaptation for bone marrow separation using density gradients and islet purification have been previously described 990,1171.

i) Optimisation of discontinuous gradient separation

Methods

The cross sectional profile adopted by the disposable bag during centrifugation at 3000 rpm was determined, by filling it with various volumes of polyacrylamide gel (density 1.02g/cm^3), which was then allowed to set during centrifugation over 10 minutes.

By radially slicing (Figure 7.3) the resulting cast, the cross sectional profile of the 2991 bag filled with 640ml, 500ml, 350 ml and 200ml volumes was obtained (Figure 7.4).

Manual graphical integration at 0.5cm radial increments, was used to determine the volume of density medium needed to produce an interface at any radius within the bag. The area of this interface could be calculated from a knowledge of the radius from the centre of the bag (r), and measurements of the height of the interface (h) on the cross sectional profiles at that point, using the formula, interface area = h x 2πr (Figure 7.5).
Figure 7.4 Showing the exact cross sectional profiles of the COBE bag as it filled.
Results

Figure 7.4 shows, the exact cross sectional profiles of the 2991 bag obtained with each of the four filling volumes (actual size). The radial measurements begin at 6cm since the 2991 bag is shaped like a doughnut, with a central 'hole' of radius 6cm (Figure 7.3). The volume of the four channels connecting the peripheral bag to the axial entry port was constant at 15 ml, and the volume of the flap at the bag periphery (beyond a radius of 12 cm) was 20 ml and again did not change with increasing fill volumes.

The results of the volume integration are shown in Table 7.1.

<table>
<thead>
<tr>
<th>Radius (cm)</th>
<th>fill volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>640 mls</td>
</tr>
<tr>
<td>6</td>
<td>620</td>
</tr>
<tr>
<td>6.5</td>
<td>613</td>
</tr>
<tr>
<td>7</td>
<td>600</td>
</tr>
<tr>
<td>7.5</td>
<td>575</td>
</tr>
<tr>
<td>8</td>
<td>535</td>
</tr>
<tr>
<td>8.5</td>
<td>480</td>
</tr>
<tr>
<td>9</td>
<td>410</td>
</tr>
<tr>
<td>9.5</td>
<td>335</td>
</tr>
<tr>
<td>10</td>
<td>260</td>
</tr>
<tr>
<td>10.5</td>
<td>185</td>
</tr>
<tr>
<td>11</td>
<td>110</td>
</tr>
<tr>
<td>11.5</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 7.1 This shows the volume contained in the centrifuge bag peripheral to the radius noted for four different filling volumes ranging from the maximum 640 ml to 200 ml.

Using this, and measurements from Figure 7.4, it is possible to calculate the area of any interface (Table 7.2). It can be seen that the maximum interface areas occur at radii between 9 and 11 cm, which for the maximum fill volume of 640 ml, corresponds to volumes of BSA of between 110ml-410 ml. Since the volume left in the 2991 after using the hydraulic 'superout' process is 130ml, in order to retrieve all the lower interface of a discontinuous
Figure 7.6 Showing the formation of a discontinuous density gradient on the COBE 2991 cell processor.
gradient, the volume of ‘high density’ pellet was chosen to be 200ml, followed by 150 ml of ‘mid density’ BSA whose density was adjustable in order to allow only islets to rise through it, and then a further 200ml made up of 50 ml ‘capping’ BSA and 150 ml of MEM (Figure 7.6). Even with this optimisation however, there remained a significant chance of overloading the gradient with as little as 20ml of digest, since if all this were to accumulate at the lower interface whose area is about 140cm$^2$, it would form a confluent layer 140μm thick, clearly capable of impeding the movement of islets into the ‘mid density’ BSA.

<table>
<thead>
<tr>
<th>Volume of high density BSA</th>
<th>r (cm)</th>
<th>h (cm)</th>
<th>Surface area of interface (cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>613</td>
<td>6.5</td>
<td>0.4</td>
<td>16.3</td>
</tr>
<tr>
<td>600</td>
<td>7.0</td>
<td>0.7</td>
<td>30.8</td>
</tr>
<tr>
<td>575</td>
<td>7.5</td>
<td>1.2</td>
<td>56.5</td>
</tr>
<tr>
<td>535</td>
<td>8.0</td>
<td>1.7</td>
<td>85.5</td>
</tr>
<tr>
<td>490</td>
<td>8.5</td>
<td>2.1</td>
<td>112.2</td>
</tr>
<tr>
<td>410</td>
<td>9.0</td>
<td>2.3</td>
<td>130.0</td>
</tr>
<tr>
<td>335</td>
<td>9.5</td>
<td>2.4</td>
<td>143.2</td>
</tr>
<tr>
<td>260</td>
<td>10.0</td>
<td>2.3</td>
<td>144.5</td>
</tr>
<tr>
<td>185</td>
<td>10.5</td>
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<td>138.5</td>
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<td>11.0</td>
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</tr>
<tr>
<td>50</td>
<td>11.5</td>
<td>1.7</td>
<td>122.8</td>
</tr>
<tr>
<td>20</td>
<td>12.0</td>
<td>0.7</td>
<td>52.8</td>
</tr>
</tbody>
</table>

Table 7.2 Showing the area of interfaces produced by loading differing volumes of high density BSA within a COBE bag during filling to 640 ml.

ii) Production of 300ml linear continuous BSA gradients

Methods
Using both a commercially produced gradient maker (Hoefer SG500) and a similarly designed custom made autoclavable glass gradient maker based on previously described two chamber gradient makers 587,596,598,1021 (Figure 7.7), we examined the linearity of a 300 ml continuous gradient of BSA, produced in a measuring cylinder.
150 ml of high density BSA (1.106g/cm³) was placed in one chamber and 150 ml of low density BSA (1.075g/cm³) in the other. The tap between the chambers was opened and a magnetic stirrer was used to ensure thorough mixing as the BSA was pumped into a measuring cylinder at 30ml/minute using a Watson-Marlow 503S pump. Sequential 10 ml fractions down the gradient were then analysed using a DMA 35 densitometer, calibrated to read absolute densities.

Results

The results showed that as long as adequate mixing was produced by the magnetic stirrer, a linear density gradient was produced from both gradient makers (r=0.998, p=<0.001) (Figure 7.8). Inadequate mixing, visibly distorted the linearity, although there was still a significant correlation between the fractions and their density, (r=0.988, p<0.001)

Figure 7.8 Showing the linear increase in density down the gradient produced using a gradient maker, and the detrimental effect of incomplete mixing.
### iii) Linearity of BSA gradients on the 2991

**Methods**

The same technique was then used to examine the possibility of producing 300ml linear continuous density gradients on the 2991. 150ml of high density BSA was pumped onto the 2991 from the gradient maker initially, to act as a buffer zone below the gradient, priming the tubing from the gradient maker to the centrifuge with high density BSA, and allowing all the gradient to be emptied from the machine using the ‘superout’ facility at the end of the procedure (*Figure 7.9*). The 2991 bag was then spun at 3000rpm and all the air was allowed to vent from the system using the ‘superout’ and ‘hold’ facilities. While continuing to spin, the gradient formed in the gradient maker was pumped at 30ml/minute onto the buffer layer of BSA, followed by 150 ml of MEM. After centrifugation at 3000rpm for 5 minutes, the MEM was removed while continuing to spin at the same rate and the gradient was collected as 10 fractions using the ‘superout’ and ‘hold’ controls. The density of each fraction was measured, and to examine the reproducibility of these gradients the procedure was repeated 5 times using different densities of BSA as the ‘high’ and ‘low’ density starting materials.

**Results**

The densities of each fraction from the five gradients was plotted graphically (*Figure 7.10*) to demonstrate that the linear increase in density down the gradient was maintained despite the changing shape of the 2991 bag during loading and unloading.

**Graph Showing the Linearity of Five 300ml Gradients Run on the 2991**

*Figure 7.10* shows the linearity in density of BSA gradients formed and emptied from the 2991 during continuous centrifugation (*r* = Pearson’s product-moment correlation coefficient, in each case *p*<0.001)
Figure 7.9 Showing the formation of a continuous density gradient on the COBE 2991 cell processor.
iv) **Human islet purification using linear continuous gradients on the 2991**

**Methods**

The same process was used to form the BSA linear continuous density gradient within the 2991, but the MEM was replaced with UW containing various volumes of human pancreatic digest (20, 40 and 60 ml) (*Figure 7.9*). This allowed us to examine the maximum volume of digest which could be purified on one gradient after ‘top’ loading. The human pancreases were removed from heart-beating brain-dead organ donors with appropriate consent, and after ductal distension with collagenase were digested using the automated process described in Chapter 3.2 iii.

In order to minimise streaming artefacts, the 100 ml of UW (density 1.042g/cm\(^3\)) containing the suspended digest was pumped slowly over a period of 5 minutes onto the top of the gradient. This allowed the islets and exocrine fragments to disperse into the gradient without building up at the UW/BSA interface. The tubing was then washed through with a further 50 ml of UW to minimise loss of digest, before centrifugation for 5 minutes to allow isopycnic equilibration.

The 150ml of UW was then collected, followed by the gradient which was again collected as 10 x 30ml fractions. The tissue distribution down the gradient was examined visually after washing each fraction. The linearity of two gradients on which digest had been loaded in this way was analysed with a densitometer to check that it had not been disrupted by the digest. The viability of islets from the first 5 pancreases purified using the linear continuous gradients on the 2991 in this way were examined by perfusion (Chapter 3.2 v)

**Results**

It was possible to ‘top’ load up to 40 ml of digest onto continuous gradients and achieve separation of islets from exocrine tissue which mirrored that observed on mini continuous gradients. Attempts to load 60 ml of digest on three occasions, resulted in a build up of digest at the UW/BSA interface which failed to enter the gradient and produced disruption of the gradient during the ‘superout’ collection process.

The linearity of the density gradients was not disturbed by the process of digest purification (*Figure 7.11*), and the significant correlation between gradient fraction and density persisted during purification of both pancreases, for HP220 (r=0.991, p=0.003) and HP221 (r=0.993, p<0.001).
Figure 7.11 Showing that loading 40 ml of pancreatic digest does not disrupt the continuous gradient.

Viable islets were produced from each of the first 5 pancreases purified, with a median stimulation index of 5.15ng/ml (range 3.27-10.43). We have now gone onto purify islets from 45 human pancreases without encountering any technical problems.

7.4 DISCUSSION

i) Advantages of the 2991

The use of the 2991 has been widely adopted for islet purification because of the ability to purify large volumes of tissue particles in a sterile closed system according to their buoyant density. The shape of the 2991 bag prevents wall effects during centrifugation, while the fact that the gradient is loaded and unloaded while spinning at a constant rate, avoids vortices created from rotational movements of the gradient media during acceleration/deceleration of the centrifuge, which would produce mixing of different zones in the gradient. It also offers the possibility of "on-line" processing of pancreatic digest directly linked to the pancreas digestion circuit, by gradual "top" loading onto pre-formed, already spinning, gradients.

ii) Advantages of continuous gradients

Discontinuous density gradients have always been utilised partly for supposed simplicity, and partly because it was felt intuitively that LCDGs could not be produced within the 2991 bag, due to the changes in shape which occur during loading. Such gradients severely limit the functional portion of the gradient which becomes that of the interface areas. Even after maximising these areas, the clogging and tissue aggregation which occurs, means...
that the digest fails to separate optimally as islets became physically trapped in the denser layers by the layer of exocrine tissue held at such interfaces. In addition, by limiting the volume of digest which can be purified on any one gradient, the 40-60 ml of digest produced from a human pancreas requires 2 or 3 gradients for purification, and this increases both the time and cost involved.

This study demonstrates that linear continuous density gradients can be run remarkably easily on the 2991 using commercially available gradient makers. It seems likely that the process of loading and unloading the gradient during centrifugation served to stabilise it and prevent distortion of the density shape of the gradient, despite changes in its actual 3 dimensional conformity. The continuous gradient has important advantages over the discontinuous one, increasing the functional volume of the gradient many fold. This in turn reduces many of the artefacts produced during density dependent centrifugation such as streaming, tissue aggregation and gradient overloading.

For the purposes of islet purification there are several additional advantages to the use of continuous gradients. The density gradient media used no longer have to be tailored to the individual density of the islets and exocrine tissue from any one pancreas. The high and low density media can therefore be made up in large batches and stored until used, shortening the processing time considerably. This reduces tissue aggregation during the period of storage.

The ability to maintain the gradient undisturbed during its fractionated removal allows only those fractions which contain sufficiently pure islets on subsequent visual analysis to be kept. This allows not only exocrine tissue denser than the islets to be removed, as was possible on the discontinuous gradients, but also allows the least dense fractions which often contain cellular and ductal debris but few islets to be discarded.

They also offer the intriguing possibility of producing gradients which not only vary continuously in density but also osmolality. Positive osmolality gradients might further improve islet purification, since the islets should never see the hypertonic areas of the gradient, which would then increase the density of the exocrine tissue selectively.

iii) Advantages of ‘top’ loading

Top loading of digest onto such gradients fails to disrupt them, and the ability to load digest onto the top of the gradient, rather than suspend it in the dense medium at the bottom, has several further advantages. Islet trapping in the pellet below the accumulation of exocrine tissue at the pellet/gradient interface is prevented, since they should never get there.

It allows the digest to be loaded slowly onto the top of the gradient while spinning, potentially straight from the digestion circuit in an on-line process. Loading the digest in UW through the same tube as the gradient served to create slight mixing at the UW/gradient interface which in theory by creating a very steep gradient at the sample-gradient interface has been suggested to minimise the possibility of streaming artefacts 587. These should be further minimised by loading the dispersed digest onto the gradient during centrifugation, avoiding any delay prior to centrifugation, and preventing tissue building up at the interface.
Loading the pellet of BSA, the gradient and the digest onto the 2991 in continuity through a single tube from the gradient maker, prevents the entry of any air into what becomes a sealed system once the air has been evacuated following loading of the pellet. Although in practice small amounts of air did not appear to disrupt the discontinuous gradients, appearing to remain at the top of the gradient during centrifugation, the elimination of any air fluid interfaces from the system is likely to reduce artefacts such as swirling and vortexing.

Islet damage during centrifugation should be minimised, since they never reach the peripheral parts of the gradient where the highest g forces occur. It also allows the digest to be loaded onto the gradient in a protective media such as UW, rather than the high density gradient media used to bottom load gradients. This would not only be expected to improve viability at 4°C, but also to improve islet purification by selectively increasing exocrine tissue density, as shown in Chapter 5.

iv) Conclusion

By increasing the functional volume of the gradient and allowing digest to be loaded gradually in this way, the amount of tissue that can be loaded onto one gradient can be increased to 40 ml, often allowing the entire pancreas to be processed on one gradient.

The data on the cross sectional area of the 2991 bag allows the process of islet purification on discontinuous gradients to be easily optimised by adjusting the volumes of the gradient media layers. The use of continuous gradients however optimises the process of islet purification on the 2991 and considerably improves on the discontinuous gradient method whose many advantages had already led to its widespread use.
CHAPTER 3
THE PRODUCTION AND PURIFICATION OF MONOCLONAL ANTIBODIES TO THE NON-ISLET COMPONENTS OF THE RAT AND HUMAN PANCREAS

8.1 INTRODUCTION
i) The rationale for using a panel of monoclonal antibodies
ii) The production, purification and concentration of antibodies

8.2 METHODS
i) The production of monoclonal antibodies
ii) Purification by gel chromatography
iii) Monitoring and evaluation
   IgM ELISA
   Polyacrylamide gel electrophoresis
iv) Concentration by ultrafiltration

8.3 RESULTS
i) The results of hybridoma formation and screening
ii) The results of LDSd9 purification and concentration

8.4 SUMMARY
8.1 INTRODUCTION

The exocrine and endocrine pancreas have been shown to express cell surface antigens which are not only pancreas specific but specifically differentiate the two pancreatic components.

Monoclonal antibodies to the β-cell component of human pancreatic islets have been produced and used by several groups to try to identify the antigen/s involved in the autoimmune aetiology of diabetes; and other islet-specific antibodies to hormones such as glucagon have been used to study the distribution of hormone producing cells not only within islets but the throughout the whole of the gastrointestinal tract. While the use of monoclonal antibodies directed towards the islet rather than the exocrine component of the pancreas would have some advantages for immunomagnetic islet purification, possibly allowing the purification of uncleaved islets from the digest, the unpredictable consequences of various antibody interactions with islet cell surface antigens on the antigenic expression and immunogenicity of islets, together with the need to remove all the beads from the positively selected islets prior to transplantation, made depletion of the exocrine component the more straightforward and logical approach to separation.

Several groups have developed monoclonal antibodies specifically against the exocrine components of the human, porcine and rodent pancreas. However, most of the antibodies described with such activity, were developed for their reactivity to other cells such as ovarian, breast and pancreatic islet tumours, T cell receptors, ABH or Lewis blood group antigens (known to be expressed only on the acinar component of the pancreas) and even sweat. Previous attempts at using immunological methods for islet purification, whether the method used was complement or light mediated cell lysis, or immunomagnetic for the negative selection of islets.

I) The rationale for using a panel of monoclonal antibodies

The process of pancreatic digestion using collagenase produces a digest which consists not only of islets and acinar fragments but also contains fragments of pancreatic ducts, vessels and possibly lymph nodes. The use of antibodies reactive only to acinar cell surface antigens will not allow the removal of the other non-islet components of the digest, many of which have been shown to express HLA class II antigens and to be responsible for the initiation of islet rejection when co-transplanted with islets. Furthermore, if immunomodulation of pancreatic islets with a view to transplantation into patients with diabetes in the absence of lifelong immunosuppression is ever to be a reality, a prerequisite is that all the contaminating class II antigen expressing tissue is removed before depletion of class II presenting cells from within the islets is performed.

The use of panels of monoclonal antibodies to produce a cocktail of Dynabeads reacting with a variety of cell surface antigens has been shown to be very effective at purging
bone marrow of heterogeneous neuroblastoma cell populations and suggests that the
use of a similar panel for islet purification might not only be more effective at removing the
acinar tissue, by attaching beads to several different acinar antigens, but could simultaneously
be used to remove all the other non-islet components of the digest.

Two monoclonal antibodies to the acinar component of the pancreas already existed
in the Department of Surgery at the commencement of this work, one to the rat pancreas
(LDSd9), and one to the human pancreas (LDSb8). The aim of the first part of the work
described in this Chapter was to use the methods described by Kohler and Milstein (see Chapter 3) to generate clones of hybridoma cells producing antibodies which, reacted
not only with the acinar components but also the vessels and ducts of both the rat and human
pancreas. A decision was made to select only IgM antibody producing clones, since these
could be directly coupled to M-450 Dynabeads without the need for a secondary antibody to
act as a spacer, making the isolation procedure as simple and economical as possible.

In order to use these monoclonal antibodies to coat M-450 Dynabeads in a reliable
and reproducible fashion, large quantities of each antibody had to be produced, purified and
quantified. The monoclonal antibody producing hybridomas were therefore used to produce
quantities of ascites in mice containing high titres of the relevant antibody. While such an
ascites contained the high concentrations of antibody required, it also contained
contaminating proteins produced by the host mouse, which could potentially block binding
sites on the Dynabeads during coating procedures limiting the reproducible nature of the
work.

The second part of the work described in this Chapter aimed to use the ascites formed
by the monoclonal hybridomas, to produce a pool of purified and concentrated IgM
monoclonal antibodies which could be stored at 4°C under sterile conditions and reliably
used in subsequent studies on immunomagnetic islet purification (Chapter 9).

Well established methods for antibody purification were set up and used to purify and
concentrate the antibodies appropriately. Gel chromatography was used to fractionate the
molecular components of the ascites according their molecular size/weight, with the IgM
containing fraction then being concentrated by ultrafiltration. The purification process was
monitored using polyacrylamide gel electrophoresis, immunohistology, UV
spectrophotometry and an IgM ELISA developed for the purpose.

8.2 METHODS

i) The production of monoclonal antibodies

The methods used for the immunisation of BALB/c mice and the subsequent
production and screening of antibody producing monoclonal hybridomas are described in
Chapter 3.4. A total of 10 fusions were performed over a period of 6 months; in 3, human
exocrine tissue was used as the stimulating antigen, in 5 rat exocrine was used and in 2 pig exocrine (Table 8.1).

<table>
<thead>
<tr>
<th>Fusion Number</th>
<th>Antigen</th>
<th>Number of positive clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>Human exocrine</td>
<td>1</td>
</tr>
<tr>
<td>90</td>
<td>Rat exocrine</td>
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</tr>
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<td>92</td>
<td>Rat exocrine</td>
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<td>0</td>
</tr>
<tr>
<td>111</td>
<td>Rat exocrine</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 8.1 showing the antigenic stimulus used before each fusion and the number of hybridoma clones producing antibody to rat or human exocrine tissue that resulted (excludes anti-islet antibodies).

The antigenic specificity of each hybridoma was examined initially by immunohistology of the cell supernatants on sections of pancreas from the same species used to immunise the mice, although in the case of mice immunised with pig exocrine, reactivity to both rat and human exocrine tissue was screened for. To avoid selection of antibodies to blood group antigens which would have limited the usefulness of anti-human monoclonals, only sections of O negative pancreases were used. Staining was performed for IgM antibodies, and positive clones were subsequently screened for cross reactivity with other species.

Once the positive hybridomas were deemed to be monoclonal on screening (Chapter 3.4iv), at least 6 million cells (3 vials) from each clone were frozen in liquid nitrogen for future use. Other cells from each clone were used to produce ascites containing high titres of the relevant antibody (Chapter 3.4v). Each pool of ascites was stored at -20°C prior to purification and cellular debris was removed by centrifugation after thawing.

ii) Purification by gel chromatography

It was decided to purify the ascites produced using just gel filtration chromatography after initial unsuccessful attempts to use dialysis against a 2mM pH 6.0 phosphate buffer as a preliminary concentrating step. The process involves the application of a protein mixture to a column of porous beads with a carefully controlled pore size. Large molecules,
Figure 8.1 Showing the equipment used for the purification of mouse ascites by gel chromatography
excluded from the pores, pass through the column unimpeded, and are eluted from the column first, in the ‘initial void volume’ \( (V_o) \). Small molecules enter the bead pores and have their passage delayed in a size dependent fashion. A variety of gels are commercially produced allowing one with a pore size appropriate to the molecule being purified to be chosen, so that the molecule is eluted between the void and total volume of the column. For the purpose purifying IgM (mol wt \( \sim 900,000 \)), Sepharose 4B (Pharmacia, Uppsala, Sweden, no.17-0120-01) was selected, which has a molecular weight fractionation range of \( 6 \times 10^4 \) to \( 2 \times 10^6 \).

The gel was loaded into a 65cm long chromatography column of internal diameter 26mm (LKB 2137) and allowed to settle overnight at room temperature. After washing upwards for 24 hours with TBS\(^1\) to remove the ethanol used to preserve the gel and any air bubbles, the column was transferred to the cold room for use at 4\(^\circ\)C. Fractionation was achieved by upward flow at 0.5ml/min through the column (Figure 8.1) and the UV absorbance of the eluant from the column was analysed continuously at 280nm using in line UV spectrophotometry (LKB Uvicord S II connected to a potentiometric chart recorder LKB 2210) before collection as 150 x 2ml fractions (LKB 2211 Superac).

![The distribution of proteins of several molecular weights in the fractions eluted from the Sepharose 4B column](image)

**Figure 8.2** showing the distribution of proteins in the fractions collected following calibration of the column.

Before using it to purify ascites, the column was calibrated using samples of known molecular weight, which were dissolved in 2 mls of TBS and introduced via an access port between the buffer reservoir and the pump. The pattern of UV absorbence recorded during each run is shown in **Figure 8.2**. Using blue dextran (Sigma D5751, mol wt \( 2 \times 10^6 \)) allowed...
V_o to be determined for the column, the total volume (V_t) of the column was determined using BSA (mol wt 67,000), and the elution volumes (V_e) of thyroglobulin (mol wt 669,000) and ferritin (mol wt 440,00) were measured.

Using the formula $K_{av} = \frac{V_e - V_o}{V_t - V_o}$

the $K_{av}$ value can be plotted against the molecular weight to produce a graph (Figure 8.3) from which the molecular weight of any protein run on the column can be determined.

**Graph Plotting the Kav for proteins run on the Sepharose 4B column against Their Molecular Weight**

From the regression equation

the mol wt = 1.09 - y / 5.38 x 10^-7

so that D9 IgM with a Kav of 0.643 has a mol. wt of 834,000

**Figure 8.3** showing the calibration of the column to allow the determination of molecular weights.

The calibrated column was used to purify 2ml aliquots of ascites at a time. These became diluted during the procedure into a total of 150 x 2 ml fractions, each collected individually. After analysis to determine which of these fractions contained pure IgM (see below), the relevant fractions were pooled and concentrated to achieve a final IgM concentration of ~1mg/ml.

**iii) Monitoring and evaluation**

During the process of gel fractionation chromatography, each fraction produced had its protein content estimated using the in-line UV spectrophotometer, the value obtained being approximately related to the protein concentration of the fraction by the formula

Protein content (mg/ml) = UV absorption at 280nm x 0.741

The amount of immunospecific IgM in each fraction was also assessed semi-quantitatively by immunohistology, using the same method as for screening hybridomas. The
intensity of the IgM staining on each section was scored by 2 independent observers on a scale from 0, 0.5, 1, 2 to 3, and the two scores for each fraction combined. By comparing the two, the peak of UV absorption resulting from the presence of IgM could be identified.

**IgM ELISA**

**Preliminary experiments to develop an accurate IgM ELISA**

In order to quantify the IgM content of both the fractions produced by the column and the final IgM concentrate, a competitive antigen modification of the indirect ELISA was established 1210,1211 (Figure 8.4).

This involved coating a plate with a pre-determined concentration of IgM, which was then labelled by reaction with anti-mouse IgM (μ-chain specific) alkaline phosphatase conjugated antibody developed in the goat (anti-IgM-AP) (Sigma, A-7784) 880. The concentration of alkaline phosphatase enzyme bound to the plate, was then quantified by the colour reaction produced on the addition of phosphatase substrate (P-Nitrophenyl phosphate disodium, Sigma, 104-0). The enzyme labelling of the IgM bound to the plate can be competitively inhibited, after blocking free binding sites on the plate, by the addition of samples containing unbound IgM which reacts with a proportion of the anti-IgM alkaline phosphatase conjugate, and which is then removed by washing the plate before the addition of the substrate. The decreased colour reaction developed by the reduced enzyme concentration left, can be measured and used to quantify the amount of IgM in the competing sample.

The initial method examined attempted to use commercially available purified monoclonal mouse IgM (Mouse IgM, k (TEPC 183) purified myeloma protein, Sigma, M2770), both to coat the plate and as the competitive standard samples. Using a 96 well ELISA plate (Linbra® EIA Microtitration plate, Flow Laboratories, Irvine, UK.), a chequer board titration experiment was performed (with each well in duplicate), to determine the
optimal concentration of IgM for coating the plate (50 or 5 µg/ml) and the optimal dilution of the anti-IgM-AP label (1:500, 1:1000, 1:2000, 1:4000, 1:7000, 1:14000, 1:28000). Standard curves were produced for serial dilutions of competitive IgM (10, 5, 2.5, 1.25, 0.625, 0.312 and 0 µg/ml) with the developing colour reaction being read through-the-plate at 10, 20, 30, 60 and 120 minutes after the addition of the phosphatase substrate, by light absorption at 405 nm using an 8-channel computer-controlled vertical-light-path filter photometer (Titertek Multiskan MCC/340, EFLAB, Finland).

Using dilutions of anti-IgM-AP of greater than 1:2000 failed to develop a satisfactory colour reaction. Standard curves were plotted for the 1:500, 1:1000 and 1:2000 dilutions using the readings from both of the duplicate wells, and the correlation between the concentration of competitive IgM and the colour reaction developed was determined using Pearson's product moment correlation coefficient. The highest correlation (r=−0.835, p<0.001) was obtained using 5 µg/ml of IgM to coat the plate, 1:1000 dilution of anti-IgM-AP and reading the colour developed at 10 minutes (Figure 8.5).

Figure 8.5 Showing the best series of standard curves obtained using the monoclonal IgM to coat the ELISA plate and as the standard competitor.

Despite the statistical correlation, in practice (as can be seen from Figure 8.5) the accuracy was not high enough to reliably determine the concentration of mouse IgM in the samples, with the light absorption by samples with different standard IgM concentrations often overlapping. A further problem was the cost of using commercially purchased IgM to coat each well.
We therefore examined whether polyclonal rather than monoclonal mouse IgM would provide a more accurate and economical system. Polyclonal IgM was obtained by purification of mouse serum obtained from BALB/c mice using the Sepharose 4B gel fractionation column. Using the monoclonal based IgM ELISA detailed above, the IgM concentration of each fraction from the column was estimated, and compared with the total protein content of each fraction measured by UV absorption (Figure 8.6).

![Graph Showing the Relative Concentration of Total Protein and Mouse IgM in Each Fraction of Mouse Serum](image)

*Figure 8.6* showing the relatively high concentration of IgM in fractions 70-100 compared with the total protein content of those fractions.

Using gel electrophoresis (see below) fractions 70-90 were shown to contain relatively pure IgM and these fractions were pooled and concentrated to a final protein concentration (measured by UV spectrophotometry) of 0.919 mg/ml, this was assumed to be the concentration of IgM.

A series of titration experiments was then performed to determine the optimal concentration of polyclonal IgM for coating the plate, in combination with either monoclonal or polyclonal standard dilutions as the competitive inhibitor. Using a concentration of 50 µg/ml of polyclonal IgM to coat the plate, with polyclonal IgM as the competitive standards and a 1:1000 dilution of anti-IgM-AP produced the best standard curve over a range of concentrations from 0-20 µg/ml when the absorbence was measured 120 minutes after the addition of phosphatase substrate (*Figure 8.7*). For wider ranges of IgM values a semi-log plot had to be used (see *Figure 8.15, p190*).
ELISA Control Curves Using Polyclonal IgM Standards and 50μg/ml polyclonal IgM for Coating the Plate

- Titertek reading at 20 mins, r=-0.920, p<0.001
- Titertek reading at 30 mins, r=-0.953, p<0.001
- Titertek reading at 60 mins, r=-0.962, p<0.001
- Titertek reading at 120 mins, r=-0.964, p<0.001

**Figure 8.7** Showing the standard curves for the optimised ELISA using polyclonal IgM to coat the plate and as the standard competitor.

In addition to improving the correlation of the standard curve, the use of polyclonal IgM derived from the same strain of mouse used to grow the monoclonal ascites was felt to have theoretical advantages over the use of monoclonal IgM derived from a myeloma cell line, in terms of the extrapolation of results between standards and the samples to be analysed, and by using a reagent produced in-house, was more economical.

The optimised ELISA parameters using polyclonal IgM were used as the established method for determining the absolute concentration of IgM in samples during the purification and concentration process.

**Established method**

The contents of each duplicated well in the 96 well EIA plate was planned to include negative controls assessing background binding of anti-IgM-AP to uncoated wells, and positive controls in which the anti-IgM-AP enzyme conjugate and its phosphatase substrate were mixed after the final wash. Polyclonal IgM was made up to a concentration of 50μg/ml in 0.3M Na₂CO₃ pH9.5 coating buffer, and 100 μl of this was pipetted into the selected wells before the plate was sealed and incubated at 4°C overnight.

The following day the coating buffer was removed and each well in the plate washed 3 times with PBS³¹. Blocking buffer (200μl of 5% BSA in PBS) was added to each well for 30 minutes, and the plates washed again with PBS x 3.

Using appropriately diluted polyclonal IgM in serial dilutions from 50 to 0.78μg/ml as standards, 50μl of either the IgM standard or the sample being assayed were added to the
appropriate wells, followed by 50μl of anti-IgM-AP diluted 1:1000 using 0.1% gel/PBS\(^1\),
(this was also added to those uncoated wells designated as negative controls). The plate was
sealed again and incubated at 22°C for 90 minutes. Each well was again washed x3 with PBS
and 100μl of phosphatase substrate (made up to 1mg/ml in glycine buffer\(^1\) was added,
(50μl of the anti-IgM-AP was also added to the positive control wells at this stage).

After 120 minutes, the colour developed in each well was measured using the
Titertek. The background count determined from the uncoated wells in practice was found to
be effectively zero, and the Titertek reading was plotted directly against the log IgM
concentration as a semi-log plot which produced a straight line relationship over the
concentrations of IgM being measured (Figure 8.15\(^12\)). The regression equation derived
from the control curve for the relationship between Titertek reading and log IgM
concentration was then used to calculate the concentration of IgM in the samples assayed.

**Polyacrylamide gel electrophoresis**

The fractions obtained from the gel chromatography column were pooled into batches
of 5 fractions (80-85, 86-90 etc.) and concentrated by ultrafiltration (see below). After
concentration, the purity of each batch of 5 fractions was assessed using gel electrophoresis
\(^{12}\). Electrophoresis refers to the transport of ions or charged molecules through a solution
by an electric field. The rate of migration of a molecule in the electric field depends on its
size, shape and charge, and allows individual species of molecules to be distinguished. The
process is carried out in a solution stabilised within a gel matrix of polyacrylamide, agarose,
cellulose acetate or starch, the supporting matrix not only improving the resolution of
proteins by minimising the random diffusion of the sample components that would occur in a
free solution, but also improving the resolution of proteins of different sizes by acting as a
molecular sieve, allowing free passage of smaller molecules while impeding the migration of
larger molecules.

Polyacrylamide is the matrix of choice for resolving proteins. The pore size of the
polyacrylamide gel is one of the major factors determining the separation of polypeptide
molecules during electrophoresis, and is determined by the acrylamide content of the gel.
This can be manipulated to achieve optimal separation, being used either as a gel of uniform
but variable acrylamide content, or as a gradient where the acrylamide content increases
(decreasing the pore size) in the direction of migration.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), was used
to eliminate the intrinsic charge of the proteins. The detergent SDS denatures the proteins and
at neutral pH most peptides become uniformly coated with the negatively charged SDS. The
electrophoretic mobility of the SDS-peptide complex then depends on the molecular weight
of the peptide.

The electrophoresis tank was filled with running buffer\(^1\), and continuously cooled to
5-6°C. Using a gel mould produced by clamping two glass plates as a sandwich, with
perspex strips as spacers, the resolution obtained with a gel of uniform 7.5% acrylamide
concentration was compared with one using a continuous acrylamide gradient from 5-12.5%, produced using a continuous gradient maker (Figure 7.7, p160). Each acrylamide resolving gel\(^1\) was allowed to set between the glass plates, before a stacking gel\(^1\) was run onto the top, allowing the samples to be run onto the gel in 10 lanes (Figure 8.8).

![Diagram of the apparatus used for examining the purity of IgM containing fractions using polyacrylamide gel electrophoresis](Image)

The 10 samples to be run on the gel which in each case included high molecular weight markers \([15.4, 18.2, 28.7, 45.8, 69.3, 107, 211.4 \times 10^3]\) and the original ascites, were boiled for 5 minutes in eppendorfs and then diluted with sample buffer\(^1\) to a final concentration of \(\sim 25 \mu\text{g} \text{ of protein/50\mu l}\). 50\mu l of sample was then loaded onto the appropriate lane, and the gel was placed in the electrophoresis tank. The voltage and current were adjusted to run the gel overnight. The following day the gel was placed in coomassie blue staining buffer\(^1\) for 5-6 hours on a rocker, and then placed in destain. Once the background had cleared to leave the visible bands of protein in each lane, the gel was analysed to determine which of the fraction pools contained IgM in the absence of other impurities.

The simpler 7.5% acrylamide gel provided equal resolution, and proved to be simpler and more robust during staining and destaining and was therefore used routinely.
iv) Concentration by ultrafiltration

Membrane ultrafiltration 1214-1216 was used to concentrate the pools of 5 fractions of TBS containing the IgM. This process involves the selective transport of solutes through a thin porous barrier under pressure. Solutes smaller than the pore size in the membrane are driven through with the solvent, while larger molecular weight solutes are retained (Figure 8.9). Mechanical stirring prevents the retained solute accumulating at the membrane interface and obscuring the selectivity of the original ultrafilter.

An Amicon 8200 stirred cell (Amicon Ltd, Stonehouse, UK, no 5123) with a YM10 Diaflo ultrafilter which retained molecules $>10,000 \text{ mol wt}$ (Amicon, no 13632) was used, (Manufacturers information suggest that this should retain $>98\%$ of IgM molecules). This was pressurised to 10-20 psi by connection to a cylinder of compressed N$_2$ gas and the volume of the IgM containing fractions was reduced by $\sim90\%$. The protein (IgM) concentration of both the retentate and the ultrafiltrate before and after ultrafiltration was monitored by UV spectrophotometry to prevent inadvertent loss of IgM should the membrane become damaged.

After analysis of each pool using gel electrophoresis, all those containing pure IgM were pooled, and filter sterilised through a 0.2$\mu$m filter (Acrodisc, Gelman Sciences UK., Product number 6224192). The IgM concentration was determined using UV spectrophotometry and the IgM ELISA, before storage in 1 ml aliquots at 4°C.
8.3 RESULTS

i) The results of hybridoma formation and screening

The sequence of cloning and screening for each of the 10 fusions is shown in the form of a family tree, identifying the lineage of each monoclonal hybridoma according to its fusion number, and then the well numbers in which it was identified during each cloning. Cell lines which were recloned are underlined (95/39/21), those cryopreserved for future use are shown in italics (95/39/17) and those grown as an ascites in bold (95/39/21/13). The species reactivity is designated in brackets as (h) = human, (r) = rat and (p) = pig.

From some fusions, to avoid exceeding the capacity to screen and maintain each clone, some of the potentially useful cell lines were cryopreserved, rather than being recloned immediately.

**FUSION 87**

<table>
<thead>
<tr>
<th>Immunised</th>
<th>14.11.90</th>
<th>5.12.90</th>
<th>17.12.90</th>
<th>3.1.91</th>
<th>17.1.91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion</td>
<td>30.1.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 87/14 Acinar cells
- 87/17 Islets
- 87/24 Occasional cell
- 87/30 Exocrine
- 87/33 Exocrine + Ducts

Hybridomas produced from Fusion 87 (Immunised with human exocrine)

**FUSION 92**

<table>
<thead>
<tr>
<th>Immunised</th>
<th>25.1.91</th>
<th>7.2.91</th>
<th>21.2.91</th>
<th>4.3.91</th>
<th>11.3.91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion</td>
<td>14.3.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 92/19 Exocrine + Ductal
- 92/19/86 92/19/27,38
- 92/19/86/27 92/19/86/10,14,28 etc

Hybridomas produced from Fusion 92 (Immunised with rat exocrine)
Figure 8.10 Immuno-photomicrograph of 92/19/86/27 (LDS35) on rat pancreas

Figure 8.11 Immuno-photomicrograph of 93/32/85/89 (LDS34) on human pancreas
Hybridomas produced from Fusion 93 (Immunised with human exocrine)
Hybridomas produced from Fusion 94 (Immunised with pig exocrine)
Hybridomas produced from Fusion 96 (Immunised with human exocrine)

Figure 8.12 Immuno-photomicrograph of 95/20/9/18 (LDS42) on human pancreas
Hybridomas produced from Fusion 97 (Immunised with rat exocrine)

Figure 8.13 Immuno-photomicrograph of 97/38/8 (LDS43) on rat pancreas
Fusions 110 and 111 failed to produce any positive hybridomas at all on the first screening, despite the fact that the serum from the immunised mice had been shown on immunohistology to contain IgM reactive to the rat pancreas, before their last intraperitoneal immunisation with exocrine antigen.

The results are summarised in Table 8.2 which also shows the LDS number assigned to each antibody once it had been grown as an ascites.

<table>
<thead>
<tr>
<th>FUSION</th>
<th>POSITIVE CLONES</th>
<th>ANTIGEN</th>
<th>LDS NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>F454A3.D9</td>
<td>Rat exocrine</td>
<td>D9</td>
</tr>
<tr>
<td>68</td>
<td>F68.4D2.27</td>
<td>Human exocrine</td>
<td>LDS b8</td>
</tr>
<tr>
<td>87</td>
<td>87/14/26</td>
<td>Human acinar cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>87/17/65</td>
<td>Human Islets</td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>92/19/86/27</td>
<td>Rat exocrine and ductal</td>
<td>LDS 35</td>
</tr>
<tr>
<td>93</td>
<td>93/1/51/2/14</td>
<td>Human &amp; rat vessel</td>
<td>LDS 41</td>
</tr>
<tr>
<td></td>
<td>93/32/85/89</td>
<td>Human exocrine</td>
<td>LDS 34</td>
</tr>
<tr>
<td>94</td>
<td>94/16/40/16</td>
<td>Human vessel</td>
<td>LDS 38</td>
</tr>
<tr>
<td></td>
<td>94/53/2/89</td>
<td>Human exocrine</td>
<td>LDS 36</td>
</tr>
<tr>
<td></td>
<td>94/66/21/9</td>
<td>Pig collagen &amp; human scattered cells</td>
<td>LDS 39</td>
</tr>
<tr>
<td>95</td>
<td>95/8/2/2</td>
<td>Rat scattered cells</td>
<td>LDS 37</td>
</tr>
<tr>
<td></td>
<td>95/8/12/92</td>
<td>Human ducts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95/17/87/16</td>
<td>Pig Islets</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95/20/9/18</td>
<td>Rat and human vessels and exocrine</td>
<td>LDS 42</td>
</tr>
<tr>
<td></td>
<td>95/39/21/13</td>
<td>Human vessel</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td></td>
<td>no positives</td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>97/38/8</td>
<td>Rat exocrine</td>
<td>LDS 43</td>
</tr>
<tr>
<td>110</td>
<td></td>
<td>no positives</td>
<td></td>
</tr>
<tr>
<td>111</td>
<td></td>
<td>no positives</td>
<td></td>
</tr>
</tbody>
</table>

*Table 8.2* Summarising the results of the fusions and designating LDS numbers to monoclonal antibodies produced as an ascites.
ii) The results of LDSd9 purification and concentration

A total volume of 12 ml of ascites produced by 5 mice each receiving an intraperitoneal injection of $5 \times 10^6$ F45.4A3.D9 monoclonal hybridoma cells was pooled, and after centrifugation to remove cellular debris, was fractionated in 6 x 2 ml aliquots using gel chromatography. This produced 150 fractions of 12 ml each, with 110 of these being voided between the $V_o$ and $V_t$ volumes of the column and therefore containing the proteins fractionated from the ascites.

The protein content of each fraction was measured using UV spectrophotometry and compared with the calibration curves for the column(Figure 8.2). Staining intensity on immunohistology was used to examine the amount of anti-rat exocrine IgM semi-quantitatively, and these two measurements are plotted for comparison in Figure 8.14.

![Graph Showing the Relative Protein and IgM Content of Each Fraction Following Gel Chromatography of LDSd9](image)

Figure 8.14  Showing the distribution of protein and IgM in the fractions produced by gel chromatography, reflecting the degree of purification.

By plotting the $K_a$ (0.643) for the peak of D9 shown here, on Figure 8.3, the molecular weight of the D9 molecule was estimated at 834,000.

An ELISA was performed to analyse the IgM concentration of each fraction of LDSd9. Standards with an IgM concentration ranging from 0.78 to 100μg/ml were analysed in quadruplicate and the standard curve was drawn as a semi-log plot of the Titertek reading against the log of the known IgM concentration (Figure 8.15).
Figure 8.15 Showing the linearity of the ELISA control curve over a range of standard IgM concentrations from 0.78 to 100μg/ml.

Using the regression equation from this standard curve (Log IgM = 2.84 - 1.62 x Titertek reading), the IgM content of each fraction of D9 was determined (Figure 8.16).

Figure 8.16 Showing the peak of IgM in fractions 70 to 110 corresponding to that shown by both the UV absorption and immunohistology in Figure 8.14.

The fractions were pooled into batches of fractions 60-64, 65-69, 70-74 to 96-100, and each 60 ml batch was concentrated by ultrafiltration to a volume of ~ 6ml. 20μl of each
batch was analysed after dilution with sample buffer by gel electrophoresis, to produce the gel shown in Figure 8.17.

Figure 8.17 Showing the result of gel electrophoresis of the fractionated LDSd9 ascites, the impurities present in the original ascites and in fractions 96-100 can be clearly seen.

Analysis of the gel showed that fractions 75-95 contained denatured IgM either in the form of unresolved light chains or as heavy chain monomers or dimers, without the contaminating protein molecules present in the initial ascites, and in concentrated in fractions from 96 upwards.

On the basis of these analyses, fractions 75 to 95 were pooled to give a final volume of 26.4 ml of purified LDSd9 with a concentration of 1.23mg/ml after filter sterilisation. This was stored in 1 ml aliquots at 4°C.

This purified monoclonal IgM was used in the series of experiments described in Chapter 9 to examine the use of M-450 Dynabeads for the immunomagnetic purification of rat islets. The other monoclonal hybridomas were used to produce pools of purified antibodies for future work using panels of antibodies to the non-islet components of the human pancreas.
8.4 SUMMARY

The work detailed in this chapter produced a total of 12 monoclonal hybridoma cell lines producing IgM antibodies specific to the non-islet components (acinar cells, ducts, vessels, etc.) of the rat and human pancreas, with the possibility of further clones being developed from cryopreserved cell lines. A method using gel filtration chromatography to effectively purify large quantities of each monoclonal antibody from ascites produced in mice, and then concentrating them by ultrafiltration was established. Quality control being performed using a combination of UV spectrophotometry, immunohistology, gel electrophoresis and an IgM ELISA developed specifically for the purpose.

The purified monoclonal antibodies produced using these methods allowed reproducible experiments to be performed optimising the coating of Dynabeads with antibody, the binding of the coated beads to target exocrine tissue, and the immunomagnetic purification of islets which are described in the following chapter.
INTRODUCTION

m) Binding of antibody coated Dynabeads to target cells
n) Removal of labelled exocrine fragments

ANTIBODY COATING OF BEADS

Methods

IMMUNOMAGNETIC SEPARATION

Summary

Density gradient purification of Dynabead labelled digest
Comparison of separation by eppendorfs or test tubes
Comparison of pull on top and bottom magnets
Use of flow or static exposure of digest to the magnetic field

CONCLUSIONS

A) Presence of digest under gravity into a bipolar magnetic field
B) Loss of flow or static exposure of digest to the magnetic field
C) Comparison of eppendorf pull and bottom magnets
D) Comparison of separation in eppendorfs or test tubes
E) Density gradient purification of Dynabead labelled digest

DYNAMARADS FOR THE PURIFICATION OF RAT ISLETS

THE DEVELOPMENT AND USE OF ANT-EXOCRINE COATED

CHAPTER 9
9.1 INTRODUCTION

This Chapter describes studies on the immunomagnetic purification of rat pancreatic islets using the purified LDSd9 monoclonal antibody (see Chapter 8), which binds to rat exocrine tissue highly specifically (Figure 9.1).

Figure 9.1 Immuno-photomicrograph of LDSd9 on rat pancreas, showing the specificity of the monoclonal antibody for exocrine tissue, and the complete absence of binding to islets.
The reliable availability of rat pancreases, in contrast to human pancreatic tissue allowed a series of experiments to be planned adapting procedures devised for separation of single cell suspensions, to the separation of multicellular tissue fragments.

Although the collagenase digestion of the rat pancreas is more reliable than that of the human pancreas, the size of the tissue fragments produced is the same, and there is no reason why methods developed in the rat model should not be directly transferable to the purification of human pancreatic islets (given suitable antibodies), and allow the use of panels of Dynabeads designed to remove ducts and vessels as well as acinar tissue.

Before methods for immunomagnetic islet purification could be examined, an effective method, for attaching the microsphere to its target cell via monoclonal antibody linkages had to be developed. This involved optimising conditions for coating beads with antibody, and for attaching them to their exocrine targets.

i) Antibody coating of microspheres

Many of the immunological methods for cell separation outlined in Chapter 2, such as affinity chromatography, panning, rosetting and the use of immunomicrospheres, require antibody linked to a solid phase to bind specific cell antigens, and trap the target cell population. Effective methods for coupling antibodies (or other ligands such as lectins, hormones, toxins, enzyme inhibitors, drugs and chemical transmitters) to the solid phase in an orientation which effectively retains their antigen binding capacity are therefore an integral part of any such process.

In order to couple antibodies to solid microspheres numerous approaches have been used (Table 2.4, p65-67), most of them initially described for the preparation of column matrices in affinity chromatography. Those used for coupling antibodies to magnetic microspheres have been described in detail in Chapter 2, p69, falling into two broad categories, in which the antibody is linked to the bead either by physical adsorption or covalent bonding, the latter often requiring initial activation of groups on the bead surface.
While physical adsorption to their hydrophobic surface was the first method used and has remained popular, Dynabeads have since been activated in several ways. Two types of sulfonyl chlorides (tosyl chloride and tresyl chloride) have been used (Equation 2.5), as has 2-fluoro-1-methylpyridinium toluene-4-sulfonate (Equation 9.1). Filling the bead pores with epoxy resins rather than polymers is a further method, allowing direct coupling of the NH₂-terminal of antibodies to epoxy groups.

The decision to select IgM monoclonal antibodies (in preference to IgG), was designed to allow the use of the simplest procedure, that of direct non-specific physical adsorption of the IgM to M-450 Dynabeads. This has been shown to be highly effective and allows the binding of 3-5µg of antibody per mg of M-450 beads. Attempts to remove the antibody bound in this way using guanidine hydrochloride or 1M sodium lauryl sulphate only reduced the amount of antibody bound by 10%, suggesting that covalent coupling to active groups as well as physical adsorption was occurring, a phenomenon which appears to increase with longer coupling times of the order of 24 hours.

The straightforward direct method in which the primary antibody is coupled directly to the bead, prior to incubation with the target antigen (Figure 2.1, p54), was used throughout this study. IgM antibodies have been shown to be ideal for this, and it seemed likely that in contrast to the steric advantages sometimes seen using the indirect method for coupling of beads to single cells, the use of the indirect method with irregular multicellular exocrine particles, would result in the effective loss of any antibody which became bound within exocrine tissue fragments, with only antibody bound to the surface being subsequently available for bead attachment.

The initial experiments performed in this study optimised the process of nonspecific adsorption of antibodies to uncoated M-450 Dynabeads (Dynal, Product number 140.01) by examining the effect of alterations in pH and buffer conditions. This was then compared with the result of covalent binding using M-450 beads activated using p-toluenesulfonyl chloride (Dynal, Product number 140.03). This has been shown to be of equal efficacy with other methods for covalent coupling of IgM to Dynabeads over incubation periods of 24 hours, but can be inferior to physical adsorption for some IgM antibodies.

ii) Binding of antibody coated Dynabeads to target cells

The binding of beads to their target cells has been shown by electron microscopy to occur in three stages, with the initial interaction of antibody and cell surface antigen becoming reinforced by the successive interaction of other antibody molecules with further antigens (the zipper mechanism), and then by movements of the cell surface to envelope parts of the particle, a series of processes which can be inhibited by both high and low target antigen densities.

The efficacy of direct bead to cell binding has been shown to depend on factors such as the concentration of antibody used to coat the beads, and the time, temperature, media and mechanical conditions used for the subsequent incubation with their cell targets. Each of
these conditions must be optimised not only to achieve maximal binding of beads to their antigenic targets, but also to minimise non-specific binding. While some of the conditions for direct negative immunomagnetic selection of cells using IgM coated Dynabeads have been defined, with antibody coating using 2-10μg antibody/mg of beads, followed by incubation with the target cells at 4°C for 30 minutes on a Rock-N-Roller being advocated for lymphocyte purification and used effectively for the selection of monocytes, T cells and eosinophils, other factors such as the media used for the incubation, and the volume of media in which cells and beads are incubated (between 2 x 10^6 and 2 x 10^8 cells/ml) have varied.

The experimental conditions that produced optimal bead to exocrine binding were defined in the second part of this study (Section 9.3, p204) using visual assessment under the light microscope of rosette formation, already a well established technique for assays using both the larger sheep red blood cells as labels and Dynabeads to determine the efficacy of binding. To make such an assessment easy, dithizone staining and a ratio of beads to exocrine tissue fragments of 1000:1 was used throughout this part of the study, and rosette formation was assessed independently by two observers.

Once conditions which gave reliable and effective rosetting of exocrine fragments had been established, methods for separating the labelled exocrine fragments from the islets were finally investigated.

iii) Removal of labelled exocrine fragments

Although the attachment of only one bead has been shown to allow effective immunomagnetic removal of cells, in practice an excess of beads is required. The ratio of beads to target cells is critical to the success of immunomagnetic cell separation, too few beads and the target cells are not removed, too many and non-specific binding, trapping of antigen negative cells, and physical blocking of antigen binding sites become a problem. The optimal ratio varies with the method of purification selected and the degree of purity required. Negative selection of cells has been shown to require higher particle to cell ratios than those employed in positive selection to achieve effective depletion of all the unwanted cells. To deplete T cells from a suspension of peripheral blood mononuclear cells (containing 50-70% T cells) 10-20 beads per cell have been recommended, although positive selection might require only 3-5 beads per target cell. The number of beads required to effectively remove the much larger exocrine fragments has never been reported, although estimates based on published data suggest the use of ratios of the order of 1000:1 significantly greater than those required to remove single cells. Titration experiments were therefore performed to establish the optimal number of beads required per exocrine fragment.

The rest of the third part of this Chapter (Section 9.4, p213) details the investigation of various methods for removing the magnetically labelled exocrine tissue by exposing the digest to magnetic fields generated by permanent magnets. Most of the studies using
Dynabeads for immunomagnetic cell separation have made use of a series of side-pull neodymium-iron-boron magnets produced by Dynal for the purpose, such as the MPC®-1 & 6 and the MPC®-E 988-960,964,965,969,970,973,1222. Following incubation with Dynabeads, the suspension of cells in either a test tube or eppendorf is placed against the magnet for 2-4 minutes, the suspending medium containing the cells not attracted to the magnet is then collected as the 'non-magnetic fraction', before removal of the tube from the magnetic field allows recovery of the 'magnetically trapped fraction'.

Single cell separations such as the positive selection of lymphocytes 962,963,975 and the T-cell depletion of bone marrow 950,951 have also been performed using flat horizontal magnets. The supernatant fluid being removed either by pipette or decantation, leaving the magnetically held cells behind. To allow this, Dynal have now begun to produce flat permanent magnets (Dynal product list 1993) for use with blood bags in bone marrow purging (MPC®-BMP, Figure 9.10, p219), and with flexible 96 well microtitre plates (MPC®-96), which allow the cell suspension to be lowered into the magnetic field.

One problem with such devices is that even using the most powerful permanent magnets the effective range of the magnetic field is reduced to a radius of ~1cm around the magnet, and this limits the capacity of static devices, making them less suitable for large scale separations.

This led to the development of separation devices, initially for automated assays 1223, in which the magnetically labelled fraction was removed during flow through a magnetic field. Kemshead et al were the first to describe the use of such a system for cell separations, using a system in which large volumes of bone marrow were purged of magnetically labelled neuroblastoma cells by flow through a series of 3 magnetic fields 896,923. This device was adopted by others 842 and formed the basis for a series of modifications by Kemshead's 1224 and other groups 893,924-926,930,934,937,938 in which the bone marrow is pumped from one blood bag to another over arrayed series of permanent magnets. Smaller scale devices using flow through flexible polyethylene 1225 or tygon 931 tubing within magnetic fields, have also been effectively used with Dynabeads and with other beads such as albumin microspheres 805, while flow through high gradient magnetic fields is an integral part of the separation procedure used with smaller particles such as colloidal dextran 844.

The application of each of these categories of magnetic separation to islet purification was examined, with the percentage of exocrine tissue and islets removed in the magnetic fraction being estimated visually, to determine the degree of purification and islet yield achieved. New methods were developed to overcome the problems encountered.
9.2 **ANTIBODY COATING OF BEADS**

Using visual assessment by two independent observers of rosette formation with exocrine particles, a series of chequerboard experiments were performed to determine the optimal conditions for coating either unactivated or tosylactivated M-450 Dynabeads with LDSd9 antibody.

i) The lowest concentration of antibody that allowed effective binding of Dynabeads to exocrine particles.

ii) The ideal buffer for incubation of the beads with LDSd9, and

iii) The relative efficacy of physical adsorption and tosyl activation, were each examined.

**General Methods**

In all the experiments it was assumed that the commercially produced solution of Dynabeads contained -4x10^8 beads/ml or 30mg/ml (Manuacturers information), and that the purified LDSd9 was at a concentration of 1.23mg/ml (Chapter 8, p191). This allowed conditions such as the concentration of antibody in µg per mg of beads to be calculated.

Certain well established methods remained constant throughout, with only the parameters under investigation being subject to variation.

*Figure 9.2* Showing the MPC®-E designed to hold 6 microcentrifuge tubes of the eppendorf type, of volumes 1.0-1.5ml.

*Adding antibody solution to beads;* The solution of Dynabeads was vortexed to render it an homogeneous suspension before the required volume (number or mg) of beads was
pipetted into an eppendorf. The preservative solution was then removed by placing the eppendorf into an MPC®-E magnetic particle separator (Figure 9.2), and after 30 seconds pipetting off the supernatant. One ml of antibody solution in the appropriate buffer was then added to 50 mg of beads to produce a standard volume of solution for incubation (e.g. for 100μl (3mg) of Dynabeads, 60μl of IgM solution was added). The concentration of LDSd9 in this standard volume of buffer solution was adjusted to give the required amount of antibody in μg/mg of beads.

**Incubation conditions**: Because of the small volumes of Dynabeads and IgM used during these experiments, to maintain contact between antibody solution and beads the eppendorfs containing the mixtures were incubated at 4°C for 24 hours upright on a rocker, rather than being rolled. After incubation, the MPC®-E was used to remove the antibody coated Dynabeads from the supernatant IgM solution. These were then washed 4 times for 5 minutes in 0.01M PBS containing 0.5% BSA, and finally suspended overnight at 4°C in PBS with 0.1% BSA to block unoccupied protein binding sites on the beads. The beads were either used immediately, or stored at 4°C in PBS/0.1% BSA with the addition of 0.02% sodium azide (conditions which have been shown to allow storage for more than 6 months with no alteration in antigen binding capacity).

**Rat pancreatic digest**: The rat pancreatic digest was obtained and processed as described in Chapter 3.3, p89. Since immunomagnetic purification of human islets is likely to be used as a secondary purification process following initial density gradient separation, the rat pancreas was purified on discontinuous density gradients of BSA as a preliminary step, before the addition of the Dynabeads, so that any effect that this process might have on antigen expression would have occurred. Standard aliquots of 100 islets handpicked from the islet layer and ~500 exocrine particles resuspended from the denser interfaces were prepared. The number of exocrine particles was estimated by counting samples from the resuspended exocrine layers and adding appropriate volumes of exocrine suspension to the aliquots of islets.

**Methods specific to section 9.2**

**Rosette formation and assessment**: It was obvious that assessing the binding of antibody to bead using rosetting required a reliable method of bead to exocrine linkage before it could be used. A preliminary series of experiments (not detailed here) similar to those described in Section 9.3 were therefore carried out to establish a working method. Based on these, the aliquots of digest were resuspended in 100μl of MEM in eppendorfs, and 500,000 beads were added to give a ratio of beads to exocrine particles of 1000:1. This ratio was found to allow reliable visual comparison of exocrine rosettes, non-specific binding to islets and numbers of unbound beads. Lower ratios resulted in 'disappearance' of bound beads which presumably became hidden within exocrine particles.
The mixture was then incubated for 45 minutes at 4°C on a rocker, before being transferred to wells in 24 well tissue culture plates (Nunc®), for assessment. In order to limit subjective errors, each experiment was performed in duplicate with negative controls using uncoated Dynabeads included, and rosette formation was assessed 'blindly' by two independent observers and categorised as None, Poor, Moderate, Good (sub-categorised with + & ++) or Excellent by one observer, and -, -/+, +, ++, ++++, or ++++ by the other, this allowed the score given by each observer to be easily identified when examining the results, and helped to standardise the early assessments by the two observers.

Variation in coating conditions: The chequerboard experiment outlined in Tables 9.1 & 2 was then performed. The LDSd9 solution was diluted with one of three buffers, (0.05M Tris-HCl pH9.5, 0.2M Na Phosphate pH7.4 and 0.5M Borate pH9.5), each of which has been described for antibody coating of either uncoated or tosylactivated Dynabeads. In each case undiluted LDSd9 stored in TBS was used to coat the beads at a concentration of 32µg/mg of beads by adding 78µl/100µl of beads. The LDSd9 was then diluted using 80µl of buffer to 160µl of LDSd9 and 60µl of this was added to 100µl of beads to give an antibody coating concentration of 16µg/mg, at the required ratio of 1ml of solution /50mg of beads. Serial dilutions were used to coat the beads at 8 and 4µg/mg.

After washing, each 100µl of beads was resuspended in 1ml of PBS/0.1%BSA. For each chequerboard experiment (uncoated and tosylactivated) 26 aliquots of digest were prepared and suspended in 87.5µl of MEM. 12.5µl of the appropriately coated beads (500,000) were then added and the mixture incubated at 4°C upright on a rocker for 45 minutes. The effectiveness of rosetting was then assessed in 24 well tissue culture plates after addition of dithizone and dilution with a further 200µl of MEM to improve visibility.

Results
The assessment by the two independent observers for rosette formation in each of the matching pairs of aliquots were combined and summarised in Tables 9.1 & 2, first for uncoated and then tosylactivated beads. The number of unbound beads showed an inverse relationship to the effectiveness of the rosetting and each score therefore reflects a visually assessed ratio, with the number of unbound beads seen at the bottom of each well decreasing as the rosettes became more obvious (Figure 9.3 &4).
From these results it can be seen that the unactivated beads produced better rosettes, particularly at borderline antibody concentrations such as 4 pg/mg than the tosylactivated, and that using an antibody concentration of 16 pg/mg of unactivated beads diluted with borate buffer, resulted in the most effective rosetting (shaded cell in Table 9.1). No non-specific binding of beads to islets was seen at any of the antibody concentrations.

<table>
<thead>
<tr>
<th>µg of antibody/mg beads</th>
<th>0.05M Tris-HCl pH 9.5</th>
<th>0.2M Na Phosphate pH7.4</th>
<th>0.5M Borate Buffer pH9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None (-)</td>
<td>None (-)</td>
<td>None (-)</td>
</tr>
<tr>
<td>4</td>
<td>Good (+++)</td>
<td>Good (+++)</td>
<td>Good++ (+++)</td>
</tr>
<tr>
<td>8</td>
<td>Good++ (+++)</td>
<td>Good+ (+)</td>
<td>Good++ (+)</td>
</tr>
<tr>
<td>16</td>
<td>Good++ (+)</td>
<td>Good+ (+)</td>
<td>Excellent (+++)</td>
</tr>
<tr>
<td>32 has to be neat</td>
<td>Excellent (++++)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 9.1 using UNCOATED BEADS**

<table>
<thead>
<tr>
<th>µg of antibody/mg beads</th>
<th>0.05M Tris-HCl pH 9.5</th>
<th>0.2M Na Phosphate pH7.4</th>
<th>0.5M Borate Buffer pH9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None (-)</td>
<td>None (-)</td>
<td>None (-)</td>
</tr>
<tr>
<td>4</td>
<td>Moderate (+++)</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
</tr>
<tr>
<td>8</td>
<td>Good (+++)</td>
<td>Good (+)</td>
<td>Good (+)</td>
</tr>
<tr>
<td>16</td>
<td>Good+ (+)</td>
<td>Good+ (+)</td>
<td>Good (+)</td>
</tr>
<tr>
<td>32 has to be neat</td>
<td>Good (+++)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 9.2 using TOSYLATED BEADS**
**Figure 9.3 Above** Negative control, using beads without antibody coating, showing the beads scattered across the bottom of the well and no rosette formation. **Figure 9.4 Below** using beads coated at 16μg/mg with LDSd9 in a borate buffer, in contrast illustrates the presence of obvious rosettes.
9.3 BEAD TO EXOCRINE BINDING

Using the method established above for physical adsorption of IgM at pH 9.5 to the uncoated Dynabeads, we then examined the effect of variations in the conditions under which the beads were incubated with the pancreatic digest in order to:

i) Better define the lowest effective concentration of antibody for coating the beads.
ii) Determine the best media for suspending the digest during incubation with the beads.
iii) Determine the ideal length of time and temperature used for the incubation, and
iv) The volume of media and degree of mechanical agitation required.

i) Optimal concentration of antibody used for bead coating

Using serial dilutions of LDSd9 in the borate buffer, beads were incubated in antibody solutions whose concentration decreased from 32 to 0.25 µg/mg. The assessment of rosette formation is shown in Table 9.3.

<table>
<thead>
<tr>
<th>CONCENTRATION OF LDSd9 in µg/mg</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (negative control)</td>
<td>None (-)</td>
<td>None (-)</td>
</tr>
<tr>
<td>0.25</td>
<td>None (+/-)</td>
<td>None (+/-)</td>
</tr>
<tr>
<td>0.5</td>
<td>None (+/-)</td>
<td>None (+/-)</td>
</tr>
<tr>
<td>1</td>
<td>Poor (+/-)</td>
<td>Poor (+/-)</td>
</tr>
<tr>
<td>2</td>
<td>Moderate (+)</td>
<td>Good (+)</td>
</tr>
<tr>
<td>4</td>
<td>Good (++)</td>
<td>Good (++)</td>
</tr>
<tr>
<td>8</td>
<td>Good ++ (+)</td>
<td>Good ++ (+)</td>
</tr>
<tr>
<td>16</td>
<td>Excellent (+++)</td>
<td>Excellent (+++)</td>
</tr>
<tr>
<td>32 (positive control)</td>
<td>Excellent (+++)</td>
<td>Excellent (+++)</td>
</tr>
</tbody>
</table>

Table 9.3 Showing the dependence of rosette formation on the concentration of antibody used to coat the beads.

These results confirmed those in Table 9.1 showing that effective binding required an antibody concentration of at least 4 µg/mg for bead coating, with increasing concentrations up to 16 µg/mg resulting in further improvements in rosette formation.

In order to allow detection of improvement in subsequent experiments, a suboptimal concentration of 8 µg/mg was usually used.
Effect of media used for incubation of beads with digest

During the preliminary experiments to achieve a working method for rosette formation it had been noted that the use of certain media, in particular University of Wisconsin solution (UW) and solutions containing BSA, appeared to inhibit the process. This was investigated by examining the effect of using several solutions not only while the digest was incubated with the beads, but also when used for washing and storing the digest before incubation with the beads.

Experiment 1

Following density gradient purification the rat islets and exocrine tissue were divided into three, and the tissue was washed and then stored in either MEM, UW or MEM+5%BSA, during preparation of the aliquots of 100 islets and 500 exocrine particles. The aliquots were then resuspended in 87.5μl of the same medium, and incubated at a ratio of 1000:1 with beads coated in 8μg/mg of LDSd9, for 45 minutes in eppendorfs at 4°C on a rocker.

The effect of the different media on rosette formation is shown in Table 9.4.

<table>
<thead>
<tr>
<th>MEDIA</th>
<th>no 1</th>
<th>no 2</th>
<th>no 3</th>
<th>no 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Good+</td>
</tr>
<tr>
<td></td>
<td>(+++)</td>
<td>(+++)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>UW</td>
<td>None</td>
<td>Poor</td>
<td>Moderate</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>(-+)</td>
<td>(-+)</td>
<td>(-+)</td>
<td>(-)</td>
</tr>
<tr>
<td>MEM+5%BSA</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Table 9.4 Showing the inhibitory effect of washing, storage and incubation in UW and MEM+BSA on rosette formation

Experiment 2

This examined the effect of using a variety of different media for the incubation period only, having washed and stored all the digest in MEM.

MEM, UW, RPMI and HBSS were used either as a stock solution, or with the addition of 1% or 5% BSA, 5% NCS (Applied Protein Products, AS-202-50, Batch AN1257) or 10% FCS (SeraLab, Batch 001116), each a common addition to tissue culture media.

Table 9.5 Shows the effect of incubation in these various media on rosette formation.
Table 9.5 Showing that when used only for incubation the effect of different media on rosette formation is minimal.

The conflicting results in Tables 9.4 & 9.5, suggest that either the length of time for which the digest is exposed to certain media, or the stage at which the exposure occurs is responsible for the inhibition seen in the first experiment but not the second.

Experiment 3

To try to determine this, a cross-over experiment was designed in which digest washed and stored for 1 hour in either UW or MEM, was then incubated with beads either in the same media or the alternative one. This gave four groups for assessment;

i) Digest stored and incubated in MEM throughout.
ii) Digest stored in MEM but incubated in UW.
iii) Digest stored and incubated in UW throughout, and
iv) Digest stored in UW but incubated in MEM.

Each groups was studied in quadruplicate (Table 9.6).

<table>
<thead>
<tr>
<th>Media</th>
<th>no</th>
<th>Binding of 16μg/mg uncoated beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM comp</td>
<td>1</td>
<td>Good+ (+++)</td>
</tr>
<tr>
<td>MEM+5% BSA</td>
<td>2</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>MEM+1% BSA</td>
<td>3</td>
<td>Good (+++)</td>
</tr>
<tr>
<td>MEM+5% NBCS</td>
<td>4</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>MEM+10% FCS</td>
<td>5</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>UW</td>
<td>6</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>UW+5% BSA</td>
<td>7</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>UW+1% BSA</td>
<td>8</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>UW+5% NBCS</td>
<td>9</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>UW+10% FCS</td>
<td>10</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>RPMI comp+Hepes</td>
<td>11</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>RPMI+5%BSA</td>
<td>12</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>RPMI+1%BSA</td>
<td>13</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>RPMI+5%NBCS</td>
<td>14</td>
<td>Poor (+)</td>
</tr>
<tr>
<td>RPMI+10% FCS</td>
<td>15</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>HBSS</td>
<td>16</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>HBSS+5% BSA</td>
<td>17</td>
<td>Good++ (++)</td>
</tr>
<tr>
<td>HBSS+1% BSA</td>
<td>18</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>HBSS+5% NBCS</td>
<td>19</td>
<td>Good+ (++)</td>
</tr>
<tr>
<td>HBSS+10% FCS</td>
<td>20</td>
<td>Good+ (++)</td>
</tr>
</tbody>
</table>
Table 9.6. Showing that better rosettes formed when digest was incubated with beads in MEM than in UW, regardless of the media used for digest storage.

Overall, these results demonstrated that incubation of beads and digest in tissue culture media such as MEM produced better binding of beads coated in LDSd9 to exocrine tissue, than incubation in cold storage media such as UW. The inhibitory effect of UW was greatest if the digest was also stored in UW (at 22°C) before incubation (at 4°C).

The mechanism responsible is unclear, but one possibility is that cell swelling in tissue culture media at 4°C which occurs within minutes but continues for 1-2 hours, could have resulted in the exposure of more LDSd9 responsive antigen, and improved rosette formation. As human digest is now stored in UW for periods of up to one hour during the process of density dependent islet purification to prevent this (Chapter 5), the fact that the inhibition of bead binding shown in Experiment 1 was at least partially reversible by simply performing the incubation with the beads in MEM is directly relevant.

Subsequent work Table 9.7 showed that at borderline concentrations of antibody coating (1µg/mg), RPMI+10%FCS improved the formation of rosettes compared with MEM (Table 9.3). Since RPMI with 10% FCS is routinely used for islet culture (Chapter 3.2v, p88) having been shown to be optimal for islet viability 305,1226,1227 it was decided to use this media for suspending the digest throughout, in order to maximise both rosette formation and islet viability.

iii) Effect of temperature and time on binding

The temperature recommended by Dynal and used by most reported studies for incubation of cells with antibody coated Dynabeads has always been 4°C. However, there seemed no reason why this should be better than incubation at higher temperatures which might not only improve the dynamics of the binding reaction but also improve viability of the tissue suspended in RPMI. A chequerboard experiment was therefore designed to examine both the effect of temperature (4, 12, 22 and 37°C) and time (10 and 60 minutes) on the formation of rosettes. Both UW and RPMI were also included as variables since it was felt if
4°C was shown to be optimal, the use of UW would minimise hypothermic tissue damage, whereas at higher temperatures RPMI would result in better viability of metabolically active tissues (see Tables 9.7 & 9.8).

Beads coated in either 16μg/mg or 1μg/mg were used in order to allow assessment of both improvement and deterioration in rosette formation. After addition of the beads, the eppendorfs were incubated for 10 or 60 minutes in water baths at the appropriate temperatures, before being promptly transferred to 24 well plates for assessment. To prevent further rosette formation following transfer invalidating the results, the experiments were organised to allow immediate assessment of rosette formation by both observers at the end of the incubation period.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>16μg/mg LDSd9 Suspended in UW</th>
<th>1μg/mg LDSd9 Suspended in UW</th>
<th>16μg/mg LDSd9 Suspended in RPMI</th>
<th>1μg/mg LDSd9 Suspended in RPMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+++)</td>
<td>Good+ (+++)</td>
</tr>
<tr>
<td>12°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+++)</td>
<td>Good+ (+++)</td>
</tr>
<tr>
<td>22°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+++)</td>
<td>Good+ (+++)</td>
</tr>
<tr>
<td>37°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+)</td>
<td>Good+ (+)</td>
</tr>
</tbody>
</table>

Table 9.7 Rosette formation after 10 minute incubation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>16μg/mg LDSd9 Suspended in UW</th>
<th>1μg/mg LDSd9 Suspended in UW</th>
<th>16μg/mg LDSd9 Suspended in RPMI</th>
<th>1μg/mg LDSd9 Suspended in RPMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>Poor (++)</td>
<td>Poor (++)</td>
<td>Good+ (+++)</td>
<td>Good+ (+++)</td>
</tr>
<tr>
<td>12°C</td>
<td>Poor (++)</td>
<td>None (c)</td>
<td>Good+ (+)</td>
<td>Good+ (+)</td>
</tr>
<tr>
<td>22°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+)</td>
<td>Good+ (+)</td>
</tr>
<tr>
<td>37°C</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
<td>Good+ (+)</td>
<td>Good+ (+)</td>
</tr>
</tbody>
</table>

Table 9.8 Rosette formation after 60 minutes

Although incubation at 37°C resulted in visible fragmentation of exocrine tissue and a deterioration in rosette formation, lower temperatures resulted in uniformly good rosette formation.
formation at both 10 and 60 minutes when RPMI was used for the incubation. The most obvious factor affecting rosette formation was the use of UW for the incubation which resulted in uniformly poor rosette formation compared with RPMI.

Since RPMI+10%FCS is not a cold storage media, it was decided to perform the incubations at 22°C for at least 10 minutes, although binding in these conditions deteriorated at 1 hour.

**iv) Conditions affecting exocrine/bead contact during binding**

The quantity and quality of contact between the antibody on the bead and its target antigen is clearly an important determinant of the ability to form rosettes, and depends on the volume of medium in which two components are incubated and the degree of mixing that occurs within it. To investigate the optimal combination of these two factors aliquots of digest were suspended in 50μl, 100μl, 200μl, 500μl, 1ml and 1.5ml of RPMI. After addition of beads coated in either 16 or 1μg/ml LDS9, these were incubated for 30 minutes in eppendorfs either relatively static upright on a rocker, or actively rolling.

To avoid subjective errors in rosette assessment due to differences in the concentration of the components, all the samples were diluted to 1.5ml with RPMI in the 24 well plates.

<table>
<thead>
<tr>
<th>Volume of media</th>
<th>Static</th>
<th>Rolling</th>
</tr>
</thead>
<tbody>
<tr>
<td>20μl</td>
<td>16μg/mg</td>
<td>16μg/mg</td>
</tr>
<tr>
<td></td>
<td>Good++</td>
<td>Good++</td>
</tr>
<tr>
<td>100μl</td>
<td>Moderate++</td>
<td>Moderate++</td>
</tr>
<tr>
<td>200μl</td>
<td>Moderate++</td>
<td>Good++</td>
</tr>
<tr>
<td>500μl</td>
<td>Good++</td>
<td>Good++</td>
</tr>
<tr>
<td>1ml</td>
<td>Good++</td>
<td>Good++</td>
</tr>
<tr>
<td>1.5ml</td>
<td>Moderate++</td>
<td>Moderate++</td>
</tr>
</tbody>
</table>

Table 9.8 Showing the effect of factors affecting bead/exocrine contact during incubation.

The results showed that both factors had a dramatic impact on the formation of rosettes, with the more dynamic agitation caused by rolling producing better mixing of beads.
and exocrine tissue, and contact being maximised at a volume of 500µl (1µl/exocrine particle). Volumes of medium in excess of this resulted in a rapid fall off in the rosette formation, presumably by reducing bead and exocrine contact and hence the chance of effective antibody to antigen interaction.

v) Summary

As a result of these experiments by optimisation of each variable the concentration of antibody required to coat the beads and still achieve optimal binding could be reduced to 8µg/mg (Table 9.10).

<table>
<thead>
<tr>
<th>Concentration of LDSd9</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None (-)</td>
<td>None (-)</td>
</tr>
<tr>
<td>0.25</td>
<td>Poor (+/-)</td>
<td>Poor (-)</td>
</tr>
<tr>
<td>0.5</td>
<td>Moderate (+)</td>
<td>Moderate (+)</td>
</tr>
<tr>
<td>1</td>
<td>Moderate (+)</td>
<td>Moderate (+/-)</td>
</tr>
<tr>
<td>2</td>
<td>Good++ (+++)</td>
<td>Good++ (++)</td>
</tr>
<tr>
<td>4</td>
<td>Good++(++++)</td>
<td>Good++(++++)</td>
</tr>
<tr>
<td>8</td>
<td>Excellent (++++)</td>
<td>Excellent (++++)</td>
</tr>
<tr>
<td>16</td>
<td>Excellent (++++)</td>
<td>Excellent (+++)</td>
</tr>
<tr>
<td>32</td>
<td>Excellent (++++)</td>
<td>Excellent (+++)</td>
</tr>
</tbody>
</table>

**Table 9.10** Showing the effect of antibody concentration using optimal conditions for rosette formation.

Using beads coated with LDSd9 at a concentration of 8µg/mg at pH 9.6 and incubating these for 30 minutes at 22°C with digest suspended in 500µl of RPMI+10%FCS on a roller at a ratio of 1000:1, the rosette formation shown in *Figures 9.5 & 9.6* was obtained routinely.
Figure 9.5 Showing the rosette formation achieved by optimising conditions, an islet is clearly visible with no attached beads.
Figure 9.6 Digest stained with dithizone showing specific formation of exocrine rosettes.

These conditions were therefore used as the standard method for immunomagnetically labelling digest for use in the separation experiments detailed below.
9.4 IMMUNOMAGNETIC SEPARATION

i) Density gradient purification of Dynabead labelled digest

Immunomicrospheres can alter the physical properties of their target cells in a variety of ways, and although most of the immunomicrospheres have been deliberately designed with a similar density to that of cells to maximise contact between bead and cell during bead attachment, many of them will produce a change in the density of the cells they attach to. By either increasing \(^{\text{741}}\) or decreasing \(^{\text{742,746}}\) the density of specific cell populations, density dependent methods have then be used to remove them.

Dynabeads have a specific gravity of 1.50g/cm\(^3\) which is significantly greater than that of both islets and pancreatic exocrine tissue. We therefore examined the possibility of using the Dynabeads to increase the density of exocrine fragments and improve the separation achieved by isopycnic centrifugation on linear continuous density gradients of BSA (Chapter 3.5, p94).

Half the digest from an entire rat pancreas was incubated with antibody coated Dynabeads at a ratio of 500 beads/exocrine fragment, the other half was treated identically but without the beads being added. The effect on density separation was then examined by centrifugation of 100μl of digest on each of four mini continuous gradients produced using 300mOsm BSA of densities 1.103 and 1.057g/cm\(^3\). Two of these used Dynabead rosetted digest and two ordinary digest from the other half of the same pancreas. Centrifugation was carried out at a reduced force of 400g for 10 minutes using an acceleration rate of 3 and no brake, to minimise shear forces between beads and attached exocrine fragments.

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>ISLETS</th>
<th>EXOCRINE</th>
<th>INSULIN</th>
<th>AMYLASE</th>
<th>DYNABEADS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>27.7</td>
<td>168</td>
<td>+/-</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>+/-</td>
<td>85.08</td>
<td>6190</td>
<td>+/-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>27.02</td>
<td>682</td>
<td>+/-</td>
</tr>
<tr>
<td>4</td>
<td>+/-</td>
<td>+++</td>
<td>29.7</td>
<td>2510</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+/-</td>
<td>+++++</td>
<td>28.98</td>
<td>5140</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>+++++++</td>
<td>66.24</td>
<td>6950</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+++</td>
<td>21.42</td>
<td>5290</td>
<td>++++</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+++</td>
<td>10.38</td>
<td>4600</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+++</td>
<td>7.42</td>
<td>2850</td>
<td>+++</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>++</td>
<td>12.5</td>
<td>2900</td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>++</td>
<td>57.34</td>
<td>3370</td>
<td>++++</td>
</tr>
</tbody>
</table>

**Table 9.11** Showing the distribution of islets, exocrine tissue and beads in the gradient fractions of the two gradients run using Dynabead labelled digest.
After division into 11 fractions, the distribution of exocrine tissue and islets down the gradient was examined both visually and by assay. The results from the two gradients using Dynabead labelled digest are shown in Table 9.11.

The visual assessment of the gradient using digest without the attachment of Dynabeads, showed an identical distribution of tissue, and comparison of the cumulative percentage distribution of amylase and insulin down the gradients (Figure 9.7) confirmed the fact that Dynabead labelling of the exocrine fragments had produced no significant improvement in isopycnic islet purification.

![Cumulative Distribution of Islets and Dynabead Labelled and Unlabelled Exocrine Tissue Down the Gradient Fractions](image)

**Figure 9.7** Showing the identical distribution of tissue down the two gradients.

From Table 9.11 it can be seen that although considerable numbers of Dynabeads remained visibly bound to the exocrine tissue, a lot became unattached and pelleted at the bottom of the gradient. The main problem however was that with the volume of a 150μm diameter exocrine fragment being 37,000 times that of a 4.5μm diameter Dynabead and the two densities being of a similar order of magnitude, coupling sufficient Dynabeads to an exocrine fragment to significantly alter its density was physically impossible.

**ii) Comparison of separation in eppendorfs or test tubes**

Following density gradient purification, aliquots of rat digest containing 100 hand picked islets and 2000 exocrine particles were prepared in eppendorfs and incubated in 0.5 ml RPMI with Dynabeads coated in 8μg/mg of LDSd9. The ratio of beads to exocrine fragments was varied from 1000:1 to 1:1 using serial dilution of the bead suspensions (1000:1 was used in the previous experimental sections, only because it allowed easy visual
evaluation of rosette formation, not because of any known effect on immunomagnetic separation).

Following incubation, the immunomagnetically labelled digest suspension was either exposed in the eppendorf to a magnetic field using the MPC®-E (Figure 9.2, p209), or was diluted with 9.5 ml RPMI in a test tube and exposed to a magnetic field using the MPC®-6 (Shown in Figure 9.15, p225), each experiment was performed in duplicate with a negative control using no beads.

After 3 minutes, the supernatant RPMI was aspirated using a pasteur pipette as the 'non-magnetic fraction', the eppendorf or test tube was then removed from the magnetic field and the magnetically trapped tissue resuspended as the 'magnetic fraction'. The two fractions were suspended in 1ml of RPMI stained with dithizone and the two observers counted the number of islets in each fraction and estimated the % of exocrine tissue in the magnetic compared with the non-magnetic fractions. The scores by both observers for the matching pairs were averaged and expressed as a percentage (Table 9.12).

Examination of the correlation and British Standards Institution repeatability coefficient of the 64 measurements between the 2 observers showed that for islet counts there was a significant correlation of $r=0.91 \ p<0.001$ with a mean difference of 1.9 islets ($+/-.10.36$) between observers. For exocrine estimation the correlation was even better, $r=0.97, \ p<0.001$, with a mean difference in estimated exocrine % of 0.05 ($+/-.9.4\%$).

<table>
<thead>
<tr>
<th>RATIO</th>
<th>EPPENDORF</th>
<th>TEST TUBE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-MAGNETIC</td>
<td>MAGNETIC</td>
</tr>
<tr>
<td></td>
<td>Isl %</td>
<td>Exo %</td>
</tr>
<tr>
<td>1000</td>
<td>51</td>
<td>4</td>
</tr>
<tr>
<td>500</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>100</td>
<td>91</td>
<td>35</td>
</tr>
<tr>
<td>50</td>
<td>92</td>
<td>66</td>
</tr>
<tr>
<td>10</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>99</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 9.12 Showing the percentage of islets and exocrine fragments in the magnetic and non-magnetic fractions.

The results clearly show that increasing numbers of beads increased exocrine removal in the magnetic fraction, but that this was at the expense of a simultaneous increase in the number of islets trapped. Assessment of the relative efficacy of any method clearly had to involve an arbitrary balance between these two factors.
Nevertheless, it was obvious that there was a reduction in the effectiveness of exocrine removal with ratios of less than 500:1 in the eppendorf and 1000:1 in the test tube. The best balance of islet yield and purity was achieved using ratios of 500:1 in the eppendorf which allowed removal of 93% of the exocrine contamination and only trapped 21% of the islets.

While the eppendorf clearly provided excellent immunomagnetic separation of small numbers of islets, attempts to scale up the separation using a 500:1 bead ratio, showed that the capacity of the system was limited to the purification of 500 islets from 10,000 exocrine particles using $5 \times 10^6$ beads. Above this all the islets became trapped in the magnetic fraction (Table 9.13).

The same experiment was repeated using the test tube, the results also being shown in Table 9.13 in terms of the % of islets and exocrine tissue trapped in the magnetic fraction. On this occasion the islets were allowed to settle under gravity over a period of 5 minutes, and the non-magnetic fraction involved removing only 4 ml of the RPMI from the bottom of the test tube using a pasteur pipette. The level of the RPMI in the test tube never fell below the level of the magnetically trapped fraction as a result, reducing disruption of this fraction and contamination of the non-magnetic fraction.

The control experiments (shaded cells) involved exactly the same conditions but used 'blank' Dynabeads which had been incubated in borate buffer with no LDSd9, before being washed in PBS + BSA.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Eppendorf</th>
<th>Test tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl no</td>
<td>Exo no</td>
<td>Isl %</td>
</tr>
<tr>
<td>100</td>
<td>2,000</td>
<td>41</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>100</td>
<td>10,000</td>
<td>64</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>100</td>
<td>20,000</td>
<td>48</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>500</td>
<td>10,000</td>
<td>53</td>
</tr>
<tr>
<td>1000</td>
<td>20,000</td>
<td>83</td>
</tr>
<tr>
<td>5000</td>
<td>100,000</td>
<td>98</td>
</tr>
</tbody>
</table>

Table 9.13 Showing the result of increasing amounts of digest and beads on immunomagnetic islet purification. Only the % of islets and exocrine tissue in the magnetic fractions are given, (the ideal result would be a 100% exocrine removal with 0% islet trapping).
Allowing the islets to settle under gravity and removing them without disturbing the magnetically held exocrine tissue gave substantially better results than those shown in Table 9.12. The use of the test tube also allowed scaling up, but the number of islets trapped in the magnetic fraction still increased significantly as greater amounts of digest and beads were involved. The results of the control experiments demonstrated the effect on tissue trapping of increasing numbers of even blank non-binding beads, which in numbers of $1 \times 10^7$ in the eppendorf were capable of trapping three-quarters of the tissue.

![Diagram](image)

*Figure 9.8* Diagrammatic representation of the mechanism of islet trapping within the magnetic fraction, and the resulting decrease in islet yield.
These separation experiments illustrated two major obstacles to the use of immunomagnetic methods for the separation of large multicellular tissue fragments rather than single cell suspension.

Firstly as many more beads are involved, despite their minimal magnetic remanence outside magnetic fields, while within the field they are capable of attracting each other, producing a cross-linking meshwork of beads and exocrine particles which traps islets within it (Figure 9.8). A problem which clearly increases with scaling up.

Figure 9.9 A diagrammatic representation of the fragility of the magnetic trapping of a 50μm exocrine particle.
The second problem is that the hold of the magnet on the magnetically labelled exocrine fragments is only as strong as the weakest link in the bead-antibody-antigen-exocrine linkage. The relatively massive size of the exocrine particles in comparison with that of single cells (Figure 9.9), magnifies the disruptive effect of even minimal shear forces on this linkage and results in an increase in the exocrine contamination of the non-magnetic fraction, as exocrine tissue becomes released from the magnetic fraction.

The difficulties posed by these two problems are increased by the fact that methods designed to minimise one, invariably result in an increase in the deleterious effect of the other.

The aim of the experiments described in the following sections was to try to achieve effective scaling up of the immunomagnetic separation procedure by simultaneously minimising these two problems.

iii) Comparison of side-pull and bottom-pull magnets

Three modifications to the above methods were examined for comparison;

i) A bottom pull magnet MPC®-BMP (Figure 9.10) was used.

ii) This allowed the magnetic field strength to be slowly increased over a period of 5 minutes by lowering the separation vessel into the magnetic field, and

iii) Re-exposure of the magnetic fraction to the magnetic field was performed.

*Figure 9.10* The MPC®-BMP magnetic particle concentrator for the Dynal bone marrow purging system
The rosette formation was carried out using Dynabeads in the standard manner, at ratios of 1000:1, 500:1 and 100:1. Separation of aliquots at a ratio of 500:1 in an eppendorf as previously described was used as a positive control, negative controls using blank beads were included, and each experiment was performed in duplicate.

After rosette formation, the aliquots were transferred to 24 well tissue culture plates. These were then either placed directly onto the MPC®-BMP magnet, or lowered into the field over 5 minutes using the ratcheted lowering handle (Figure 9.10). By gently sloping the particle separator, the non-magnetic fraction could be carefully removed from each well using a pasteur pipette. The 24 well plate was then removed from the magnet and half of the magnetic fractions were analysed, the other half were resuspended and exposed to the magnetic field for a second time.

The % of islets and exocrine tissue trapped in the magnetic fractions after each sequence of events is shown in Table 9.14.

<table>
<thead>
<tr>
<th>Ratio of Beads</th>
<th>PLACED DIRECTLY ONTO MAGNET</th>
<th>LOWERED INTO FIELD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 exposure</td>
<td>2 exposures</td>
</tr>
<tr>
<td></td>
<td>Isl %</td>
<td>Exo %</td>
</tr>
<tr>
<td>1000:1</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>500:1</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>100:1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>-ive cont</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 9.14 Showing the effect of rate and number of exposures to a flat magnetic field on the % of tissue in the magnetically trapped fractions.

In comparison, the positive controls separated in eppendorfs using ratios of 500:1, trapped 83% of the exocrine tissue and 26% of the islets in the magnetic fraction.

Using the flat magnet did not achieve comparable results, with a decrease in the amount of exocrine removed by the magnetic field, and ratios of 1000:1 were required to remove more than 50% of the exocrine tissue. This was almost certainly due to unavoidable disruption of the magnetically trapped tissue during removal of the non-magnetic fraction.

Re-exposure to the magnet dramatically decreased the % exocrine removal, and examination of the digest after the first exposure to the magnet showed that the rosette formation was no longer satisfactory preventing effective immunomagnetic separation.

Comparison of the separation achieved by rapid and gradual exposure to the magnetic field failed to demonstrate an obvious advantage of one over the other.
iv) **Use of flow or static exposure of digest to the magnetic field**

The use of a flow system for immunomagnetic separation had 2 attractions, firstly, it might decrease islet trapping by carrying the islets through the magnetic field in a direction tangential to that taken by the magnetically attracted exocrine fragments. Secondly, it would allow scaling up of the procedure with minimal modification.

In order to examine this the MACS magnetic cell separator was adapted by removing the mesh from a Type C column (*Figure 9.11*).

*Figure 9.11* The MACS Type C separation column with a cross-sectional area of 1.6 cm² and an inner surface area of 200cm², designed for the HGMS of 2 x 10⁸ cells. The mesh which only allowed particles <50μm in diameter through was removed before use.

Larger aliquots of 500 islets and 10,000 exocrine particles were prepared, and incubated with Dynabeads at ratios of 1000:1, 500:1 and 100:1. Each aliquot was then suspended in 5 ml of RPMI + 10% BSA in a 5 ml syringe. The modified Type C column was placed in the magnetic field generated by the MACS separator (*Figure 9.12*), and filled with RPMI +10%BSA, the syringe containing the digest was then connected to the top of the column and the suspension of digest was emptied through the magnetic field at the rate of 1ml/min.

During this, the effluent from the bottom of the column was collected as non-magnetic fraction 1 (NMF 1). Using the same syringe, two column volumes (2 x 10ml) of
RPMI + 10%BSA was flushed down the column at 1 ml/min and the effluent collected as NMFs 2 & 3 respectively. The Type C column was then removed from the magnetic field and flushed to retrieve the magnetic fraction.

Figure 9.12 Showing the MACS separator with its bipolar magnet.

After centrifugation at 400g for 2 minutes to pellet the tissue in each fraction, they were resuspended in 1 ml RPMI and assessed in the usual way (Table 9.15).

The best results were achieved using a ratio of 500:1, after flushing the column x2 (pooling NMFs 1+2+3). This produced results which were similar to those using a test tube to separate identical amounts of digest (Table 9.13, p216). However the negative control illustrated one of the problems of using this system which trapped almost a quarter of both the islets and exocrine in the magnetic fraction (twice the % trapped in the test tube using the same ratio and number of blank beads).
One of the obvious problems with all the systems was that increasing the ratio of beads also increased the trapping of islets in the magnetic fraction. To investigate whether increasing the concentration of LDSd9 used to coat the beads might increase the effective hold of the beads on their exocrine target (by amplifying the ‘zipper mechanism’ of binding) and allow a reduction in the ratio of beads needed to achieve the removal of the bead-exocrine complex, the experiment was repeated using beads coated at concentrations of LDSd9 of 32 rather than 8μg/mg.

The results are shown in Table 9.16.

### Table 9.15

| Ratio of beads | NMF 1 | | | NMF 2 | | | NMF 3 | | | MF | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | |
| 1000:1 | 25 | 10 | 33 | 16 | 44 | 23 | 56 | 80 | |
| 500:1 | 30 | 8 | 48 | 11 | 67 | 35 | 80 | 85 | |
| 100:1 | 48 | 40 | 69 | 64 | 88 | 80 | 12 | 20 | |
| -ive control | 48 | 45 | 69 | 68 | 79 | 75 | 21 | 25 | |

### Table 9.16

| Ratio of beads | NMF 1 | | | NMF 2 | | | NMF 3 | | | MF | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|   | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | Isl % | Exo % | |
| 1000:1 | 18 | 8 | 47 | 12 | 57 | 14 | 43 | 86 | |
| 500:1 | 24 | 7 | 52 | 11 | 61 | 19 | 48 | 87 | |
| 100:1 | 2 | 8 | 44 | 30 | 55 | 45 | 45 | 55 | |
| -ive control | 18 | 18 | 53 | 42 | 58 | 47 | 42 | 63 | |

These results are almost identical to those using 8μg/mg of LDSd9 to coat the beads and show that increasing the concentration of LDSd9 used to coat the Dynabeads beyond 8μg/mg had no effect on the removal of immunomagnetically labelled exocrine tissue.

This result validated the experimental protocols used in 2 ways.

i) It demonstrated the reproducible nature of the results, and

ii) It supported the hypothesis, that the results of the earlier experiments examining rosette formation, would accurately predict the strength of the immunomagnetic exocrine labelling and the ability to remove the labelled tissue in a magnetic field, since increasing the concentration of LDSd9 beyond 8μg/mg had also had no effect on this (Table 9.10).
**Figure 9.13** Using iron filings to show the shape of the magnetic field created by two repelling magnets placed either side of the tube, this contrasts with that created using a single side-pull magnet such as the MPC®-6 shown below in **Figure 9.14**.
v) Release of digest under gravity into a bipolar magnetic field

This experiment was designed to evaluate the effect of using a bipolar magnetic field (Figure 9.13), created using a pair of repelling neodymium-iron-boron permanent magnets (Magnet developments, Swindon, UK. Product no BLNI 00680), in comparison with the unipolar field created by the MPC®-6 (Figure 9.14).

Figure 9.15 Showing the digest being released from a pasteur pipette into a test tube held in the MPC®-6. The magnetically held fraction can be clearly seen midway down the back wall of the tube.

The effect of applying this field across a test tube (external diameter 12mm), or a 30ml universal container (external diameter 22mm, Sterilin, UK.) was compared, and in contrast to previous experiments, the digest instead of being suspended in the RPMI at the time of exposure to the magnetic field, was released from a pasteur pipette into the test tube.
or universal which had been 3/4 filled with RPMI and placed in the magnetic field. The
digest was seen to drift downwards under gravity into the magnetic field which was
concentrated midway down the tube (Figure 9.15).

Aliquots of 500 islet and 10,000 exocrine particles were prepared and rosetted with
8µg/mg Dynabeads. They were then separated using one of three systems, and each system
was compared over 6 separations.

i) A test tube using a side-pull MPC-6 magnet.

ii) A test tube using a bipolar magnetic field, and

iii) A universal using a bipolar magnetic field.

The islets were allowed to sediment over 10 minutes and then using a fine pasteur
pipette they were carefully aspirated from the bottom of the tube in about 1 ml RPMI (as
NMF-1). The RPMI was then completely removed from the tube using a pasteur pipette as
NMF-2, and finally the tube was removed from the magnetic field and the magnetic fraction
was resuspended in RPMI.

The results were assessed on this occasion by assaying the amount of insulin and
amylase containing tissue in each of the fractions (Appendix 2). It was found that combining
NMFs 1&2 gave an increased yield of islets with very little increase in exocrine
contamination. The % of tissue present in the magnetic fraction of each separation is
therefore shown in Table 9.17.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test tube Side-pull magnet</th>
<th>Test tube Facing magnets</th>
<th>Universal Facing magnets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isl %</td>
<td>Exo %</td>
<td>Isl %</td>
</tr>
<tr>
<td>1</td>
<td>44.148</td>
<td>96.723</td>
<td>44.059</td>
</tr>
<tr>
<td>2</td>
<td>62.560</td>
<td>99.052</td>
<td>45.976</td>
</tr>
<tr>
<td>3</td>
<td>40.312</td>
<td>93.908</td>
<td>50.555</td>
</tr>
<tr>
<td>4</td>
<td>31.786</td>
<td>98.134</td>
<td>45.488</td>
</tr>
<tr>
<td>5</td>
<td>46.621</td>
<td>89.804</td>
<td>38.569</td>
</tr>
<tr>
<td>6</td>
<td>53.230</td>
<td>87.812</td>
<td>30.038</td>
</tr>
<tr>
<td>mean</td>
<td>46.44</td>
<td>94.24</td>
<td>42.45</td>
</tr>
</tbody>
</table>

Table 9.17 Showing the % of insulin and amylase containing tissue in the
magnetic fraction for each experiment determined by assay.

The results are remarkably uniform and overall the methods removed a mean of
44.4% of the islets and 93.0% of the exocrine tissue. This is very similar to the results
obtained by visual assessment of separation of suspensions of 500 islets and 10,000 exocrine
fragments in a test tube shown in Table 9.13, where the magnetic fractions contained 42% of
the islets and 92% of the exocrine tissue.
Comparison using the Mann-Whitney U test of the results shown in Table 9.17 demonstrated no significant differences between the systems in the % of either islets trapped or exocrine tissue removed. There was a tendency however for the universal container to remove less exocrine tissue (p=0.17), presumably due to the increased distances and decreased magnetic field strengths involved.

9.5 CONCLUSIONS

The work detailed in this chapter demonstrated that IgM monoclonal antibodies could be simply coupled by physical adsorption to M-450 Dynabeads at pH 9.5, and that at a concentration of 8μg of antibody/mg of beads, these Dynabeads will form rosettes very specifically with the exocrine components of pancreatic digest.

This rosette formation is effective enough to allow rapid, non-traumatic removal of more than 90% of the exocrine tissue by a single passage through a magnetic field generated by permanent magnets. However islets are trapped non-specifically within the magnetic fraction reducing the yield to 79% (Table 9.12), a figure which falls to 44% with increasing quantities of digest and beads (Table 9.17).

A variety of methods were examined to optimise islet yield and purity. The direction of the magnetic force appeared important, with methods involving the use of side-pull magnets producing better islet purification than bottom-pull ones. Other factors such as the strength of the magnetic field at the point of separation (related to the distance from the magnet to the centre of the separation container), and the degree of dispersion of the digest at the moment of separation also appeared to be important and interrelated. This meant that while reducing the diameter of the separation device increased the magnetic force it also reduced digest dispersal and increased islet trapping, so that using an eppendorf produced islet trapping that rapidly approached 100% as scaling up was attempted, while using a universal container started to reduce exocrine removal. With the strongest currently available permanent magnets, a distance of about 6mm (produced by the test tube) appeared optimal. While the best dispersal of tissue was achieved by pre-suspension of the digest in medium before exposure to the magnetic field, release from a pipette into medium, allowing the digest to disperse and drift through the field, produced similar results, and could be used to introduce digest in a flow through system permitting application of the method to large scale separations.

Assuming a good digestion of the pancreas, immunomagnetic islet purification by negative selection and depletion of the non-islet components of the pancreas fulfils all the requirements for a simple, rapid and atraumatic alternative or adjunct to density dependent methods. The use of permanent magnets and a single (rather than multiple) passage through the magnetic field, reduces the cost of the procedure considerably, and should help to make it easy to adopt once a suitable panel of antibody coated Dynabeads is available.
CHAPTER 10
SUMMARY, CONCLUSIONS AND FUTURE WORK

10.1 DENSITY DEPENDENT ISLET PURIFICATION
i) Optimisation of differences in islet and exocrine tissue densities
ii) Use of large scale continuous density gradients on the COBE
iii) Future work on density dependent islet purification

10.2 IMMUNOMAGNETIC ISLET PURIFICATION
i) The results of immunomagnetic islet purification in the rat
ii) Further work in the rat model
iii) Development of a panel of antibody coated Dynabeads for human islet purification
10.1 DENSITY DEPENDENT ISLET PURIFICATION

One of the major obstacles to human islet transplantation remains the purification of large numbers of islets from donor pancreases, with current yields of ~200,000 being inadequate for successful transplantation in type 1 diabetics on immunosuppression. The islet yields are not only inadequate but they are also extremely variable, being dependent on the difference in density between the islets and the denser exocrine components of the collagenase digested pancreas. The work described in the first half of this thesis aimed to identify some of the reasons for this variation and to improve density dependent islet purification. The second half examined the possibility of using a much more specific immunomagnetic method of islet purification based on differences in cell surface antigens.

I) Optimisation of differences in islet and exocrine tissue densities

The work described in Chapters 4, 5 and 6 show that the effect of cold storage of tissue particularly after digestion of the pancreas can have a profound effect on the density of tissues and a significant influence on the results of subsequent density dependent islet purification. It is well known that hypothermic inactivation of cell membrane metabolic processes results in isosmotic cell swelling unless the extracellular environment is very carefully balanced in terms of its permeant and impermeant molecular species. This has led to the development of cold storage solutions such as hyperosmolar citrate and University of Wisconsin solution which contain large impermeant molecules and in the case of UW solution a colloid.

Chapter 4 compared the use of these 2 cold storage solutions for the in situ vascular perfusion and subsequent storage of the donor pancreas for periods of up to 6 hours cold ischaemia. Perhaps not surprisingly in view of the proven benefits of both solutions compared with Euro-Collins, there was no significant difference in the yield, purity or viability of the islets obtained in the two groups. However the greater cost of UW and the presence of collagenase inhibitors necessitating the use of greater amounts of collagenase enzyme effectively doubled the cost of islet purification.

Chapter 5 went on to examine the effect of storing the pancreatic digest at 4°C either in tissue culture medium (used by all groups world-wide for this) or in UW, which as a cold storage solution appeared more logical. The results showed that in MEM the tissue fragments became significantly less dense presumably due to cell swelling and that this produced a significant decrease in islet purity. In UW in contrast, the tissue increased in density producing a significant improvement in islet purification compared with MEM (p=0.035). When components in UW were added to MEM, the most important was the colloid HES which minimised any change in density, again improving purity.

To decrease the exposure of islets to damaging periods of warm ischaemia Chapter 6 examined the effect of performing density gradient purification at 4°C instead of 22°C. The results showed no significant difference despite the fact that the density gradient medium
used (500mOsm BSA) was not designed for cold storage. This led to the refrigeration of the COBE 2991 cell processor to allow large scale islet purification to be performed at 4°C.

**ii) Use of large scale continuous density gradients on the COBE**

Further optimisation of the process of large scale density gradient purification was described in Chapter 7, which described the development of a method for producing linear continuous density gradients on the COBE. The use of a continuous rather than a discontinuous gradient had numerous advantages, significantly increasing the capacity of the gradient and allowing the isopycnically distributed digest to be collected as a series of fractions after centrifugation, with only those containing islets of suitable purity being kept. The absence of wall effects on the COBE allowed the digest to be top loaded onto the continuous gradient at 4°C suspended in UW, combining the optimal conditions for digest storage and density gradient purification.

**iii) Future work on density dependent islet purification**

With the obvious effect of hypothermia and appropriate storage solutions on density dependent islet purification, a solution based on UW needs to be developed which can be used not only for the cold storage of the pancreas and the pancreatic digest as described here, but also for delivery of the intraductal collagenase at 4°C at the time of pancreas retrieval and as the basis of a gradient medium designed appropriately for use at 4°C. This is a concept which has begun to be explored in a limited way in the last year in large animal models 1228,1229.

Such a solution must avoid the use of collagenase inhibitors which prevent the use of UW for intraductal delivery of the enzyme and must also be non-toxic when warmed to 37°C during the digestion process. The identification of the components in UW which inhibit collagenase and the development of a solution without these, with an extracellular concentration of Na⁺ and K⁺ ions and including both impermeant molecules and a colloid such as HES is therefore a logical and so far unexplored progression of the work described here.

10.2 IMMUNOMAGNETIC ISLET PURIFICATION

Despite a considerable amount of research into ways of optimising the purification of islets from exocrine pancreatic fragments on the basis of physical differences in their density, it seems likely that there will always be a considerable overlap, preventing reliable purification of high islet yields. Immunomagnetic cell separation procedures provide an alternative, much more specific method based on cell surface specific antigens.
The results of immunomagnetic islet purification in the rat

Chapter 8 describes the production and purification of mouse IgM monoclonal antibodies specific to cell surface antigens expressed by the non-islet components of the rat and human pancreas. Using a monoclonal antibody to rat exocrine tissue Chapter 9 describes a series of experiments to optimise the attachment of 4.5μm Dynabeads® to exocrine fragments and use this specific immunomagnetic labelling to remove the exocrine fragments by passage through a magnetic field. Reliable removal of 90% of the exocrine fragments could be accomplished by a single exposure to a magnetic field generated by permanent magnets. The yield of islets varied however, decreasing from 79% to 56% with increasing quantities of tissue and beads.

While there is clearly scope for considerable further improvement, the adaptation of immunomagnetic cell separation techniques for islet purification looks promising, not only are the yields achieved here probably better and certainly more reliable than those achieved in human islet isolation by density dependent methods, but by combining the two methods in sequence, immunomagnetic purification offers the possibility of retrieving 60% of the islets currently discarded as contaminated following isopycnic centrifugation.

This has exciting implications for human islet transplantation, since by retrieving potentially large numbers of islets from the contaminated fractions of the continuous density gradient now used on the COBE, one to one transplantation with better HLA matching and higher success rates would become a reality.

In order to achieve this goal requires further work in two directions;

Further work in the rat model

Work on optimising the physical process of immunomagnetic islet purification using the now well-established rat model needs to continue to examine ways of further improving islet yields. One of the critical factors reducing yields as larger scale separations are attempted, appears to be the concentration of digest and beads released into the magnetic field. In order to allow the required increase in dilution of the immunomagnetically labelled digest, a system in which the digest is allowed to flow through the magnetic field appears unavoidable, but will have to be designed to prevent shear forces disrupting the attachment of the exocrine tissue to the magnet, and minimise tissue losses within the system itself.

Although the method developed appears to be rapid and atraumatic with the digest being suspended in tissue culture medium at 22°C throughout, a qualitative assessment of the viability of Dynabead purified islets remains necessary. Reversal of streptozotocin induced diabetes by syngeneic transplantation of islets in the rat model is the most valid method for assessing this, and by comparing the numbers of islets required, would allow direct comparison of the quality of Dynabead purified islets and those purified on density gradients.
iii) Development of a panel of antibody coated Dynabeads for human islet purification

IgM monoclonals such as those shown in Figures 8.11 (p182), 8.12 (p186) and 10.1 among others, were produced with the aim of combining them, to allow removal not only of the acinar tissue, but also the immunogenic vascular and ductal elements of human pancreatic digest.

Figure 10.1 Immuno-photomicrograph of LDSh8 on human pancreas
Titration experiments using visual assessment of the percentage of tissue removed by increasing ratios of each antibody coated bead should define a cut-off point above which increasing numbers of beads no longer improves separation. By combining the lowest effective ratios of each, a panel of Dynabeads with varying antibody coatings could be assembled to simultaneously remove of all the non-islet components of the digest, using the system developed and scaled up in the rat model.

In conclusion the work described in this thesis makes a significant contribution to solving one of the major remaining obstacles to successful human islet transplantation, that of islet yield. By optimising the process of density gradient purification and combining it with the much more specific process of immunomagnetic separation, reliable purification of large numbers of islets could become a reality, allowing islet transplantation from a single donor who perhaps also simultaneously donates a kidney, a procedure which would allow better matching and treatment of rejection episodes.
APPENDIX 1

Chapter 3

Chromogenic substrate pH 8.2 for Immunohistology

100ml H₂O
1.21g Trizma Base (Sigma, T1503)

pH corrected to 8.2 using 1M HCl.

Just before use
1mg/ml Naphthol AS-BI phosphoric acid (Sigma, N-5000).
1mg/ml Fast red (Sigma, F-1500)
were added and then
24mg/ml Levamisole (Sigma, L-9756, f wt 240.8).
filter through Whatman paper prior to use.

Collagenase for rat islet purification

100ml MEM
100mg Collagenase (Batch 03092C, Serva) 1mg/ml
1.5ml 1M CaCl₂
10ml DNA-ase (Sigma, DN25)

After sonication for 20 minutes this was filter sterilised.

Dithiophenylcarbazone (Dithizone) for islet staining

190mg Dithizone (Sigma D5130, f wt.256.3) dissolved in
30ml Dimethyl sulphoxide (DMSO).

After mixing overnight, filter and add 20 ml of the filtrate to 180 ml MEM containing 2%
NCS mixing continuously during the addition.

50:50 medium for hybridoma formation

300ml DMEM
300ml RPMI-1640
20 ml Penicillin/streptomycin 100U/ml
10 ml L-glutamine 2mmol
24 ml HEPES 24mmol
2 ml Sodium pyruvate 1mmol

For growing cells add 15% fetal calf serum (001010)
For HAT medium add
HAT (Sigma H0262) was added to the medium for growing cells to give
0.1mmol Hypoxanthine
0.01mmol Aminopterin
0.02mmol Thymidine

Fluorescence medium for immunohistology
100ml MEM
5ml FCS
5ml/ml 10% sodium azide

Gelatinising slides
1.5g Gelatin powder (BDH Chemicals, 44045)
300ml Warm H2O
0.15g Chromic potassium sulphate (Fisons C/5720)
The slides are immersed in slide baskets in this solution of gelatin for 10 minutes before being air dried

Gey and gey buffer for perfusion
6.5g NaCl 110mmol
2.27g NaHCO3 27mmol
0.37g KCl 5mmol
0.2g MgCl2.6H2O 1mmol
0.07g MgSO4.7H2O 0.28mmol
0.03g KH2PO4 0.2mmol
0.113g Na2HPO4 0.8mmol
this solution is gassed for 5 minutes with 95% air and 5% CO2 while adding
2ml 1M CaCl2, reducing the pH to 7.4.
0.03g glucose /100ml to give 1.7mM glucose
0.45g glucose /100ml to give 25mM glucose

MEM for use during human islet isolation
Was made up in 4 litre containers
400ml 10 x MEM
80ml Penicillin & Streptomycin 100 U/ml
40ml Fungizone
40ml HEPES 10mmol
added to 3360ml sterile water.
For MEM used in the circuit during phase 1, 2 & 3 2% NCS was added.

For washing and storing digest 10% NCS was added

For dynamic incubations

1.7mmol glucose was prepared by diluting the stock MEM 1:3 with balanced electrolyte solution to lower the glucose appropriately.

The high glucose medium was prepared by the addition of 50% glucose to the MEM.

**RPMI 1640 for islet culture**

500ml RPMI 1640

50ml FCS 10%

10ml Penicillin & streptomycin 100 U/ml

1ml Sodium pyruvate 1mmol

0.5ml 2-mercaptoethanol 0.1mmol

0.5ml Hydrocortisone 0.01mmol

10ml 1M HEPES 10mmol

1ml L-glutamine/100ml was added just before use.

**Tris buffered saline (TBS) pH 7.6 for use in immunohistology and gel filtration chromatography**

60.55g Trizma base (Sigma T1503) 50mmol

85.2g NaCl (Fisons S/3160) 150mmol

Dissolve in 500ml of H2O and adjust pH to 7.6 using 2M HCl

Make up to 1 litre for storage.

Dilute 10:1 with dd H2O for use.
### Chapter 2

**GEL ELECTROPHORESIS**

**Running buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base</td>
<td>15.14g</td>
</tr>
<tr>
<td>Glycine</td>
<td>72.00g</td>
</tr>
<tr>
<td>Sodium Lauryl Sulphate (SDS)</td>
<td>5.00g</td>
</tr>
</tbody>
</table>

Make up to 5 litres with dd H$_2$O and pH 8.3 with HCl

### Resolving gel

**FINAL ACRYLAMIDE CONCENTRATION**

<table>
<thead>
<tr>
<th>Component</th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>14.78</td>
<td>13.28</td>
<td>11.78</td>
<td>10.28</td>
<td>8.78</td>
</tr>
<tr>
<td>Resolving Buffer</td>
<td>11.25</td>
<td>11.25</td>
<td>11.25</td>
<td>11.25</td>
<td>11.25</td>
</tr>
<tr>
<td>50% Acrylamide</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.5</td>
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<tr>
<td>10% SDS</td>
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<td>0.3</td>
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<tr>
<td>TEMED</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>1.5% AM Persulphate</td>
<td>0.68</td>
<td>0.68</td>
<td>0.68</td>
<td>0.68</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**TEMED** (N,N,N',N'-Tetramethyl-ethylenediamine) (Sigma T-8133)

10% SDS (Sodium Lauryl Sulphate) 10g/100ml H$_2$O (Fisons S/5202)

1.5% Ammonium Persulphate 150mg/10ml H$_2$O (Fisons A/6160)

Resolving buffer 121.1g/l Trizma base (1M) pH8.8 with HCl

50% Acrylamide 50g Acrylamide (BDH Prod No 44313, f wt 71.08)

1.3g bisacrylamide (Sigma M-7256, mol. wt 154.2)

Make up to 100ml with dd H$_2$O

### Stacking gel

**FINAL ACRYLAMIDE CONCENTRATION**

<table>
<thead>
<tr>
<th>Component</th>
<th>7.7</th>
<th>7.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
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<td></td>
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<tr>
<td>Stacking buffer</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>50% acrylamide</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>10% SDS</td>
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<td>0.1</td>
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<tr>
<td>TEMED</td>
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<td>0.01</td>
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<tr>
<td>1.5%AM.Persulphate</td>
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<td>0.35</td>
</tr>
</tbody>
</table>

Stacking Buffer 158g/l Tris HCl (1M) pH6.8 with concentrated HCl
Sample Buffer
0.125M Tris-HCl pH 6.8
50% Sucrose (FSA Lab Supplies S/8600, mol wt 342.36)
0.005% Bromophenol Blue

Staining buffer.
0.0525g Coomassie brilliant blue G Sigma B-1131
0.0525g Coomassie brilliant blue R Sigma B-0630
2.50mls Methanol (BDH, prod no 26129)
25mls Acetic acid
225mls water

Destain.
75mls Acetic acid
50mls Methanol
875mls Water

ELISA

0.1M Glycine Buffer for ELISA
For 1 litre
7.507g Glycine
0.2033g MgCl2 0.001mmol
0.1363g ZnCl2 0.001mmol
pH 10.4

PBS for ELISA
3.402g KH2PO4
5.706g K2HPO4.3H2O
1.461g NaCl
made up to 500ml with dd H2O
pH 7.2

0.3M Na2CO3 Carbonate buffer
26.5g Na2CO3
made up to 500ml with dd H2O
pH 9.5
Chapter 2

0.5M Borate Buffer pH 9.5 for antibody binding to Dynabeads
21.02 g Citric acid (10081, BDH Chemicals Ltd., Poole, UK)
6.18 g Boric acid (Fisons B/3800)
13.8 g NaH₂PO₄·H₂O (22,352-2, Aldrich Chemical Co Ltd)
Dissolve each reagent in 100 ml of distilled water and mix to produce 300 ml.
250 ml of this stock solution is diluted to 400 ml with distilled water.
Adjust pH to 9.5 with 10 M NaOH (Fisons S/4840) and make up to 500 ml.

0.05M Tris-HCl Buffer pH 9.5 at 4°C for antibody binding to Dynabeads
6.055 g Trizma Base (Sigma T1503, f wt 121.1)
Add 500 ml of distilled water at 4°C and stir until dissolved.
Using pH meter add 1 M HCl to bring pH down to 9.5 at 4°C
Make up to a total of 1 L with distilled water.
Use at 4°C as pH changes significantly with temperature for this buffer.

0.2 M Na-Phosphate Buffer pH 7.8
5.48 g of NaH₂PO₄⁻ (Aldrich Chemicals, Catalogue number 22,352-3. f wt 137.99)
28.4 g of Na₂HPO₄ (Fisons A.R. grade S/4520. mol wt 141.96)
Make up the 5.48 g of NaH₂PO₄ in 200 ml of water produces a solution of pH about 4.7.
Make up the 28.4 g of Na₂HPO₄ in 1000 ml of water to produce a solution of pH about 9.4.
Add about 190 ml of the acid monobasic NaH₂PO₄ to the 1 litre of Na₂HPO₄ until the pH
falls to 7.5

0.01 M Phosphate buffered saline (PBS) pH 7.4
1.19 g of Na₂HPO₄ 8.4 mmol alkali
0.22 g of NaH₂PO₄ 1.6 mmol acid
8.76 g of NaCl 150 mmol
Dissolve in 1000 ml of H₂O to produce a solution of pH 7.5.

Washing and storage media
For PBS+0.1% BSA add 1.43 ml of 35% BSA to 500 ml of PBS.
For PBS+0.5% BSA add 7.16 ml of 35% BSA to 500 ml of PBS.
For 0.02% Sodium azide add 0.1 g to 500 ml of PBS0.1% BSA.
For PBS+FCS add 1.0% fetal calf serum.
These were filter sterilised before storage using a 0.22 μm filter (Falcon 7105 bottle top filter,
Becton Dickinson Labware, New Jersey, USA.)
APPENDIX 2

INSULIN RADIOIMMUNOASSAY

Reagents

RIA Buffer
Radio-immunoassay buffer. 0.5% BSA (Sigma A7030, Lot 127F-0384) in PBS.

Serum
Guinea pig anti-bovine serum (ICN Biomedicals Ltd., High Wycombe, Bucks).

125I insulin
3,1231 Iodotyrosyl A14 Insulin human, recombinant freeze dried solid. (IM166 activity 1.85MBq, Amersham International Ltd., Amersham, UK.), 20μl aliquots in 5-6 ml of RIA buffer gave 20-30K counts per tube.

Sac cell
Donkey anti-guinea pig coated cellulose suspension (IDS, Washington, UK), diluted 1:2 in PBS.

Wash solution
0.1% Triton X-100 (Sigma T6878) in distilled water.

Human Insulin Standards
Human serum (Novo Biolabs Ltd., Cambridge, UK). 40πl of human serum in 960πl of RIA buffer is 8ng/ml, serial dilutions were carried out to give 4,2,1,0.5,0.25,0ng/ml.

Equipment

LP3 Tubes and stoppers (SS.483, Sarstedt, Numbrecht, Germany)
Centrifuge (CR422, Jouan, Tring, UK)
Gamma counter (1282 Compugama CS, LKB, South Croydon, UK)
Vortex-Genie (Scientific Industries, New York, USA)

Methods

1) Samples were diluted appropriately with RIA buffer and each tube was assayed in duplicates.

LP3 Tubes were used, and for each assay the same series of controls, standards and samples were used

- Tubes 1&2 = Total counts (50μl of 125I insulin).
- Tubes 3&4 = Blanks (50μl of serum).
- Tubes 5-19 = Human Insulin Standards + 50μl of serum.
- Tubes 20-181 = 50μl of sample + 50μl of serum.

2) Each tube was vortexed and stored at 22°C for 30 minutes.
3) 50\mu l of \(^{125}\)I insulin was added to each tube and left after vortexing for 90 minutes at 22°C.

4) 50\mu l of Sac cell was added to tubes 3-181, vortexed again, and left for a further 30 minutes at 22°C.

5) 1 ml of wash solution was added to each tube, and the tubes centrifuged at 3,500g for 6 minutes (no brake). The supernatant was tipped off and the tubes capped.

6) The samples were then counted in a gamma counter which calculated a standard curve and the insulin content of the samples in ng/ml, using the Multicalc Data Management Package (Pharmacia, Milton Keynes, UK). The principle involved subtracting the mean blank count rate from the mean count rate of each set of duplicate samples. By comparison with a log plot of the % inhibition of the total counts in the insulin standards, inhibition of the total count to was used to determine the insulin concentration in the samples.

**PHADEBAS AMYLASE ASSAY**

**Reagents**

- Phadebas tablets
- Phadebas® Amylase Test (Pharmacia Diagnostics, AB Upsala, Sweden).
- 0.5M NaOH (S/4845/60, Fisons)

**Equipment**

- 12 ml conical test tubes (144AS, Sterilin)
- Cuvettes (67.742, Sarstedt, Nuembrecht, Germany)
- Spectrophotometer (LKB Ultrospec 4050, LKB Biochrom, Cambridge, UK)

**Method**

1) 4 ml of dd H\(_2\)O was pipetted into the appropriate number of conical tubes.

2) Tube 1 = MEM as a blank

200\mu l of sample appropriately diluted was added to each tube.

3) All the tubes were pre-incubated at 37°C for at least 5 minutes in a waterbath, and then 1 Phadebas tablet of starch polymer was added to each tube using forceps. The tube was then vortexed immediately for 10 seconds and placed in the waterbath.

4) Each tube was incubated for exactly 15 minutes at 37°C and 1ml of NaOH was added and the tube vortexed to stop the reaction.
5) The tubes were centrifuged at 1500g for 5 minutes. The colour reaction resulting from the release of blue dye from the water-insoluble starch polymer by the action of amylase, was assessed by measuring the absorbance at 620nm against distilled water, using semi-micro cuvettes with a 1 cm light path in the spectrophotometer.

6) The absorbance of the blank was subtracted from that of the samples and the amylase value in U/L was measured from the date coded standard curve supplied with each Phadebas kit.
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