AN INVESTIGATION INTO THE FACTORS
INFLUENCING THE PURIFICATION OF MAMMALIAN
PANCREATIC ISLETS FOR TRANSPLANTATION

by

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Statement of Originality

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

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Publications/Presentations Arising from this Thesis

Publications


Presentations

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'Islet Purification: Defining the optimal composition of islet isolation solutions'
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'Improvements in Islet Isolation Media'
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Posters

'Porcine pancreatic islet purification: the benefits of UW solution and its mechanism of action'
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'Does exocrine enzyme discharge influence islet purification?'
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COMMONLY USED ABBREVIATIONS

IDDM Insulin-Dependent Diabetes Mellitus
CSII Continuous Subcutaneous Insulin Infusion
UCT Unchanged Conventional (Insulin) Treatment
ICT Intensified Conventional (Insulin) Treatment
GFR Glomerular Filtration Rate
UAE(R) Urinary Albumin Excretion (Rate)
HbA1c Glycosylated Haemoglobin Concentration

MHC Major Histoincompatibility Complex
HLA Human Leucocyte Antigen system
ALG (Polyclonal) Anti-Lymphocyte Globulin
ATG (Polyclonal) Anti-Thymocyte Globulin
OKT3 Monoclonal mouse antibody to CD3 T-cell antigen
APC Antigen-Presenting Cell

UW University of Wisconsin Solution
HOC Hyperosmolar Citrate Solution
HBSS Hank's Balanced Salt Solution
NBCS New-Born Calf Serum
MEM Minimum Essential Medium
HEPES Hydroxyethyl Piperazine Ethane Sulphonic Acid
BSA Bovine Serum Albumin
EF Euro-Ficoll
RPMI Roswell Park Memorial Institute-1640 Tissue Culture Medium
HES HydroxyEthyl Starch
ATP Adenosine TriPhosphate

95% CI Statistical 95% Confidence Interval
Dedication

To Lucy - for her patience and understanding throughout the production of this thesis
ABSTRACT

The treatment of Type 1 (Insulin-Dependent) Diabetes Mellitus by the transplantation of isolated islets of Langerhans is an attractive concept, but one which has been difficult to realise in clinical practice. Insulin-independence has been relatively rare following human islet allotransplantation, due partly to immunological rejection of islets, but also due largely to the problems inherent in the isolation of sufficient numbers of purified islets from the native pancreas.

The purification of islets prior to transplantation is desirable for several reasons, and is usually achieved using large-scale density gradients. Current techniques of density-dependent separation of islets are inefficient, however, and result in an unacceptable loss of islet yield. The purpose of the work described in this thesis was therefore to examine the possible factors limiting the efficiency of islet purification using density gradients, and to investigate ways in which these factors may be usefully modified.

Using a standardised, quantitative system for assessing the efficiency of islet purification on density gradients, it was demonstrated that the separation of purified human and porcine islets was highly dependent upon the physico-chemical environment of pancreatic tissues throughout the isolation process. In particular, it was shown that islet purity is compromised by exocrine tissue swelling occurring during the isolation procedure, but that this swelling can be reversed, and islet purity thereby markedly improved, by suspension of the dispersed, collagenase-digested pancreatic tissues in appropriate storage solutions. The optimal composition of such solutions was defined, and their combined use both for storage of the dispersed pancreas and as a solvent for novel density gradient media was examined.

The results obtained in this way have contributed to an improved understanding of the factors potentially influencing islet purification, and may therefore assist in expediting the clinical application of islet transplantation as a therapy for diabetes.
CHAPTER 1

Endocrine Replacement Therapy in Human Insulin-Dependent Diabetes Mellitus

Section A
Insulin Therapy, Diabetic Complications and Glycaemic Control

Section B
Vascularised Pancreatic Transplantation

Section C
Pancreatic Islet Tissue Transplantation
It is now widely accepted that Type 1 (Insulin-Dependent) Diabetes Mellitus (IDDM) is an auto-immune disease, producing destruction of the insulin-secreting \( \beta \)-cells of the pancreatic islets of Langerhans, and that the metabolic disturbances characteristic of the disease are a result of this process.

The discovery of insulin in 1922, and its subsequent introduction into clinical use revolutionised the treatment of this previously universally fatal condition but, despite many advances in the intervening years, the current treatment of IDDM with parenterally administered exogenous insulin remains unsatisfactory, particularly with regard to the degree of metabolic control which can be achieved and to the ever-present risks of hypoglycaemia and ketoacidosis.

In addition, patients with Type 1 diabetes mellitus are susceptible to the long-term development of certain complications, in particular nephropathy, retinopathy, neuropathy and vascular disease, and evidence has begun to accumulate in recent years that the development of these complications is related, at least in part, to the long-term control of the metabolic disturbance in diabetes. Considerable effort has therefore been made to develop alternative methods for replacing the endocrine function of the \( \beta \)-cell, namely by transplantation of the pancreas, either as a vascularised intact pancreatic graft or as isolated islets of Langerhans. Islet transplantation has a number of theoretical advantages over transplantation of the whole pancreas, particularly with respect to the relative safety of the two procedures, but it has taken many years to develop the techniques of islet isolation sufficiently to allow clinical trials of islet transplantation in human diabetes to be conducted. Nevertheless, these trials have now been initiated, with promising early results.

The purpose of this chapter is to examine the rationale behind islet transplantation for the treatment of human diabetes.

Firstly, in Section A, the evidence that diabetic complications can be prevented or ameliorated by optimising glycaemic control is reviewed and the limitations of insulin therapy in achieving such optimal control are discussed.

In Section B the clinical results of vascularised pancreatic transplantation are reviewed, with particular reference to the degree of metabolic control which can be maintained by a pancreatic allograft, the influence that this has upon diabetic complications, and the factors limiting the application of this technique in the treatment of human diabetes.

Finally, in Section C the potential advantages of islet transplantation are discussed and the preliminary results obtained from clinical trials reviewed.
SECTION A
INSULIN THERAPY, DIABETIC COMPLICATIONS AND GLYCAEMIC CONTROL

Insulin Therapy

For many years the standard method of insulin administration has been by once or twice-daily subcutaneous injection. Such regimes of insulin therapy, however, do not restore normal metabolism (MacGillivray et al, 1982). Even when using a mixture of purified insulins with rapid and prolonged absorption profiles, the normal pattern of insulin release (meal-time peaks, superimposed upon pulsatile basal insulin release; pulsatile release being important in maximising the metabolic effects of secreted insulin (Lang et al, 1979; Matthews et al, 1983; Rosario et al, 1986)) is not approximated. Recently, therefore, a number of developments in insulin therapy have been made, largely directed towards more closely matching physiological insulin delivery, in an attempt to achieve normal metabolism.

Multiple Injection Therapy

It has been theoretically possible for many years to produce more physiological insulin delivery by providing basal insulin supply with 'long-acting' insulin and meal-time supply with injections of 'short-acting' insulin immediately prior to meals. However, it has only been with the introduction of home blood glucose monitoring (Sønksen et al, 1978; Walford et al, 1978), enabling diabetic patients to tailor individual insulin doses to prevailing blood glucose concentrations, that the true potential of this treatment method has been realised. Meanwhile, the use of pen injectors rather than more cumbersome insulin syringes and vials has increased the popularity of this method with patients, and the introduction of clinical measurements (such as glycosylated haemoglobin concentration) which reflect long-term glycaemic control (Koenig et al, 1976; Gabbay et al, 1977; Gonen et al, 1979) has enabled the potential benefits of such therapy to be assessed. Unfortunately, achieving optimal glycaemic control with this system requires considerable training and effort on the part of the patient, needs specialised equipment, and carries an increased risk of hypoglycaemic coma (Unger, 1982; Leslie et al, 1986; Reichard et al, 1991a).

Artificial Endocrine Pancreas

In 1974 Albisser (Albisser et al, 1974) described the use of a closed-loop system which acted as an artificial endocrine pancreas, continuously analysing blood glucose levels
via a dual-lumen intravenous catheter and using a computer to maintain physiological blood glucose levels by the controlled intravenous infusion of insulin or dextrose. Using such a system normoglycaemia can be maintained during in-patient treatment, but its widespread application is currently limited by the great bulk and expense of each machine, and by the need for intravenous access, with its attendant problems of thrombosis and infection. Although the possibility is being examined of developing glucose sensors which are miniaturised and capable of monitoring subcutaneous rather than intravenous glucose concentrations (Meyerhoff et al, 1992), these applications are unlikely to be available for clinical use in the near future.

**Continuous Insulin Infusion**

Continuous delivery of insulin was initially developed using externally-worn, battery-driven syringe pumps, with subcutaneous administration of insulin (Pickup et al, 1978). These devices deliver a constant basal insulin supply, supplemented by preprandial boluses upon manual operation of a switch on the side of the pump, and have been demonstrated to improve glucose, amino acid and lipid metabolism in in-patients previously managed by conventional insulin therapy (Pickup et al, 1979; Tamborlane et al, 1979).

Direct comparison, however, between continuous subcutaneous insulin infusion (CSII) systems and optimised conventional insulin therapy in the out-patient setting have demonstrated only modest improvements in glycaemic control with CSII (Marshall et al, 1987; Helve et al, 1987). Furthermore, significant complications can result from CSII therapy (Mecklenburg et al, 1984; Chantelau et al, 1989), in particular local infection/inflammation at the infusion site and ketoacidosis, which may rapidly follow dislodgement of the infusion catheter due to the lack of any subcutaneous depot of insulin.

Continuous delivery of insulin by the intravenous route has been attempted, but long-term use of these devices is limited, again by the thrombotic and infective complications of long-term vascular access (Irsgler et al, 1979).

The need to carry the infusion device at all times has also been approached by long-term implantation of remote-controlled pumps and in most such cases insulin delivery has been via the intra-peritoneal route (Irsgler et al, 1981; Point Study Group, 1988). Although this method can achieve good metabolic control over prolonged periods of time, considerable problems remain, particularly related to infection at the implantation site, occlusion of the infusion catheter and the need for replacement of batteries and malfunctioning devices.
In terms of short-term glycaemic control, there is little to choose between these methods of intensified insulin therapy (Rizza et al, 1980). However, considerations of cost-effectiveness, patient preference and simplicity of use have meant that multiple injection therapy and CSII have become the most commonly used techniques in patients aiming to achieve 'optimal' glycaemic control. Alternative routes of insulin delivery (eg. oral, nasal) have also been investigated, but to date none has shown sufficient promise of improvement over the subcutaneous route (Home et al, 1989). It is apparent, therefore, that, despite the advances mentioned above, no technique of exogenous insulin delivery is yet available which provides long-term normoglycaemia safely in the out-patient population of Type 1 diabetics.

**Diabetic Complications**

In addition to the need for insulin-replacement therapy, Type 1 diabetes mellitus is characterised by a tendency to develop certain chronic complications, most importantly retinopathy, nephropathy, neuropathy and vascular disease, and despite recent improvements in clinical management of diabetes, these complications continue to represent a major source of morbidity and mortality amongst the diabetic population. The remainder of this section will therefore review the pathology and natural history of these conditions, and the influence of treatment upon their development.

**Retinopathy**

Retinopathy is the commonest long-term complication of IDDM and is characterised by a well-defined progression of pathological changes in the retinal microvasculature, taking place over many years. Two stages in the development of retinopathy are described (Kohner, 1991), namely the background and proliferative stages. The earliest changes of *background* retinopathy are generally not immediately sight-threatening. Initially, capillary dilatation is observed, followed by loss of capillary pericytes, resulting in the formation of microaneurysms, visible as small red dots on fundoscopy. Progression of retinopathy is then accompanied by areas of capillary occlusion; an increase in the number of microaneurysms; increased microvascular permeability resulting in the formation of retinal oedema and lipid-rich hard exudates; and the appearance of intraretinal haemorrhage.

These changes are not usually associated with significant visual loss, unless the macula is directly affected. Continuing capillary occlusion, however, results in the development of areas of retinal ischaemia, which in turn stimulates the growth of abnormal, pre-retinal new vessels and associated fibrous tissue. These *proliferative*
changes may then result in visual loss through pre-retinal haemorrhage or through fibrous tissue retraction causing distortion, and ultimately detachment, of the retina. Blindness may also result from proliferative changes affecting the anterior chamber of the eye, due to the development of secondary, neovascular glaucoma.

Visual prognosis in diabetic retinopathy is generally poor once proliferative changes have developed, but has been improved by the recognition of changes which are highly predictive of imminent new vessel formation (cotton-wool spots and intra-retinal microvascular abnormalities, IRMA) and the introduction of treatment with laser photocoagulation during this 'pre-proliferative' stage.

The development of retinopathy is related to the duration of diabetes (Palmberg et al, 1981; Klein et al, 1984). The initial, background changes are rare during the first five years of diabetes, but develop rapidly thereafter and are almost ubiquitous in patients with IDDM of more than 20 years standing (Figure 1). Progression to proliferative disease is not inevitable, however, once background changes have developed. Indeed, even after 40 years of diabetes only up to 62% of patients suffer proliferative retinopathy, of whom about 10% have severe visual loss (Klein et al, 1984).

Nephropathy

Diabetic nephropathy is also characterised by a progression of pathological changes, in this case influencing the structure and function of the renal glomerulus (Deckert et al, 1991).

Morphologically, these changes consist of thickening of the glomerular capillary basement membrane, expansion of the mesangium with accumulation of basement membrane-like, electron dense material, and hyalinosis of the afferent and efferent arterioles.

Clinically, diabetic nephropathy is characterised by changes in urinary protein (particularly albumin) excretion. Initially, urinary albumin excretion is within the normal range (<30 mg/24 hours), although the kidneys are often enlarged and the glomerular filtration rate (GFR) increased above normal ('hyperfiltration'). This stage may then be followed by a prolonged phase of 'incipient nephropathy', during which there is a gradual increase in urinary albumin excretion, in the range 30-300 mg/24 hours. Although above the normal range, this degree of albumin excretion ('microalbuminuria') is not sufficient for detection using routine dip-stick tests for urinary protein (eg. Albustix), and sensitive radioimmunoassays for albumin must therefore be used.

A large proportion of patients with microalbuminuria will then progress into overt diabetic nephropathy, defined by a urinary albumin excretion rate consistently greater than 300 mg/24 hours (equivalent to a total urinary protein of >500 mg/24 hours), in
Figure 1
Cumulative Risk of Diabetic Complications According to Duration of Type 1 Diabetes Mellitus

Cumulative Risk (%)

Duration of Diabetes (Years)

- Background Retinopathy
- Nephropathy
- Proliferative Retinopathy
- Atherosclerosis
A. Diabetic Retinopathy
PLATE 1.2
CHRONIC COMPLICATIONS OF DIABETES MELLITUS

B. Haemodialysis for End-Stage Diabetic Nephropathy

C. Peripheral Gangrene: Contributed by Peripheral Macro- and Micro-Vascular Disease and by Diabetic Somatic Neuropathy
the absence of other renal disease, urinary infection or cardiac failure. During this phase blood pressure becomes elevated and there is a gradual reduction in GFR, at a rate of approximately 10 ml/min/1.73 m² per year, which progresses inexorably towards the final phase, that of uraemia and end-stage renal failure.

Not all Type 1 diabetic patients are susceptible to the development of nephropathy, so that even after more than 40 years duration of diabetes only 40-45% of patients will progress to persistent proteinuria (Andersen et al, 1983; Krolewski et al, 1985; Krolewski et al, 1987). However, amongst this subgroup of patients prognosis is extremely poor. Other long-term complications, such as retinopathy, are almost ubiquitous, and progression to end-stage renal failure occurs in the majority of cases within the following 10-15 years (Krolewski et al, 1985). Uraemia is the cause of death in about two-thirds of these patients, and may account for about 15-20% of premature deaths in juvenile-onset diabetes overall (Deckert et al, 1978; Morsley et al, 1983; Krolewski et al, 1985). Mortality from cardiovascular causes is also markedly increased in patients with nephropathy compared to diabetic patients who do not develop proteinuria (Andersen et al, 1983; Borch-Johnsen et al, 1985; Krolewski et al, 1987). Overall, therefore, life expectancy is considerably reduced once clinical nephropathy develops, due to a combination of renal failure and accelerated atherosclerosis, and median survival is only about 7-10 years.

**Neuropathy**

Neurological abnormalities in diabetes are highly heterogeneous, both clinically and pathologically, and for this reason it has proven difficult to define accurately the pathology, epidemiology and natural history of diabetic neuropathy. Histological studies of human diabetic neuropathy have been largely confined to investigation of autopsy material or small biopsies from easily accessible nerves whose removal does not result in serious sequelae (eg. the sural nerve). The pathological changes described have been highly variable, but have generally included elements of axonal atrophy (Yagihashi et al, 1979), segmental demyelination and remyelination (Dyck et al, 1980; Sima et al, 1988; Dyck et al, 1988), deformation of myelin sheath-nerve complexes at the nodes of Ranvier ('axo-glial dysjunction'; (Sima et al, 1988)), and abnormalities of endoneurial capillaries (Fagerberg, 1959; Williams et al, 1980; Dyck et al, 1985).

More recently, attention has also focused upon biochemical abnormalities identified within the nerves of diabetic subjects, and in particular to derangements in the activity of the polyol pathway, by which glucose is metabolised to sorbitol (Greene et al, 1987; Dyck et al, 1988). However, the contribution of these changes to the development of clinical neuropathy is not fully established.
A number of clinical syndromes accompany these pathological changes (Thomas, 1991), the two most important being: a symmetrical, distal sensory polyneuropathy, maximal in the lower limbs, and contributing to diabetic foot ulceration and neuropathic arthropathy; and autonomic neuropathy, associated with a range of symptoms, such as postural hypotension, gastric stasis, diarrhoea and impotence, and possibly contributing to reduced awareness of insulin-induced hypoglycaemia. Autonomic neuropathy may also contribute to the increased mortality of patients with IDDM, particularly if clinical tests of cardiovascular reflex function are abnormal (Ewing et al, 1980).

Estimates of the prevalence of neuropathy differ widely, depending upon the definition employed. Its development does appear, however, to be related to the prior duration of diabetes (Gregersen, 1967; Pirart, 1978), and evidence of neuropathy may be present in about 40% or more of long-term diabetic patients.

**Macroangiopathy**

The increase in cardiovascular mortality of diabetic patients with nephropathy has been mentioned above. Even in the absence of nephropathy, however, patients with IDDM are at greatly increased risk of morbidity and mortality from cardiovascular and cerebrovascular disease (angina, myocardial infarction and stroke) and from peripheral vascular disease (intermittent claudication, diabetic foot disease, amputations). Overall, cardiovascular mortality is increased about 2-3 fold in men and about 6-7 fold in women with IDDM, compared to age-matched controls (Garcia et al, 1974; Krolewski et al, 1987), and similar relative risks have been observed for the consequences of peripheral vascular disease.

Pathologically, the changes of diabetic macroangiopathy appear to be identical to those of atherosclerosis affecting non-diabetics. Presumably, however, the atherosclerotic process may be accelerated in patients with IDDM due to the association with diabetes of certain risk factors also important in the pathogenesis of atherosclerosis (Steiner, 1981; Jay et al, 1991). Such factors may include hyperlipidaemia, hypertension, haemostatic abnormalities (such as elevated fibrinogen levels and abnormal platelet function) and hyperinsulinaemia (Stout, 1987; Jarrett, 1988; Eschwege et al, 1992), although the relative contributions of these factors is unknown.

Unfortunately, the aetiology and pathogenesis of these diverse complications are not fully established. Although the obvious metabolic abnormalities of IDDM have long been assumed to be important, there is evidence that genetic and possibly other factors may play an important role (Krolewski et al, 1987; Rosenstock et al, 1988). Nonetheless, the observation that the non-diabetic identical twins of patients with Type
1 diabetes do not develop microvascular complications (Pyke et al, 1973; Leslie et al, 1982; Steffes et al, 1985) suggests that the metabolic abnormalities of diabetes are of primary importance. Therefore, implicit in the development of improved techniques for insulin administration has been the assumption that tighter 'control' of metabolism, in particular blood glucose, might result in prevention, arrest or even reversal of these long-term microvascular complications in diabetics. Indeed, evidence in support of this hypothesis has begun to accumulate in recent years, partly from studies in experimental diabetes in animals, and partly from clinical trials in diabetic patients. Resolution of this issue has important implications for any form of endocrine replacement therapy in IDDM, and this evidence will, therefore, be reviewed in some detail.

**The Influence of Glycaemic Control on Complications**

**Experimental Diabetes**

Micro-vascular lesions, similar to those seen in the organs affected by long-term complications in human diabetes, can be induced in experimental animals rendered diabetic through the use of β-cell toxins (such as streptozotocin or alloxan), pancreatectomy, or through spontaneous diabetes. The advantages of such an approach are that the development of diabetic microvascular complications can be studied under controlled laboratory conditions; the use of inbred strains of animals allows some degree of control over genetic influences on the development of complications; and the shorter time-scale involved in the evolution of experimental lesions expedites results. The relevance of the microvascular pathology induced in these animals to that seen in human diabetes is unknown, however. For instance, whilst diabetic rats develop glomerular changes histologically similar to those seen in human diabetic nephropathy, they do not undergo the progressive decline into end-stage renal failure seen in the human disease. Likewise, experimental diabetes in dogs leads to 'background' retinopathy similar to that seen in humans, but not to the sight-threatening pre-retinal proliferative disease of human diabetes. Furthermore, the development of vascular lesions in experimental diabetes has been induced in most studies using levels of hyperglycaemia which would be unacceptable in human diabetes. Finally, whilst many such studies have provided support for the role of hyperglycaemia in the development of diabetic complications, few have addressed the question of when, and to what extent, the metabolic disturbance must be corrected, in order to influence the subsequent progression of such complications once diabetes has been induced. This, however, is the question of greatest relevance to human diabetes. In view of the controversy concerning the relevance of experimental diabetes to the human
disease, only those studies which have directly addressed this issue will be discussed further.

Engerman (1977) examined the development of retinopathy in outbred adult alloxan-diabetic dogs. Following induction of diabetes, twenty dogs were randomised into two equal groups: a poor-control group, in which the administration of insulin was insufficient to prevent severe hyperglycaemia and glycosuria; and a good-control group, given sufficient insulin to minimise hyperglycaemia/glycosuria. The development of retinopathy was examined over 5 years in each group, and in a control group of non-diabetic animals, by fundoscopy and by light and electron-microscopy of eyes removed at necropsy upon completion of the study. Significant differences in plasma glucose levels and urinary glucose excretion were successfully maintained between the two treatment groups. After 5 years, retinopathy in the good-control group, assessed as the number of capillary microaneurysms per eye, and the density of acellular capillaries and pericyte ghosts, was comparable to that seen in the non-diabetic group and significantly less than that of the poor-control group. Although the non-diabetic group was unusual in this study, in containing three alloxan-treated but non-diabetic dogs, the results clearly indicated that the development of retinopathy could be prevented by control of hyperglycaemia using exogenous insulin in diabetic dogs, and that glycaemic control did not have to approximate to that of non-diabetic animals in order to achieve this (mean urinary glucose excretion was still 4 g/day in the good control group). However, even in achieving this level of glycaemic control hypoglycaemia was a problem, resulting in the death of one animal in the good-control group.

A similar design of study was employed by Rasch (Rasch, 1979a; Rasch, 1979b), in which the progression of glomerular changes over 6 months in streptozotocin-diabetic rats was examined. Inbred rats were either well or poorly-controlled using subcutaneous insulin injection and were compared with a non-diabetic group. Glomerulopathy in the rat (as in the human) is characterised by increases in glomerular capillary basement membrane thickness, mesangial volume and the amount of basement membrane-like material in the mesangium, and these parameters were assessed by electron microscopy of kidney sections. After 6 months there were no significant differences in glomerular structure between the non-diabetic group and the well-controlled group, whilst all parameters of nephropathy were markedly and significantly increased in the poorly-controlled group.

More recently Kern (Kern et al, 1990) also reported the effects of improved glycaemic control on the development of glomerulopathy in alloxan diabetic dogs over 5 years. In addition to the poorly-controlled and well-controlled groups employed in the study mentioned above, a further group of animals underwent poor control for 2.5 years,
followed by good control for 2.5 years, and long-term metabolic control was more accurately compared between groups using glycosylated haemoglobin estimations. Good glycaemic control from onset of the study largely prevented the development of glomerular changes which were present to a marked extent in the poor control group. However, institution of good control after 2.5 years of poor control only arrested, but did not reverse these glomerular changes.

Similar results were obtained by Petersen (Petersen et al, 1988), who examined the influence of improved glycaemic control using continuous subcutaneous insulin infusion upon established changes of glomerulopathy in the rat. Again, after six months of diabetes existing mesangial changes could be arrested, but not reversed by improved control, although the level of control achieved using their insulin infusion system was far from optimal.

The impact of normoglycaemia upon nephropathy in streptozotocin-diabetic rats was studied by Mauer (Mauer et al, 1975). In this study light and immuno-fluorescent microscopy of renal biopsies were used to assess glomerulopathy (localisation of periodic acid-Schiff-positive material and immunoglobulins in the mesangium) in rats six to eight months following induction of diabetes, and subsequently at three time intervals following normalisation of glucose metabolism by pancreatic islet transplantation. Mesangial thickening, present after six months of diabetes, was reversed by islet transplantation within two weeks, indicating that normalisation of metabolism (in contrast to merely improved metabolism produced by insulin treatment) might be able not only to prevent the development, but also to reverse certain established changes of glomerulopathy in the rat. Interestingly, however, regression of the mesangial changes was also observed in three animals which remained diabetic following islet transplantation. Insulin levels were within the normal range in these animals and it was suggested therefore that insulin deficiency may be more important than hyperglycaemia in the pathogenesis of mesangial changes in the rat.

Islet transplantation was also used to establish normoglycaemia in streptozotocin-diabetic mice (Hoffman et al, 1983), and the development of glomerulopathy was compared with non-diabetic controls, and with diabetic animals, both untreated and managed using insulin injection therapy. Glomerular changes were largely prevented by good diabetic control using insulin (resulting in a normal glycosylated haemoglobin level, but mean fasting blood glucose levels significantly higher than non-diabetic animals), and were completely prevented by islet transplantation (associated with normal blood sugars and normal HbA1c).

Increased urinary albumin excretion, accompanying these morphological changes of nephropathy, has also been reversed by pancreatic transplantation in streptozotocin-diabetic rats (Otsu et al, 1992). Urinary albumin was restored to normal if successful
transplantation was undertaken within 4 months of induction of diabetes, but only partially corrected if transplantation was performed after 8 months, again suggesting that improved glycaemic control may only influence the progression of diabetic complications if instituted at an early stage.

Long-term follow-up studies of glomerular changes in rats treated by vascularised pancreaticoduodenal transplants or intraportal islet transplants were undertaken by Orloff (Orloff et al, 1987; Orloff et al, 1988). Strict normoglycaemia was maintained for 2 years by the vascularised pancreatic grafts, and in this group of animals glomerular histology was indistinguishable from non-diabetic control animals. In contrast, intraportal islet grafts were able to maintain normoglycaemia for only three months, following which mild hyperglycaemia ensued; although blood glucose levels in these animals were significantly lower than untreated diabetic animals, glomerulopathy developed at a similar rate in these two groups. These findings suggested that glycaemic control may need to approximate that of non-diabetic animals to prevent nephropathy over more prolonged periods of follow-up. The failure of islet transplants to maintain normoglycaemia for greater than three months was also of concern, and this topic will be discussed in more detail in Chapter 2.

One problem in interpretation of studies of experimental diabetes using β-cell toxins, however, has been the observation that these agents may themselves cause renal damage. A novel approach to this problem was employed by Lee (Lee et al, 1974). Kidneys from non-diabetic rats were transplanted into syngeneic rats rendered diabetic with streptozotocin six months previously, and kidneys from streptozotocin diabetic rats were also transplanted into non-diabetic rats. Glomerular changes were assessed in renal biopsies two and four months after transplantation and compared with biopsies obtained prior to transplantation.

Normal kidneys transplanted into diabetic rats developed light- and immunofluorescent-microscopy changes of diabetic glomerulopathy within two months. Kidneys from diabetic animals, however, demonstrated arrest or regression of these changes two months after transplantation into non-diabetic recipients, whilst the (contralateral) kidneys remaining in the diabetic animals continued to deteriorate. Thus, the development of diabetic glomerulopathy in rats appears to be entirely attributable to the metabolic abnormalities of diabetes, rather than the inducing agent, and is reversible if these abnormalities are fully corrected.

The influence of glycaemic control on neuropathy in experimental diabetes has been less extensively investigated, partly due to the paucity of well-defined morphological abnormalities in the somatic nerves of diabetic animals. However, histological changes have been observed in the mesenteric autonomic nerves of diabetic rats which may be ameliorated by improved glycaemic control using islet transplantation or insulin therapy.
Moreover, CSII therapy over 8 weeks in rats has been found to prevent the development of certain biochemical abnormalities of experimental somatic neuropathy, namely accumulation of glucose, sorbitol and fructose in nerves, with depletion of myoinositol (McCallum et al, 1986).

In conclusion, it appears that microvascular disease in experimental diabetes can be prevented by improved glycaemic control and arrested, or even reversed, by restitution of normoglycaemia at a suitably early phase.

**Clinical Trials**

At best, the experimental data summarised above is only suggestive that improved glycaemic control might prevent or ameliorate long-term complications in diabetic patients. Providing direct evidence in favour of this hypothesis in humans has proven difficult, however, for several reasons.

Firstly, all patients with IDDM are, of course, treated with insulin in order to avoid profound hyperglycaemia/glycosuria: there is, therefore, no 'untreated control' group (nor deliberately poorly-controlled group) with which to compare the results of optimised insulin therapy, unlike the situation with experimental diabetes. Maintenance of differences in long-term metabolic control between treatment groups, sufficiently large to allow detection of differences in the development of complications, has therefore been dependent upon the improvements in methods of insulin delivery mentioned above. Furthermore, even these methods do not result in normal metabolism, so that most clinical trials have essentially compared two sub-optimal methods of glycaemic control, thereby reducing the sensitivity to detect any beneficial influence of improved metabolism.

Secondly, meaningful assessment of long-term metabolic control cannot be made on the basis of intermittent plasma/urinary glucose estimations, and has therefore been dependent upon the development of assays reflecting mean concentrations of blood sugar eg. glycosylated haemoglobin or fructosamine levels.

Thirdly, assessment of the long-term complications of diabetes is problematical. Although the retina is accessible to direct inspection, using fundoscopy and fluorescein angiography, it is clearly unethical to obtain tissue routinely for histological examination from the other organs affected by long-term complications (with the exception of skeletal muscle biopsy to assess capillary basement membrane thickening), so that indirect measures of function are necessary to assess these organs. Any improvement in these measures cannot then be automatically assumed to result in prevention of long-term clinical sequelae.
Despite these and other limitations, however, a number of recent studies have been performed which suggest that improved glycaemic control may have a beneficial influence upon the progression of long-term diabetic complications. Those studies which have examined the influence of pancreatic transplantation on complications in diabetic patients will be discussed in Section B of this chapter. The remaining studies will be the subject of the following discussion.

Retrospective Studies
Many studies have been performed which suggest that the development of long-term complications is related to quality of glycaemic control (Keiding et al, 1952; Hardin et al, 1956; Johnsson, 1960). In most cases, however, these have involved cross-sectional analysis of diabetic populations, comparing complication rates in patients retrospectively ascribed to either well-controlled or poorly-controlled groups. In many cases the number of patients studied is small, and the data used to assess complications and control inadequate (Kaplan et al, 1973), so that only limited conclusions can be drawn from these investigations.

One such study is, however, notable due to the large number of patients examined. Pirart (1978) examined the development, over up to 40 years, of long-term complications in 4,398 patients (approximately 25% of which were insulin-dependent) retrospectively ascribed to groups having good, fair and poor glycaemic control. Although the aetiology of diabetes was heterogeneous and there was a considerable reduction with time in the number of patients continuing to be studied, a clear correlation was observed between the level of glycaemic control and the prevalence of retinopathy, nephropathy and neuropathy, particularly in insulin-dependent patients.

The principal limitation of retrospective analyses, however, is that the finding of an association between control and the development of complications does not establish a causal relationship between the two. As mentioned above, the crucial question is whether institution of improved metabolic control influences the subsequent onset of complications - this can only be answered by prospective controlled trials.

Prospective Controlled Trials: Retinopathy
Job (Job et al, 1976) examined the progression of retinopathy in forty-two insulin-dependent diabetic patients randomly assigned to either single insulin injection/day or multiple insulin injection/day groups. Initial follow-up was for three years, and the results of a fourth year of follow-up were subsequently reported (Eschwege et al, 1979). Although the reported progression in microaneurysm counts was statistically significantly higher in the single-injection group, this study suffered numerous flaws which make interpretation of these results difficult. For instance, 5/21 patients in the
single-injection group were changed during the course of the study onto multiple-injection therapy, whilst in the multiple-injection group 2/21 patients actually received single-injection therapy for at least three months and only 4/21 patients received the planned three injections/day regime for the whole of the study. Furthermore, there were only marginal differences between the two groups in the parameters of diabetic control studied (mean fasting blood sugar and urinary glucose excretion) and there was quite marked deterioration of retinopathy even in the multiple-injection group.

Holman (Holman et al, 1983) studied 74 patients with background retinopathy, randomised to receive either their usual diabetic care (the majority being on single insulin injection/day regimes) or alternative therapy involving adjustment of insulin doses in response to home-monitored blood glucose levels, more frequent clinic visits and individual dietary advice. Follow-up was for two years, and retinopathy was assessed by fundoscopy, a retinopathy index being calculated to include features such as number of cotton-wool spots and areas of haemorrhage and exudation, in addition to simple microaneurysm counts. Although glycosylated haemoglobin concentrations were significantly lower in the alternative therapy group at 1 year, the size of this improvement was small, and improvement in the 'usual-care' group over the final year resulted in similar final concentrations in the two groups. Interestingly, although long-term glycaemic control was far from optimal by present day standards, retinopathy did not progress significantly in either group, nor were there any significant differences between the two groups upon completion of the study.

These studies essentially only confirmed the difficulty in maintaining meaningful differences in long-term glycaemic control between two treatment groups using conventional methods of insulin delivery. However, the introduction of continuous subcutaneous insulin infusion (CSII) via insulin pumps enabled such differences to be sustained throughout a clinical trial, and several such trials were subsequently performed.

Lauritzen and colleagues of the Steno Study Group in Denmark (Lauritzen et al, 1983) studied 30 insulin-dependent diabetics with background retinopathy, randomised to receive either unchanged conventional treatment (UCT- the majority receiving two insulin injections/day) or optimised insulin delivery via CSII. Significant differences in glycaemic control were maintained between the two treatment groups over the course of the study, fasting blood glucose and HbA1c levels being substantially improved in the CSII group compared to levels at baseline and in the UCT group. After 1 year follow-up retinopathy had progressed in both groups of patients, but surprisingly the frequency of deterioration was greatest in the CSII group, and was particularly high amongst those patients attaining the lowest mean blood glucose levels. During a further 1 year follow-up, however, improvement in retinopathy was more common in the CSII
group and deterioration was commoner in the UCT group, so that the two groups did not differ significantly upon conclusion of the study (Lauritzen et al, 1985). Similar findings were obtained in a larger, multicentre prospective trial involving 68 diabetic patients with background retinopathy randomised again to either unchanged conventional insulin therapy or CSII with frequent self-monitoring of blood glucose levels (Kroc Collaborative Study Group, 1984). Sustained improvement in long-term glycaemic control using CSII was achieved throughout the study, whilst no change was observed in the UCT group. Retinopathy was assessed using slightly different methods to those used in the Steno study, but again, after 8 months deterioration in retinopathy scores had occurred more frequently in the CSII group compared to the group receiving conventional therapy. This unexpected finding again stimulated a further assessment after a final follow-up period of 2 years (Kroc Collaborative Study Group, 1988). Unfortunately, only about 70% of patients continued to be treated according to the original protocol. However, optimised glycaemic control with CSII was now associated with an improvement in retinopathy, such that retinal morphology was indistinguishable between the two groups upon completion of the study.

In a further study performed in Oslo (Dahl-Jorgensen et al, 1985; Dahl-Jorgensen et al, 1986), the progression of early retinopathy was assessed during treatment with CSII, conventional and intensified insulin injection regimes. Unfortunately, the numbers of patients in each group were small, but once again there was evidence of an early acceleration of retinopathy, followed by stabilisation, with CSII, compared to steady deterioration in the conventionally treated group. After two years micro-anneurism counts and haemorrhages were in fact less frequent in the two 'well-controlled' groups than in the 'poorly-controlled' group. After seven years (Brinchmann-Hansen et al, 1992), reduction in glycosylated haemoglobin continued to be associated with lesser progression of retinopathy, but multiple patient cross-overs between treatment groups made further interpretation of this study difficult.

Interestingly, rapid deterioration of retinopathy has also been described in a number of non-controlled studies and case-reports, when tight blood glucose control has been instituted using CSII or intensified conventional insulin therapy (Daneman et al, 1981; Lawson et al, 1982; Lorenzi et al, 1983; van Ballegooie et al, 1984). Specifically, deterioration has involved the appearance of cotton-wool spots, intra-retinal microvascular abnormalities (IRMA) and new vessels, changes which reflect retinal hypo-perfusion. Hyperglycaemia is associated with an increase in retinal blood flow (Kohner et al, 1975). It has been postulated, therefore (van Ballegooie et al, 1984; Keen, 1984), that rapid or large reductions in blood glucose concentration, by reducing retinal perfusion, may precipitate a deterioration in retinopathy, particularly in areas of the retina susceptible to hypoperfusion by virtue of pre-existing vascular changes. If
this is the case, then such deterioration will depend upon both the degree of hyperglycaemia and the severity of the vascular changes prior to the onset of improved control. Differences in these parameters between individual trials may, therefore, explain why transient deterioration in retinopathy has not been a universal finding upon the institution of tight glycaemic control (Tamborlane et al, 1982).

In all three of these investigations utilising CSII therapy, baseline retinopathy was relatively advanced (although still of 'background' type). In the study performed by Beck-Nielsen (Beck-Nielsen et al, 1985; Beck-Nielsen et al, 1990), however, 24 diabetic patients with minimal or no background retinopathy at onset were examined, receiving either conventional insulin treatment or CSII. Significant improvement in glycaemic control was achieved with CSII and was associated with less progression in the number of microaneurysms compared to the conventionally-treated group, after 1, 3 and 5 years of follow-up. This difference did not reach statistical significance, although only small numbers of patients were studied, so that a clinically significant effect cannot be excluded.

Fifty-four patients were followed-up for over 30 months in the Dallas study (Friberg et al, 1985; Rosenstock et al, 1986). Improved glycaemic control with CSII was associated with less progression in microaneurysm counts and a retinopathy grading score than treatment with conventional insulin therapy. The principal limitation of this study, however, was that allocation to treatment groups was determined by patient choice, rather than by randomisation.

Finally, in the Stockholm Diabetes Intervention Study (Reichard et al, 1991b) optimal glycaemic control was attempted using intensified conventional insulin therapy (ICT, involving home blood glucose monitoring and multiple insulin injections), rather than CSII. Compared to a group receiving standard insulin treatment, the ICT group nevertheless maintained significant differences in glycosylated haemoglobin concentrations, which were associated with a significant, but small, reduction in the rate of progression of retinopathy over 5 years. However, even in the ICT group proliferative changes occurred in at least one in ten cases.

In Table 1 an attempt is made to summarise this rather heterogeneous group of trials. A number of problems exist in interpretation of these studies. Of particular concern are: the small number of patients studied in most cases; patient cross-over between treatment groups; the relatively short lengths of follow-up employed, compared to the previous length of exposure to diabetes and the long time-course of development of retinopathy; and differences between trials in the methods used to assess the severity of retinopathy. Despite these problems, however, there are a number of tentative conclusions which can be drawn.
### Table 1: Effect on Diabetic Rehospitalization of Intensive Insulin Therapy: Summary of Prospective Trials

| Differences in Outcomes | RCT-ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT | ICT vs. ICT |
|-------------------------|---------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Hospitalization in Patients | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Mortality | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Cardiac Event | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Stroke | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Renal Failure | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Non-Cardiac Event | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| Total Rehospitalization | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |

**Legend:**
- ICT = Intensive Control Therapy
- SII = Standard Insulin Infusion
- RCT = Randomized Controlled Trial
Firstly, long-term normoglycaemia was not achieved in any of the studies discussed, and in each case some progression of retinopathy was observed even in the groups with improved glycaemic control. Secondly, transient deterioration in retinopathy may accompany the onset of improved control in some patients. Finally, modest improvements in metabolic control are nevertheless associated with a reduction in the rate of progression of retinopathy, although this beneficial effect has often been marginal and its influence upon long-term visual prognosis is unknown.

Prospective Controlled Trials: Nephropathy

As with retinopathy, a considerable number of trials, generally involving relatively small numbers of patients, have been performed to assess the effect of improved glycaemic control upon the development of diabetic nephropathy.

Viberti (Viberti et al, 1981) examined eight diabetic patients with normal resting urinary albumin excretion, treated consecutively either with conventional treatment or with CSII for 3 weeks. Improved glycaemic control with CSII was associated with a reduction in exercise-induced albuminuria which is often seen in otherwise normoalbuminuric diabetics and which may represent the earliest functional changes of nephropathy in these patients. Similarly, hyperfiltration, another early feature of diabetic nephropathy, could be reversed after 1 year of intensified insulin therapy with CSII, but not with conventional insulin therapy, although neither treatment influenced early changes in kidney volume (Wiseman et al, 1985; Christensen et al, 1986).

In contrast, however, improved metabolic control using CSII did not appear to have any beneficial influence upon glomerular filtration rate or urinary albumin excretion in patients with advanced kidney disease ie. either established diabetic nephropathy (Viberti et al, 1983) or microalbuminuria and intermittent clinical proteinuria (Bending et al, 1986).

The Steno study group examined changes in GFR and urinary albumin excretion rate (UAER) in 29 Type 1 diabetic patients with only microalbuminuria, randomised to CSII or UCT (Deckert et al, 1983). Again, hyperfiltration was reversed in the CSII group, but no changes in GFR were observed in those patients receiving conventional therapy. More importantly, a significant increase in urinary albumin excretion rate occurred over the two years of the study in the conventionally-treated group, whilst no change was seen in the CSII group. Furthermore, of patients with an initial UAER >70 \( \mu g/\text{min} \) (previously established by the Steno group as being highly predictive of the later development of clinical nephropathy in their diabetic population (Mathiesen et al, 1984)), 5/5 from the UCT group went on to develop overt nephropathy, whilst only 1/3 in the CSII group did so. Unfortunately, however, the two groups were not
identical at onset of the trial, GFR being significantly higher in the CSII group, and albumin excretion being higher in the UCT group.

In a separate study from the same centre (Feldt-Rasmussen et al, 1986a; Feldt-Rasmussen et al, 1986b), thirty-six diabetic patients with microalbuminuria were again randomised to receive either CSII or UCT. After 1 year, significant improvements in glycaemic control were observed in the CSII group, although albumin excretion remained unchanged in both groups. After two years follow-up, however, UAER had significantly increased in the conventionally-treated group, but remained stable in the CSII group in which metabolic control remained superior. Again, progression to overt diabetic nephropathy was observed in 5/18 conventionally-treated patients, compared to 0/18 of the CSII-treated patients.

More recently, the patients included in both the Steno studies have been re-assessed (Feldt-Rasmussen et al, 1991), after 5-8 years of follow-up. Unfortunately, approximately one-third of patients originally randomised to conventional treatment had elected to undergo more intensive insulin therapy upon completion of the formal two-year studies. Nevertheless, progression to clinical nephropathy continued to be less common in the CSII groups than in the UCT groups in both studies.

Many of the studies, outlined above, concerning metabolic control and retinopathy also contain information on the progression of nephropathy. Holman (Holman et al, 1983) observed a reduction in creatinine clearance in the conventionally-treated group, compared to stable renal function with intensive treatment. Unfortunately, however, no data was supplied concerning baseline urinary albumin excretion rates, so that few conclusions can be drawn from this observation.

In the Kroc Collaborative study (Kroc Collaborative Study Group, 1984), complete data concerning urinary albumin excretion were available in 59 patients, 20 of whom had microalbuminuria, the remainder having normoalbuminuria. In the latter patients, UAER remained constant over 8 months, irrespective of treatment modality. In patients with baseline microalbuminuria, however, albumin excretion was reduced almost to normal by CSII, but remained elevated in the UCT group. Unfortunately, further information on urinary albumin excretion was not obtained beyond the initial 8 month study.

Patients in the trial conducted by Beck-Nielsen (Beck-Nielsen et al, 1985) initially had either normal or only slightly elevated urinary albumin excretion rates. After 1 year, hyperfiltration was reversed only by intensified insulin therapy with CSII, glomerular filtration being unchanged in the conventionally-treated group. Urinary albumin excretion was unaffected by conventional insulin therapy and slightly reduced by CSII treatment, although this difference did not reach statistical significance. After 5 years (Beck-Nielsen et al, 1990), UAE remained low in both groups of patients, and
hyperfiltration had also reduced in the conventional-treatment group, so that in the long term no statistically significant benefit of intensified insulin therapy was observed. Similar findings were also observed in the Oslo study (Dahl-Jorgensen et al, 1986; Dahl-Jorgensen et al, 1988) in which patients had normoalbuminuria or mild microalbuminuria at baseline. After two years the only significant change observed was a small reduction in hyperfiltration in the CSII group, whilst GFR was unchanged in the two conventional treatment groups. After 4 years, urinary albumin excretion was also reduced with CSII therapy, and to a lesser extent in the multiple insulin injection group, but the magnitude of these changes was very small. Finally, in the Stockholm Diabetes Intervention Study (Reichard et al, 1991b) the majority of patients also had normo- or micro-albuminuria at onset, although 3 patients in each treatment group had clinical nephropathy. After five years 8/50 patients in the conventionally-treated group had progressed to clinical nephropathy (four of whom were normoalbuminuric at baseline), compared to 0/44 in the intensively-treated group. In addition, mean UAER was stable in the intensively-treated group over 5 years, but was increased in the conventionally-treated group.

The findings of these trials are further summarised in Table 2. Despite the limitations, mentioned above, inherent in these studies, their findings are generally in agreement concerning the development of diabetic nephropathy: Firstly, in the earliest stages of nephropathy (with normal urinary albumin excretion or only mild micro-albuminuria) hyperfiltration can be reversed by intensified insulin therapy, although kidney volume is unaffected. The long-term influence of this effect is unknown, however. Glomerular filtration rate can be elevated in non-diabetics by infusion of glucose, so that changes in GFR early in the evolution of diabetic nephropathy may merely reflect the prevailing level of hyperglycaemia (Christiansen et al, 1981). Reduction in GFR, without the appearance of microalbuminuria, may also occur spontaneously in patients with hyperfiltration (Jones et al, 1991; Bognetti et al, 1993). Nonetheless, hyperfiltration may be important in the pathogenesis of glomerulopathy (Hostetter et al, 1982), and its reduction may therefore be of some long-term benefit. Secondly, institution of improved glycaemic control in the late stages of nephropathy (intermittent or established proteinuria) appears to be of no benefit, implying that there is a 'point of no return' (similar to the situation in experimental diabetes), beyond which renal damage is irreversible. In most cases, however, there was also evidence that improved glycaemic control could influence the progression of urinary albumin excretion before this stage is reached. Three trials contained patients progressing from incipient to overt diabetic nephropathy,
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<th>Reference</th>
<th>Prior Design</th>
<th>No. of Patients</th>
<th>Design Duration</th>
<th>Baseline U/A</th>
<th>Follow-up</th>
<th>Reduction in U/A</th>
<th>Reduction in Albuminuria</th>
<th>Hypertension Outcome</th>
<th>Progression of Nephropathy</th>
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<td>MA = Monotherapy; U/A = Urinary Albuminuria</td>
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and in each case the majority of these patients were conventionally-treated. Microalbuminuria was also more likely to increase with conventional treatment and decrease with intensified treatment, although in many cases the changes in albumin excretion rates were quite modest. Furthermore, in an individual patient urinary albumin excretion may vary widely from day to day (Feldt-Rasmussen et al, 1984), so that several urine collections may be required to establish the true pattern of changes in UAER. In many of the above studies baseline UAER was established on the basis of only a single 24-hour urinary collection and in three trials only two or three further collections were made throughout the rest of the investigation. The long-term significance of these small changes in microalbuminuria is also uncertain. Although microalbuminuria is predictive of the later development of diabetic nephropathy, there is disagreement concerning the threshold value of urinary albumin excretion rate above which the risk of nephropathy is increased (Walker, 1991). If, for an individual patient, the initial urinary albumin excretion is below this threshold, their risk of developing nephropathy may already be low, so that there may be little advantage gained by further reduction in UAER. Similarly, by potentially containing many patients at low risk of nephropathy, the power of these trials to detect a beneficial effect, should one be present, will also be reduced. Estimates of this threshold value vary up to 70 µg/min (ie. 100 mg/24 hours; Mathiesen et al, 1984), and many of the patients in the above trials had baseline urinary albumin excretion rates considerably below this. Much longer follow-up than that employed in these trials would therefore be necessary to determine the long-term effects of these small changes in albuminuria.

Renal transplantation studies have provided perhaps the most convincing evidence that diabetic nephropathy may be reversible by normalisation of metabolism. Thus, the transplantation of kidneys from a long-standing diabetic donor into non-diabetic recipients has been reported to reverse almost completely both clinical and histological features of established diabetic nephropathy within 7 months (Aboua et al, 1983). In contrast, the transplantation of normal kidneys to diabetic recipients is associated with the development of histological features of diabetic nephropathy within 2 years (Mauer et al, 1976; Mauer et al, 1983). It should be pointed out, however, that the correlation is generally poor between the histological features of diabetic nephropathy and its clinical course (Mauer et al, 1984; Chavers et al, 1989), so that the long-term significance of these findings is also not entirely clear.

It is also relevant to note at this point that metabolic abnormalities are not the only factors influencing the course of diabetic nephropathy. In recent years the importance of aggressive management of hypertension in preserving renal function in diabetic patients with nephropathy has been increasingly recognised (Björck et al, 1986; Parving et al,
1987; Parving et al, 1988). Furthermore, the benefits of antihypertensive drugs, in particular the angiotensin converting enzyme (ACE) inhibitors, are not limited to those patients with hypertension. Thus, the use of ACE inhibitors in normotensive patients delays the onset of overt nephropathy in patients with microalbuminuria (Marre et al, 1988; Mathiesen et al, 1991; Wiegmann et al, 1992), and reduces the rate of decline in renal function and the rate of increase in albumin excretion once nephropathy is established (Parving et al, 1989). Finally, dietary protein restriction may also be of some benefit in preservation of renal function in established nephropathy (Zeller et al, 1991).

Prospective Controlled Trials: Neuropathy
In 1980, Pietri (Pietri et al, 1980b) reported a small improvement in motor nerve conduction velocity after 6 weeks of CSII therapy in ten diabetic patients. Glycaemic control with CSII was superior to a conventionally-treated group of 5 patients, in whom nerve conduction velocities did not change during the 6-week period. A similar non-randomised comparison of CSII and conventional therapy was undertaken by Ehle (Ehle et al, 1986). In this study, follow-up was after 1 year, but again, mild improvements in motor nerve conduction velocity accompanied improved glycaemic control with CSII, whilst no change occurred in the conventionally-treated group.

At the Mayo Clinic, two trials were performed to assess the effect of improved glucoregulation upon peripheral nerve function. In the first study (Service et al, 1983) patients with both non-insulin-dependent diabetes (NIDDM) and IDDM were studied, randomised to receive either conventional treatment or intensified insulin therapy with home blood glucose monitoring. Unfortunately, it did not prove possible to maintain significant differences in glycaemic control between the two groups using these methods, so that, not surprisingly, no beneficial effects upon nerve function were observed. In the second study (Service et al, 1985), however, only IDDM patients were examined, and significant improvement in glycaemic control was achieved using CSII, compared to standard insulin therapy. Although there were only six patients within each treatment group, small but significant improvements were seen in the CSII group after 8 months (but not at 4 months), in both sensory and motor nerve conduction velocities, and in vibration sensory threshold. In four of the trials mentioned in the above sections on retinopathy and nephropathy, information was also obtained on changes in neurological function. Holman observed a deterioration in vibration sensory threshold in the conventionally-treated group, compared to a slight improvement in the intensively-treated group.
In the Oslo study motor nerve conduction velocities also deteriorated with conventional therapy, were stable or mildly improved with multiple injection therapy, and were significantly improved in the CSII group. In the Stockholm Study nerve conduction velocities were reduced after 5 years of unchanged insulin therapy but remained stable with intensified treatment, although vibration threshold deteriorated in both groups. Furthermore, the number of patients developing new signs of neuropathy during this study was 3/44 intensively-treated patients, versus 17/52 conventionally-managed patients. In the Steno study (Lauritzen et al, 1%5) no changes were observed in vibration sense in either treatment group, but there was a deterioration in beat-to-beat variation of heart rate in the conventionally-treated group only. Changes in autonomic function were also assessed by Jakobsen (Jakobsen et al, 1988), in a randomised 2-year study comparing CSII with conventional insulin therapy. Again, heart rate variation remained stable in the CSII group, and deteriorated in the conventional treatment group (despite reasonably good glycaemic control, with an HbA1c level of 8.6%).

Thus, optimised glycaemic control appears consistently to improve certain neurophysiological indices of diabetic neuropathy. The long-term influence of these effects must remain speculative, however, particularly as it is known that nerve conduction velocities can be acutely influenced by short-term changes in blood glucose levels (Gregersen, 1968).

Prospective Trials: Macrovacular Disease
No prospective trials have been performed directly examining the influence of glycaemic control on atherosclerosis. Indeed, the long time course of this process, and the lack of any reliable early marker for its presence make such a study impractical. However, improved glucose regulation with intensive insulin therapy is accompanied by potentially beneficial changes in plasma lipid and lipoprotein levels (Pietri et al, 1980a; Rosenstock et al, 1987). In particular, long-term reduction can be achieved in total and LDL-cholesterol, the levels of which are considered important risk factors for the development of atherosclerosis. Reduction in these risk factors may, in turn, slow the development of diabetic macrovascular disease, although this hypothesis remains far from proven.
In summary, the cumulative evidence of these diverse trials suggests that intensified insulin therapy may reduce the rate of progression of (but not reverse) existing background retinopathy; may prevent or delay progression to clinical nephropathy and renal failure, if instituted at a sufficiently early stage; and may ameliorate certain neurophysiological indices of neuropathy and improve risk factors predisposing to atherosclerotic vascular disease.

It is apparent from the above discussion, however, that these investigations are all unsatisfactory in many respects, so that a firm conclusion regarding the beneficial effects of improved glycaemic control upon long-term complications cannot yet be made. Many of the problems inherent in these trials have been addressed in the design of a recently-initiated, multicentre prospective trial in the USA (Diabetes Control and Complications Trial; (DCCT Research Group, 1986, 1987 and 1990)). In this study conventional insulin therapy will be compared with intensive therapy using either CSII or multiple daily injections, the intensively-treated group using frequent home blood glucose monitoring in an attempt to achieve as near normal glycaemic profiles as possible. The major outcome studied will be the development of retinopathy, although assessment will also be made of changes in glomerular filtration rate, urinary albumin excretion rate, nerve conduction velocities, autonomic nervous function tests and quality of life estimation. With respect to retinopathy, patients have also been separated into a primary intervention group, studying the development of retinopathy in those without any initial signs of retinal involvement, and a secondary intervention group, in which the progression of existing background changes will be assessed.

The results of the DCCT will be uniquely valuable for several reasons. Firstly, the large number of patients randomised (n=1441) and the projected length of follow-up (5-10 years, median 7 years) should enable statistically valid conclusions to be drawn concerning the influence of intensified control upon long-term complications. Secondly, at the latest analysis the vast majority of subjects continued to be treated according to their original randomisation (>99.5%), and data collection has been meticulous. Thirdly, it has proven possible, to date, to maintain differences in glycaemic control (HbA1c) between treatment groups which would be expected to produce a clinical effect upon the development of complications (estimated to be at least a 1.5% improvement in HbA1c; DCCT Research Group1986. NB. this is, therefore, the threshold selected in Tables 1 & 2). Finally, no other trial has adequately examined the role of glycaemic control in the primary prevention of complications, particularly retinopathy. Thus, until the results of this trial are available hard evidence that improved glycaemic control favourably influences complications is lacking.

It is equally noteworthy, however, that patients in the experimental group of this trial have suffered a 2-3 fold increased risk of severe hypoglycaemic attacks and
hypoglycaemic coma (DCCT Research Group, 1991). Therefore, even in selected, well-motivated patients, closely monitored in a carefully-conducted trial there is still a considerable risk attached to intensified regimes of exogenous insulin delivery. Furthermore, even in this setting such regimes do not result in long-term normoglycaemia: HbA1c levels in the experimental group of the DCCT were still significantly higher than in a reference, non-diabetic population (7.2% versus 6.05%).

Section A: Conclusions

The hypothesis that the chronic complications of diabetes can be delayed, or even prevented, by long-term normoglycaemia has not been conclusively proven, although current evidence is highly suggestive that this is the case. Unfortunately, no currently available method of exogenous insulin delivery is capable of achieving this goal safely in large numbers of diabetic patients, even when these individuals are highly motivated and carefully monitored.

SECTION B

VASCULARISED PANCREATIC TRANSPLANTATION

The shortcomings of parenteral insulin therapy have led to interest in alternative methods of replacing the endocrine function of the β-cells lost in Type 1 diabetes. One logical approach to this problem is the replacement of the β-cells themselves by transplantation of the pancreas. Early attempts to achieve this by subcutaneous implantation of non-vascularised pancreatic pieces were unsuccessful (reviewed by Downing, 1984). Attention therefore became focussed upon the transplantation of primarily-vascularised pancreatic grafts. Following extensive investigation of this possibility in experimental animals the first vascularised pancreatic transplants in humans were performed in 1967 (Kelly et al., 1967), with sufficient graft function to permit temporary discontinuation of exogenous insulin. Following this initial success there has been an enormous increase in the number of pancreatic transplantation procedures performed, at numerous institutions around the world, and results have continued to improve. These results have been carefully recorded by an International Pancreas Transplant Registry, based at the University of Minnesota Medical School, Minneapolis, USA. Up to November 1992, 4,285 pancreas
transplants have been reported to this registry from more than 140 institutions, 64% of these transplants being performed in the USA (Sutherland et al, 1993).

A number of techniques for transplantation may be employed. In the vast majority of cases the donor pancreas is transplanted extraperitoneally into the iliac fossa of the recipient (contralateral to any simultaneous or previously transplanted kidney), with anastomosis of the pancreatic vasculature to the iliac vessels. Transplantation may involve either the whole pancreas, with or without the duodenum, or only the body and tail (segmental graft). Furthermore, a number of approaches exist for managing the exocrine secretions of the transplanted pancreas, the commonest being obliteration of the main pancreatic duct by intraductal injection of polymers, and anastomosis of the duct to the recipient's gastro-intestinal tract or bladder. The advantage of the latter approach is that rejection episodes may be detected earlier and effectively monitored using estimations of urinary amylase levels (Prieto et al, 1987), whilst biopsy of the pancreas is also facilitated at cystoscopy.

Pancreatic transplantation must be accompanied by long-term immunosuppression to prevent graft rejection and for this reason, most pancreatic transplants have been performed either simultaneously with, or following a previous kidney transplant for which immunosuppression is already required. However, pancreatic transplantation alone has also been performed in non-uraemic patients whose diabetic problems were felt to outweigh the potential hazards of the subsequently necessary immunosuppressive therapy (Sutherland et al, 1988a; Sutherland, 1992b).

Finally, the majority of pancreatic grafts have been obtained from cadaveric organ donors, although a number of segmental grafts from living related donors have also been transplanted (Sutherland et al, 1984).

The most recent report of the International Pancreas Transplant Registry (Sutherland, 1991) demonstrates the extent of improvement in the results of pancreatic transplantation since the 1960s. Overall, patient survival at one year post-transplant has improved from 41% in the era 1966-1977 to 91% between 1988-1990. Graft survival (insulin-independence) at 1 year has also risen from 5% in 1966-1977 to 68% in 1988-1990. These data are also consistent with a recent report from the United Network for Organ Sharing (UNOS), detailing the results of pancreatic transplantation in the USA alone (Sutherland et al, 1993), and are similar to the results currently obtained with transplantation of other solid organs. Pancreatic graft survival is optimal in recipients of a simultaneous-pancreas-kidney transplant compared to other recipient categories, and is higher using the bladder-drainage technique compared to other duct management methods.
The quality of metabolic control achieved by successful pancreatic transplantation is unrivalled by any other method of insulin replacement therapy. Complete insulin-independence is achieved on an unrestricted diet and fasting blood glucose levels are equal to those of healthy, nondiabetic individuals (Pozza et al, 1985; Robertson et al, 1989; Morel et al, 1992a; Tibell et al, 1992; Sousa-Castello et al, 1992). In addition, most studies report glycosylated haemoglobin concentrations within the normal, nondiabetic range (Nathan et al, 1991; Morel et al, 1992a; Sousa-Castello et al, 1992), although others have noted a slight increase (usually <0.5% HbA1c) relative to nondiabetics, presumably because of the mild hyperglycaemic effect of immunosuppressive drugs, particularly steroids (Morel et al, 1991; Tibell et al, 1992). However, in these cases HbA1c levels are equivalent to those of non-diabetic kidney transplant recipients who also receive these drugs, and remain considerably better than insulin-treated diabetics (Nathan et al, 1991; Morel et al, 1991). Furthermore, these normal or near-normal levels of HbA1c are maintained for many years.

Other mild abnormalities of metabolism have also been recorded in recipients of successful pancreatic transplants. The rate of decline in blood glucose levels following an intravenous glucose tolerance test may be slightly reduced compared to non-diabetics (Morel et al, 1992a; Tibell et al, 1992), although many patients have completely normal rates (Robertson et al, 1989). Similarly, blood glucose levels at 2 hours during an oral glucose tolerance test may be mildly elevated or normal. Furthermore, serum insulin levels, both basal and following meals or glucose challenge, are elevated compared with those of healthy non-diabetics (Pozza et al, 1985; Robertson et al, 1989) and of non-diabetic kidney transplant recipients (Ostman et al, 1989), largely due to the delivery of insulin from the graft into the systemic, rather than the hepatic portal circulation (Diem et al, 1990). The importance of these mild metabolic abnormalities remains to be established, however.

Pancreatic Transplantation and Diabetic Complications

Despite the extraordinary degree of metabolic control achieved, pancreatic transplantation has had limited influence upon the long-term complications of diabetes. This is perhaps not surprising, as this procedure has generally been reserved for patients also requiring renal transplantation for established diabetic nephropathy, or whose problems in diabetic management are considered greater than the risks of long-term immunosuppression (Sutherland et al, 1988a; Sutherland, 1992a; Sutherland, 1992b). In both groups of patients chronic complications of diabetes are well-advanced, and perhaps therefore irreversible, prior to transplantation. In addition, many
of the trials investigating this issue have involved small numbers of patients and a relatively short period of follow-up.

**Retinopathy**

Further problems are apparent in interpretation of those trials examining the influence of pancreatic transplantation upon retinopathy. Firstly, in many cases only changes in visual acuity have been examined. This may, however, be influenced by non-retinal disease eg. cataract formation, whilst in the late stages of retinopathy gross visual acuity may remain stable for prolonged periods, despite continuing deterioration in more sensitive indices of retinal function (Ramsay et al, 1977). Also, it should be remembered that transient deterioration in retinopathy may in any case accompany improved metabolic control in the short term.

Secondly, stabilisation of retinopathy is known to follow pan-retinal photocoagulation (Diabetic Retinopathy Study Research Group, 1976), which is often used in patients prior to pancreatic transplantation, due to the advanced nature of their retinopathy. Finally, an ideal control group is lacking. Many patients have received pancreatic transplants in association with a kidney, so that the effects of pancreatic transplantation may be modified by co-existent reversal of anaemia (Najarian et al, 1979b; Bentley et al, 1985). For this reason, comparisons have necessarily been made between diabetic patients receiving either a combined pancreas-kidney transplant or a kidney transplant alone, or alternatively between patients with a successful, compared to a failed, pancreatic transplant. Even these control groups are not ideal, however, as in the first instance treatment is not allocated at random, and in the second, factors associated with loss of the pancreatic graft may be co-incidentally associated with development of retinopathy.

Nevertheless, in the majority of studies pancreatic transplantation has conferred no beneficial effect upon the development of advanced retinopathy, despite maintenance of euglycaemia for up to 4 years (Ramsay et al, 1988; Petersen et al, 1990; Scheider et al, 1991; Baudello et al, 1991; Zech et al, 1991; Bandello et al, 1992). A slight beneficial effect has been observed by other investigators, although in these studies control groups have either been omitted (Ulbig et al, 1987; Landgraf et al, 1989; Esmatjes et al, 1992) or have contained significantly fewer patients with functioning renal grafts (Konigsrainer et al, 1991), so that the influence of the pancreatic transplant itself is difficult to assess.

**Nephropathy**

As mentioned above, renal allografts transplanted from normal individuals into diabetic recipients develop, within two years of transplantation, histological changes typical of
diabetic glomerulopathy (Mauer et al, 1983). Transplantation of the pancreas, however, prevents these changes from occurring if carried out simultaneously with the renal allograft (Bohman et al, 1985), and arrests their progression if carried out 1-7 years after kidney transplantation (Bilous et al, 1989).

Furthermore, transplantation of the pancreas alone in non-uremic diabetics has been associated with reversal of certain lesions of established glomerulopathy within the patients' native kidneys, and with some reduction in albumin excretion rates (Bilous et al, 1987). The long-term beneficial effects of these changes remain to be determined, however, and may be limited by the nephrotoxic effects of cyclosporin used in recipients of pancreatic grafts (Bilous et al, 1987; Sutherland et al, 1988a).

**Neuropathy**

As with retinopathy, any beneficial effect of pancreatic transplantation upon neuropathy may be influenced by the co-existing reversal of uremia in patients receiving combined pancreas-kidney grafts. Neuropathy is common in uraemia and some aspects of nerve function improve following successful renal transplantation alone (Bolton, 1976; Ibrahim et al, 1974; Nielsen, 1974; Solders et al, 1986), although there is some evidence that this short-term benefit is followed by continued deterioration in diabetic patients (van der Vliet et al, 1988b). For this reason, most trials have included similar control groups to those described above.

In general, only mild improvements in peripheral nerve function attributable to the pancreatic graft itself have been observed (Solders et al, 1987; Solders et al, 1991; Solders et al, 1992; Kennedy et al, 1990; Comi et al, 1991; Muller-Felber et al, 1991; Naouri et al, 1992) and these improvements have often been limited to small increases in nerve conduction velocities. Improvements in motor and sensory action potential amplitude, which may more closely reflect nerve fibre regeneration, have usually been more modest and restricted to the upper limbs (Kennedy et al, 1990; Comi et al, 1991; Muller-Felber et al, 1991; Naouri et al, 1992). Presumably this is again due to the relatively advanced, and therefore irreversible, nature of the peripheral neuropathy, especially in the lower limbs, of these patients.

In most studies, no convincing amelioration of autonomic neuropathy has followed the additional transplantation of a pancreas to subjects undergoing renal transplantation (Solders et al, 1987; Solders et al, 1991; Boucek et al, 1991; Nusser et al, 1991; Naouri et al, 1992). A slight benefit was, however, observed in two studies involving larger numbers of patients. Kennedy (Kennedy et al, 1990) demonstrated stable autonomic function in patients with successful pancreatic transplants, compared with a steady deterioration in diabetics on insulin treatment alone, or in patients whose pancreatic graft had failed early after transplantation. A greater effect was observed by
Navarro (Navarro et al, 1990; Navarro et al, 1991), who demonstrated improvement in cardiorespiratory reflexes occurring after 1-5 years in recipients of a functioning pancreatic graft. Interestingly, these changes were associated with a significantly reduced mortality rate for patients with a successful pancreatic transplant, amongst patients with initially abnormal autonomic function tests.

Therefore, regarding the long-term complications of diabetes the effects of pancreatic transplantation appear limited to: the prevention of nephropathy in a simultaneously transplanted kidney; a reduction in nephropathy in the native kidneys of non-uraemic patients; and a modest amelioration of peripheral neuropathy.

In contrast to this limited benefit, however, successful pancreatic transplantation has been repeatedly demonstrated to improve quality of life. In particular, successful grafting ensures freedom from dietary restrictions and the need for insulin injections; relieves anxiety related to 'control' of diabetes; facilitates increased physical activity; improves patients' perceptions regarding their future health prospects; and increases the number of patients able to resume full-time occupation (Nakache et al, 1989; Johnson et al, 1990; Nathan et al, 1991; Zehr et al, 1992). The extent of this improvement is such that in one centre the majority of patients receiving solitary pancreas transplants felt that being insulin-independent, although immunosuppressed, was preferable to life prior to the transplant (Zehr et al, 1990; Zehr et al, 1991).

Pancreatic transplantation is, however, attended by the complications expected of a major surgical procedure. Mortality is considerably increased in those patients whose pancreatic graft fails early due to technical reasons such as thrombosis, haemorrhage, graft infection and pancreatitis. In this group of patients 1 year survival is 79%, compared to 93% in patients with a 'technically-successful' graft (Moudry-Munns et al, 1992). Excess mortality attributable to transplantation of the pancreas appears confined mostly to those aged over 45 years, and is much less apparent in younger recipients (Sutherland et al, 1989a; Nathan et al, 1991; Cheung et al, 1992). However, in recipients of technically successful pancreas transplants mortality is little different than in diabetic recipients of kidney grafts alone, and the majority of deaths in these patients are due to cardiovascular disease and infection resulting from immunosuppression (Sutherland et al, 1989a).

A considerable increase in morbidity is also associated with pancreatic transplantation. Complications related to the graft, such as infection, fluid collections, pancreatic-cutaneous fistulae (and urological complications and metabolic acidosis with the bladder drainage technique: (Elkhammas et al, 1992; Ketel et al, 1992)) are common,
necessitating increased post-operative hospitalisation (Sutherland et al, 1989a; Nakache et al, 1989; Nathan et al, 1991; Cheung et al, 1992). Furthermore, it is likely that chronic immunosuppression, with its attendant risks, will always be required in recipients of vascularised pancreatic transplants. Finally, it may be argued that transplantation of the intact pancreas as a treatment for diabetes is somewhat illogical, in that the majority of tissue transplanted (i.e. the exocrine component) is not only unnecessary, but is coincidentally the cause of most complications related to the procedure.

Section B: Conclusions

Long-term normoglycaemia can now be regularly achieved in Type 1 diabetic patients by transplantation of the pancreas as a vascularised graft. Unfortunately, the potential benefits of this degree of metabolic control upon the microvascular complications of diabetes are limited when angiopathy is already well established. Furthermore, wider application of this technique will always be limited by the risks of the procedure itself and the need for chronic immunosuppression. Therefore, it is unlikely that transplantation of the whole pancreas will ever be applicable to young diabetics prior to the onset of microangiopathic complications, in whom optimal metabolic control might be expected to have the greatest benefit.

SECTION C

PANCREATIC ISLET TISSUE TRANSPLANTATION

The transplantation of pancreatic endocrine tissue alone has numerous theoretical advantages over transplantation of the intact pancreas. Firstly, the transplantation procedure itself is relatively minor and free from the hazardous complications associated with drainage of exocrine secretions from the intact organ. Secondly, the ability to store islet tissue for prolonged periods of time using cryopreservation techniques allows transplantation to be performed on an elective basis and may facilitate optimal donor-recipient cross-matching. Thirdly, the need for long-term immunosuppression may be reduced, or even avoided altogether, by in vitro manipulation of grafted tissue prior to transplantation in order to reduce its immunogenicity, or by immunoisolation techniques using selectively permeable membranes to protect islet tissue from immune attack. Finally, potential exists for the use of xenogeneic tissue, thereby avoiding problems due to shortage of suitable human organ donors.
Over the last 20 years the feasibility of reversing diabetes by endocrine tissue transplantation has been firmly established in a variety of experimental animals (Sutherland, 1981), employing a number of approaches, including the use of foetal/neonatal pancreatic tissue and adult pancreata in the form of fragments; dispersed, unpurified tissue; purified islets of Langerhans; and purified β-cells.

Following this success several clinical trials of islet tissue transplantation in humans have been initiated, and the results of these trials will be reviewed in the remainder of this chapter. Many of the above approaches have been employed in these studies, although transplantation of purified endocrine cells obtained from isolated islets has been attempted only in rodents (Tze et al, 1984; Pipeleers-Marichal et al., 1991; Pipeleers et al., 1991a; Pipeleers et al., 1991b), and will not therefore be discussed further.

**Foetal Tissue Transplantation**

The use of foetal, rather than adult pancreatic tissue for transplantation has some theoretical advantages (Tuch et al., 1992). In particular, foetal pancreas contains a high proportion of endocrine cells and little exocrine tissue, so that purification steps are unnecessary, whilst foetal endocrine tissue undergoes considerable growth and differentiation following transplantation.

The results of clinical trials using foetal tissue have been somewhat disappointing, however. A review of the world literature in 1988 (Hering et al., 1988) summarised the results obtained by foetal pancreatic tissue transplantation in 398 diabetic patients. In the majority of cases human tissue obtained from therapeutic abortions was used, although bovine and porcine xenogeneic tissue was also employed in some centres. Insulin independence was achieved in only five cases, however, and this was short-lived in at least two patients. In one case transplantation was performed shortly after the diagnosis of diabetes was made (Chastan et al., 1980), and it was not clear therefore whether insulin independence in this patient resulted from graft function or from the well-recognised phenomenon of spontaneous temporary remission of diabetes.

Similarly, in three other patients there was inadequate documentation of pretransplant diabetic status to establish the contribution of the transplant to insulin independence. Finally, in many cases only modest reductions in post-transplant insulin requirements were achieved and graft function was not formally established eg. by assessment of serum C-peptide levels.

Since 1988 research using human foetal material has continued in several centres (Kondratiev et al., 1989; Djordjevic et al., 1992; Hu et al., 1992; Farkas et al., 1992;
Rückert et al, 1992), insulin-independence being achieved in a limited number of cases (Federlin et al, 1992). Particularly remarkable have been reports from China (Hu et al, 1992) claiming insulin independence in 48 of 755 patients receiving human foetal pancreas transplanted to a variety of sites (including intracerebral!), often without any immunosuppression. Unfortunately, in many of these cases pretransplant C-peptide levels were either unavailable or suggestive of residual pancreatic endocrine function in the recipient, so that these results are difficult to interpret.

One problem with foetal pancreas transplantation concerns the ethical issues surrounding the use of human foetal tissue obtained from therapeutic abortions. Indeed, in the USA ethical considerations led to a government ban upon the use of human foetal tissue for research (Marwick, 1988), which has only recently been lifted.

In order to avoid some of these ethical problems, interest in foetal xenotransplantation has recently been rekindled. In Sweden eight diabetic patients have recently been transplanted intraportally with porcine foetal 'islet-like cell clusters' (Groth et al, 1993). However, despite the transplantation of up to 1,020,000 cell clusters (obtained from about 70 foetal pancreata) the only evidence of graft function has been the appearance of small amounts of C-peptide in the urine of three patients, serum C-peptide and insulin requirements being essentially unchanged. Furthermore, a marked humoral response to the transplanted tissue was observed (Kumagai Braesch et al, 1992).

In summary, insulin independence following foetal pancreas transplantation has been reported, but interpretation of the results of this procedure have been hampered by the large variety of approaches used (source of tissue, site of transplantation etc.) and by often inadequate documentation of pre- and post-transplant function. Therefore, the potential role of foetal pancreas transplantation in the treatment of human diabetes remains uncertain.

**Adult Islet Transplantation**

Two broad approaches have been employed in clinical trials involving the transplantation of islets obtained from the mature pancreas, namely: *autotransplantation* of islets in order to prevent post-operative diabetes following pancreatectomy for primary pancreatic disease (usually chronic pancreatitis); and *allotransplantation* of islets, in some cases for the prevention of surgical diabetes following pancreatectomy, but primarily for the treatment of Type 1 diabetes mellitus.

Although this thesis is mostly concerned with allotransplantation of islets, autotransplant studies have provided much of the evidence that normoglycaemia can
follow the transplantation of islet tissue to an ectopic site in humans. For this reason the clinical results of both approaches to islet transplantation will be reviewed.

**Islet Autotransplantation**

Human islet autotransplantation following pancreatectomy for chronic pancreatitis was first reported by investigators at the University of Minnesota, Minneapolis (Najarian et al, 1979a). Since then various groups have undertaken this procedure, achieving insulin-independence in a number of patients, as summarised in Table 3. However, some important points need to be made concerning the interpretation of these studies. Firstly, although insulin-independence has followed islet autotransplantation, it has not always been firmly established that this state has resulted from the function of the autograft itself, rather than that of the residual, unresected pancreas, as in many of the reported cases less than 95% of the pancreas has been resected. The incidence of diabetes after pancreatectomy alone depends upon the extent of resection, and even after 95% pancreatectomy about 8% of patients may retain sufficient islet function to remain insulin-independent (Child et al, 1969), whilst lesser degrees of resection result in a requirement for insulin in only 50-70% of cases (Grodzinsky, 1980; Rossi et al, 1987; Dafoe et al, 1990). Establishing the contribution of the islet autograft following subtotal pancreatectomy therefore depends upon documentation of the site of insulin secretion using selective venous catheterisation techniques.

In three patients undergoing subtotal pancreatectomy and intraportal islet autotransplantation selective catheterisation of portal and hepatic veins has been performed (Cameron et al, 1980; Grodsinsky et al, 1981; Dafoe et al, 1990). In all cases some function of the pancreatic remnant was apparent: in one patient this appeared to be solely responsible for insulin-independence; in another, insulin-independent, patient a small contribution from the islet autograft was documented; and in the third case the function of the intrahepatic autograft clearly contributed greatly to the state of insulin-independence.

Following total pancreatectomy long-term insulin-independence can be assumed to be due to the function of the autotransplanted islets. Unfortunately this has been achieved in a limited number of patients. Thus, of 29 patients undergoing total pancreatectomy and islet autotransplantation, reported in the literature, only 7 have achieved insulin-independence for any period of time (Lorenz et al, 1988; Farney et al, 1992), and in only 5 cases has this state continued long-term. In these cases, however, islet autotransplantation has been able to maintain fasting blood glucose and glycosylated haemoglobin concentrations within the normal range and on occasions to preserve normal oral glucose tolerance (Pyzdrowski et al, 1992).
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**Table 3. Reported Cases of Clinical Liver Autotransplantation**
Selective catheterisation studies have also been performed in patients with intraportal islet autotransplants following total pancreatectomy. Insulin secretion by the intrahepatic islets was established in one of the two insulin-dependent patients studied by Traverso (Traverso et al, 1981) and in all 6 insulin-independent patients investigated by Valente (Valente et al, 1980a) and Pyzdrowski (Pyzdrowski et al, 1992), whilst significant C-peptide secretion in response to feeding has also been observed in several other patients (Dobroschke et al, 1981; Lorenz et al, 1981; Altman et al, 1984).

These promising results are made more remarkable by the observations that islet function is often abnormal preoperatively in patients with chronic pancreatitis attending for pancreatectomy (Grodsinsky, 1980; Rossi et al, 1987) and that digestion/dispersal of the chronically inflamed pancreas is more difficult than in the normal gland (Mehigan et al, 1980b). It is likely therefore that the yield of functional islets obtained in these studies has been limited by the quality of the pancreata from which they have been isolated. Furthermore, in most cases relatively traumatic methods for pancreatic dispersal (chopping/mincing, often followed by a period of incubation of the resulting tissue with collagenase) have been used. It is possible, then, that the use of newer, improved techniques which have recently been developed for islet isolation prior to allotransplantation (such as the 'automated' digestion system introduced by Ricordi (Ricordi et al, 1988)) may improve further the clinical results of islet autotransplants. Indeed, using such techniques one group has already reported insulin-independence in both of their first two autograft recipients (Fontes et al, 1992).

The late failure of many grafts, initially able to maintain insulin independence, is also of great concern. The reasons for this deterioration of graft function with time are not fully established, although the transplantation of a marginal islet mass, which subsequently becomes 'exhausted' may be contributory. This topic will be discussed in more detail in Chapter 2.

Finally, in the majority of islet autografts dispersed pancreatic tissue has been used without an attempt at purification of islets. It may therefore be more appropriate to describe these grafts as dispersed pancreas transplants, rather than as islet transplants. This is more than a purely academic point, as the transplantation of exocrine tissue along with islets may contribute to the complications of islet autotransplantation. Thus, in most of the series outlined above the intraportal infusion of unpurified pancreatic tissue was associated with a transient increase in portal venous pressure, sometimes accompanied by systemic hypotension and postoperative elevation of liver enzymes. Although these abnormalities have usually been transient, more serious problems have arisen in other patients. Severe portal hypertension and disseminated intravascular coagulation have been reported (Mehigan et al, 1980a; Dafoe et al, 1990), as has thrombosis of the superior mesenteric (Dafoe et al, 1990) and portal veins, the latter
resulting in delayed bleeding from oesophageal varices in one patient (Memsic et al., 1984). In three patients death has even occurred, following hepatic infarction (Walsh et al., 1982; Toledo-Pereyra et al., 1984b) and disseminated intravascular coagulation (Mittal et al., 1981).

In conclusion, autograft studies have established that normoglycaemia can follow the transplantation of islets to an ectopic site in humans, and that this function can continue for prolonged periods of time. Furthermore, adequate function can result from transplantation of islets isolated from less than a whole human pancreas. These findings suggest that the concept of islet transplantation as a treatment for diabetes is sound.

**Islet Allotransplantation**

Clinical transplantation of isolated adult, allogeneic islets for the treatment of diabetes was also first reported by the University of Minnesota group in 1977 (Najarian et al., 1977). Ten transplant procedures were performed in seven diabetic patients with established renal allografts. Islet tissue was obtained from six infant and four adult pancreata, the islets from the adult pancreata being purified on Ficoll density gradients prior to transplantation. Insulin requirements were temporarily reduced by 26-75% in 6/7 patients, although none achieved insulin independence, and no increase in serum C-peptide was observed in any patient post-transplantation.

These initially disappointing results were attributed to the transplantation of an inadequate islet mass. Therefore, between 1977 and 1981 a further series of transplants was performed in Minneapolis, utilising unpurified, dispersed adult pancreatic tissue, the density gradient purification step being omitted to increase the mass of islet tissue transplanted (Sutherland et al., 1980a; Gores et al., 1992). Ten procedures were performed in nine diabetic patients. Six patients had established renal allografts and in a further three patients islets were transplanted simultaneously with a kidney transplant from the same cadaveric donor. In two cases islets were obtained from living related donors following hemipancreatectomy.

After transplantation insulin therapy could be discontinued in two patients, between 2-4 days and 13-17 days respectively. Unfortunately, insulin requirements rapidly increased in both cases to pre-transplant values and it was not entirely clear, therefore, whether this temporary amelioration of diabetes was due to insulin 'leakage' from dying islet tissue or to genuine graft function. In four other patients graft function was suggested by an increase in fasting serum C-peptide levels to >1 ng/ml (normal level ~1.5 ng/ml), although none of these cases were C-peptide negative prior to transplantation and insulin requirements remained essentially unchanged.
During this period transplantation of unpurified, dispersed pancreatic tissue was also undertaken in a number of other centres. In Zurich (Largiader et al, 1980; Kolb et al, 1981) transplantation was performed into the portal vein of four patients and by direct puncture into the spleen in three cases. Graft function was evident in only one of these latter patients, but remarkably this patient became completely insulin-independent eight months after transplantation of approximately 200,000 islets, and remained so until her death from intestinal haemorrhage a year later. The late onset of function of this graft despite severe rejection of the simultaneously transplanted kidney from the same organ donor may have been due to the relative immaturity of the islet tissue transplanted (obtained from a 2½ year old donor) which may therefore have retained some capacity for regeneration following partial rejection. Graft function was not formally established by determination of C-peptide secretion, but the clinical course of this patient was highly suggestive that insulin independence resulted from the transplant alone.

In a further study in Genoa, Italy (Valente et al, 1980b) it was attempted to overcome the problem of islet rejection by implantation of unpurified islets within 14-mm-diameter diffusion chambers, sealed with Millipore filters. Chambers (between one and five per patient) were implanted subcutaneously in 12 patients and intraperitoneally in one. Remarkably, given the small number of islets transplanted in each case (between 10,000 and 90,000), insulin-independence was claimed in two patients from 3-10 and 3-19 months post-transplantation and insulin requirements in the remaining patients were reduced by up to 78%. No data concerning pre- or post-transplant C-peptide secretion were presented, however, so that it is not clear whether insulin-independence resulted solely from function of the grafted islets.

Transplantation of unpurified, dispersed pancreas to the spleen and to the renal subcapsular space was also attempted in the early 1980's, by a group in Detroit (Toledo-Pereyra, 1986; Bandlien et al, 1987). In two patients receiving renal subcapsular transplants significant quantities of C-peptide were demonstrated within the first two weeks of transplantation and temporary insulin independence was claimed in one report from this centre (Toledo-Pereyra, 1986) but not in a subsequent communication (Bandlien et al, 1987). However, long-term insulin independence was not achieved and C-peptide levels had fallen to 0.4-0.7 ng/ml by 3 months post-transplantation. Furthermore, significant complications occurred in three renal subcapsular transplant recipients, including the development of perinephric and subhepatic abscesses in one.

In 1985 Lorenz reported the transplantation of unpurified islets in six patients, but only minimal function in terms of C-peptide secretion was observed and there were no changes in any patients' insulin requirements (Lorenz et al, 1985).
In all of these initial trials islet tissue was obtained from pancreata using methods directly adapted from those which had been successful in isolating islets from rodent pancreas. These methods involved mincing/chopping of the pancreas, with or without a previous intraductal distension with a balanced salt solution, usually followed by collagenase digestion of the minced tissue and various forms of tissue filtration. Unfortunately, the yields of islets obtained by these methods were poor when applied to the more fibrous pancreata of larger mammals, including humans. However, a considerable improvement followed the observation that these pancreata could be more effectively dispersed when the collagenase was administered by intraductal injection (Horaguchi et al, 1981; Noel et al, 1982; Gray et al, 1984). Using this technique the St. Louis group were able to isolate an average of 240,000 islets/human pancreas, which were transplanted into the spleen of six diabetic patients with established renal allografts (Scharp et al, 1989). Unfortunately, however, only limited islet function was demonstrated in three patients after transplantation, and even this function was rapidly lost after four weeks. Similarly, in five transplant procedures in Miami, no patients were rendered insulin-independent, although early graft function was established in all cases and maintained for 26 weeks in one (Alejandro et al, 1987b; Mintz et al, 1988). Subsequently, further modifications of the isolation process involved the introduction of gentler methods of dispersal of the collagenase-digested pancreas, previously achieved by relatively traumatic means, such as tissue macerators or trituration through needles. In addition, improved methods for the density gradient purification of islets were introduced. These changes will be discussed in more detail in Chapter 3, but their adoption enabled several groups to increase the yield and viability of islets obtained from the human pancreas and thereby to improve the results of continuing clinical trials of islet allotransplantation.

Thus, in the next series of nine (intraportal) transplants of purified islets performed in St. Louis insulin-independence was achieved for the first time in a patient documented to be C-peptide negative prior to transplantation (Scharp et al, 1990). Unfortunately, the function of this graft was also lost 25 days after transplantation, but a further patient remained off insulin for 308 days and significant levels of C-peptide were produced in all cases, for periods of two weeks to over 1 year (Scharp et al, 1991).

In a second series of intraportal transplants in Miami (Alejandro et al, 1992a) the mass of islets transplanted was maximised by sequential grafting, via an indwelling portal cannula, of fresh, purified islets isolated from multiple donor pancreata. Between 2 and 4 donor organs per recipient were used, resulting in the transplantation of up to 21,000 islet equivalents per kg recipient weight. Five patients had established, functioning renal allografts, whilst the sixth underwent simultaneous islet-kidney transplantation from the same donor. In all six patients grafted significant levels of C-peptide secretion...
were observed for 23 to >375 days, and two patients remained normoglycaemic off insulin therapy for periods of 36 and 38 days.

In Edmonton five patients underwent simultaneous transplantation of kidneys with purified islets from the same organ donors (Warnock et al, 1992b; Warnock et al, 1992c; International Islet Transplant Registry Report, 1992). In three cases the fresh islet preparation was supplemented with previously cryopreserved islets from 4, 5 and 10 donors. In all three patients receiving multiple donor grafts prolonged function was observed after transplantation, C-peptide rising from undetectable pretransplant levels to fasting values of up to 5 ng/ml, with insulin independence being achieved in two cases. One of these patients remained normoglycaemic without exogenous insulin for over two years, the longest period of insulin-independence yet produced by islet allotransplantation, and currently only intermittently requires low doses (4 units/day) of insulin.

In Pittsburgh, Pennsylvania, a remarkable series of islet transplants in combination with liver transplantation were performed in 1990 (Tzakis et al, 1990; Ricordi et al, 1992a; Ricordi et al, 1992d). Ten patients of this series underwent upper abdominal exenteration, entailing removal of the liver, pancreas, stomach, spleen, duodenum, proximal jejunum and terminal ileum for extensive malignant disease. Orthotopic liver transplantation was then performed and islets, obtained generally from the pancreas of the same cadaveric donor, were transplanted into the liver graft via the portal vein. In one patient islets from a third party pancreas donor were used, and in four cases islets from one or two additional donors were used to supplement those initially transplanted. Apart from the extent of the surgery undertaken this series of 'cluster transplants' was notable for the functional results of the islet allografts. Insulin independence was achieved in six patients for at least 8 months, and of those patients currently surviving without tumour recurrence two remain insulin independent, more than 22 and 24 months post-transplantation.

Insulin independence following combined liver-islet transplantation was also observed in two of the seven patients described by Altman et al., when islets were transplanted into a surgically-constructed epiploic flap (Altman et al, 1992b). One of these patients has continued off insulin for over 30 months, although diabetes in this case was secondary to haemochromatosis, rather than Type 1 diabetes mellitus.

Three other patients at Pittsburgh also received combined liver-islet grafts. These were patients with Type 1 diabetes who also required a liver transplant for primary liver disease, but insulin-independence was not attained in any of these patients, nor in a group of nine patients receiving combined kidney-islet transplants for end-stage diabetic nephropathy, although C-peptide levels of >1 ng/ml were observed in 5 cases (Ricordi et al, 1992a).
In an attempt to avoid immunologically-mediated damage to grafted islets, one group has adapted to human islet transplantation the technology of immunoisolation, whereby islets are encapsulated within selectively-permeable poly-aminoacid-alginate membranes. In this trial (Calafiore et al, 1991; Calafiore, 1992) encapsulated islets were then incorporated into the wall of a vascular chamber which was subsequently anastomosed to the recipients' vessels as an axillo-femoral artery graft in one case, and as a humeral artery-axillary vein graft in another. Only one of these reported patients was a (C-peptide negative) Type 1 diabetic and, although significant levels of C-peptide were observed in this case for over six weeks post-transplantation, insulin independence was not achieved.

More recently, however, further progress in islet allotransplantation has been made in two other centres. In Milan six patients were reported to have received purified islet allografts following or simultaneously with a kidney allograft (Socci et al, 1991). In all six patients significant graft function, in terms of C-peptide secretion, was observed for periods up to 20 months. Of particular note was the occurrence of insulin independence in one patient, lasting for over six months, as this was the first unequivocal description of the prolonged reversal of diabetes in a C-peptide-negative Type 1 diabetic patient using purified allogeneic islets obtained from a single adult pancreas, in the absence of a concomitant liver graft. Since this report a further patient from this centre has been able to discontinue insulin following purified islet allotransplantation (International Islet Transplant Registry Report, 1992), although in this case multiple donors were used. Finally, whilst most groups have determined to transplant increasingly pure islet preparations, on the understanding that improved purity will optimise islet engraftment and may reduce immunogenicity, the Minneapolis group have recently reported further success using completely impure dispersed pancreatic allografts (Gores et al, 1992; Gores et al, 1993). Indeed, in both patients most recently reported (Gores et al, 1993) insulin independence has now been achieved (Sutherland, personal communication), again using islet tissue from a single donor pancreas.

Encouraged by the improving results of islet allotransplantation we have also recently initiated a clinical trial of human islet transplantation for the treatment of Type 1 diabetes in Leicester (London et al, 1992b).

Purified islets obtained from adult cadaveric organ donors have been transplanted intraportally into three C-peptide negative diabetic patients, immunosuppressed for a previous or simultaneous renal allograft. In all three cases basal serum C-peptide has risen to significant levels post-transplantation and further increases have followed stimulation during a Sustacal challenge test. No patient has been rendered insulin-independent, and the C-peptide response has, unfortunately, been short-lived (<1
month) in two patients. However, basal C-peptide secretion of >1 ng/ml continues in the third patient, accompanied by an approximately 50% reduction in insulin dose, more than 7 months following transplantation of islets from a single donor pancreas.

Table 4 summarises the results of the above trials, using data primarily from published reports (supplemented with up-to-date information supplied by the International Islet Transplant Registry centred in Giessen, Germany). Comparison between the results of adult islet autografts and allografts is further summarised in Figure 2.

It is apparent from these results that islet allotransplantation is far from established as a method of treatment in human Type 1 diabetes. Long-term insulin independence is a rare event and therefore the potential benefits of islet transplantation upon quality of life and the chronic complications of diabetes cannot even begin to be explored. Furthermore, the obvious immunogenicity of allogeneic islets has essentially limited the use of this technique to patients receiving generalised immunosuppression for another (previous or simultaneous) organ transplant. Nevertheless, two important findings have been made:

Firstly, islet transplantation appears to be a safe procedure. In only two studies were complications directly attributable to the islet graft observed. In the Detroit series of renal subcapsular transplants (Toledo-Pereyra, 1986) one patient suffered oedema of the kidney receiving the transplanted islets, one patient required treatment for an episode of systemic sepsis, and one developed perinephric and subhepatic abscesses. In Minneapolis (Gores et al, 1992; Gores et al, 1993) intraportal transplantation of unpurified, dispersed pancreatic tissue has been associated with the development of transient portal hypertension, similar to that seen in the autograft studies mentioned above. In one patient this rise in portal pressure was sufficient to cause an intraoperative rupture of the spleen, necessitating splenectomy (Gores, personal communication). It is interesting, however, that unpurified tissue was used in both of these studies. In contrast, no complications have been observed in any centre following the transplantation of purified islets. Meanwhile, the increasing adoption of percutaneous techniques for islet infusion (Rilo et al, 1992; London et al, 1992b) has reduced islet transplantation to a simple, local anaesthetic procedure and improved its potential safety even further.

Secondly, it has now been established unequivocally that insulin independence can follow the allotransplantation of islets into an ectopic site in C-peptide negative Type 1 diabetic patients and that this can be achieved using islets from a single pancreas. Therefore, the concept of islet transplantation in humans appears to be sound and, although considerable problems remain to be overcome, there is justifiable enthusiasm.
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Figure 2
Comparison of Adult Islet Autografts and Allografts

- Temporary Insulin Independence
- Continuing Insulin Independence
- All Cases
- Data Incomplete

![Bar chart showing comparison of adult islet autografts and allografts.](chart_image)
CONCLUSIONS

For many years an important hypothesis in diabetic management has been that long-term normoglycaemia may prevent or ameliorate the chronic complications of diabetes. Whilst this appears to be the case in experimental animals, it has proven difficult to establish in humans due to the inability to maintain prolonged normoglycaemia safely using insulin injections. This degree of metabolic control can now be regularly achieved by transplantation of the pancreas as a vascularised graft, which also confers an improved quality of life on diabetic patients, due primarily to freedom from the need for insulin injections. However, the wider application of this procedure will always be limited by the morbidity and mortality of a major surgical operation, and by the need for immunosuppressive drugs to prevent rejection, which restrict its use to patients in whom chronic complications are already advanced. The initial results of adult islet transplantation suggest that this may represent a suitable alternative treatment. Most importantly, islet transplantation appears to be safe, and potential exists, at least theoretically, to avoid the need for long-term immunosuppression. However, prolonged insulin independence has so far been difficult to achieve, so that further improvements are necessary before the most promising application of this technique can be realised - namely, the transplantation of islets to reverse diabetes soon after diagnosis and thereby prevent the debilitating complications of this disease.
CHAPTER 2

Adult Islet Allotransplantation: The Problems
The initial results of clinical allotransplantation of islets into diabetic patients are encouraging, but considerable improvement will be necessary if this technique is ever to become an established method for treating Type 1 diabetes. The purpose of this chapter is to analyse the factors limiting the success of islet transplantation in clinical trials, and to examine ways in which some of these factors may be modified.

ISLET GRAFT FAILURE: THE FACTORS RESPONSIBLE

Failure of islet transplants to reverse diabetes in the long-term may, theoretically, result from a number of causes: firstly, the number or viability of the islets initially transplanted may be inadequate to supply the necessary insulin requirements of the recipient; secondly, engraftment of islets, with the establishment of a new blood supply in the transplant site, may be poor; and finally, the continued survival and function of the islets after implantation may be compromised by a variety of causes, both immune- and nonimmune-mediated.

Potentially, any or all of these factors may have contributed to the high failure rate observed in the clinical trials outlined in Chapter 1. Unfortunately, attempting to determine the relative contributions of these factors by analysis of the clinical data is difficult, due to the extraordinary variety of approaches used in different centres. Thus, almost all reported series differ from each other with respect to the number and purity of islets transplanted; the site of transplantation; the categories of graft recipient studied; the methods used to avoid graft rejection; and even in the documentation of graft function.

In the absence of any histological evidence to establish the reasons for graft failure in these clinical series, the relative contributions of these factors must remain speculative. However, some important information may be obtained, using a combination of experimental and clinical data, and each potentially important factor will therefore be considered separately in the following discussion.

The Number of Islets Transplanted

When discussing the number of islets required for transplantation three important points must be made.

Firstly, islets vary considerably in size, and therefore in their content of β-cells. In an individual human pancreas islet diameters range between 15-500 μm (Kaihoh et al, 1986), corresponding to a greater than 30,000-fold range in islet volume (because volume is related to the cube of the diameter). Between about 18 and 82% of an islet's volume is in turn made up of insulin-containing cells, the exact proportion depending
upon the location of an individual islet within the pancreas (Stefan et al., 1982).
Furthermore, the number of islets within the pancreas also varies between individuals
(Hellman, 1959; Saito et al., 1978), and similar considerations apply to the islets of
experimental animals (Marchetti et al., 1990; Davis et al., 1988). Therefore, it is clear
that the results of islet transplantation will depend upon the volume (or mass) of islet
tissue transplanted, rather than the absolute islet number, and that there is not
necessarily a direct relationship between these two latter variables (Alderson et al,
1987a).
Secondly, the viability of the transplanted islets is clearly important. Unfortunately, this
variable is difficult to assess accurately prior to transplantation (Ricordi et al, 1990a)
and therefore remains a considerable source of error in interpreting the number of islets
required to reverse diabetes.
Finally, whilst the ability of an islet graft to reverse diabetes at all must depend upon its
content of insulin-secreting β-cells, and therefore upon the mass of islets transplanted,
there is also evidence that the long-term function of such a graft may be affected, in a
more subtle manner, by the number of islets initially transplanted. Furthermore, whilst
several studies have suggested the optimal number of islets necessary for early function
in experimental animals, only limited information is available concerning the
requirements for prolonged function.
It is apparent from the above discussion that future progress will depend in part upon
standardisation in reporting the quantity and quality of islets transplanted. Graft size
should preferably be expressed as an absolute volume of (pure) islet tissue or as the
number of 150μm islet-equivalents (i.e. the number of islets of 150μm diameter which
would occupy the same volume as the graft; 150 μm being the approximate mean
diameter of islets in the human pancreas), with some indication of in vitro viability also
being provided (Ricordi et al, 1990a; Ricordi et al, 1992b).

Islet Mass and the Reversal of Diabetes: Animal Studies
The concept of a 'critical islet mass' above which initial normoglycaemia can be
consistently achieved, and below which it cannot, has been confirmed in a number of
experimental animals. Mice require about 200 syngeneic islets consistently to reverse
diabetes (Gotoh et al, 1988a), whilst 800-1000 islets (representing about one-third of
those present within the native pancreas) are generally required in immunocompetent
rats receiving syngeneic islets or in 'nude' rats receiving allo- or xeno-geneic tissue
(Lake et al., 1988; Lake et al., 1989b; Keymeulen et al, 1992).
In initial experiments involving intrasplenic autografts of dispersed pancreas in dogs,
the critical mass also appeared to represent about one-third of the islet tissue contained
in the native pancreas (Alderson et al, 1984a; Alderson et al, 1984b; Griffin et al,
1986). However, these studies also demonstrated that the method of islet isolation used can profoundly influence the estimate of critical mass obtained in an individual study (Griffin et al, 1986), presumably due to differences in islet viability between isolation techniques. Moreover, following successful transplantation mild abnormalities in glucose clearance may still occur, despite insulin independence being achieved, and these are not necessarily reversed by transplantation of increasing quantities of islet tissue, once the critical mass has been exceeded (Alderson et al, 1984a).

The most useful animal studies for estimating the critical islet mass required in humans have been those in which the methods of transplantation have been similar to those used in clinical trials i.e. purified islets, transplanted into the portal vein; in which islet mass has been accurately quantified, as discussed above; and in which the critical islet mass has been corrected for the weight of the animal. Such studies have been performed in dogs (Warnock et al, 1988a; Warnock et al, 1990a), suggesting that diabetes may be consistently reversed using autotransplantation of 4.1-4.5 μl/kg body weight, i.e. 2,300-2,500 (150μm)-islet equivalents/kg, or approximately 5000 (150μm)-islet equivalents/kg. In other studies, estimates of the critical graft size in dogs have been obtained, both higher (up to about 8.4 μl/kg or 4,700 (150μm)-islet equivalents/kg) and lower (4,380 dog islets/kg, diameters not stated) than these, using slightly different methods (Alderson et al, 1987a; Munn et al, 1988).

In dogs, therefore, the critical graft volume for reversal of diabetes following autotransplantation is between about 4 and 8 μl/kg. If these results can be directly extrapolated to human beings, this would imply that 160,000 to 320,000 islet equivalents (150 μm diameter) would be required in a diabetic patient of average weight (70kg), in the absence of allograft rejection.

However, animal studies have also demonstrated that an increased number of islets is necessary to reverse diabetes following allotransplantation, compared to autotransplantation, presumably due to the effects of early allograft rejection (Finch et al, 1977; Selawry et al, 1983). In dogs, this increase may need to be 40% or more (Warnock et al, 1990a). On this basis, therefore, it might be expected that the initial reversal of diabetes could be achieved in humans by allotransplantation of 224,000 to 448,000 islet equivalents.

**Islet Mass and the Reversal of Diabetes: Clinical Evidence**

Following partial pancreatectomy in humans normoglycaemia can quite often be maintained by a pancreatic remnant representing 10-20% of the original pancreatic mass (Child et al, 1969; Farney et al, 1992). As the human pancreas contains around 1-1.5 million islets, this again suggests that about 200,000 islets is the minimum number required for reversal of diabetes following islet transplantation in the absence of
rejection. However, these estimates do not take account of the concomitant reduction in glucagon secretion and nutrient absorption following pancreatectomy, nor of the potential for regeneration of islets within the pancreatic remnant, which, at least in rodents, is not inconsiderable (Weir et al, 1986). Therefore, extrapolation from pancreatectomy studies will tend to underestimate the critical mass of isolated islets required in Type 1 diabetic patients, where glucagon secretion is high, nutrient absorption normal, and the regenerative capacity of transplanted islets potentially compromised by the preceding trauma of the isolation process. Similar arguments apply when considering the results of islet autotransplantation, when, following total pancreatectomy, insulin independence has been achieved using as few as 110,000 islets (approximately 3,100 islets/kg), whilst recipients of lower numbers have universally remained diabetic (Farney et al, 1992).

However, as documented in Chapter 1, Type 1 diabetic patients can be rendered insulin independent by transplantation of segmental pancreatic allografts representing about 50% of the original pancreas. On this basis, 500,000 to 750,000 islets (or about 7,000 -11,000 islets/kg) might be expected to achieve normoglycaemia consistently in Type 1 diabetes following allotransplantation.

The most relevant data, however, is that which is beginning to accumulate following clinical trials of adult islet allotransplantation. In Table 1 those patients achieving insulin independence for any length of time after adult islet allotransplantation are summarised with respect to the number of islets transplanted. It is clear that there is considerable variation between studies, but that the above estimates of the critical islet mass appear to be consistent with the clinical results obtained so far, in that insulin independence has generally followed transplantation of 200,000 to 1,200,000 islets. More specifically, in those cases where diabetic status and islet graft function have been adequately documented by C-peptide assay, the minimum number of islets required to reverse Type 1 diabetes using adult islets from cadaveric donors has been 250,000 with a concomitant liver transplant, and 300,000 without.

**Islet Mass and Long-term Graft Function**

Insulin independence has been achieved by adult islet allotransplantation, but in a number of these cases initial graft function has been followed by progressive failure and a return to hyperglycaemia, often presumed to result from allograft rejection. Undoubtedly, rejection has been very important in limiting the long-term success of islet allografts, but a similar pattern of early function and delayed failure has also been described following islet autotransplantation in large animals and humans, a situation presumably unaccompanied by such immunological damage.
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**TABLE 1**

Class of insulin independence following islet allotransplantation.
Thus, in 1986 Alejandro and colleagues reported that, in dogs undergoing successful intraportal islet autotransplantation after pancreatectomy, initial insulin independence was followed by a spontaneous return to hyperglycaemia in 80% of cases over 4-15 months (Alejandro et al., 1986). Furthermore, in each of these animals there was a progressive reduction in intravenous glucose tolerance prior to clinical graft failure. Biopsies from dogs reverting to hyperglycaemia revealed marked β-cell degeneration and degranulation. The authors suggested that, amongst other mechanisms, the chronic stimulation of an initially marginal β-cell mass might have been responsible for these late graft failures.

Subsequently, late failure of islet autografts was observed in a number of other studies. Following successful intraportal islet autografts in monkeys, 7/10 recipients became hyperglycaemic between 3-18 months post-transplantation (Sutton et al., 1987). Significantly, late graft failure was related to poorer initial function, as reflected by glucose clearance during intravenous tolerance tests (IVGTT K-values). A similar broad relationship between early graft function and late failure was also observed by Kneteman, in a study of intrasplenic islet autografts in dogs (Kneteman et al., 1989). Furthermore, Kaufman established a correlation between the number of islets initially transplanted and the duration of function of intrahepatic and intrasplenic autografts in dogs (Kaufman et al., 1990a). Interestingly, despite this, there was no link between long-term survival and initial graft function (IVGTT K-values) in this latter study. A similar delayed deterioration in β-cell function had previously also been observed following extensive partial pancreatectomy in rats (Martin et al., 1963) and dogs (Sun et al., 1974; Yasugi et al., 1976). In these experiments a small pancreatic remnant (5-30% of the pancreas) was initially able to maintain normoglycaemia, but diabetes subsequently ensued after about 6 weeks, accompanied by histological changes of β-cell degranulation and degeneration. In humans a similar pattern of appearance of diabetes has also been reported following extensive pancreatic resection (Rossi et al., 1987).

Hyperglycaemia per se may contribute to this impairment of β-cell function. Thus, permanent diabetes may be induced in dogs undergoing partial pancreatectomy by maintaining hyperglycaemia for two weeks with a glucose clamp (Imamura et al., 1988). In this situation diabetes occurs even in animals whose extent of pancreatectomy would not normally result in a delayed onset of diabetes. Also, exposure of islets to high glucose concentrations both in vitro (Davalli et al., 1991) and in vivo (Weir et al., 1986; Leahy et al., 1988; Leahy et al., 1992) produces abnormalities in glucose-induced insulin secretion, associated with various changes within islets at the molecular level. These include defects in the transport, storage and metabolism of glucose and in the production of the secondary messengers which usually lead to insulin release (Leahy et
Finally, in rodents, β-cell loss has been associated with chronic hyperglycaemia following the deliberate implantation of an islet mass insufficient to reverse diabetes (Gray et al., 1989a; Jansson et al., 1990; Warnock et al., 1990d; Montana et al., 1992).

There is, therefore, some evidence to support the concept that islet grafts containing a marginal islet mass may be susceptible to long-term failure through chronic stimulation by even transient or mild degrees of hyperglycaemia. Indeed, this hypothesis has also been advanced to explain ongoing deterioration in β-cell function following initial autoimmune damage in the pathogenesis of Type 1 diabetes (Unger et al., 1985; Weir et al., 1986).

However, β-cell 'exhaustion' is not the only mechanism responsible for long-term islet graft failure. In dogs receiving segmental, vascularised autografts of approximately one-third of the pancreas normoglycaemia has been maintained for up to 4 years, without deterioration in glucose tolerance (Cutfield et al., 1985). Furthermore, initial graft function in these animals was very similar to that of the intrahepatic islet autografts, mentioned above, which ultimately failed (Alejandro et al., 1986). Also, a number of studies have suggested that the incidence of late failure of islet autografts is dependent upon the site of transplantation. In particular, late failure appears to be more frequent after intraportal autotransplantation than in other sites (Warnock et al., 1989a; Hiller et al., 1991; Scharp et al., 1992b). Unfortunately, the relative importance of transplant site and islet mass on the long-term function of human islet autotransplants is impossible to assess, because almost all have been performed via the portal vein.

**Number of Islets Transplanted: Summary**

The mass of islets grafted is critical to the success of clinical islet allotransplantation in achieving and maintaining normoglycaemia. A recent review of all adult islet autografts (International Islet Transplant Registry Report, 1992) suggested that transplantation of greater than 8,000 islet equivalents/kg recipient weight correlated with increased success (insulin independence for >1 week), and this figure is in broad agreement with estimates obtained from large animal studies. However, for prolonged function in Type 1 diabetic patients considerably more islets may be required, in order to allow for any ongoing losses and to prevent long-term graft 'exhaustion'. Unfortunately, obtaining this mass of viable islets continues to be problematical, and in many centres has only been achieved by pooling islets isolated from multiple donors. Indeed, even in the last 3 years, 54% of all adult islet autografts, and 57% of those in which insulin independence has been achieved, have been performed using multiple donors. Although the facility for culture or cryopreservation of islets makes this
approach feasible, its immunological consequences are unknown, and, given the international shortage of cadaveric organ donors, it does not represent an efficient use of a scarce resource. Thus, improvements in islet isolation techniques are necessary before the full potential of islet allotransplantation can be realised. Finally, it is important to note that implantation of a large islet mass does not guarantee success. In three centres cases have been reported where transplantation of >10,000 islet equivalents/kg has failed to reverse diabetes (Scharp et al, 1991; Socci et al, 1991; Wannock et al, 1992c), even temporarily. Other factors are therefore clearly important in establishing graft function.

Islet Engraftment

Islets are highly vascular structures. Within the native pancreas islets contain a rich capillary network, perfused directly from nearby arterioles, through which they may cumulatively receive 10-20% of the blood flow of the whole pancreas (Carroll, 1992). Thus, it is likely that re-establishment of some form of vascular supply following transplantation will be necessary for continuing islet function. Following intraportal transplantation of isogeneic islets in rodents, islets have been found to impact within the interlobular portal venules of the liver, in which location revascularisation does indeed occur, by the ingrowth of new capillaries, derived predominantly from the hepatic arterial system. This process begins on the second day post-transplantation and is generally complete by the 8th-11th day (Griffith et al, 1977; Andersson et al, 1989). A similar sequence of events occurs following transplantation of rodent islets to the renal subcapsular site, although in this location revascularisation is slower, occurring between the 6th and 34th day after transplantation (Rooth et al, 1989).

Unfortunately, despite its undoubted importance, there is relatively little information available on the factors which influence this revascularisation process following transplantation. Potentially, the conditions in which islets are stored prior to transplantation might be of relevance, as it is known that deterioration in the islet capillary bed may occur in this period, for instance during short-term tissue culture (Stagner et al, 1990). However, it appears that, despite these changes, revascularisation may still occur successfully following culture of islets for seven days at either 24°C or 37°C (Menger et al, 1990c), and also following cryopreservation (Menger et al, 1992).

The site of implantation may also be important. However, whilst engraftment of islets has been documented in each of the transplant sites commonly used in clinical trials, i.e.
the liver (Griffith et al, 1977; Andersson et al, 1989), spleen (Bryer-Ash et al, 1983) and renal subcapsular space (Rooth et al, 1989), there have been no histological studies directly comparing these sites solely with respect to islet engraftment. Following transplantation, the engraftment process may also be adversely affected by immunological mechanisms. In hamsters, xenogeneic islets are initially revascularised identically to syngeneic islets, but whilst the microcirculation of the latter tissue continues to develop, that of the former undergoes degeneration and occlusion by white cells (Menger et al, 1990a). This degeneration may be partially ameliorated by administration of the novel immunosuppressive agent 15-deoxyspergualin, but not by cyclosporin (Menger et al, 1991). Presumably immune mechanisms may also exert a similar adverse influence upon islet revascularisation following allotransplantation. Thus, early graft failure associated with rejection may result partly from ischaemic damage to islets, in addition to the specific immune destruction of endocrine tissue. Although the extent to which this actually occurs in clinical transplants is unknown, it is likely to be an important factor, and this process may therefore explain the high rate of primary graft failure seen in clinical trials of islet allotransplantation.

Factors related to the diabetic state may also potentially compromise islet engraftment. For instance, early graft function is improved, and less islets are required to reverse diabetes, if normoglycaemia is maintained with insulin treatment in the immediate post-transplant period (Hoffman et al, 1981; Hayek et al, 1988; Korsgren et al, 1989; Yumiba et al, 1992). Indeed, the 'tight' control of blood glucose using intravenous insulin infusions in this period is one factor which has been correlated with greater success after clinical islet allotransplantation (International Islet Transplant Registry Report, 1992). However, hyperglycaemia itself does not appear to inhibit the ingrowth of new capillaries into transplanted islets (Menger et al, 1990b), so that its harmful effects are probably due to altered regulation of blood flow through established vessels (Jansson et al, 1989), or to the direct effects on B-cell function mentioned above. In theory, islet engraftment might also be impaired by pre-existing diabetic microangiopathy. Unfortunately, the importance of this issue is difficult to assess, as clinical islet transplantation has been largely restricted to patients with advanced small vessel disease, whilst results from animal studies have been contradictory (Koulmanda et al, 1987; Gray et al, 1989a; Cuthbertson et al, 1990). Finally, the immunosuppressive drugs used in clinical trials of islet transplantation are inhibitory to the revascularisation process. Certainly, this has been established for prednisolone (Menger et al, 1990d) and for cyclosporine, although the detrimental effects of the latter may be reversed by treatment of the recipient with verapamil (Rooth et al, 1989).
One further feature of islet engraftment, worthy of mention, concerns the re-innervation of islet tissue. Following islet transplantation to the liver, spleen and kidney capsule, branches of nerve fibres from the surrounding parenchyma have been observed growing into the grafted tissue (Griffith et al, 1977; Korsgren et al, 1991; Korsgren et al, 1992; Noda et al, 1993). Whether these nerve fibres serve any physiological function is not known, although in normal subjects neural stimuli are thought to be important in causing anticipatory release of insulin from pancreatic islets upon ingestion of a meal. Denervation of isolated islets prevents this from occurring and may be of some relevance to clinical islet transplantation, as a delay in insulin release until blood glucose levels start to rise produces greater degrees of hyperglycaemia than with anticipatory release, even when the amount of insulin delivered is identical (Kraegen et al, 1981). Furthermore, denervation may contribute to systemic hyperinsulinaemia following pancreatic transplantation (Bewick et al, 1981). Unfortunately, the factors influencing islet re-innervation remain unknown at present.

**Post-Transplantation Islet Function**

**Transplantation Site**
The site selected for islet transplantation may profoundly influence graft function. In rodents, a bewildering number of sites have been employed (Bretzel et al, 1992), islets being implanted into muscle; subcutaneous tissue; the peritoneal cavity; the spleen; the portal vein; and beneath the renal capsule, in addition to more exotic sites, such as into the lung, testis, prostate, salivary glands, anterior chamber of the eye, and into the brain.

The large variety of approaches has made it difficult to establish the relative advantages of these various locations. Potentially, certain sites may be beneficial by conferring protection from allograft rejection. However, these 'immunologically privileged' sites, such as the testis, eye and brain, are unsuitable for use in clinical islet transplantation, and there is little evidence that the other commonly-used sites differ significantly from each other in terms of immunological advantage. The unique exception to this, of relevance to clinical transplantation, is the apparent protective effect conferred by implantation of islets into a simultaneously transplanted liver, syngeneic with the islet graft (Reece-Smith et al, 1983). This phenomenon may explain the greater success, and lesser islet mass required, in the transplant series performed in Pittsburgh and Paris (Table 1).

Transplantation sites may also differ in their ability to sustain engraftment of transplanted tissue, and to facilitate the metabolic effects of the graft. Concerning the latter point, it is generally accepted that delivery of insulin from the graft into the hepatic
portal circulation is advantageous, both to maximise the effects of insulin on glucose utilisation, and to avoid systemic hyperinsulinaemia (Brown et al., 1979; Reece-Smith et al., 1982; Nason et al., 1988; Diem et al., 1990).

For these and other considerations, the commonest sites employed in large animal studies have been the hepatic portal vein, spleen and, to a lesser extent, the renal subcapsule; and the majority of clinical islet grafts in humans have been performed via the portal vein, although the spleen, renal subcapsule and epiploic flap have all been used in recent years.

Determining which of these sites is optimal for human islet transplantation on current evidence is essentially impossible, given the low success rate at any site, and the predominance of intraportal transplants. However, some information has been provided by studies in large animals. Thus, in dogs and monkeys only limited success has been obtained following renal subcapsular implantation of islets. In all cases in which direct comparison has been made, this site has yielded results inferior to those following intrasplenic transplantation of both autologous and allogeneic islets (Hesse et al., 1986b; Cattral et al., 1988; Kaufman et al., 1990a; Gray, 1990). Indeed, consistent reversal of diabetes in dogs has only been achieved in one centre using this route of islet implantation (Toledo-Pereyra et al., 1984a), in contrast to the universally successful use of this site in rodents. Some success has also followed intraperitoneal islet transplantation in dogs, although the number of islets required to establish normoglycaemia is higher than at other sites (Warnock et al., 1992a; Ao et al., 1992).

In the majority of studies, therefore, comparison has been between the intraportal and intrasplenic sites. Initial graft function at these locations is essentially indistinguishable (with few exceptions (Kretschmer et al., 1977a)), and similar islet numbers are required to reverse diabetes. However, as mentioned above, late failure of islet autografts appears to intervene more often and earlier after transplantation into the portal vein, compared with the spleen, and this is the case in both dogs (Warnock et al., 1989a; Kaufman et al., 1990a; Motojima et al., 1992; Scharp et al., 1992b) and monkeys (Gray, 1990).

The reasons for the increased rate of graft loss following intraportal transplantation are unknown, although various possibilities have been suggested. Thus, it has been repeatedly observed that canine islets undergo a degree of fragmentation after transplantation into the liver, individual β-cells coming to lie adjacent to, and even to form junctional complexes with hepatocytes (Griffith et al., 1977; Alejandro et al., 1986; Blech et al., 1990; Hiller et al., 1991). It has been suggested that this fragmentation may, in turn, be associated with loss of islet function, although fragmentation has also been observed following intrasplenic autotransplantation (Bryer-Ash et al., 1983).

Alternatively, islet damage may follow the local release of inflammatory mediators,
such as nitric oxide, which can be released from appropriately stimulated hepatocytes (Nussler et al, 1992). Finally, portal venous blood may contain toxic components, such as bacterial endotoxins, gastrointestinal peptides (Warnock et al, 1992a) and even glucose. The latter is, of course, present in higher concentrations in portal than in systemic blood, so that β-cell 'exhaustion' may occur earlier in intrahepatic than intrasplenic grafts, despite transplantation of a similar islet mass.

The advantage of the intraportal site for clinical islet transplantation is the facility for percutaneous implantation under local anaesthesia, which is clearly important if islet transplantation becomes more widely applicable. In this respect it is important to note that the results of intrasplenic transplantation in dogs are improved by reflux of islet tissue into the splenic veins at laparotomy, rather than by direct percutaneous injection into the splenic pulp (Warnock et al, 1983; Hesse et al, 1986a), and that this technique has therefore been the most widely used in large animal studies. In addition, only limited success has been achieved following direct intrasplenic implantation in humans. Clearly, the ideal site for islet transplantation in humans is uncertain, although the established safety of the intraportal site (at least when using purified islets) will probably ensure its continuing popularity in future clinical trials.

**Immune Injury to Islets**

Theoretically, two immunological processes may contribute to the failure of allotransplanted islets in patients with Type 1 diabetes mellitus, namely recurrence of autoimmune β-cell destruction, and allograft rejection.

Selective destruction of β-cells, presumably due to recurrence of autoimmune disease, has been observed in segmental pancreatic grafts transplanted between monozygotic twins or HLA-identical siblings (Sutherland et al, 1988b; Sutherland et al, 1989b). However, in these cases recipient immunosuppression has been minimal, or omitted altogether, whilst disease recurrence has never been observed unequivocally in patients receiving standard immunosuppressive regimes for organ transplantation from cadaveric donors. Thus, it is unlikely that autoimmune recurrence has contributed significantly to the poor results of clinical islet allotransplantation, as the majority of these patients have been fully immunosuppressed.

Allograft rejection, on the other hand, is widely held to be of considerable importance in limiting the results of clinical islet allotransplantation. Certainly, in animals allogeneic islets are rejected within days in the absence of immunosuppression (Bretzel et al, 1992; Warnock et al, 1992a), and isolated islets appear to be equally, or even more immunogenic than vascularised pancreatic grafts in this respect (Nash et al, 1977; Sutherland et al, 1980b; Perloff et al, 1980; Gray et al, 1985; Hiller et al, 1989).
Unfortunately, the diagnosis of islet rejection in clinical transplantation trials is highly problematical. By the time blood glucose levels start to rise, or C-peptide levels to fall, the majority of the graft has already undergone irreversible damage, associated with extensive lymphocytic infiltration (Schulak et al, 1977; Franklin et al, 1979; Schulak et al, 1985). Thus, recurrent hyperglycaemia is a late marker of rejection, in addition to being non-specific for immunological causes of graft loss. The lack of a suitably specific rejection marker not only impairs interpretation of the factors contributing to graft loss in clinical trials, but also prevents appropriately-timed treatment to reduce rejection when it occurs.

In vascularised pancreatic transplantation this problem has been addressed in two principal ways. Firstly, the transplantation of a pancreas simultaneously with a kidney from the same organ donor has allowed pancreatic rejection to be anticipated, and therefore appropriately treated, by monitoring for concurrent renal allograft rejection (Sollinger et al, 1988). In this way pancreatic graft survival has been improved, compared to the results of pancreatic transplantation alone or following a previous kidney transplant from an unrelated donor; overall 1 year graft survival rates being 68%, 37% and 45% respectively, in the years 1986-1990 (Sutherland, 1991).

Secondly, methods have been developed to monitor exocrine tissue rejection, which occurs concurrently with rejection of endocrine tissue. These include the use of bladder drainage techniques of duct management, with analysis of changes in urinary amylase (Prieto et al, 1987); and direct biopsy of the graft, either at cystoscopy for bladder-drained grafts (Perkins et al, 1990; Brayman et al, 1992) or percutaneously (Allen et al, 1990). Again, these techniques are associated with improved graft survival (Sutherland, 1991; Sutherland, 1992c).

In addition to these improvements in the monitoring of graft rejection, the results of vascularised pancreatic transplants have also been enhanced by measures designed to reduce the incidence of rejection. Thus, reducing the number of HLA-DR mismatches improves graft survival, although this effect is only significant in recipients of pancreas transplants alone (Sutherland, 1991). In this group of patients, 1 year graft survival in the era 1986-1990 was 54% with both DR antigens matched, 44% with one DR antigen matched, and 29% with complete DR mismatch. In addition, the use of potent anti-T-cell agents, such as polyclonal Anti-Lymphocyte Globulin (ALG) or the monoclonal antibody OKT3, for induction immunosuppression improves pancreatic graft survival. One year graft survival is increased from 59% in patients receiving standard immunosuppression with cyclosporine, azathioprine and prednisolone, to 63% and 71% if ALG or OKT3 respectively are used in immunological prophylaxis (Sutherland, 1991).
Some of these factors, such as the effect of simultaneous renal transplantation, DR matching and anti-T-cell induction immunosuppression, might therefore be expected to influence the success of clinical islet transplantation. Unfortunately, analysis of these factors individually, based upon the available clinical data, is difficult, due to the small number of patients in each relevant subgroup; the large number of different approaches used in various centres; and to the use of multiple pancreatic donors.

Thus, of those adult islet allografts, excluding the Pittsburgh 'cluster' transplant series, performed between January 1990 and June 1992 (International Islet Transplant Registry Report, 1992), simultaneous renal transplantation was undertaken in 30 cases, and other recipient categories accounted together for a further 26 cases. Insulin independence of at least 1 week was achieved in 13% of the simultaneous-islet-kidney group, compared to 15% of the 'other' recipient category group; the corresponding figures for achieving positive C-peptide levels (≥ 1 ng/ml for ≥ 1 month) were 57% and 62%. These data suggest that simultaneous renal transplantation does not benefit islet grafts, in the way that it benefits whole pancreas grafts. However, in the majority of cases islets were obtained from multiple donors, so that only part of the islet graft was syngeneic with the simultaneously transplanted kidney, and the remainder of the graft may therefore have been rejected without any change in renal function. The potential benefits of a simultaneous renal transplant upon islet graft survival are therefore unknown, and will only be realised if single pancreatic donors are used.

Similarly, the use of multiple donors makes interpretation of HLA-DR matching data essentially impossible, particularly as only 3 recipients have received islets with both HLA-DR antigens matched, in the 1990-1992 era.

The role of induction immunosuppression with anti-T-cell agents is also difficult to interpret. Between 1990 and June 1992, insulin independence and positive C-peptide levels respectively were achieved in 13% and 75% of those receiving OKT3 induction, compared to 23% and 62% receiving polyclonal sera; and 24% and 60% receiving neither therapy. It should be pointed out, however, that no patient in the Pittsburgh 'cluster' series, which may represent a unique immunological situation, received prophylactic anti-T-cell therapy: exclusion of these cases from the final group produces revised figures of 0% achieving insulin independence, and 47% achieving positive C-peptide levels, and suggests therefore that anti-T-cell induction therapy may indeed be of benefit in prolonging islet graft survival (Figure 1).

Macrophages and Inflammatory Mediators

Macrophages may be involved in the autoimmune destruction of islets in spontaneous diabetes (Walker et al, 1988; Ihm et al, 1990), and are certainly capable of releasing cytokines which are known to injure islets in vitro (Mandrup Poulsen et al, 1986;
Figure 1

Adult Islet Allografts: The Effect of Induction Immunosuppression

- Insulin independence ≥ 1 week
- C-Peptide ≥ 1 ng/ml for ≥ 1 month
- All Cases

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<tr>
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<td>6</td>
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</tbody>
</table>

Number of Patients

30

20

10

0
Mandrup Poulsen et al., 1987; Sandler et al., 1987; Pukel et al., 1988). Thus, these cells may contribute to islet damage following transplantation, in addition to the role of classical T-cell mediated immune rejection. Furthermore, this damage may occur very early in the post-transplant period, and thereby contribute to the high rate of primary nonfunction often seen following islet allografting.

Thus, in mice primary nonfunction of islet allografts (i.e., the failure to reverse diabetes, even temporarily, after transplantation) has been associated with macrophage infiltration of the graft; moreover, pretransplant depletion of the recipient macrophage population with silica completely prevented this phenomenon (Kaufman et al., 1990b).

Other agents which depress macrophage function, such as 15-deoxyspergualin, also prolong islet allograft survival in rats and dogs (Walter et al., 1987; Gores et al., 1992), results which have led one group to use this agent in their human islet allotransplantation programme (Gores et al., 1993). However, the role of macrophages in primary nonfunction of islet allografts in human recipients is not yet established, and indeed universal success has not been observed in all animal experiments in which attempts at macrophage depletion have been made (Mellert et al., 1992).

**Drug Effects**

In order to avoid rejection of transplanted islets, the majority of islet allografts in humans have been performed in patients receiving immunosuppressive drugs. Unfortunately, many of these drugs are known to have adverse effects upon islet function, in addition to the effects upon islet engraftment, mentioned above. Thus, insulin release from islets may be inhibited by cyclosporin A (Gunnarsson et al., 1983; Nielsen et al., 1986; Robertson, 1986; Alejandro et al., 1992b) and by FK506 (Ricordi et al., 1991b; Alejandro et al., 1992b), both of which also cause increased peripheral resistance to insulin (Alejandro et al., 1992b). Similar effects are produced by prednisolone (Kaufman et al., 1991; Morel et al., 1992b), whilst azathioprine appears to reduce insulin content of islets, as well as inhibiting insulin release (Viviani et al., 1989).

The combined effect of these drugs in islet transplant recipients may therefore seriously compromise graft function (although interestingly, prednisolone may protect against the adverse effects of cyclosporin and azathioprine (Viviani et al., 1990)), particularly when it is considered that insulin resistance is already present in Type 1 diabetic patients, even prior to transplantation (DeFronzo et al., 1982).
Islet Purification

The need to purify islet tissue before transplantation is a contentious issue, and will be considered separately here, as islet purity may potentially influence each of the factors mentioned above. Thus, all methods of islet purification are associated with some reduction in islet yield, and therefore in the volume of islets transplanted. Furthermore, purification procedures are potentially traumatic, and may therefore be associated with a reduction in islet viability. These considerations have led some investigators to abandon islet purification prior to transplantation, in the interests of maintaining optimal islet yield (Gores et al, 1992). However, most centres involved in ongoing clinical studies continue to use purified tissue, as post-transplantation graft function may be influenced by islet purity. In addition, loss of islets in culture, prior to transplantation, occurs much more rapidly in islet preparations containing large quantities of acinar tissue, compared to pure islets (Sever et al, 1992). Thus, when using unpurified tissue, maintenance of islet viability can only be assured if transplantation is performed immediately after islet isolation. Confirmation of islet sterility cannot then be achieved prior to transplantation, which may be a considerable disadvantage, given the known potential for microbial contamination of islet preparations (Scharp et al, 1992a).

Islet Purity and Engraftment

Contamination of transplanted islets with large quantities of acinar tissue, capable of releasing potentially damaging proteases, might reasonably be expected to inhibit islet engraftment. Despite its importance, however, few studies have been performed which directly address this issue. Allotransplant studies in pigs have demonstrated some relationship between initial graft function and islet purity (Mellert et al, 1991) when an adequate mass of islets are transplanted. However, as we have seen, initial function in the allogeneic situation may also be influenced by allograft rejection. Thus, the influence of purification alone upon engraftment can only truly be assessed in iso- or auto-graft studies.

In a rat isograft model, Downing (Downing et al, 1986) demonstrated a significant negative correlation between the amylase content of tissue transplanted to the portal vein and the function of the graft after 12 weeks, although no histological data was available to establish the fate of the transplanted tissue and the cause of its reduced function. In another study, highly purified rat islets, obtained by hand-picking, were deliberately contaminated with various known volumes of exocrine tissue, prior to transplantation into the renal subcapsular space of syngeneic rats (Gray et al, 1988). After two weeks, histological examination revealed marked scarring and loss of islet tissue in those grafts...
originally contaminated with large numbers of acinar cells, whilst grafts comprising highly purified islets appeared to undergo successful engraftment. In addition, the final insulin content of the former grafts was reduced, compared to the latter, again suggesting damage to the islet tissue of impure grafts. Similar observations were also made following renal subcapsular transplantation of human islets into nude rats, and following islet autografts in monkeys (Gray et al, 1988; Gray, 1990). These studies, therefore, clearly demonstrated the adverse effects of exocrine tissue contamination upon islet engraftment, at least when tissue was implanted beneath the renal capsule. It must be conceded, however, that transplantation to less confined sites, such as the spleen or portal vein, may reduce these effects by achieving wider dispersal of transplanted tissue, thereby reducing the number of acinar cells which come to lie adjacent to islets upon implantation (Gray, 1989). Indeed, experience with human islet autotransplants suggests that successful engraftment can occur following transplantation of completely unpurified dispersed pancreatic tissue to these sites. However, since purified tissue has not been used in these studies an adverse effect of exocrine contamination upon the percentage of islets successfully engrafting, and upon their long-term function, cannot be excluded. Clearly, further experimental investigation of this issue will be necessary before a firm conclusion can be drawn.

Islet Purity and Allograft Rejection
That the purity of transplanted islet tissue might influence the rate of its rejection was originally suggested following investigations in rodents, examining the effects of graft immunomodulation by the depletion of passenger leucocytes from islets prior to transplantation (Lacy et al, 1979b; Faustman et al, 1981; Lau et al, 1984a; Selawry et al, 1984). In these studies uniquely pure islets were obtained by 'hand-picking' from the digested pancreas, and it was observed that the survival of such preparations could often be prolonged, even in the absence of immunomodulatory treatment. Also, in dogs, whilst it had not previously been possible to induce immune unresponsiveness to impure islet allografts using cyclosporin A, this could be achieved when highly purified islets were used (Alejandro et al, 1985). This raised the possibility that the immunogenicity of allografted pancreatic tissue may be largely due to the presence of pancreatic acinar and other cells, rather than due to the islets themselves. Unfortunately, however, only a few studies have been performed specifically to examine this hypothesis, and these have been restricted to rodents. Studies in mice initially suggested that partially purified islets were indeed rejected more rapidly than hand-picked islets (Gotoh et al., 1986b). Subsequently, it was demonstrated that contaminating lymphoid tissue was largely responsible for this phenomenon, and that ductal, vascular and exocrine tissue did not contribute
significantly to graft immunogenicity (Gotoh et al, 1986a). Interestingly, however, graft function was impaired if exocrine tissue was transplanted beneath the renal capsule together with islets, rather than in the contralateral kidney, again suggesting a local inhibitory influence upon islet engraftment.

Similar findings were made in further studies in mice, where the addition of acinar cells to hand-picked islets did not influence the rate of rejection, which was however accelerated by the addition of donor-specific splenocytes (Gores et al, 1986a).

In contrast, some investigators have found that the contamination of rat islets with exocrine tissue and vascular fragments may increase graft immunogenicity (Hiller et al, 1990). More recently, this issue has also been examined using mixed lymphocyte-islet co-culture techniques, in which the proliferative response of lymphocytes to allogeneic islets is assessed in vitro. In these studies, increased contamination of human islets with acinar cells was associated with an increased response of co-cultured lymphocytes (Ulrichs et al, 1990), although the possibility could not be excluded that coincident contamination with lymphoid or ductal tissue was contributory.

Again, there is clearly a need for further investigation into this issue, in particular to study the immunological implications of islet purity in large animals, using a range of purities which can be achieved on a scale suitable for clinical transplantation.

Islet Purity and the Safety of Islet Transplantation

As mentioned in Chapter 1, significant complications, and even mortality, have followed the transplantation of unpurified pancreatic tissue into the portal vein in man, whilst no such problems have accompanied the use of even moderately pure islet preparations.

Similar observations have also been made in animals, when using the intraportal route of transplantation. Deaths following this procedure have been reported in rats (Downing et al, 1986) and dogs (Mehigan et al, 1980a). Furthermore, in both dogs (Kretschmer et al, 1978) and monkeys (Gray, 1990) marked portal hypertension has been a common feature, often accompanied by systemic hypotension (Traverso et al, 1982) and even disseminated intravascular coagulation (Mehigan et al, 1980a). Interestingly, transplantation of unpurified pancreas into the spleen in dogs appears to be relatively safe (Mirkovitch et al, 1976; Mirkovitch et al, 1977), although even here complications may result from the necessarily large volume of tissue implanted (Kretschmer et al, 1977b).

These complications appear related in part to the methods of preparation of tissue prior to transplantation. Thus, cell-free supernatants obtained during processing of the pancreas are capable of inducing the haemodynamic changes (Traverso et al, 1982) and the features of disseminated intravascular coagulation (Mehigan et al, 1980a; Miller et
al, 1983) seen following transplantation of impure tissue in dogs. The toxic components responsible appear to be proteases released from damaged acinar cells, although collagenase itself, carried over into the transplanted tissue, may also be contributory (Miller et al, 1983). Protease inhibitors, such as aprotinin, and heparin largely prevent the clotting abnormalities induced by these factors (Mehigan et al, 1980a), but do not appear to influence the haemodynamic changes, at least in primates (Traverso et al, 1982) and man (Gores et al, 1993).

Clearly, if islet transplantation is ever to be extended to newly-diagnosed young diabetic patients, its safety will be of primary concern, and this is perhaps the most convincing argument in support of the need for highly purified islets.

**Islet Graft Failure: Summary**

It is clear from the above discussion that several factors have been responsible for limiting the success of clinical islet allotransplantation, and that improvements in each of the areas discussed are necessary.

The first requirement for successful islet transplantation is a sufficient mass of purified, viable islets. Although autotransplantation of 110,000 islets has reversed diabetes in humans after total pancreatectomy, considerably greater numbers will be required for allotransplantation into patients with Type 1 diabetes mellitus: in order to overcome the insulin resistance present in these patients; to prevent islet loss from the 'exhaustion' suffered by grafts of marginal mass; and to allow for some loss of islets through poor engraftment and subsequently through immunological/inflammatory damage.

Secondly, at present, islet engraftment may only be beneficially influenced in clinical studies by tight control of blood glucose with exogenous insulin in the immediate post-transplant period, and possibly by administration of verapamil to patients receiving cyclosporine. Further research is therefore undoubtedly needed to establish ways in which islet engraftment may be improved. In addition, the optimal site for implantation of islets, particularly with respect to the maintenance of long-term islet function, remains to be established.

Finally, the problems of allograft rejection must be overcome. Several recent advances have raised the possibility that islet transplantation in the absence of long-term generalised immunosuppression may be achievable. This issue is clearly of importance regarding the wider application of islet transplantation in diabetes, and these developments are therefore discussed in more detail below.

At present, however, islet transplantation is restricted to patients requiring immunosuppression for another organ transplant. In this group of patients...
improvements may still be made, by better prospective HLA matching of donor and recipient; by using islets from single donor pancreata; by the use of OKT3/ALG for induction immunosuppression; and possibly by increasing the proportion of islet transplants performed simultaneously with a syngeneic renal graft. Unfortunately, achieving even these aims will also depend upon further improvements in islet isolation.

There is already clinical evidence that the success of islet grafts in immunosuppressed patients is dependent upon a combination of criteria including islet mass and purity, transplant site, induction immunosuppression, and peri-transplantation glucose control (International Islet Transplant Registry Report, 1992; Hering et al, 1993a): such criteria are currently used by the International Islet Transplant Registry to define so-called 'state of the art' cases, whose success rate is significantly greater than cases in which these criteria are not satisfied (Figure 2). Thus, further improvements in these key areas can reasonably be expected to result in enhanced clinical islet graft function.

**STRATEGIES FOR PREVENTING ALLOGRAFT REJECTION**

**Immunomodulation**

Theoretically, cellular grafts, such as pancreatic islets, have certain advantages over vascularised whole organ allografts, in that firstly they may be less susceptible to antibody-mediated hyperacute rejection, being largely revascularised by host-derived endothelium after transplantation (Calcinaro et al, 1992); and secondly, by virtue of their size and facility for storage in culture or by cryopreservation, they are more amenable to manipulation in vitro, prior to transplantation, in order to reduce their immunogenicity. Islet allografts are nevertheless highly susceptible to cell-mediated immune destruction in the absence of immunosuppression (Reckard et al, 1973; Franklin et al, 1979), so that some form of immunological manipulation of graft or recipient will be essential in order to realise the potential of islet transplantation.

Allograft rejection is a modification of the normal cellular immune response to endogenous or exogenous antigens (Figure 3). The initiation of this response involves interaction between various T-lymphocyte subsets and specialised antigen-presenting cells (APC's), represented by macrophages, Langerhans' cells of the epidermis, and lymphoid dendritic cells. These cells present processed antigens to T-cells in association with proteins encoded by the Major Histocompatibility Complex (MHC). Recognition of antigens in association with MHC Class I molecules by CD8+ lymphocytes leads to the generation of cytotoxic T-cells, capable of killing cellular
Figure 2

Adult Islet Allografts: 'State-of-the-Art' Cases versus Other Cases

- Insulin Independence $\geq$ 1 week
- C-Peptide $\geq$ 1 ng/ml for $\geq$ 1 month
- All Cases

'State-of-the-Art' Cases:
- Islet Mass $\geq$ 8,000 IEQ/kg; Purity $\geq$ 50%
- Intraportal Transplant Site; ATG, ALG or OKT3 Induction

Number of Patients

State-of-the-Art' Cases

Others

22
19
7
1

35
17

1

'State-of-the-Art Cases: Islet Mass $\geq$ 8,000 IEQ/kg; Purity $\geq$ 50%; Intraportal Transplant Site; ATG, ALG or OKT3 Induction
Figure 3

Schematic Diagram Illustrating the Generation of Cell-Mediated Immunity to an Antigen.
targets which bear the same antigen-MHC Class I complex on their cell surface. However, activation of this process is dependent upon the provision of 'help' from CD4+ T-cells which have been stimulated by binding antigen-MHC Class II complexes at the surface of the APC, and which receive a co-stimulatory signal (interleukin-1 and probably other, less well defined, stimuli) from the APC, presumably in response to T-cell binding of the latter's MHC Class II molecules.

The generation of a normal cellular immune response to an antigen therefore depends upon the interaction of T-cells with specialised APC's, expressing Class I and Class II MHC molecules upon their surface, and capable of providing an appropriate co-stimulatory signal. Furthermore, the effector phase of this immune response is restricted to the killing of cells expressing antigen in association with the same MHC Class I molecules as the APC. Class I molecules of the MHC are expressed on all nucleated cells, which are therefore susceptible to these effector mechanisms. Class II molecules, however, are constitutively expressed only on the specialised APC's mentioned above, on B-lymphocytes, blood monocytes and thymic epithelial cells, although T-lymphocytes and vascular endothelial cells may express these molecules under certain circumstances.

Allograft reactivity results from the ability of allogeneic MHC molecules, expressed on the surface of transplanted cells, to be themselves recognised as foreign antigens by T-cells of the recipient. This may occur through two distinct pathways (Golding et al., 1984; Singer et al., 1984; Stock et al., 1991). Firstly, intact MHC molecules may directly stimulate allogeneic T-cells whose receptors recognise determinants unique to these molecules (Figure 4). In this situation donor-specific MHC molecules are presented to recipient T-cells by donor APC's present within the graft (so-called 'passenger leukocytes' (Snell, 1957; Steinmuller, 1967)). The ability of these cells to initiate allograft reactivity is due to their expression of Class I and Class II molecules at the cell surface, and their ability to provide the appropriate stimulation to CD4+ T-cells in order to generate T-helper activity. Parenchymal cells bearing donor Class I MHC are then destroyed by cytotoxic effector cells restricted to recognise these molecules. In the second pathway (Figure 5) donor MHC molecules are processed and presented to recipient T-cells indirectly, by recipient APC's, in much the same way that any foreign antigen is processed. In this way cytotoxic effector cells capable of destroying parenchymal cells bearing the same allogeneic MHC determinants may again be produced.

Both direct and indirect pathways are probably involved in the initiation of allograft rejection of islet tissue (Stock et al., 1989b; Stock et al., 1991). Thus, islets contain dendritic cells and macrophages (Plate 2.1), many of which express surface Class II molecules (Alejandro et al., 1982; Ulrichs, 1985; Shienvold et al., 1986) and which may
Figure 4

Schematic Diagram Illustrating the Direct Pathway for the Generation of Alloimmunity

Killing

CD8+ T-Cell

Allo- T-Cell

Allogeneic APC

MHC II

MHC I

Allo- MHC I

II-1

II-2

Th

Panethelial Cell

Allogeneic Cell

MHC I
Figure 5

Schematic Diagram Illustrating the Indirect Pathway for the Generation of Alloimmunity
PLATE 2.1

Localisation of MHC Class II +ve cells ('passenger leucocytes') within an Islet of Langerhans, using an appropriate monoclonal antibody-staining technique

Microencapsulation of human islets of Langerhans
function as antigen-presenting cells in the direct pathway (Stock et al., 1991). Class II MHC molecules may also be expressed on intra-islet vascular endothelial cells, at least in human and pig islets (Alejandro et al., 1982; Shenvold et al., 1986), and there is also some evidence that endothelial cells may be capable of functional antigen presentation to T-cells (Vetto et al., 1972; Hirschberg et al., 1980; Ashida et al., 1981). However, although Class II expression may also be induced on islet endocrine cells by incubation with various cytokines (Pujol-Borrell et al., 1987; Wright et al., 1987; Markmann et al., 1989), and even by the process of islet isolation itself (Swift et al., 1992), this expression does not confer antigen-presenting function upon these cells (Markmann et al., 1988; Markmann et al., 1989), and should not therefore contribute to lymphocyte activation via the direct pathway. Class II molecules may, however, be processed by recipient APC's in the indirect pathway, as may Class I molecules which are constitutively expressed on islet endocrine cells, and these antigens may then act as targets for cytotoxic effector cells.

As mentioned above, the advantage of islet allografts, compared to vascularised whole organ transplants, is the enhanced facility for graft modification to reduce immunogenicity prior to transplantation. The potential of this approach was initially suggested by studies demonstrating prolonged survival of allogeneic ovarian and thyroid tissue with minimal immunosuppression, after depletion of passenger leukocytes by a period of pretransplantation tissue culture (Jacobs, 1974; Lafferty et al., 1975). Following these observations a number of studies have been performed, attempting to deplete islets of passenger leukocytes prior to transplantation, utilising the differential sensitivity of islet endocrine cells and lymphoid elements to several physical and chemical treatments.

Thus, short-term culture of islets at low temperature (22-24°C) has been employed in some studies (Lacy et al., 1979a; Woehrle et al., 1990; Markmann et al., 1990), as it is known that lymphoid cells are inactivated by this treatment (Opelz et al., 1974). Similarly, attempts at islet immunomodulation have involved: culture at high oxygen tensions (Gill et al., 1988); cryopreservation, particularly involving rapid cooling rates (Höll et al., 1985; Taylor et al., 1988; Taylor et al., 1992); ultraviolet irradiation (Lau et al., 1984a; Lau et al., 1984b); and gamma-irradiation (James et al., 1989; Kanai et al., 1989). More specific means for the elimination of APC's from islet grafts have also been utilised, including treatment of islets with anti-MHC Class II antibodies (Faustman et al., 1981) or anti-dendritic cell antibodies plus complement (Faustman et al., 1984).

These treatments have resulted in prolongation of islet allograft survival in the absence of long-term immunosuppression of recipient animals in most, but not all (Gores et al., 1986b) studies. It is important to note, however, that in many cases, particularly in
large animals (Alejandro et al, 1987a; Kenyon et al, 1990), islet pretreatment has only been successful when used in conjunction with a short period of immunosuppression in the peri-transplant period, using cyclosporin A or anti-lymphocyte serum, agents which occasionally lead to prolonged survival of untreated islet allografts (Selawry et al, 1983; Alejandro et al, 1985; Gotoh et al, 1988b). This, and the fact that islets depleted of MHC Class II+ cells remain immunogenic in vitro (Stock et al, 1989a; Stock et al, 1989b), suggests that mechanisms other than simple removal of donor APC's from the islet graft are involved in long-term allograft survival. Presumably this is due to the influence of the indirect pathway of allogeneic MHC presentation. Interestingly, certain pretreatment protocols, such as low-temperature culture of islets may also reduce immunogenicity by reducing the expression of MHC Class I on islet endocrine cells, and this may even be more important in determining islet survival than the degree of APC depletion (Markmann et al, 1989; Markmann et al, 1990).

The immunological relationship between the recipient and those islet allografts achieving long-term survival in these studies is also of interest. In some cases challenge of the recipient with donor-specific splenocytes or dendritic cells resulted in acute rejection of the grafts (Faustman et al, 1981; Taylor et al, 1992), suggesting that the graft was in a metastable state, accepted by the recipient, but still susceptible to rejection by the addition of appropriate APC's; in other cases rejection did not occur, implying that an established state of donor-specific tolerance had been induced (Gill et al, 1988; Kanai et al, 1989). Furthermore, in one study this tolerant state was only observed in recipients of unmodified islets given cyclosporin, and not after transplantation of immunomodulated islets, even in the presence of cyclosporin (James et al, 1989). Thus, it appears that standard regimes of islet immunomodulation, aimed at eliminating passenger leukocytes, may not necessarily induce true immunological tolerance, despite being able to prolong islet survival in the absence of chronic immunosuppression.

The Induction of Tolerance

True immunological tolerance is defined as the inability of a recipient to reject an established allograft, in the absence of generalised immunosuppression, despite immunisation of the recipient with donor-specific lymphoid cells, whilst the ability to reject grafts from genetically different third party donors is retained.

It has been known for many years that such a state may be induced by transplantation of donor-specific bone marrow stem cells into foetal or neonatal animals (Billingham et al, 1953). In adults, complete replacement of the recipient's immune system with that of the donor, by bone marrow transplantation, also produces donor-specific tolerance, which has allowed the transplantation of solid organ allografts in humans without
immunosuppression (Sayegh et al, 1991). The production of mixed allogeneic (and even xenogeneic) chimeras, in which bone marrow cells derived from both donor and recipient coexist, can also be achieved in adult animals using a variety of approaches (Ildstad et al, 1992; Oluwole et al, 1992), and again donor-specific tolerance results. However, the treatments at present necessary for attaining these states of bone marrow chimerism are impractical when considering the possible use of islet transplants in otherwise healthy diabetic patients.

Normal tolerance to self-antigens is induced by maturation of T-cell precursors in the thymus. IntrathyMIC injection of allogeneic antigens or cells might therefore be expected to induce tolerance to these antigens. Indeed, there is evidence that donor-specific unresponsiveness can be produced by intrathyMIC injection of allogeneic cells, if peripheral, mature T-cells are simultaneously depleted eg. by a peri-transplant course of anti-lymphocyte serum. Tolerance has been induced in this way using donor-specific splenocytes, providing protection of skin and other grafts (Ohzato et al, 1992; Oluwole et al, 1992); using glomerular cells to protect renal allografts (Remuzzi et al, 1991); and using islets to protect subsequent extrathyMIC islet grafts (Posselt et al, 1990).

Interestingly, intrathyMIC islet transplantation in diabetes-prone animals also seems to protect against the subsequent development of auto-immune insulitis, by development of tolerance to B-cell antigens (Posselt et al, 1992; Kœvary et al, 1992; Gerling et al, 1992), so that this technique may be of particular value for islet transplantation into Type 1 diabetic patients.

Finally, extrathyMIC tolerance may develop with time, following transplantation of islets depleted of APC's by the methods described above (Bowen et al, 1981; Gill et al, 1988; Faustman, 1992), although the exact mechanisms responsible are not fully established. One possibility is that, whilst normal Th-cell activation requires the provision of at least two signals (T-cell receptor/CD4 binding, with a co-stimulatory signal from the APC- Figure 3), provision of a single signal eg. by binding of the T-cell receptor alone may induce a state of antigen-specific T-cell anergy (Schwartz, 1990; Sprent et al, 1990; Faustman, 1992). Tolerance may certainly be induced in this way if antigens are presented to T-cells by chemically modified APC's or by purified MHC Class II molecules inserted in vitro into planar lipid membranes (Quill et al, 1987; Jenkins et al, 1987). Theoretically, then, this may also occur following transplantation of immunomodulated islets, if these islets contained non-functional (but still MHC Class II+) APC's, or if Class II molecules were expressed on islet endocrine cells, which are incapable of APC function (Markmann et al, 1988). In contrast, tolerance would not necessarily be expected if islet pretreatment led to the complete elimination of all Class II-expressing cells from the graft: such a graft may then be susceptible to rejection if expression of Class II antigens on endocrine cells was subsequently induced.
eg. by the release of cytokines during an episode of non-specific inflammation. This may explain the results, mentioned above, which were observed by James et al. (1989). This 'single signal' mechanism of tolerance may also be responsible for the prolonged survival of islet allografts induced by pre-transplantation administration to the recipient of donor-specific blood or dendritic cells treated with anti-MHC Class II antibodies and complement or ultraviolet irradiation (Faustman et al, 1982; Agostino et al, 1984; Oluwole et al, 1992). Finally, it may partly explain the state of donor-specific unresponsiveness produced by anti-CD4 monoclonal antibody treatment of allograft recipients (Sablinski et al, 1991; Shizuru et al, 1992).

The induction of extrathymic tolerance to islets may therefore be achievable, using combinations of graft immunomodulation and more specific treatment of the recipient, aimed at preventing the generation of Th activity, in particular to bypass the indirect pathway of alloimmunogenicity. Clearly this is an area worthy of further research.

**Immunoisolation**

An alternative approach to the use of graft immunomodulation or recipient immunosuppression is the encapsulation of islets within selectively permeable membranes, which permit the flux of glucose and insulin necessary for islet function, whilst protecting islets from humoral and cellular immune attack (Plate 2.1). In addition to obviating the requirement for recipient immunosuppression, such devices may also facilitate the use of xenogeneic tissue.

Thus, vascular shunts have been designed, incorporating an islet-containing chamber separated from the blood stream by a selectively permeable membrane. Implantation of such shunts into large animals (Monaco et al, 1991; Lanza et al, 1992b; Calafiore et al, 1992) and even into humans (Calafiore, 1992) has facilitated function of islet allografts and xenografts in the absence of immunosuppression, although significant complications may occur, including the inevitable problems associated with long-term vascular access, namely thrombosis and infection (Maki et al, 1991; Lanza et al, 1992b).

Alternatively, islets can be encapsulated within hollow cylindrical fibres (Altman et al, 1992a; Lanza et al, 1992a), or individually within microcapsules (Lim et al, 1980; O'Shea et al, 1986; Calafiore et al, 1990a; Soon-Shiong et al, 1992), each composed of a suitable, selectively permeable membrane. However, despite the theoretical attractiveness of this approach, several problems remain to be solved before its wider application can be considered. Firstly, engraftment of islets clearly does not occur, so that oxygen is supplied to islets purely by diffusion: the inefficiency of this process
across the additional thickness of an artificial membrane may lead to loss of islet tissue
(Schrezenmeir et al., 1992).
Secondly, an intense cellular and fibrotic reaction may occur around encapsulated islets,
particularly in spontaneously diabetic animals (Mazaheri et al., 1991; Darquy et al., 1990;
Weber et al., 1990a; Weber et al., 1992b). This may be related, to some extent, to the
composition of the capsule itself (Soon-Shiong, 1992), although in many cases empty
capsules have not elicited such a reaction. Furthermore, in spontaneously diabetic
animals this cellular reaction has often resulted in destruction of the islets contained
within the capsules (Weber et al., 1990a; Darquy et al., 1990; Weber et al., 1990b). This
may be due to the release of antigens from islets, with activation of an immune reaction
and the local release of cytokines, potentially toxic to islets: currently used membranes
may not exclude these relatively low molecular weight molecules, so that encapsulated
islets may remain susceptible to immune attack (Weber et al., 1992a; Weber et al.,
1992b).
Finally, the size of the encapsulated islets limits the sites available for transplantation.
The intraperitoneal site has been most widely used in experimental studies of
encapsulated islet transplantation, but more islets are required to reverse diabetes in
this, compared to other sites (Kemp et al., 1973; Yasunami et al., 1983; Hayek et al,
1990; Ao et al., 1992).
However, should these problems be overcome, immunoisolation of islets could become
a widely applicable means for avoiding immunological damage to transplanted islets.

**ISLET XENOTRANSPLANTATION**

One universal problem concerning the transplantation of human organs is the relative
shortage of supply of these organs from cadaveric donors. This problem has therefore
led to increased interest in recent years in the possibility of using organs from other
animal species.
There is an increasing consensus that the pig may represent the ideal donor animal for
this purpose (Niekrasz et al., 1992), because: it is easy to breed, produces large litters
and grows rapidly, so that suitable numbers of animals could always be made available;
it adapts well to different environments, and may therefore be suitable for rearing under
germ-free conditions; and marked similarities in physiology exist between pig and man.
Of particular relevance to islet transplantation is the fact that porcine insulin differs from
human insulin by only one amino acid, and has been widely used for many years in the
treatment of human diabetes. Furthermore, porcine islets respond to the same
secretagogues as human islets, releasing insulin primarily in response to glucose, and
They maintain plasma glucose levels within a very similar range to that seen in humans. Finally, the use of pigs may circumvent the potential problems associated with the use of primate organs, namely the ethical issues involved, the lower numbers of animals available, and the risk of transmitting pathogenetic viruses to human recipients. However, considerable immunological problems exist in the use of organs transplanted between species (Auchincloss, 1988; Platt et al., 1991). Firstly, vascularised xenotransplants may be susceptible to hyperacute rejection, similar to that seen in presensitised recipients of vascularised allografts, due to the presence of pre-formed natural antibodies to xenogeneic tissue. Secondly, xenografts which have avoided hyperacute rejection remain susceptible to cell-mediated rejection processes. Considerable advances have recently been made in understanding the processes leading to hyperacute xenograft rejection (Platt et al., 1991). At least in the pig-to-primate situation, this reaction appears to be mediated by binding of naturally occurring xenoreactive antibodies in the recipient to specific glycoproteins on vascular endothelial cells of the graft. This is followed by the activation of complement and the induction of changes in endothelial cells, which together result in vessel thrombosis and loss of the graft (Bach et al., 1992). The inability of the normal porcine endothelial complement-inhibitory proteins to prevent the activation of human complement may also contribute to this process.

Fortunately, progress has also been made in devising the means to bypass this process. Thus, it has been observed that xenograft survival may be prolonged following temporary depletion of xenoreactive antibodies from the circulation of the recipient, despite the eventual return of these antibodies to the circulation. This phenomenon is referred to as accommodation or adaptation (Fischel et al., 1991; Bach et al., 1992; Hasan et al., 1992), and may also be observed in appropriately treated recipients of ABO-incompatible allografts (Alexandre et al., 1987). Such temporary depletion of antibodies may be achieved by plasma exchange, ex vivo perfusion of xenogeneic organs, or by immunosuppression aimed at reducing humoral immunity (Bach et al., 1992). However, more specific depletion may also be achievable by identification of the carbohydrate structures recognised by xenoreactive antibodies, and the use of these carbohydrates to adsorb antibodies ex vivo, or to inhibit their action in vivo (Bach et al., 1992; Good et al., 1992). The inhibition of complement activation may also be of benefit in preventing hyperacute rejection. This has been achieved by the administration of soluble complement inhibitory proteins to xenograft recipients (Pruitt et al., 1992; Xia et al., 1992). However, the ability to insert human complement inhibitory proteins, such as decay accelerating factor (DAF) or CD59 antigen, into endothelial cell membranes, with demonstrable inhibition of complement function in vitro (Bach et al., 1992; White et al,
1992; Akami et al, 1992) may represent a more widely applicable alternative. Thus, the production of transgenic animals expressing the human inhibitory protein upon their endothelial cells may ultimately provide the answer to the problems of hyperacute rejection.

It is not known to what extent islet xenografts, which are primarily revascularised from donor endothelium, are susceptible to classical hyperacute rejection. However, islets may potentially be damaged by humoral mechanisms (Frangipane et al, 1977; Perloff et al, 1981), and natural xenoreactive antibodies present in human sera may be capable of directly recognising islet endocrine cells (Eckstein et al, 1992), so that removal of these antibodies may still be of relevance in islet xenotransplantation.

Cellular immune mechanisms are also important in the rejection of xenogeneic tissue, including islet xenografts (Auchincloss, 1988). As might be expected, the induction of this immune response appears to depend primarily upon the indirect pathway of antigen presentation, involving recipient APCs (Gill, 1992). The use of islet pretreatment protocols aimed at the elimination of donor-specific passenger leukocytes are therefore unlikely to be of benefit in the xenogeneic situation (Hering, 1992). Conversely, however, it may prove easier to prolong xenograft than allograft survival by treatment of the recipient aimed more specifically at the function of CD4+ T-cells (Lacy et al, 1989; Hering, 1992). In addition, xenograft tolerance may be achieved by the production of xenogeneic chimeras (Zeng et al, 1992) using the same methods applied to allogeneic chimerism, whilst potential also exists for avoiding cellular immune mechanisms by encapsulation of islet xenografts, as mentioned above.

Thus, whilst further developments will clearly be required before islet xenografting is applicable to human beings, there is cause for optimism that the problem of donor organ shortage may ultimately be bypassed using porcine pancreata, enabling the full application of islet transplantation in human diabetes to be achieved.

CONCLUSIONS

The potential for wider application of islet transplantation in the treatment of human diabetes is obvious. Equally clear, however, is that considerable improvements in islet isolation and purification are still required. Thus, while islet transplantation remains restricted to patients receiving immunosuppression for other organ transplants, improved islet yields will be necessary to ensure the consistent reversal of diabetes and to permit transplantation of islets from single donors, thereby improving prospective HLA matching. Meanwhile, improved purity will be required to optimise the safety of the procedure, and to enhance islet engraftment.
Most importantly, however, it will only ever be possible to realise the exciting advances recently made in understanding and avoiding islet graft rejection, and thereby to extend the potential application of islet transplantation to non-immunosuppressed patients, if large yields of highly purified islets are available. Achieving this aim is a considerable challenge, and is the subject of the remainder of this thesis.
CHAPTER 3

Islet Isolation and Purification
The first demonstration that islets of Langerhans could be isolated intact from the pancreas of experimental animals was made over 80 years ago when Bensley obtained islets by microdissection from the guinea-pig pancreas (Bensley, 1911). Although this technique was modified many years later (Hellerström, 1964), the number of islets obtained was sufficient only to allow morphological and biochemical studies.

The modern era of islet isolation really began with the discovery that islets could be liberated from the surrounding acinar tissue by incubation of the chopped pancreas with the enzyme collagenase (Moskalewski, 1965). Subsequently, Lacy and Kostianovsky (1967) adapted this technique into a method for the isolation of relatively large numbers (200-300) of islets from the rat pancreas. This method involved in situ disruption of the pancreatic acinar tissue by distension of the pancreas with a balanced salt solution via the common bile duct, following which the pancreas was excised, chopped with scissors and the resulting tissue incubated in a collagenase solution. Liberated islets were then retrieved by either 'hand-picking' with a glass loop, or by centrifugation of the digested tissue on sucrose density gradients.

The general principles of this technique remain fundamental to modern islet isolation methods. Thus, the pancreas must firstly be dissociated into its separate components, using a combination of enzymatic and mechanical means; following this, islets must then be purified from the resulting dispersed tissue by various means. Therefore, when considering the ways in which islet isolation may be improved it is convenient to examine these two phases separately.

**DISSOCIATION OF THE PANCREAS**

Despite the success of Lacy's technique in isolating rat islets, adaptation of this method to the pancreas of humans and large experimental animals produced generally poor results in terms of islet yield and viability (Ashcroft et al, 1971; Ballinger et al, 1972; Sutherland et al, 1974; Najarian et al, 1977; Sutherland, 1981), due to the more fibrous, and therefore less distensible, nature of these organs than the rodent pancreas (van Suylichem et al, 1987). Fortunately, it was later demonstrated in dogs that this problem could be overcome by incorporation of the collagenase into the ductal distension solution (Horaguchi et al, 1981; Noel et al, 1982). In this way the enzyme was delivered directly to the pancreatic exocrine tissue, which was therefore selectively broken down upon incubation of the distended pancreas, leaving the islets intact.

Further modifications then allowed this technique to be applied successfully to the human pancreas (Gray et al, 1984), allowing for the first time the isolation of large numbers of intact human islets, free from adherent exocrine tissue and therefore
suitable for subsequent purification. Furthermore, this method also enabled islets to be obtained from other primate pancreata (Gray et al., 1986), and appeared superior to the initial technique of Lacy et al. when reapplied to the rat pancreas (Sutton et al., 1986). The established superiority of the intraductal collagenase digestion technique has ensured its continued use in the isolation of islets from large mammalian pancreata. However, since its original description further modifications have been necessary in order to obtain consistently high yields of islets. Thus, it has long been known that the time required for collagenase digestion to reach an optimal end-point varies markedly between different pancreata and between different batches of collagenase (Moskalewski, 1965; Scharp et al., 1975; Shibata et al., 1976). If digestion is arrested prior to reaching this end-point islets will not be separated (or 'cleaved') from adherent acinar cells, and cannot therefore be adequately purified. On the other hand, excessive exposure to collagenase ultimately results in damage to the islets themselves, which must therefore be removed from the incubation medium immediately upon liberation from the pancreas. Until recently, assessing this optimal digestion time has proven difficult, due to the lack of rapid and accurate methods for the identification of islet tissue released during this process. Fortunately, however, following the rediscovery of the zinc-binding dye, diphenylthiocarbazone (or 'dithizone'), which rapidly and specifically stains islets red when applied in vitro (Latif et al., 1988), this can now be achieved more readily. In addition, it has been increasingly recognised that islets are susceptible to mechanical trauma. Thus, initially favoured methods for the dispersal of the pancreas, such as chopping or shaking; passage through tissue macerators (Ricordi et al., 1986; Scharp et al., 1987; Alderson et al., 1987b); or even trituration (i.e. the repeated aspiration of tissue through graded needles) (Sutherland et al., 1974; Marchetti et al., 1988) were almost certainly responsible for some degree of fragmentation of islets, with inevitable loss of viability. The most important modifications of the digestion procedure have therefore involved the introduction of methods to determine the ideal length of digestion for an individual pancreas and to disperse the pancreas more gently once this optimal point is reached. In the method described by London et al. the optimal digestion time of the human pancreas was determined by the sequential examination of dithizone-stained pancreatic tissue biopsies obtained from, and incubated simultaneously with the collagenase-distended pancreas (London et al., 1990b). As soon as well-cleaved islets were seen within these biopsies the pancreas was gently teased apart using forceps, and the digestate was passed through a 500μm mesh into cold medium, in order to terminate the action of collagenase. Similar methods were employed by Warnock, using

More recently, a semi-automated system for digestion of the human pancreas has also been described (Ricordi et al, 1988). In this procedure the collagenase-distended pancreas is incubated within a specially designed digestion chamber, through which balanced salt solution is continuously recirculated at a controlled temperature. The progress of digestion is assessed by intermittent sampling of the chamber effluent, whilst gentle mechanical dissociation of the pancreas is achieved simply by shaking, aided by the inclusion within the chamber of several glass marbles. The potential advantage of this system over the above 'manual methods' is that some allowance can be made for variation in digestion rate between different areas of the same pancreas. Thus, relatively underdigested pancreatic fragments are retained within the chamber by a mesh of defined pore size, allowing digestion of these particles to continue; meanwhile, well cleaved islets, which require no further digestion, are immediately flushed from the chamber and thereby removed from the injurious action of collagenase. Furthermore, the use of a closed system reduces the risk of microbiological contamination.

Recent comparisons between the automated and manual methods of digestion suggest that higher yields of islets can indeed be obtained using the automated technique, at least from canine (Wamock et al, 1990b) and porcine (Toomey et al, 1993) pancreata. Thus, the automated method has become the most widely used technique for digestion of the human pancreas prior to transplantation, although some centres continue to prefer the relative simplicity of the manual approach (Gray et al, 1992).

The Factors Influencing Pancreatic Digestion/Dissociation

The isolation of islets from the pancreata of cadaveric human organ donors involves several stages (Figure 1), each contributing variables which may influence the final yield of islets obtained.

Donor Variables
The brain-dead, heart-beating organ donor represents the primary source of human pancreata for islet isolation and clinical trials of islet transplantation. Considerable variation exists between such donors, however, and this variability may influence the process of islet isolation.

Firstly, the age of the donor appears to be important, islet yields from younger donors (<20 years) being generally lower than those from older donors (Scharp, 1988; Ricordi et al, 1991a; Klitscher et al, 1993; Socci et al, 1993). A similar effect has been
FIGURE 1
Factors Involved in the Efficient Isolation of Human Pancreatic Islets

CADAVERIC ORGAN DONOR

ORGAN DONATION

EXCISED PANCREAS

COLD STORAGE AND TRANSPORT

COLLAGENASE-DISTENDED PANCREAS

DIGESTION

PANCREATIC DIGEST

PURIFICATION

PURIFIED ISLETS

Age
Body Mass Index
Past Medical History
Predonation Hospitalisation

In situ vascular perfusion
Warm Ischaemia
Maintenance of intact capsule

Cold Ischaemia
Cold Storage Solutions

Method/Timing of Collagenase Delivery
Composition of Distending Solution

Collagenase
Temperature, pH, Calcium
Method of Dispersal

Cold Ischaemia/Storage Solutions
Density Gradient Medium
observed in the isolation of islets from porcine pancreata (Socci et al., 1990), and it has been suggested that this is due to changes in the distribution or maturation of collagen with age. Thus, islets from older donors may be better delimited from acinar tissue by the presence of a thicker peri-insular capsule (van Deijnen et al., 1992), thereby preventing fragmentation of islets during digestion. Alternatively, increased collagen deposition around the pancreatic duct may prevent its rupture during distension or its collapse during cold storage of the pancreas, thereby enhancing the distribution of collagenase following intraductal distension. Similar differences in collagen distribution may also explain the association between body mass index and islet yield, lower numbers of islets being obtained from lean donors than from those of normal weight (Klitscher et al., 1993).

Profound haemodynamic and metabolic changes occur in brain-dead patients (Novitzky et al., 1987; Robertson et al., 1989; Quesada et al., 1991; Thicope et al., 1991), and, at least theoretically, these may also be of relevance to subsequent islet isolation. Thus, acinar cell content of exocrine enzymes may be affected by these changes and by predonation starvation or the influence of drugs administered to the donor, and these changes in enzyme content may subsequently influence pancreatic digestion (Scharp, 1988). Furthermore, hyperglycaemia and the need for dopamine as inotropic support prior to organ retrieval have been associated with a slight impairment in the results of vascularised pancreatic allografts (Gores et al., 1990). The effects of such variables upon islet isolation have not been systematically investigated, however, so that their importance is difficult to assess.

Finally, the occurrence of chronic pancreatitis in the donor markedly reduces the numbers of well-cleaved islets obtained using the above-mentioned techniques. However, in the absence of overt chronic pancreatitis, it is not clear to what extent islet isolation is affected by other pancreatico-biliary diseases or agents such as alcohol, which may contribute to lesser degrees of pancreatic inflammation.

Variables During Organ Retrieval and Cold Storage

Pancreata used for human islet isolation are generally obtained from brain-dead, heart-beating organ donors in association with the retrieval of other organs (liver, kidneys, and often heart and lungs) for transplantation. Despite the intimate anatomical relationship between the pancreas and the vascular/biliary structures which must be excised en bloc with the liver, with appropriate care combined retrieval of liver and pancreas can usually be achieved (Sollinger et al., 1989; Ricordi et al., 1992c). In removing the pancreas which is to be used for islet isolation, however, it is particularly important to maintain the integrity of the pancreatic capsule, as damage to this structure
may preclude successful distension, and therefore digestion, of the gland (Scharp, 1988; Brandhorst et al., 1993).

All organs removed from cadaveric donors for transplantation are subject to an inevitable period of ischaemia, between excision from the donor and implantation into the recipient. The harmful effects of this ischaemia upon organ function are reduced at low temperature (Belzer et al., 1988), and for this reason it has become standard practice to cool organs in situ, prior to excision, by perfusion of the vascular system with various cold storage solutions at 4°C, and to continue hypothermic storage of the organ in these solutions, up to the point of its implantation into the recipient. Organ retrieval is therefore associated with a short period of warm ischaemia and a more prolonged period of cold ischaemia, both of which may influence post-transplantation organ function.

In certain respects, the tolerance of the pancreas to warm ischaemia appears to be better than that of many other organs (Jones et al., 1975), periods of between 45 and 90 minutes being tolerated before irreversible histological and electron-microscopic changes occur in pancreatic acinar cells (Slater et al., 1975; Medvetskii et al., 1978). Interestingly, in these studies islets of Langerhans appear even more resistant to warm ischaemia than acinar cells, whilst other investigations have also shown that islet function in vascularised pancreatic allografts in dogs may be maintained after exposure of the graft to warm ischaemia of up to one hour (Cerra et al., 1970; Florack et al., 1984).

Unfortunately, however, the isolation of islets from the pancreas may be adversely affected by relatively short periods of warm ischaemia. Thus, in rats, 30-60 minutes of warm ischaemia is sufficient to reduce markedly the yield of islets obtained following collagenase digestion of the pancreas (Fonseca et al., 1987; Ohzato et al., 1989), and in pigs islet yields are similarly reduced by ischaemic periods of as little as 18-20 minutes (Ricordi et al., 1990b). Warm ischaemia may also occur during retrieval of the human pancreas: firstly during the period between initiation of in situ vascular perfusion and achieving effective cooling of the pancreas; and secondly during the period between termination of hypothermic perfusion and completion of the pancreatectomy. Unfortunately, few studies have directly examined the influence of these ischaemic episodes upon the results of human islet isolation, although the available evidence suggests that even periods of less than 20 minutes warm ischaemia may profoundly reduce the number and quality of islets obtained (Abri et al., 1987; Brandhorst et al., 1993).

The enhanced susceptibility to warm ischaemia of the pancreas used for islet isolation, compared to vascularised pancreatic grafts, presumably reflects the additional insult of pancreatic digestion, which may also itself be influenced by the release of proteolytic
enzymes from acinar cells exposed to ischaemia. Whatever the mechanism, however, it is clear that warm ischaemia prior to islet isolation must be minimised: this may ultimately require excision of the pancreas prior to that of other organs.

As mentioned above, warm ischaemia during organ retrieval may also be reduced by in situ vascular perfusion of the abdominal organs with cold storage solutions. However, there is also evidence that such perfusion may itself compromise subsequent islet isolation. Thus, Ohzato demonstrated that islet yields were lower from rat pancreata excised following in situ vascular perfusion with modified Sacks' solution or Hank's balanced salt solution than from pancreata excised without a vascular flush, when both were processed immediately, without significant cold ischaemia (Ohzato et al, 1990). Similar results were obtained by Kneteman, again in the rat, using Euro-Collins or University of Wisconsin (UW) solutions for vascular perfusion (Kneteman et al, 1990).

In dogs vascular flushing with UW or silica-gel-filtered plasma (SGF) resulted in similar islet yields, but increased islet fragmentation during collagenase digestion, compared to non-flushed organs (Zucker et al, 1989). Also, dispersed pancreatic autografts in dogs were universally unsuccessful after in situ vascular perfusion of the pancreas with Collins' solution or SGF, whilst some success was observed following simple cold storage of the non-flushed pancreas in these solutions (Hesse et al, 1987). Furthermore, in pigs in situ perfusion with cold Euro-Collins solution produced vastly inferior islet yields following subsequent pancreatic digestion, even compared to pancreata suffering 20 minutes of warm ischaemia due to excision from non-heart-beating abattoir animals (Ricordi et al, 1990b).

Finally, in humans a slight (but statistically non-significant) reduction in islet yield has been observed from pancreata flushed in situ with UW solution, compared to those excised prior to vascular perfusion of the abdominal viscera (Kneteman et al, 1992). Thus, vascular perfusion with a variety of solutions has been associated with impaired isolation of islets from the pancreata of several species. The mechanism of this effect has not been investigated, but a number of theoretical possibilities exist. Firstly, it is possible that islets may be damaged simply by hydrostatic pressure exerted during perfusion, to which they may be more susceptible due to their direct connection with the arteriolar system of the pancreas (Carroll, 1992). Fragmentation of these islets during subsequent digestion may then result in reduced yields. Secondly, vascular perfusion may produce physical changes (such as interstitial oedema) in the pancreas which impair the efficiency of subsequent intraductal distension. Finally, the solutions used for in situ perfusion may contain inhibitors of collagenase, thereby impairing the subsequent digestion of the pancreas. Preliminary experience in this laboratory certainly suggests that this is the case with UW solution, at least in the human pancreas.
Robertson et al, 1993a; Contractor et al, 1993): digestion of organs perfused with UW produces an increased proportion of uncleaved islets, unless a higher concentration of collagenase is used for intraductal distension. Interestingly, this may also explain the paradoxical reduction in purified islet yield with short, compared to longer cold ischaemia observed by Kneteman in human pancreata perfused with UW (Kneteman et al, 1992): islet purification after short cold ischaemia may have been reduced by the presence of uncleaved islets, which cannot be purified on density gradients, whilst the increased release of endogenous protease activity during digestion of pancreata with long cold ischaemia might have overcome the inhibitory effects of UW and resulted in an increased proportion of cleaved islets.

The length of the cold ischaemic period may also influence the efficiency of collagenase digestion. In rats, islet yields are reduced following cold storage of the pancreas for 2-6 hours, compared to those obtained by immediate pancreatic digestion (Munn et al, 1989; Ohzato et al, 1990; Kneteman et al, 1990). The rate of this deterioration appears to be reduced, but not completely prevented, by in situ vascular perfusion and preservation in certain cold storage solutions such as UW (Kneteman et al, 1990). Interestingly, however, islet yields can be maintained at control levels for up to 48 hours if intraductal injection of collagenase in balanced salt solutions or in UW is performed prior to, rather than following, cold storage (Munn et al, 1989; Ohzato et al, 1990). Similarly, in dogs, distension of the pancreas with collagenase in UW at the time of organ retrieval maintains islet yields almost at control values following subsequent hypothermic storage for up to 24 hours, in contrast to the marked deterioration seen at 12 hours following vascular perfusion with the same preservation solution (Munn et al, 1989). The mechanism of this effect appears to be related largely to deterioration in the pancreatic ductal system during cold storage of the organ. Thus, after 6 hours of cold ischaemia the volume of collagenase solution which can be injected into the rat pancreatic duct prior to its rupture is reduced, compared to fresh pancreata (Ohzato et al, 1991), whilst the penetration of intraductally injected barium sulphate solution into the peripheral acinar tissue of the pancreas is also impaired.

Finally, the temperature used during hypothermic organ preservation may also be relevant. Although 4°C is the temperature most commonly used for cold storage of organs prior to transplantation, this temperature may be damaging to cell membranes. Milder degrees of hypothermia may therefore be preferable for organ preservation, although the optimal temperature for storage may be tissue-specific (Wang et al, 1989; Kasiske et al, 1990; Astarcioglu et al, 1993). At least in the rat pancreas, however, the yield and function of islets isolated following 24 hour cold storage in UW solution is greater using a storage temperature of 7-10°C, compared to both 4°C and 15°C (Lakey et al, 1991).
The influence of organ preservation upon islet isolation from the human pancreas is less well defined, as few studies have directly investigated this issue. Furthermore, in those studies which have been performed, the recovery of viable islets is not markedly reduced during preservation periods of up to 17 hours, following vascular perfusion with solutions such as UW (Kneteman et al, 1992), which are appropriate for prolonged cold storage of pancreatic tissue (Wahlberg et al, 1987; Belzer et al, 1988). Nevertheless, it has recently been demonstrated that digestion of the human pancreas can also be significantly improved by intraductal injection of collagenase in Hank's solution at the time of organ retrieval, compared to pancreata perfused with UW but distended following cold storage (Socci et al, 1993). Presumably, therefore, some deterioration, at least in the ductal system, of the human pancreas must occur during cold ischaemia, even when UW is used as the preservation solution.

Finally, the viability of isolated islets may be affected by the cold ischaemic interval. Prolonged hypothermia has several adverse effects upon cellular function, which are limited by the use of appropriate cold storage solutions (Belzer et al, 1988). The mechanism of action and the relative advantages of the solutions commonly used for pancreatic preservation will be discussed in more detail later in this and other chapters. However, numerous studies have demonstrated, in various animal species and in humans, that islet viability following isolation reduces progressively with increasing cold ischaemia of the pancreas, even when stored in such solutions (Hesse et al, 1987; Warnock et al, 1988a; Field et al, 1989; Heise et al, 1989; Zucker et al, 1989; Kneteman et al, 1990; Kneteman et al, 1992).

Variables Associated with Collagenase Digestion

The integrity of the pancreas is maintained by a combination of direct intercellular adhesion and an extracellular matrix containing various collagen subtypes (McShane et al, 1990a; van Deijnen et al, 1992; Gray et al, 1992). The aim of pancreatic digestion during islet isolation is to break down these structures, in order to liberate intact islets. Theoretically, therefore, it is important that enzymatic digestion should be directed to the interface between islets and acinar cells, whilst preserving the structures responsible for maintaining the integrity of the islets themselves.

The nature of this interface appears to vary between animal species (van Deijnen et al, 1992): in dogs islets are almost completely separated from acinar cells by a well-defined, collagen-rich capsule, whereas in pigs this capsule is deficient, and the islet-acinar interface is maintained largely by direct cell-cell adhesion. In rats and humans, the situation is intermediate between these two extremes. Furthermore, there is broad correlation between the degree of development of the peri-insular capsule in these species and the ease with which intact islets can be isolated by collagenase digestion.
Optimising the digestion phase of islet isolation must therefore depend either upon improving the distribution of collagenase specifically into the islet-acinar interface or increasing the enzymatic specificity of the collagenase for the structures contained within this interface.

As mentioned above, the intraductal injection of collagenase appears to be crucial in obtaining large numbers of islets from the fibrous pancreata of large animals and humans, and it has been suggested that this is due to the relatively selective delivery of the enzyme to the pancreatic acinar tissue (Gray et al, 1992). However, studies in which India ink has been injected into the pancreatic duct of various animal species have demonstrated that some penetration of the distending solution into the islets does occur using this method (van Suylichem et al, 1992). The extent to which this occurs may depend upon the volume of the distending solution injected. In the rat pancreas, intraductal injection of a low volume (0.5 ml) of solution results in the presence of ink particles around, but not within islets, whilst the injection of a larger volume (10 ml) is associated with the presence of ink particles within the islets themselves, particularly if the injection is undertaken rapidly. Adequate macroscopic distension of the human pancreas is generally achieved using a volume of about 2 ml/g pancreatic weight, but even using this volume of distending solution, some penetration into the islets has been observed (van Suylichem et al, 1992).

The pressure achieved during intraductal injection may also be relevant. Extravasation of intraductally injected solutions may occur between acinar cells and through the duct wall at remarkably low pressures (40-50 mmHg i.e. within the physiological range of intraductal pressure found during stimulated pancreatic secretion (Anderson et al, 1968; Pirola et al, 1970; Bockman et al, 1971; Armstrong et al, 1985)), whilst the pressures achieved during pancreatic distension prior to islet isolation may be very high (200 mmHg to 560 mmHg (Mumm et al, 1989; Ohzato et al, 1991)).

Thus, using a single intraductal injection for distension of the pancreas, the distribution of collagenase may be significantly influenced by the volume of solution injected, the rate of injection and the intraductal pressure achieved. Unfortunately, no studies have been performed to establish the optimum combination of these variables for islet isolation from large mammalian pancreata, and this is therefore an area worthy of further research.

An alternative approach to the use of a single intraductal injection for pancreatic distension is constant perfusion of the pancreatic duct with collagenase solution. Using this technique one group has reported a significant increase in islet yield from the human pancreas, compared to the single injection method (Warnock et al, 1988a; Warnock et al, 1989b). It is not clear, however, to what extent this improvement is due
to changes in the variables mentioned above: improvement in these variables during single ductal injection might equally well result in similarly increased islet yields. Perhaps the greatest variability in islet isolation is related to the activity of the collagenase enzyme itself, which is derived from cultures of the bacterium *Clostridium histolyticum*. Unfortunately, commercially available batches of collagenase vary greatly in their efficiency in islet isolation (Toledo-Pereyra et al., 1979b; Wolters et al., 1988), necessitating extensive testing of batches to find one which is suitably effective. Furthermore, the reasons for this variability are not fully established. For example, only broad correlation has been observed between the collagenolytic activity of the preparation in vitro and its efficacy in islet isolation (Gray et al., 1992). This is probably because commercial 'collagenase' contains a number of other enzymes, including various proteases, which may also be important in dissociation of the pancreas, in addition to true collagenolytic activity (Wolters et al., 1988; McShane et al., 1989; Wolters et al., 1992). A suitable enzyme may therefore be one which contains an appropriate balance of collagenolytic, proteolytic and possibly other enzymatic activities (Wolters et al., 1992), but at present the optimal composition of enzymes for islet isolation is not established. Excessive proteolytic activity does, however, appear to be harmful, resulting in fragmentation of the islets. In this respect it is also important to note that components present in commercial collagenases may be responsible for the activation of endogenous pancreatic protease activity (Traverso et al., 1978; Wolters et al., 1990), which may therefore contribute to increased variability of collagenase digestion. This activation may be eliminated by the addition of 10% Bovine Serum Albumin (BSA) to the distending solution (Wolters et al., 1989). This in turn increases the yield of islets obtained from the rat pancreas, although the influence of BSA upon the isolation of islets from the human pancreas, in which the activation of endogenous proteases is less marked, remains uninvestigated.

Collagenase requires the presence of calcium ions for its enzymatic function (Soru et al., 1972). Moreover, calcium ions are known to be important in the intercellular and cell-matrix interactions responsible for maintaining tissue integrity (Garrod et al., 1981). It might therefore be expected that the calcium concentration within the collagenase solution would influence the results of islet isolation. Indeed, complete chelation of calcium by ethylenediamine-tetraacetic acid (EDTA) is capable of terminating the action of collagenases in digestion of the pancreas (Gray et al., 1992). In addition, the yield of intact islets obtained from the rat pancreas appears to be dependent upon the addition of calcium, when using collagenase administered in UW solution (Dono et al., 1992a), which also contains molecules capable of binding calcium (Burgmann et al., 1992). However, in the absence of chelating agents islet yields do not appear to be markedly
influenced by calcium concentrations in the range 0-50 mM, and a concentration of 2.5 mM is probably optimal (Wolters et al, 1990).

The activity of collagenase, as with many enzymes, is also dependent upon temperature and pH (Soru et al, 1972). Again, however, only limited information is available concerning the optimal conditions for islet isolation. In most centres a temperature of 37-39°C is used during initial incubation of the pancreas, although interestingly, advantages have been claimed for the use of temperatures both higher (Sutton et al, 1990) and lower (Ricordi et al, 1986; Dono et al, 1992b) than this. Within a relatively broad range of pH (6.2-8.0) pancreatic digestion is satisfactory (Wolters et al, 1990), and most investigators therefore continue to use solutions within the physiological pH range.

ISLET PURIFICATION

Collagenase digestion of large mammalian pancreata produces a considerable volume of dispersed pancreatic tissue (e.g. up to 70 ml of packed tissue from the human pancreas). Although this digest contains all the cellular elements present within the native pancreas, including ductal, vascular and lymphoid tissues, the vast majority is composed of exocrine tissue fragments varying in size from single acinar cells to large, multicellular aggregates measuring up to 500 \( \mu \)m in diameter. As the islets of Langerhans may represent only about 1-2% of this tissue, it is clear that if islet tissue substantially free from contamination by exocrine and other tissue is to be produced, a considerable degree of purification must be achieved. A large number of methods of islet purification have been reported (Table 1), but in only a few cases is the efficiency of islet separation sufficiently high for use in the primary purification of islets from crude pancreatic digest.

Hand-picking was the first method described for the purification of islets following collagenase digestion of the pancreas (Lacy et al, 1967), and human islets have been isolated using this method (Ashcroft et al, 1971). However, although islets prepared in this way are uniquely pure, the rate at which islets can be handpicked is clearly inadequate for islet purification prior to clinical transplantation, where the isolation of several hundred thousand islets is required.

Simple filtration and trituration have also been widely employed, but are capable only of removing large fragments of exocrine and ductal tissue. Reduction in the size of the filters or needles in order to achieve greater purification results in an unacceptable loss of islet tissue, whilst both methods are also associated with a significant degree of mechanical trauma which may damage islets. Similarly, the use of elutriation devices...
<table>
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<tr>
<th>METHOD</th>
<th>ADVANTAGE</th>
<th>DISADVANTAGE</th>
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<tr>
<td>Handpicking</td>
<td>Simple, Highly Specific, Relatively Atraumatic</td>
<td>Low Capacity, Slow, Labour Intensive</td>
</tr>
<tr>
<td>Filtration</td>
<td>Simple, Rapid, High Capacity</td>
<td>Very low Specificity, Mechanical Trauma</td>
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<td>Trituration</td>
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<td>Elutriation</td>
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<td>Tissue Culture</td>
<td>Simple</td>
<td>Islet Toxicity if initially highly impure; therefore limited capacity</td>
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<td>Cryopreservation</td>
<td>High Capacity</td>
<td>Islet Toxicity, Limited degree of Purification</td>
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<td>Irradiation Toxins</td>
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<td>Lectin/Antibody-Directed Toxicity</td>
<td>High Capacity</td>
<td>Islet Toxicity</td>
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<tr>
<td>Immunomagnetic</td>
<td>Highly Specific, Nontraumatic, Relatively Simple</td>
<td>Capacity currently limited by islet trapping, Dependent upon quality of precedent digestion</td>
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<tr>
<td>Separation</td>
<td></td>
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<tr>
<td>Fluorescence-Activated Cell Sorting (FACS)</td>
<td>Specific, High Capacity, Potential to recover uncleaved islets</td>
<td>Slow, Optimum stain unknown for human islets</td>
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<tr>
<td>Density Gradient</td>
<td>High Capacity, Simple, Moderate specificity, Rapid</td>
<td>Variability between pancreata, Dependent upon quality of precedent digestion</td>
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<td>Centrifugation</td>
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results only in the removal of cellular debris and single acinar cells, and may also produce fragmentation of islet tissue (Scharp, 1988).

Many alternative methods of islet purification have employed the differential sensitivity of exocrine tissue and islets to various physical and chemical conditions. For instance, acinar cells tend to degenerate in tissue culture during periods which are relatively well tolerated by islets, so that short-term tissue culture has been used in islet purification (Weber et al, 1977; Matas et al, 1977). However, islet viability during short-term culture may be impaired in the presence of large amounts of exocrine tissue (Sever et al, 1992), presumably due to the release of damaging proteases from degenerating acinar cells, so that use of this method is associated with an unacceptable loss of islet tissue if the level of exocrine tissue contamination is initially high. Similarly, some degree of islet purification results from the enhanced susceptibility of acinar cells to cryopreservation (Evans et al, 1987), irradiation (Nason et al, 1986) and to certain toxins (McShane et al, 1990b), but again these methods may be associated with considerable loss of functional islet tissue (Nason et al, 1986; McShane et al, 1990b; Rich et al, 1993), and the degree of purification achieved is often modest.

Increased specificity of toxic agents for exocrine tissue has been achieved using anti-acinar cell monoclonal antibodies with complement (Soon-Shiong et al, 1990), and using exocrine-binding, fluorescein-labelled lectins for the selective photodynamic destruction of acinar cells using laser energy (Brunicardi et al, 1992), although, even using these methods, coincident damage to islets still occurs. However, the specificity of anti-acinar monoclonal antibodies and lectins may also be employed by their attachment to magnetic microspheres, which facilitate the subsequent removal of exocrine tissue by the simple application of a magnetic field (Soon-Shiong et al, 1990; Winoto-Morbach et al, 1990). The advantages of this method are the specificity, relative simplicity and nontraumatic nature of islet purification. However, at present the efficiency of islet separation, and therefore the potential for use in the primary purification of crude pancreatic digest, is limited by trapping of islets within masses of acinar tissue cross-linked by magnetic forces (Robertson et al, 1993e).

Highly specific purification of islets may also be achieved using the technique of fluorescence-activated cell sorting (Gray et al, 1989b; Specht et al, 1990). Existing cell sorters can be modified in order to separate particles the size of islets, and this method also has the theoretical potential for the purification of uncleaved islets, making it less dependent upon the quality of the preceding digestion of the pancreas. However, a suitable fluorochrome for the staining of human islets has yet to be found. The use of intravital dyes, such as neutral red in the initial description of this technique, has clear limitations in the clinical application of this method. Furthermore, although the combined use of neutral red and dithizone staining in vitro may facilitate this application
Density gradient centrifugation was initially applied to the isolation of rat islets in 1967 (Lacy et al., 1967), and shortly thereafter its potential in the purification of islets from the pancreata of large animals (Sutherland et al., 1974; Scharp et al., 1975; Noel et al., 1982) and humans (Ballinger et al., 1972) was also confirmed. Initially, the use of this method for large-scale islet purification required the formation of multiple density gradients in test-tubes. Subsequently, however, Lake et al. described a method for the formation of large volume discontinuous density gradients using the COBE (formerly IBM) 2991 cell separator (Lake et al., 1989a), which allowed the purification of large numbers of islets to be achieved in a much more rapid and less labour intensive manner: Using such discontinuous density gradients up to 20 ml of crude pancreatic digest can be processed, involving minimal human intervention, within about 20 minutes (Alejandro et al., 1990; London et al., 1992a). In addition, using appropriate media, a considerable degree of purification of human islets can be achieved using density gradients (Scharp et al., 1987; Lake et al., 1989a; O'rack et al., 1991). Thus, the combined advantages of density gradients, namely their relative simplicity, facility for rapid processing, and high capacity, coupled with a considerable world-wide experience in their use, have led to this technique becoming the most widely-used method for the purification of human and other large mammalian islets prior to transplantation.

However, whilst density gradients undoubtedly have the potential to produce highly purified preparations of viable islets, there is considerable variation in the efficiency of purification between pancreata using this technique, particularly with human pancreata (Warnock et al., 1990c; Vives et al., 1992; London et al., 1992a), so that on occasions islet yields are disappointingly low.

It is clear, therefore, that no technique of islet purification is without limitation, so that the provision of highly purified islets for transplantation may ultimately require the use of a combination of purification techniques. However, for the reasons mentioned above, density gradient separation is likely to be the most suitable method for primary purification of the crude pancreatic digest, and this technique will therefore be the subject of the remainder of this chapter.

**Cell Separation Using Density Gradient Centrifugation**

The separation of different types of animal cells by centrifugation is a technique which has been available for many years, and a number of excellent reviews on this topic have
been published (Shortman, 1972; Harwood, 1974; Pretlow et al, 1975; Pretlow et al, 1982). Unfortunately, the majority of experimental and theoretical work on cell separation using this technique has been largely restricted to the isolation of single, regularly shaped cells, particularly erythrocytes, lymphocytes and haemopoietic cells, and little attention has been paid to the separation of larger, irregular, multicellular particles such as islets and exocrine tissue fragments. Nevertheless, the general principles underlying the separation of all these types of cells are similar. Thus, the movement of any particle in a centrifugal field is dependent upon the size and density of the particle and upon the resistance to movement provided by the surrounding medium. For an ideal particle (ie. one which is smooth, spherical, rigid, uncharged, unhydrated and of constant size and density), the velocity at any point in the centrifugal field is given by the following equation (Pretlow et al, 1969):

\[
V = \frac{\frac{\partial r}{\partial t} \cdot a^2(D_p-D_m) \cdot \omega^2}{18\eta}
\]

where \( V \) = velocity of the particle at a distance, \( r \) from the centre of revolution; \( a \) = diameter of the particle; \( D_p \) = density of the particle; \( D_m \) = density of the medium, and \( \eta \) = viscosity of the medium at point \( r \); \( \omega \) = angular velocity.

It is clear from a consideration of this equation that cell separation techniques using centrifugation may be subdivided into two categories:

**Velocity sedimentation** methods separate cells on the basis of differences in their rates of sedimentation, and in general this is achieved by centrifugation of the cell mixture for short periods of time (insufficient for the cells to reach a point of equilibrium), in a medium whose density is markedly different to that of the cells. As the rate of sedimentation is proportional to the square of the diameter, these methods rely principally upon differences in cellular diameter, although they may also be used to separate cells of similar diameter and different density.

**Isopycnic (Density-Dependent) Centrifugation**, on the other hand, separates cells solely on the basis of differences in their density, by centrifugation in a medium with a density range close to that of the cells being separated and for sufficient time for the cells to reach the point of equilibrium at which their density is equal to that of the surrounding medium.

In the separation of different populations of single cells, velocity sedimentation is generally more effective than isopycnic centrifugation (Pretlow et al, 1982), and has the
advantage of shorter exposure to centrifugal forces of lower magnitude than those used in density-dependent separation methods. However, the separation of islets from crude pancreatic digest represents an exception to this general rule, mainly because the range of diameters of the multicellular exocrine tissue fragments resulting from collagenase digestion of the pancreas varies greatly between pancreata, and overlaps completely with the range of diameters exhibited by intact islets. Furthermore, pancreatic acinar cells belong to a small group of cell types (including mature mast cells and cardiac myocytes) which have densities much higher than the normal range of cellular densities (Blackmon et al, 1973), permitting their effective purification on isopycnic density gradients, even as single cells.

In general, therefore, isopycnic gradients have been used in the purification of islets from collagenase-digested pancreata. Some investigators have employed sequential isopycnic and isokinetic separation (a particular form of velocity sedimentation, in which the increased centrifugal force exerted on cells as they move away from the centre of revolution is exactly balanced by a gradually increasing viscosity of the gradient medium, so that cells are made to sediment at a constant velocity), in order to remove particles of similar density but smaller size than islets, such as fragments of ductal/vascular tissue and individual lymphoid cells (Hering et al, 1993b). However, in the majority of centres isopycnic centrifugation is the sole method utilised for islet purification, and the remainder of this chapter will therefore be confined to a consideration of the factors currently limiting the efficiency of islet purification using this technique.

These factors may be divided into two broad categories, namely the methodological problems associated with the gradient separation of any type of cell, and those problems which are relatively specific to islet isolation.

**Methodological Problems in Isopycnic Centrifugation**

Isopycnic density gradient separation of cells may be achieved in a number of ways (Figure 2): firstly, a single density of medium may be used to separate cells simply on the basis of whether their density is greater than or less than that of the medium (neutral density separation). An extension of this approach is the use of discontinuous density gradients, comprising multiple layers of media, each with a different density, in which cells of different density are isolated within the interfaces between separate layers. Finally, continuous gradients may be constructed, in which there is a gradual change in density of the medium along the gradient, and in which all individual cells come to rest at their point of equilibrium.

All three methods have been employed in the isolation of islets, although for large-scale islet purification the most commonly used method has been the discontinuous density
Demonstrating the types of isopycnic density gradients

- Continuous Density Gradient
- Discontinuous Density Gradient
- Neutral Density Separation

More Dense Particle e.g. Exocrine Tissue
Less Dense Particle e.g. Livers

FIGURE 2
gradient, due to the facility for its production on the COBE 2991 cell separator.
However, the efficiency of cell separation using density gradients is highly dependent
upon the type of gradient used, due to the occurrence of certain well-described
confounding effects during sedimentation.
For instance, it is well known that all density gradients have a limited capacity with
respect to the number of particles which can be loaded onto the gradient without
inducing abnormal patterns of sedimentation. If this capacity is exceeded gradients can
become locally unstable, and loss of resolution of previously well defined cell
concentrations may occur (Pretlow et al, 1975), presumably reflecting the inability of
some particles to reach their point of bouyant density due to the presence of excessive
numbers of other particles within that area of the gradient. In neutral density separation
and discontinuous gradients this effect is clearly exaggerated, due to the accumulation
of particles at narrow interfaces, the intervening portions of the gradient being
essentially unused. This accumulation may also prevent completely the effective
migration of some particles through certain interfaces, leading to their trapping in an
inappropriate interface, with potential loss of resolution in cell separation. In
continuous gradients, however, the even distribution of particles throughout the
gradient ensures that the entire gradient is functional, with the result that continuous
gradients have a considerably higher capacity than these other methods, whilst trapping
occurs to a much lesser extent. When using continuous gradients, capacity also
depends upon the effective volume of the gradient (which is not necessarily equivalent
to the actual volume - vide infra), and upon the density range covered by the gradient,
increases in both of these variables being associated with a higher gradient capacity.
Another technical problem encountered in the density gradient separation of cells
concerns cell aggregation, a phenomenon which occurs to some extent with all viable
cells in suspension (Shortman, 1972; Pretlow et al, 1975). Aggregated cells clearly
cannot be separated from each other by density-dependent means, and in addition the
density of cell aggregates may differ from that of the unaggregated cells, so that the
results of cell separation may be markedly affected by this phenomenon. Aggregation
may be reduced by the addition of low concentrations of bovine serum albumin, by
lowering the pH, by thorough dispersal of cells before centrifugation, by the use of
higher centrifugal forces, and by operation of gradients at a temperature of 4°C
(Shortman, 1972; Pretlow et al, 1982). It is, however, increased at high cell
concentrations, and again this is therefore more pronounced within the interfaces of
discontinuous gradients than with cells relatively well dispersed throughout a
continuous gradient.
Continuous gradients therefore offer considerable advantages compared to the other
methods of isopycnic separation. However, at the time of initiation of this thesis, the
methods for the construction of large-scale, sterile continuous gradients for islet purification were not available, so that for the purification of large numbers of islets the disadvantages of discontinuous gradients had to be accepted.

Another common artifact encountered during the separation of cells by centrifugation is the 'wall effect', which results from the fact that the force vectors of a centrifugal field are directed radially outwards from the centre of revolution (Figure 3): thus, when using gradients contained within cylindrical tubes these vectors are not parallel to, but rather intersect the walls of the tube, with the result that many cells may hit the side of the tube before reaching their point of equilibrium. These cells may then be prone to agglutination or simple adherence to the side of the tube, with a consequent loss of resolution in cell separation.

The wall effect artifact is also responsible for reducing the effective volume of gradients formed in cylindrical tubes. Thus, as cells tend to migrate towards the walls of such tubes, gradient capacity at any point is related, not to the cross-sectional area of the tube, as might be expected, but to its circumference (Pretlow et al, 1975). Paradoxically, therefore, gradient capacity is proportionally higher in relation to gradient volume if small, rather than large tubes are used, as the former have a relatively high ratio of circumference to volume. The separation of large numbers of cells using cylindrical tubes may therefore be best achieved using several small gradients rather than a single large gradient.

Wall effects may also be reduced by loading the cells at the bottom, rather than the top of the gradient, because the centrifugal force vectors become more parallel to the sides of the tube with increasing distance from the centre of revolution (Figure 3). Bottom-loading does, however, lead to an exposure of cells to increased centrifugal forces. Top-loading, on the other hand, has the advantage of lower centrifugal forces and the potential to maintain cells in more physiological media for the maximum possible time, but has the additional disadvantage of susceptibility to 'streaming', where cells at the sample-gradient interface aggregate and stream through the gradient as globular masses, rather than single particles.

Alternatively, wall effects may be reduced using sector-shaped tubes, the walls of which run parallel to the centrifugal force vectors. Essentially this is achieved during islet purification on the COBE 2991 cell separator, as the processing bag in which the gradient is formed adopts an approximate sector shape during centrifugation. During density gradient centrifugation problems may also arise from disruption of the gradient by swirling induced by acceleration and deceleration of the rotor, and by thermal convection (Pretlow et al, 1975). These may, however, be reduced by the use of low acceleration/deceleration rates (or the use of the COBE cell separator, as
FIGURE 3
Wall Effects and the Influence of Distance from the Centre of Revolution
gradients may be unloaded without deceleration), careful control of temperature, and steeper density gradients.

Finally, effective cell separation using isopycnic density gradients relies upon centrifugation for sufficient time and with sufficient centrifugal force for cells to reach their points of bouyant density. However, excessive centrifugation should be avoided, as it is damaging to cells, and this is particularly so with cells which have already reached their isopycnic point in the gradient (Pretlow et al, 1982).

Theoretically, therefore, the optimal conditions for cell separation in cylindrical tubes are achieved using multiple small continuous gradients and bottom-loading. The avoidance of wall effects when using large-scale gradients on the COBE cell separator might permit top-loading, although at present the formation of continuous gradients has not been attempted using this machine. In all circumstances, however, purification is also optimised by the use of the minimum necessary centrifugal force, by rapid processing, operation at low temperature, and by avoiding overloading of the gradient.

**Density Gradient Purification of Islets: The Problems**

Theoretically, islet purification may be influenced by variables arising from each of the preceding stages of islet isolation (Figure 1). Unfortunately, however, only limited information is available on the influence of many of these factors, as most investigations concerning islet purification have focussed solely upon the role of various density gradient media. Although a large number of such media have been used in islet purification, including sucrose (Lacy et al, 1967), Ficoll (Lindall et al, 1969; Scharp et al, 1973; Nash et al, 1976), Hypaque-Ficoll (Tze et al, 1976), Percoll (Scharp et al, 1987), Bovine Serum Albumin (Lake et al, 1989a; Alejandro et al, 1990; Vives et al, 1992), Dextran (van der Vliet et al, 1988a), Metrizamide (Raydt, 1977), and Nycodenz (Hering et al, 1990), no gradient medium appears able to provide consistent purification of islets from large mammalian, and particularly human, pancreata. Indeed, using any of these media there is considerable variability in the efficiency of islet purification between pancreata, which appears to be due to marked variability in the density of the exocrine tissue fragments resulting from collagenase digestion of different pancreata (London et al, 1992c). Using isopycnic gradients, no medium can separate cells of identical density, so that it is necessary firstly to consider the potential reasons for this variability in exocrine tissue density before addressing the means to improve islet purification.

**Tissue Density and Cell Content**

Although, by definition, the density of an individual cell must be related to its mass and volume, heterogeneity in density between different types of mammalian cells appears to
be related much more closely to their relative contents of cytoplasmic material, rather than their relative cellular volumes (Shortman, 1972; Pretlow et al, 1975). Thus, many large cells with expansive nuclei and little cytoplasmic material (eg. lymphoid blast cells and testicular cells) are relatively light, whilst, at the other extreme, erythrocytes, which are relatively small and contain no nucleus but large amounts of cytoplasmic protein, are amongst the densest mammalian cells. As mentioned above, several other cells (eg. mature mast cells, parotid epithelial cells, cardiac myocytes, and pancreatic acinar cells) also exist whose densities exceed the normal range for mammalian cells, and in each case these cells are known to be actively involved in the synthesis and storage of cytoplasmic protein. Indeed, in the case of mast cells direct correlation has been observed between cellular density and the extent of cytoplasmic granulation (Pretlow et al, 1970).

The relatively high density of the pancreatic acinar cell may therefore be related to its storage of exocrine enzymes within large numbers of high density cytoplasmic granules, and variability in density between acinar cells may be related to differences in their content of these enzymes, resulting either from natural variation between pancreata or from degranulation in response to a variety of stimuli.

Potentially, a number of stimuli may result in acinar cell degranulation during islet isolation. As previously mentioned, the metabolic changes associated with brain-stem death, the period of fasting prior to organ donation, and the effects of drugs administered to the organ donor may all be important, whilst pancreatic dissection itself, and the use of electrical diathermy in the vicinity of the splanchnic nerve supply during organ donation may also theoretically result in acinar cell degranulation. Finally, it is likely that the further insults of warm and cold ischaemia, coupled with the effects of collagenase digestion may also contribute to exocrine enzyme discharge, and thereby to variable acinar cell density.

It is also important to note that the exocrine tissue fragments produced by collagenase digestion of the pancreas are multicellular aggregates whose density will depend partly upon the amount of connective tissue and interstitial fluid present, in addition to the density of individual acinar cells. Thus, the quality of pancreatic digestion and dispersal, with respect to the size of exocrine fragments produced, is one factor which is also likely to have a profound effect upon exocrine tissue density.

Tissue Density and Cell Volume

Whilst the densities of different types of mammalian cell are related more to cellular content than volume, the density of an individual cell may nevertheless be profoundly influenced by changes in its volume with time, associated with the uptake or loss of water and other molecular species. Indeed, for cells such as erythrocytes, which behave
as almost perfect osmometers in solutions of varying tonicity, these changes in density can be predicted mathematically. Thus, Leif has derived, from the original equation described by Ponder, the relationship between changes in the buoyant density of erythrocytes and changes in the volume of cellular water resulting from alterations in the tonicity of the surrounding medium (Leif, 1970). This equation can be re-expressed as:

\[ \Delta \rho = \frac{-\Delta V/V_{iso}(\rho_{iso} - \rho_w)}{(\Delta V/V_{iso} + 1)} \]

Where \( \Delta \rho \) = the change in density of the cell resulting from an increase in tonicity of the surrounding medium from isotonic conditions, and associated with a change in volume, \( \Delta V \); \( \rho_{iso} \) = the density of the cell under isosmotic conditions; \( \rho_w \) = the density of water at 4°C; and \( V_{iso} \) = the volume of the cell under isosmotic conditions.

The maintenance of cell volume under normal conditions is dependent upon certain properties of the surface membrane of cells, and upon the concentrations of various molecular species on either side of this membrane (MacKnight et al, 1977). Usually, all cells are bathed in extracellular fluid, in which sodium and chloride are the principal ionic species. Within the cell potassium is the main cation, whilst the anions principally comprise the polyvalent sidegroups of intracellular macromolecules (proteins and nucleic acids), and other ions such as phosphate and sulphate. In addition, the cell membrane is generally freely permeable to water and to small ions such as sodium, potassium and chloride (by virtue of the presence of specific ionic channels), but is impermeable to the intracellular macromolecules and other anions. In any situation where charged molecular species are restricted to one side of a membrane permeable to all other ions and to water, there is a redistribution of the permeable ionic species, resulting in an excess of diffusible ions in the compartment containing the impermeable species, and in the development of an electric charge across the membrane. For instance, in the situation depicted in Figure 4, in which the membrane is permeable to potassium and to chloride, but not to the anion \( A^- \), and in which the initial concentration of potassium is equal on both sides of the membrane, chloride will tend to enter the cell down its concentration gradient. This will in turn generate a negative charge within the cell, which also results in a redistribution of potassium ions and the net uptake of solute by the cell (the Gibbs-Donnan hypothesis).
FIGURE 4
The Redistribution of Permeable Ions Resulting from the Presence of Impermeable Anions Restricted to One Side of a Cell Membrane
Furthermore, the situation is unaltered if sodium, rather than potassium, is the principal cation (MacKnight et al, 1977).

Under physiological conditions, however, the tendency for the principal cations, sodium and potassium, to enter or leave respectively down their concentration gradients is counteracted by the action of a specific sodium-potassium pump, located at the cell membrane, which extrudes sodium ions in exchange for the entry of potassium ions (Skou, 1965), using energy derived from the hydrolysis of ATP (adenosine triphosphate) in order to overcome the relevant electrochemical gradients. The action of the sodium pump effectively renders the cell membrane impermeable to extracellular sodium. Importantly, this therefore prevents the net entry of chloride ions into the cell, as the entry of these anions is counteracted by the generation of an increasing electrical gradient in the absence of the simultaneous entry of extracellular cations. Therefore, the presence of impermeable anions within cells effectively results in the generation of an excess of intracellular osmotic pressure, which has been calculated to be equivalent to about 130 mOsm/kg H$_2$O (MacKnight et al, 1977). This is usually overcome by the active pumping of sodium ions from the cell, resulting in a state (the so-called double-Donnan 'equilibrium') in which the intracellular fluids are isosmotic with the extracellular fluid.

This 'equilibrium' can be disturbed in a number of ways. Thus, it has long been known that inhibition of cellular metabolism results in marked swelling of cells, accompanied by a net gain of cellular sodium and chloride and a loss of potassium, which is rapidly reversible upon the restitution of cell respiration (Elshove et al, 1963; MacKnight et al, 1977). These changes are due to inhibition of membrane sodium pump activity, which is dependent upon cellular metabolism. In these circumstances the cell becomes effectively permeable to extracellular cations (ie. sodium), and chloride is therefore able to enter the cell accompanied by these ions, resulting in the net uptake of solute by the cell. This in turn leads to the influx of water, in order to maintain constant osmolality. The overall effect, therefore, is the uptake of isosmotic extracellular fluid by the cell (Figure 5).

Similar changes may also be induced by hypothermia, which directly inhibits the sodium pump (Martin et al, 1972). Hypothermic-induced cell swelling is potentially damaging to cells, and indeed appears to be one of the most important factors limiting cell viability during cold storage, at least in the short term (Daniel et al, 1976; Belzer et al, 1988; Marsh et al, 1989b; Lindell et al, 1989; Southard et al, 1990). Therefore, one vital function of solutions used for hypothermic preservation of organs is the prevention of this swelling.

Many solutions have been used in cold storage preservation of the pancreas (Southard et al, 1988), including Collins' solution (Collins et al, 1969) and variants based upon
A. Metabolising Cell:
Redistribution of extracellular solutes, due to the presence of intracellular impermeant anions, is prevented by the action of the membrane sodium pump: sodium is effectively an extracellular impermeant.

B. Sodium pump inhibition permits the entry of extracellular solute, accompanied by water. In addition, there is exchange of intracellular potassium for sodium, down the appropriate concentration gradients.

C. Net entry of solute and water during inhibition of sodium pump activity is prevented by extracellular impermeants, although exchange of intracellular potassium for sodium may still occur.
it, such as Euro-Collins; the hypertonic citrate solution of Ross and Marshall (Ross et al, 1976); and the University of Wisconsin (UW) solution, developed by Belzer and colleagues (Belzer et al, 1988). Although the composition of these solutions is markedly different, the mechanism by which they inhibit hypothermic-induced cell swelling is primarily related to their content of impermeant molecules. By replacing permeable extracellular ions (in particular chloride) these molecules prevent the influx of solute and water into cells during the period of inactivation of the membrane sodium pump (Downes et al, 1973; Southard et al, 1980; Wahlberg et al, 1986), as illustrated in Figure 5. The volume of cells in aqueous solutions is therefore dependent, not only upon the function of their membrane sodium pumps, but also upon the composition of the surrounding media, particularly with respect to the concentrations of the permeable ions sodium and chloride.

Cells may also be induced to swell under osmotic conditions by changes in the permeability of the cell membrane to sodium ions by various physical or chemical treatments, and by other alterations in the extracellular ionic composition eg. the replacement of extracellular sodium with potassium (MacKnight et al, 1977). The volume of cells is also dependent upon the osmolality of the extracellular fluid. The permeability of cell membranes to water is much higher than the permeability to inorganic ions. Therefore, when placed in hypertonic media, for instance, cells initially shrink, due to the rapid movement of water out of the cell down an osmotic gradient (the reverse occurs, resulting in cellular swelling, if the medium is hypotonic). In actively metabolising cells these changes persist, at least in the short term, for as long as the cell is exposed to anisosmotic conditions. In non-metabolising cells, or those in which the sodium pump is inactivated, however, subsequent changes in cell volume depend upon the concentration gradients mentioned above, and these cells therefore exhibit a biphasic response upon being placed for example in hypertonic solutions of sodium chloride, an initial loss of cellular free water being followed by a slower gain of isosmotic fluid (Davey et al, 1971).

It is apparent, therefore, that the volume (and hence the density) of cells suspended in aqueous solutions is dependent upon the total osmolality of the solution, the activity of the sodium pump, the ionic permeability of the membrane, the composition of the solution, and the time spent by the cell within the solution.

During islet isolation pancreatic cells are exposed to ischaemia, hypothermia and to a variety of storage solutions. Furthermore, the membrane permeability of pancreatic cells might also be altered by exposure to ischaemia, or through the damaging effects of collagenase, which has been shown to influence membrane permeability, and therefore the degree of cellular swelling, in separated renal tubules (Burg et al, 1964).
Potentially, therefore, several steps in the procedure of islet isolation might contribute to variability in exocrine tissue density by inducing changes in cellular volume.

At present, it is unclear to what extent exocrine enzyme discharge or changes in acinar cell volume contribute to the observed variability in the density of exocrine tissue fragments in the dispersed pancreas. Some recent observations suggest, however, that both may be important. Thus, previous work in this department, in which electron-microscopy was used to examine human pancreatic tissues isolated from the low and high-density parts of linear density gradients of Bovine Serum Albumin (BSA), has suggested that those acinar cells separating into the low density fractions contain fewer exocrine granules than those in the high density fractions (unpublished observations).

Furthermore, it has also been established that the densities of human and porcine pancreatic tissues isolated on gradients of BSA are dependent upon the osmolality of BSA used: most importantly, the use of hypertonic BSA leads to a differential increase in the density of exocrine tissue over that of islets, resulting in an improvement of islet purification (London et al., 1992c; Chadwick et al., 1993).

Interestingly, the purification of other cell types has also been shown to depend upon gradient medium osmolality (Williams et al., 1972a; Timonen et al., 1982; Boyum, 1983), and again, this is due to a differential effect of osmolality upon the densities of different cells. Presumably this effect is due partly to differences in the ionic permeabilities or the content of exchangeable intracellular water between different cells.

In addition, consideration of the above equation demonstrates that the change in density of a cell produced by a given change in volume (of cellular water) is also dependent upon the original density of the cell under isosmotic conditions. Thus, the density of cells, such as pancreatic acinar cells, whose isosmotic density is relatively high will be influenced by a given change in cellular volume to a greater degree than relatively 'light' cells (so long as the isosmotic volume of these cells is similar), and this effect may therefore be of use in the separation of these cells using density gradients.

Thus, hypertonicity of the gradient medium may also explain the improved islet purity observed with Ficoll gradients using Euro-Collins solution as a diluent ('Euro-Ficoll') in place of Hank's solution (Olack et al., 1991), although the relative concentrations of inorganic ions and glucose within these media may also be important.

Finally, little information is available concerning the influence, specifically upon islet purification, of the solutions used for in situ perfusion and cold storage of the pancreas prior to islet isolation. However, recent evidence has suggested that islet purification may be profoundly influenced by the solution used to store the dispersed, collagenase-
digested pancreas prior to density gradient centrifugation. Thus, storage of canine pancreatic digest in UW solution has been associated with a marked improvement in the subsequent yield and purity of viable islets obtained on dextran density gradients (van der Burg et al, 1990a; van der Burg et al, 1991a), presumably resulting from the reversal of pre-existing acinar cell swelling.

SUMMARY AND PROSPECTS FOR IMPROVING ISLET ISOLATION

It is clear from the above discussion that many factors exist which may influence the number and quality of islets obtained from the pancreas, using existing techniques of islet isolation. Potentially, therefore, considerable improvement may be achieved by attention to each of these factors.

Variables related to the organ donor are difficult to control, and shortage of such donors precludes undue selectivity with respect to factors such as age, body mass index and past medical history. However, it may be possible to influence the degree of acinar cell granulation by administration of anti-secretory agents such as somatostatin to the organ donor, prior to organ retrieval, in order to improve the ultimate purification of islets.

During organ retrieval, optimal results depend upon minimisation of warm ischaemia, but further improvement may follow development of alternative techniques to the use of in situ vascular perfusion, for instance by dissection and retrieval of the pancreas prior to that of other organs during multi-organ procurement.

Distension of the pancreas with collagenase solution prior to cold storage will undoubtedly be necessary to reduce the harmful effects of cold ischaemia, and thereby optimise islet yields. However, the optimal medium for delivery of collagenase in this way has yet to be established, at least for human pancreata: media such as Hank's solution, whilst commonly used as a vehicle for collagenase delivery immediately prior to pancreatic digestion, are unsuitable for prolonged cold storage of organs by virtue of their electrolytic composition (high concentrations of sodium and chloride). On the other hand, solutions such as UW, whilst appropriate for prolonged cold storage, contain components which inhibit collagenase digestion of the human pancreas. Indeed, previous attempts by investigators in this department to use UW for the delivery of collagenase at the time of organ retrieval demonstrated a significant reduction in the yield of viable islets compared to the use of Hank's solution (Toomey et al, 1991). Thus, it will be necessary to develop solutions suitable both for digestion and cold storage, in order to maximise the potential of this approach.

The method of collagenase delivery may also need to be modified, paying attention, for instance, to the volumes of collagenase solution used, and to the pressures attained.
within the pancreas, in order to optimise the distribution of enzyme to the interface between islets and acinar cells. Likewise, research into the components of collagenase responsible for lysis of this interface, and the optimal conditions necessary for this to occur, will also be vital in improving the consistency of the results of islet isolation.

Finally, islet purification may be improved by attention firstly to the physical requirements for effective density gradient separation of any cells; and secondly to the mechanisms underlying the marked variability in the density of exocrine tissue between pancreata, and the ways in which acinar tissue density may be manipulated.

Theoretical and existing experimental evidence suggests that the solutions used for storage of pancreatic tissues during each of the stages of islet isolation are of paramount importance. Thus, one approach likely to improve markedly upon the present results of islet isolation is the development of solutions specifically optimised for each phase of the isolation process.

With this in mind, the aims of this thesis are: firstly, to establish the means to assess accurately and reproducibly the density of pancreatic tissues and the efficiency of islet purification from large mammalian and human pancreata using density gradients; secondly, to use these methods to determine the efficiency of islet purification by the techniques currently in widespread use; and thirdly to examine some of the mechanisms potentially contributing to variable exocrine tissue density, and the means by which the densities of pancreatic tissues may be manipulated in order to improve islet purification.
CHAPTER 4

Methods
In the preceding chapters it has been demonstrated that the isolation and purification of human islets of Langerhans is highly problematical. Unfortunately, investigation of the factors influencing this process is also hampered by the relative shortage of suitable organ donors. The use of an appropriate animal model is therefore necessary in order to expedite such investigations.

In this work the pig was chosen as an appropriate large animal model in which to study the factors influencing islet isolation and purification, for a number of reasons: first, it is well established that the problems involved in the isolation of porcine islets are similar to those affecting human islet isolation. Thus, the marked fragility of porcine islets, particularly from young pigs, necessitates considerable care during pancreatic digestion, in order to separate these islets effectively from adherent exocrine tissue, whilst avoiding undue islet fragmentation (Ricordi et al., 1986; Calafiore et al., 1990b; Ricordi et al., 1990d). Furthermore, the purification of porcine islets using density gradients similar to those used in human islet isolation is also variable, allowing substantial room for improvement (Ricordi et al., 1990c; Ricordi et al., 1990d; Thomas et al., 1992).

Second, porcine islets obtained using similar techniques to those employed for human islet isolation have been demonstrated to retain their functional capacity, assessed both in vitro (Ricordi et al., 1986; Crowther et al., 1989; Calafiore et al., 1990b; Marchetti et al., 1991) and in vivo (Ricordi et al., 1986; Ricordi et al., 1987; Ricordi et al., 1990c; Calafiore et al., 1990b; Ricordi et al., 1990d; Marchetti et al., 1991), thus confirming their suitability for study, and permitting investigation of the effects of changes in the isolation process upon islet viability.

Third, should the problems of xenograft rejection be overcome, the porcine pancreas probably represents the most appropriate source of xenogeneic islets for transplantation into diabetic patients (Ricordi et al., 1986; Marchetti et al., 1989; Ricordi et al., 1990c; Calafiore et al., 1990b), due to the similarity between the two species: in the distribution of the various hormone-secreting cells within islets (Crowther et al., 1989; Marchetti et al., 1990); in the secretory response of β-cells to glucose and other secretagogues (Marchetti et al., 1989; Calafiore et al., 1990b; Marchetti et al., 1991); and in the structure of human and porcine insulins. Improving the results of porcine islet isolation may therefore be a useful goal in its own right, in addition to supplying information relevant to human islet isolation.

Finally, a readily available supply of suitable porcine pancreata were available from a local abattoir, and the methods necessary for processing these pancreata were already partly established within the department.

In this thesis, therefore, the purification of both porcine and human pancreatic islets was studied. The purpose of this chapter is firstly, to outline the methods for the
purification of islets from these species, established within the Department of Surgery upon initiation of this thesis in June 1991; and secondly, to describe the development and evaluation of the method, subsequently used throughout this work, for assessment of pancreatic tissue densities and the efficiency of islet isolation using density gradients.

THE ISOLATION OF HUMAN PANCREATIC ISLETS

Donor Operation

Human pancreata used for islet isolation were obtained with appropriate consent from brain-dead, heart-beating organ donors. In the majority of cases retrieval of the pancreas took place as part of a multi-organ procurement in which the liver, heart/lungs and kidneys were also removed for transplantation; in the remaining cases only the kidneys and pancreas were removed.

In each case a cannula was sited within the abdominal aorta, to allow perfusion of the viscera with 2-4 litres of either Hyperosmolar Citrate solution (HOC, Baxter Healthcare Ltd., Thetford, Norfolk, UK) or University of Wisconsin solution (Viaspan Belzer UW solution, Du Pont Critical Care Inc., Waukegan IL, USA) at 4°C, decompression of the venous system being performed either through a further cannula placed into the inferior vena cava, or simply by division of this vessel. If the liver was also retrieved perfusion of the hepatic portal circulation with 2-3 litres of cold UW solution was also achieved via a cannula advanced into the main portal vein from a superior mesenteric vein tributary. During in situ perfusion of the viscera in this way pancreatic venous hypertension was avoided by venting the splenic vein (Marsh et al, 1989a). Following removal of the liver and kidneys the whole pancreas was excised separately from the duodenum (in order to minimise microbiological contamination), care being taken to maintain the integrity of the pancreatic capsule. The pancreas was then immediately transferred to a sterile polythene transport bag containing HOC at 4°C, for transport to the laboratory.

Pancreatic Digestion

Following transport, all pancreata were processed immediately in order to minimise the cold ischaemic time, and all procedures were carried out within a Class II Microbiological Safety Cabinet (Medical Air Technology, Manchester). Pancreata were cleaned of fat, blood vessels and lymphoid tissue and divided transversely at the junction between the body and neck of the organ. Both segments were then weighed,
and the main pancreatic duct in each cannulated with an appropriately sized catheter (Abbocath-T®, Abbott Ireland Ltd., Sligo, Republic of Ireland), which was secured with a silk suture.

Pancreata were then distended by the intraductal injection of 2 ml/g pancreatic weight of Hank's Balanced Salt Solution (HBSS, Northumbria Biologicals Ltd., Cramlington, Northumberland, UK) containing collagenase (Serva Feinbiochemica, Heidelberg, Germany) at a concentration of 2.5 mg/ml for donors aged <20 years; 3 mg/ml for HOC-perfused pancreata from older donors; and 4 mg/ml for UW-perfused pancreata. Collagenase solutions were manually injected at 22°C, using a 50 ml syringe, the intraductal cannula being slowly withdrawn during injection in order to obtain effective distension of the entire gland.

Following distension, pancreata were then digested using an automated digestion-filtration system (Figure 1), similar to that described by Ricordi (Ricordi et al., 1988). In this system digestion of the collagenase-distended pancreas was achieved within the lower portion of an enclosed, stainless steel digestion chamber, divided by a steel mesh of 500 μm pore size. Both segments of the distended pancreas were loaded into the chamber, with ten stainless steel ball-bearings of 1cm diameter. By means of a polyvinylchloride tubing circuit (COBE Laboratories, Gloucester, UK), Minimal Essential Medium (MEM, Northumbria Biologicals Ltd.) was recirculated through the chamber by means of a peristaltic pump (503S, Watson-Marlow Ltd., Falmouth, UK), a heat exchanger being used to control the temperature within the chamber. Once the circuitry had been primed with MEM, the initial phase of digestion was accomplished by continuous recirculation of MEM at a rate of 100 ml/min, the temperature being maintained at 37-38°C and monitored by means of a temperature probe within the chamber. Intermittent gentle agitation and inversion of the chamber ensured that liberated islets were not retained within the chamber by gravity.

At 2-5 minute intervals, 2 ml 'biopsy' samples of the medium emerging from the chamber were taken from a side port of the tubing, stained with 1-2 ml of dithizone (Diphenylthiocarbazone, Sigma Chemical Co. Ltd., Poole, Dorset, UK; 1.2 mM in 5% (v/v) Dimethylsulphoxide/MEM/1% BSA), and examined for the presence of cleaved islets (ie. those free from adherent exocrine tissue). Once several such islets were seen within an individual sample the flow of medium was redirected to allow fresh MEM to enter from a 4L reservoir, whilst the chamber outflow was collected into 1L sterile bottles containing 100 ml of 10% New-Born Calf Serum (NBCS, Advanced Protein Products Ltd., Brierley Hill, West Midlands, UK) at 22°C, thereby protecting the liberated islets from the further action of collagenase, whilst allowing digestion within the chamber to continue. During this phase of the digestion, the flow rate was increased to 200 ml/min, and the temperature maintained at 37-38°C. Dispersal of the pancreas
Schematic Diagram of the Circuitry used in the Automated Digestion-Recirculation System

FIGURE 1
was aided by manual agitation of the chamber, whilst intermittent sampling of the chamber effluent was also continued.

If at any stage there was evidence of islet fragmentation in the biopsy samples, ongoing digestion within the chamber was terminated by replacing the prewarmed reservoir MEM with MEM at 22°C, increasing the flow rate to 300 ml/min and bypassing the heat exchanger, thereby rapidly reducing the temperature within the chamber to 22°C. Meanwhile, collection of the digested tissue continued until no further islets were seen within the biopsies from the chamber effluent.

The dispersed tissue collected during the digestion process was pooled and washed in MEM, then centrifuged at 200g for 2 minutes, followed by resuspension to a total volume of 200 ml in MEM at 4°C. This suspension was then stored at 4°C prior to islet purification.

**Large-Scale Density Gradient Purification of Islets**

The purification of human islets was achieved using large-scale discontinuous density gradients of either Bovine Serum Albumin (BSA) or EuroFicoll (EF), constructed on the COBE 2991 cell processor (Lake et al, 1989a; London et al, 1992a).

For each pancreas miniature test gradients of both media (20 ml discontinuous gradients for EF, and 11 ml continuous gradients for BSA- see below and Chapter 5) were initially run, in order to select the most appropriate medium in each case, and to determine the optimal densities of the pellet and isolation layers of the BSA gradients, if this was the medium to be used.

Sterile solutions of 35% BSA, osmolality 500 mOsm/kg H2O were obtained (Advanced Protein Products Ltd., Brierley Hill, West Midlands, UK) and diluted to the appropriate densities using MEM adjusted to an osmolality of 500 mOsm/kg H2O by the addition of sodium chloride. Density measurements were made using a digital densitometer (DMA 35, Paar Scientific Ltd., London), and osmolality measurements were made using a vapour pressure osmometer (Wescor 5500, Wescor Inc., Logan, Utah, USA).

Stock EF solutions were prepared by the addition of 353 g/l of Ficoll 400-DL (Sigma Chemical Co. Ltd., Poole, Dorset, UK) to EuroCollins solution (Prevenius AG, Bad Homburg, Germany), followed by the addition of 5.9 g/l of HEPES powder (Hydroxyethyl-piperazine ethane sulphonic acid, Sigma) and the adjustment of pH to 7.4 using 20M sodium hydroxide. These solutions were then filtered through a 0.22 μm bottle top filter (Gelman Sciences Ltd., Northampton, UK) prior to autoclaving. Solutions of densities 1.108, 1.096 and 1.087 g/cm³ were then produced by dilution of the stock EF with complete EuroCollins solution.
Large-scale density gradients were constructed on the COBE 2991 cell processor using the standard blood processing sets and the following volumes of density gradient media:

<table>
<thead>
<tr>
<th>Layer</th>
<th>EuroFicoll</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet</td>
<td>300 ml</td>
<td>200 ml</td>
</tr>
<tr>
<td></td>
<td>(1.108 g/cm³)</td>
<td>(Variable Density)</td>
</tr>
<tr>
<td>Isolation</td>
<td>100 ml</td>
<td>150 ml</td>
</tr>
<tr>
<td></td>
<td>(1.096 g/cm³)</td>
<td>(Variable Density)</td>
</tr>
<tr>
<td>Capping</td>
<td>100 ml</td>
<td>50 ml</td>
</tr>
<tr>
<td></td>
<td>(1.037 g/cm³)</td>
<td>(1.069 g/cm³)</td>
</tr>
<tr>
<td>Medium</td>
<td>75 ml</td>
<td>150 ml</td>
</tr>
<tr>
<td></td>
<td>EuroCollins</td>
<td>MEM</td>
</tr>
</tbody>
</table>

When using BSA the densities of the pellet and isolation layers were determined on the basis of the test gradients, so that the majority of the islets were less dense than the isolation layer, and the majority of the exocrine tissue was more dense than the pellet layer.

Up to 20 ml of pancreatic digest was suspended in the appropriate volume of high density medium, to form the pellet layer, which was loaded into the processing bag of the COBE 2991 under gravity. Air was then eliminated from the processing bag by utilisation of the 'supernatant out' facility of the machine during centrifugation at 1200 rpm. The remaining layers of the gradient were then sequentially pumped onto the machine during centrifugation at 1200 rpm, using a peristaltic pump (Watson-Marlow) at a rate of approximately 40 ml/min. The centrifugation speed was then slowly increased to 2200 rpm, and any residual air within the bag allowed to escape slowly by releasing the pressure on the waste line of the processing system.

Following centrifugation at 2200 rpm for 5 minutes (at 4°C for EuroFicoll and 22°C for BSA—see Chapter 5) the supernatant out facility of the COBE processor was used to
pump off defined volumes of media to produce three fractions containing the interfaces of the discontinuous gradients (Figure 2). These fractions were then washed in MEM, and samples from each were stained with dithizone and examined for the presence of islets. With EF, the purest islets were found within interface 1, although significant numbers of less pure islets were often found in interface 2. With BSA the densities of the lower two layers were selected to produce a single band of pure islets in the first interface.

Samples containing suitably purified islets were then pooled as necessary, for islet quantification, prior to placing the purified islets into tissue culture.

Islet Quantification

The number of islets present in the crude pancreatic digest and following density gradient purification were routinely estimated. Samples from the pancreatic digest were taken following suspension in 200 ml of MEM: from this volume 5 x $100 \mu l$ samples were taken, maintaining the tissue in suspension between samples by continuous agitation. Each of these samples was then thoroughly mixed with 900 $\mu l$ of MEM in five separate universal containers, from each of which a further 2 x $100 \mu l$ aliquots were taken and separately placed upon a Petri dish, to give a total of ten aliquots. These were then stained with dithizone, and the number of islets $>50 \mu m$ in diameter counted in each. The total number of islets in the digest was then calculated by multiplying the mean number of islets per sample by the dilution factor of 20,000.

The mean volume of an islet was then estimated by measuring 200 consecutive islet diameters using an eyepiece micrometer and using these data to calculate the volume of each islet ($0.524 \times \text{diameter}^3$, assuming islets are spherical). Islet yield was then calculated as both the total volume of islet tissue (total number of islets multiplied by their mean volume), and as the number of 150 $\mu m$-equivalent islets (total volume of islet tissue divided by the volume of an islet of 150 $\mu m$ diameter $= 17.7 \times 10^{-4} \text{mm}^3$).

In addition, a cleavage index (ie. the percentage of the total number of islets which were completely free of adherent exocrine tissue) was also determined by recording the number of uncleaved islets during measurement of the islet diameters.

Following density gradient purification the same variables were calculated for the purified islet fraction, by examination of 5 x $100 \mu l$ samples taken from a total volume of 200 ml in MEM. In addition, the % purity of the islet preparation (ie. the volume of islet tissue as a percentage of the total tissue present) was estimated visually.
FIGURE 2
Illustrating the Volumes of Fractions Collected from Discontinuous Gradients of EF or BSA from the COBE Cell Processor

EuroFicoll

- 75 ml
- 100 ml
- 100 ml
- 100 ml Interface 1
- 100 ml Interface 2
- 300 ml
- 125 ml Waste

BSA

- 150 ml
- 50 ml
- 150 ml Interface 1
- 150 ml Interface 2
- 200 ml
- 160 ml Waste
THE ISOLATION OF PORCINE PANCREATIC ISLETS

Pancreas Procurement

The problems, mentioned above, inherent in porcine islet isolation may be reduced by the use of adult, rather than juvenile pigs as donor animals (Socci et al, 1990). Furthermore, islet yields from the pancreata of abattoir animals may be improved by instituting changes in the normal slaughtering process to allow excision of the pancreas from the heart-beating animal, and thereby reduce warm ischaemic time (Ricordi et al, 1990b). However, in this study the majority of porcine pancreata were obtained from juvenile pigs under normal conditions of abattoir operation, firstly because this ensured an abundant supply of pancreata from a local abattoir; and secondly because it was felt that the additional problems associated with the isolation of islets from these pancreata made these studies more applicable to the considerable difficulties also encountered in human islet isolation.

Donor pigs were thus predominantly of Large White/Welsh cross-breed, 6-8 months of age, weighing 80-100 kg. Following slaughtering and evisceration of the animals the splenic lobe of the pancreas was rapidly excised and immersed in HOC at 4°C, the warm ischaemic interval being limited to approximately 7 (range 5-9) minutes. The splenic lobe alone was used, partly because this lobe contains the highest proportion of islets within the porcine pancreas (Marchetti et al, 1990), and partly to minimise warm ischaemia by reducing the time required for dissection of the more proximal pancreas, which is closely applied to the main portal vein.

Pancreata were then transported to the laboratory in HOC, dissected clean of fat and lymphoid tissue and distended by the intraductal injection of collagenase in Hank's solution, just as with the human pancreas, except using a concentration of collagenase of 2 mg/ml.

Digestion of the pancreas was then achieved in an automated digestion-filtration system similar to that used for digestion of the human pancreas, with some modifications. Thus, a separate, smaller digestion chamber was employed, the pore size of the mesh also being reduced to 355 μm. In addition, the enhanced fragility of porcine islets required manual shaking of the chamber to be kept to an absolute minimum, whilst the yield of intact islets also appeared to be improved by operation of the recirculation phase at a lower temperature of 35-36°C.

As with the human pancreas, digested tissue was collected into 10% NBCS, washed and resuspended in MEM, and stored at 4°C in MEM prior to islet purification.
DEVELOPMENT OF A MINI-CONTINUOUS GRADIENT ASSAY SYSTEM

Theoretical Considerations

In order to investigate the factors potentially influencing the separation of islets using density gradients it was necessary to adopt a system capable of providing both a quantitative measure of the efficiency of islet purification, allowing meaningful comparison to be made between different gradients and conditions; and an estimate of the individual densities of islets and exocrine tissue, to provide information concerning the mechanisms by which various manipulations may affect this efficiency.

In order to achieve these aims it was elected to examine the use of an existing method for the production of small scale, continuous, linear density gradients, for the following reasons:

First, as outlined in Chapter 3, continuous density gradients have numerous advantages over the other types of isopycnic gradient available, due to their reduced propensity for the production of artifacts. Indeed, it has been stated that 'density gradient centrifugation in a continuous gradient is the analytical method 'par excellence'", lending itself to "an entirely objective assessment of the frequency-distribution curves of certain physical properties (of cells), such as density" (de Duve, 1971).

Second, continuous density gradients have previously been used to estimate the buoyant density distributions of pancreatic tissues (van Suylichem et al, 1990; London et al, 1992c) and other cell types (Leif, 1970; Shortman, 1972). Indeed, these estimates can only be provided using continuous gradients, as only in this method of isopycnic centrifugation do all tissues reach equilibrium at their point of buoyant density. The form of these gradients is however important when used for this purpose. For instance, the number of cells separating into an individual fraction of a continuous gradient is dependent upon the slope of the gradient (ie. the density increment with distance from the centre of revolution) at that point. The use of linear gradients (in which the slope is uniform throughout the gradient) therefore greatly assists in the analysis of the buoyant density of cells, as in these gradients the density distribution of cells can be directly extrapolated from their actual distribution within gradient fractions of identical volume (Shortman, 1972). In addition, because the density of cells is dependent upon the osmolality of the surrounding medium (Chapter 3) it is also essential that osmolality is constant throughout gradients used to assess the density of different cell types (Leif, 1970).

Finally, the efficiency of islet purification under different conditions can only be consistently compared using continuous gradients. Thus, whilst the purification of
islets by any means may be conveniently expressed as the islet yield (i.e. the recovery of islets in the purified fraction as a percentage of those present before purification) and purity (i.e. the volume of purified islet tissue as a percentage of the total volume of the purified fraction), it is important to note that there is a complex reciprocal relationship between these two variables: with any density gradient islet yield can always be increased at the expense of reduced purity, and likewise purity may be optimised by accepting a lesser islet yield (Figure 3).

Furthermore, the exact relationship between islet yield and purity in a given gradient is also dependent upon the relative numbers of islets and exocrine tissue fragments present, which in turn may vary between different pancreata. For example, if only 2% of the unpurified digest is made up of islets, and the remainder is exocrine tissue, then even if all islets are recovered (i.e. the yield is 100%), contamination of this preparation with 2% exocrine tissue will result in a final islet purity of approximately 50%.

Thus, it is apparent that comparison between different gradients can only be achieved by measurement of one of these variables at a constant value of the other variable e.g. islet purity at a fixed islet yield. This is not consistently possible when using discontinuous density gradients, as both variables are predetermined by the densities chosen for the various layers. Theoretically, however, this may be achieved by using continuous linear density gradients in order to plot the density distribution curves of each tissue type, allowing extrapolation to be made to any given value of islet yield or purity.

For these reasons the method employed to study islet purification during this work involved the production of small scale linear continuous density gradients. Bovine Serum Albumin was predominantly used as the density gradient medium because the osmolality of BSA, unlike that of other media such as Ficoll (Williams et al, 1972a), does not vary significantly over the density range used for islet isolation (Lake et al, 1987), thereby permitting continuous gradients of fixed osmolality to be constructed.

**Method for the Production of 11 ml Linear Continuous Density Gradients**

For the study of human islet isolation BSA solutions of osmolality 500 mOsm/kg H₂O were used (London et al, 1992c), whilst for porcine islets an osmolality of 400 mOsm/kg H₂O was employed (Chadwick et al, 1993). Solutions of BSA of appropriate high and low density were produced by dilution of stock BSA with MEM adjusted to the relevant osmolality by the addition of sodium chloride. Density measurements were made using a digital densitometer calibrated to read absolute density (i.e. 1.000 g/cm³).
FIGURE 3
Demonstrating the Reciprocal Relationship Between Islet Yield and Purity in Continuous Density Gradients

NB. The degree of overlap in the densities of islets and exocrine tissue is identical in both gradients
using distilled water at 4°C. The final osmolality of BSA solutions was measured using a vapour pressure osmometer (Wescor).

Gradients were formed in 12 ml round-bottomed test tubes. In each case 100 μl of pelleted pancreatic digest was first suspended in 1 ml of high density BSA, which was then overlaid with a 10 ml continuous linear density gradient generated by a commercially-produced two chamber gradient maker (SG100, Hoefer Scientific Instruments, San Francisco, CA, USA- Figure 4 + Plate 4.1) containing within its chambers 5.2 ml each of high and low density BSA (the extra 0.4 ml being used to counter the effect of losses of BSA in the gradient maker and pump tubing) and emptied by means of a peristaltic pump at a rate of 2 ml/min.

Following centrifugation of these gradients (CR422 centrifuge, Jouan, Tring, Herts.) at 500g (for porcine pancreata) or 800g (for human pancreata) at 22°C for 5 minutes, 11 x 1ml sequential aliquots were carefully aspirated from top to bottom of the tubes using a 1 ml Gilson pipette (Gilson, Villiers, France). Each aliquot was then washed twice in MEM, in order to dilute out the BSA and to eliminate free amylase and insulin released into the medium during centrifugation. Qualitative assessment of the continuous gradients could then be made by visual examination of these fractions, following dithizone staining (Plates 4.2 to 4.4). In this way the optimal densities of BSA could also be selected for use in the large-scale purification of human islets using discontinuous gradients on the COBE processor (see above).

Alternatively, in order to provide quantitative information of gradient function the tissue contained in each washed fraction of a gradient was resuspended in 2ml of MEM and disrupted by sonication for 20 seconds. One ml of each fraction was then stored for amylase assay (see Appendix) to assess the distribution of exocrine tissue, whilst the remaining 1ml underwent acid-alcohol extraction of insulin, using the method of Ziegler et al. (1985), prior to radio-immunoassay (see Appendix) to assess islet distribution.

Assessment of the Continuous Density Gradient Assay System

The Relationship Between Gradient Fraction and Density

For each density range of BSA used an initial series of 6 identical gradients was performed, and the gradients fractionated as described above. Corresponding fractions from each gradient were then pooled and the density of the pooled samples was measured using a digital densitometer. Graphical plots of gradient fraction versus density were then used firstly to establish the linearity of the gradients; and secondly to provide a measure of the mean density of each fraction by linear regression analysis (Figure 5).
With the taps open and the pump emptying the high density chamber at a fixed rate, high density medium is gradually diluted with low density medium under the influence of gravity, thereby producing a continuous linear density gradient.

**FIGURE 4**

Demonstrating the Principal Features of the Linear Continuous Gradient Maker.
Gradient makers and multi-channel peristaltic pump, permitting the simultaneous formation of multiple 11ml continuous density gradients

Tissue distribution within 11ml continuous density gradients following centrifugation
PLATE 4.2

Visual Analysis of Fractionated 11ml Continuous Density Gradient Following Dithizone Staining of Islet Tissue Fractions 1-4
PLATE 4.3

Visual Analysis of Fractionated 11ml Continuous Density Gradient Fractions 5-8
PLATE 4.4

Visual Analysis of Fractionated 11ml Continuous Density Gradient
Fractions 9-11
FIGURE 5

Graph showing the density of the gradient fractions for the density range 1.074-1.102 g/ml.
In addition, the pH and osmolality of the pooled fractions were measured, confirming that these both remained constant throughout the gradient.

**Quantitative Assessment of Pancreatic Tissue Distribution and the Efficiency of Islet Purification**

Using the above data each gradient was assessed by constructing plots of cumulative percentage of amylase and insulin against density (Figure 6), representing the density distributions of exocrine tissue and islets respectively. Because these tissues did not generally exhibit a Gaussian frequency distribution with density, it was elected to use the median of each distribution as an estimate of the buoyant density of each tissue. These values were calculated simply by extrapolation to the density axis from the 50th percentile of each of the above curves (Figure 6). In addition these curves could be used to provide a standardised measure of the efficiency of islet purification for each gradient, by allowing the exocrine contamination of any given islet yield to be calculated (Figure 6). An islet yield of 60% was chosen as suitable for investigation because it was felt that consistently achieving this yield of highly purified islets would be both a realistic and clinically useful goal.

The value of this quantitative assessment had previously been examined by comparison with assessment using a visual scoring system, following dithizone staining of the gradient fractions from duplicate gradients (Robertson et al, 1993b). In 14 pairs of gradients close correlation (using Pearson's product-moment correlation coefficient) was observed between the two methods in estimating the distribution of exocrine tissue ($r = 0.806$ (median), $p < 0.001$). The correlation between the two methods was less marked when used to estimate the islet distribution ($r = 0.657$, $p < 0.022$), largely because it is difficult to estimate visually the proportion of the total number of islets present within those gradient fractions containing large quantities of exocrine tissue: visual scoring systems inevitably underestimate islet numbers within these fractions (van der Burg et al, 1993), and thereby tend to overestimate the islet yield in the purified fractions. In view of this, it was felt that the quantitative assay system described above provided a more objective assessment of tissue distribution with which to compare gradients than that produced by visual assessment, in addition to providing a more standardised measure of the efficiency of islet purification.

Having elected to use this system as a means of investigating the purification of islets, it was then necessary to examine the reproducibility of the measurements provided. This was achieved by comparison of paired values obtained from identical duplicate gradients during the initial series of experiments described in later chapters. Twenty-eight pairs of gradients were used in this analysis, using digest from seven different
Demonstrating the Cumulative Percentage Pairs of Amylase and Insulin Against Density

Figure 6
pancreata and a variety of gradient conditions, in order to provide a wide range of values for the three variables measured in each case. Thus, the %exocrine contamination of a 60% islet yield varied between 0.5-53%; the median density of exocrine tissue varied between 1.091-1.097 g/cm³; and the median islet density ranged between 1.083-1.094 g/cm³.

The most appropriate methods for graphical representation and interpretation of this type of data have been described by Bland and Altman (1986). Using these methods a repeatability coefficient can be calculated, based upon the standard deviation of the differences in values between paired measurements. This coefficient can then be used to express the 95% confidence limits of individual measurements. For the 28 paired gradients investigated here the repeatability coefficients (twice the standard deviation of the paired differences) were: ± 9.2% for the values of %exocrine contamination of a 60% islet yield; ± 13 x 10⁻⁴ g/cm³ for the median exocrine tissue density; and ± 45 x 10⁻⁴ g/cm³ for the median density of islets. Thus, we can be 95% confident that the value of each of these variables lies within these limits of the value provided by a single gradient.

These results are further expressed in Figure 7, in which the differences in values of these three variables between paired duplicate gradients are plotted against the mean values obtained from each pair. Examination of these plots shows that for each variable the mean difference between duplicates is reassuringly close to zero (0.4% for exocrine contamination; 4.6 x 10⁻⁴ g/cm³ for exocrine density; and 3.9 x 10⁻⁴ g/cm³ for islet density). Furthermore, there does not appear to be any obvious relationship between inter-duplicate differences and the absolute value of each variable, suggesting that the measurements obtained are equally reliable over the whole range of values studied for these variables. The variability of the values for islet density is more marked than that for exocrine tissue density, but appears to be within reasonable limits, given that the limit of resolution of the digital densitometer used was ± 0.001 g/cm³ (range).

Discussion
The most commonly reported method for assessing islet purification by isopycnic centrifugation has been comparison between the number of islets present in the crude pancreatic digest with the number in the purified fractions obtained from discontinuous density gradients, in conjunction with a visual estimate of islet purity in the latter sample.

This method is unsatisfactory, however, for the systematic investigation of factors influencing islet purification, for several reasons. First, as mentioned above, visual assessment is prone to underestimation of islet numbers within samples containing large quantities of exocrine tissue, and particularly within crude pancreatic digest
FIGURE 7
Bland-Altman Plots Demonstrating the Variability in the Measurements Obtained From 28 Paired Continuous Density Gradients

%Exocrine Contamination

Median Exocrine Density (g/cm³)

Median Islet Density (g/cm³)
because islets comprise such a small percentage of the total tissue present. This therefore inevitably results in an overestimate of islet yield following purification. Second, the efficiency of islet purification cannot be estimated from the yield and purity of the final islet preparation alone, as the relative proportions of islets to other tissues in the unpurified fraction must also be known. For example, the recovery of a 100% yield of 50% pure islets from an original digest containing only 1% islet tissue clearly represents a different efficiency of separation than an identical yield and purity obtained from digest in which islets made up 10% of the original tissue. Because the relative content of islets and other tissues within the unpurified fraction is highly variable, this is an important issue when attempting to quantify the efficiency of islet separation. Third, meaningful comparison between gradients can only be achieved if one variable in the yield/purity equation is controlled. This is difficult to achieve when simply comparing purified and unpurified fractions from different gradients: unless one set of conditions results in both higher yield and purity than another, the relative efficiency of separation cannot be compared.

In addition, this system is susceptible to considerable observer bias; does not provide information on the changes in tissue density resulting from different isolation conditions, which may be useful in determining the mechanisms by which islet purification may be improved; and is prone to the production of those artifacts inherent in the use of discontinuous gradients. In contrast, the method described above for the production of linear, isosmotic continuous density gradients may be used to provide a more objective, and reasonably reliable, estimate of pancreatic tissue densities and a standardised measure of the efficiency of islet purification. It should be noted at this point that objections have been raised to the use of insulin/amylase ratios in expressing post-purification islet yields and purity (Gray et al., 1987). Indeed, it is clear that the expression of the absolute insulin/amylase content of the purified islet preparation is valueless in this respect, due to the differences between laboratories in methods of insulin extraction and of insulin/amylase assay, in addition to the loss of insulin/amylase activity between digest and purified preparation due simply to the release of these molecules from cells into the washing medium. In the method described above, however, these assays are used to assess the proportion of the total gradient insulin/amylase present after washing, within each gradient fraction, which therefore reflects the relative mass of islet/exocrine tissue isolated in these fractions: certainly close correlation exists between insulin content and β-cell mass, within both the pancreas (McCulloch et al., 1991) and individual islets (Wolters et al., 1980), and a similar relationship between amylase content and acinar tissue mass is suggested by the results outlined above.
One disadvantage of the system outlined is that assessment of purification is limited to the separation of islets from exocrine tissue only, the distributions of other pancreatic tissues eg. ductal and lymphoid tissue, being uninvestigated. At present, however, the principal problem in density-dependent purification of islets remains the removal of large quantities of contaminating exocrine tissue, whilst the most immediate benefits of islet purification (improving the safety of clinical transplantation) are also most closely linked with this aim.

Finally, one further advantage of the method described results from the ability to perform multiple gradients using tissue from each individual pancreas, thereby reducing the confounding influence of the marked variation in islet purity observed between different pancreata using apparently identical conditions of isolation. Using a series of six gradient makers and a multi-channel peristaltic pump (Gilson, Villiers, France) samples of digest from a single pancreas may be simultaneously purified under a variety of conditions. For every pancreas, each gradient may then be compared with an appropriate control gradient, so that comparisons are always made only between different gradient conditions, rather than involving comparison between different pancreata. Combination of these results using a statistical test suitable for paired data (e.g. the Wilcoxon signed rank test for paired data) thus largely eliminates the influence of interpancreatic variability. This methodology and experimental design will therefore form the basis for much of the work described in the following chapters.
CHAPTER 5

Islet Purification: A Comparison of Euro-Ficoll and Bovine Serum Albumin, and the Influence of Density Gradient Temperature and Glucose Concentration
Gradient media used in the density-dependent separation of most cells are based upon aqueous solutions of various macromolecules, and a very large number of these media have been used for cell purification and the measurement of cellular density (Shortman, 1972; Pretlow et al, 1975).

Certain of these media have also been applied to the purification of islets of Langerhans. Sucrose was the first such example to be used in the purification of rat islets (Lacy et al, 1967), although the viability of the islets obtained with this medium appeared to be impaired, possibly due to its marked hyperosmolality over the density range required.

Shortly thereafter the use of an alternative medium, Ficoll, was investigated for rat islet isolation (Lindall et al, 1969). This molecule is a branched chain copolymer of epichlorohydrin and sucrose, with an approximate molecular weight of 400,000, and was first employed in cell separation techniques over 30 years ago, for the isolation of rat peritoneal mast cells (Uvnäs et al, 1959). Initial experience with Ficoll for rat islet purification demonstrated improved viability of the isolated islets compared to the use of sucrose gradients, provided that low molecular weight contaminants, potentially toxic to islets directly or through their influence upon gradient osmolality, were excluded by dialysis or by use of particular batches of Ficoll (Ballinger et al, 1972; Scharp et al, 1973; Nash et al, 1976). Subsequently, this medium was successfully used in the purification of viable islets from large experimental animals (Sutherland et al, 1974; Noel et al, 1982; Ricordi et al, 1990c; Warnock et al, 1990a; Scharp et al, 1992b) and humans (Sutherland et al, 1974; Gray et al, 1984; Alderson et al, 1987b; Scharp et al, 1987; Warnock et al, 1988a), leading to its widespread use for this purpose in many centres.

Bovine serum albumin (BSA) was first employed in the density-dependent separation of cells in 1946 (Ferreebe et al, 1946), and later used by Leif and Vinograd for estimation of the bouyant density of erythrocytes through the construction of linear continuous, isosmotic gradients (Leif et al, 1964). Its use in the purification of islets was introduced by investigators in this Department in 1987 (Lake et al, 1987), and it was using this medium that large-scale discontinuous density gradients constructed on the COBE 2991 cell processor were first employed for islet isolation (Lake et al, 1989a). Subsequently, the efficacy of BSA for islet isolation has also been confirmed by other groups (Alejandro et al, 1990; Vives et al, 1992).

In general terms there is little to choose between Ficoll and albumin as media for cell separation (Shortman, 1972; Pretlow et al, 1975). Albumin has the advantage of a relatively low viscosity, in addition to a general protective effect on cells and the ability to reduce cell aggregation. It is, however, difficult to prepare and, being derived from animal sources, susceptible to microbiological contamination. In contrast Ficoll is
entirely synthetic and relatively easy to handle, but leads to enhanced cell aggregation, is more viscous, and at high concentrations increases the osmotic activity of media by a direct water-binding effect (Williams et al., 1972a).

Few direct comparisons have been performed between these two media for islet purification. Some investigations have demonstrated superior yields of intact islets in rats (Lake et al., 1987) and dogs (Alejandro et al., 1990) using albumin compared to standard Ficoll, although other workers have noted little difference between these media for rat islet isolation (van Suylichem et al., 1990), and no information exists upon their relative efficiency in human islet purification.

In addition, developments in the use of both gradient media, leading to improved islet yields, have recently been reported. Thus, in 1991, improvement in the results of canine and human islet purification using Ficoll was achieved by employing Euro-Collins cold storage solution as a diluent for the gradient medium (producing 'Euro-Ficoll'), in place of balanced salt solutions such as Hank's solution (Olack et al., 1991). Meanwhile, results obtained with BSA for porcine and human islet isolation have been optimised by increasing the osmolality of this medium, to 400 mOsm/kg H$_2$O in the former instance (Chadwick et al., 1993) and to 500 mOsm/kg H$_2$O in the latter (London et al., 1992c).

No comparison has therefore been performed in any species between these two optimised media i.e. Euro-Ficoll (EF) and hyperosmolar BSA. Despite this, however, these were the two media most commonly used for large-scale human islet purification within this and the majority of other centres, at the outset of the present investigation. The initial part of this study was therefore concerned firstly with determining the results currently attainable using these two media for human islet isolation, and secondly with a comparison of their relative efficiency for this purpose.

Upon initiation of this work, the choice of medium used for large-scale purification of human islets was based upon the results of mini test gradients of BSA and EF, as outlined in the previous chapter, 20 ml discontinuous gradients being used for EF, and 11 ml continuous gradients for BSA. The use of different types of test gradient (discontinuous versus continuous) for these media was related to the water-binding effects of Ficoll on gradient osmolality, mentioned above. Although continuous density gradients of EF can be constructed, the osmolality of such gradients increases with density, which may in turn produce significant changes in the density distribution of islets and exocrine tissue. Osmolality also increases with density in discontinuous gradients, but the influence of this upon the density distribution of pancreatic tissues is reduced by the arrest of tissue at discrete interfaces, preventing exposure of cells to the osmolality of the adjacent medium. Extrapolation of results obtained with continuous test gradients to those obtained using large-scale discontinuous gradients is therefore
difficult using EF, making it necessary to accept the theoretical disadvantages of discontinuous test gradients for this purpose. The osmolality of BSA does not, however, vary over the density range used for islet isolation, so that either type of gradient could be used for predicting the results obtained with large-scale discontinuous gradients on the COBE cell processor. Thus, as discontinuous gradients were invariably used for large-scale islet isolation at the beginning of this work, this was the form of test gradient of EF used in this investigation, the quantity of tissue separated on each gradient (100 μl) being kept well below their maximum capacity (0.5-1 ml) in order to minimise the production of artifacts with these gradients. Continuous gradients were then used with BSA, in order to allow islet purity to be compared at an identical islet yield to that obtained with EF, as described in Chapter 4, thereby enabling the efficiency of islet purification with these two media to be more accurately collated.

Methods

Ten consecutive human pancreata were studied, obtained with appropriate consent from brain-dead, heart-beating organ donors of median (range) age 42 (13-56) years, following in situ vascular perfusion with HOC (n=7) or UW (n=3), as outlined in Chapter 4. Following excision, pancreata were transported to the islet isolation laboratory in the appropriate cold storage solution at 4°C, with a median (range) cold ischaemic time of 3.6 (2.2-7.0) hours. Following collagenase digestion of the pancreas, the dispersed pancreatic tissues were washed in MEM at 4°C and finally centrifuged at 200g for 2 minutes, prior to density gradient separation. BSA solutions of density 1.075 and 1.106 g/cm³ and osmolality 500 mOsm/kg H₂O, and EF solutions of density 1.108, 1.096 and 1.037 g/cm³ were produced, as described in Chapter 4.

For each pancreas, duplicate discontinuous density gradients of EF were constructed. In an Universal 100 μl of digest was thoroughly suspended in 8 ml of EF, density 1.108 g/cm³, and this was then carefully overlaid with successive 4 ml layers of EF at densities 1.096 and 1.037 g/cm³ and finally by 4 ml of Euro-Collins solution. After centrifugation at 800g for 5 minutes at 4°C, the tissue at the 1.037-1.096 interface (designated interface 1) and at the 1.096-1.108 interface (interface 2) was aspirated with a 1 ml Gilson pipette, care being taken to ensure that all the tissue present at each level was removed. These samples and all the tissue remaining within the Universal
after aspiration (ie. the gradient 'pellet') were then separately washed twice in MEM, giving three tissue samples from each gradient. The samples from one of the duplicate gradients were then assessed visually by incident white light microscopy after staining of islets with dithizone, whilst those from the other gradient were each resuspended in 2 ml of MEM, sonicated for 20 seconds each, and separated for amylase and insulin assay, as described for BSA continuous gradients in Chapter 4. For each pancreas duplicate linear continuous density gradients of BSA were also run, using a density range of 1.075-1.106 g/cm$^3$ and centrifugation at 800g for 5 minutes at 22°C. Following fractionation into 11 x 1 ml aliquots, each aliquot was washed twice in MEM, those from one duplicate gradient being dithizone-stained for visual assessment, while those from the other gradient were sonicated and processed for amylase/insulin assay, as above.

Islet yield and purity were assessed for the EF gradients by expressing the insulin/amylase content of each interface as a percentage of the total insulin/amylase content of the gradient (interfaces + pellet) after washing. For the BSA gradients plots of cumulative percentage insulin/amylase against density were then used, as described, to calculate the % exocrine contamination at islet yields equivalent to those obtained from the EF gradients (Figure 1). As both gradient media were used for every pancreas, statistical comparison between the two was performed using the Wilcoxon signed rank test for paired data.

**Results**

In the EF gradients purified islets were present in both interfaces aspirated. The upper interface (1.037-1.096; interface 1) usually contained highly purified islets, although significant numbers of less pure islets were often present in interface 2, so that overall islet yield could be improved by pooling the tissue from both interfaces. In practice, therefore, the final preparation of purified islets was obtained by use of the tissue contained in either interface 1 alone or in interfaces 1 and 2 together. The islet yield (% insulin recovery) and purity (% amylase contamination) obtained in this way from the EF gradients are summarised in Table 1 with the values of islet purity at corresponding islet yield obtained from the BSA gradients. At the low islet yield obtained with EF gradients using interface 1 alone, amylase contamination was significantly lower than at an equivalent islet yield in the BSA gradients (95% Confidence Interval of the difference in amylase contamination between EF and BSA = -1 to -21%, p=0.013). At the higher yield obtained by pooling
TABLE 1

Insulin Recovery in the Interfaces of EF Gradients, and the Corresponding Amylase Contamination for both EF and BSA

<table>
<thead>
<tr>
<th>Pancreas Number</th>
<th>% Insulin Interface 1</th>
<th>% Amylase-EF</th>
<th>% Amylase-BSA</th>
<th>% Insulin Interface 1+2</th>
<th>% Amylase-EP</th>
<th>% Amylase-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.4</td>
<td>0.1</td>
<td>0.0</td>
<td>37.2</td>
<td>48.7</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>33.0</td>
<td>0.8</td>
<td>25.0</td>
<td>66.0</td>
<td>48.8</td>
<td>52.0</td>
</tr>
<tr>
<td>3</td>
<td>30.0</td>
<td>1.3</td>
<td>2.5</td>
<td>66.0</td>
<td>35.1</td>
<td>29.0</td>
</tr>
<tr>
<td>4</td>
<td>71.0</td>
<td>3.4</td>
<td>36.0</td>
<td>76.0</td>
<td>46.0</td>
<td>41.0</td>
</tr>
<tr>
<td>5</td>
<td>45.0</td>
<td>3.0</td>
<td>37.0</td>
<td>65.0</td>
<td>50.0</td>
<td>49.0</td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>0.3</td>
<td>1.0</td>
<td>36.0</td>
<td>65.0</td>
<td>49.0</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>0.0</td>
<td>0.0</td>
<td>16.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>39.1</td>
<td>2.3</td>
<td>11.0</td>
<td>69.7</td>
<td>49.8</td>
<td>33.0</td>
</tr>
<tr>
<td>9</td>
<td>25.6</td>
<td>0.8</td>
<td>10.0</td>
<td>80.5</td>
<td>28.1</td>
<td>48.0</td>
</tr>
<tr>
<td>10</td>
<td>24.0</td>
<td>0.65</td>
<td>2.5</td>
<td>43.0</td>
<td>45.0</td>
<td>9.5</td>
</tr>
<tr>
<td>MEDIAN</td>
<td>27.8</td>
<td>0.8</td>
<td>6.3</td>
<td>65.5</td>
<td>45.5</td>
<td>31.0</td>
</tr>
</tbody>
</table>
interfaces 1 and 2, however, purity tended to be better with BSA than with EF, although this difference was not statistically significant overall (95% CI of difference in amylase contamination = -2 to 25%, p=0.155).

Although these were the overall trends, the optimal medium for islet isolation appeared to differ between pancreata. For example, in pancreas No. 1 islets were not purified to any extent with EF, whilst a reasonable yield could be obtained without any amylase contamination using BSA. In contrast, in pancreas No. 4 little purification was achieved by BSA, whilst EF performed relatively well.

In 8/10 pancreata a reasonable yield and purity (>30% insulin yield, <5% amylase contamination) could be obtained: in two of these cases BSA was the optimum medium; in four cases EF was optimal; and in two cases purification was similar with both media (although for one of these, pancreas No. 7, exact determination of the purity obtained at an islet yield of >30% was only possible for BSA).

In the remaining 2/10 pancreata this efficiency of islet purification was achieved by neither medium. In one of these cases a lesser yield of pure islets was obtainable with EF, but not BSA; in the other case there was little to choose between the two media at any islet yield.

Overall, therefore, results were best with EF on 5/10 occasions, with BSA on 2/10 occasions, and similar with both media in 3/10 cases. Visual assessment of the test gradients, used to determine rapidly the optimal medium for large-scale islet isolation, confirmed these observations, BSA being used on the COBE cell processor on 4/10 occasions, and EF in 6/10 cases.

**Discussion**

The results outlined above suggest that, whilst EF and BSA are both able on occasions to isolate large numbers of purified islets, the efficiency of purification is highly variable and, in general, disappointingly low. At low islet yields, purity was greater with EF than with BSA, and at higher yields purity tended to be better with BSA than with EF, so that overall there was little to choose between the two media. Indeed, the optimum medium for islet purification appeared to differ between pancreata, BSA being best in some cases and EF in others. The factors determining which medium is most suitable for individual pancreata are unknown, and it is therefore preferable to have both media available, and to select the best medium for use in large-scale islet purification from each individual pancreas by visual assessment of test gradients similar to those described above.
The performance of EF in this study appears to be significantly inferior to that experienced by Olack et al., in their original description of this gradient medium (Olack et al., 1991). In that investigation it was stated that islet yields of >70% with >90% purity were obtained from discontinuous EF gradients using the upper interface only. Islet yield was, however, expressed as the percentage recovery of counted islets in the purified fraction, compared to the digest. As discussed in Chapter 4, this method is prone to overestimation of the efficiency of islet purification, largely through underestimation of islet numbers within the crude digest. In addition theirs was not a consecutive series, and the present study has demonstrated that whilst EF may indeed produce these high yields of purified islets on occasions, the overall efficiency of purification is much less than this. Finally, even using these methods for estimation of islet yield, other investigators have more recently reported results closer to those of the present study, with a mean islet yield of 39% with 72% purity using EF (Lakey et al., 1992).

Perhaps the most important finding of the present study therefore was the confirmation that neither BSA nor EF was consistently able to provide highly purified islets at the yield likely to be necessary for successful clinical transplantation using islets from a single donor pancreas. Visual examination of test gradients and the pellet of large-scale gradients constructed on the COBE cell processor demonstrated, however, that considerable numbers of intact, and largely cleaved, islets were present within gradient fractions below those selected for their content of highly purified islets. This suggests, therefore, that most of the loss of islets occurring during purification is due simply to inefficient density-dependent separation of cleaved islets from exocrine tissue, rather than to inadequate pancreatic dispersal or islet fragmentation. This in turn suggests that further improvements in islet purification using density gradients may be possible, but that in order to achieve this the factors influencing the densities of pancreatic tissues during islet isolation must be investigated. As discussed in Chapter 3, these factors are likely to include the composition of any media in which pancreatic tissues are suspended in this process, including density gradient media. Therefore, the next important step in this study was to investigate and compare the compositions of the existing density gradient media, EF and BSA.

Determining the Composition of Gradient Media

The concentrations of the major ionic species within stock solutions of EF and BSA were determined using an inductively coupled optical emission spectrometer (ARL 3580 ICP OES, performed by Fisons Scientific Equipment Division, Loughborough,
UK), capable of detecting ionic concentrations of \(<1 \times 10^{-3}\) parts per million in aqueous solutions. Stock solutions were diluted 1:100 with double-distilled water, to provide sample solutions of sufficiently low viscosity to be processed, and the ionic concentrations (in ppm) within the samples and the ddH₂O diluent were determined. Thus, the ionic concentrations within the original stock solutions could be calculated. In addition, measurements were made of pH (pHM 62 meter, Radiometer A/S, Emdrupvej 72, Copenhagen, Denmark) and osmolality (Wescor vapour pressure osmometer). The results of these determinations are given in Table 2, from which it can be seen that the two media differ from each other in a number of respects. First, the osmolality of EF is greater than that of BSA. Potentially this is an important difference, given the known influence of osmolality upon the density-dependent separation of islets, outlined in Chapter 3. However, as EF is less hypertonic at lower density, only the lowermost fraction of EF gradients (density 1.108 g/cm³) has an osmolality significantly higher than that of BSA, so that this factor alone is unlikely to explain the differences observed between the two media with respect to islet purification.

Second, the principal cation in BSA is sodium, whilst in EF it is potassium. Cold storage solutions such as Euro-Collins (the diluent in EF) were originally designed to simulate intracellular fluid, as it was believed that cellular damage during cold storage was related to the loss of intracellular potassium, which was reduced in media containing high concentrations of this cation (Collins et al., 1969). Subsequently, however, it has been demonstrated that the damaging effects of hypothermia are mediated largely by cellular swelling, and that reduction of this swelling in effective cold storage solutions is dependent upon their content of cellular impermeants, but not upon their relative contents of sodium and potassium (Downes et al., 1973; Daniel et al., 1976; Southard et al., 1980). Indeed, effective hypothermic preservation of whole organs, including the pancreas, has been achieved using solutions containing impermeants and a high Na⁺:K⁺ ratio (Moen et al., 1989; Griffin et al., 1993). Thus, to the extent that islet purification is dependent upon the control of cellular volume, it appears unlikely that differences in the results obtained between EF and BSA are due to their disparate Na⁺:K⁺ ratios.

The anionic content of the two media may, however, be important in this respect. Although the concentrations of anions could not be directly measured, they may be inferred from a knowledge of the diluents used in preparation of the stock density gradient solutions. For EF, the anions present in the Euro-Collins diluent are phosphates, bicarbonate and sulphate, to which cells are largely impermeable (Downes et al., 1973), whilst permeable chloride anions are present in relatively low concentrations (15 mmol/l). In hyperosmolar BSA, however, chloride is the principal anion. In addition, EF contains large concentrations (= 160 mmol/l) of an uncharged
TABLE 2
The Composition of Stock Solutions of Euro-Ficoll and BSA

<table>
<thead>
<tr>
<th></th>
<th>Euro-Ficoll</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/cm³)</td>
<td>1.115</td>
<td>1.106</td>
</tr>
<tr>
<td>pH</td>
<td>7.21</td>
<td>7.07</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg H₂O)</td>
<td>560</td>
<td>500</td>
</tr>
<tr>
<td>Na⁺ (mM)</td>
<td>8</td>
<td>189</td>
</tr>
<tr>
<td>K⁺ (mM)</td>
<td>91</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca²⁺ (mM)</td>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>Mg²⁺ (mM)</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Zn²⁺ (mM)</td>
<td>0.003</td>
<td>0.14</td>
</tr>
</tbody>
</table>
molecule, glucose, whilst the glucose concentration in stock BSA is very low (manufacturers' information). Theoretically these differences may be important when using these density gradient media under hypothermic conditions, due to their influence upon cellular swelling and therefore upon cell density. However, whilst EF was routinely used in our laboratory at 4°C (logically a suitable temperature at which to undertake an anaerobic process such as density gradient purification), BSA gradients were usually run at 22°C. Differences between the two media for islet purification may therefore reflect the combined effects of the temperature used in each case and the concentrations of anions and glucose in each medium.

The aim of the following investigations was therefore to study the influence of these variables upon islet purification using BSA, with the prospect of improving both the results obtained using this medium and understanding of the mechanisms influencing islet purification by density gradients. In these studies porcine pancreata were used, as outlined in Chapter 4, in order to provide a reliable and uniform source of material which was relevant to investigating the problems of human islet isolation.

THE EFFECT OF TEMPERATURE ON PORCINE ISLET PURIFICATION USING BSA DENSITY GRADIENTS

Methods

Seven porcine pancreata were studied. These were obtained and processed as described in Chapter 4, warm ischaemic time being limited to approximately 5-10 minutes, and cold ischaemia to 30-60 minutes.

Following collagenase digestion, dispersed pancreatic tissues were pooled and washed in MEM, then resuspended in MEM at 4°C, immediately prior to density gradient separation.

The density range of BSA used was 1.072-1.098 g/cm³, and the osmolality of both high and low density BSA solutions was adjusted to 400 mOsm/kg H₂O by the addition of sodium chloride. As the density of any aqueous solution, including BSA, is dependent upon temperature, separate high and low density solutions of BSA were used for each temperature studied, and density measurements were made with solutions at the relevant temperature in each case, using a densitometer (DMA 35) calibrated to read absolute density (i.e. 1.000 g/cm³ with ddH₂O at 4°C).

Gradients were then constructed as previously described, either at 22°C or in a cold-room at 4°C. In each case 100 µl of pelleted digest was separated following bottom-
loading into the gradient, and gradients were centrifuged at 500g for 5 minutes in Jouan centrifuges previously equilibrated to 22 or 4°C, as appropriate. Following centrifugation the gradients were fractionated and assayed as described in Chapter 4, in order to estimate the individual densities of exocrine tissue and islets, and the %exocrine contamination at a fixed islet yield of 60%. Statistical comparison between gradients at the two temperatures was performed using the Wilcoxon signed-rank test for paired data, expressed as the value, T, of the test statistic, the significance level, p, and 95% confidence intervals (95% Cl).

Results

As illustrated in Figure 2, the densities of both exocrine tissue and islets were influenced by temperature. Median (range) values for the density of exocrine tissue were 1.095 (1.093-1.097) g/cm³ at 22°C, compared to 1.092 (1.091-1.097) g/cm³ at 4°C, representing a significant reduction in density at the lower temperature (T=28, p=0.022, 95% CI = 14 to 41 x 10⁻⁴ g/cm³ reduction in density at 4°C). However, whilst on two occasions islet density was reduced to a similar extent to that of exocrine tissue at 4°C, the overall changes in islet density with temperature were less marked than for exocrine tissue. Median (range) values were 1.087 (1.085-1.095) g/cm³ at 22°C, compared to 1.086 (1.084-1.092) g/cm³ at 4°C (T=24, p=0.108, 95% CI = -3 to 32 x 10⁻⁴ g/cm³ reduction in density at 4°C). At 4°C, therefore, there was a greater degree of overlap in the density distributions of exocrine tissue and islets than at 22°C, and as a result the amylase contamination at a fixed 60% islet yield was greater at the lower temperature (Median 29% versus 8% at 22°C), representing a significant deterioration in islet purity (T=21, p=0.036, 95% CI = 1 to 24% greater contamination at 4°C). These changes are further outlined in Figure 3, in which temperature-dependent changes in tissue density and islet purity are summarised as the differences (Median and 95% CI) between paired gradients (ie. 4°C versus 22°C) from each pancreas. By expressing the results in this way the confounding effects of interpancreatic diversity in these variables are reduced, whilst the relative magnitude and statistical significance of the changes can be readily appreciated: any column wholly to one side of the baseline represents, by definition, a significant change (at p<0.05), and even if not 'statistically significant' overall, trends in one or other variable may also be detected. In fact, this has recently been recommended as the method of choice when expressing the results of comparison between experimental groups (Gardner et al, 1989), and is preferable to
FIGURE 2

The Effect of Temperature on the Median Densities of Islets and Exocrine Tissue and upon Exocrine Contamination in a 60% Islet Yield.
FIGURE 3
Summarising Temperature-Dependent Changes in Tissue Density and Exocrine Contamination

<table>
<thead>
<tr>
<th>Exocrine Tissue Density, 4°C vs 22°C (g/cm$^3$ x 10$^{-4}$)</th>
<th>Islet Density 4°C vs 22°C (g/cm$^3$ x 10$^{-4}$)</th>
<th>% Exocrine Contamination 4°C vs 22°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columns = 95% CI of Difference Between Paired Gradients</td>
<td>Horizontal Bars = Median Difference</td>
<td></td>
</tr>
</tbody>
</table>
simple hypothesis testing (and the use of p-values alone), particularly with small sample sizes.

**Discussion**

The results outlined above suggest that the densities of porcine exocrine tissue and islets in density gradients of hypertonic BSA are differentially affected by gradient medium temperature, which may therefore influence the efficiency of islet purification using this medium.

As discussed in Chapter 3, cells in which the membrane sodium pump is inactivated (e.g. by hypothermia) demonstrate a biphasic response upon being placed in hypertonic solutions of sodium chloride, such as BSA, an initial loss of cellular free water being followed by a slower gain of isosmotic fluid. The lower density of pancreatic tissues at 4°C compared to 22°C is therefore likely to reflect reduced activity of the sodium pump of these tissues at the lower temperature, with consequent cellular swelling. The difference in temperature-dependent density changes between islets and exocrine cells may then represent tissue-specific differences in the temperature-sensitivity of their sodium pumps, or in the permeability of their membranes to extracellular ions. Such differences between these tissues may in turn pre-exist or alternatively result from ischaemic damage during organ retrieval and islet isolation, to which acinar cells have an enhanced susceptibility compared with islets (Slater et al, 1975; Jones et al, 1975; Medvetskii et al, 1978).

These results suggest, therefore, that the control of hypothermic cellular swelling is indeed likely to be of great importance during density gradient islet purification. Consequently, media used at 4°C should be designed specifically to reduce hypothermic cellular swelling, whilst better results are obtained at 22°C when using media such as BSA, which contain high concentrations of ions to which cell membranes are permeable.

However, even at 22°C the ability of pancreatic cells to maintain their volume in aqueous density gradient media may be compromised, as the sodium pump may not be fully active at this temperature: in the porcine liver, for instance, membrane Na/K ATPase activity is much less at 20°C than at 37°C (Martin et al, 1972), so that cellular swelling may still occur at this temperature, although it is likely to be less marked than at 4°C. Furthermore, this effect will be augmented by depletion of cellular ATP, necessary for sodium pump activity, due to the anaerobic conditions prevalent during density gradient centrifugation. Finally, the permeability of pancreatic cells to
extracellular ions may also be increased due to the damaging effects of the isolation process, leading to a tendency for such cells to take up extracellular fluid. The composition of BSA may therefore be important, even at 22°C, and investigation of this possibility was the subject of the following study, in which the effect of glucose concentration upon islet purification using BSA was assessed.

THE EFFECT OF GLUCOSE CONCENTRATION ON PORCINE ISLET PURIFICATION USING BSA DENSITY GRADIENTS

As discussed above, one factor potentially contributing to the different results obtained with EF and BSA is the widely disparate concentration of glucose and permeable anions in these two media. In attempting to improve the results obtained with BSA, therefore, it was considered important to examine the effects of altering the concentrations of glucose and anions in this medium, even when used at 22°C. Previous work in this Department had indeed suggested that increasing the glucose concentration within BSA could improve the results achieved (London et al., 1992c), although the mechanism of this effect, and the optimal concentration required had not been investigated.

The glucose concentration within BSA density gradient solutions prepared by diluting stock BSA with MEM had previously been determined to be approximately 2 mmol/l (Contractor, personal communication). In this investigation, therefore, it was elected to study concentrations of 2 mM (present in BSA solutions currently in use); 5 mM (representing a more physiological concentration); 160 mM (the approximate concentration in EF); and 50 mM (as a suitable, arbitrary intermediate concentration).

Methods

Solutions of BSA at the above glucose concentrations were prepared by the addition of appropriate quantities of powdered glucose (D-Glucose, 95% a-anomer, G-8270, Sigma Chemical Co., Poole, Dorset, UK), weighed out on a sensitive balance (Sartorius 1712MP8 balance, Sartorius Instruments Ltd., Belmont, Surrey, UK), to stock BSA of osmolality 300 mOsm/kg H2O (for 2, 5 and 50 mM glucose-solutions) or 200 mOsm/kg H2O (for 160 mM glucose-BSA). Density adjustments were made in each case using MEM to which powdered glucose was added to attain the same glucose concentration as that of the BSA to be diluted, and all solutions were adjusted to the same final osmolality of 400 mOsm/kg H2O, using sterile sodium chloride.
The final glucose concentrations of high and low density BSA solutions were then determined using a commercial enzymatic glucose assay (GOD-Perid glucose assay kit, Boehringer-Mannheim, Mannheim, Germany). In this assay, 100 µl samples of BSA were initially de-proteinised by the addition of 1 ml of 5% TCA (trichloroacetic acid), followed by centrifugation at 2000 rpm for 12 minutes. From the resulting supernatants 40 µl aliquots were then taken, and to each of these aliquots and to 40 µl samples of a distilled water blank and a standard glucose solution (5.55 mg/ml), 1 ml of GOD-Perid buffer was added, and these solutions were then incubated for 30 minutes at 22°C. The absorbances at 610 nm of the samples and glucose standard were then measured in a spectrophotometer (Unicam SP1800, Pye Unicam Ltd., Cambridge, UK) against the distilled water blank, allowing the glucose concentrations of the original BSA solutions to be calculated.

This assay was firstly calibrated to measure glucose concentration within BSA solutions, as follows. A stock solution of 35% BSA containing 0 mM glucose was obtained (Advanced Protein Products Ltd.), and its density at 22°C (1.104 g/cm³) determined using a digital densitometer. Samples of 11.040 g (=10 ml) of this solution were then weighed out (Sartorius 1712MP8), to which powdered glucose was added to produce solutions at seven defined concentrations between 5 and 200 mM. At each concentration duplicate samples were produced, left overnight in order to ensure complete dissolution of the glucose, and assayed as above. In this way the accuracy of glucose concentrations determined by this assay when applied to BSA could be estimated. As demonstrated in Figure 4, the results suggested that assay data in fact consistently underestimated the concentration of glucose within BSA. However, the extent of this underestimation was approximately proportional to the absolute value of the actual glucose concentration, so that linear regression analysis could reasonably be used to determine the actual concentrations present in the above experimental BSA solutions from their assay results.

The ionic composition of the experimental BSA solutions was also determined by optical emission spectrometry, as for the stock solutions mentioned above, and the results are given in Table 3, along with the glucose concentrations estimated by the above method. The pH was between 7.00 and 7.05 for all solutions.

Islet purification using these BSA solutions was then studied using pancreatic digest from seven consecutive porcine pancreata, processed as above. Digest was washed and stored in MEM at 4°C immediately prior to density gradient separation, and gradients at each glucose concentration were constructed and centrifuged (500g for 5 minutes) simultaneously at 22°C before fractionation and assay as previously described.
FIGURE 4
Calibration of the GOD-PERID Glucose Assay for BSA

A. Bland-Altman Plot Demonstrating the Discrepancy Between Measured and Actual Glucose Concentration Over the Range 0-200 mM

B. Linear Regression Plot Demonstrating the Relationship Between Measured and Actual Glucose Concentration
<table>
<thead>
<tr>
<th>Glucose</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>K⁺</th>
<th>Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.27</td>
<td>1.82</td>
<td>2.3</td>
<td>0.5</td>
<td>1.53</td>
</tr>
<tr>
<td>0.44</td>
<td>1.55</td>
<td>2.3</td>
<td>0.5</td>
<td>1.53</td>
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<tr>
<td>0.27</td>
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<td>0.44</td>
<td>1.55</td>
<td>2.3</td>
<td>0.5</td>
<td>1.53</td>
</tr>
</tbody>
</table>

All values are in mM/L.

The composition of BSA solutions used to determine the influence of glucose concentration upon kcat.

**Table 3**
Results

Using standard BSA (400 mOsm/kg H₂O and 2 mM glucose), the median (range) values for exocrine tissue density, islet density and the % exocrine contamination at 60% islet yield were 1.092 (1.086-1.098) g/cm³; 1.091 (1.086-1.093) g/cm³; and 40% (12-59%) respectively.

The (paired) differences between these values and those obtained using BSA at 5, 50 and 160 mM glucose concentrations are summarised in Figures 5 and 6. At 5 mM glucose there were no significant differences observed in any of the three variables, compared to the corresponding values using 2 mM glucose-BSA (p>0.25).

At 50 mM glucose, significant increases in the densities of both exocrine tissue (p=0.022) and islets (p=0.036) resulted. The change in islet density (Median 7; 95% CI = 2 to 10 x 10⁻⁴ g/cm³) was, however, less than that of exocrine tissue (Median 9; 95% CI = 4 to 24 x 10⁻⁴ g/cm³), resulting in a small, but significant improvement in islet purity compared to that at 2 mM glucose (p=0.036).

At 160 mM glucose, exocrine tissue density was further increased, but the effect upon islet density, and therefore upon islet purity, was highly variable. In general, however, there was a trend towards reduced exocrine contamination at 160 mM than at 2 mM glucose (p=0.063).

Discussion

The results outlined above suggest that islet purification may be influenced by changes in the composition of a given gradient medium (BSA), even when the total osmolality and temperature of the medium are kept constant. As discussed above, this is most likely to be due to alterations in cellular volume, and therefore to depend principally upon the relative concentrations of impermeant and permeant molecules in the density gradient medium.

In the above study the addition of glucose to BSA solutions not only increased glucose concentration, but, because the total osmolality was kept constant, also resulted in a reduction in the concentrations of sodium and chloride ions, the reduction in each ion being, of course, approximately half the increase in glucose concentration. This is documented in Table 3, in which changes in sodium ion concentration represent the principal difference between the BSA solutions, the concentrations of the other major cations being similar, particularly at 2, 5 and 50 mM glucose.

Increased cell density with glucose concentration in BSA may therefore merely reflect a concomitant reduction in the concentration of permeable chloride anions, resulting in a
FIGURE 5

Changes in Tissue Density with BSA Glucose Concentration

- Exocrine Tissue
- Islets

Columns = 95% CI of Difference Between Paired Gradients
Horizontal Bars = Median Difference
FIGURE 6

Changes in % Amylase Contamination at 60% Insulin Yield with BSA Glucose Concentration

Columns = 95% CI of Difference Between Paired Gradients
Horizontal Bars = Median Difference

5 vs 2 mM Glucose
50 vs 2 mM Glucose
160 vs 2 mM Glucose
reduced tendency to cellular swelling under the conditions of hypoxia and mild hypothermia experienced during density gradient purification. Although it has previously been demonstrated that glucose is not an effective impermeant for pancreatic cells over 24 hours of cold storage (Wahlberg et al, 1986), the above results suggest that such cells are at least less permeable to glucose than to chloride, so that, in the short period during which they are exposed to the density gradient medium, cell swelling is reduced by replacing chloride with glucose.

The cause of the large variability in islet density using BSA at 160 mM glucose is also of interest. For 5/7 pancreata, gradients at each glucose concentration were assessed by visual examination of dithizone-stained fractions. In addition to confirming that islet purity was slightly improved at 50 mM glucose, these gradients revealed that fragmentation of pancreatic tissues, including islets, occurred to a variable degree using BSA at 160 mM glucose, whilst the morphology of these tissues was normal at the other concentrations studied. The 160 mM glucose-BSA solutions therefore appeared to be toxic to pancreatic tissues, this toxicity presumably being the cause of the enhanced variability in tissue density observed above.

The presumed detrimental effects of 160 mM glucose-BSA solutions may have been due to unmeasured contaminants present in the stock 200 mOsm/kg BSA from which they were produced, or due to their higher content of cations such as calcium, which may be an important mediator of tissue injury following exposure to ischaemia (McCord, 1985; Cheung et al, 1986). With respect to the latter explanation, however, it should be pointed out that the values given in Table 3 are the total calcium concentrations within each solution: albumin readily binds free calcium, so that the ionised calcium levels within all these solutions are likely to be much lower than those documented in this Table.

Alternatively, a direct toxic effect of glucose may be postulated, although a similar degree of fragmentation to that observed in this study has not usually been problematical when using density gradients of EF, which contain a similar glucose concentration. Whatever the cause, however, this toxicity appears to limit the benefit achieved by modifying existing BSA solutions using glucose.

CONCLUSIONS

The studies described in this Chapter have revealed a number of important points concerning the density gradient purification of islets. First, whilst on occasions highly purified islets can be obtained using currently available density gradient media, the general efficiency of purification is low, due
principally to the marked overlap in the density distributions of islets and exocrine tissue.

Second, the densities of exocrine tissue and islets may be differentially affected, and islet purification thereby improved, by manipulation of various physical and chemical conditions operational during density gradient centrifugation. In addition to the known influence of osmolality upon islet purity, the temperature and molecular composition of density gradient media appear to be of primary importance in this respect, presumably through their effects upon cellular volume.

Finally, modification of the composition of commercially-available BSA solutions has proven problematical. Even when stock solutions of well-defined osmolality are obtained, considerable variation may occur in their precise ionic make-up, which may have profound effects upon the results of islet purification. The use of BSA in the generation of linear continuous gradients at constant osmolality is, however, of undoubted benefit.

Fortunately, islet purity may also be influenced by the solutions in which the dispersed pancreas is suspended prior to density gradient centrifugation (van der Burg et al, 1990b). Potentially this provides an alternative means for establishing the optimal environment for pancreatic tissues, whilst retaining the advantages of continuous linear gradients of BSA in quantifying the efficiency of islet purification. Further investigation of this issue will therefore be the subject of the following chapters of this thesis.
CHAPTER 6

Storage of Pancreatic Digest Prior to Islet Purification: The Influence of Storage Period and Preservation Solution
Most investigations concerned with improving the density-dependent purification of islets have concentrated, not unnaturally, upon the various density gradient media employed. However, as discussed in the preceding chapters, the efficiency of islet purification using these media may also be profoundly influenced by variables arising during the many phases of islet isolation prior to density gradient centrifugation, and this may largely explain the considerable variation in results obtained with individual gradient media, as outlined in Chapter 5. Further improvement in islet purification will therefore depend upon addressing the influence of these variables.

One potentially important variable in this respect is the use of hypothermic storage of pancreatic tissues. Hypothermia is essential to the successful preservation of any organ exposed to an obligatory period of ischaemia (above a certain organ-specific limit), maintaining organ viability by reducing the rate of cellular metabolism which otherwise leads to cell damage in ischaemic tissues (Belzer et al, 1988). However, as previously discussed, hypothermia may also lead to cellular swelling, which may in turn contribute to changes in the density of cells and thereby influence density-dependent separation techniques. The use of appropriate cold storage solutions, which reduce this swelling during exposure to hypothermia, is therefore one means by which the detrimental effects of hypothermia may be reduced, and the results of islet purification improved.

In recent years much attention has focussed upon the use of the cold storage solution developed at the University of Wisconsin (UW solution) for organ preservation. This solution was originally designed to provide effective preservation of the liver and pancreas, which had previously been difficult to achieve using solutions effective at the time for preservation of kidneys (Belzer et al, 1988). Although other solutions, such as Collins and Hyperosmolar Citrate (Nolan et al, 1983) and a number of plasma-based solutions (Toledo-Pereyra et al, 1979a; Toledo-Pereyra et al, 1988; Abouna et al, 1988; Florack et al, 1989) had also allowed reasonably prolonged cold storage of the pancreas, use of UW solution allowed for the first time successful preservation of pancreata from experimental animals to be extended to 72 hours (Wahlberg et al, 1987). Following this initial success, UW was rapidly adopted into clinical practice for in situ vascular perfusion and subsequent cold storage not only of the pancreas and liver, but also of the kidney prior to transplantation (D'Alessandro et al, 1989; D'Alessandro et al, 1990a; D'Alessandro et al, 1990b). Since then, several direct comparisons between UW and other commonly used cold storage solutions have suggested that UW is at least equally, and often considerably more effective in preservation of the liver (Jamieson et al, 1988b; Badger et al, 1990; Cooper et al, 1990; Olthoff et al, 1990; Cofer et al, 1990), kidney (Ploeg, 1990; Moukarzel et al, 1990) and even the lung (Kawahara et al, 1993), particularly during prolonged cold storage.
Direct comparisons in preservation of the pancreas prior to transplantation as a vascularised graft suggest that, whilst the difference between solutions is less marked, UW again appears to represent the 'gold standard' against which other solutions can be compared (Barr et al, 1990; Leonhardt et al, 1993), although certain novel solutions containing combinations of histidine and lactobionate have more recently provided slightly superior preservation to that achieved with UW after 48 hours (Sumimoto et al, 1992).

Regarding cold storage of the pancreas prior to islet isolation, the situation is complicated by the adverse effects of in situ perfusion itself, discussed in Chapter 3, and by the paucity of studies directly comparing the influence of preservation solution upon islet purification. Those studies which have been performed, however, suggest that UW is at least more effective than solutions such as Euro-Collins in maintaining the yield of purified islets (Kneteman et al, 1990) or isolated $\beta$-cells (Korbett et al, 1992) during cold storage.

More recently, however, it has been reported that the use of UW solution for storage of the canine pancreas following collagenase-digestion and dispersal markedly improves the efficiency of subsequent islet purification on Dextran density gradients, compared to storage in tissue culture medium (van der Burg et al, 1990a; van der Burg et al, 1990b). Prior to these reports little attention had been paid to this phase of islet isolation, and most groups had stored the dispersed pancreas at $4^\circ$C in tissue culture media or other balanced salt solutions (eg. MEM in this Department), despite the fact that the ionic composition of such solutions is theoretically likely to contribute to cellular swelling under these conditions. Indeed, it had generally been accepted, although not proven, that prolongation of this phase of islet isolation resulted in a deterioration of islet yield, so that islet purification was usually performed as soon as possible after pancreatic digestion. Unfortunately, even when using large-scale discontinuous density gradients on the COBE cell separator, only 15-20 ml of digest may be effectively purified at any one time, whilst dispersal of the human pancreas may result in 60-70 ml of digest. Thus, part of the pancreatic digest may require storage for up to 1 hour, if more than one gradient needs to be constructed.

The aim of the work described in this chapter was therefore to investigate the changes occurring in the densities of porcine pancreatic tissues during this storage period in both MEM and UW solutions, and the influence of these changes upon the subsequent purification of islets on density gradients of BSA.
Methods

Ten consecutive porcine pancreata were obtained and collagenase-digested, as previously described. Following pancreatic digestion, the dispersed pancreatic tissues were collected concurrently into either MEM (Minimum Essential Medium with Hanks' salts, Northumbria Biologicals Ltd., Cramlington, Northumberland, UK) with 10% New-Born Calf Serum (NBCS) or into UW (Viaspan Belzer UW solution, Du Pont Critical Care Inc., Waukegan IL, USA) +10% NBCS at 4°C. Once collection was complete, the tissue in each sample was then washed twice in MEM or UW (without NBCS), as appropriate, and centrifuged at 100g for 2 minutes at 4°C. From each resulting pellet of tissue 100 µl aliquots were then taken for immediate separation on linear continuous density gradients of BSA. The remaining tissue was then resuspended in the relevant storage solution and kept at 4°C for 1 hour, following which further aliquots of 100 µl of pelleted tissue were taken from each sample, for density gradient separation, as before (Figure 1).

The UW solution was used, as in the study of van der Burg and colleagues, without addition of insulin, dexamethasone or antibiotics. Solutions of BSA were made up as described in Chapter 4, without addition of glucose (final glucose concentration 2 mM). The density range of BSA used was 1.074-1.103 g/cm³, and the osmolality was 400 mOsm/kg H₂O throughout.

All gradients were constructed and centrifuged (500g for 5 minutes) at 22°C, then fractionated and analysed as previously described, statistical comparison between storage conditions being performed using the Wilcoxon signed-rank test for paired data.

Results

The median (range) densities of exocrine tissue and islets in BSA gradients run immediately after washing the digest in MEM (samples MEM, t=0) were 1.094 (1.090-1.097) g/cm³ and 1.091 (1.085-1.095) g/cm³ respectively, resulting in an exocrine contamination at 60% islet yield of 39% (5-64%); results which are comparable to those obtained following MEM wash and using similar BSA gradients in the studies of Chapter 5.

The (paired) differences between these values and those obtained by washing the digest in UW (UW, t=0), and following 1 hour storage in MEM (MEM, t=60) or UW (UW, t=60) are summarised in Figures 2 and 3.
FIGURE 1
Protocol for Comparison of UW and MEM for Storage of Pancreatic Digest Prior to Islet Purification

AUTOMATED DIGESTION

DISPERSED PANCREATIC TISSUES

WASH MEM

Sample MEM, t=0

Store MEM 4°C 60 Mins

Sample MEM, t=60

WASH UW

Sample UW, t=0

BSA DENSITY GRADIENT

Sample UW, t=60

Store UW 4°C 60 Mins
FIGURE 2

Changes in Tissue Density with Storage in MEM or UW

- Exocrine Tissue
- Islets

Columns = 95% CI of Difference Between Paired Gradients
Horizontal Bars = Median Difference

UW, t=0 vs MEM, t=0
MEM, t=60 vs MEM, t=0
UW, t=60 vs UW, t=0
FIGURE 3

Changes in % Amylase Contamination at 60% Insulin Yield with Storage in MEM or UW

Columns = 95% CI of Difference Between Paired Gradients
Horizontal Bars = Median Difference
It is clear that simply washing the pancreatic digest in UW, rather than MEM, caused a
marked increase in the buoyant density of exocrine tissue on subsequent BSA density
gradients (\(p=0.009\)). The density of islets was not, however, influenced to the same
extent by washing in UW, so that no significant differences were observed, compared
to washing in MEM (\(p=0.76\)). Therefore, the overlap in the density distributions of
exocrine tissue and islets was reduced by washing in UW, resulting in a profound
improvement in islet purity (Median = 14%, 95% CI = 6 to 31% reduction in amylase
contamination, \(p=0.011\)).

Following 1 hour storage in MEM the densities of both exocrine tissue and islets on
BSA density gradients were significantly reduced, compared to the values obtained
from gradients run immediately after washing in MEM (\(p=0.014\)). However, the
change in density for each tissue was similar, so that islet purity was not significantly
influenced by 1 hour storage in MEM (95% CI = -6 to 2% change in amylase
contamination, \(p=0.529\)).

During 1 hour storage in UW the densities of both exocrine tissue and islets continued
to increase, although the change in density tended to be greater for islets than for
exocrine tissue, so that no further improvement in islet purity was observed, compared
to simply washing the digest in UW (\(p=0.386\)).

Overall, therefore, the most efficient separation of islets from exocrine tissue was
achieved during both storage periods when digest was suspended in UW (Figure 4).

Discussion

This study has confirmed the importance of the storage period between pancreatic
collagenase digestion and islet purification, and shown that the densities of islets and
exocrine tissue, and therefore the efficiency of subsequent islet purification, are
dependent upon the length of this period and the medium in which they are stored.
Storage of the dispersed pancreas in UW, rather than MEM, brought about a marked
improvement in islet purity, by differentially increasing the density of exocrine tissue
compared to that of islets. Indeed, much of this improvement was evident after simply
collecting and washing the digest in UW. The magnitude of this effect in terms of the
final purity of an islet preparation is considerable, and readily appreciated when it is
considered that the vast majority (over 95%) of the pancreas is composed of exocrine
tissue: a reduction in median exocrine contamination of 25% by storage in UW
therefore represents an almost four-fold improvement in the purity of a given islet yield.
The most likely explanation for these changes is that pancreatic tissues undergo cellular
swelling during retrieval, cold storage and collagenase digestion of the pancreas, and
FIGURE 4

%Amylase Contamination at 60% Insulin Yield with Storage in MEM or UW

Values are Median + Upper Limit of 95% CI
Comparing UW with MEM: p = 0.011 at t=0; p = 0.030 at t=60
that this swelling is reversed by washing and storage of the dispersed tissues in UW. As previously discussed, exocrine cells may be more susceptible than islets to swelling during previous phases of the isolation procedure, whilst given changes in their cell volume may result in larger changes in density than an equivalent volume change in islet cells. The beneficial effects of UW on cellular volume may therefore be more pronounced for acinar cells, resulting in a differential effect upon the densities of exocrine tissue and islets.

During cold storage in MEM for 1 hour, the densities of both acinar tissue and islets were reduced, presumably due to further swelling of these tissues. Interestingly, however, the density changes in this situation were similar for both types of tissue, so that no effect upon islet purity was observed.

In contrast, during storage in UW the densities of acinar tissue and islets were not only maintained, but continued to increase, presumably due to the continued reversal of pre-existing cell swelling, and perhaps also to shrinkage of cells below their original physiological volume (Mees et al, 1982). Islet density appeared to increase to a greater extent than that of exocrine tissue, although it must be pointed out that after even brief exposure to UW the majority of exocrine tissue was often found within the lowermost fraction (or 'pellet') of the BSA gradients, implying that the density of much of this tissue was greater than the maximum density of BSA used. Thus, because this tissue may have been prevented from reaching its true isopycnic point (by reaching the bottom of the tube), the values of exocrine tissue density after washing the pancreatic digest in UW may have been substantially underestimated, and of course further increases with storage in UW could not have been detected. Similarly, any beneficial influence of an additional period of storage in UW upon islet purity may also have been underestimated.

These results, obtained with porcine pancreata, are consistent with those observed in dogs by van der Burg and colleagues, in which the purity of islets obtained on Dextran density gradients was improved from 31% to 91% by prior storage of digest in UW rather than RPMI, accompanied by an increased density of exocrine tissue and islets (van der Burg et al, 1990b). They are also consistent with results obtained in this Department using pancreata retrieved from human organ donors following in situ perfusion with HOC and cold storage for <3 hours (Robertson et al, 1992). The changes in tissue densities and islet purity with storage in MEM or UW for such pancreata were almost identical to those observed in the present investigation, but interestingly the purity of human islets was further improved by exposure to UW for 1 hour, compared with immediate separation. Indeed, it was found that, on some occasions when human islets could not be adequately purified immediately, storage for 1 hour in UW usually permitted successful islet separation.
The apparently different time course of improvement in islet purity between porcine and human pancreata using UW, however, probably only represents differences in experimental design between these studies. Thus, in the present investigation dispersed pancreatic tissues were collected into MEM or UW during ongoing automated digestion, and were therefore exposed to these media for 20 minutes or more prior to the initial BSA gradients (at time 0) being run, whereas in the above-mentioned study on human islet purification tissues were originally collected in MEM, and only briefly exposed to UW prior to the initial gradient separation. In general, therefore, it appears that the beneficial effects of UW storage of pancreatic digest are very similar between species.

In addition to the potential for improving islet purification, the findings of this study have further implications. Firstly, it is clear that, because the densities of pancreatic tissues may change markedly during relatively short periods of storage in various media, the use of predetermined densities for the layers of large-scale discontinuous gradients is inappropriate; and that, even when the densities of these layers have been determined from small-scale test gradients, the optimal density for separation may change if there is any delay in density gradient centrifugation of tissue. Such large-scale discontinuous gradients should therefore be run as soon as possible after running any test gradients, irrespective of the medium used for storage of the pancreatic digest. Secondly, because viability may be compromised by cellular swelling, it is likely that the reversal of this process by storage of digest in UW will better maintain, and may even improve, islet viability. This issue was not directly assessed in the present study, although in the study of van der Burg et al. normal islet function was maintained both in vitro and in vivo following UW storage and Dextran gradient separation (van der Burg et al, 1990a; van der Burg et al, 1991a).

Finally, it is also clear that the period of tissue storage between pancreatic digestion and islet purification represents a unique opportunity to study the factors influencing the relative densities of islets and exocrine tissue, because during this period tissues are suspended in a relatively large volume of media, the composition of which can be more readily manipulated than that of many gradient media (particularly BSA). Furthermore, the changes occurring within this storage period appear to persist during separation on gradients of BSA, at least for sufficient time for such gradients to be used in their measurement.

As discussed in Chapter 5, however, cellular swelling may occur during density gradient separation using BSA, so that some of the beneficial effects of UW may have been reversed during islet purification with this medium. It is possible, therefore, that once the conditions most appropriate for storage of the dispersed pancreas are elucidated, the results of islet purification may then be further improved by the design

180
of new density gradient media, with compositions based upon the optimal storage solution. In order to achieve this goal, the mechanisms by which UW influences pancreatic tissue densities and islet purity must firstly be examined in more detail, and this is the purpose of the investigations outlined in the following chapter.
CHAPTER 7

Storage of Pancreatic Digest Prior to Islet Purification: The Mechanisms of Action of UW Solution
In Chapter 6 the marked improvements in porcine islet purification resulting from storage of the pancreatic digest in UW, rather than in MEM, were documented and compared with similar effects previously observed using UW to improve islet purification from the pancreata of other species. The mechanism of this effect is undoubtedly related to differences in the composition of these two media. However, simple comparison between them provides little information concerning the components of UW most likely to contribute to its beneficial effects, as these media differ with respect to every constituent present (Table 1). Therefore, in order to investigate further the manner in which UW improves islet purity it is necessary to reconsider the theoretical and experimentally documented advantages of each of its separate components, and the potential mechanisms by which storage solutions might influence pancreatic tissue densities and thereby affect islet purification.

The development of UW was based largely upon theoretical requirements for the maintenance of tissue viability during cold storage (Belzer et al, 1988), and, as described in Chapter 6, this solution has proven highly effective in the prolonged hypothermic preservation of many organs. Since its introduction, however, a number of studies have demonstrated that several of its constituents, despite their theoretical advantages, can, in fact, be omitted without altering the efficacy of the preservation solution. It is therefore important to consider which of these constituents have proven essential, and which have been dispensable, for effective tissue preservation in these experimental studies.

Impermeant Molecules
As described in Chapter 3, the damaging effects of hypothermic ischaemia are largely mediated by the induction of cellular swelling, due to inhibition of the cell membrane ATPase-dependent sodium pump in the presence of extracellular anions to which the cell membrane is highly permeable. The replacement of these anions by impermeant molecules might therefore be expected to contribute considerably to the efficacy of cold storage solutions, and this was the reason for the inclusion of lactobionate and raffinose in the original formulation of UW solution.

The swelling of tissue slices during hypothermic incubation in vitro is certainly highly dependent upon the presence of such molecules in the incubation medium (Southard et al, 1980; Wahlberg et al, 1986). With canine kidney slices, the prevention of cellular swelling is proportional to the ratio of lactobionate:chloride in the cold storage solution, swelling being maximal at a ratio of 0:140, and prevented by ratios of 80:60 or greater (Southard et al, 1980). With slices of canine pancreas, prevention of tissue oedema is also dependent upon the replacement of chloride anions by impermeant molecules, the
TABLE 1

The Composition of MEM and UW used for Storage of Porcine Pancreatic Digest

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>MEM</th>
<th>UW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>138</td>
<td>20</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.8</td>
<td>140</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>145</td>
<td>-</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>0.4</td>
<td>25.0</td>
</tr>
<tr>
<td>HPO₄²⁻</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactobionate⁻</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Glutathione</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>HES (g/l)</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vitamins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>7.35</td>
<td>7.53</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg H₂O)</td>
<td>300</td>
<td>315</td>
</tr>
</tbody>
</table>

Values are mmol/l, unless otherwise stated.

Compositions are based upon Data Sheet Information supplied by Northumbria Biologicals Ltd. (MEM) and by Du Pont Pharmaceuticals (UW). Osmolality measurements were made using a vapour pressure osmometer (Wescor), and pH measurements were made at 4°C with a pH probe (Radiometer pHM62).
most effective anions being those of highest molecular weight, so that lactobionate is, for example, more effective in this respect than gluconate (Wahlberg et al., 1986). In view of these observations, it is not surprising that the presence of impermeant anions within UW-based cold storage solutions has also been found essential in the preservation of whole organs. Thus, preservation of rat kidneys for up to 48 hours prior to transplantation is only successful in such solutions if chloride anions are replaced by anions of larger molecular weight: lactobionate is highly effective for this purpose, although gluconate and citrate have similar efficacy (Marshall et al., 1990; de Mel et al., 1990). Similar considerations also apply to preservation of the rat and rabbit liver, although in these cases lactobionate appears slightly more effective than gluconate (Jamieson et al., 1988a; Yu et al., 1990; Sumimoto et al., 1990a; Sumimoto et al., 1990b). In the tissue slice experiments mentioned above cellular oedema was also reduced by inclusion of various saccharides, acting as uncharged impermeant molecules, in the cold storage solution, and again the beneficial influence of these molecules appeared to be proportional to their molecular weight: raffinose (M₄ 594) was more effective than sucrose (M₄ 348), which was in turn more effective than mannitol (M₄ 180). The effects of saccharides such as raffinose in whole organ preservation are somewhat variable, however. In the rat kidney cellular swelling is slightly increased if raffinose is replaced by mannitol (de Mel et al., 1990), and initial function after transplantation is impaired if raffinose is substituted by sucrose or chloride, but not if replaced by extra lactobionate (Marshall et al., 1990). Similarly, in the isolated perfused rabbit liver model, hepatocyte function following 48 hour cold storage is reduced when raffinose in the storage solution is replaced by mannitol or sucrose (Jamieson et al., 1988a). In contrast, however, survival following cold storage and transplantation of the rat pancreas appears to be unaffected by omission of raffinose and its replacement by glucose or even by chloride (Sumimoto et al., 1990a; Yu et al., 1990); in addition, the function of rat pancreata cold-stored for 48 hours before transplantation is not impaired if raffinose is substituted by glucose (Urushihara et al., 1992). The additional benefit resulting from the use of such impermeant saccharides therefore appears to be limited, so long as the cold storage solution contains other impermeants and therefore few permeable anions.

**Colloids/Hydroxyethyl Starch**

The inclusion of the colloid Hydroxyethylstarch (HES) in UW solution was designed to prevent the development of interstitial oedema, resulting from hydrostatic pressure exerted across capillary walls during continuous perfusion or initial vascular flush of the graft prior to simple cold storage. The putative advantages of preventing this oedema were that distribution of the storage solution and the flush-out of blood from
the organ would be more effective. The importance of this phenomenon in influencing post-transplantation function of organs is not fully established, however, whilst the tendency to develop interstitial oedema in this way may also vary greatly between different organs.

Thus, in kidney preservation omission of HES is associated with an initial increase in graft oedema (Wahlberg et al, 1989; Schlumpf et al, 1991), although organ function after transplantation appears to be unaffected (Biguzas et al, 1990b; Schlumpf et al, 1991). Similarly, inclusion of a colloid in the preservation solution reduces the degree of interstitial oedema produced by flushing and cold storage of the whole liver (Ar'Rajab et al, 1991); however, colloids appear not to influence the subsequent development of cellular swelling in liver slices (Ar'Rajab et al, 1991), and their omission from cold storage solutions has not generally influenced liver function, as assessed following transplantation (Sumimoto et al, 1989; Howden et al, 1990; Yu et al, 1990) or using the isolated perfused liver model (Jamieson et al, 1988a; Ar'Rajab et al, 1991), although in one study liver function was reduced slightly after prolonged preservation using a UW-like solution lacking HES (Adam et al, 1990).

The role of colloids in preservation of the pancreas has been somewhat more controversial. Initial studies using simple flush and cold storage techniques, without continuous perfusion, suggested that optimal preservation required the presence of a colloid (Toledo-Pereyra et al, 1979a; Toledo-Pereyra et al, 1988; Abouna et al, 1988; Florack et al, 1989), the beneficial effects of which were again mediated by the prevention of interstitial oedema during initial vascular flushing of the organ (Ruka et al, 1989). However, the solutions used in these studies were generally based upon plasma, and contained few other impermeant molecules. In more recent studies using UW-like solutions the influence of colloids such as HES has been much less marked (Urushihara et al, 1992; Griffin et al, 1993), suggesting that they are of lesser importance when used in solutions which are otherwise well adapted for cold storage of organs. Even in these solutions, however, interstitial oedema during initial vascular flushing of the pancreas is reduced when HES is included, and one group of investigators have demonstrated improved graft function as a result (Ploeg et al, 1992).

Glutathione/Allopurinol

Glutathione and allopurinol were originally included in UW solution in order to reduce the potentially harmful effects of oxygen-derived free radical formation, which is liable to occur in cells reoxygenated following a period of ischaemia.

Even in normally metabolising cells such radicals are spontaneously produced, by 'leakage' of electrons from the mitochondrial cytochrome oxidase system and their interaction with molecular oxygen (Fuller et al, 1988b) (Figure 1). Usually, however,
FIGURE 1
Oxygen Free Radical Generation and Degradation in Normal Cells and in Cells Following Ischaemia/Reperfusion

Normal Cell

Mitochondrial Electron Transport Chain

\[ \begin{align*}
O_2 & \rightarrow O_2^+ \\
& \rightarrow H_2O_2 \\
& \rightarrow H_2O
\end{align*} \]

Superoxide Dismutase
GSH Glutathione Peroxidase
GSSG

Following Ischaemia/Reperfusion

Mitochondrial Electron Transport Chain

\[ \begin{align*}
O_2 & \rightarrow O_2^+ \\
& \rightarrow H_2O_2 \\
& \rightarrow H_2O
\end{align*} \]

Catalase

Hypoxanthine Xanthine Oxidase Xanthine

ADP ATP Xanthine Dehydrogenase

\[ \begin{align*}
Fe^{2+/3+}/Mn^{2+/3+} & \rightarrow OH^-
\end{align*} \]

Free Radical Chain Reactions With Membrane Phospholipids
the superoxide radicals so produced are rapidly degraded: firstly by superoxide dismutase to produce hydrogen peroxide, and then by reduction of this otherwise toxic molecule to water by various peroxidases, one of the most important of which uses reduced glutathione (GSH) as a substrate. The presence within cells of adequate supplies of antioxidants such as reduced glutathione is therefore potentially of great importance.

During ischaemia and cold storage of cells a number of changes occur which may disturb this delicate balance once oxygen is reintroduced to the system (McCord, 1985; Fuller et al, 1988b). Firstly, the cellular enzyme xanthine dehydrogenase is converted to a form (xanthine oxidase) which is capable of directly reducing molecular oxygen (rather than the usual substrate, nicotinamide adenine dinucleotide, NAD+) during the conversion of hypoxanthine to xanthine or of xanthine to uric acid. This results, therefore, in an increased production of superoxide radicals, which may also be stimulated by the increased availability of hypoxanthine, resulting from the depletion of ATP during ischaemia. Secondly, during cold storage cellular glutathione levels are rapidly depleted (Vreugdenhil et al, 1990), so that complete degradation of toxic oxygen-derived metabolites does not occur: instead, hydrogen peroxide may accumulate and react with transition metals such as iron, liberating the hydroxyl radical. This radical is one of the most reactive species known, and can rapidly initiate free radical chain reactions involving cell membrane phospholipids, leading to cellular damage.

Thus, the inclusion of the xanthine oxidase inhibitor, allopurinol, in UW was intended to reduce this damage by inhibition of the initial generation of superoxide radicals, whilst glutathione was included to stimulate the regeneration of intracellular reduced glutathione levels, thereby accelerating the degradation of hydrogen peroxide. Unfortunately, reduced glutathione in commercially available bags of UW solution is itself unstable, and is rapidly converted to its oxidised form (Astier et al, 1989; Postaire et al, 1991). It is known, however, that extracellular glutathione is degraded into compounds which are taken up by cells and used in the resynthesis of intracellular reduced glutathione (Puri et al, 1983). The oxidation state of glutathione in the storage solution may not therefore be important for mediation of its proposed action.

The presence of glutathione in the preservation solution certainly appears protective to isolated rabbit renal tubule cells during 48 hour cold storage (Southard et al, 1990). Interestingly, the mechanism of this effect is due largely to the degradation of glutathione to glycine, which itself has a direct protective influence upon hypoxic cells, independent of the regeneration of intracellular glutathione levels (Weinberg et al, 1987; Weinberg et al, 1989; Marsh et al, 1991). It is possible, therefore, that the beneficial
effects of glutathione are in fact unrelated to the influence of this agent upon free radical formation as outlined above.

In general, however, the beneficial effects of glutathione upon preservation of whole organs have been modest, and have usually been observed only after prolonged cold storage. Thus, in one study, initial post-transplantation function of dog kidneys following 48 hour cold storage was improved if glutathione was present in the preservation solution, although after 14 days renal function was similar to that of kidneys stored without glutathione (Southard et al, 1990). Also, during 48 hour storage of rat kidneys, glutathione alone did not appear important (Wahlberg et al, 1989), although a modest synergistic effect with adenosine was observed in another investigation (Biguzas et al, 1990a).

In liver preservation for periods of up to 48 hours glutathione has been universally beneficial (Jamieson et al, 1988a; Yu et al, 1990). Interestingly, however, one group has found that for cold storage of canine and human livers for longer than 20-24 hours, the reduced form of glutathione is more effective than the oxidised form (Boudjema et al, 1990; Boudjema et al, 1991), suggesting that the mechanism of action of this agent may vary between different organs. Indeed, this may explain why preservation of the rat pancreas, even for 48 hours, appears to be unaffected by the omission of glutathione (Urushihara et al, 1992).

The potentially beneficial effects of allopurinol upon kidney preservation were first suggested many years ago (Owens et al, 1974; Toledo-Pereyra et al, 1974), although in these initial investigations allopurinol was used also for pretreatment of the donor, whilst the solutions used for cold storage of the transplanted organs were otherwise poorly adapted to prolonged hypothermic storage. In contrast, preservation of the kidney, liver and pancreas in UW-like solutions does not appear to be influenced by the presence or absence of allopurinol (Jamieson et al, 1988a; Wahlberg et al, 1989; Sumimoto et al, 1989; Biguzas et al, 1990a; Yu et al, 1990; Urushihara et al, 1992).

Adenosine

The inclusion of adenosine in UW solution was intended to stimulate the regeneration of ATP (adenosine triphosphate) upon organ reperfusion, in order to restore the energy supply of stored cells, necessary for restitution of normal metabolism.

In isolated rabbit renal tubules and rat hepatocytes, for instance, ATP synthesis can be stimulated by the presence of adenosine in the cold storage solution, resulting in a concomitant improvement in cellular viability assessed in vitro, particularly if glutathione is also present (Southard et al, 1990). Similarly, ATP synthesis by dog kidneys following prolonged periods of continuous hypothermic perfusion is enhanced.
if adenosine is included in the perfusate, and this is associated with an improvement in some aspects of renal function upon normothermic reperfusion (Southard et al, 1985). For simple cold storage of whole organs before transplantation, however, the role of adenosine appears to be modest. In one investigation, addition of adenosine appeared to be of some benefit for 48 hour preservation of the rat kidney, although its most significant effects were only observed when glutathione was also present (Biguzas et al, 1990a). In the majority of studies, however, the omission of adenosine has been without effect upon preservation of the kidney, liver or pancreas (Jamieson et al, 1988; Wahlberg et al, 1989; Sumimoto et al, 1989; Yu et al, 1990; Urushihara et al, 1992).

Other Components

The theoretical advantages of magnesium sulphate, dexamethasone, insulin and antibiotics were less clearly defined in the initial description of the principles underlying the design of UW solution. However, magnesium has been postulated to act as a metabolic inhibitor (Kamiyama et al, 1970) and may also be involved in the reduction of hypothermic cellular swelling (Downes et al, 1973); indeed, its inclusion in Collins solution has been claimed to be the most important feature of this cold storage medium (Collins et al, 1972). Furthermore, dexamethasone is known to stabilise cell and lysosomal membranes (Bowman et al, 1980), although the importance of this action in protecting cells from cold-induced injury is unknown.

Few studies have directly addressed the role of magnesium in UW solution, the majority of investigators apparently feeling intuitively that this agent is an essential component. In certain studies, however, its omission has not been detrimental, at least for liver preservation (Yu et al, 1990; Ar'Rajab et al, 1992).

Dexamethasone, insulin and antibiotics have also been found unimportant in most studies (Jamieson et al, 1988a; Sumimoto et al, 1989; Biguzas et al, 1990a), and in fact one investigation has even suggested that the omission of insulin from UW may be beneficial (Yu et al, 1990).

Finally, the use of a high potassium:sodium ratio in UW might be expected to reduce the exchange of intracellular potassium for extracellular sodium during cold storage, although, as explained in Chapter 3, cellular swelling should be unaffected. In general, however, the cation ratio does not seem to influence the efficacy of UW-like solutions to a great extent (Biguzas et al, 1990b; Urushihara et al, 1992; Griffin et al, 1993), and in fact the use of a high sodium, low potassium solution may be of greater benefit (Sumimoto et al, 1989; Moen et al, 1989).
Summary

Although based upon sound theoretical principles, the inclusion of many of the above-mentioned components in UW has not universally been found necessary in preserving organs prior to transplantation. Indeed, simplified solutions, from which several of these components have been omitted, have been used in the cold storage of organs in clinical transplantation programmes, with equivalent success to that achieved with 'complete' UW solution (Jamieson et al, 1990; Baatard et al, 1993).

Whilst organ- and species-dependent differences in the requirements for various components may exist, in general only the presence of a suitable impermeant anion has been found consistently essential for simple hypothermic storage of organs prior to transplantation, although for storage periods greater than 24-48 hours agents such as glutathione may also be important.

The effects of UW storage of pancreatic digest on islet purification are mediated by differential changes in the densities of islets and exocrine tissue, the density of acinar cells in particular being markedly increased following storage. As outlined in Chapter 3, these density changes may, in turn, be mediated either by alterations in the volume of pancreatic cells, or by changes in the rate of release of high density cellular contents, e.g. the discharge of exocrine enzymes from acinar cells.

As previously suggested, alteration in cellular volume is the most likely mechanism for the changes in density observed in the studies described in Chapter 6, particularly as the density of exocrine tissue was greater after 1 hour storage in UW than after washing in this medium: thus, although a reduction in exocrine enzyme discharge in UW, compared to MEM, might explain the initial improvement in islet purity observed in UW, the irreversible nature of exocrine enzyme discharge from cells already washed in UW implies that further increase in the density of such cells must be due to other factors. Also, if the control of cellular volume is indeed the principal mechanism by which UW storage of pancreatic digest improves islet purification, it is then even more likely that the impermeant molecules are the most important components responsible, and that a simplified solution with several of the other above-mentioned components omitted might be equally efficacious.

The aim of the experiments described below was therefore firstly to determine the relative contributions of cellular volume changes and exocrine enzyme discharge to the observed density changes during storage of digest; and secondly to examine the contribution of the various components of UW, and in particular its anionic composition, to its beneficial effects upon islet purification.
Methods

Five storage solutions (A-E), similar in composition to UW, were produced, containing the concentrations of lactobionate, raffinose and ions outlined in Table 2. All solutions were produced by addition of the appropriate reagents to double-distilled water, analysis of which had demonstrated suitably low levels of contaminating ions (ARL3580 ICP OES, Fisons Scientific Equipment Division, Loughborough: concentrations < 1 x 10⁻⁵ mM for sodium, potassium, calcium, magnesium, and numerous other metal ions, including copper, iron, manganese, lead and zinc). Insulin, dexamethasone and antibiotics were omitted from all solutions (including UW), as these components had not been present in the UW solutions used in the experiments of Chapter 6, and were not expected to contribute to improved islet purity. In addition, solutions A-E did not contain hydroxyethyl starch, as it was felt unlikely that this component would be important in the storage of dispersed tissue, where the problems of hydrostatic forces acting across a vascular endothelial barrier do not apply. Glutathione was also omitted because, as discussed above, this agent has only been consistently important for prolonged cold storage (24-48 hours) of organs, whilst the benefits of UW on islet purity were observed during very brief storage of the dispersed pancreas. Finally, adenosine and allopurinol were also excluded from these solutions as they have generally not been found necessary, even during relatively prolonged hypothermic preservation of organs. In order to limit the number of variables studied, however, phosphate buffers and magnesium sulphate were included, somewhat empirically, in all solutions.

For each solution lactobionate was added as lactobionic acid (Cat. No. 15,351-6, Aldrich, Gillingham, Dorset, UK), and the pH was subsequently adjusted by addition of sodium and potassium hydroxide in proportions appropriate to maintain the Na⁺:K⁺ ratio at approximately 20:140, as in UW solution (Viaspan™ Data Sheet, Du Pont Pharmaceuticals). Raffinose was added as D(+)-raffinose, obtained from Sigma Chemical Co. Ltd., Poole, UK (Cat. No. R-7630). Finally, all solutions were filter-sterilised (0.22 μm bottle-top filter, Falcon 7105, Becton Dickinson Labware, New Jersey, USA) to facilitate storage before use.

Solutions A-D were designed to differ from each other only in their anionic composition, by altering the ratio of lactobionate:chloride ions from 0:100%, as in MEM, up to 100:0%, as in UW. Furthermore, in solution E raffinose was omitted, and replaced by extra lactobionate.

For the storage of pancreatic digest, seven solutions were then compared, namely MEM, solutions A-E, and UW. All solutions were adjusted to the same final osmolality (± (range) 5 mOsm/kg H₂O, measured using a vapour pressure osmometer (Wescor...
Values are given in mmoL, unless otherwise stated.

<table>
<thead>
<tr>
<th></th>
<th>Solution A</th>
<th>Solution B</th>
<th>Solution C</th>
<th>Solution D</th>
<th>Solution E</th>
<th>Mm</th>
<th>Mem</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES (g/l)</td>
<td>56 5.6</td>
<td>56 5.6</td>
<td>56 5.6</td>
<td>56 5.6</td>
<td>56 5.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cae+</td>
<td>1.26 1.26</td>
<td>1.26 1.26</td>
<td>1.26 1.26</td>
<td>1.26 1.26</td>
<td>1.26 1.26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.78 0.78</td>
<td>0.78 0.78</td>
<td>0.78 0.78</td>
<td>0.78 0.78</td>
<td>0.78 0.78</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MgSO4</td>
<td>0.81 0.81</td>
<td>0.81 0.81</td>
<td>0.81 0.81</td>
<td>0.81 0.81</td>
<td>0.81 0.81</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Raffinose</td>
<td>1.45 1.45</td>
<td>1.45 1.45</td>
<td>1.45 1.45</td>
<td>1.45 1.45</td>
<td>1.45 1.45</td>
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<tr>
<td>K+</td>
<td>145 145</td>
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<td>145 145</td>
<td>145 145</td>
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<td>Net+</td>
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<td>MEM</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The composition of Storage Solutions A-E, compared with MEM and UW.

Table 2
using sodium chloride for MEM, and appropriately increased concentrations of lactobionate/chloride for solutions A-E. In addition, all solutions were corrected to the same final pH (at 4°C, ± (range) 0.04 pH units, measured using a pH meter (Radiometer pHM62)).

Ten porcine pancreata were collagenase-digested, as previously described, and the dispersed pancreatic tissues were pooled and washed in MEM at 4°C. The pooled digest was then divided equally between the seven storage solutions, and each sample of tissue was washed twice by resuspension in its respective solution after centrifugation at 200g for 2 minutes at 4°C. Following the final centrifugation, 1 ml aliquots of pelleted tissue were then taken from each sample for the studies on changes in tissue volume/exocrine enzyme discharge, as detailed below. The remaining tissue in each sample was then resuspended in its appropriate storage solution, and stored at 4°C for 10 minutes, prior to density gradient separation (Figure 2).

Density gradients of BSA were constructed and analysed as described in the previous chapters: the density range used was 1.074-1.103 g/cm³; the osmolality was 400 mOsm/kg H₂O throughout; and gradients were run at 500g for 5 minutes at 22°C.

**Tissue Volume/Enzyme Discharge Study**

In order to measure the changes in pancreatic tissue volume produced during storage in the study solutions, modified 'capillary tubes' were produced from glass Pasteur pipettes, by heat-sealing one end and subsequently calibrating the resulting narrow tube using known volumes of water (Plate 7.1). In this way, changes of volume within these tubes could be measured, in increments of 50 μl.

The 1 ml aliquots of tissue taken from each storage solution were then placed into seven of these capillary tubes, and 1 ml of the appropriate storage solution was added to each tube. The tubes were then simultaneously centrifuged at 200g for 3 minutes, and the volume of pelleted tissue in each was recorded (Vol, t=0). This tissue was then resuspended in the supernatant solution, and the tubes stored at 4°C. Tissue volumes were then recorded in an identical manner both 10 and 60 minutes later (Vol, t=10 and t=60).

Finally, following 60 minutes storage, 100 μl aliquots of supernatant were taken from each capillary tube, diluted 1:10 in MEM, and stored for subsequent amylase assay (Phadebas test kit- see Appendix). In addition, 100 μl aliquots of the pelleted tissue from each tube were taken into 1900 μl of MEM, sonicated for 20 seconds, and further diluted 1:10 in MEM, again prior to amylase assay.

These data were then used to calculate the percentage change in the initial volume of the tissue pellets occurring during storage in each of the solutions studied, and the percentage of the total amylase content of each capillary tube which had been released.
FIGURE 2
Protocol for Studying the Effects of Storage Solutions A-E

Dispersed Pancreatic Tissues

Storage Solutions

MEM  A  B  C  D  E  UW

1 ml Pelleted Tissue + 1 ml Storage Solution
200g, 3 minutes

Store 4°C 10 Minutes

BSA Density Gradients

Vol, t=0
Resuspend, Store 10 minutes, Re centrifuge

Vol, t=10
Resuspend, Store 50 minutes, Re centrifuge

Vol, t=60

Supernatant → Amylase Assay
Pellet
PLATE 7.1

Modified Pasteur Pipettes Calibrated for the Measurement of Changes in Pancreatic Tissue Volume During Storage
into the supernatant during storage, as a measure of the degree of exocrine enzyme
discharge. These variables could then be correlated with the densities of exocrine tissue
estimated using the BSA density gradients.
Statistical comparison between the results obtained using different storage media was
by the Wilcoxon signed-rank test for paired data, expressed as the p-value and 95% CI.
Correlation between tissue densities and changes in tissue volume/enzyme discharge
was assessed using Pearson's product-moment correlation co-efficient, r or
Spearman's rank correlation coefficient, rho.

Results

The bouyant densities (Median and 95% CI) of exocrine tissue and islets on BSA
density gradients following storage of pancreatic digest in each of the seven storage
solutions are summarised in Figure 3.
The densities of both types of tissue were similar after storage in MEM and Solution A,
both of which contained high concentrations of permeable chloride anions (95% CI of
the difference in density between Solution A and MEM = -7 to 31 x 10^{-4} g/cm^{3} for
exocrine tissue, and -6 to 29 x 10^{-4} g/cm^{3} for islets). Following storage in all other
solutions, however, these densities were significantly increased, compared to storage in
MEM (p<0.05). Furthermore, the density of exocrine tissue was once again influenced
to a greater extent than that of islets, so that islet purity was dependent upon the
medium used for storage of the pancreatic digest. Median (range) exocrine
contamination at 60% islet yield was 41% (6-60%) using MEM, and the (paired)
differences between these values and those obtained using the other six storage
solutions are outlined in Figure 4.
Following storage of digest in Solutions A and B, exocrine contamination at 60% islet
yield was similar to that obtained using MEM (p=0.906 and 0.799 respectively). Using
Solutions C, D and E, however, there was a trend towards improved islet purity
(although not statistically significant overall; median contamination = 26%, 27% and
29%; p=0.154, 0.114 and 0.286 respectively), whilst storage in UW was associated
with the lowest values for exocrine contamination (Median 16%, p=0.014 compared to
MEM).
The increase in exocrine tissue density responsible for these changes in islet purity was
most closely associated with a reduction in the volume of stored tissue. In Figure 5, the
values for exocrine tissue density obtained from each of the 70 gradients analysed in
this experiment are plotted against the respective percentage changes in tissue volume
occurring during storage for 10 and 60 minutes in the various solutions, and against the
FIGURE 3

The Bouyant Densities of Percutaneous Tissues on BSA Density Gradients following Storage of Dipeptin in MPM, UW and Solutions A-E. Columns = 95% CI. Horizontal Bar = Median Density. Horizontal Tissue. Exocrine Tissue.
Figure 4

Columns = 95% CI
Horizontal bars = Median Changes in Contamination

Changes in % Amylase Contamination at 60°C Insulin Yield Following Storage
FIGURE 6

Relationship Between Median Exocrine Tissue Density and Median Changes in Amylase Release and Tissue Volumes for Each Storage Medium (MEM, UW and Solutions A-E).
FIGURE 7
Median Exocrine Tissue Density as a Function of Anionic Composition of Solutions A-D.
percentage amylase release after 1 hour storage. These data demonstrate that there was a significant negative correlation between exocrine tissue density and changes in tissue volume ($r = -0.466, p<0.001$ for the tissue volume change at 10 minutes; and $r = -0.405, p<0.001$ at 60 minutes), but that there was no correlation between exocrine tissue density and changes in amylase discharge during storage ($r = 0.114, p>0.10$).

This relationship is further summarised in Figure 6, in which the median values of exocrine tissue density and the changes in tissue volume/amylase discharge are plotted for each individual storage solution: again, only for changes in tissue volume is the expected negative correlation with exocrine tissue density observed ($\rho = -0.652, p>0.10$ at 10 minutes, and $\rho = -0.813, p<0.05$ at 60 minutes), whilst there is no significant association between density and amylase discharge ($\rho = 0.045, p>0.10$).

Finally, changes in pancreatic tissue density were associated most closely with the anionic composition of the storage media. This is demonstrated in Figure 7, which documents the densities of islets and exocrine tissue following storage in Solutions A-D, which differed from each other only in the ratio of lactobionate:chloride anions. It is apparent that, for these solutions, the densities of both types of tissue were dependent upon the replacement of chloride anions by lactobionate, and also that an increase in the lactobionate:chloride ratio caused exocrine tissue to increase in density more than islets.

**Discussion**

This study has again demonstrated the importance of the storage period between collagenase digestion of the pancreas and density gradient purification of islets, and confirmed the beneficial influence of UW during this phase of islet isolation. In addition, it has also provided some information concerning the components of UW responsible for these effects, and the likely mechanism of their action.

Thus, improved islet purity following storage of pancreatic digest results from a differential increase in the density of exocrine tissue compared to that of islets. In this study, increased exocrine tissue density was most closely associated with a reduction in the volume of the stored digest, the vast majority of which is composed of exocrine tissue. Although it may be argued that the discharge of exocrine enzyme granules from acinar cells might contribute to changes in their volume, amylase discharge did not differ significantly between storage solutions. These results therefore suggest that the control of acinar cell swelling, rather than changes in exocrine enzyme discharge, is the most important factor in determining the density of exocrine tissue, and therefore influencing islet purity, at least during storage of the dispersed pancreas.
However, although statistically significant, the strength of this relationship between exocrine tissue density and the measured changes in digest volume was relatively low, and potentially this may have been due to a number of factors. Firstly, some of the changes in tissue volume produced by the storage solutions may have occurred prior to the initial volume measurements (Vol, t=0) i.e. during washing of the dispersed pancreas in the experimental solutions, during separation of the washed tissue aliquots into the capillary tubes, and during the initial 3 minute centrifugation of these tubes. Indeed, it was observed that the initial volumes of tissue following this first period of centrifugation were significantly lower in the groups washed in Solutions C, D, E and UW than those washed in MEM (p<0.01), suggesting that volume changes were occurring to some extent during centrifugation (Figure 8). The true change in tissue volume produced by these solutions may therefore have been underestimated using the method described. In addition, because of the time required to construct and run the density gradients, estimations of exocrine tissue density were necessarily restricted to a single measurement for each solution in each experiment. Thus, it was only possible to compare the change in tissue volume occurring during 10 minutes storage with the final density of exocrine tissue after this time period, rather than with the change in density occurring between washing and the end of the storage interval. Again, some of the density changes produced by the storage solutions may also have occurred during the initial wash phase, so that use of the final exocrine tissue density alone may have resulted in an overestimation of the changes occurring during storage in the capillary tubes.

Secondly, the narrow bore of the capillary tubes, selected to facilitate the measurement of small changes in tissue volume, produced significant wall effects during centrifugation, which occasionally made estimation of tissue volumes difficult. Thus, pilot experiments indicated that if insufficient centrifugal force was used an adequate tissue pellet was not produced, whilst excessive centrifugation resulted in compression of the tissue pellets, producing a uniform volume independent of the storage solution used. In general, however, centrifugation at 200g for 3 minutes proved adequate on most occasions.

Finally, with Solutions D and E, and with UW, the density of exocrine tissue was often increased to such an extent that the majority of the amylase content was found in the pellet of the corresponding gradients. As discussed in Chapter 6, this implies that the true densities of these tissues were greater than the maximum density of the BSA used, and therefore greater than the estimated values outlined above (unfortunately, it was not possible to increase the upper density of BSA solutions used in this study, as this was already equal to the density of the stock, undiluted BSA at an osmolality of 400 mOsm/kg H2O).
FIGURE 8

Differences Between Storage Solutions in the Initial Tissue Volumes: Recalculated (Vol. Eq.)

Error Bar = Upper Limit of 95% CI

Tissue Volume (μl)

0 200 400 600 800 1000

MEM

Solution A

Solution B

Solution C

Solution D

Solution E

UW
Despite these problems, however, the results still support the conclusion that exocrine tissue density is influenced primarily by changes in acinar cell volume, rather than enzyme discharge. In addition, this conclusion is also consistent with two other observations. Firstly, as documented in the experiments of Chapter 6, exocrine tissue density is greater after 1 hour storage in UW than after washing in this medium: enzyme discharge from acinar cells is irreversible, and so cannot explain this change in density, whilst the above experiments also show that tissue volume continues to decrease between 10 and 60 minutes storage in this medium (median = 2%; 95% CI = 0 to 11% further reduction in volume, p=0.093). Secondly, similar changes (although of lesser magnitude) occur in islet density, islet cells also being susceptible to swelling during isolation and hypothermic storage, but capable of discharging only a small fraction (about 10%) of their stored insulin in response to physiological stimulation (Schnell Landstrom et al, 1988; Carroll, 1992).

Moreover, this conclusion is further supported by the observation that the replacement of permeable chloride anions by lactobionate in the storage medium was the most important factor in determining pancreatic tissue densities and therefore islet purity, as cellular swelling is the feature most likely to be influenced by the anionic composition of cold storage media. Thus, islet purity was similar after storage in MEM and Solution A, which both contained chloride as the principal anion, but which differed markedly in their other constituents (calcium, magnesium, buffers, Na+:K+ ratio, glucose and amino acids), whilst increasing only the lactobionate:chloride ratio in Solutions B-D produced a marked improvement in islet purity.

At 60% islet yield there was little difference in exocrine tissue contamination between Solutions C, D and E. As mentioned above, however, the tendency for much of the exocrine tissue to pellet following storage in Solutions D and E may have resulted in an underestimation of the beneficial effects of these solutions on islet purity using this density range of BSA. This problem may be partly overcome by examination of the exocrine tissue contamination at a lower islet yield for each solution. Accordingly, the values for amylase contamination at islet yields of both 30% and 60% are compared in Figure 9. At 30% islet yield contamination using Solution D is significantly improved compared to MEM (p=0.044), and now slightly better than using Solution C, although this latter difference is not statistically significant. In addition, the replacement of raffinose by extra lactobionate does not result in any significant change in purity at either islet yield.

Interestingly, however, islet purity at both 30% and 60% yields was optimal following storage of digest in UW, and despite the similarity in anionic composition of UW and Solution D, exocrine tissue contamination tended to be lower using the former solution (Median = 1%, 95% CI = -1 to 10% reduction in amylase contamination at 30% yield,
compared to Solution D, p=0.205; and median = 8%, 95% CI = -4 to 20% reduction at 60% yield, p=0.124). Thus, one of the components omitted from Solution D but present in UW must exert an additional beneficial effect, and further investigation of this issue was therefore undertaken in the following experiments.

THE ROLE OF COLLOIDS AND SODIUM: POTASSIUM RATIO IN SOLUTIONS USED FOR STORAGE OF PANCREATIC DIGEST

The investigations detailed above suggested that the beneficial effects of suspending the pancreatic digest in solutions such as UW were mediated by the reversal of pre-existing acinar cell swelling and the prevention of further swelling during hypothermic storage prior to density-dependent islet purification. In addition, although the presence of impermeant anions such as lactobionate was of primary importance, this was not the only factor responsible for the positive effects of UW. The first aim of the following investigations was therefore to determine which of the components present in UW, other than the impermeant molecules lactobionate and raffinose, were responsible for further improving islet purity.

In addition, the role of the sodium:potassium ratio of storage solutions was also investigated. Comparison of MEM with Solution A in the studies outlined above suggested that this factor was relatively unimportant, and indeed this would be expected on purely theoretical grounds, if changes in exocrine tissue density were determined solely by the control of cellular swelling, which is independent of the nature of extracellular cations at 4°C. This issue was felt to be important, however, because of the potential for using similar storage solutions during other phases of the islet isolation process (eg. for intraductal delivery of the collagenase solution). Such solutions may require modification in order to be appropriate for use both at 4°C and at 37°C, and one factor likely to be important in this respect is the Na+:K+ ratio, as high extracellular potassium levels induce cellular swelling and may therefore be toxic at 37°C.

Predicting on theoretical grounds which of the other components of UW might be of additional benefit was difficult, because, as discussed above, none of the components omitted from Solution D (glutathione, allopurinol, adenosine and hydroxyethyl starch) have been found universally essential for preservation (particularly in the short-term) of whole organs prior to transplantation. However, independent work within this department suggested that the colloid, hydroxyethyl starch, was important, and that the presence of this component improved islet purity, even when using solutions such as MEM, which are otherwise poorly adapted to hypothermic storage of tissue (Robertson et al, 1993d). This finding was unexpected, due to the lack of a vascular endothelial
barrier perfused under increased hydrostatic pressure in this experimental system. Nonetheless, it was elected to investigate further the influence of hydroxyethyl starch in digest storage solutions, and in addition to determine whether a similar effect was observed using other colloids (the term colloid in this context being used to describe a substance of large molecular weight, capable of exerting an oncotic pressure across physiological membranes).

Methods

The composition of the storage solutions studied in these experiments are detailed in Table 3. In addition to UW, six phosphate-buffered solutions were studied, containing lactobionate and raffinose in equimolar concentrations to those present in UW and containing either no colloids (Lactobionate-Raffinose Solution; LRS) or 0.2 mM concentrations of the colloids Hydroxyethyl starch (HES 'Pentafraction, Du Pont Critical Care Inc.), Dextran of molecular weight 40,000 (Cat. No. D4133, Sigma) or 250,000 (Cat. No. 38014, BDH Chemicals Ltd., Poole, UK), or Ficoll 400DL (Cat. No. F9378, Sigma). Finally, one lactobionate-raffinose based solution was produced containing HES and a more physiological Na+:K+ ratio. Solutions were produced by the addition of lactobionate, raffinose, MgSO₄ and K/Na H₂PO₄ to double-distilled water from the same source as that used in the above experiments, followed by adjustment of pH (at 4°C) using appropriate combinations of sodium and potassium hydroxide. The relevant colloid was then added to each solution, which was then filter-sterilised (0.22 μm filter) before use.

Seven porcine pancreata were obtained and processed as previously described. Following collagenase-digestion the dispersed pancreatic tissues were pooled and washed in MEM at 4°C, and subsequently divided equally between the seven storage solutions. Each sample was washed twice by resuspension in its respective solution after centrifugation at 200g for 2 minutes at 4°C. Following the final centrifugation tissues were resuspended in the relevant solution and stored for 20 minutes at 4°C, prior to the removal of aliquots for separation on continuous linear density gradients of BSA. The density range of BSA used was 1.076 to 1.103 g/cm³, and in order to reduce the problems previously encountered with excessive 'pelleting' of tissue, the osmolality was adjusted to 350 mOsm/kg H₂O throughout. Gradients were centrifuged at 500g for 5 minutes at 22°C, and were fractionated and analysed as previously described.

In addition, seven human pancreata were also studied. These were obtained, with appropriate consent, from brain-dead, heart-beating organ donors of median (range)
Values are given in mmoL/l unless otherwise stated.

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The composition of solutions used for investigating the influence of colloids on liver function.

**Table 3**
age 53 (30-61) years, following in situ vascular perfusion with HOC. Following excision, pancreata were transported to the laboratory in HOC at 4°C, with a median (range) cold ischaemic time of 4.25 (1.75-5.5) hours. Following collagenase-digestion of the pancreas, the dispersed tissues were treated exactly as described above for porcine pancreata, except that only six storage solutions were studied (the LRS + HES solution with a normal Na⁺:K⁺ ratio being omitted). Continuous, linear density gradients of BSA were used, with a density range of 1.075 to 1.106 g/cm³ and an osmolality of 500 mOsm/kg H₂O throughout. Gradients were centrifuged at 800g for 5 minutes at 22°C, and were fractionated and analysed as above.

Results

Porcine Pancreata
Following storage of porcine digest in UW, median (range) values for exocrine tissue and islet density on BSA density gradients (osmolality 350 mOsm/kg H₂O) were 1.096 (1.093-1.099) g/cm³ and 1.093 (1.090-1.097) g/cm³ respectively, resulting in an exocrine tissue contamination at a 60% islet yield of 49% (8-54%). The paired differences between these values and those obtained following storage of digest in the six experimental solutions are summarised in Figures 10 and 11. The densities of both types of tissue were slightly reduced (although not to a statistically significant extent) following storage in plain LRS (without a colloid), compared to storage in UW (p=0.142 for the change in exocrine tissue density; and p=0.151 for the change in islet density). In all solutions containing a colloid, however, densities were similar to those obtained using UW, although in the presence of HES and a normal Na⁺:K⁺ ratio the densities of both exocrine tissue and islets tended to be greater than using UW (p=0.052 and 0.076 respectively). Likewise, exocrine tissue contamination at 60% islet yield was similar following storage in all solutions, although in the absence of a colloid there was a trend towards increased contamination, whilst in the presence of a colloid this trend was reversed, and purity tended to be slightly better than using UW. This trend was greatest for the solution containing Ficoll 400, and this was the only solution for which exocrine tissue contamination was statistically significantly less than that using LRS alone (median = 9% reduction in contamination compared to LRS, p=0.036).
Figure 10

Differences in Pancreatic Tissue Densities Following Storage in the Experimental Solutions Compared to LW

Porcine Pancreata

Bars
- Blank
- Exocrine Tissue
- Colons = 95% CI
- Horizontal lines = Median Difference

Change in Density (g/cm^3 x 10^-4)

Legend
- Ficol + LRS
- Dextran 250
- LRS + LRS
- NAc + LRS
- LRS + HES
- LRS + HES
- Normal
Change in % Amylase Contamination

Porcine Pancreata

Solutions Compared to UW

Differences in % Amylase Contamination at 60% Islet Yield Following Storage in the Experimental

FIGURE 11
Human Pancreata

Following storage of human pancreatic digest in UW, the median (range) values for exocrine tissue and islet density on BSA density gradients (osmolality 500 mOsm/kg H$_2$O) were 1.105 (1.099-1.105) g/cm$^3$ and 1.095 (1.090-1.099) g/cm$^3$ respectively, resulting in an exocrine tissue contamination at 60% islet yield of 4% (0-6%). The densities of both exocrine tissue and islets were similar after storage in all the solutions studied, and there were no significant differences compared to those values obtained using UW. As a result, the exocrine contamination at a 60% islet yield did not differ markedly between the storage solutions, although there was a slight trend towards reduced islet purity with LRS alone, and also with LRS containing Dextran 40, compared to UW, whilst this trend was not observed in the presence of HES or Ficoll. These changes are summarised in Figure 12.

Discussion

The results outlined above confirm the findings of the series of experiments described in the first section of this chapter, which suggested that storage of digest in solutions containing only the impermeant molecules lactobionate and raffinose resulted in slightly reduced islet purity, compared to UW. However, they also suggest that the addition of low concentrations of a colloid to such solutions produces results comparable to those obtained using UW. Therefore, in addition to these impermeants, HES appears to be a further essential component of UW when used for storage of pancreatic digest prior to islet purification, whilst glutathione, adenosine and allopurinol may be safely omitted. Furthermore, at least for the porcine pancreas, the addition of certain other colloids (eg. Ficoll 400) may even produce results slightly superior to those achieved with UW. The effects of a colloid were similar in the two species, although in this series of human pancreata islet purity was high after storage in all the experimental solutions, including LRS alone, thereby limiting the potential for improvement by the addition of colloids. The effects of colloids in this series of experiments were relatively modest, and were limited to statistically non-significant trends in islet purity. Nonetheless, differences between digest storage media with or without colloids were sufficiently large to be observed upon visual assessment of the respective density gradients in both species. Furthermore, these data are also consistent with independent observations, obtained in this Department, that the addition of 50 g/l of HES to MEM prevents the reduction in tissue densities which otherwise occurs during storage of digest in this medium, and thereby maintains islet purity during subsequent density gradient centrifugation (Robertson et al, 1993d).
FIGURE 12

Change in % Amylase Contamination

Horizontal lines = Median Difference
Columns = 95% CI

Differences in % Amylase Contamination at 60% Islet Yield Following Storage in the Experimental Solutions Compared to UW

Human Pancreata

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With porcine pancreata, lower islet purity using LRS alone was generally associated with reduced values for exocrine tissue density compared to those using UW or colloid-containing LRS, and these changes are similar to those obtained in the above-mentioned studies using MEM for storage of human pancreatic digest (Robertson et al, 1993d). Similar changes in density were not observed for human pancreata in this study; however, this may have been due to the problems, previously described, of detecting changes in density when much of the exocrine tissue reaches the pellet of density gradients, as was the case in the present investigation, even when tissue was stored in LRS alone.

It is likely, therefore, that the effects of colloids are also mediated by reducing the degree of cellular swelling, particularly of acinar tissue. The mechanism by which this is achieved is, however, difficult to explain. The possibility that extracellular colloids may act simply by counteracting the effects of intracellular colloid osmotic pressure is an unsatisfactory explanation, as their effects are present in solutions containing large concentrations of other effective impermeant molecules. It seems unlikely, therefore, that the presence of 0.2 mM concentrations of any colloid could be noticeably effective in a solution already containing at least 130 mmol/l of such impermeant molecules. In addition, the fact that certain colloids, such as Ficoll, have slightly greater effect than others when used in equimolar concentrations also argues against this mechanism for their action.

One potential effect of colloids, however, may be related to their capacity to bind free extracellular water. Indeed, this is the likely explanation for the fact that the addition of 0.2 mM concentrations of colloids increased the osmolality of the storage solutions by 20 mOsm/kg H$_2$O for HES, and by 2, 12 and 30 mOsm/kg H$_2$O for Dextran 40, Dextran 250 and Ficoll respectively (Table 3). This effect on osmolality is not due to the presence of contaminating ions, as analysis (Fisons ICP OES) of colloid solutions made up in ddH$_2$O alone revealed combined concentrations of sodium, potassium, calcium and magnesium ions of < 0.03 mmol/g colloid in each case. The reduction in extracellular water activity resulting from the water-binding effects of colloids may therefore contribute to protection from cellular swelling; moreover, differences between various colloids in their ability to bind water may partly explain the variation in their beneficial effects on islet purity.

In addition to the studies outlined at the beginning of this chapter, in which the effects of colloids on organ preservation prior to transplantation were examined, a number of studies have also investigated the role of colloids in controlling cell swelling during cold storage, with variable results. Thus, in one such investigation the swelling of rat liver slices during 24 hour cold storage was uninfluenced by the presence or absence of hydroxyethyl starch or dextran 40 in the preservation solution (Ar'Rajab et al, 1991).
Similarly, the use of solutions containing albumin and other plasma proteins did not prevent an increase in wet weight of the rat pancreas during simple cold storage for up to 96 hours (Ruka et al, 1989). In contrast, however, the use of the colloid polyethylene glycol (PEG, molecular weights between 6,000 and 20,000) consistently reduced cell swelling in tissue slices of rat kidney (Davey et al, 1971; Robinson, 1971) or heart (Ganote et al, 1977), and in isolated hepatocytes and kidney cells (Daniel et al, 1976; Marsh et al, 1989b). In these studies the mechanism of action of PEG also appeared unrelated simply to its colloid osmotic properties. Indeed, it has been suggested that this particular colloid may act primarily by interacting with cell membranes, perhaps thereby influencing their ionic permeability (Daniel et al, 1976). Furthermore, this action may be of particular importance in the protection of isolated cells, due to the potential influence of the isolation process, which usually involves enzymatic dissociation of tissue, upon membrane permeability (Marsh et al, 1989b).

It is possible, therefore, that the colloids studied above may also have a similar 'membrane-stabilising' effect upon dissociated pancreatic tissues. Interestingly, however, although UW-like solutions containing PEG of molecular weight 20,000 (PEG 20M) have been used very successfully in the preservation of the intact pancreas (Zheng et al, 1991) and heart (Wicomb et al, 1990), pilot studies using lactobionate-raffinose solutions containing PEG 20M (at concentrations of both 50 g/l and 0.2 mM) for storage of porcine pancreatic digest did not appear to improve islet purity (using visual assessment of BSA density gradients for two pancreata). The interaction of colloids with cell membranes may, however, be organ-specific, which may again explain the different effects of the colloids studied above upon islet purification.

One further observation made in the present study was that the densities of porcine pancreatic tissues were significantly greater using a solution containing impermeants and HES with a normal sodium:potassium ratio than in a similar solution with a reversed sodium:potassium ratio. The densities of both exocrine tissue and islets were increased to a similar extent (Median increase in density using a normal Na⁺:K⁺ ratio, compared to a reversed ratio = 21 x 10⁻⁴ g/cm³ for exocrine tissue, p=0.035; and 15 x 10⁻⁴ g/cm³ for islets, p=0.022), so that islet purity was unaffected. Nonetheless, it is likely that these changes in density reflect a reduction in cell swelling, and potentially therefore an improvement in cell viability, in solutions with a normal Na⁺:K⁺ ratio, possibly consistent with previous observations that such solutions are at least as effective as UW (with high potassium), and sometimes further improve preservation of the liver, kidney and pancreas prior to transplantation (Sumimoto et al, 1989; Moen et al, 1989; Urushihara et al, 1992).

This effect is difficult to reconcile with the mechanisms involved in the regulation of cell volume, outlined in Chapter 3, as theoretically, in the absence of sodium pump

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activity, cellular swelling should not be influenced by the nature of the extracellular cation, and in addition, swelling should not occur in solutions containing only impermeant anions. By implication, therefore, the cell membranes of dispersed pancreatic tissues must be permeable to some of the anions present in LRS, and less permeable to extracellular sodium than potassium cations. However, whilst enhanced permeability to certain anions, e.g. sulphate and hydrogen phosphate anions, might easily be produced by the effects of collagenase digestion upon cell membranes, the putative differences in cation permeability are more difficult to explain. The presence of some sodium pump activity would provide one explanation, although it seems unlikely that the degree of activity present at 4°C would be sufficient to cause such significant changes in tissue density. Presumably, therefore, sodium ions in LRS are rendered effectively impermeant to cell membranes by another, undefined mechanism, for instance by enhanced binding to lactobionate or HES, and this reduced permeability may therefore prevent the entry of permeable anions into cells, and so reduce cellular swelling.

Whatever the mechanism, however, the observation that digest storage solutions with a high sodium:potassium ratio do not compromise islet purity may facilitate their use at temperatures other than 4°C, e.g. during some of the preceding phases of the islet isolation procedure.

SUMMARY

The experiments outlined above suggest that storage of pancreatic digest in UW improves subsequent islet purification by reversing pre-existing acinar cell swelling, and by preventing further swelling during storage. Furthermore, these beneficial effects are mediated by the replacement of permeant chloride anions by impermeant lactobionate anions, and by the presence of a colloid. Glutathione, adenosine and allopurinol do not appear to contribute, whilst the sodium:potassium ratio may be increased to physiological levels without impairing the efficacy of storage solutions. Solutions containing buffers, impermeants and colloids are therefore most suitable for storage of digest prior to islet purification. It may, however, be possible to optimise such solutions further, by modification of other factors, such as pH, which has documented effects upon the densities of certain cells, and which was not examined in the above experiments. Thus, before attempting to extend the benefits of these storage solutions into the density gradient or other phases of the islet isolation procedure, it was considered important firstly to examine the influence of their pH upon islet purification,
using the methods of analysis established above. These experiments are outlined in the following chapter.
CHAPTER 8

Further Optimisation of Digest Storage Media:
The Influence of pH
Theoretically, the control of pH during cold storage of the dispersed pancreas may be considered relevant for two reasons: firstly, islet viability may be influenced by intracellular pH changes occurring during cold storage; and secondly, the densities of pancreatic tissues might be affected by variations in the pH of the storage media in which they are suspended, thereby influencing islet purification.

During ischaemia profound changes in pH occur within all metabolically active cells: the activation of anaerobic metabolism and the hydrolysis of ATP lead to the accumulation of hydrogen ions, and thereby to a reduction in intracellular pH (Sehr et al, 1979), and potentially these changes in pH may then influence cellular viability following ischaemia. The nature of this influence is, however, the subject of debate, some investigators suggesting that intracellular acidosis is itself a source of tissue damage during ischaemia (Williamson et al, 1976; Bore et al, 1981), whilst others maintain that reduced pH may in fact be protective (Pentilla et al, 1975; Bonventre et al, 1985).

Furthermore, the majority of studies investigating the effects of pH have been concerned primarily with those changes occurring during normothermic ischaemia. Intracellular pH changes occur more slowly during cold storage of tissues than at normothermia (Sehr et al, 1979), and their effects on cellular viability may therefore be modified. Unfortunately, however, few studies have addressed the importance of pH during hypothermic preservation of tissues, and those which have been performed have been somewhat contradictory. Thus, in one study, hypothermic preservation of isolated rat hepatocytes for up to 72 hours was optimal using storage media of pH between about 7.0 and 7.6 (Fox et al, 1989), whilst in other investigations involving whole organ preservation, the use both of alkaline (pH 7.9-9.0 (Lie et al, 1984; Abouna et al, 1988)) and mildly acidic (pH 6.8 (Calne et al, 1972)) perfusates have been suggested to improve preservation of the liver and pancreas, compared to those at pH 7.4.

In addition, interpretation of the effects of pH on cell viability during cold storage is further complicated by the observation that commonly used organ perfusates have relatively low buffering capacity, so that final tissue pH levels in whole organs may be similar after preservation with different perfusates, irrespective of their initial pH (Fuller et al, 1988a). Indeed, in one study improved renal preservation resulted simply from the addition of extra buffers to the storage medium, without change in its initial pH (Besarab et al, 1984). It may be concluded, therefore, that the influence of pH on cell viability during cold storage is not fully established, although it is likely to be of some importance.

However, possibly of greater relevance to the present series of experiments concerning islet purification is the potential influence of pH upon cell density. In 1968, Legge and Shortman demonstrated that the volume and buoyant density of erythrocytes in isotonic
albumin density gradients was dependent upon the pH of the gradient medium; as pH was reduced, cells became swollen and their density was reduced. Changes in the ionisation state of intracellular haemoglobin with pH were thought to be responsible for these effects, the production of a positive charge at low pH permitting the entry of extra chloride anions (and therefore water) into the cell. Shortly thereafter, however, these results were criticised (Kneece et al., 1971) on the basis that pH adjustments had been made using sodium hydroxide, thereby producing an increase in extracellular sodium ion concentration at higher pH: since sodium is largely excluded from erythrocytes, it was then argued that the apparent effect of pH was merely the result of an increase in extracellular tonicity. Adjustment of pH at a fixed concentration of extracellular cations, using different ratios of chloride:bicarbonate revealed that the change in buoyant density of erythrocytes with pH was much less than previously observed, and that increased density with increasing pH was largely explained by the impermeant nature of the bicarbonate anion, rather than an effect of pH itself.

The possibility therefore existed that the observed changes in cellular density were due to a combination of the particular ion permeabilities of erythrocytes and the use of albumin (which is negatively charged above pH 5.1, and which may therefore influence extracellular sodium concentrations) as a density gradient medium. Subsequently, however, it was demonstrated that the buoyant densities of lymphocytes and antibody-forming cells were also reduced at low pH (reducing pH from 7.2 to 5.1 at 4°C producing an increase in density of approximately 0.01 g/cm³), and that this also occurred in density gradients of Ficoll (Williams et al., 1972b).

The density of other nucleated cells at 4°C might therefore be influenced by pH, and theoretically cellular swelling would be expected to result from a reduction in extracellular pH; the entry of hydrogen ions into cells, and the subsequent neutralisation of intracellular anions allowing the net entry of chloride or other permeant extracellular anions into the cell, water following to maintain osmotic equilibrium. Equally, it might be expected that cellular swelling could be reversed by an increase in extracellular pH. This effect may, however, depend upon the anionic composition of the extracellular medium, and upon the particular characteristics of the cells under investigation.

The influence of pH upon the densities of pancreatic cells has not previously been investigated, and it was felt important, therefore, to study the potential for reversing cellular swelling further by increasing the pH of the storage media described in the previous chapter.
Methods

The compositions of the storage solutions studied are outlined in Table 1. The control solution was based upon Solution D, described in the previous chapter, the pH of which in this series of experiments was 7.45 at 4°C. Three other experimental solutions were then produced by the addition of 35 mmol/l of HEPES (Hydroxyethylpiperazine ethane sulphonic acid, Cat. No. H9136, Sigma) to this basic solution, followed by the adjustment of pH (measured at 4°C) to 7.45, 7.95 and 8.45 respectively, using 10M NaOH. All solutions were then filter-sterilised before use.

Seven porcine pancreata were collagenase-digested, and the dispersed pancreatic tissues were pooled and washed in MEM at 4°C, as previously described. The pooled digest was then divided between the four storage solutions, and each sample of tissue was washed twice in its respective solution and stored at 4°C for 20 minutes prior to the removal of aliquots for separation on BSA density gradients. The density range of BSA used was 1.076-1.103 g/cm³, the osmolality 350 mOsm/kg H₂O throughout, and the gradients were run at 500g for 5 minutes at 22°C, and analysed as previously described.

Results

The median (range) densities of exocrine tissue and islets on BSA density gradients following storage of digest in the 'control' solution were 1.102 (1.091-1.102) g/cm³ and 1.098 (1.092-1.099) g/cm³ respectively, resulting in an exocrine tissue contamination of 9% (4-62%) at 60% islet yield, and 6% (0-28%) at 30% islet yield. The paired differences between these values and those obtained using storage solutions A-C are summarised in Figures 1 and 2. The densities of exocrine tissue and islets tended to be higher following storage in solutions A-C than using the control solution, although only the increase in islet density with solution B was statistically significant (p=0.036), and there were no significant differences between the three solutions varying only in pH. Similarly, exocrine tissue contamination at both 60% and 30% islet yield was similar between all four solutions studied, although the lowest values were obtained at pH 7.45 in both cases.
### TABLE 1

The Composition of Storage Solutions Used to Study the Effect of pH on Islet Purification

<table>
<thead>
<tr>
<th></th>
<th>Control Solution</th>
<th>Solution A</th>
<th>Solution B</th>
<th>Solution C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobionate</td>
<td>115</td>
<td>115</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td>Raffinose</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>H₂PO₄</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Na⁺</td>
<td>20</td>
<td>30</td>
<td>44</td>
<td>54</td>
</tr>
<tr>
<td>K⁺</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>HEPES*</td>
<td>-</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>pH</td>
<td>7.45</td>
<td>7.45</td>
<td>7.95</td>
<td>8.45</td>
</tr>
<tr>
<td>Osmolality</td>
<td>323</td>
<td>355</td>
<td>358</td>
<td>364</td>
</tr>
</tbody>
</table>

Values are in mmol/l, unless otherwise stated

* Total Concentration of HEPES (Acid + Base).
Figure 1: Differences in Porcine Pancreatic Tissue Densities Following Storage in Solutions A-C Compared to the Control Solution.

Columns = 95% CI
Horizontal Bar = Median Difference
Blocks = Exocrine Tissue

Change in Density (g/cm³ x 10⁻⁴)
Change in % Amylase Contamination

**Figure 2**

Solutions A-C, compared to the Control Solution, showed differences in % Amylase Contamination at 30% and 60% yield. Following storage in...
Discussion

The experiments described above were designed to minimise some of the problems inherent in studying the effects of pH during cold storage of tissues. Thus, in order to maintain differences in pH during storage, small volumes of tissue (200-300 µl) were suspended in a relatively large volume of storage medium (10 ml), the buffering capacity of which was increased considerably by the addition of 35 mM HEPES. This buffer was selected partly because its dissociation constant (pK_a = 7.5) was close to the range of pH under investigation, thereby maximising the buffering capacity of the solutions studied; and partly because its relatively high molecular weight (M_r = 238) and hydrophilic nature made it likely that the anionic form of this molecule was impermeant to the cell membrane, thereby maintaining the beneficial effects of high impermeant concentrations within the storage solutions. Increasing the pH of such solutions by the addition of sodium hydroxide clearly increases the concentration of extracellular sodium, and also increases the total osmolality (Table 1). However, by comparing the HEPES-containing experimental solutions with a control solution lacking HEPES but also containing predominantly impermeant anions (lactobionate), it was expected that the purely osmotic effects on cellular density resulting from increased sodium concentration and osmolality at higher pH might be separated from the effects of pH alone.

Tissue densities were indeed higher following storage in the experimental solutions A-C than in the control solution, whilst density differences between the experimental solutions (i.e. at different pH) were minimal, suggesting that the majority of the changes observed were the result of purely osmotic effects, and that pH itself had little influence upon the density of pancreatic tissues.

Even after storage in the control solution and with the use of BSA at an osmolality of 350 mOsm/kg H_2O, the median density of exocrine tissue was close to the maximum density range of the gradients. As previously discussed, therefore, the changes in exocrine tissue density outlined in Figure 1 represent an underestimate of the true changes in density produced by the experimental solutions. Equally, this may have partly obscured any pH-dependent changes in exocrine tissue density, although the fact that islet density also differed only slightly with varying pH suggests that such changes were probably limited in magnitude. The lack of any influence of pH upon pancreatic tissue densities is presumably therefore due to the anionic composition of the storage solutions studied, the diffusion of hydrogen ions across the cell membrane in either direction being limited by the lack of an accompanying permeant anion.

The tendency for exocrine tissue to 'pellet' may also explain the apparent trend towards increased exocrine tissue contamination at 60% islet yield using solutions A-C,
compared to the control solution. Comparing these solutions at an islet yield of 30% may partly overcome this problem, and in this study revealed that islet purity was indeed comparable between all solutions studied.

Storage solutions at lower pH than 7.4 were not investigated, as the theoretical considerations discussed above suggested that only alkaline solutions would have a beneficial influence upon cellular swelling, and therefore upon islet purification. In addition, no information was provided in this study concerning the effects of pH upon the viability of islets, although the morphology of dithizone-stained islets within two groups of visually-assessed density gradients was grossly normal after storage in solutions at all three pH values. Nevertheless, the observations that islet purity was greatest using solutions at 'physiological' pH (7.4), and that hypothermic preservation of other types of isolated cells appears to be optimal at this pH (Fox et al., 1989), suggests that this is the most suitable pH of those studied for storage of pancreatic digest prior to islet purification.

**SUMMARY**

The results of this study and those described in the previous chapters have shown that the density-dependent purification of islets is improved by storage of the pancreatic digest in solutions which reverse pre-existing acinar cell swelling, and which prevent further swelling during storage. Furthermore, this can be achieved using a relatively simple solution containing magnesium sulphate, phosphate buffers, impermeants and a colloid, at physiological levels of pH and sodium:potassium ratio.

Using such storage solutions, however, the density of exocrine tissue often approaches the upper limit of the density ranges in which commercially-available hypertonic BSA solutions are provided. Further optimisation of digest storage media is therefore hindered, as it becomes difficult to measure changes in exocrine tissue density, and to estimate exocrine contamination at a suitably high islet yield. It is also likely that some of the beneficial effects of the digest storage solutions used in the preceding studies were reversed by using BSA (the ionic composition of which may contribute to cellular swelling) as a density gradient medium. Additional improvements in islet purification will therefore require the design of new density gradient media, the composition of which is easier to manipulate than that of BSA, and which is based upon that of the optimal storage solution. The production of such a medium and initial observations concerning its efficacy in islet isolation are therefore the subjects of the following chapter.
CHAPTER 9

From Storage Solution to Density Gradient Medium: Initial Observations in Porcine Islet Purification
As discussed in Chapter 3, in order to improve the efficiency of islet purification using density gradients attention must be paid both to the physical requirements for the density gradient separation of any cells, and to the biochemical factors which influence the relative densities of pancreatic tissues during islet isolation. The work described in this thesis has been concerned primarily with these latter, biochemical factors, although during the course of this work advances in the physical methods of large-scale islet isolation have also been made, some of which also influence the biochemical requirements for optimal islet separation.

One such advance has been the facility for producing large-scale continuous linear density gradients on the COBE 2991 cell processor (Robertson et al., 1993c), thereby extending to large-scale gradients the advantages of an increased gradient capacity, more rapid processing, and a reduced incidence of artifacts arising from tissue aggregation which otherwise occurs in the interfaces of discontinuous gradients. In addition, recently-introduced technology for controlling the temperature of large-scale density gradients on the COBE processor has allowed such gradients to be constructed and run at 4°C. The advantages of this approach are that the metabolic demands of tissues may be reduced during this period of obligatory hypoxia, thereby potentially improving islet viability, and that tissue aggregation may also be inhibited at low temperature. This facility, however, requires the production of density gradient media suitable for use at low temperature, which in turn depends upon the biochemical considerations outlined in the preceding chapters. Thus, the work already described has demonstrated that the purification of islets is profoundly influenced by the composition of solutions in which pancreatic tissues are suspended during the islet isolation procedure, optimal purification being achieved using solutions designed to prevent or reverse acinar cell swelling. The density gradient media currently used for islet purification from large mammalian pancreata do not possess such an ideal composition, particularly when used at 4°C, which may therefore explain their relative inefficiency in this process. Nonetheless, the results obtained with these media may be markedly improved by relatively modest changes in their osmolality and ionic composition, suggesting that further advances in islet purification might be achieved using density gradient media specifically optimised for low-temperature isolation.

Furthermore, although the foundation solute of density gradient media (ie. that which provides the density of these solutions, eg. BSA or Ficoll) has been the focus of attention in most studies in which media have been compared for islet purification, the results outlined in the preceding chapters suggest that the nature of this solute is of much lesser importance in determining the efficiency of islet purification than the solvent in which it is dissolved. Improved islet isolation is therefore more likely to follow the use of gradient media specifically designed to maximise density differences.
between islets and other pancreatic tissues at 4°C, i.e. those in which the solvent is based upon the optimal digest storage solutions outlined above. Thus, the purpose of this chapter is to describe the design of such a medium, and to examine its use in the isolation of porcine islets using large-scale continuous linear density gradients at 4°C on the COBE cell processor. In addition to providing information on the efficiency of gradient separation, the experiments described will also be used to establish the viability of islets isolated using this medium, following suspension of digest in an 'optimised' storage solution. Although the foundation solute of a density gradient medium may not be of primary importance in determining the efficiency of islet purification, its influence upon certain other features of the gradient medium makes selection of an appropriate solute relevant. Thus, ideally such a compound should be non-ionic, of relatively high molecular weight, and have a low water-binding capacity, in order to limit its influence upon gradient medium osmolality and thereby facilitate the production of isosmotic gradients and the independent manipulation of the ionic composition of the medium. Furthermore, it would be preferable if such a solute had only a limited influence upon gradient medium viscosity (particularly if the medium is to be used at 4°C, at which temperature the viscosity of all media is increased), in order to reduce the exposure of cells to excessive centrifugal forces. Finally, the compound selected should be water soluble; stable in aqueous solution over the temperature range at which it will be used; non-toxic to cells; free from contaminating ions; and preferably also stable at high temperature, so that the final gradient medium may be sterilised by autoclaving. The selection of a synthetic solute, rather than one derived from animal sources, may also be advantageous, in order to reduce the risks of microbial contamination. Compounds which satisfy these criteria to a large degree are the iodinated contrast media (Rickwood, 1978), the most promising of which (due to their reduced influence upon gradient osmolality) are the non-ionic media, Metrizamide and Nycodenz, both of which have been used successfully in the purification of many types of cells, including islets (Raydt, 1977; Ford et al, 1982; Boyum, 1983; Hering et al, 1990). For these reasons, therefore, Nycodenz was selected as an appropriate solute for the production of the gradient medium described below.

Methods

The composition of the storage solutions used in this series of experiments was based upon those solutions associated with the lowest exocrine tissue contamination in the preceding studies (Table 1). Thus, a combination of Lactobionate, Raffinose and Ficoll.
### TABLE 1

The Composition of the Storage Solution (LRF) Selected for Use in the Large-Scale Isolation of Porcine Islets

<table>
<thead>
<tr>
<th>Component</th>
<th>LRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobionate</td>
<td>100</td>
</tr>
<tr>
<td>Raffinose</td>
<td>30</td>
</tr>
<tr>
<td>Ficoll 400DL (g/l)</td>
<td>80</td>
</tr>
<tr>
<td>Na⁺</td>
<td>144</td>
</tr>
<tr>
<td>K⁺</td>
<td>4</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>25</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg H₂O)</td>
<td>324</td>
</tr>
<tr>
<td>pH</td>
<td>7.45</td>
</tr>
</tbody>
</table>

Values are in mmol/l, unless otherwise stated.
400DL formed the basis of the solution (LRF), which was produced, as previously described, adjusting the sodium-potassium ratio and pH to 'physiological' levels. Nycodenz solutions of high and low density were then produced by the addition of Nycodenz powder (5-(N-2,3-dihydroxypropylacetamido)-2,4,6- triiodo-N,N' bis(2,3-dihydroxypropyl) isophthalamide; supplied by Nycomed AS Diagnostics Ltd., Torshov, Oslo, Norway) to aliquots of this solution, the addition of approximately 50 g/l and 130 g/l producing solutions of density 1.075 and 1.115 g/cm³ (measured at 4°C) respectively, with osmolalities of 380 and 490 mOsm/kg H₂O.

Due to the cessation of supply of porcine pancreata from the source used in the previous experiments, those used in this study were obtained from adult Large White sows, aged 2-3 years, and weighing 200-300 kg. Pancreata were retrieved as previously described, except that warm ischaemic time was more prolonged, at 15-20 minutes (range), and cold ischaemia was between 60 and 90 minutes.

Ten consecutive porcine pancreata were studied, according to the experimental protocol outlined below and summarised in Figure 1. Following the dissection of fat and connective tissue from the pancreas, each gland was weighed prior to intraductal distension with 2 ml/g of HBSS containing collagenase at 2 mg/ml. Each pancreas was then digested using a semi-automated digestion-filtration system, as previously described.

The dispersed pancreatic tissues were collected into 10% NBCS at 22°C, then washed in MEM at 4°C, and the volume of packed tissue recorded. This tissue was then resuspended to a total volume of 200 ml in MEM, from which 5 x 100 µl samples were taken for islet quantification, using a wide-bore, positive-displacement sampling pipette (Drummond microdispenser, Drummond Scientific Co., Broomall, PA, USA), tissue being maintained in suspension between samples by continuous agitation. Each of these samples was 'streaked' onto a Petri dish, stained with dithizone, and the number of islets >50 µm in diameter counted in each. The total number of islets in the digest was then calculated by multiplying the mean number of islets per sample by the dilution factor of 2,000.

In addition, using an eyepiece micrometer, the diameters of 200 consecutive islets within these samples were measured, allowing the mean volume of an islet, the total volume of islet tissue, and the number of 150 µm-equivalent islets within the digest to be calculated, as described in Chapter 4. Finally, a cleavage index (i.e. the percentage of the total volume of islets which were free from adherent exocrine tissue) was calculated by recording the number and diameters of uncleaved islets during the measurement of the 200 islet diameters.

Meanwhile, the pooled digest was washed twice in the experimental storage solution (LRF), and stored at 4°C for 20 minutes prior to density gradient separation of islets,
FIGURE 1
The Experimental Protocol Used in the Study of Large-Scale Porcine Islet Purification

**Protocol**

Porcine Pancreas → Collagenase Digestion

Dispersed Pancreatic Tissues → Wash x2 in LRF, Store 4°C, 20 mins

Stored Digest → Islet Purification

Nyco-LRF Gradient on COBE
1100 rpm, 5 mins

Purified Islets → Culture RPMI
24 hours

Cultured Islets → Culture RPMI
6 Days

Cultured Islets

**Data Recorded**

Weight of Pancreas

Volume of Digest
Islet Quantification

Total Volume
Islet Quantification
Estimated Purity

Islet Perfusion

Islet Perfusion
Microfluorometry
which was achieved for each pancreas using a single large-scale, continuous, linear density gradient of Nycodenz-LRF at 4°C, as follows (Figure 2):

**Islet Purification**
All solutions were loaded onto the COBE cell processor (pre-cooled to 4°C, using a specially-designed cooling system provided by COBE Laboratories, Denver, Colorado, USA), using a peristaltic pump (503S, Watson-Marlow Ltd., Falmouth, UK - not shown in Figure 2), from the chambers of a custom-made, autoclavable glass gradient maker, of similar design to that described in Chapter 4. Initially, 100 ml of high density (1.115 g/cm³) Nycodenz-LRF medium was loaded into the high-density chamber of the gradient maker, and this medium was pumped into the processing bag of the COBE 2991, the air within the pump tubing firstly being allowed to escape to waste before diverting the medium into the bag. In this way the pump tubing and processing bag were primed with the gradient medium, and a buffer zone of medium was created which would allow the whole continuous density gradient to be collected at the end of the procedure. Excess air present within the bag was then expelled to waste by using the supernatant out facility of the COBE 2991 while spinning at 1100 rpm.

Having 'reset' the machine and restarted centrifugation at 1100 rpm, 150 ml each of high (1.115 g/cm³) and low (1.075 g/cm³) density Nycodenz-LRF were then loaded into the appropriate chambers of the gradient maker. With both taps of the gradient maker open, and with the magnetic stirrer adjusted to ensure adequate mixing of the contents in the high-density chamber, the medium contained in the gradient maker was then pumped onto the COBE 2991 at 30 ml/minute during centrifugation at 1100 rpm, resulting in the formation of a 300 ml continuous linear density gradient within the processing bag (Figure 2, Panel C).

The stored digest was then resuspended to a total volume of 100 ml in LRF at 4°C, and this tissue was pumped at 30 ml/minute immediately on top of the (spinning) continuous gradient. During this procedure considerable care was taken to avoid visible aggregation of tissue at the LRF-gradient interface, the pump being stopped temporarily if this was observed to occur, in order to allow tissue to disperse into the gradient.

Finally, a further 50-100 ml of LRF was used to flush the pump tubing, in order to ensure that all the digest had been loaded into the processing bag. Centrifugation at 1100 rpm and 4°C was then continued for a further 5 minutes, to allow all tissues to reach their points of bouyant density.

After centrifugation the supernatant out facility of the COBE 2991 was used firstly to expel the 'waste' LRF from the top of the gradient (volume = (total volume of LRF + digest suspension loaded) - volume of digest, as previously recorded), and then to allow the collection of 10 x 30 ml sequential fractions encompassing the 300 ml
FIGURE 2
Schematic Diagram Illustrating the Formation of Large-Scale Continuous Density Gradients on the COBE Cell Processor

A. Priming

B. Loading the Continuous Density Gradient
C. Loading the Pancreatic Digest

D. Centrifugation
continuous density gradient. From each of these fractions, 100 μl samples were then taken, stained with dithizone and examined by light microscopy, in order to establish the distribution of islets and exocrine tissue within the gradient. On the basis of this examination the fractions considered to contribute islets of optimal yield and purity were then pooled, washed in MEM, and finally resuspended in 100 ml of MEM. From this volume, 5 x 100 μl samples of tissue were then taken for quantification of the purified islets and for visual estimation of the islet purity, as described in Chapter 4. In addition, following a further centrifugation at 200g for 2 minutes, the volume of the purified tissue pellet was also recorded, by marking the level of the pellet on the outside of the centrifuge tube, and, following removal of the tissue, determining the volume of water required to fill the tube up to this level.

Assessment of Islet Viability
Purified islets were cultured free-floating in RPMI-1640 medium (Northumbria Biologics Ltd., Cramlington, UK - see Appendix) at 37°C in 95% air/5% CO₂ for 1 week. Islet viability was assessed by islet perifusion both within 24 hours of isolation, and again after 7 days of culture; and by microfluorometry after 7 day culture.

Islet Perifusions
The perifusion system used was based upon that originally described by Lacy et al (Lacy et al, 1972), and assessed the ability of islets to respond to an increase in extracellular glucose concentration by the biphasic release of insulin.
In each case, 2 groups of 50 islets were handpicked using a drawn-out Pasteur pipette, and trapped within a Millipore perifusion chamber by a plug of glass wool. Using a peristaltic pump this chamber was then perfused at 1 ml/min with Gey and Gey buffer (see Appendix) containing 1.7 mM glucose, at 37°C. After 1 hour, during which the islets were left to re-establish their basal insulin secretion rate, the effluent from each perifusion chamber was sampled during 1 minute intervals (1 ml samples) at the times indicated below, whilst islets were perfused with Gey and Gey buffer containing 1.7 mM glucose during the first 20 minutes, 25 mM glucose over the next hour, and 1.7 mM glucose during the final 60 minutes.

<table>
<thead>
<tr>
<th>Sample Times</th>
<th>Glucose Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,10,19</td>
<td>1.7 mM</td>
</tr>
<tr>
<td>21,23,25,27,29,40,50,60,70,79</td>
<td>25 mM</td>
</tr>
<tr>
<td>90,100,110,120,130,140</td>
<td>1.7 mM</td>
</tr>
</tbody>
</table>
The insulin content of these samples was then assayed (see Appendix), and the data used to plot the profile of insulin release over time for each group of islets (Figure 3). From these plots two stimulation indices were calculated, as a measure of islet viability. The first index was obtained simply by dividing the peak concentration of insulin during stimulation by the mean level during both basal periods. The second was obtained by initially calculating the area under the curve during the period of stimulated insulin release and that during both basal periods, providing a measure of the total amount of insulin released during these periods. By dividing each of these figures by the duration of each period, the mean rate of insulin release during stimulation and under basal conditions could then be determined, allowing a stimulation index (SI) to be calculated as follows:

\[ SI = \frac{\text{Stimulated Rate of Insulin Release}}{\text{Mean Basal Rate of Insulin Release}} \]

For each period in culture, 2 groups of islets were perifused and the mean value of each stimulation index was therefore used as the estimate of islet viability.

Microfluorometry
The membrane integrity of 1-week cultured islets was assessed using a quantitative microfluorometric assay, originally described by London et al. (1990a). In this assay, islets are incubated with two fluorochromes, propidium iodide (PI) and fluorescein diacetate (FDA). The former of these is excluded from cells with an intact plasma membrane, but enters dead or dying cells where it binds to nucleic acids and produces a bright red fluorescence. The latter fluorochrome, however, enters cells and is hydrolysed by intracellular esterases, producing a form which cannot then pass through intact cell membranes and which therefore accumulates within living cells to produce a green fluorescence. The ratio of green to red fluorescence therefore gives an indication of the proportion of cells within islets which have an intact plasma membrane.

The working mixture of fluorochromes was prepared immediately before use from stock solutions of PI (188 µl of a 75 µM solution in phosphate-buffered saline, PBS) and FDA (125 µl of a 24 µM solution in acetone), diluted into 4190 µl of PBS. For each set of cultured islets assessed, ten islets were randomly hand-picked onto glass slides using a drawn-out Pasteur pipette, and incubated with 50 µl of the fluorochrome solution within a humidified darkened case for 100 minutes. Following incubation, the red and green fluorescence of each islet, corrected for the background fluorescence of each slide, was then measured photometrically using an
Typical Example of Insulin Release Profile During Islet Perfusion, Demonstrating the Biphasic Release of Insulin in Response to Glucose-Stimulation

Glucose Concentration of Perfusate

1.7 mM  25 mM  1.7 mM

Insulin Release (ng/min)

Time (Mins)

Area = Total Insulin Release During Stimulation
Area = Total Insulin Release During Basal State

(Area Stimulation Index = 2.07
Peak/Basal Index = 4.2)
Olympus BH2 RFL microscope equipped for fluorescence microscopy, as previously described (London et al, 1990a).

These data could then be used to provide a quantitative estimate of the percentage of cells having an intact plasma membrane within each islet, as follows:

\[
\text{% 'viability' = } \frac{\text{FDA}}{\text{FDA} + (\text{PI}) - (0.04 \times (\text{FDA} - \text{PI}))}
\]

where FDA = fluorescein diacetate fluorescence, PI = propidium iodide fluorescence.

**Results**

The results obtained for the principal variables studied are summarised in Table 2, from which it is apparent that there was considerable variation in the efficiency of both pancreatic digestion and density gradient separation of islets.

The density gradient purification of islets was generally satisfactory (median 52% recovery of islet volume, and median purity 85%), although there was marked variation between pancreata in the efficiency of this process. Because of the reciprocal relationship between islet yield and purity, discussed in Chapter 4, and in the absence of a means to fix one of these variables at a constant value, this efficiency may best be expressed as the product of yield and purity for each pancreas (London et al, 1992a). Median (range) values of gradient efficiency obtained in this way were 45% (18-82%), confirming that islet separation was highly variable, although very effective on occasions (82% efficiency, for instance, was equivalent to a yield of 97% islet volume, with a purity of 85% - Table 3).

Digestion and dispersal of the pancreas, however, was generally disappointing: the mean volume of islets within the digest was less than the mean islet volume determined histologically in the native pancreas of donor pigs (17.4 x 10^{-4} mm^3), suggesting that many islets had become fragmented during collagenase digestion. In addition, the cleavage index (i.e. the percentage of the total islet volume within the digest contributed by cleaved islets) was often poor, indicating that a considerable proportion of the total islet volume also remained trapped within exocrine tissue.

In turn, these factors may have influenced the efficiency of density gradient purification of islets. Thus, the presence of large numbers of uncleaved islets might theoretically compromise the recovery of islet volume, although in this study the relationship between cleavage index and gradient efficiency was relatively weak, and not statistically significant (Spearman's rank correlation coefficient, rho = 0.251, p>0.10; see Figure 4). In addition, however, the mean volume of purified islets was significantly higher.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean w/Vol of Purified Islets</td>
<td>136-2872</td>
</tr>
<tr>
<td>Purity</td>
<td>92%</td>
</tr>
<tr>
<td>Volume of Purified Islets (µL)</td>
<td>1.61</td>
</tr>
<tr>
<td>Yield</td>
<td>52%</td>
</tr>
<tr>
<td>Mean W/Vol of Pellet (µL)</td>
<td>85</td>
</tr>
<tr>
<td>Purification Viability (µL)</td>
<td>92%</td>
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TABLE 3

Islet Yield, Purity and Calculated Gradient Efficiency for Each Pancreas Undergoing Large-Scale Islet Isolation

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<th>Gradient Efficiency (%)</th>
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FIGURE 4
The Relationship Between Gradient Efficiency and the Percentage and Volume of Cleaved Islets in the Pancreatic Digest
than that of islets present within the crude pancreatic digest (Median Difference in Volume = 3.2 x 10^4 mm^3; 95% CI of the difference = 0.4 to 14.1 x 10^4 mm^3, p=0.044; Wilcoxon signed rank test for paired data). Furthermore, analysis of the frequency distributions of islet diameters within the crude pancreatic digest and in fractions of purified islets often demonstrated that, whilst in both tissue samples the majority of islets were of 50-100 μm diameter, these smaller islets comprised a much higher proportion of the total islets in the crude digest, compared to purified tissue which therefore contained a correspondingly higher proportion of larger islets (>200 μm diameter) (Figure 5). Finally, there was also a broad correlation between gradient efficiency and the mean volume of cleaved islets within the crude digest (rho = 0.442, (95% CI from -0.260 to 0.838), p>0.10 - see Figure 4). Taken together these data suggest: firstly that a proportion of those islets less than 100 μm in diameter in the pancreatic digest were in fact fragments of larger islets, produced by overdigestion of the pancreas; secondly that these fragments were selectively lost during purification using the system described; and thirdly that the efficiency of islet purification was therefore dependent partly upon the quality of the preceding digestion and in particular upon maintaining the integrity of larger islets.

**Discussion**

Formal comparison between the Nycodenz-LRF density gradient medium described above and other gradient media available for islet purification has not been performed in this study. Nonetheless, the results outlined above have provided useful information concerning the efficiency of islet separation which can be achieved using storage and density gradient media specifically designed for this purpose, in conjunction with the use of large-scale continuous linear density gradients at 4°C on the COBE cell processor. In particular, it has been possible to translate the improvements in gradient efficiency, documented in the preceding chapters in terms of the percentage exocrine tissue contamination at 60% islet yield, into the recovery of islet volume and the fractional purity of the final preparation, parameters which are more readily appreciated conceptually than the percentage exocrine contamination (although the latter method is superior for quantitative comparison between matched pairs of gradients, as previously discussed). Finally, important data has also been supplied concerning the viability of islets isolated using the solutions optimised in the preceding work. Using the system described above, continuous linear density gradients of Nycodenz-LRF were formed rapidly and easily on the COBE cell processor, and the entire
FIGURE 5

A Representative Frequency Distribution of Ilets Within Foci of Lumped Pancreatic Ilets
pancreatic digest could be purified within 30-40 minutes, following top-loading onto these gradients. Potentially, there are several advantages to this approach: Firstly, as discussed in Chapter 3, continuous density gradients have a reduced propensity for the production of artifacts during centrifugation, and have an increased gradient capacity, compared to discontinuous gradients, thereby reducing the time and cost involved in processing large volumes of tissue. In this study, for instance, the packed volume of crude pancreatic digest was often greater than 20 ml, a volume which would usually necessitate the formation of more than one discontinuous gradient in order to avoid exceeding its capacity, but which was readily processed using a single continuous gradient.

Secondly, with continuous gradients islet purification can be optimised for each individual pancreas using gradient media of predetermined density, merely by selecting the gradient fractions containing islets of the desired purity following separation. Using discontinuous gradients, however, this can only be achieved by the use of preliminary test gradients in order to select for each pancreas the appropriate density of each gradient layer, a process which then adds considerably to the time required for purification.

Thirdly, the one or two fractions of continuous gradients immediately below those selected for their content of highly purified islets often contain the majority of the residual islet volume, and a relatively small percentage of the total pancreatic volume of exocrine tissue. The salvage of islets from these fractions using a secondary islet purification technique might therefore be feasible. In discontinuous gradients, however, these islets are contaminated with the entire residual volume of exocrine tissue not present in the purified fractions, and are therefore less suitable for secondary purification procedures, which, as discussed in Chapter 3, are often of limited capacity.

Finally, top-loading the pancreatic digest onto the gradient has the advantages that dispersed pancreatic tissues can be maintained for a longer period of time in more physiological storage media, and that islets are exposed to lesser centrifugal forces than those experienced during bottom-loading, because they are never situated distal to their point of buoyant density within the gradient. Similarly, when using media such as Ficoll, in which osmolality increases with density, the exposure of islets to the more hypertonic parts of the gradient may also be avoided.

Despite these advantages, however, the efficiency of islet purification using gradients of Nycodenz-LRF, although generally satisfactory, was highly variable, and not apparently greater than that reported for other gradient media used for porcine islet isolation (Ricordi et al, 1986; Ricordi et al, 1990c; Marchetti et al, 1991; Finke et al, 1991). Potentially, however, several factors unrelated to the gradient medium itself may have contributed to these findings.
Firstly, as discussed in Chapter 4, assessment of gradient efficiency by analysis of islet quantification data is subject to considerable error. In particular, the true efficiency of islet separation cannot be accurately estimated from the islet yield and purity alone, as the relative proportions of islets to the other pancreatic tissues in the unpurified digest are unknown.

Furthermore, the process of islet quantification is itself subject to marked inter-observer error and a tendency to overestimation of islet volume, particularly within the purified fraction of islets. In this study the extent of this latter error was estimated by comparing the values for the final volume of purified islets obtained by direct measurement of the purified fraction volume (corrected for islet purity) and by estimation from the islet quantification data. As demonstrated in Figure 6, although there was a close correlation between these two estimates (Pearson's product-moment correlation coefficient, $r^2 = 0.89$, $p<0.001$), the total islet volume determined from islet quantification data tended to be higher than that determined by direct measurement, particularly for larger values of islet volume. Although the method used for measuring the volume of the final tissue pellet (see above) is subject to some underestimation of the true volume, due to the presence of residual fluid within the centrifuge tube following removal of the tissue sample, this error is generally small, and becomes less significant at higher tissue volumes. Islet quantification data therefore appear to overestimate total islet volume, the extent of this overestimation being approximately 41% (95% CI = 14 to 68%) of the mean value obtained by both quantification and measurement (Figure 6).

Secondly, the efficiency of islet separation is also dependent upon the quality of the preceding digestion of the pancreas. Uncleaved islets, for instance, cannot be separated efficiently from exocrine tissue by density-dependent techniques, so that gradient efficacy may be compromised by under-digestion of the pancreas, with the production of large numbers of uncleaved islets. In this study, the median cleavage index was only 74%, whilst a broad correlation did exist between cleavage index and gradient efficiency, suggesting that the true efficiency of islet separation using the Nycodenz-LRF gradient medium was higher than that estimated above (if, for example, the yield of purified islets is corrected for each pancreas, to allow for the cleavage index of the digest, the estimated (median + range) recovery of cleaved islets in this series becomes 74% (41-100%)).

In addition, the data outlined above suggest that islet recovery from the density gradient is also compromised by overdigestion of the pancreas, fragmented islets being selectively lost during purification. Digestion of the porcine pancreas is in turn dependent upon factors such as the age and breed of pigs used (Stocci et al, 1990), warm and cold ischaemic intervals (Ricordi et al, 1990b), and the batch of collagenase.
FIGURE 6
The Discrepancy Between Total Islet Volume Determined by Direct Measurement and That Estimated from Islet Quantification Data.

A. Correlation Analysis

B. Bland-Altman Analysis
used, so that comparison of gradient efficiency between reported series is highly problematical.

It is clear, therefore, that formal comparison between Nycodenz-LRF and other gradient media, using experiments similar in design to those detailed in the preceding chapters, will be necessary to establish the relative benefits of these media. The data from the present series, however, suggest that Nycodenz-LRF is a relatively efficient density gradient medium, particularly considering the quite prolonged period of warm ischaemia suffered by pancreata during retrieval, and the comparatively poor results of pancreatic digestion alone.

Furthermore, the viscosity of Nycodenz-LRF gradient solutions is markedly less than that of media such as BSA and Ficoll: for example, viscosity measurements (Brookfield Microviscometer, Brookfield Engineering Labs. Inc., Stoughton, Massachusetts, USA) on Nycodenz-LRF and BSA solutions of appropriate density for islet isolation revealed values of 0.78 and 0.90 cps for Nycodenz-LRF solutions of densities 1.080 g/cm\(^3\) and 1.115 g/cm\(^3\) respectively, compared to 1.30 and 5.90 cps for BSA solutions of density 1.070 g/cm\(^3\) and 1.106 g/cm\(^3\). This low viscosity allowed Nycodenz-LRF gradients in this series of experiments to be operated at lower centrifugal forces than those usually employed with BSA or Ficoll, and therefore represents one advantage in the use of this medium over those currently used for islet isolation.

Finally, the viability in vitro of porcine islets purified following storage of digest in LRF solution was uniformly good. Even after 1 week in tissue culture, over 80% of islets could still be recovered, and 95% (median) of cells within randomly-selected islets demonstrated normal plasma membrane integrity, as assessed by microfluorometry. In addition, in every case islets were able to respond to an increase in glucose concentration during in vitro perifusion with a biphasic release of insulin (Figure 3).

Several methods have been described for calculating stimulation indices from perifusion data, but the most commonly used involve either division of the peak insulin release by the mean basal level of release or a comparison of the total insulin released (area under the insulin release profile with time) during stimulation with the total released during the basal periods (Warnock et al, 1989b; Clayton et al, 1990). The majority of studies have employed the former (peak/basal) index, although the latter index may be more indicative of islet viability, as it also incorporates assessment of the second phase of glucose-stimulated insulin secretion, and the ability of islets to terminate insulin release rapidly upon discontinuation of the stimulus. Both indices were therefore calculated in the present investigation: the former facilitated comparison with other studies, and thereby demonstrated that insulin release from islets isolated in the present investigation was at least as good as that observed from porcine islets in many studies (Ricordi et al,
1986; Marchetti et al, 1989; Calafiore et al, 1990b; Finke et al, 1991), and superior to that observed in other cases (Marchetti et al, 1991; Davalli et al, 1992). The latter index allowed more accurate comparison to be made of changes in islet viability during culture, and demonstrated that viability was uniformly good, and that no significant changes occurred during culture between 24 hours and 7 days.

SUMMARY

The effective purification of islets from porcine pancreata undergoing prolonged warm and cold ischaemia is considerably challenging, and such pancreata therefore represent a suitable model for investigating factors likely to improve human islet isolation. Reasonably efficient purification can now be achieved by optimising both the physical procedures of density gradient separation, using large-scale continuous linear gradients at 4°C on the COBE cell processor; and the biochemical milieu of pancreatic tissues during storage of the dispersed pancreas and during density gradient purification. Furthermore, the viability of islets isolated in this way is maintained at a high level. Nonetheless, the efficiency of islet purification remains variable, and further room for improvement clearly exists. The results of this study suggest that such improvement will require not only the development of optimised storage and density gradient solutions, but also the enhancement of techniques for collagenase-digestion and dispersal of the pancreas, which may itself influence the efficacy of subsequent islet purification. Theoretically, future developments in both of these areas of research may be influenced by the findings detailed in this thesis, and this potential will therefore be examined in the following chapter.
PLATE 9.1
Porcine Islets Purified on Nycodenz-LRF Density Gradients Following Storage in LRF Solution

A. Islets stained with dithizone to demonstrate islet morphology and the high degree of purification achieved

B. A single islet stained with Propidium Iodide (left) and Fluorescein Diacetate (right) to demonstrate the proportion of cells with respectively damaged or intact membranes
CHAPTER 10

Summary and Prospects for Future Work
One of the principal factors limiting the clinical application of islet transplantation as a therapy for insulin-dependent diabetes mellitus is the poor yield of viable islets obtained using current techniques of isolation. The process of islet purification is responsible for much of the loss of islets during isolation. It is likely, however, that a reasonably high degree of islet purification will be necessary before transplantation to enhance islet engraftment; to facilitate islet culture and immunomodulation; and to ensure the safety of transplantation, thereby potentially facilitating the most promising application of islet transplantation, namely the prevention of diabetic complications by reversing diabetes soon after diagnosis in non-immunosuppressed patients.

The work described in this thesis has confirmed that the efficiency of purification of human and porcine islets using current techniques of density gradient centrifugation is disappointingly low. It has also demonstrated, however, that the results of islet purification do not depend solely upon the conditions used at the stage of purification itself, but that changes occurring during the preceding phases of islet isolation may also have a profound influence. Indeed, an appreciation has developed that the densities of pancreatic tissues, and in particular the relative densities of acinar tissue and islets, are dependent upon the physico-chemical environment in which these tissues are maintained throughout the isolation process, and that the control of cellular volume is the principal mechanism responsible for determining changes in tissue density during isolation.

Using a standardised, quantitative system for assessing the efficiency of density gradient purification of islets, it has been demonstrated that purification is improved by storage of the dispersed pancreas in solutions, such as UW, which reverse pre-existing acinar cell swelling. Furthermore, impermeant anions and a colloid are the components of these solutions principally responsible for this effect: simplified solutions containing only these components, magnesium sulphate and a buffer are at least as effective as more complex solutions designed for prolonged cold storage of whole organs. The sodium:potassium ratio and pH of such solutions do not, however, have a marked influence, and optimal islet purification is compatible with the use of 'physiological' values for both of these variables.

In addition to their documented benefit when used for storage of the dispersed pancreas, it is possible then that further improvements in islet purification may follow the use of such solutions during the other phases of islet isolation. Their use as a solvent for density gradient media is one such approach, and the preliminary observations made in this study using this approach have been quite encouraging, as have those made recently by other groups using 'complete' UW as the solvent in various media (van der Burg et al., 1991b; van der Burg et al., 1992; Arbet-Engels et al., 1992). The results obtained remain limited, however, by the quality of the
preceding digestion of the pancreas, with the production of well-cleaved, but intact islets. It is therefore likely that advances in digestion and dispersal of the pancreas will be necessary before optimal islet purification can be achieved. Although such advances will depend largely upon the development of collagenases optimal for the digestion of large mammalian pancreata, and an improved understanding of the actions of these enzymes, the use of storage solutions similar to those developed in this thesis may also have an important role to play.

Thus, because deterioration in the integrity of the pancreatic duct occurs during cold storage, optimal digestion will probably depend upon intraductal distension of the pancreas with collagenase solution prior to preservation. However, whilst in dogs UW solution appears to be the best medium for delivery of collagenase in this way (Munn et al, 1989), similar use of UW in the human pancreas markedly reduces the yield of viable islets obtained (Toomey et al, 1991). This is probably due to the fact that UW contains certain components which inhibit collagenase digestion of the human pancreas. Thus, pancreata undergoing in situ vascular perfusion with UW subsequently require a higher concentration of collagenase for effective digestion, even if this is itself administered in Hank's solution (Robertson et al, 1993a). In addition, the digestion of portions of human pancreas incubated in vitro is markedly delayed if collagenase is dissolved in UW, rather than Hank's solution (Contractor et al, 1993). Interestingly, this effect is only observed with the human pancreas, the digestion of porcine pancreata being unaffected by UW (Contractor et al, 1993), thereby potentially explaining the above-mentioned species-dependent differences in optimal collagenase delivery solutions.

Although Hank's solution has been used for the intraductal administration of collagenase prior to cold storage of the human pancreas (Socci et al, 1993), this medium is not likely to provide optimal islet purification or viability, as its composition (with high concentrations of sodium and chloride ions) will contribute to cellular swelling under hypothermic conditions. For the human pancreas, therefore, it will be necessary to develop solutions which do not inhibit collagenase digestion, but which remain suitable for use during cold storage. In this respect, the work described in this thesis, in conjunction with other studies performed concurrently in this Department, may contribute to the development of such solutions. Thus, it has been observed that the inhibitory action of UW in human pancreatic digestion is largely due to the glutathione, allopurinol and low Na⁺:K⁺ ratio present (Contractor et al, 1993). In contrast, however, the present investigation suggests that these components are unimportant in determining the relative densities of islets and acinar tissue, and therefore in determining islet purity, following cold storage. It is possible therefore that the simplified solutions described in this thesis, containing only impermeants, colloids
and buffers may also represent the most appropriate media for collagenase administration prior to pancreatic preservation. Thus, by using these (or similar) solutions for cold storage of the intact and the dispersed pancreas, and during density gradient centrifugation, the densities and viability of pancreatic tissues may be better maintained, resulting in improved islet purification. Future research may then be directed towards further optimising such solutions for the individual phase of isolation during which they will be used.

In order to optimise solutions for collagenase delivery and storage of the whole pancreas, the collagenase-inhibitory influence of the important components, such as impermeants, colloids and buffers, alone and when used in combination must be further examined. In addition, the use of several other potentially beneficial agents may be worthy of research: for instance, the amino acid, glycine has been reported to have a profound protective effect upon other types of cells isolated by collagenase digestion (Weinberg et al, 1987; Marsh et al, 1991), and may therefore be of benefit during islet isolation. In preservation of the whole pancreas, the inclusion of another amino acid, histidine, in lactobionate-containing solutions has also improved results (Sumimoto et al, 1992), and investigation of similar effects upon islet isolation therefore seems appropriate. The calcium concentration of such solutions is also likely to be important, given the known influence of calcium in the control of cellular volume (MacKnight et al, 1977; Lindell et al, 1989); in the maintenance of intercellular adhesion (Garrod et al, 1981), particularly during collagenase digestion (Dono et al, 1992a); and in suppressing lipid peroxidation in isolated cells during hypothermic preservation (Umeshita et al, 1988). Finally, the effects of agents such as BSA (Wolters et al, 1989) and benzamidine (Korbutt et al, 1992), which limit the release of excessive protease activity during collagenase digestion, and the influence of oxygen free-radical scavengers other than glutathione (eg. superoxide dismutase, which has improved islet yield when used for pretreatment of rat pancreas donors (Fonseca et al, 1987)) may also be worthy of investigation.

Digest storage solutions and density gradient media may also be improved by the inclusion of some of these agents (eg. calcium and histidine), due to their potential effects upon cellular volume/density and viability during cold storage. Further investigation into the mechanism of action of colloids in influencing cell volume, and determination of the optimal concentration necessary for this action is also likely to be important.

Finally, further optimisation of density gradient media will require research into the most suitable solute to be used, and possibly also into the effects of altering other features of continuous density gradients, for instance by the production of osmolality gradients. In such gradients, the tonicity of the gradient solution is made to vary
continuously between high and low density regions, thereby producing changes in the bouyant density distributions of cells during centrifugation. When populations of one cell type (e.g. erythrocytes) are separated on such gradients, their density distribution curves are simply either broadened or narrowed, depending on whether the osmolality gradient is positive (i.e. osmolality increases with increasing density) or negative (i.e. osmolality decreases with increasing density) respectively (Leif, 1970). It has been established, however, that the densities of pancreatic exocrine tissue and islets can be differentially affected by an increase in gradient medium osmolality, the density of exocrine tissue increasing to a greater degree than that of islets (London et al, 1992c; Chadwick et al, 1993). Theoretically, therefore, the introduction of an osmolality gradient might improve the separation of these two cell types in a continuous density gradient, although its influence may also depend upon whether tissue was top or bottom-loaded onto the gradient. During this study, preliminary experience in the use of such gradients (using visual assessment of dithizone-stained fractions) suggested that this was indeed the case, and that islet separation was optimal following top-loading of digest into a positive osmolality gradient. On theoretical grounds alone this might be expected, as exocrine tissue initially overlapping in density with islets might increase in density in response to an increment in tonicity more rapidly than islets, thereby moving into ever more hypertonic areas of the gradient, with further consequent increases in density and therefore separation from islets. Indeed, the presence of a positive osmolality gradient in the Nycodenz-LRF gradient described in Chapter 9 may have been partly responsible for the relatively high efficiency of islet purification achieved following adequate pancreatic digestion. Unfortunately, top-loading of digest in test-tubes results in unacceptable aggregation of tissue in the upper fractions of the gradient. Further investigation of this issue will therefore require the formation of gradients varying continuously both in density and osmolality on the COBE cell processor.

In conclusion, the work described in this thesis has contributed to an improved understanding of the factors potentially influencing the relative densities of pancreatic tissues during islet isolation. With further research this knowledge may be instrumental in enhancing the efficiency of islet purification from human and other large mammalian pancreata, and thereby advancing the application of islet transplantation as a clinical therapy for diabetes.
APPENDIX

ASSAY METHODS AND COMPOSITION OF COMMONLY USED REAGENTS

Amylase
Amylase was measured using a Phadebas® Amylase Test Kit (Pharmacia Diagnostics AB, Uppsala, Sweden). The substrate for this assay is a water-insoluble, cross-linked starch polymer, the hydrolysis of which is catalysed by amylase within the test sample to yield water-soluble fragments labelled with a blue dye, which is subsequently detected by spectrophotometry.

Assays were performed as follows:

1. Four ml of distilled water were pipetted into 10 ml conical test-tubes, incubated in a water bath at 37°C.
2. To each tube 200 μl of the test samples were added, including plain MEM as a control.
3. Following incubation in the water bath for at least 5 minutes one tablet of the polymer substrate was added to each tube, and the tube vortexed for 10 seconds before replacement in the water bath. Multiple test samples were assayed by serial addition of tablets to sequential tubes at exactly 15 second intervals.
4. All tubes were incubated for exactly 15 minutes, following which 1 ml of 0.5% sodium hydroxide was added serially to each of the tubes (to stop the hydrolysis reaction), which were again vortexed for 10 seconds, as above.
5. All tubes were centrifuged at 1500g for 5 minutes, and 1 ml samples of the supernatants were aspirated into cuvettes (67.742, Sarstedt, Numbrecht, Germany).
6. Supernatant absorbance was measured at 620 nm (LKB Ultrospec 4050 spectrophotometer, LKB Biochrom, Cambridge, UK), against the MEM blank. These data were then used to calculate the amylase activity of the test samples, using a batch-coded standard curve supplied with the test kit.

Experience in the use of this assay with the fractions aspirated from the 11 ml continuous gradients described in Chapter 4 revealed that obtaining accurate results was dependent upon selecting an appropriate dilution of the original sample. Thus, because the content of amylase within pancreatic acinar tissue is so high, even when gradients
were loaded with only 100 μl samples of pancreatic digest, certain gradient fractions could contain sufficient amylase to hydrolyse all the assay substrate in less than 15 minutes. As this would result in underestimation of the true amylase content of these fractions, it was therefore necessary to dilute the original samples obtained from these gradient fractions until their amylase content came within the assay range.

As it could not be assumed that the adjusted amylase values would remain constant at all sample dilutions using MEM as a diluent, all samples from an individual gradient were diluted to the same extent prior to assay. This was generally best achieved using a dilution of 1:10 to 1:20 with MEM. At this dilution the amylase levels of samples from both acinar cell-rich and from acinar cell-depleted gradient fractions (which may differ several hundred-fold from each other in amylase content) fell within the assay range.

Insulin

Insulin was measured using a standard radioimmunoassay. In this assay system the binding of guinea-pig antibodies to radiolabelled $^{125}$I-insulin is competitively inhibited by the insulin present within the test sample. Antibody-bound insulin is subsequently precipitated by the addition of anti-guinea-pig antibodies attached to cellulose microspheres. The radioactivity of the precipitate is therefore inversely related to the insulin content of the test sample, which can therefore be calculated from a standard curve produced by concurrent assay of standard samples containing known quantities of insulin.

The reagents used and procedure followed are outlined below.

Reagents

RIA Buffer: 0.5% BSA (A7030, Sigma) in Phosphate-buffered saline (PBS)

Guinea-pig anti-bovine serum (ICN Biomedicals Ltd., High Wycombe, Bucks.), 1:20,000 in RIA Buffer

Radiolabelled Insulin: $^{125}$I-iodotyrosyl A14 Human Insulin solid (1.85 MBq, Amersham International Ltd., Amersham), dissolved in 500 μl of RIA Buffer and divided into 20 μl aliquots. Each 20 μl aliquot diluted into 5-6 ml RIA Buffer before use, to give 20-30 x 10^3 CPM per tube

Second Antibody-Coated Cel lulose (SAC-CEL): Donkey anti-guinea-pig coated cellulose (IDS, Washington, UK), 1:2 in RIA Buffer
Human Insulin Standards: 8 ng human insulin in 40µl serum (Novo Biolabs Ltd., Cambridge, UK), diluted into 960 µl RIA Buffer and then serially diluted to concentrations of 4, 2, 1, 0.5, and 0.25 ng/ml.

Procedure

1. All samples were run in duplicate, in LP3 tubes (SS.483, Sarstedt) and test samples were first diluted appropriately in RIA Buffer. As with the amylase assay, all samples from an individual gradient were diluted to the same extent prior to assay.

2. To each 50µl of test sample was added 50 µl of guinea-pig serum. The following duplicate control tubes were also included in each assay:

   - Total Counts: (50 µl of ¹²⁵I-Insulin only)
   - Blanks: (100 µl RIA Buffer; no guinea-pig serum)
   - Standards (50 µl of each standard concentration including RIA Buffer alone (0 ng/ml) + 50 µl of guinea-pig serum)

3. Each tube was vortexed and incubated at room temperature for 30 minutes.

4. Each tube received a total of 50 µl of ¹²⁵I-Insulin, prior to vortexing and incubation for 90 minutes at room temperature.

5. 50 µl of SAC-CEL was added to all tubes (except the Total Count tubes) and each was incubated for a further 30 minutes.

6. To each tube (except the Total Count tubes) 1 ml of 0.1% Triton X-100 in distilled water was added. These tubes were then centrifuged at 3500g for 6 minutes, following which the supernatants were carefully poured off, the tubes capped, and subsequently counted in a gamma counter (1282 Compgama CS, LKB, Croydon, UK) connected to a computer terminal for automatic calculation of insulin content in the test samples.
**Common Reagents**

**Minimum Essential Medium (MEM) for Islet Isolation**

Made up in 4 litre containers:

- 400 ml x10 MEM (Northumbria Biologicals Ltd.)
- 40 ml 10 mM HEPES
- 80 ml Penicillin + Streptomycin (100 U/ml)
- 40 ml Fungizone

**Phosphate-Buffered Saline (PBS, pH 7.4)**

Made up in ddH₂O:

- 8.4 mmol/l Na₂HPO₄
- 1.6 mmol/l NaH₂PO₄
- 150 mmol/l NaCl

**Gey and Gey Buffer for Islet Perifusion**

- 110 mmol/l NaCl
- 27 mmol/l NaHCO₃
- 5 mmol/l KCl
- 1 mmol/l MgCl₂.6H₂O
- 0.28 mmol/l MgSO₄.7H₂O
- 0.2 mmol/l KH₂PO₄
- 0.8 mmol/l Na₂HPO₄
- 2 mmol/l CaCl₂

+ 1.7 or 25 mmol/l Glucose

Gassed with 95% air/5% CO₂ to maintain pH = 7.4
**RPMI-1640 Medium for Islet Culture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640 (Northumbria Biologicals Ltd.)</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td>10% Fetal Calf Serum (S-0001a, SeraLab, Sussex, UK)</td>
<td>50 ml</td>
<td></td>
</tr>
<tr>
<td>Penicillin + Streptomycin (100 U/ml)</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>1 mmol/l</td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.1 mmol/l</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.01 mmol/l</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>10 mmol/l</td>
<td></td>
</tr>
</tbody>
</table>

1 ml of 200 mM L-Glutamine added immediately before use
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