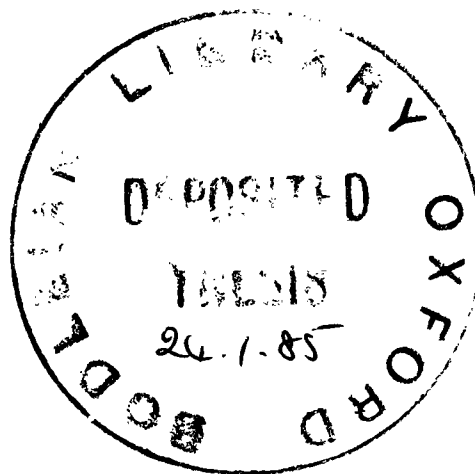


EXPERIMENTAL PANCREATIC ISLET TRANSPLANTATION

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Two major problems preventing the clinical application of pancreatic islet transplantation were investigated. The problem of allograft rejection was studied in rats, made diabetic by streptozotocin treatment. It was shown that DA rats given LEW renal allografts and treated with cyclosporine accepted their grafts, and subsequently developed a strain-specific unresponsive state that allowed successful transplantation of LEW islets without further immunosuppression, whilst BN islets were rejected normally. The effect was demonstrated to be independent of the site of islet transplantation, and, once an islet allograft had been accepted, it was possible to remove the original renal allograft without affecting the transplanted islets. The effect was shown to apply to another strain combination (LEW into PVG), and also to animals made unresponsive to renal allografts by another method (donor-specific blood transfusion).

The problem of separation of adequate numbers of viable islets from the pancreas was studied in the rat, dog, pig and human. To aid the investigation, supravital staining techniques were developed, using neutral red to identify the islets, and fluorescein diacetate and ethidium bromide to assess islet viability. A variety of islet isolation techniques were investigated, and a new technique for isolation of islets from the dog pancreas, yielding up to 160,000 islets from 1 pancreas with a maximum purity of 80%, was developed. The structural integrity and in vitro function of the isolated islets was demonstrated, but it was not possible to prevent diabetes by autotransplantation of islets to the portal vein of pancreatectomised dogs.

A method for isolation of islets from the human pancreas was developed from that used in the dog, yielding up to 80,000 islets from a whole pancreas, with a maximum purity of 40%. The technique was shown to be both simple and reliable. The structural integrity and in vitro function of the isolated islets was demonstrated, and the viability of the islets proven by successful transplantation under the kidney capsule of nude mice.

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If I have achieved anything, it is due to the encouragement, trust and patience shown by my parents. Lastly, and most importantly, I should like to thank my wife Anne, for being herself.

LIST OF ABBREVIATIONS

ALS	Antilymphocyte serum
BN	Brown Norway
DA	Dark Agouti
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
F1	First cross
FDA	Fluorescein diacetate
FG	French guage
HBSS	Hank's balanced salt solution
HG	High glucose (20 mmol/litre)
ISRG	Insulin secretion in response to glucose stimulation
IV	Intravenous
Ia ⁺	I-region associated antigens present
LEW	Lewis
LG	Low glucose (2 mmol/litre)
MHBSS	Modified Hank's balanced salt solution
RT-1	Rat transplantation antigen 1
SD	Standard deviation
SEM	Standard error of the mean
°C.	Degrees Centigrade
u	Microns
ul	Microlitres
w/v	Weight to volume
x G	Times the force of gravity

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INTRODUCTION AND HISTORICAL REVIEW

DIABETES MELLITUS

It seems likely that the disease we now call diabetes mellitus has been an affliction of man for as long as records have been kept. One of the most obvious features of diabetes is the presence of polyuria, and clear reference to such a condition is made in ancient texts such as the Ebers Papyrus, dated approximately 1500 B.C.(Ebell 1937). The fascinating history of diabetes has been accurately documented elsewhere (Papaspoulos 1952, Wrenshall et al. 1962, Wellmann and Volk 1977, Labhart 1978, Bliss 1983) and it is intended to present only the more important events here. The first full description of diabetes is ascribed to Arateus of Cappadocia (A.D.81-138), who also coined the term "diabetes", which means "to run through a siphon" in Ionic Greek (Wrenshall et al. 1962). Diabetes was recognised in many civilisations. Japanese and Chinese physicians described diabetes in the early centuries A.D. (Marble et al. 1971). Indian physicians in the 5th and 6th centuries A.D. referred to "urine of honey" (Malins 1968), and Arabian physicians, of whom the best known was Avicenna (A.D. 960-1037), observed all the diabetic symptoms, and described the complications of gangrene and loss of sexual function (Wellmann and Volk 1977).

The condition was not properly recognised in Europe until much later, the first real description being ascribed to the magnificently named Swiss physician: Aureolus Theophrastus Bombastus von Hohenheim (A.D. 1493-1541), who affected the title Paracelsus (Wrenshall et al. 1962). Apart from describing the symptoms, he also evaporated the urine and obtained a white powder which he mistook for salt. The rediscovery of sugar in the urine is attributed to Thomas Willis (1621-1675), who also distinguished a form of diabetes without sugar in the urine - diabetes insipidus (Pordage 1684). The adjective mellitus was subsequently added by William Cullen. (Wellmann

and Volk 1977).

The association of diabetes with the pancreas was not made for a further 2 centuries. Although J.C. Brunner (1653-1727) performed total pancreatectomy in dogs and noted polydipsia and polyuria, he failed to recognise diabetes (Labhart 1978). In 1889 von Mering and Minkowski repeated the experiment of total pancreatectomy in dogs to see if survival was possible (Von Mering and Minkowski 1890). Their attention was drawn to presence of sugar in the urine by an assistant mentioning that the urine of these dogs attracted flies (Busnardo et al. 1983). Following this discovery it was initially assumed that the exocrine secretions of the pancreas in some way prevented diabetes, but this was disproved by Minkowski (1892,1893) who showed that dogs did not develop diabetes following pancreatectomy if a portion of pancreas was embedded, with it's vascular supply, in the abdominal wall. Removal of the tissue caused diabetes to ensue, thus proving that the pancreas prevented diabetes by a factor other than the exocrine secretions. These findings led to the suggestion that diabetes was prevented by some form of internal secretion, and the same year Laguesse (1893) suggested that small clusters of cells within the pancreas, originally described (without knowledge of their function) by Paul Langerhans in 1869, might be the source. Laguesse called them the islets of Langerhans. Confirmation that the islets were the source of the internal secretion was provided by Ssobelow (1902), who showed that duct ligation caused atrophy of the exocrine tissue but left the islets intact and the animals did not develop diabetes, whilst Opie (1901) showed that subsequent removal of the duct-ligated gland caused diabetes to ensue.

Insulin

From the beginning of the 20th century the hunt was on to isolate the

internal secretion of the pancreas. So sure were investigators of its existence that it was even given a name - insulin - by de Mayer in 1909.

Numerous investigators tried a variety of extraction methods, but only succeeded in producing preparations that were either ineffective or highly toxic, and a general air of despondency settled over the subject, with suggestions that the internal secretion was not extractable. In 1921 Banting and Best started a series of experiments to try and extract the internal secretion from the pancreas of the dog. It is said that they were unaware of much of the work (and gloomy reports) of previous investigators. They decided to use duct-ligated dog pancreas and, for reasons which remain unclear, used a cold, acidic solution for extraction. By choosing these conditions they avoided the pitfalls that had caught previous investigators: digestion of insulin by exocrine enzymes and denaturation in alkaline solution. Their extract was able to reverse diabetes on injection into diabetic dogs, and so dawned the era of insulin therapy for diabetes.

Banting and Best rapidly adapted their method to extract insulin from calf pancreas and first injected their extract into a diabetic patient in 1921, with dramatic results (Banting and Best 1922(a)). From there the use of insulin spread rapidly. At first the effects of the available preparations were erratic, but the development of a more reliable large-scale extraction method from the bovine pancreas by Collip led to much better control (Banting et al. 1922(b)), and insulin was in general use for diabetes throughout Europe and North America by 1925.

Diabetic complications

The most dramatic features of diabetes prior to insulin therapy were progressively increasing thirst, polyuria and weight-loss, progressing eventually to coma and death. However, many patients survived for some

years even before the advent of insulin, particularly if they adhered to the diet therapy first advocated by Rollo (1797), and in these long-surviving patients other complications of diabetes were noted. Gangrene was a well-known complication, even recognised by Avicenna (A.D. 960-1037) (Wellmann and Volk 1977), and arteriosclerosis was a recognised associated condition (Leyton 1936(a)). Diabetic cataract and retinopathy with retinal haemorrhages and exudates were also well known (Leyton 1936(b)). Diabetic neuropathy was described by Marchal de Calvi in 1864.

The effect of insulin on the most obvious features of diabetes was dramatic, leading to a rapid reduction in the number of deaths from coma and an apparent return to normal health of previously cachectic individuals. The realisation that the other complications of diabetes were not similarly receding came only slowly. Joslin (1934) noted that the incidence of gangrene in diabetics was rising - from 5.1% in 1914 to 10.4% in 1934. Also in 1934, O'Brien et al. found that young diabetics were developing cataracts, despite insulin treatment, and the complex continuing effects of diabetes on the nervous system were recognised by Jordan in 1936.

Also in 1936, Braun documented the continuation of diabetic retinopathy, and noted it's relationship to vascular changes. He also noted a new feature of diabetes, correlating retinopathy with renal changes. The same year Kimmestiel and Wilson presented a report of 8 cases of diabetes with a common clinical syndrome of renal failure associated with characteristic pathological changes in the kidney. These features were subsequently confirmed by Allen (1941). Since these early reports the long-term complications of diabetes treated with insulin have been amply documented, and the problem continues today. Thus in 1978 Pirart reported that juvenile-onset diabetes of 20-25 years duration is associated with a 45% incidence of clinical neuropathy, 55% incidence of

retinopathy and 15% incidence of nephropathy. These complications are associated with a considerable mortality, thus the lifespan of juvenile diabetics is shortened by an average of 20 years (Deckart 1978), and, in the United Kingdom, diabetes was the cause of 448 deaths in patients under 50 years in 1979 (Tunbridge 1981). Furthermore, diabetes is a cause of considerable morbidity, being now the commonest cause of blindness between the ages of 30 and 64 (Kohner et al. 1982), and an increasing proportion of patients require surgery for peripheral vascular disease and gangrene as a result of diabetes (Jarrett et al. 1982). Diabetes was the cause of 1.55 % of United Kingdom hospital beds occupied in 1981 (D.H.S.S. 1983). The number of diabetics in the U.K. is estimated at between 1 and 2% of the total population (Watkins 1982), the exact figure being uncertain, but estimated at a total of 1,200,000 by the British Diabetic Association (1984). Many of these have a fairly mild form of the disease, usually of the late-onset type, and may escape the complications described. Nevertheless diabetes is clearly a major problem in health care in Great Britain today.

The aetiology of diabetic complications

Since it has been realised that insulin therapy does not prevent the long-term complications, considerable effort has been directed towards defining their aetiology in the hope that preventive measures will be found. Numerous mechanisms have been proposed, but it is not within the scope of this discussion to mention them, except to point out that central to most theories is the idea that the basic cause of the complications is inadequate control of the blood glucose; the corollary of this being, that if the blood glucose were to be maintained within the limits achieved by normal homeostatic mechanisms this should prevent, halt or possibly reverse diabetic complications. There is evidence to support this idea (see Tchobroutsky 1978 for a recent review). Diabetes can be induced in

animals by a variety of manoeuvres, an example being the use of drugs such as alloxan and streptozotocin, which are toxic to insulin-producing B cells. Animals made diabetic by alloxan develop glomerular lesions with increased glomerular basement membrane thickness, similar to that seen in human diabetics (Bloodworth and Hamwi 1956, Steen-Olsen et al. 1966, Mauer et al. 1972). Similar lesions are seen in streptozotocin-induced diabetic rats, where typical mesangial thickening of the glomerulus has been described (Lee et al. 1974). Lee et al. also showed that the progression of renal changes seen in diabetic rats was halted by transplanting the affected kidney into a normal rat, whilst a normal kidney transplanted into a diabetic rat began to develop diabetic changes. Similarly, correction of diabetes in rats by transplantation of insulin-secreting tissue normalises the blood glucose and prevents the development of renal changes (Mauer et al. 1975, Bretzel et al. 1979). Perfect control of blood sugar by insulin treatment is difficult, even in laboratory animals, but nevertheless it has been shown that insulin therapy can certainly prevent the renal changes from developing in streptozotocin-diabetic rats (Rasch 1979). In dogs, diabetes can be induced by alloxan treatment, and Engermann et al. (1977) showed that treatment with insulin, where there was poor control of blood glucose, produced microvascular lesions in the kidneys and eyes, whilst careful control of the blood glucose led to a decrease in the number and severity of lesions.

Studies in humans are more difficult to perform for obvious reasons, but Miki et al. (1969) and Job et al. (1976) have reported that diabetics with good control of blood sugar have a lower incidence of retinopathy. Takazara et al. (1975) also correlated the progress of renal lesions inversely to the control of blood sugar. Pirart (1978) has shown that the incidence of peripheral vascular disease is related to poor control. In the same study Pirart also reviewed 60 previous studies, of

which 41 were considered to be "serious" and noted that only one found no relationship between the incidence of complications and control of blood glucose. More recently, controlled randomised trials have compared normal control with more intensive control of blood glucose and again correlated progression of complications with the degree of control (Holmann et al. 1983).

EXPERIMENTAL MODELS OF DIABETES

Research into the aetiology and prevention of the late complications of diabetes has been greatly facilitated by the development of a number of models of the condition in laboratory animals.

Pancreatectomy

Complete removal of the pancreas is the classic model for the experimental production of diabetes (Minkowski 1893, Banting and Best 1922(a)). The technique has been well described in the dog (Markowitz et al. 1964), in which species it is a relatively easy operation, producing reliable onset of diabetes immediately, with a survival ranging from 1 to 12 days, usually 6 days (DuToit et al. 1982). Pancreatectomy can be used to produce diabetes in other species including the rat (Scow 1957, Reece-Smith et al. 1982(b)) and the monkey (Mirsky 1937). For the reliable production of diabetes it is important to remove all the pancreatic tissue: early attempts at total pancreatectomy in the rat (a procedure made difficult by the multiple pancreatic ducts and poor blood supply to the bile duct) failed to produce diabetes in 20% of animals, even though 99% of the gland was removed, as estimated by the amylase content of the remaining tissue (Treadwell and Roe 1954). It follows that, ideally, proof of function of any transplanted islet tissue in an animal made diabetic by total pancreatectomy should include confirmation that the graft is the source of insulin by removing the graft and demonstrating recurrence of diabetes.

Chemical diabetes

Dunn et al. (1943(a)) first noted that alloxan given to rabbits induced, first, hyperglycaemia, then hypoglycaemia and death, and

subsequently Dunn et al. (1943(b)) showed that alloxan could be used to produce permanent diabetes in the rat. Numerous studies followed showing that alloxan could induce diabetes in a variety of species, and many analogues of alloxan were found to be similarly active, but like alloxan itself, rather toxic. An excellent review of the effects and mechanism of action of alloxan and its derivatives has been published by Okamoto (1970).

In 1963 an antibacterial agent called streptozotocin, isolated from fermentation cultures of streptomyces achromogenes, was reported to be diabetogenic (Rakieten et al. 1963). The compound is the 1-nitroso-1-methylurea derivative of 2-deoxyglucose, is unrelated to alloxan, and, interestingly, it is the 1-nitroso-1-methylurea portion of the molecule which is diabetogenic (Gunnarsson et al. 1974). Streptozotocin has been proven diabetogenic in a variety of species including the rat (Rakieten et al. 1963) mouse (Findlay et al. 1973) dog (Schein et al. 1973) and monkey (Pitkin and Reynolds 1970, Schein et al. 1973).

The response to intravenous injection of streptozotocin in the rat is triphasic, with initial hyperglycaemia 1-2 hours after injection, hypoglycaemia with increased plasma insulin levels at 6-12 hours and permanent hyperglycaemia with markedly reduced pancreatic and circulating insulin from 24 hours onwards (Junod et al. 1967). Histological examination at 7 hours shows specific B cell necrosis. The mechanism of action is thought to involve NADH depletion since nicotinamide administration blocks the effect of streptozotocin (Schein and Loftus 1968).

Dose response studies by Junod et al. (1969), in the rat, established that the severity of diabetes produced is directly related to the dose injected. A dose of 100 mg/Kg IV produced severe hyperglycaemia, ketosis and death within 3 days, whilst a dose of 35 mg/kg IV resulted in a

mild diabetes with spontaneous recovery observed in 25% of animals. A dose of either 55 mg/Kg or 65 mg/Kg IV resulted in moderately severe diabetic state with hyperglycaemia, polyuria, moderate ketosis and weight-loss which was stable and compatible with survival for several weeks.

There are some problems with administration of streptozotocin. The compound is unstable and rapidly inactivated at neutral pH and so must be dissolved in saline and the pH adjusted to 4.5 immediately before injection (Junod et al. 1967). The agent is relatively ineffective by routes of administration other than intravenous, (Junod et al. 1969) although it has been used to produce a mild diabetes by intraperitoneal injection (Whiting et al. 1982). The half life in serum is 5 minutes, and none is detectable in the circulation 2 hours after IV injection (Schein and Loftus 1968). Because the compound is relatively unstable and especially if IV injection is difficult, perhaps with some leakage of the injection outside the vein, the severity of diabetes produced by injection is not always predictable. A dose of 65 mg/kg IV may produce only mild diabetes in a few rats in an identically injected batch. Furthermore, animals with eventually mild diabetes may show initially high blood glucose values but later return to only slightly raised or even normal (Junod et al. 1969). Animals that have only mild diabetes have usually shown a reduction of serum glucose by 7 days, compared to more severely diabetic animals where hyperglycaemia increases. To ensure complete reliability of the diabetic state it is usual to use only animals which have remained diabetic for at least 14 days after injection. The diabetic state in such animals has been shown to be permanent (Rakieta et al. 1963, Schein et al. 1973).

In some circumstances it may be necessary to repeat streptozotocin injection. Junod et al. (1965) showed that 2 separated

doses of streptozotocin 25 mg/Kg IV were additive and produced a permanent diabetes of severity between that produced by a single dose of either 45 mg/Kg or 55 mg/kg IV. Multiple (5) small doses can also be used to induce diabetes, though it has been suggested that the mechanism is rather different, with the production of an insulinitis, characterised histologically by lymphocytic infiltration of the islets (Like and Rossini 1976).

A variety of other drugs and hormones have been shown to produce diabetes in experimental animals, either as a main or side effect, examples being the benzothiadiazine diuretics, quinolones, glucagon, growth hormone, glucocorticoids, adrenalin and others. An excellent review is provided by Dulin and Soret (1977).

Virally induced and spontaneous diabetes

Certain virus infections are relatively specific for pancreatic B cells, particularly in rodents, an example being encephalomyocarditis virus which produces diabetes in mice. A full review of the topic is provided by Craighead (1977).

A number of spontaneous diabetic syndromes arise in laboratory and domestic animals, some of which have been reproduced by specific inbreeding with selection for the diabetic characteristic. The syndromes vary in severity with different timing of onset and some are associated with high circulating levels of insulin, some with low levels of insulin. It is certain that they represent a variety of different aetiologies, with hyperglycaemia as a common feature. A review of the topic is provided by Like (1977). A particularly interesting model is seen in the "BB" rat (Nakhoda et al. 1975), where young affected rats develop diabetes characterised by hyperglycaemia, hypoinsulinaemia, ketosis, weight-loss and histological features of insulinitis similar to that seen in juvenile diabetes.

PREVENTION OF DIABETIC COMPLICATIONS

If the concept that poor glucose control causes complications is correct, it should be possible to prevent their development by improving control, and a variety of ways have been proposed to achieve this aim.

Improved insulin therapy

The most obvious way is by using a method of glucose control already available: insulin. For many years the practical management of diabetes was based on urine glucose measurement. This gave only a crude measure of blood glucose over several hours, and close control was not possible until the advent of simple methods for measuring blood glucose, based on fingerprick blood samples. Over the last 10 years attempts have been made to improve control of blood glucose by using multiple subcutaneous doses of insulin, guided by blood glucose measurements. A recent prospective randomised trial comparing unchanged conventional therapy to intensified therapy, over a 2 year period, showed that a significant improvement in blood glucose control could be achieved by intensive therapy, with associated improvement in neuropathy. Deterioration of renal function was halted, whereas the conventionally-treated group continued to progress. However, retinopathy continued to worsen in both groups (Holman et al. 1983).

In 1978, improvements in miniaturisation allowed the introduction of a portable pump for delivery of insulin subcutaneously (Pickup et al. 1978) and since then pumps have become smaller and more efficient. They have the advantage of being able to deliver a constant basal rate of insulin as well as pulses of insulin when required. Initial studies reported greatly improved control of blood glucose using portable pumps, and rapid improvement in neuropathy was reported (Pietri et al. 1980). Disappointingly Tamborlane et al. (1982) reported no effect on

nephropathy or retinopathy, and Lauritzen et al. (1983) reported significant deterioration of retinopathy following constant subcutaneous insulin infusion. Full assessment of this method of insulin delivery will require much longer studies, particularly in patients before development of complications. Control of glucose using currently available pumps requires regular blood glucose measurement by the patient, and control is still not perfect. Attempts are being made to develop glucose sensors that will allow direct adjustment of insulin dosage, with the entire device being implantable. Unfortunately a glucose sensor, reliable and stable enough for long term implantation, seems presently a long way off.

Transplantation of insulin secreting tissue

The logical alternative way of obtaining good control of glucose metabolism is to replace the cells that secrete insulin, since these will hopefully retain the homeostatic mechanisms that keep the blood glucose within the normal range. Like all forms of transplantation, the problems encountered fall into 2 divisions: technical problems associated with the technique of transplantation and problems associated with immunologic rejection of the transplanted tissue. In the case of transplantation of insulin-secreting tissue, the technical problems have been as great as those associated with rejection.

Insulin is secreted by B cells in the islets of Langerhans, but these cells can be obtained from a variety of sources, all of which have been extensively investigated.

1. Vascularised pancreatic grafts.

The first pancreatic transplants were autografts performed in dogs by Minkowski (1892) to investigate the role of the pancreas. In these experiments the vascularity of the gland was maintained by transplanting a

segment of the pancreas to the abdominal wall on a vascular pedical, allowing secondary revascularisation to occur, then severing the initial vascular connection. Similar procedures were performed by many investigators to examine physiological function, but interest in transplantation as a way of replacing insulin secretion only came after the realisation that insulin treatment failed to prevent the complications of diabetes. Sporadic attempts at experimental transplantation of the whole pancreas in the 1930's and 1940's were followed by more methodical studies in the 1950's such as those of Lichtenstein and Barschak (1957), and Brookes and Gifford (1959), who described allografts in dogs, noting that the major problems seemed to be thrombosis and pancreatitis. De Jode and Howard (1962) performed pancreaticoduodenal allografts in dogs and again noted vascular thrombosis and also graft rejection, with a mean survival of 6 days. Since then numerous studies, mainly in the dog, have investigated ways of overcoming these fundamental problems (These studies have been summarised by Sutherland 1981(a)).

Following experimental animal studies, and encouraged by the success of renal transplants, the first human pancreatic transplant was performed by Kelly et al. in 1966 (Kelly et al. 1967). Since then many transplants using adult vascularised grafts have been performed (total 335 to June 1983, Sutherland 1983). The major problems identified have been vascular thrombosis, pancreatitis, exocrine leakage and rejection; the problem of exocrine leakage being perhaps the major difficulty (see Sutherland 1981(b) for a detailed summary). A variety of techniques have been tried to deal with the exocrine drainage, including transplanting the whole gland attached to the duodenum (Lillehei et al. 1970), transplanting the tail segment alone, creating a cutaneous fistula (Bortagaray et al. 1970), duct ligation (Gliedman et al. 1973), pancreatic ductostomy (Gliedman et al. 1973), leaving the duct open with intraperitoneal drainage (Sutherland et al. 1979), roux-en-Y

ductojejunosomy (Groth et al. 1980(a)) and injecting a variety of polymers into the duct to produce occlusion (Dubernard et al. 1978). The number of methods attest to the lack of superiority of any single method. The results of vascularised pancreas transplantation remain poor. The early transplant series (before June 1977) showed very high mortality of around 50% and graft function of 5% at 1 year (Sutherland et al. 1981(b)).

The introduction of methods such as duct occlusion with polymers led to a great improvement in the mortality rate, but the 1 year graft survival remains poor at 15% (Sutherland 1983). The reasons for this failure rate remain unclear. Many grafts still fail because of thrombosis or other technical complications. The vascularised pancreatic allograft is subject to rejection, and despite currently available immunosuppressive regimens part of the failure is probably due to rejection. Many failures of apparently established grafts have been ascribed to fibrosis, particularly following the use of ductobliteration techniques. However, in most cases of late failure the exact cause remains uncertain (See Calne 1983 for review).

The concept of vascularised pancreas transplantation for the treatment of diabetes is unsound, since it is illogical to transplant an organ where 99% of the tissue is unwanted, and which produces highly deleterious effects. Furthermore, even if the operation were to be made safer and more effective it would always remain a major procedure, justifiable in patients with severe life-threatening complications, especially renal failure, but arguably unjustifiable in diabetics before the development of complications, which must be the eventual aim. Since the exocrine tissue is unwanted, other lines of research have investigated methods of minimising or removing the exocrine tissue, leaving the insulin-secreting tissue intact.

2. Foetal pancreas grafts

The foetal pancreas contains both exocrine and endocrine elements, but Coupland (1960) showed that transplanted foetal tissue undergoes a fortuitous atrophy of exocrine tissue, whilst the epithelial cells of the ducts continue to form islets of Langerhans, so that eventually the transplanted tissue consists mainly of islets. This finding led many investigators to attempt transplantation of foetal tissue, but without success in reversing experimental diabetes, until Brown et al. (1974) showed that rat foetal pancreas placed under the kidney capsule would grow and differentiate, as described above. 2-3 pancreases from 15-18 day old fetuses, placed under the kidney capsule, could reverse diabetes in syngeneic streptozotocin-diabetic recipients.

Since then, Mullen et al. (1977) have shown that reversal of diabetes can be obtained using only 1 foetal rat pancreas, and Mandel et al. (1983) showed that foetal mouse pancreas behaves similarly. Furthermore, Mandel has shown that foetal mouse pancreas benefits from a period in organ culture, by increasing in size and insulin content, with subsequent increased efficiency in reversal of diabetes in syngeneic, experimentally diabetic, mice.

It was initially hoped that foetal pancreas would be less immunogenic than adult pancreatic tissue, but this has been proven not to be the case (Garvey et al. 1979), at least for the rat. Attempts to overcome rejection of foetal rat pancreas allografts using cyclosporine proved unsuccessful (Garvey et al. 1980(b)), though more complex regimens (such as donor liver extract and procarbazine pretreatment followed by anti-lymphocyte serum administration and also total lymphatic irradiation followed by bone marrow transplantation) were reported successful in weak strain combinations (Mullen et al. 1982,1983).

Most interesting was a report of tissue culture of foetal mouse pancreas allografts in 90% oxygen for 21-28 days, resulting in indefinite survival after transplantation (Mandel et al. 1982), although this was not confirmed in the rat (Garvey et al. 1980).

Unfortunately there have been no successful studies reported using larger animal models. Mandel et al. (1982) has demonstrated that human foetal pancreas survives organ culture and transplantation to athymic mice. Minced human foetal pancreas allografts have been performed clinically (Groth et al. 1980(b), Valente et al. 1980(b)), though without proven success. It is clear that the techniques developed in the rodent need to be extended to large animal models before clinical foetal pancreas transplants are likely to be successful.

There remains some doubt about the availability of foetal pancreas for transplantation. Only pancreas from fetuses of a fairly narrow developmental age seem to be suitable, at least in the rodent, which may create problems, and the moral and ethical issues may prove more of a hurdle than some investigators have suggested. Nevertheless, foetal pancreas transplantation has exciting possibilities.

3. Neonatal pancreas grafts

Neonatal pancreas, not unexpectedly, shows similarities in structure to foetal pancreas with a relatively high proportion of endocrine to exocrine tissue (Leonard et al. 1974), but the actual mass of tissue is larger, and therefore might be expected to be more effective than foetal tissue. However, the larger mass does mean that it is usually necessary to disperse the tissue in some way, to allow transplantation and implantation. Leonard et al. (1974) first demonstrated successful transplantation of minced, collagenase digested, neonatal rat pancreas to the peritoneal cavity of diabetic rats, a finding since confirmed by many

others (see Sutherland 1981(a) for other studies). Hegre et al. (1976) and Axen and Pi-Sunyer (1981) used neonatal rat pancreas which was minced, cultured for a short period, then transplanted to the intramuscular and intraperitoneal sites, with reported success. The number of donors needed for the intraperitoneal and intramuscular sites was large, around 20 - 30. Injection of dispersed neonatal rat pancreas into the portal vein has been found more efficient, requiring only 6 donors to reverse diabetes (Woo and Pi-Sunyer 1982). In some cases it has not been necessary to disperse the pancreas: Woo and Pi-Sunyer (1982) found that 5 whole rat neonatal pancreases transplanted under the renal capsule would survive and reverse diabetes as efficiently as dispersed preparations placed into the portal vein.

There have been relatively few studies on the rejection of neonatal pancreas, though it is established that neonatal pancreas does reject in a manner similar to foetal pancreas (Leonard et al. 1974) and attempts at preventing rejection have not met with markedly greater success than similar methods used for foetal pancreas (see Sutherland 1981(a) for summary). The overall impression is that neonatal pancreas has little, if any, advantage over foetal pancreas, and it is probable that the problems of supply would be insurmountable.

4. Insulinoma

A superficially attractive source of insulin-secreting tissue is insulinoma, since this tissue can be maintained in experimental animal transfer systems or even tissue culture, and large quantities can be grown. A Syrian Hamster insulinoma has been maintained by transfer between hamsters and athymic mice, and transplants of this tissue have been shown to reverse experimentally induced diabetes in syngeneic hamsters (Reintgen et al. 1980). A similar tumour from a rat source has also been

demonstrated to reverse diabetes in allogeneic rats with prevention of rejection by cyclosporine treatment (Gance et al. 1983). However, it should be noted that, in the long-term, successful recipients died of hypoglycaemia, and this demonstrates the fundamental weakness of this approach; unfortunately insulinoma tissue secretes insulin in an uncontrolled fashion, whereas the function required is a controlled response. There are also serious questions about the eventual metastasising potential of such tissue.

5. Adult dispersed pancreas and isolated islets.

The adult pancreas has one immediate advantage as a source of insulin-secreting tissue: it is readily available, both in experimental animals and also from human renal transplant cadaver donors. Probably for this reason sporadic attempts were made to transplant fragments of adult pancreatic tissue, perhaps the first being Watson Williams, a Bristol physician, who transplanted 3 pieces of sheep pancreas to the subcutaneous tissue of a 15 year old diabetic boy, without beneficial effect, as long ago as 1893.

i) Studies in rodents

Successful functional transplants of adult pancreatic tissue were not possible until Moskalewski (1965) reported the action of collagenase on minced guinea pig pancreas. The enzyme caused the islets to separate from the surrounding exocrine tissue, and the method was further perfected by Lacy and Kostianovsky (1967) allowing separation of large numbers of islets. Subsequently Moskalewski (1969) reported successful transplantation of rat islets prepared by this method.

Further modifications led to the technique reported by Ballinger and Lacy (1972) which has now become the standard method of preparation of rat

islets and, with modifications, mouse islets. A considerable number of studies of islet transplantation in the rodent have been reported, from which the following important points have become clear.

a) The site of transplantation

Ballinger and Lacy (1972) first showed partial reversal of experimental diabetes in the rat using islets implanted into the peritoneal cavity, but full reversal of diabetes was not regularly achieved until Kemp et al. (1973) demonstrated that intraportal injection of islets resulted in the rapid and permanent cure of diabetes in syngeneic rats. Since then a number of other sites have also been investigated and successful reversion of diabetes claimed: amongst them the hepatic parenchyma (Eloy et al. 1975) , the spleen (Finch et al. 1977(c)), the testis (Bobzien et al. 1983), the omentum (Yasunami et al. 1983(b)) and under the renal capsule (Bowen et al. 1979), Reece-Smith et al. 1981(a)).

It has become clear that more vascular, or, preferably, intravascular sites are more effective than sites with a relatively poor blood supply, and that a site with venous drainage into the portal system is more efficient than a site with systemic venous drainage. Thus Kemp et al. (1973) found that profoundly diabetic rats reverted to normoglycaemia with as few as 400 islets injected into the portal vein.

b) The metabolic effects of islet transplantation

It has been proven beyond doubt that adult isolated islets survive transplantation in the rodent and reverse the immediate effects of experimental diabetes, whether the animal is made diabetic by total pancreatectomy (Helmke et al. 1975), alloxan (Boyles et al. 1975), streptozotocin (Gray et al. 1976, Feldman et al. 1977) or naturally occurring diabetes (Naji et al. 1979(a)).

Experimental diabetes in rodents produces a variety of immediate metabolic consequences and, later, secondary complications, all of which have been shown to be reversed by adult islet transplantation. Islet transplantation normalises the plasma glucose, abolishes glycosuria and with it the associated features of polydipsia, polyuria and polyphagia. (Kemp et al. 1973). The circulating plasma insulin is increased (Feldman et al. 1977) and the intravenous glucose tolerance test can be normal (Gray et al. 1976). Weight gain is restored (Finch et al. 1977(c)) and, in the longer term, serum lipids (which are raised in diabetic rats), return to the normal range (Ziegler et al. 1974). In rats, islet transplantation also prevents and reverses the development of long term diabetic complications, such as renal glomerular lesions (Mauer et al. 1975) and neuropathy (Schmidt et al. 1983).

c) Islet allografts: the problem of rejection

After the success of the early experimental and clinical kidney transplants, many investigations of the transplantation of other tissues were performed. These early experiments included attempts to transplant endocrine tissues, such as thyroid, parathyroid, ovarian and pancreatic tissue. Encouragingly, there were some early signs that not only might such endocrine tissue be transplantable , but also that endocrine grafts appeared to produce a less severe rejection than that seen with other tissues (Russell et al. 1959). It was suggested that pancreatic tissue was less immunogenic than non-endocrine tissue (Lillehei et al. 1970).

Unfortunately this sense of optimism was rapidly dispersed as several separate investigators reported that allogeneic isolated adult islets are rapidly rejected (Slijejevick et al. 1975, Finch et al. 1977), and, indeed, appear to be more rapidly rejected than any other tissue. Transplantation of allogeneic islets intraportally typically results in rejection in 4-6 days whether transplantation is across a major or minor

histocompatibility barrier (Morris et al. 1980).

d) Mechanisms of rejection

The mechanism of the rapid rejection of islet allografts has been the subject of investigation. It was initially suggested that the rapid onset of rejection was, in fact, only due to the low functional reserve of insulin-secreting tissue; indeed, increasing the number of islets transplanted does delay rejection of intraportally transplanted islets in immunosuppressed rats (Finch and Morris 1977(a)). However, Sutherland et al. (1979(a)) found that immediately vascularised pancreas grafts, that were deliberately cut into segments containing equivalent quantities of islet tissue to islet grafts, still survived twice as long as intraportal islets, even though apparently the same mass of islet tissue was transplanted.

The rejection of allogeneic islets is so rapid as to suggest that mechanisms other than cellular immunity may be operative, and Najj (1979(b)) showed that long-established islet grafts in neonatally tolerant rats are vulnerable to injected hyperimmune serum, and Perloff et al. (1981(a)) showed that fresh islets are vulnerable to donor-specific antibody given to otherwise tolerant recipients. Another mechanism, suggested by Nash et al. (1979), was that islets are rapidly destroyed by macrophages, possibly by opsonisation with antibody, and that blocking the phagocytic action of macrophages might be one way of overcoming rejection.

Despite these studies, based on the assumption that islet rejection is too fast for cellular mechanisms, histological examination of intraportal islet allografts (Franklin et al. 1979) shows that at first the graft remains intact and appear similar to isografts, but after 2 days the graft starts to be infiltrated by small lymphocytes. By 4 days the cellular invasion develops into an extensive infiltrate of mononuclear cells with

relatively abundant cytoplasm - a picture typical of cellular rejection in other organs. Naji et al. (1979(b)) also showed that long established islet allografts in neonatally tolerant rats were promptly rejected if the animal was injected with hyperimmune spleen cells from non-tolerant animals. There now seems little doubt that rejection of intraportal islets is mainly cell-mediated and is similar, though more rapid, to rejection in other tissues. The speed of the rejection process may be related to the way the islets are exposed to immune competent cells. Reece-Smith et al. (1981(a)) showed that simply transplanting islets to a possibly less exposed site, the kidney capsule, prolonged the survival of islet allografts by 3-4 days, so that the time taken for rejection was similar to that seen with vascularised organ allografts.

e) The effect of immunosuppression on rejection

Once it was established that islet allografts underwent rejection, attempts were made to overcome the rejection process using immunosuppressive agents that had been shown to be very effective in prolonging the survival of skin, heart and, particularly, renal allografts in the rat. Disappointingly, most of these agents had little effect on the rejection of isolated islet allografts.

Azathioprine and prednisolone, the standard drugs used for immunosuppression of clinical renal transplants, were virtually ineffective (even in combination) when given to rats following islet transplantation (Bell et al. 1980). Cyclophosphamide, enhancing serum and antilymphocyte serum (ALS) are very effective in preventing rejection of vascularised organ allografts in the rat, and a number of studies attempted to use these agents to prolong the survival of islet allografts. The results obtained in a series of studies from the Oxford group are representative (a summary of results from other series is provided by

Sutherland et al. 1981(a)). Enhancing serum was found to be effective only in weak strain combinations (Finch and Morris 1976), but not across a strong histocompatibility barrier (Morris et al. 1980(b)). Cyclophosphamide was moderately effective in weak strain combinations (Reece-Smith et al. 1983), but, again, ineffective in strong strain combinations. ALS was more effective, showing prolongation of survival, even in strong strain combinations (Morris et al. 1980(b)), but even here long-term survival (over 100 days) was unusual. Even the combination of ALS and enhancing serum, a very potent combination for renal allografts, failed to show synergism for islets (Finch and Morris 1977(b)). Similar, rather disappointing, results were obtained by other investigators using the same agents, and these results led to alternative approaches being tried.

Based on the concept that macrophages might be responsible for the rapid destruction of isolated islets, Nash et al. (1980) used the macrophage-specific toxin particulate silica, and demonstrated prolonged survival in a strong strain combination, though this was not confirmed in a subsequent paper from Reece-Smith et al. (1981(c)).

The cyclic fungal polypeptide cyclosporine was found to have immunosuppressive properties (Borel et al. 1977) and was subsequently found to prolong the survival of heart grafts in the rat (Kostakis et al. 1977) and pig (Calne et al. 1978), and renal allografts in the dog (Calne and White 1977) and man (Calne et al. 1979). Cyclosporine is particularly effective in preventing rejection of renal allografts in the rat, where a dose of 10 mg/kg/day for 14 days leads to prolonged survival of renal allografts in both strong and weak strain combinations (Homan et al. 1979), and this is associated with the development of a specific unresponsive state (Homan et al. 1980). Disappointingly, cyclosporine given as a 14 day course failed to produce long-term survival of islets,

even using doses up to 40 mg/kg/day (Garvey et al. 1980), though survival was prolonged slightly. If cyclosporine administration is continued there is only slight prolongation of islet allograft survival across a major histocompatibility barrier, although islets transplanted across a minor barrier can be prolonged, in some cases, using the somewhat toxic dose of 20 mg/kg/day (Rynasiewicz et al. 1980). However, once the cyclosporine is stopped even long surviving grafts are rejected, thus an unresponsive state does not seem to be produced in the same way as with renal allografts. Similar findings were reported by Bell et al. (1980).

f) The use of tissue culture to alter immunogenicity.

Whilst evidence was growing that straightforward immunosuppressive agents were not successful in abrogating the rejection of allogeneic isolated islets in the rat, an alternative approach based on the possibility of altering the tissue immunogenicity (Lafferty and Woolnough 1977) was being investigated. Following work by Lafferty et al. (1976) on thyroid tissue treated by culture in an atmosphere of 95% oxygen and transplanted into allogeneic mice without rejection, Bowen et al. (1980) (working with Lafferty) showed that islets could be similarly cultured in 95% oxygen, provided the islets were first allowed to cluster together into "megaislets", and subsequently these could be transplanted into allogeneic mice without rejection. The rationale behind this approach was that the immunogenicity of islet (and other) allografts was due to their content of "passenger leucocytes" and not the islet tissue itself. Culture of the tissue in high oxygen tension was one way of removing these "passenger leucocytes" (Talmage and Dart 1978).

This effect has been confirmed in the rat (Tucker et al. 1983), and more recently it has been shown that culture in normal tissue culture conditions also results in prolonged survival of mouse (Andersson et al.

1982) and rat (Rabinovitch et al. 1982, Yasunami 1983(a)) islet allografts. Again the mechanism is thought to be a reduction of immunogenic cells within the graft. Currently it is thought that these "passenger leucocytes" are probably the Ia^+ dendritic cells described by Hart et al. (1983), and, in support of this idea, Rabinovitch et al. (1982) has shown a reduction of Ia^+ cells in islets after culture. Based on the same approach Faustman et al. (1981) treated mouse islets with anti-Ia antibody and complement and demonstrated prolongation of survival of islet allografts in mice. Again the mechanism is thought to be the removal of "passenger leucocytes", and Reece-Smith et al. (1983(a)) found that treatment of islets with a non-complement-fixing antibody did not prolong survival, suggesting that simply masking the Ia determinant of these cells is not sufficient to produce the effect.

ii) Dispersed pancreas and isolated islet transplantation in large animals

In man, and animals larger than the rodent, the adult pancreas is a compact, fibrous organ, and the techniques that are moderately successful in separating the islets from the rodent pancreas are relatively ineffective: a problem which will be examined in detail later. A further difficulty in the experimental use of large animals is the lack of inbred species, so transplantation experimenters are left with the dilemma of either using multiple donors (and facing the formidable problems of rejection), or using an autograft model, with the necessity for performing pancreatectomy (with its attendant problems), and also the likelihood that the number of islets transplanted may be too few to have a significant effect on the diabetic state.

Notwithstanding these problems, attempts have been made to transplant isolated islets in several species. It is perhaps worth pointing out, before these studies are discussed, that the degree of difficulty of a

procedure or experiment is not sufficient reason for accepting inadequate proof of the conclusions drawn from the study. Unfortunately this truism has not always been applied to studies of isolated islet transplantation in animals larger than the rat.

In order to draw the conclusion (from a study) that islets of Langerhans have been isolated as a relatively pure preparation, and transplanted successfully, there are several criteria which should be fulfilled as a minimum. The islet preparation must be shown to contain islets of Langerhans by histological examination, with evaluation of structural integrity by both light and electron microscopy. The purity of the preparation should be assessed: the relative insulin and amylase content has been used by some authors but another, possibly more reliable, way is by histological section. The number of islets or volume of islet tissue transplanted should be estimated by a reliable method, which will require some means of differentiating islet from exocrine tissue. The islets isolated should be shown to be viable by in vitro tests such as intravital staining, appropriate insulin secretion in response to a glucose challenge or, possibly, other metabolic criteria.

The success of transplantation can only be judged if the animal receiving the transplant is proven to be diabetic. The most reliable way of producing diabetes is by total pancreatectomy. Other models of "mild" diabetes, particularly those using drugs or partial pancreatectomy, require parallel evaluation studies to prove the reliability and long-term outcome of the diabetic state. Successful transplantation can only be defined as the absence (for a reasonable period) of glycosuria, glycosaemia and the other gross metabolic alterations associated with acute diabetes, such as weight loss and polyphagia. Relative changes in blood glucose or insulin requirement are not reliable enough to allow conclusions to be drawn. For this reason, the use of allograft models is not ideal, since return of

glycosuria may be due to either graft failure or rejection.

Transplantation of islets should preferably be to a site where function can be confirmed by demonstrating raised concentrations of insulin in the draining veins and, preferably, a site which allows subsequent removal of the transplanted islets: return of diabetes being good proof of graft function. Finally, it should be possible to demonstrate islet tissue at the transplant site by histological examination, in quantities commensurate with the function shown by the transplant.

When the reported studies of the successful transplantation of isolated islets are examined by these criteria, most are found to be inadequate in some way.

a) Studies of purified adult islets

Sutherland et al. (1974) reported islet transplants in pigs after total pancreatectomy, both as autografts and allografts. The histological appearance of the preparation was not described, and no comment was made as to the purity of the preparation or the numbers of islets transplanted. Islet transplantation failed to obviate diabetes, and these transplants were not claimed to be successful.

Scharp et al. (1975) described transplantation of isolated islets in rhesus monkeys, but used an unproven technique for producing diabetes: 70% pancreatectomy followed by streptozotocin administration. Islets were given as allografts (with immunosuppression) into the portal vein. The histological appearance, purity and number of islets was not reported. Glycosuria was only reversed after 3 weeks (in 3 of 5 monkeys), which is difficult to explain, and the long-term fate of the animals was not reported.

Jonasson et al. (1977) presented an essentially anecdotal study,

reporting autografts of collagenase-isolated, cultured islets in young monkeys submitted to total pancreatectomy. No details of the islet preparation were given, other than an assessment of viability using trypan blue exclusion. Intraportal injection of the preparation failed to obviate diabetes, and, although it was claimed that significant endogenous insulin production was shown, data was not presented to support this conclusion.

Lorenz et al. (1979(a)) claimed successful transplantation of isolated islet allografts between partially inbred dogs (without immunosuppression). Diabetes was induced by the unsatisfactory method of partial pancreatectomy and low-dose streptozotocin administration. The histological appearance, number and purity of islets transplanted was not stated, and the islets were injected into the portal vein, so could not be removed to prove function. Claims of success were based on reversal of diabetes for approximately one week after injection: the subsequent slow rise in serum glucose was interpreted as rejection, but this could easily have been the effect of an insulin-containing bolus on borderline diabetes. Subsequent studies (Lorenz et al. 1979(b)) used the same technique, and looked at the duration of normoglycaemia in 2 groups of dogs, where there was thought to be either a major or minor histocompatibility barrier between donor and recipient (as determined by an unique in vitro test, the accuracy of which was not documented). "Well matched" grafts showed prolonged normoglycaemia, but, again, transplant function could not be proved as all grafts were intraportal.

Horaguchi et al. (1981) reported islet autotransplants in dogs following total pancreatectomy. The number of islets and purity of the preparation were estimated by a totally unproven method (staining with Turk's solution: more usually used as a stain for haematological slides). Histological appearance was not reported, but the insulin content and

insulin/amylase ratio were measured, and from this a yield of 60% of the amylase content of the pancreas with a sixfold purification estimated. The islet preparation was transplanted into the portal vein, with obviation of diabetes in 3 of 5 dogs, and into the spleen in 2 dogs, with normoglycaemia in both, although splenectomy to prove function was not performed.

Warnock et al. (1983) described a similar, slightly simplified, technique to that of Horaguchi, and reported normoglycaemia in 9 of 13 pancreatectomised dogs given autografts injected into the spleen via the splenic vein (Interestingly, direct injection to the splenic pulp was ineffectual). The histological appearance pretransplant and the number of islets transplanted was not reported. The islet yield by insulin content represented 25% of the pancreas, but the amylase content was also 25%, thus these cannot be considered purified islet transplants.

Noel et al. (1982) reported isolated islet autografts into the spleen of dogs made diabetic by subtotal pancreatectomy and administration of streptozotocin. The number of islets was calculated indirectly by insulin content of the complete yield compared to that of individual islets, with confirmation by counting of islets in preliminary studies. No histological examination of the islets was presented and the purity of the preparation was not estimated. Reversion of diabetes was reported in 2 of 3 transplanted dogs, and compared to 5 untransplanted controls. However, the spleen was not removed to prove islet function, and the long term-fate of the transplants was not reported.

Matas et al. (1977) used a short period of tissue culture to deplete the enzyme content of minced dog pancreas, followed by autotransplantation to the portal vein of partially pancreatectomised, streptozotocin-treated dogs. Intraportal injection was limited by the development of portal hypertension in every case. The histological appearance of the

preparation was not presented, but a sixfold increase in the insulin/amylase ratio was claimed. 7 of 15 transplanted dogs became normoglycaemic, but so did 1 of 14 control dogs. The long-term fate of successful transplants was not reported. 1 dog underwent total pancreatectomy followed by intraportal allotransplantation and remained normoglycaemic for 1 week, before rejecting.

Jolly et al. (1982) reported allotransplantation of inbred rabbit islets, using a previously described, non-enzymic, isolation technique (Hinshaw et al. 1981). This study was unsatisfactory in almost every aspect. No histological examination of the islet preparation was reported. The number of islets isolated was estimated at the remarkable figure of 1.8×10^6 per pancreas, yet the details of how islets were identified or counted were omitted. Insulin secretion in response to glucose was estimated by an inadequately reported assay. Transplantation was by intraportal injection into rabbits, made diabetic by streptozotocin, and given donor-specific blood transfusion and antilymphocyte serum to prevent rejection. The duration of normoglycaemia following transplantation was not recorded, but inferred from the survival time. There were no untreated controls, and no dose response studies quoted for the effect of streptozotocin in the rabbit.

Wise et al. (1982, 1983) described autotransplantation of islets into the spleen of pigs made diabetic by partial pancreatectomy and streptozotocin treatment. The islets were prepared by an unusual non-enzymic method based on physical separation. No histological examination of the islet preparation was reported, and the number and purity of the islets was not stated. Islet grafts were either fresh or cryopreserved, and viability assessed by insulin secretion in response to glucose (the results presented, in fact, suggesting very poor viability). Following transplantation of autogenous cryopreserved islets into the

spleen, diabetes was reversed in 9 of 15 animals, and recurred on splenectomy (which was performed after an inadequate period of normoglycaemia). Islet tissue could not be found on histological examination of the removed splenic tissue.

Long et al. (1983) described a more satisfactory study of islet transplants in puppies made diabetic by total pancreatectomy, and given autografts of isolated islets into the spleen. The number of islets was not calculated, but histological studies were presented, and, although the purity of the preparation was not discussed, approximately 10% of the tissue (seen in the histological section presented) appeared to consist of fragmented islet tissue. Viability was confirmed by trypan blue exclusion, but no insulin release data was presented. 4 dogs were transplanted, 3 of which remained normoglycaemic, in contrast to untransplanted controls. Splenectomy was performed on 1 animal, with only partial return of diabetes: this being attributed to embolisation of some of the islets to the liver, during splenic injection of the transplanted islets.

b) Studies of dispersed pancreas

The difficulty in obtaining a sufficient quantity of purified islets from the pancreas of large animals led some investigators to abandon attempts at purification, and to simply transplant the dispersed pancreas (indeed the experiments of Horaguchi et al. 1981, Warnock et al. 1983, Matas et al. 1977, Long et al. 1983, Jolly et al. 1982 and Wise et al. 1982 should probably also come into this category). This approach was pioneered by Mirkovitch and Campiche (1977) who reported that, following total pancreatectomy in the dog, diabetes could be obviated by autotransplantation of the tail of the pancreas. Before transplantation into the spleen, the tissue was prepared by a combination of mechanical

chopping and collagenase digestion. Success was reported in 20 of 25 transplants performed, and confirmed by the development of diabetes in 7 dogs that subsequently underwent splenectomy. Histological examination of the transplanted tissue showed groups of endocrine cells within the spleen, along with clumps of intact acinar tissue. This work has been confirmed by similar studies by Kolb et al. (1977), Kretschmer et al. (1977), Hanson et al. (1981), Mehigan et al. (1981), Dutoit et al. (1982) and Alderson and Farndon (1984). The success of the technique has allowed detailed metabolic studies in transplanted dogs. Although normoglycaemia is permanent, the intravenous glucose tolerance test remains abnormal, as does the circulating insulin level, which is often very low, even when the whole pancreas is transplanted (Kretschmer et al. 1977, Alderson and Farndon 1984). However, other parameters of carbohydrate and lipid metabolism associated with diabetes, and present in pancreatectomised dogs, are completely obviated by autotransplantation (Alderson and Farndon 1984). Limited allograft studies have also been performed. Kretschmer et al. (1979) described allografts of dispersed pancreas between totally pancreatectomised, outbred beagle dogs immunosuppressed with prednisone and azathioprine. 8 of 22 dogs achieved normoglycaemia after 10 days, and remained normoglycaemic until death (mean survival 33 days). The study is marred by the lack of an untreated control group, since the survival of all dogs, without rejection, is rather unexpected. DuToit et al. (1982) performed a similar study in outbred mongrel dogs, and failed to achieve normoglycaemia in either untreated intrasplenic dispersed pancreatic allografts, or similarly transplanted dogs treated with cyclosporine, although all dogs given autografts became (and remained) normoglycaemic.

Despite these successful reports the technique of transplanting unpurified dispersed pancreas does have serious defects. Intrasplenic injection in dogs is frequently accompanied by significant intraportal embolisation of tissue, producing severe systemic hypotension, bradycardia

and cyanosis, with congestion of the portal vein often causing death (DuToit et al. 1982). These features were also noted in studies where the dispersed pancreas was injected directly into the portal vein (Kolb et al. 1977, Kretschmer et al. 1978). The effect was thought to be related to the exocrine contamination, as well as the quantity of tissue injected, and was reported to be ameliorated by administration of heparin and aprotonin in dogs (Mehigan et al. 1980), although Traverso et al. (1982) found this regimen less effective.

Attempts have been made to perform transplants of unpurified dispersed pancreas in other species. Using a similar technique to that described in the dog, Mieny and Smit (1978) reported intraportal autotransplantation of dispersed pancreas in baboons, made diabetic by total pancreatectomy. Diabetes was obviated in 3 of 4 transplants. In a larger series, Nash et al. (1981) reported intraportal transplants of dispersed pancreas in baboons after total pancreatectomy. Nontransplanted controls all became diabetic and rapidly died, whilst most autograft recipients survived up to 100 days, but were not entirely normoglycaemic. Recipients of allografts were initially normoglycaemic, but rejected rapidly, and treatment with both total lymphoid irradiation and donor bone marrow failed to prevent rejection. The effect of intraportal injection of dispersed pancreas on the systemic and portal circulation was not reported.

c) Conclusions

It is clear from the number of confirmatory reports that the technique described by Mirkovitch and Campiche (1977) allows transplantation of sufficient insulin-secreting tissue to obviate the onset of diabetes following total pancreatectomy in dogs. Using tissue derived from half the pancreas, enough islets are obtained to maintain normoglycaemia and prevent most of the metabolic sequelae of diabetes. The amount of insulin

secreted is not normal, even if all the pancreas is transplanted, and this is reflected by an abnormal glucose tolerance test. Nevertheless, the technique has allowed allograft studies, the results of which are conflicting, but the more rigorously controlled studies of DuToit et al. (1982) in outbred mongrel dogs probably reflect the true picture, showing dog islets to be as sensitive to the rejection process as rodent islet allografts. However, the Mirkovitch technique has severe drawbacks, which relate to the systemic hypotension and portal hypertension produced by intraportal embolisation of tissue, even after initial injection into the spleen. Much less adequate studies in the primate suggest that a similar technique with intraportal injection can also obviate diabetes (though less successfully than in the dog). The systemic effects of intraportal injection in the primate are not commented upon.

Attempts to separate the islets from the exocrine tissue and transplant them as a pure preparation have been much less effective. The most successful techniques have been those where the purity of the preparation seems so poor as to make the transplant little different from the Mirkovitch technique. Studies where increased purification of the islets has been attempted have only worked in animals where diabetes has been induced by partial pancreatectomy. It is clear, from the work on the Mirkovitch technique, that, at least in the dog, there is no difficulty in performing total pancreatectomy for the production of diabetes (the time-honoured model), and that such animals can live happily with exocrine enzyme supplements, provided they have a functioning source of insulin. There is no excuse for using any other model for induction of diabetes, and total pancreatectomy would, in fact, provide more tissue for islet isolation. The studies which claim to have successfully transplanted purified islets have used a suspect model of diabetes and, furthermore, have presented no convincing data that the tissue transplanted contains viable islet tissue in any degree of purity. In the end, the success of a

method can be judged by the number of confirmatory studies that follow it: viz. the Mirkovitch technique. None of the published methods for purified islet transplantation has been repeated by other workers, and often only small numbers of successful transplants are claimed by the authors of the published method themselves. It would seem, therefore, that a reliable method for transplantation of purified, isolated islets in large animals has not been developed.

iii) Isolated islet and dispersed adult pancreas transplantation in humans.

There is a time-honoured tradition of "jumping the gun" in transplant surgery. This has not always been a bad thing; indeed, the current success of kidney transplantation is based on the early attempts at clinical renal transplantation, made before experimental immunology had confirmed that such a procedure would be possible. Attempts were made to transplant adult human pancreas as far back as 1924, when Pybus and Durh transplanted slices of fresh human cadaver pancreas to the abdominal wall of 2 diabetic patients, without success. In the late 1960's the reports of successful islet isolation from the pancreas of rodents re-stimulated interest, and raised the possibility of transplanting adult islets separated from exocrine tissue.

a) Transplantation of purified human islets

Anecdotal reports of human islet isolation, using the method developed for the rat pancreas, were encouraging (Ballinger et al. 1972), and prompted workers in Minneapolis to attempt islet allografts in humans (Najarian et al. 1977). Islets were isolated from cadaver human pancreases by first dispersing the pancreas by mechanical chopping, and then incubating with collagenase. Islets were separated from exocrine tissue by centrifugation on a density gradient. The number of islets isolated was never actually reported, but the yield was later stated to be less than 5% (Sutherland et al. 1981(b)). The purity of the preparation, as assessed by histology, was not good, even though insulin/amylase ratios suggested purification of 28 to 116 fold in 3 of 5 pancreases (the other 2 pancreases showing no purification). 4 transplants of allogeneic isolated islets were performed in diabetic patients already in receipt of a kidney transplant, and, therefore, also immunosuppressed. The islets were transplanted subcutaneously in 3 cases, and intramuscularly in 1 case; none showed any

definite evidence of function, but no ill effects were recorded. This single report is the only attempt at clinical transplantation of purified adult human islets recorded; the poor outcome has presumably discouraged further transplants using this method of islet isolation, and, since then, an improved method for isolation of islets from the human pancreas has not emerged.

b) Dispersed cadaver pancreas allografts

The finding that islet purification was not necessary, at least in the dog (Mirkovitch and Campiche 1977), encouraged several groups to attempt transplantation of dispersed cadaver pancreas. The method used to disperse the pancreas by most groups was, basically, a variant of the Mirkovitch technique: a combination of mechanical chopping of the pancreas followed by collagenase digestion. For reasons that have never been clear, perhaps because it was felt that the human spleen was too small and fragile to take the relatively large volume of tissue transplanted, the method of transplantation favoured by most groups has been injection of the dispersed tissue into the portal vein. Nevertheless, intrasplenic transplantation has been performed by 1 group without apparent complications. Sutherland et al. (1980(b)) reported 8 transplants of dispersed cadaver pancreas into the portal vein of 7 donors, all of whom had already received a cadaver renal transplant, and had stable renal function: thus, these patients were already immunosuppressed. Following transplantation there was a transient drop in insulin requirement, but no patient could be withdrawn from insulin therapy. Despite the injection of large volumes of tissue into the portal vein, no permanent ill-effects were noted, although portal hypertension (up to 60 cm H₂O in 1 patient) was noted immediately after injection of the tissue. The outcome of this series was particularly disappointing, because, apparently, 20-60% of the islet mass of the pancreas was transplanted, as judged by insulin content.

The actual number of islets, the histological appearance, and the viability of the tissue were not commented upon.

Kolb and Largiader (1980) transplanted dispersed cadaver pancreas into the portal vein of 4 patients, and also into the spleen of 3 patients, with a renal transplant from the same donor being performed at the same time. 6 transplants had no effect on the patients' diabetes, but 1 recipient (with tissue transplanted into the spleen) was withdrawn from insulin therapy 8 months later, the delayed function being attributed to the fact that the donor was an infant (Largiader et al. 1980). This patient was even more remarkable since diabetes did not return even after rejection of the kidney. It remains uncertain if this graft was really functioning: it is possible that immunosuppression produced reversion of the diabetic state, although the patient was a long-standing diabetic, and this phenomenon has not been previously reported. The patient eventually died, still normoglycaemic, and, at autopsy, evidence of functioning grafted tissue could not be found (Sutherland et al. 1981(b)). Thus, whether this was indeed a functioning graft remains uncertain.

Valente et al. (1980(c)) reported transplantation of dispersed, cultured, adult pancreas, enclosed within diffusion chambers, into 13 patients; 12 being implanted subcutaneously and 1 intramuscularly. The number of islets transplanted was claimed to be 10,000 to 90,000, but exactly how this figure was calculated was not described. 2 patients were withdrawn from insulin therapy after transplantation, but the diabetic status of the patients remains in doubt. No histology of the tissue pre- or post-transplant was presented, and inadequate data was presented to support the conclusion that the grafts were actually the source of insulin in these patients.

Sporadic attempts at transplantation of dispersed adult cadaver pancreas have been reported to the pancreas and islet transplant registry

since 1980 (Sutherland 1984), but no other documented series has appeared in the literature. The studies presented above are disappointing, particularly those studies where there has been an accurate estimate of the quantity of tissue transplanted. Failure may have been due to rapid rejection of the islet tissue, but other reasons can not be excluded; for instance, the viability of the dispersed tissue was not properly assessed in any of the studies.

c) Autotransplantation of dispersed human pancreas

As a rather separate consideration, much interest has been raised in the possibility of autotransplantation of islet tissue in the human, the commonest indication for this being in patients undergoing pancreatectomy for chronic pancreatitis. In these patients the pancreas is usually fibrotic, and islet isolation using the method described for the rat pancreas is even less successful than with normal human pancreas. The reported transplants have, therefore, used dispersed pancreas prepared by a combination of mechanical tissue chopping and collagenase treatment. When interpreting the results of autotransplantation of islets it is important to remember that most patients submitted to this procedure undergo subtotal pancreatectomy: thus, the absence of diabetes is not a reliable indicator of graft function. Furthermore, invasive investigations to prove graft function are probably not ethically justifiable in these patients (who have a non-life threatening condition) although some centres have carried them out. Graft function can be inferred only in those patients undergoing total pancreatectomy, and even then it is possible for small pieces of pancreas to be left behind.

Najarian et al. (1977,1980) first reported autotransplantation of dispersed pancreas into the portal vein of 10 patients, 1 of whom underwent total pancreatectomy. Following transplantation of the tissue a moderate

rise in portal pressure (30 cms water maximum) was noted, but no further ill-effects were described. 3 patients were insulin independent after the procedure (all after subtotal pancreatectomy), of whom 2 were thought to have functioning transplants. 1 other patient died of unrelated causes, and surviving islet tissue was demonstrated in the liver. This moderately enthusiastic report was later played down by 1 of the co-authors (Sutherland et al. 1981(b)), who described only 1 patient as metabolically normal, possibly due to residual pancreatic tissue.

Valente et al. (1980(a)) described 10 patients given intraportal autotransplants of dispersed pancreas following "total or near-total" pancreatectomy. The number of islets transplanted was stated to range from 25,000 to 400,000, although how this was calculated was not described. No mention was made of the effect of transplantation on portal pressure, and no other ill-effects were noted. Following transplantation 9 out of the 10 patients were stated to be insulin-independent, and function of the graft was stated to be supported by portal and IVC venous samples in 6 patients. However, the data presented does not support this view, and the results could have been due to a functioning pancreatic remnant.

Dobroschke et al. (1978) reported a case of intraportal autotransplantation following total pancreatectomy, claiming transplantation of 100,000 islets, but again the method for counting the islets was not adequate. Severe portal hypertension was noted during infusion of the dispersed pancreas, preventing transplantation of all the tissue. The patient became permanently diabetic immediately after the operation.

Cameron et al. (1981) reported intraportal autotransplantation of 8 patients following 95% pancreatectomy. 3 cases were insulin-independent after the procedure, and venous sampling studies on 1 patient were claimed to show a functioning graft (Cameron et al. 1980), although, in fact,

portal vein insulin concentrations were always higher than those recorded in the hepatic vein. Portal hypertension was much more of a problem in this series than previously reported, both at the time of operation and postoperatively, directly causing the death of 1 patient (Mehigan et al. 1980(b)).

Traverso et al. (1981) reported total pancreatectomy and intraportal autotransplantation in 4 patients. All developed severe systemic hypotension and portal hypertension, preventing transplantation of the complete graft. All patients became diabetic after the procedure.

Recently, Toledo-Pereyra (E.A.S.D. 1984) has described a further 7 cases of intraportal autotransplantation following subtotal pancreatectomy. Again, major problems with portal hypertension were noted, contributing to 3 deaths. 2 patients are now insulin-independent, but this may be because of residual pancreatic function. In an attempt to overcome the problems associated with intraportal transplantation, 1 patient has been given an autograft of dispersed pancreas transplanted under the kidney capsule, without complications. This case is reported to be insulin-independent, but again this has not been proven to be due to a functioning graft.

d) Conclusions

The overall conclusions derived from these studies of autotransplantation of dispersed human pancreas are, firstly, that no case of autotransplantation following total pancreatectomy has been proven to be insulin-independent and, secondly, no case of autotransplantation following partial pancreatectomy has been proven to have a functioning graft rather than residual pancreas. It is not unreasonable to conclude that these studies have actually shown that the technique does not work, and, further, that the technique is dangerous, at least when intraportal injection is used. One erroneous assumption, made when the technique was first put

forward, was that the autografted dispersed pancreas would behave in the same manner as dispersed pancreas in animal experiments. However, the animal experiments used normal pancreas, whereas nearly all the autografts in humans have been dispersed, fibrotic pancreas from patients with chronic pancreatitis. Only 1 study, in dogs (Mehigan et al. 1980(a)), has performed intraportal transplantation of dispersed fibrotic pancreas (induced by pancreatic duct ligation), and found that only 2 of 27 dogs were successful, and that portal hypertension was a real problem. Portal hypertension was not noted to be such a major problem in the early studies (Najarian et al. 1977), and this has been attributed to the use of heparin. There is some support for this idea from work in dogs (Mehigan et al. 1980(b)), but heparin did not prevent the complications seen in the most recent series reported by Toledo-Pereyra (E.A.S.D. 1984). It would seem logical to use a different site of transplantation, but, unfortunately, subtotal or total pancreatectomy almost always necessitates splenectomy. The kidney capsule offers an attractive alternative site, but remains totally uninvestigated as a site for transplantation of adult pancreas.

SUMMARY OF THE HISTORICAL REVIEW

There is reasonable evidence that the complications associated with long-term insulin therapy for diabetes mellitus are caused by poor control of blood glucose or metabolic processes associated with poor glucose control. Transplantation of insulin-secreting tissue has been demonstrated to be a feasible way of returning glucose control to normal, with experimental evidence that the development of diabetic complications will be at least halted, and possibly reversed. The most readily available source of insulin secreting tissue is adult cadaver human pancreas, and this may be transplanted, either as a vascularised whole organ, or as some form of dispersed pancreatic preparation. Vascularised

pancreas transplants have had the advantage of requiring the introduction of little new technology, and functioning vascularised grafts have been attained, but the mortality and morbidity associated with the procedure is still considerable, and the success rate poor, with no recent sign of significant improvement.

Transplantation of dispersed preparations of the pancreas has been proven successful in rodents, where it is possible to transplant purified islets of Langerhans with reversion of diabetes and return of the associated metabolic abnormalities to normal, provided both donor and recipient are isogeneic. However, islet allografts have proven very susceptible to the rejection process and immunosuppressive regimens that are routinely successful for other organ transplants are disappointing when applied to islet allografts.

Application of islet transplantation to larger mammals has been confounded by the lack of a successful method for isolation of purified islets from the more fibrous pancreas, and there is no convincing evidence of a reliable method for transplanting purified islets in animals larger than laboratory rodents. As a less demanding alternative, transplantation of dispersed pancreas without islet purification has been proven to provide acceptable islet cell function, particularly in the dog. Preliminary studies in this model also suggest that islets are very susceptible to the rejection process, as found in rodents.

In humans, allotransplantation of purified islets has been attempted, despite the lack of a working large animal model, and has proven unsuccessful, stumbling on the same problem as islet transplantation in large mammals: the lack of a method for isolation of islets from the fibrous human pancreas. Based on the success of unpurified dispersed pancreas transplants in dogs, several attempts have been made to transplant dispersed cadaver pancreas in humans: all have been unsuccessful. The

reasons for failure have been unclear, but at least the procedure has been relatively harmless. As a sideline to these last-mentioned studies, autografts of dispersed pancreas have been attempted in patients with chronic pancreatitis. Not only have these attempts been largely unsuccessful, but the technique has also been proven dangerous.

It is clear that clinical trials of purified islet or dispersed pancreas allotransplantation should cease until they are based on a more solid scientific foundation: preferably a reliable allograft technique in a relevant large animal model. Further clinical trials of autotransplantation for chronic pancreatitis are unjustified without further research, since the technique is not based on any applicable animal studies and is being performed for a relatively non-life-threatening condition.

The potential of islet transplantation is clear: it could be a minimally traumatic procedure, possibly performed with little or no immunosuppression, with the promise of a true cure for diabetes mellitus. This potential makes islet transplantation worth striving for, but major problems must first be overcome. The two major advances required are, firstly, a reliable method for overcoming the rejection process and, secondly, a method for isolation of islets of Langerhans applicable to the human pancreas. The work described in this thesis falls into two areas of study corresponding to each of these aims, and will be described in two parts.

2. MATERIALS AND METHODS

EXPERIMENTAL ANIMALSRats

Lewis (LEW, RT-1^l) and Dark Agouti (DA, RT-1^a) rats were obtained from an inbred colonies bred at the John Radcliffe Hospital, Oxford. These colonies were both derived from animals obtained from the Charing Cross Hospital, London in 1980, which, in turn, originated from colonies held at the Radcliffe Infirmary, Oxford in 1977. The strain purity was checked by skin grafting at regular intervals. (LEW x DA) F1 hybrids were obtained direct from Charing Cross Hospital. Black hooded (PVG, RT-1^c) and Brown Norway (BN, RT-1ⁿ) inbred rats were obtained from O.L.A.C. Ltd, Bicester, Oxfordshire.

Nude mice

Non-inbred nude mice, based on a CBA strain, were supplied from a breeding colony held at the National Institute for Medical Research, Mill Hill, London.

Dogs

Mongrel dogs weighing between 14-20 kg of both sexes were obtained from Park Farms Ltd., Oxford.

CHEMICALS AND REAGENTSHank's balanced salt solution

Sterile Hank's balanced salt solution (HBSS) was supplied by Flow Labs Ltd., Irvine, Scotland. The constituents of HBSS were as follows (mg/L): CaCl₂.2H₂O 185.5, KCl 400, K₂HPO₄ 600, MgSO₄.7H₂O 200, NaCl 8000, NaHCO₃ 350, Na₂HPO₄ 47.5, Glucose 1000. Modified Hank's solution (MHBSS) was made up in the laboratory to the same formula, but using a lower glucose

concentration of 300 mg/L.

Albumen

Bovine serum albumen (fraction V) was obtained from Miles Laboratories Ltd, Slough, Buckinghamshire, and added to HBSS in a concentration of 4 gm/L.

Tissue culture medium

RPMI 1640 tissue culture medium was supplied as sterile solution by Flow Labs Ltd., Irvine, Scotland, and cultures were kept in an atmosphere of 95% air, 5% CO₂ to maintain correct pH.

Buffer

Phosphate albumen buffer contained 32.0 mmol/litre Na₂PO₄.2H₂O, 32.0 mmol/litre Na₂HPO₄ and 1.0 gm/litre bovine albumen.

Collagenase

Collagenase (type I or type V), derived from cultures of clostridium histolyticum, was obtained from Sigma Chemicals Ltd., Poole, Dorset.

Ficoll

Ficoll 400 powder was purchased from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden, and 30gm dissolved in 100 ml saline or HBSS, corresponding to a density of 1.08. Other densities were prepared by diluting with HBSS, using a densimeter as a check.

Streptozotocin

Streptozotocin was obtained as powder from Upjohn Co., Kalamazoo, U.S.A. and dissolved in acetate buffer (pH 4.5) immediately prior to use.

Neutral red

Neutral red dye (colour index 50040) was obtained from BDH Chemicals Ltd., Poole, Dorset, and dissolved in 0.9% saline to a concentration of 2%.

Fluorescein diacetate

Fluorescein diacetate was obtained from Sigma Chemicals Ltd. and prepared as a stock solution of 200 mg/ml in acetone.

Ethidium bromide

Ethidium bromide was obtained from BDH Chemicals Ltd. Poole, Dorset, and prepared as a stock solution of 200 ug/L in HBSS.

Cyclosporine

Cyclosporine was obtained as a gift from Sandoz Ltd., Basle, Switzerland, made up in olive oil to a concentration of 10 mg/ml, and administered to rats by gavage tube under ether anaesthesia.

STERILISATION

Glassware and instruments were sterilised by autoclave. Solutions (collagenase, ficoll, HBSS etc.) were passed through a millipore filter, pore size 0.45u. Plastic-ware was either obtained pre-sterilised, or sterilised by gamma irradiation or exposure to ethylene oxide vapour.

OPERATIVE PROCEDURES

Rodent anaesthesia

Animals to be sacrificed were anaesthetised by injecting 100 mg/kg pentobarbitone intraperitoneally. Surgical anaesthesia was induced by ether inhalation supplemented by intraperitoneal 5% chloral hydrate. Care was taken to keep animals warm using a heated operating board.

Dog anaesthesia

Animals were first sedated with morphine 0.4 mg/kg and chlorpromazine 1.5 mg/Kg. Anaesthesia was induced by intravenous hexobarbitone, an endotracheal tube inserted and anaesthesia maintained by inhalation of nitrous oxide, oxygen and halothane vapour. Attention was paid to fluid balance and temperature control, use being made of intravenous fluids, a heated pad and a heat reflective blanket.

Rat kidney transplants

Orthotopic renal transplantation was performed using a binocular operating microscope. Through a midline incision the donor left kidney was dissected free, ligating vessel branches, until the renal vessels were clean. The ureter was then divided in its lower third. The recipient was prepared in similar fashion, dividing the ureter in the upper third, then vascular clamps were applied to both renal vessels, and the kidney removed. The donor rat was heparinised (200 units heparin IV), the kidney removed, and rapidly cooled in cold saline. The donor kidney vessels were then anastomosed to the appropriate recipient vessels using interrupted 10/0 nylon sutures (Ethicon Ltd). The vascular clamps were removed to restore blood flow and any leakages dealt with. An end-to-end uretero-ureterostomy was constructed, using interrupted 10/0 nylon sutures, and the wound then closed using 2 layers of 4/0 chromic catgut.

Rats given auxiliary renal transplants underwent anastomosis of the donor renal vessels end-to-side onto the recipient lower abdominal aorta and inferior vena cava, using continuous 10/0 nylon sutures. The ureter was then implanted directly into the bladder through a small puncture hole and secured to the bladder with interrupted 10/0 nylon sutures.

Total pancreatectomy in the dog

Through a midline incision the duodenum was mobilised by Kocher's manoeuvre, and the right limb of the pancreas, which hangs free in the

meso-duodenum, was gently separated from the pancreatico-duodenal vessels that run over the gland (giving rise to vessels that penetrate the surface). Ligatures were placed round the pancreatic vessels (but left untied), until all the branches to the limb were secured. A tie was then placed around the junction of the right limb with the lower part of the angle segment. The ligatures were then rapidly tied and the right limb segment removed and immediately cooled in ice cold HBSS. A similar procedure was performed with the left limb of the pancreas, which lies suspended in the dorsal mesogastrium, and must first be separated from the splenic vessels, before ligating the pancreatic branches. Finally the angle segment, which is closely applied to the duodenum and pylorus, was removed, again dissecting out all the vessels and placing ligatures. The dissection in this region was difficult and had to be taken slowly, great care being taken to preserve the vessels of the duodenal arcade, especially the fine branches to the lower bile duct. The pancreatic duct was ligated with 4/0 silk and divided, then all the pancreatic vessels were rapidly tied, the angle segment removed, and cooled as before. A careful check was made for untied fine branches of vessels, since these tended to bleed later. Finally the abdomen was closed in 2 layers, using 2/0 nylon. Post-operatively, no food or drink was allowed for 2 days, fluid balance being maintained by intravenous saline infusions. Water was then introduced over the next day, followed by small quantities of dog meat when the dog was drinking normally. Later, 3 scoops of Pancrex granular fines (Paines and Byrne, Greenford, Middlesex) were mixed with the dog meat.

ISLET PREPARATION TECHNIQUES

The techniques for islet isolation from the dog and human pancreas are the subject of subsequent chapters and will not be presented here. The technique of islet preparation used for rat transplants is similar to that described by Ballinger and Lacy (1972). 6 donors were used for each

transplant. Through a midline incision, the bile duct of each donor was cannulated with a 2 FG plastic cannula at the point of entry into the duodenum and tied in position with 4/0 silk. A further tie was placed around the bile duct at the level of the hilum of the liver. 10 ml of cold MHBSS was then slowly injected, distending the pancreas (all MHBSS used contained 4 gm/L albumen). The distended pancreas was excised, placed in a 100 ml beaker with 10 ml of MHBSS, and then chopped into 1-2mm fragments with scissors, transferred to a 10 ml test-tube and the fragments allowed to settle. The supernatant, often containing fat particles, was aspirated and discarded, then the tissue was placed in a 25 ml conical flask containing 10 mg collagenase and incubated at 37° C. for 12-16 mins in a shaking water bath. The end-point of digestion was determined by observing characteristic disintegration of the tissue on shaking, at which point the digestion was halted by adding 10 ml of cold MHBSS, and further dispersion of the tissue obtained by aspiration through a 16G needle.

The tissue was washed twice in MHBSS and then allowed to settle for 3 minutes, the supernatant being then discarded. The sedimented tissue was re-suspended in MHBSS and then centrifuged at 750 x G for 10 seconds to give a firm pellet, the supernatant being discarded by inversion. The pellet was suspended in 25% (w/v) ficoll and a density gradient of 23%, 20% and 11% ficoll carefully layered on top. The gradient was centrifuged for 10 minutes at 800 x G, after which the islets were normally found located at the 20%/11% and 23%/20% interfaces. The islets were removed by aspiration, passed through a filter, pore size 500u, then washed twice with MHBSS. The islets were placed in a 35mm plastic petri dish and examined through a stereo-microscope against a black background with side illumination. Islets were easily distinguishable from contaminating vascular fragments and small lymph nodes, and, using a siliconised glass micropipette, were rapidly hand picked clean, with the yield being 600-1200 islets, and the purity greater than 95%.

ISLET TRANSPLANTATIONSubcapsular islet transplants in the rat

The clean islets were accumulated in the bottom of a V-shaped plastic container, then aspirated into a siliconised glass micropipette, held in approximately 10 ul of MHBSS. The recipient kidney was exposed through a midline incision, the capsule incised, elevated and a pocket created beneath it. The islets in the micropipette were deposited in the pocket and the capsule replaced, no suture being necessary to hold the islets in position.

Intraportal islet transplants in the rat

The hand-picked islets were suspended in MHBSS and drawn into a 1 ml syringe. Through a midline incision the superior mesenteric vein was exposed and the islets injected through a 25G needle, washing twice by aspirating back blood. A small piece of sterispon (Allen and Hanbury) was placed on the injection site and pressure applied to stop bleeding.

Intraportal islet transplants in the dog

All animals were previously anaesthetised, having undergone total pancreatectomy. A 3 FG plastic cannula was inserted into a tributary of the superior mesenteric vein and threaded up to the portal vein, the tip being placed 2 cm below the porta hepatis (by palpation). Portal pressure was measured by connecting a saline-filled manometer, correcting the level to that of the catheter tip. The islet preparation was suspended in 10 mls of HBSS and infused over 5 minutes, flushing the tube once. Portal vein pressure was measured immediately before and after infusion, and 10 minutes later. The catheter was then removed and the vein ligated.

BLOOD SAMPLING

Blood samples were obtained from rats by amputating the tail tip (usually taking only a fine slice) under ether anaesthesia and collecting 0.2 ml of blood in a 0.4 ml plastic centrifuge tube (Beckman R.I.I.C. Ltd., High Wycombe, Buckinghamshire). The serum was separated by centrifugation in a microfuge. Venous blood samples from dogs were taken from the cephalic vein and placed in a heparinised tube, centrifuged at 1000 x G for 3 minutes then the serum was aspirated into a plastic tube. Samples for glucose estimation were assayed immediately, whilst samples for insulin estimation were stored at -40° C.

BIOCHEMICAL ASSAYS

Serum glucose analysis.

Serum glucose was measured on a Beckman Mark 2 Glucose Analyzer (Beckman RIIC Ltd., High Wycombe, Buckinghamshire). This instrument measures the fall in oxygen tension when a sample of 10 μ l is introduced to a cuvette containing glucose oxidase, which oxidises the glucose to glucuronic acid and hydrogen peroxide. The instrument is first calibrated against a standard 8.3 mmol/L glucose solution and is accurate to 1% in the range 1-25mmol/litre.

Insulin secretion of islets in response to glucose stimulation

2 different experimental systems were used. For rat and dog experiments, islets were hand-picked, in groups of 5 islets, into 2 ml plastic tubes containing 1 ml of HBSS, modified to contain glucose at a concentration of either 2 mmol/litre or 20 mmol/litre. Care was taken to distribute large and small islets randomly between tubes, without prior knowledge of the eventual glucose concentration to be used. The tubes were capped and incubated at 37° C. for 2 hours, then centrifuged at 50 G

for 2 minutes. A sample of 200 μ l was taken, added to 800 μ l phosphate albumen buffer and stored at -40° C. before assay for insulin content.

Human islets were hand-picked, in groups of 5 islets, into microtitre-plate wells containing 100 μ l of RPMI 1640 medium, modified to contain glucose at a concentration of 0, 10 or 20 mmol/litre. The islets were incubated at 37° C. in an atmosphere of 95% air, 5% CO₂ for 6 hours. A 20 μ l sample was taken, diluted to 250 μ l in phosphate albumen buffer, and stored at -40° C.

Assay of the ATP content of islets

The ATP content of islets was measured using chemiluminescence (Wettermark et al. 1970, Ashcroft et al. 1973). 5 islets were placed in 1 ml of 3% trichloroacetic acid, homogenised by sonication then neutralised with 8.4% sodium bicarbonate solution. ATP content was measured by adding the sample to luciferin and luciferase and measuring the light emitted in a luminometer (Model 1250, LKB-Wallac, Turku, Finland). ATP content was corrected against standards prepared by an identical method.

Insulin radio-immunoassay

i) Principles of the technique.

The insulin present in serum samples and samples of medium was assayed by the method of Albano et al. (1972). The method is a single antibody technique, in which a fixed quantity of antibody is allowed to react with the unknown sample in the presence of I¹²⁵ labelled pure insulin. There is competitive binding between the labelled insulin and the insulin in the unknown sample, thus, at equilibrium, the quantity of unbound labelled insulin is inversely proportional to the quantity of insulin in the sample. The free and bound I¹²⁵-labelled insulin is separated by charcoal

absorption of unbound insulin and the radioactivity of the charcoal (gamma emission) measured.

ii) Technical details

Standards

Standard rat, dog or human insulin (Novo laboratories, Denmark) was diluted to concentrations of 0, 2.5, 5, 10, 20, 30, 40, 60 and 80 microunits/ml. I^{125} iodinated insulin (Radiochemical Centre, Amersham, Buckinghamshire) was dissolved in phosphate albumen buffer to a concentration of 0.5 uCi in 10 ml.

Guinea pig anti-human-insulin serum (Wellcome Laboratories, London), 0.5 ml, 1:1000 lyophilised, was diluted to 20 ml in phosphate albumen buffer. Insulin free plasma was produced by charcoal extraction of fresh pig plasma.

Technique

Triplicate 100 ul samples of the unknown sample were added to 50 ul of phosphate albumen buffer. Triplicate samples of the standards were added to 50 ul of insulin free plasma. 50 ul of guinea pig anti-human-insulin serum was then added to both standards and unknown samples, and 50 ul of phosphate albumen buffer added to a further set of control tubes. All samples were incubated for 90-120 minutes, 50 ul of I^{125} -iodinated insulin added, and the samples incubated overnight at 4° C.. 1 ml of charcoal solution was then added, agitated then centrifuged at 2000 x G for 20 minutes. The supernatant was discarded and the tubes, containing charcoal pellets, loaded on a gamma counter (LKB 1282 Compugamma), which measured the gamma emission from each tube, calculated the mean of 3 tubes and compared the mean to a curve constructed from the standard values. The results were expressed in microunits of insulin per ml. The assay can detect insulin to a minimum concentration of 2.5 microunits/ml and is

accurate to within 20% for concentrations up to 80 microunits/ml.

Concentrations above this require subdilution before assay.

Processing of tissue for histological examination

Electron microscopy

Tissue for electron microscopy was fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer for 2 hours, with secondary fixation in osmium tetroxide. The tissue was then stained with uranyl acetate, embedded in araldite, sections cut on an ultramicrotome, and examined in a Philips EM-200 electron microscope.

Light microscopy.

Tissue was fixed in 10% formal-saline, embedded in wax, and 2u sections cut and mounted. Sections were stained with Harris' haematoxylin and eosin and also with Gomori's aldehyde fuchsin stains (counterstained with haematoxylin and orange G).

Immunoperoxidase stain for insulin was by the indirect peroxidase technique of Nakane and Pierce (1967), with primary antibodies to insulin (Miles diagnostics) and 3,3' diaminobenzidine (Sigma) as the peroxidase substrate. Controls for the staining were: omission of the primary substrate and preabsorption of the antisera with purified antigen.

3. PART 1

TRANSPLANTATION OF PANCREATIC ISLET ALLOGRAFTS IN RATS RENDERED
IMMUNOLOGICALLY UNRESPONSIVE TO VASCULARISED ORGAN ALLOGRAFTS

PART 1, CHAPTER 1.INTRODUCTION

The correction of experimental diabetes in the rat has been accomplished by the transplantation of syngeneic isolated pancreatic islets to a variety of sites. However, transplantation across a major histocompatibility barrier results in rejection, which has proved very difficult to suppress using a variety of regimens (Morris et al. 1980(b)). Allogeneic islets injected into the portal vein of untreated rats are rejected rapidly, but an interesting effect was described by Reece-Smith et al. (1981(a)): simply transplanting allogeneic islets to another site, under the renal capsule, resulted in slight, but significant, prolongation of survival. Disappointingly, allogeneic islets transplanted to the renal subcapsular site, followed by cyclosporine treatment of the rat, did not achieve long-term survival, although some delay in rejection was noted compared to intraportal islets similarly treated (Reece-Smith et al. 1981(a)). A further discovery was that islets placed under the kidney capsule of a diabetic syngeneic host for a few days, could then be transplanted, along with the kidney, into an allogeneic host. Subsequent cyclosporine treatment would then induce long-term survival (Reece-Smith et al. 1981(b)), in similar fashion to the effect of cyclosporine on renal allografts (Homan et al. 1980). This model, though interesting, is unlikely to have any practical application to the clinical situation. Furthermore, there are some inexplicable oddities about the model: the intermediate host must be a streptozotocin diabetic rat, since the use of a normal rat, or even a pancreatectomised rat, does not always prevent subsequent rejection of the islets (Reece-Smith et al. 1983).

A most interesting discovery was the finding by Reece-Smith et al. (1982) that allogeneic islets could be transplanted into rats made

unresponsive by renal allografts. In these experiments DA (RT-1^a) rats were given orthotopic LEW (RT-1^l) renal allografts, and treated with cyclosporine (5 mg/kg/day for 14 days). Contralateral nephrectomy was performed 7 days later, and a high proportion of animals went on to long-term survival (Homan et al. 1980). At times varying from 100 to 200 days after kidney transplantation the animals were made diabetic by streptozotocin, and then LEW islets were transplanted under the transplant kidney capsule, without further immunosuppression. All 7 animals transplanted became normoglycaemic, and 3 animals survived 100 days (the others died normoglycaemic: probably of natural aging processes). Graft function in these animals was proven by nephrectomy, demonstrating return of hyperglycaemia before death by uraemia. The effect was shown to be strain-specific by use of third party PVG (RT-1^c) islets, which were rejected normally.

This description was exciting, because the phenomenon described does have potential for clinical application. At present, most diabetic patients who are candidates for pancreatic transplantation, are also suffering from renal failure and require a kidney transplant. It may become possible to isolate islets from a donor pancreas and store them, probably by cryopreservation (long-term cryopreservation followed by successful transplantation has already been proven for rat islets by Rajotte et al. 1981). It may then be possible to, firstly, transplant a kidney alone into the uraemic diabetic patient, which we know from experience will be accepted in many cases, using immunosuppressive regimens currently available. Whilst renal transplantation is being performed, islets could be isolated from the pancreas of the same donor and cryopreserved. At a later date, when kidney function is stable, the islets could be transplanted into the patient, perhaps without the need for increased immunosuppression.

This scenario, whilst not an impossible dream, assumes several advances in human islet isolation and cryopreservation that have yet to be made. Furthermore, it also assumes that the long-surviving human kidney transplant is subject to a similar unresponsive state to that seen in the rat: yet there are obvious differences, notably the requirement for continual immunosuppression in the human (although often minimal in the "accepted" human renal allograft). Nevertheless, the phenomenon described in the rat is worthy of full investigation, since it appears to allow long-term survival of islets without rejection: a goal rarely attained for islets, using more conventional immunosuppressive regimens.

The description, by Reece-Smith, of long-term survival of islet allografts in rats rendered unresponsive to renal allografts, was particularly remarkable because similar studies had claimed an opposite result. Nash et al. (1978) reported that (AS x AUG)F1 islet allografts, transplanted to AS rats bearing long-surviving enhanced (AS x AUG)F1 heart allografts, showed no increase in survival: indeed, accelerated rejection was seen. Reckard et al. (1979) described (LEW x BN)F1 islet allografts transplanted into LEW rats, bearing long-surviving enhanced (LEW x BN)F1 renal allografts, without prolongation of survival. Perloff et al.

(1981) transplanted BN islet allografts into LEW rats, bearing long-surviving enhanced BN heart allografts, and noted only slight prolongation of survival. The successful experiments of Reece-Smith et al. (1982), therefore, warranted repetition, and also further careful investigation to answer the following questions:

1. Can the experiment be repeated, and is the effect consistent, using rats transplanted at identical times?
2. Can the experiment be repeated in another strain combination?
3. Can strain specificity be confirmed?
4. Is the effect due to the site of transplantation (perhaps the transplant

kidney is a relatively "privileged" site, a possible reason being loss of normal lymphatic channels)? Does unresponsiveness extend to islets transplanted to another site?

5. Is the effect related to the use of cyclosporine, or does it extend to islets transplanted into rats bearing long-surviving renal allografts induced by another method?

6. If the effect is due to a true unresponsive state, will removal of the renal allograft (which originally induced the unresponsive state) result in rejection of the islets?

PART 1, CHAPTER 2.ISOLATED PANCREATIC ALLOGRAFTS IN RATS RENDERED IMMUNOLOGICALLY
UNRESPONSIVE TO RENAL ALLOGRAFTS BY CYCLOSPORINE TREATMENTMaterials and methods

Islets were transplanted from LEW (RT-11), PVG (RT-1^C) or BN (RT-1^N) rats into DA (RT-1^a) or PVG rats of the same sex. Long-surviving kidney allografts were produced by a course of cyclosporine (10 mg/kg body weight by gavage immediately after the transplant and daily for 14 days). The kidney was placed orthotopically, and a contralateral nephrectomy performed 7 days after transplantation. Diabetes was produced by intravenous injection of streptozotocin, using 65 mg/kg for normal rats, and 50 mg/kg, given 75 days after transplantation, for rats bearing renal transplants (streptozotocin is more potent in transplanted rats). Diabetes was confirmed, by repeated serum glucose estimation above 22mmol/L, 2 weeks after injection.

Islets were isolated from 6 donors by the collagenase digestion/ficoll separation technique, and then hand-picked under a dissecting microscope. Islets were either injected into the portal vein suspended in 1 ml of Hank's solution, or placed under the transplanted kidney capsule in 10 ul of Hank's solution. After islet transplantation all animals underwent daily estimation of serum glucose and body weight, then thrice weekly for 4 weeks, and weekly thereafter.

After death of the animal all grafted tissues, and also pancreases from streptozotocin treated rats, were examined histologically using aldehyde fuchsin stain to identify islet tissue.

The following experiments were performed:

- 1) DA rats were made diabetic and given LEW islets into the portal vein, with no other treatment.
- 2) DA rats were made diabetic and given LEW islets under the kidney capsule, with no other treatment.
- 3) DA rats were given LEW kidneys and treated with cyclosporine. Long-survivors were made diabetic, and then allocated to 4 groups;
 - (a) no treatment (controls);
 - (b) intraportal LEW islets;
 - (c) renal subcapsular LEW islets.
 - (d) renal subcapsular BN islets.
- 4) PVG rats were made diabetic and given LEW islets beneath the renal capsule, with no further treatment.
- 5) PVG rats were given LEW renal allografts and treated with cyclosporine. Long-survivors were made diabetic, and given LEW islets beneath the renal capsule. Animals that did not reject their islet allograft after 100 days then underwent retransplantation, replacing the LEW kidney and islets with a normal PVG kidney.

Results

The results are summarised in table 1-2-1.

- 1) Transplantation of LEW islets into the portal vein of a diabetic DA rat, with no other treatment, resulted in rejection in less than 6 days (Fig. 1-2-1).
- 2) Transplantation of islets to the renal subcapsular site delays rejection to around 7 days (range 6-8 days) (Fig. 1-2-2).
- 3) From an original total of 45, technically satisfactory, LEW kidney transplants into DA recipients, 15 grafts were lost from rejection, and a further 7 were lost for non-immunological reasons. 23 rats were successfully made diabetic, and divided between the following groups:

TABLE 1-2-1. SURVIVAL OF ISOLATED ISLET ALLOGRAFTS
TRANSPLANTED INTO DIABETIC RATS

<u>GROUP</u>	<u>N</u>	<u>DIABETIC</u> <u>RECIPIENT</u>	<u>LONG</u> <u>SURVIVING</u> <u>KIDNEY</u>	<u>DONOR</u> <u>OF</u> <u>ISLETS</u>	<u>SITE</u>	<u>TIME TO REJECTION</u> <u>OR DEATH (DAYS)</u>
1	5	DA	-	LEW	PV ^a	3,4,4,4,5
2	5	DA	-	LEW	SC ^b	6,6,7,7,8
3	7	DA	LEW	-	-	All diabetic until death
4	5	DA	LEW	LEW	PV	15,90,>100 x 3
5	5	DA	LEW	LEW	SC	83,>100 x 4
6	3	DA	LEW	BN	SC	7,7,9
7	5	PVG	-	LEW	SC	5,6,6,7,7
8	7	PVG	LEW	LEW	SC	70,>100 x 6

^a PV = Portal vein
^b SC = Renal subcapsular

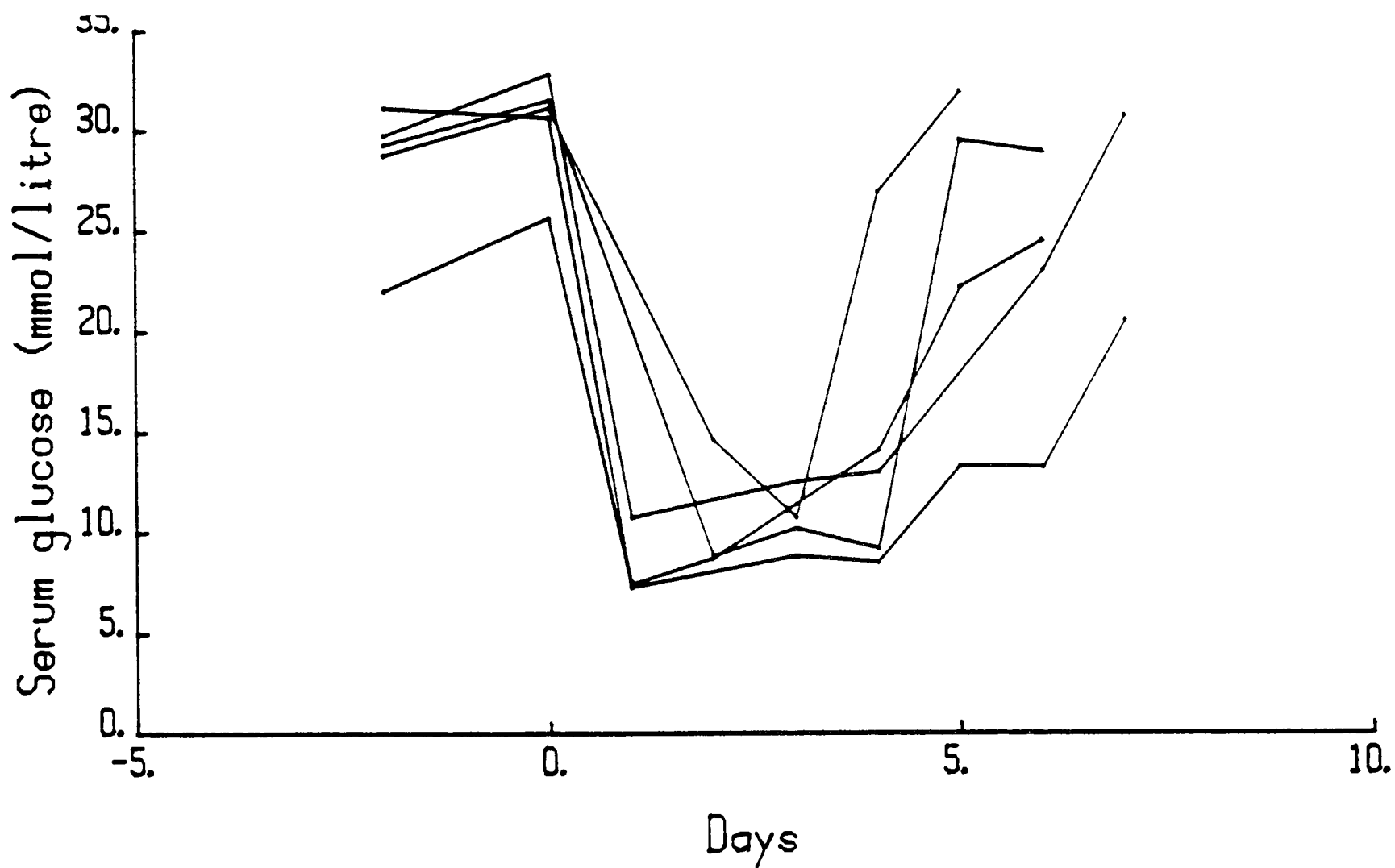


Fig. 1-2-1. Serum glucose of 5 diabetic DA rats given LEW islets into the portal vein on day 0.

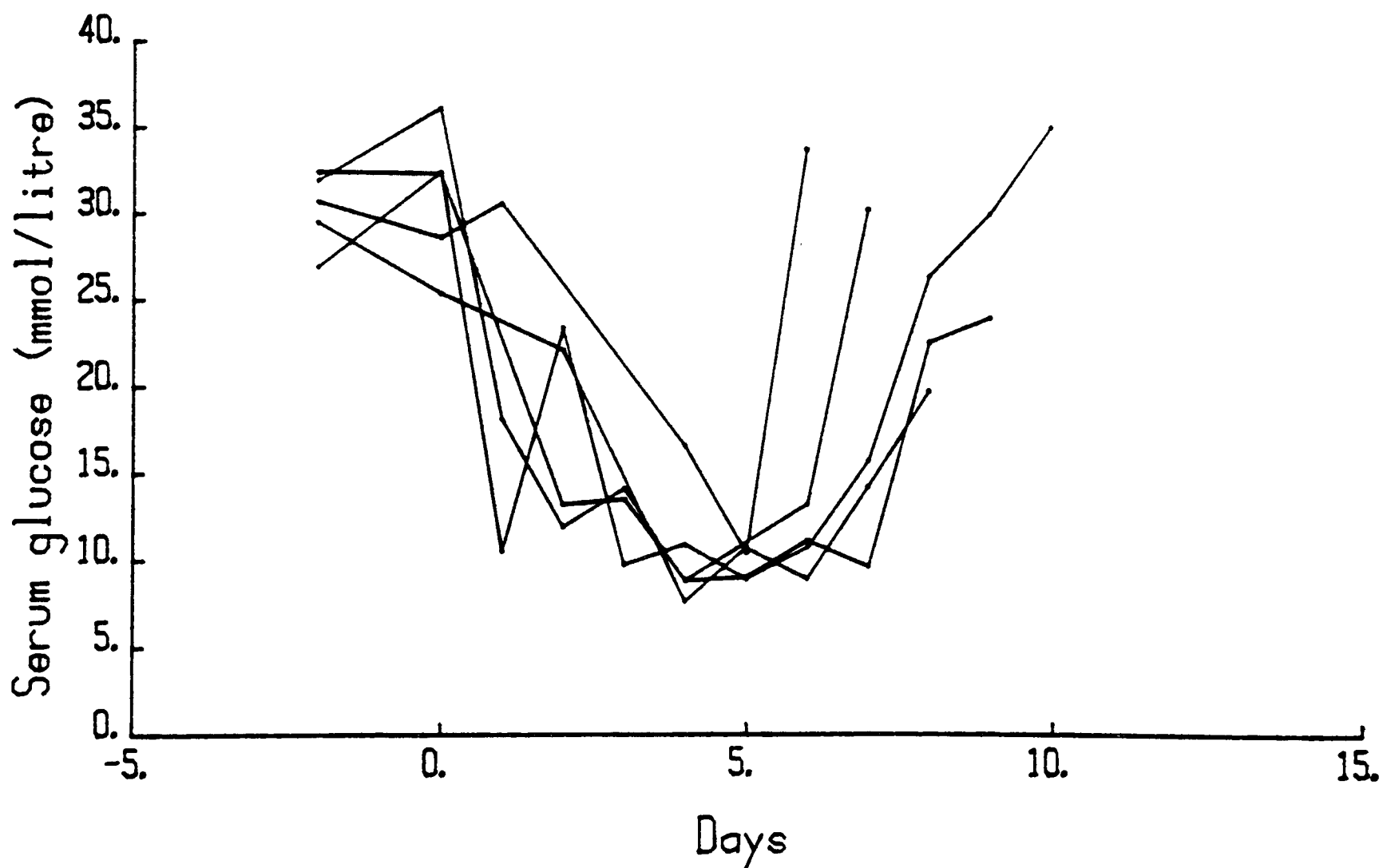


Fig. 1-2-2. Serum glucose of 5 diabetic DA rats given LEW islets under the renal capsule on day 0.

a) 7 rats were given no further treatment after being made diabetic (Table 1-2-1, group 3). All these rats remained diabetic, with serum glucose consistently more than 22 mmol/litre, obvious polyuria and loss of weight (Fig. 1-2-3). 2 rats died at 29 and 56 days, the others survived for 100 days, but in a very poor state. Histology of their pancreases showed virtually no pancreatic islets, with no beta cells in those remaining islets that could be identified.

b) 5 rats, bearing a LEW renal allograft, were given intraportal LEW islets (Table 1-2-1, group 4). 1 subsequently rejected at 15 days, but the others accepted their grafts, gained weight, and reversed their polyuria. 1 died, normoglycaemic, at 90 days, the others surviving to 100 days (Fig. 1-2-3). Histological examination of the liver revealed intact intraportal islets (Plate 1-2-1), whilst the appearance of the pancreas was similar to group 3.

c) 5 rats, bearing a LEW renal allograft, were given LEW islets under the capsule of the long-surviving kidney (Table 1-2-1, group 5). All became normoglycaemic, gained weight, and reversed their glycosuria (Fig 1-2-3). 1 rat died, normoglycaemic, at 83 days, the rest surviving to 100 days (Fig. 1-2-3). Histology of the transplanted kidney and islets showed well-preserved, subcapsular islets, with no rejection (Plate 1-2-2). The pancreas showed the same histology as in group 3.

d) 3 DA rats, bearing a LEW renal allograft, were given BN islets beneath the capsule of the long-surviving LEW kidney. Hyperglycaemia was reversed in every case, but all became diabetic again by 9 days (Table 1-2-1, group 6). Renal function in the transplanted LEW kidney was unaffected.

4) 5 PVG rats were made diabetic, and given LEW islets transplanted under the renal capsule (Table 1-2-1, group 7). All reversed their diabetes, but promptly rejected the islets in under 8 days (Fig. 1-2-4).

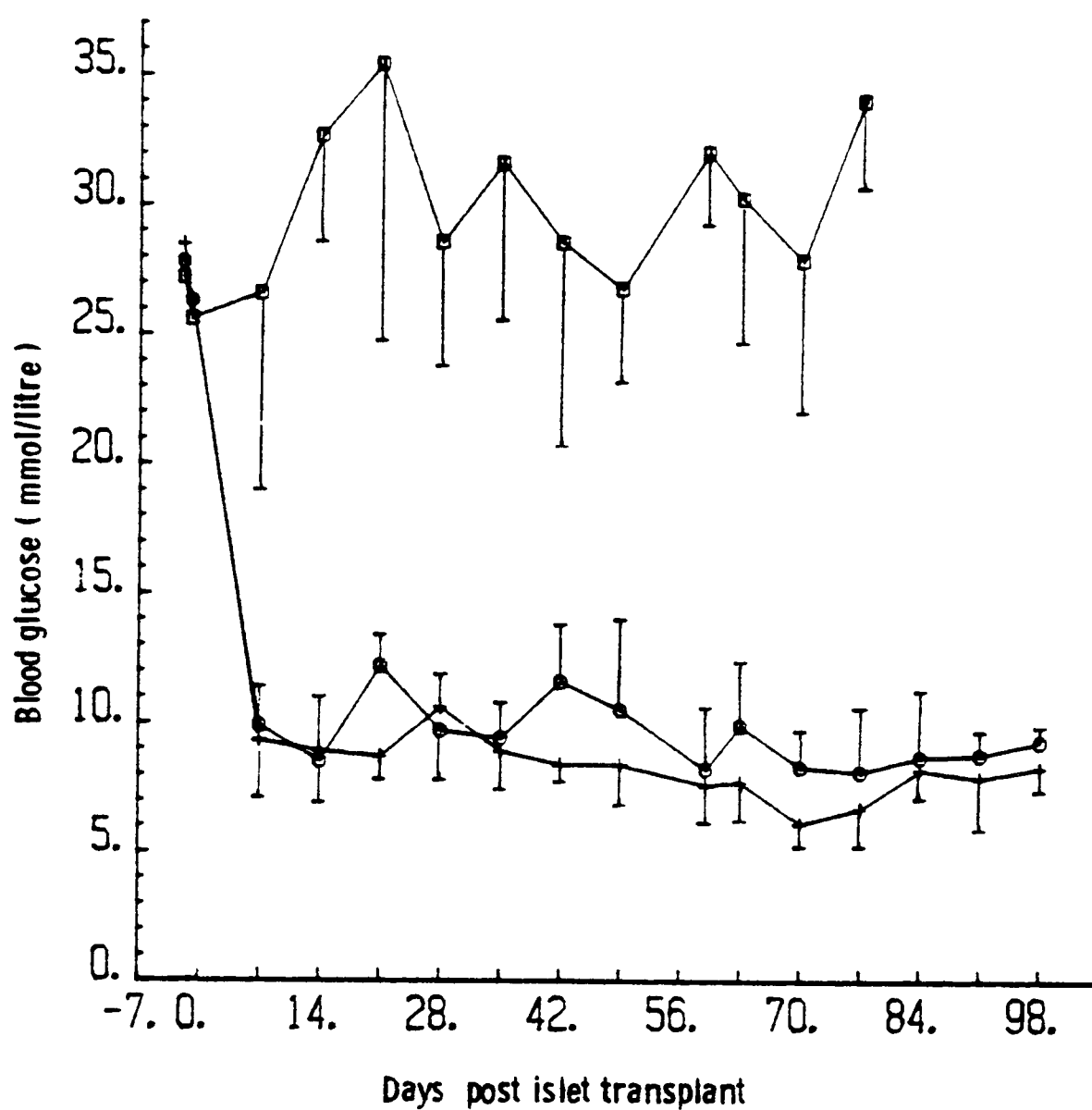


Fig. 1-2-3. Mean serum glucose ($\pm$ S.D.) of DA rats bearing long-surviving LEW kidneys, made diabetic, and given the following treatment:

□ = No further treatment.

○ = LEW islets transplanted into the portal vein on day 0.

+ = LEW islets transplanted under the renal capsule day 0.

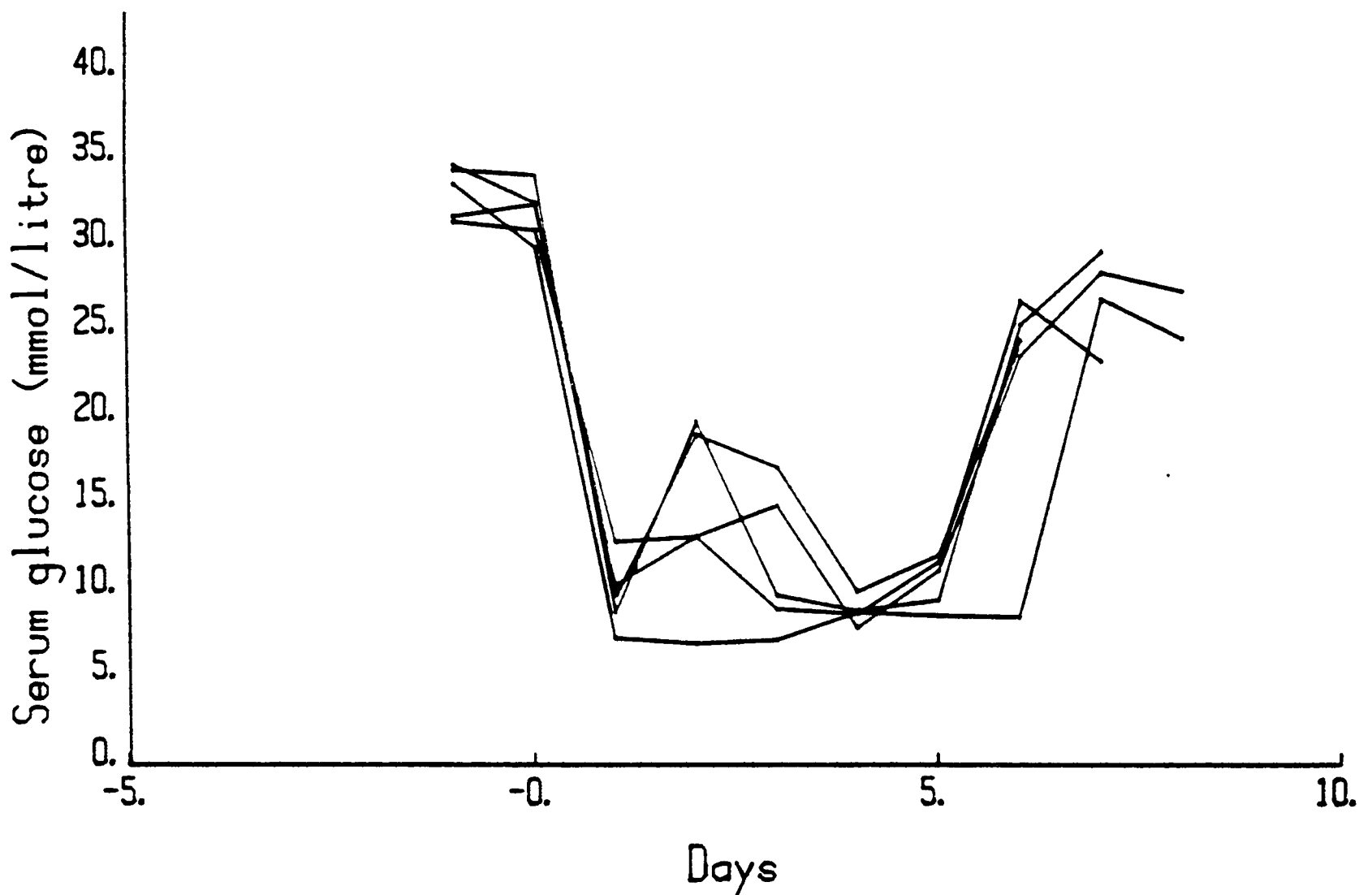


Fig. 1-2-4. Serum glucose of 5 diabetic PVG rats given LEW islets under the kidney capsule on day 0.

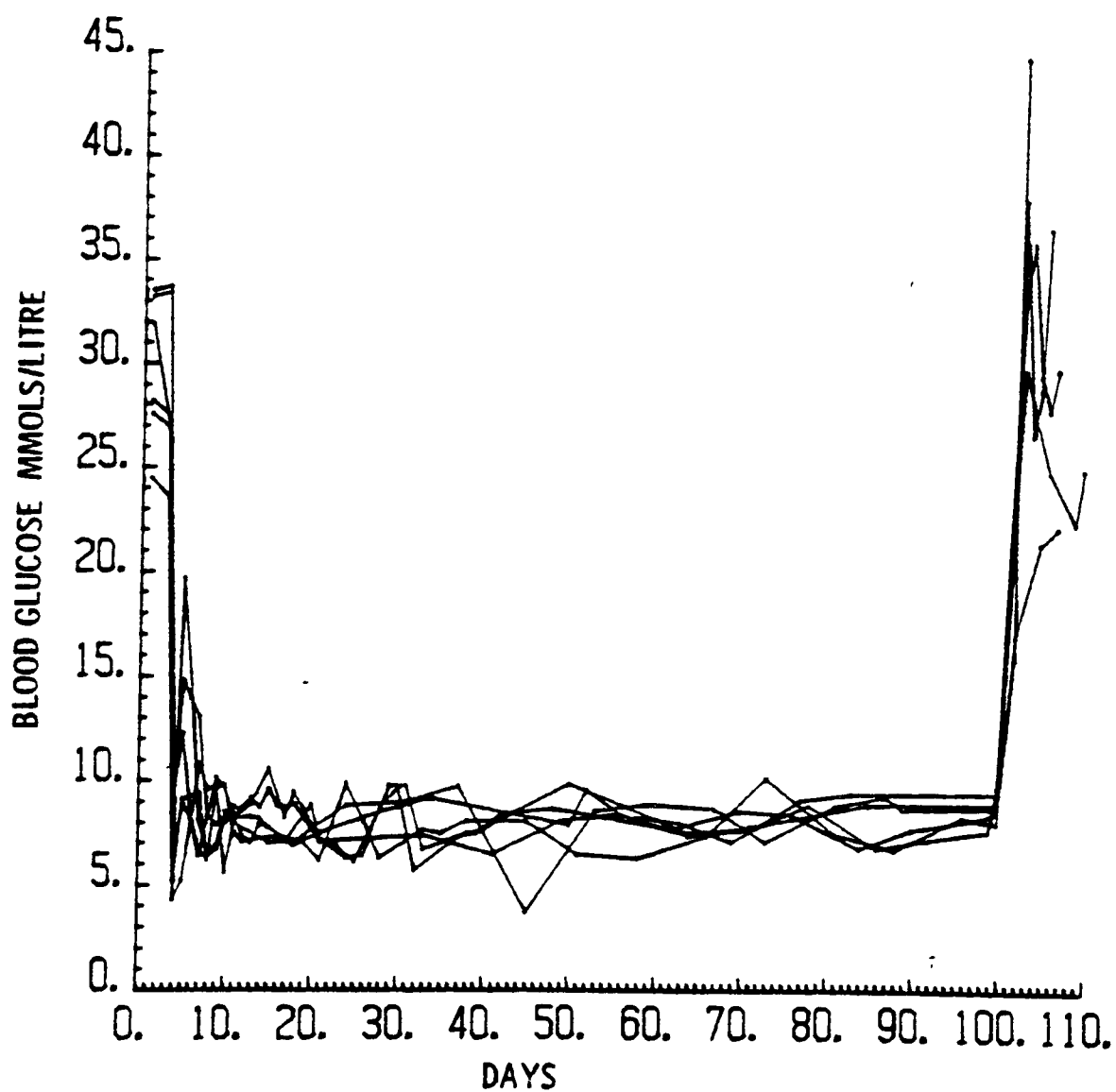


Fig. 1 2-5. Serum glucose of 6 PVG rats, bearing long-surviving LEW kidneys, made diabetic, then given LEW islets under the kidney capsule on day 0. After 100 days the LEW kidney and islets were removed, and replaced by a normal PVG kidney.

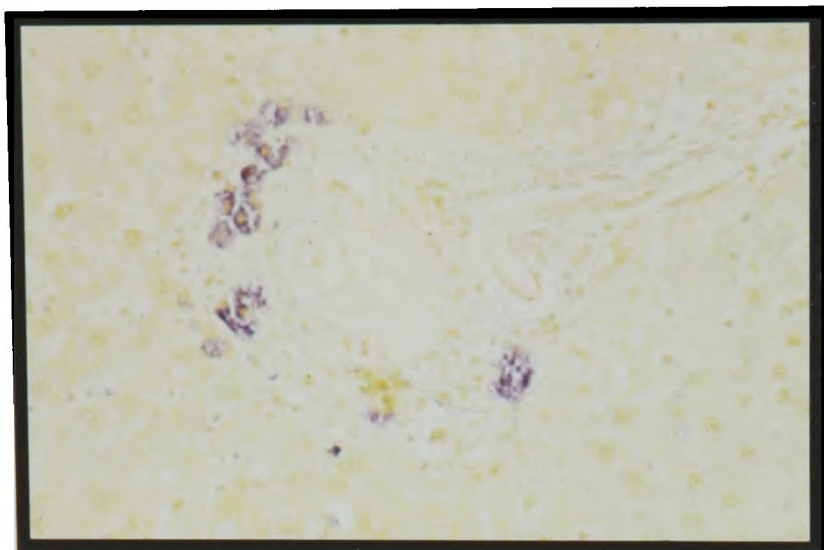


Plate 1-2-1

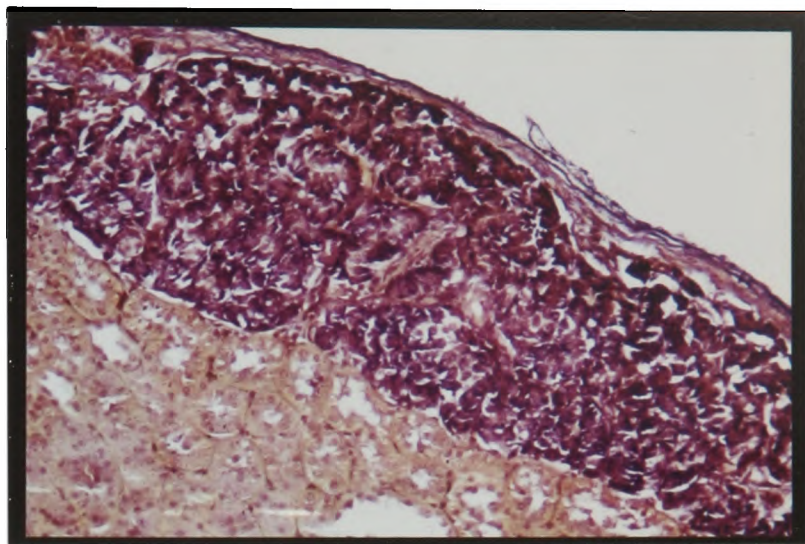


Plate 1-2-2

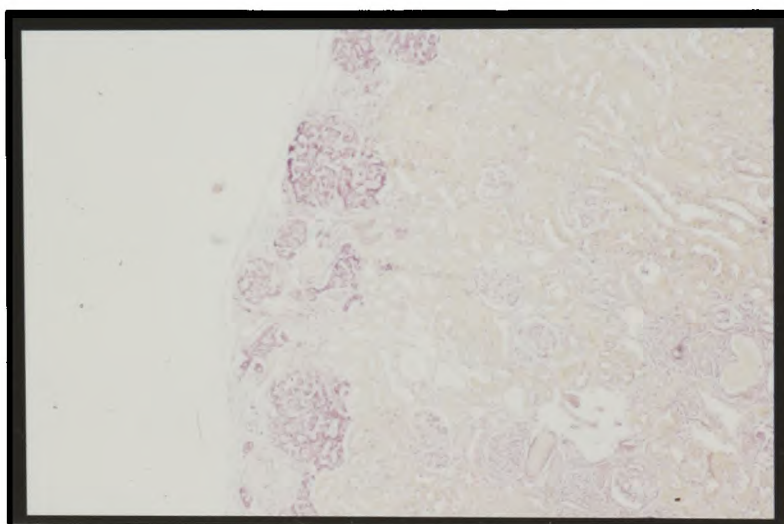


Plate 1-2-3

Plate 1-2-1. Photomicrograph of a section of liver from a DA rat, bearing a long-surviving LEW renal allograft, made diabetic and then given LEW islets into the portal vein. Tissue taken 100 days after islet transplantation. Islet tissue is easily identified surrounding the recanalised portal venule, by the blue-staining granules of the B cells. Aldehyde fuchsin. Magnification x 150.

Plate 1-2-2. Photomicrograph of a section of kidney from a DA rat bearing a long-surviving LEW kidney, made diabetic then given LEW islets under the kidney capsule. Tissue taken 100 days after islet transplantation, showing structurally intact islet tissue containing B cells with blue staining granules under the kidney capsule. There is no evidence of rejection. Aldehyde fuchsin. Magnification x 150.

Plate 1-2-3. Photomicrograph of a section of kidney from a PVG rat bearing a long-surviving LEW kidney, made diabetic, then given LEW islets under the kidney capsule. Tissue taken 100 days after islet transplant, showing well preserved islet tissue, containing B cells with blue-staining granules. There is no evidence of rejection. Aldehyde fuchsin. Magnification x 60.

5) 16 PVG rats received (technically successful) LEW renal allografts. 3 grafts were lost from rejection, and 3 from non-immunological causes, leaving 10 long-survivors. 7 long-survivors were successfully made diabetic, and received LEW islets under the kidney capsule. 1 rat died normoglycaemic at 70 days, the others all surviving, normoglycaemic, to 100 days (Table 1-2-1, group 8, Fig. 1-2-5). At that time, all 6 rats underwent retransplantation, in which the LEW kidney bearing LEW islets was removed and replaced by a PVG kidney. 1 rat died during this procedure. All animals surviving the operation immediately became diabetic once more (Fig. 1-2-5), thus proving that the subcapsular islets were responsible for the maintenance of normoglycaemia. Histological examination showed well-preserved islets with no evidence of rejection (Plate 1-2-3).

Discussion

These experiments confirm that permanent acceptance of allogeneic islets can be produced in the adult animal by implantation of the islets beneath the capsule of a long-surviving allogeneic kidney of the same strain as the islets. The effect is specific, and also reproducible in another strain combination. That this effect is not due, in part, to a protective effect of the renal subcapsular site is shown by the similar behaviour of islets placed in the liver. In other words, a rat which has been made tolerant to a renal allograft by a short course of cyclosporine will specifically accept isolated islets of the same strain, implanted either beneath the renal capsule of the transplanted kidney, or in the host liver. This suggests that no tissue-specific alloantigens are exposed on islets by their preparation (other than those shared by the kidney). Furthermore, it does suggest that the early failure of allogeneic islets transplanted to the liver is due to an immunological response, rather than to non-specific destruction of damaged islets in an organ with an active

reticulo-endothelial system (a conclusion also reached in experiments on islet allografts in neonatally tolerant rats by Naji et al. 1975, Frangipane et al. 1977).

These findings are in contrast to the previously reported inability of long-surviving heart allografts to protect allogeneic islets of the same strain (Nash et al. 1978, Perloff et al. 1981(b)). This might imply the presence of tissue-specific alloantigens, common to the kidney and islets, but not present on heart tissue. However, the differences are more likely to be related to the method of induction of graft tolerance in those experiments, namely passive enhancement.

There is also a previous report of the inability of long surviving renal allografts to protect allogeneic islets of the same strain (Reckard et al. 1979), although that report was based on only 2 rats, again where tolerance had been induced by passive enhancement.

The mechanism, by which a long-surviving renal or cardiac allograft is maintained after a short course of cyclosporin A, is thought to be due to the generation of a suppressor T cell population (Morris et al. 1983, Bordes-Aznar et al. 1983). In the case of passive enhancement, the mechanism by which a graft is maintained has not been clearly established, but there is good evidence for a suppressor cell mechanism (Morris et al. 1980(a), Barber et al. 1984, Batchelor et al. 1984).

Whatever the mechanism, it is clear from the experiments presented here that this approach presents a powerful way of avoiding rejection of allografted isolated adult islets, at least in the rodent.

PART 1, CHAPTER 3.ISLET ALLOGRAFTS IN RATS MADE TOLERANT OF RENAL ALLOGRAFTS
BY WHOLE BLOOD TRANSFUSION COMPARED TO ISLET ALLOGRAFTS
FOLLOWING WHOLE BLOOD TRANSFUSION ALONEIntroduction

The previous experiments have shown that, once a rat has accepted a renal allograft under the influence of cyclosporine treatment, islet allografts, of the same strain as the renal allograft, can be transplanted without further immunosuppression, with long-term survival in a large proportion of cases. It is possible that this effect is related to the use of cyclosporine to induce renal allograft acceptance, and it is therefore of some interest to investigate this effect in rats bearing long-surviving kidneys, produced by a method other than cyclosporin A treatment. Donor-specific blood transfusion has been shown to be an effective way of preventing renal allograft rejection in the rat (Fabre and Morris 1972), dog (Obertop et al. 1978), monkey (Van Es et al. 1977) and man (Salvatierra et al. 1983). The effect of donor-specific whole blood transfusion on islet allografts in rodents seems less successful. In rats, donor-specific blood transfusion alone is reported to produce no delay in rejection (Selawry et al. 1981), and in mice it produced accelerated rejection (Faustman et al. 1983). We therefore felt that this system would be a good test of whether the unresponsiveness to islets produced by a long-surviving renal allograft was confined to rats treated with cyclosporine.

Materials and methods

The rat strains used were DA(RT1^a), LEW (RT1^l), and (LEW x DA)F1. Donor-specific blood transfusion was given by injecting LEW rats with 1 ml

DA heparinised fresh whole blood, intraperitoneally, twice weekly for 6 weeks prior to transplantation. Orthotopic renal allografts were performed with end-to-end anastomosis of the renal vessels and ureter. The contralateral kidney was removed 7 days after transplantation. Long-survivors underwent blood urea estimation at 39 days. Diabetes was produced by intravenous injection of streptozotocin; 65 mg/kg was given to normal rats and 50 mg/kg to rats bearing long-surviving renal allografts (streptozotocin appears to be more toxic in transplanted rats). Rats were considered diabetic if the serum glucose was above 22mmol/L on 2 occasions 2 weeks after injection of streptozotocin.

Pancreatic islets were isolated from 6 (LEW x DA)F1 donors by the collagenase digestion/ficoll separation technique and then hand-picked under a dissecting microscope. Islets were transplanted to LEW recipients under the capsule of either the left kidney or the long-surviving renal allograft, as appropriate. The following experimental groups were performed:

- 1) LEW rats were made diabetic and were given (LEW x DA) F1 islet transplants with no other treatment.
 - 2) LEW rats were given 6 weeks DA whole blood transfusion and were made diabetic then transplanted with (LEW x DA)F1 islets.
 - 3) LEW rats were given 6 weeks DA blood transfusion then transplanted with a (LEW x DA)F1 kidney. Long-survivors were made diabetic 100 days after kidney grafting and were then transplanted with (LEW x DA)F1 islets.
- Following islet transplantation blood sugars were performed daily for 2 weeks, thrice weekly for 2 weeks then weekly up to 100 days. Body weight was recorded weekly. Rejection was defined as a blood sugar elevated above 11 mmol per litre on 2 consecutive days. At 100 days, long-surviving islet grafts underwent removal of the kidney bearing the islets, an isogeneic (LEW) kidney being transplanted in it's place in those rats having only the single (allograft) kidney. Blood sugar and urea estimations were then performed for a further 3 days.

After rejection or death of the recipient all grafted tissues and the host pancreas were examined histologically using haematoxylin/eosin stain and aldehyde fuchsin stain to identify islet tissue.

Results

- 1) Diabetic LEW rats transplanted with (LEW x DA)F1 islets failed to achieve normoglycaemia (Fig. 1-3-1). So unusual was this finding compared to our results in other strain combinations (where normoglycaemia is usually achieved for at least a few days) that a separate group of controls were repeated (making 8 in all). Histology showed a large mass of transplanted tissue with total degranulation of the islets, necrosis and infiltration with lymphocytes and macrophages.
- 2) 5 LEW rats were transfused with DA blood and made diabetic, then transplanted with (LEW x DA)F1 islets. 4 rats failed to achieve normoglycaemia. 1 rat became normoglycaemic at 5 days, then rejected at 8 days (Fig. 1-3-2). Histology showed similar features to group 1.
- 3) 8 LEW rats were transfused with DA blood then given (LEW x DA)F1 renal allografts. There were no technical failures and all became long-survivors, with a mean urea of 80.5 mg% (S.D. 32.5, range 49-112). Injection of streptozotocin killed 3 rats, the others all became diabetic. 5 rats received (LEW x DA)F1 islet transplants. 1 transplant was technically inadequate (islets lost in transfer) and was repeated successfully. All rats reversed their diabetes, and remained normoglycaemic for 100 days. At this time the graft bearing the islets was removed and replaced by a LEW kidney transplant. All 5 rats showed reversion to the diabetic state (Fig. 1-3-3). Histological examination of the graft kidney showed an almost normal kidney histology in 2 grafts, mild focal lymphocytic infiltration in 2 grafts, and more diffuse moderate lymphocytic infiltration in 1 graft. Histological examination of the islets showed clumps of obvious islet tissue in all 5 grafts (Plate 1-3-1). 1 graft showed a mild lymphocytic infiltrate, and in 1 graft the infiltrate was moderately heavy.

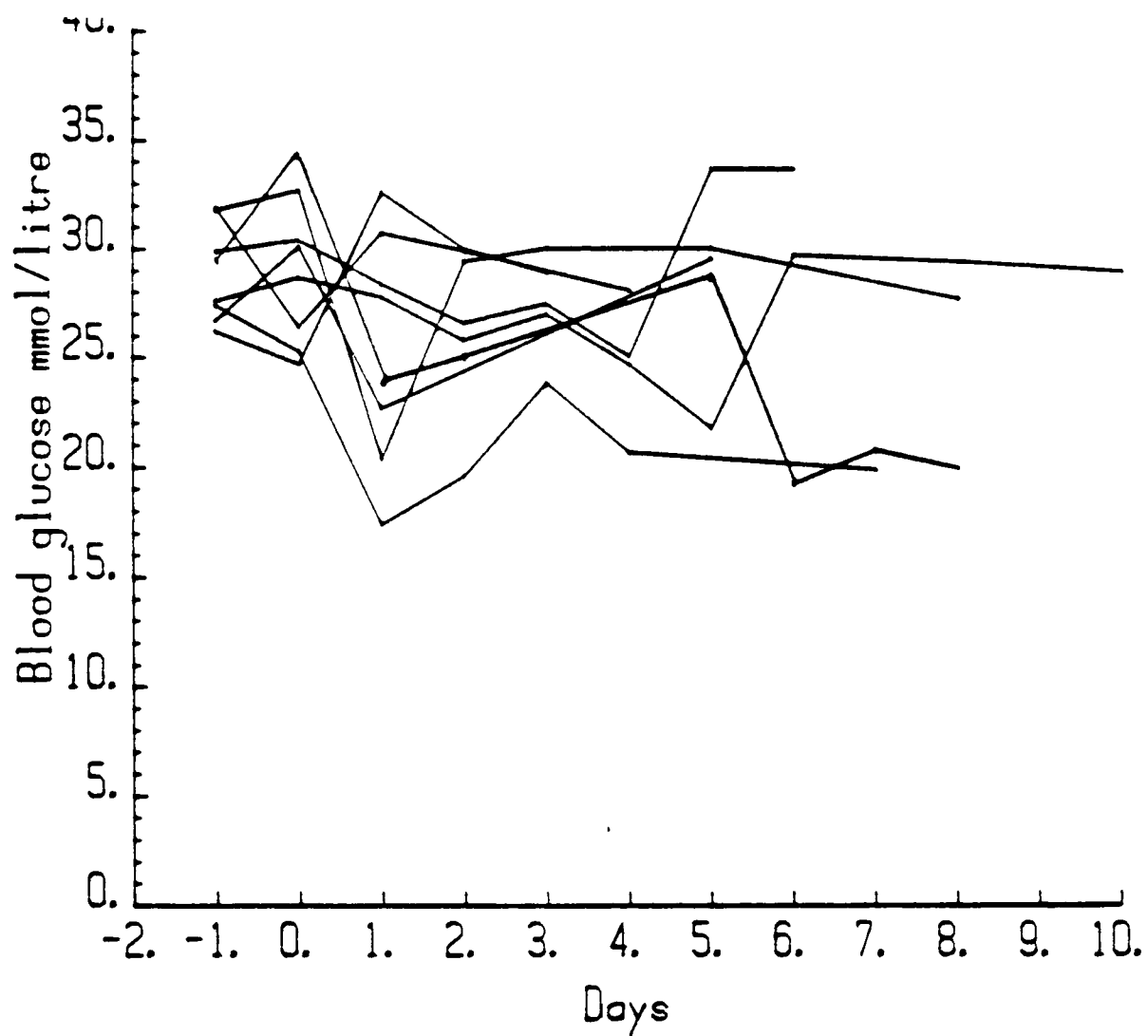


Fig. 1-3-1. Serum glucose of 8 diabetic LEW rats given (LEW x DA)F1 islets under the kidney capsule on day 0.

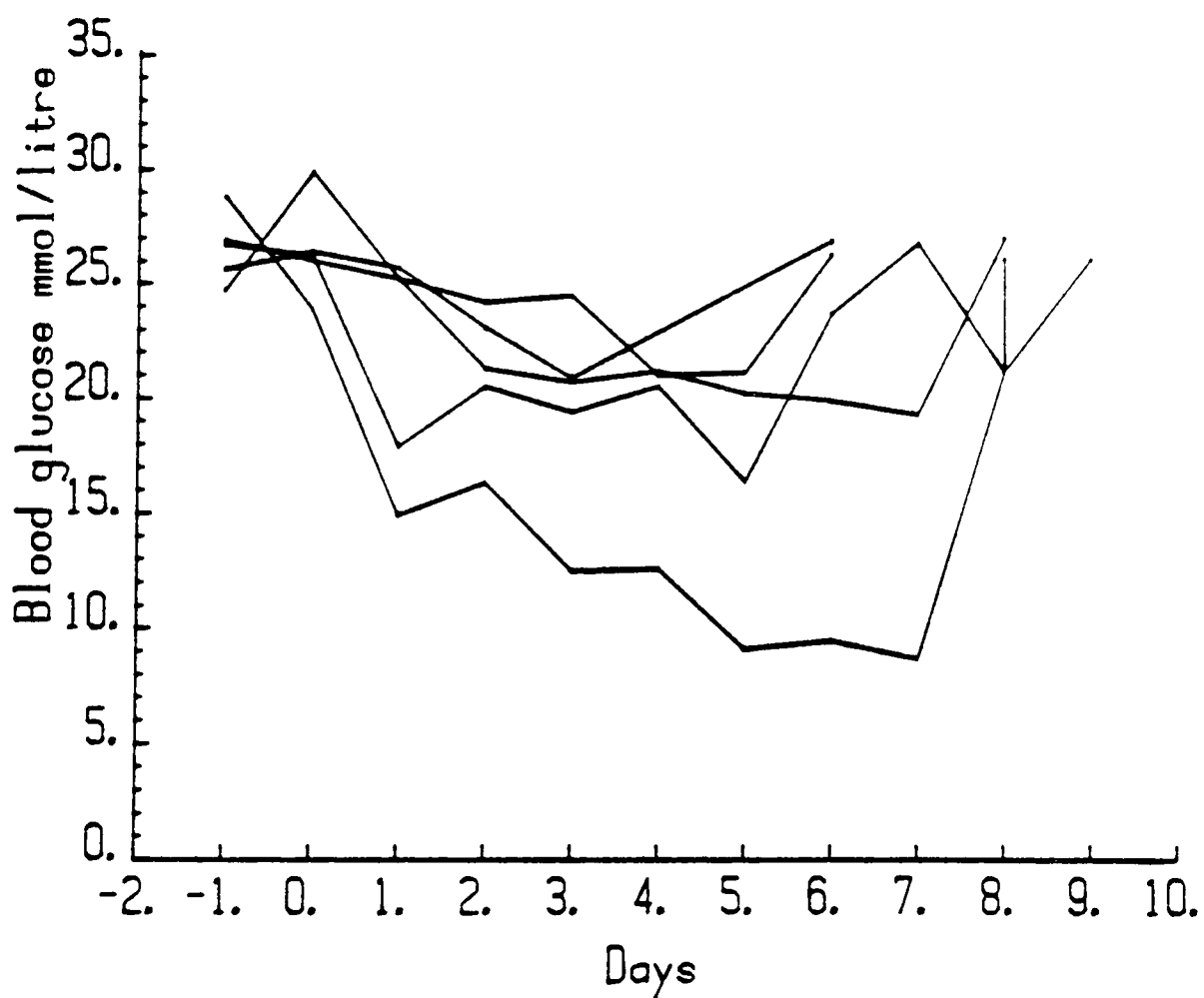


Fig. 1-3-2. Serum glucose of 5 diabetic LEW rats transfused with DA blood twice weekly for 6 weeks, then given (LEW x DA)F1 islets under the renal capsule on day 0.

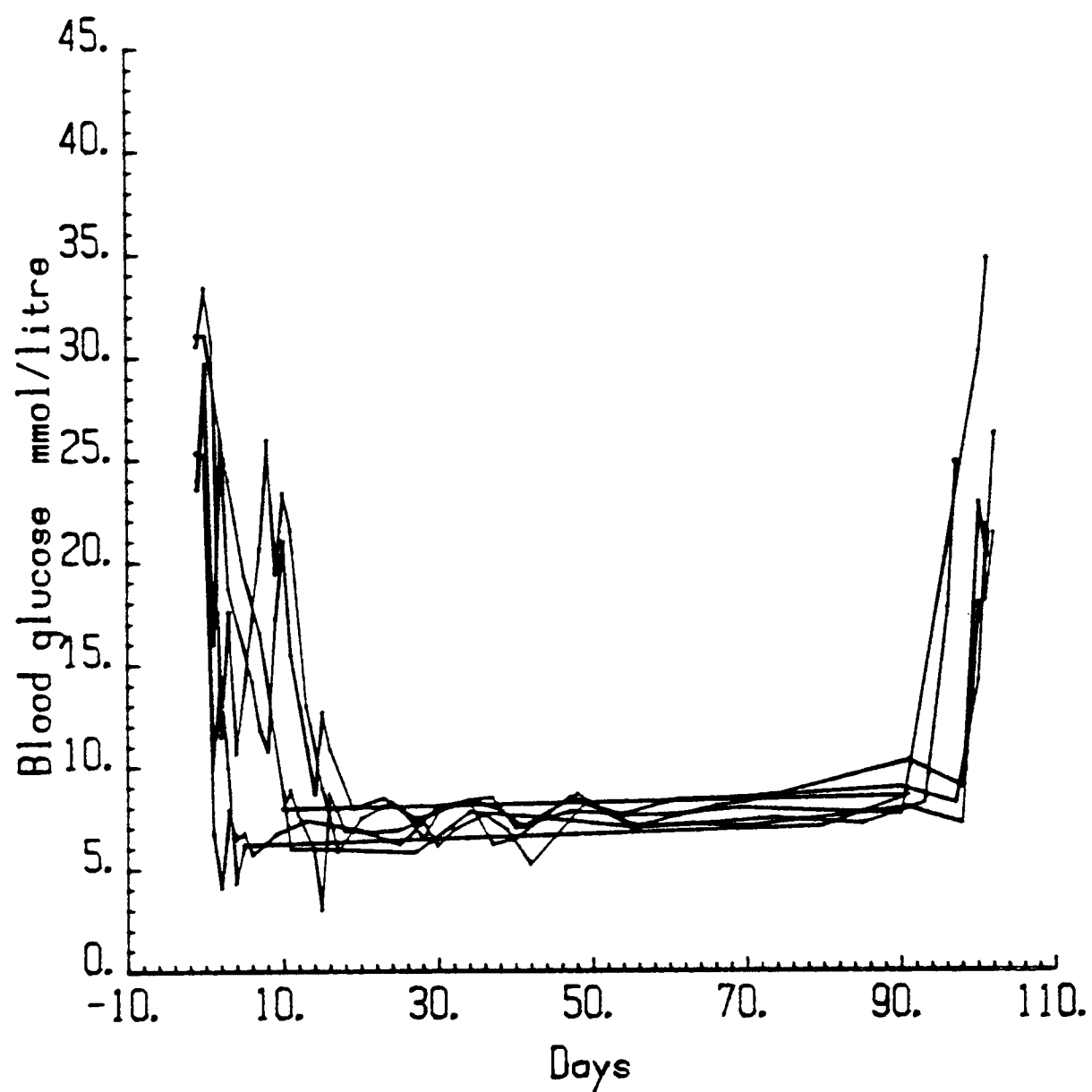


Fig. 1-3-3. Serum glucose of 5 LEW rats, bearing long-surviving (LEW x DA)F1 kidneys, and given (LEW x DA)F1 islets under the kidney capsule on day 0. After 100 days the (LEW x DA)F1 kidney and islets were removed and replaced by a normal LEW kidney.

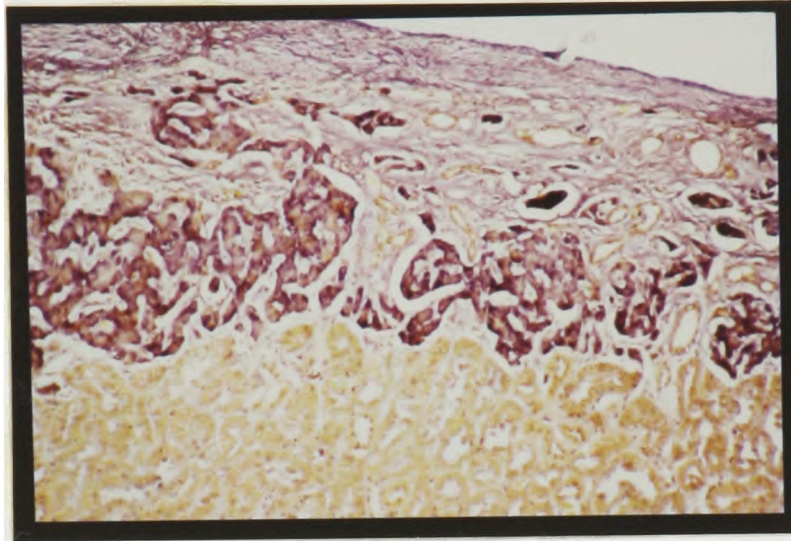


Plate 1-3-1. Photomicrograph of a section of kidney from a LEW rat bearing a long-surviving (LEW x DA)F1 kidney, made diabetic, then given (LEW x DA)F1 islets under the kidney capsule. Tissue taken 100 days after islet transplantation, showing structurally intact islet tissue with B cells containing blue-staining granules under the kidney capsule. There is no evidence of rejection. Aldehyde fuchsin. Magnification x 150.

Discussion

This experiment confirms the ability of whole blood transfusion to produce long-term acceptance of renal allografts. As previously reported, there appears to be no beneficial effect of whole blood transfusion on islet allograft survival. An interesting finding, requiring explanation, was that, in the strain combination used (LEW x DA)F1 into LEW), untreated rats failed to achieve normoglycaemia before rejection, raising the possibility of inadequate transplantation. The transplantation method used in these rats was identical to that used, with regular success, in several other strain combinations in previous experiments. There seemed to be no difference in the number or size of islets transplanted, and to guard against this, the number of donors was increased to 8, with similar results in 2 rats. Of 8 LEW rats given (LEW x DA)F1 islets, none reduced their blood sugar below 17 mmol/litre. These safeguards suggest that the cause of failure to achieve normoglycaemia in these rats was not inadequate transplantation. Furthermore, an identical technique of islet transplantation in group 3 was successful in every case. The cause of this unusual effect remains unexplained. Animals that received blood transfusion and successful kidney transplants all accepted subsequent islet allografts, proof of function being confirmed at 100 days by removing the islets (by retransplantation), and by histological examination.

This study shows that whole blood transfusion has a different outcome in islet and kidney transplantation, and that rats made unresponsive to kidneys by blood transfusion will then accept islet allografts, in a similar manner to rats made tolerant of kidneys by cyclosporine treatment. The mechanism of unresponsiveness following cyclosporine treatment has been suggested to be the production of suppressor cells (Morris 1981, Bordes-Aznar et al. 1983). The mechanism of unresponsiveness following donor-specific blood transfusion is still in dispute, but suppressor cells are also favoured by the majority of investigators (Opelz and Van Rood

1983). In both cyclosporin A treatment and donor-specific blood transfusion the effect on kidney transplants is powerful, but there is little or no effect on islet allograft survival. In both systems, however, once a renal allograft has been accepted there ensues an unresponsive state to islets of the same strain. Although there is no direct proof, this may be circumstantial evidence that the 2 effects operate through the same mechanism.

PART 1, CHAPTER 4.THE EFFECT OF REMOVAL OF THE RENAL ALLOGRAFT ON ISLET
ALLOGRAFTS GIVEN TO RATS MADE SPECIFICALLY UNRESPONSIVE
BY RENAL ALLOTRANSPLANTATION AND CYCLOSPORINE TREATMENTIntroduction

The previously described experiments have shown that rats bearing long-surviving renal allografts (as a result of treatment with either cyclosporine or donor-specific blood transfusion) develop an unresponsive state that allows acceptance of islets of the same donor strain, without further immunosuppression. It was of some interest, then, to determine the effect of removal of the original renal allograft, leaving the islet allograft in situ. If islets are unable to maintain an unresponsive state it may be expected that rejection of the islets would follow. Not only may this throw some light on the mechanisms associated with rejection of isolated islets, but, were clinical combined islet and renal transplantation to become a reality, it may give some information on the likely outcome of kidney graft removal, leaving the islets in place (as may be required on occasions).

Unfortunately, the model used previously (orthotopic renal transplantation with contralateral nephrectomy and islet transplantation under the transplant renal capsule) was not suitable for answering the above question, since removal of the renal allograft also necessitated removal of the transplanted islets. Therefore, it was necessary to use the alternative kidney transplant model described below.

Materials and methods

DA rats were given LEW renal allografts and treated with oral cyclosporine 10 mg/kg/day for 14 days. Renal transplantation was performed as an auxiliary graft, anastomosing the renal vessels end-to-side onto the aorta and inferior vena cava, with direct implantation of the ureter into the bladder. The recipient kidneys were not removed. Acceptance of the graft was confirmed by laparotomy at 28 days. Animals bearing long-surviving grafts were made diabetic by IV injection of streptozotocin 50 mg/kg at 75 days, with a repeat injection in some cases. Diabetes was confirmed in all cases by a repeated serum glucose level of 22mmol/l at least 14 days after the last streptozotocin injection. Islet transplantation was then performed, islets being prepared from 6 donors by collagenase digestion, ficoll separation and hand-picking under a dissecting microscope before transplantation under the recipient's own left kidney capsule. The islet donor strains used were LEW (Group 1), PVG (Group 2) or BN (Group 3).

Following islet transplantation, all animals underwent daily estimation of serum glucose for the first week, then thrice weekly for 4 weeks, and weekly thereafter. Rats that survived and remained normoglycaemic for 100 days after islet transplantation were then submitted to removal of the original renal allografts, and the serum glucose again monitored, as above, for a further 60 days. Finally, a further nephrectomy was performed, removing the recipient's own left kidney (bearing the islet allograft). Serum glucose was again monitored to confirm the return of diabetes. All allografted tissues, as well as the pancreases of diabetic recipients, were submitted to histological examination using both haematoxylin/eosin and aldehyde fuchsin stains.

Results

The results are summarised in table 1-4-1.

18 technically satisfactory LEW auxiliary renal transplants were performed into DA recipients, and 17 grafts were found to be well-vascularised and healthy at laparotomy 1 month later. Diabetes was successfully induced in all 17 rats, although 6 rats required repeat injections of streptozotocin: 3 rats in both groups 2 and 3.

Group 1. 6 rats were given LEW islets transplanted to the recipient left kidney capsule. All 6 became normoglycaemic and remained normoglycaemic up to 100 days. Removal of the LEW renal allograft performed at this time was followed by a continuance of normoglycaemia for a further 60 days, at which time left recipient nephrectomy (removing the LEW islets) resulted in prompt return of diabetes (Fig. 1-4-1). Histological examination of the islet allografts showed healthy, vascularised tissue with no evidence of rejection (Plate 1-4-1).

Group 2. 5 rats were given PVG islets transplanted to the recipient left kidney capsule. All 5 rats became normoglycaemic, but 3 rats rapidly rejected the islets with return of hyperglycaemia. However, 2 rats did not reject and remained normoglycaemic for 100 days, at which time the grafts were proven to be functioning by removal of the recipient left kidney, which resulted in return of diabetes (Fig. 1-4-2). Histological examination confirmed the presence of healthy islet tissue with no evidence of rejection.

Group 3. 6 rats were given BN islets transplanted to the recipient left kidney capsule. Normoglycaemia was achieved in each case, but all 6 rejected rapidly, with return of hyperglycaemia in under 10 days (Fig. 1-4-3). Histological examination confirmed features typical of rejection.

TABLE 1-4-1. SURVIVAL OF ISOLATED ISLET ALLOGRAFTS TRANSPLANTED
UNDER THE KIDNEY CAPSULE OF DIABETIC DA RATS BEARING
LONG-SURVIVING AUXILLARY LEW KIDNEYS.

<u>N</u>	<u>RECIPIENT</u>	<u>KIDNEY</u> <u>DONOR</u>	<u>ISLET</u> <u>DONOR</u>	<u>INITIAL ISLET</u> <u>SURVIVAL (DAYS)</u>	<u>ISLET SURVIVAL</u> <u>AFTER NEPHRECTOMY^a</u>
5	DA	-	LEW	6,6,7,7,8 ^b	-
6	"	LEW	LEW	>100 X 6	>60 X 5 ^c
5	"	LEW	PVG	6,7,12,>100 X 2	-
6	"	LEW	BN	6,7,8,8,9,9	-

^a 100 days after islet transplantation the long-surviving auxillary LEW renal allograft was removed.

^b Results from experiment described in Part 1, Chapter 2.

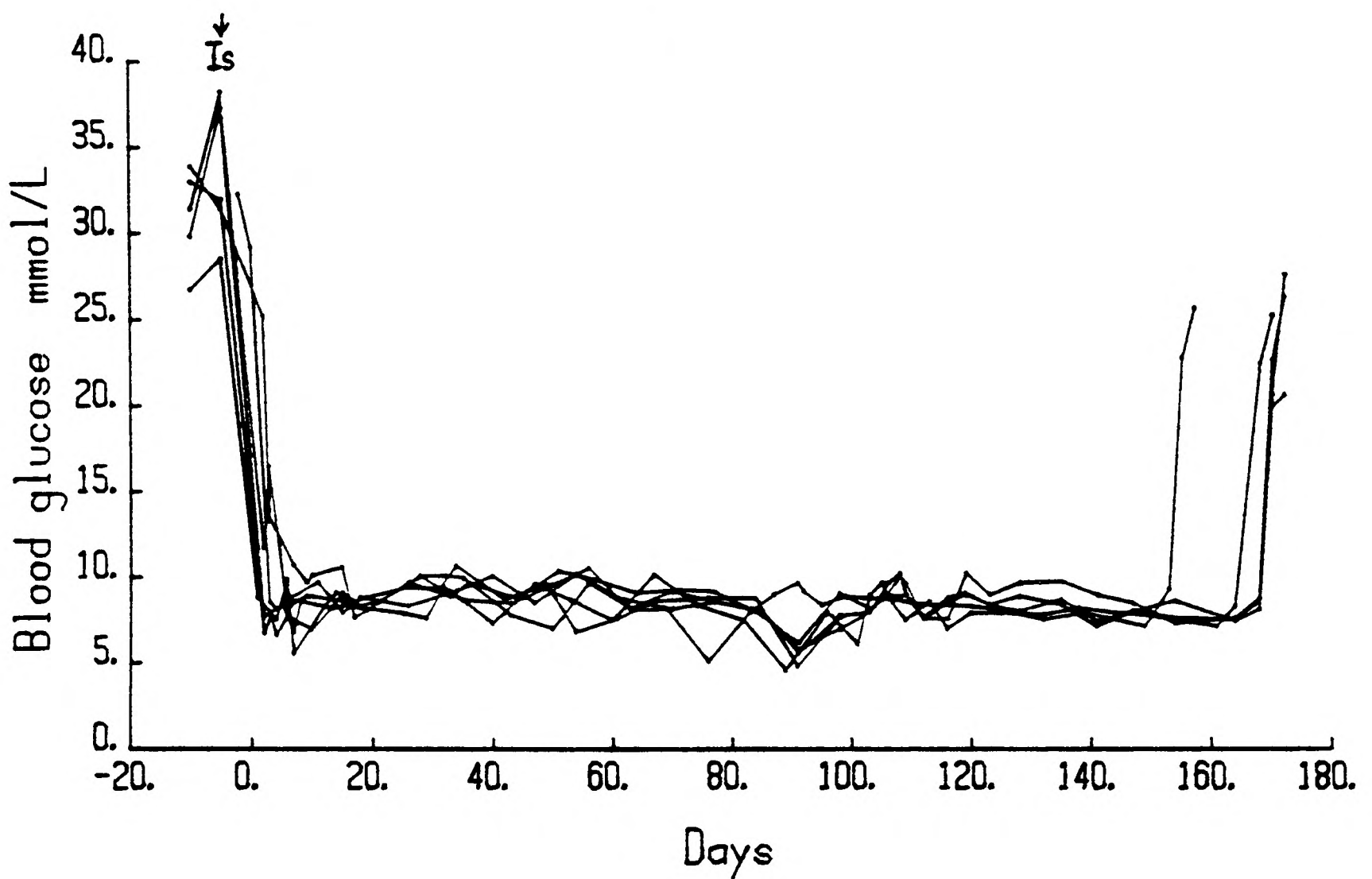


Fig. 1-4-1. Serum glucose of DA rats, bearing an auxiliary long-surviving LEW kidneys, made diabetic, then given LEW islets under the left kidney capsule at day 0. After 100 days the LEW auxiliary kidney was removed. 60 days later the left kidney (bearing LEW islets) was removed.

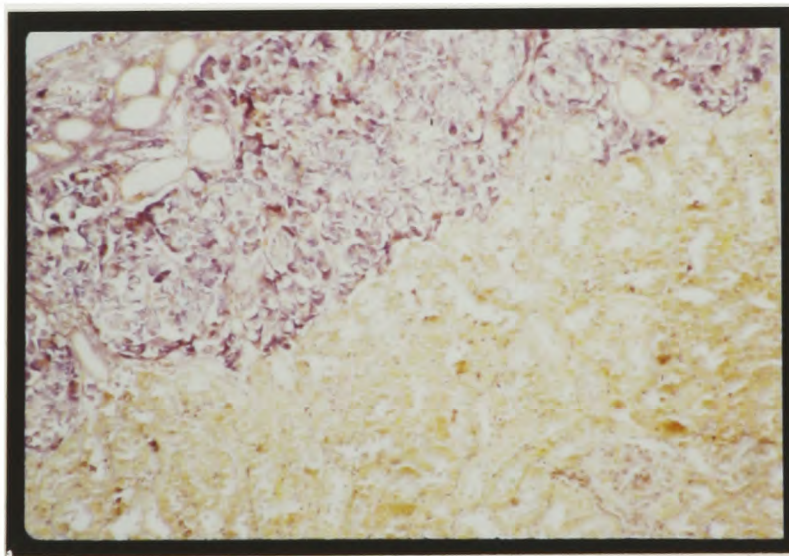


Plate 1-4-1. Photomicrograph of a section of the left kidney of a DA rat, bearing a long-surviving auxiliary LEW kidney, made diabetic, then given LEW islets under the left kidney capsule. The left kidney was removed 100 days after islet transplantation, and the section shows structurally intact islet tissue under the kidney capsule, with B cells containing numerous blue-staining granules. There is no evidence of rejection. Aldehyde fuchsin. Magnification x 150.

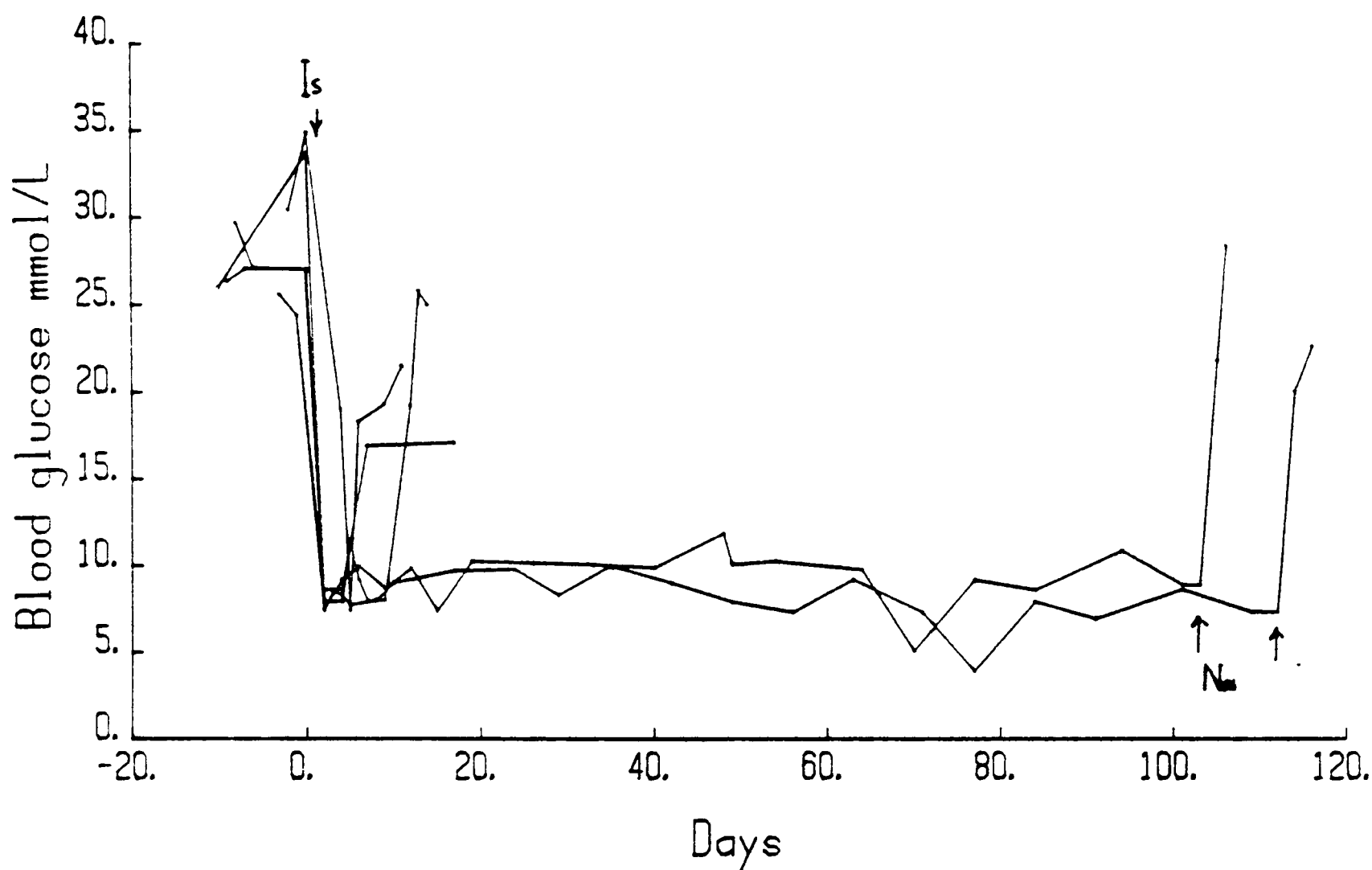


Fig. 1-4-2. Serum glucose of DA rats, bearing long-surviving auxillary LEW kidneys, made diabetic, then given PVG islets under the left kidney capsule at day 0. Animals that failed to reject the islets underwent left nephrectomy (Nx) at 100 days.

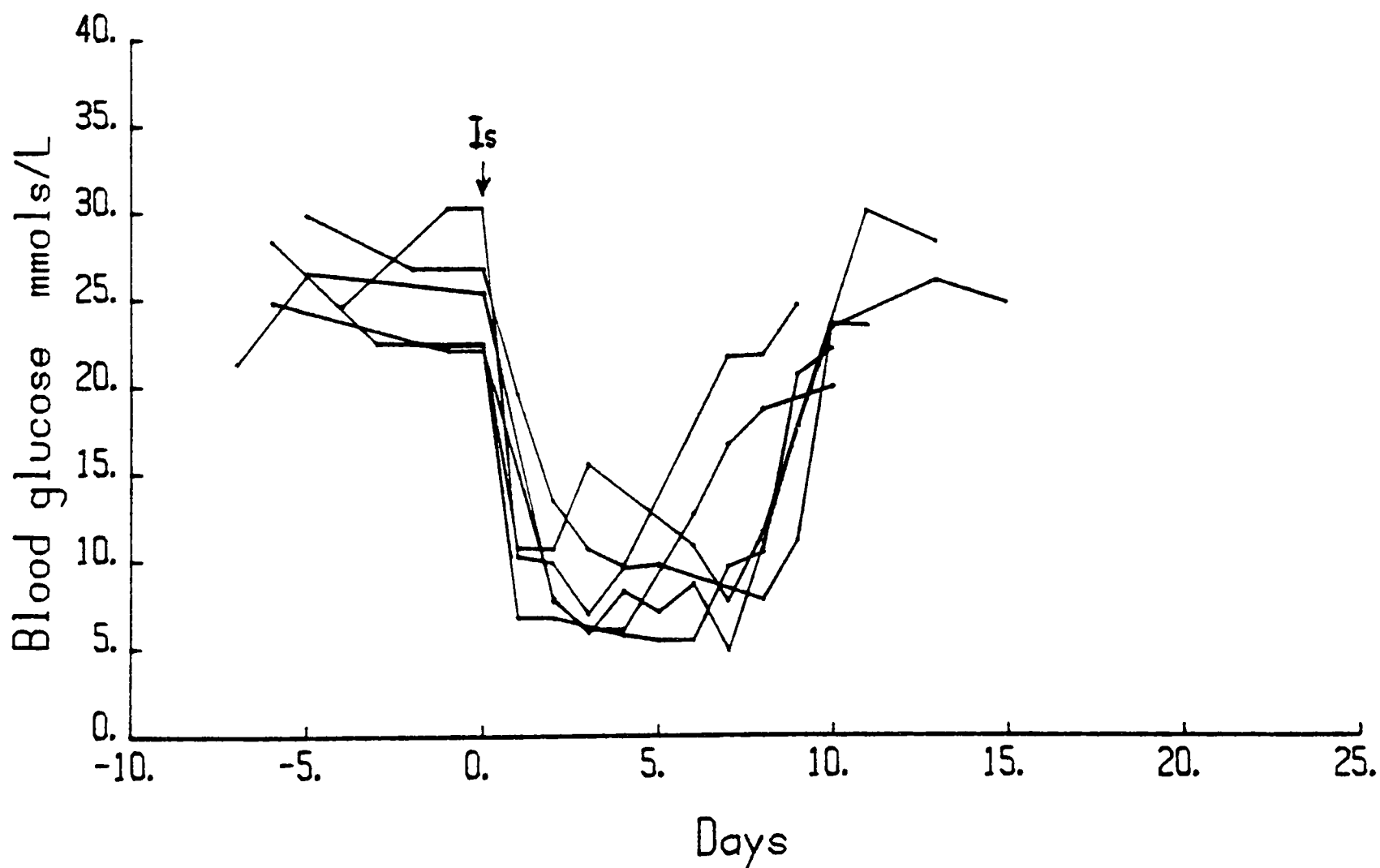


Fig 1-4-3. Serum glucose of DA rats, bearing long-surviving auxillary LEW kidneys, made diabetic, then given BN islets under the left kidney capsule at day 0 (Is).

Discussion

The experiments described have, again, shown that rats bearing long-surviving renal allografts as a result of cyclosporine treatment develop an unresponsive state. Transplantation of islets of the same donor strain is then possible, without further immunosuppression, this time in an auxiliary renal transplant model. Furthermore, these experiments show that, once an islet allograft has been accepted, it is then possible to remove the original renal allograft without causing rejection of the islets.

The nature of this unresponsive state remains uncertain, although the generation of a suppressor-cell population is thought to play a role in unresponsiveness following cyclosporine treatment (Morris 1983, Bordes-Aznar et al. 1982).

Nagao et al. (1982) studied the kinetics of unresponsiveness in rats given heart allografts with cyclosporine treatment for 14 days, and has found that unresponsiveness in these animals falls into 3 phases. The first stage coincides with the period of cyclosporine treatment, when there is non-specific unresponsiveness. The second stage lasts for around 6 weeks after cessation of cyclosporine, and, during this period, responsiveness to all donors is almost normal. The third stage is entered from 6 weeks after cessation of cyclosporine, with the development of a specifically unresponsive state. It is this third stage unresponsiveness that allows islet transplantation in the presently reported experiments.

The question arises as to why islet allografts treated with cyclosporine are not accepted in the same way as renal allografts. It is possible that islet grafts are rejected because they are unable to induce unresponsiveness (possibly through the failure of some mechanism operating in the first phase described by Nagao et al.). Another possibility is

that the the induction phase is satisfactory, but islets are unable to maintain an unresponsive state through the failure of a mechanism operating in the second or third phase. The experiments presented here suggest that islets are able to maintain a state of unresponsiveness on their own, although final proof of this idea would require transplantation of a further, fresh organ transplant, and the demonstration of acceptance in a strain-specific manner. The other mechanism suggested, that islets lack some factor important at the induction stage, cannot be concluded or excluded from these results.

Strain specificity was investigated in this model to confirm the similarity with unresponsiveness seen in the orthotopic model. Surprisingly, only 3 of 5 rats bearing long-surviving LEW kidneys rejected PVG islet grafts, in contrast to the report of Reece-Smith et al. (1982) in the orthotopic model. LEW and PVG rats share a few minor histocompatibility antigens, and it may be that the mechanism of unresponsiveness induced by an accepted organ graft is so powerful that shared minor antigens may, on occasion, affect responsiveness to major histocompatibility antigens. This hypothesis is supported by the uniform rejection of BN islets (which share few minor antigens) in this experiment.

4. PART 2

THE DEVELOPMENT OF A METHOD FOR ISOLATION OF ISLETS
OF LANGERHANS FROM THE LARGE MAMMALIAN PANCREAS

PART 2, CHAPTER 1.INTRODUCTION

There is little doubt that the idea of islet transplantation in man would have foundered years ago, were it not for the success of islet isolation techniques using the laboratory rodent. As described in the historical review, numerous attempts to adapt islet isolation to the pancreas of large mammals, including man, have been made, but no reliable technique has emerged other than that of crude pancreatic dispersion used in the dog, and this has proven inappropriate for clinical use.

Clearly there must be structural differences between the pancreas of the rodent and that of larger mammals. It is pertinent, therefore, to first compare the histological appearance and structure of the pancreas in 4 species: the rat, dog, pig and man. No such comparison could be found in the literature; the following description is therefore derived from a personal study. Normal pancreas was processed for histological examination and stained with haematoxylin/eosin, aldehyde fuchsin to show insulin-containing islet tissue and Masson trichrome to demonstrate connective tissue.

In all 4 species the gland is enclosed in a thin connective tissue capsule, and is composed of numerous lobules divided by connective tissue septa. There are considerable condensations of connective tissue around the main duct of the organ and its larger branches. The lobules are composed of numerous exocrine acini packed together irregularly, with a fine lattice of reticular fibres and capillaries between them. The exocrine duct and its side branches have a structural function and, when stripped clean of other tissues, the duct system resembles a herring bone. The side branches of the main duct give rise to intralobular ducts which in turn branch into numerous intercalated ducts that enter directly into the

acini.

The islets are dispersed throughout the pancreas, varying greatly in size and shape, with a tendency to be associated with larger vessels. The islets are remarkably delicate structures, composed of secretory cells of several types arranged in cords and clumps around numerous interspersed capillaries. There is no true capsule, but only a fine lattice of reticulin. These basic features are common to all 4 species: what are the differences between them?

In the rat, the lobules are small, often less than 50 acini appear in cross section, with a very thin single layer of connective tissue identifiable around each lobule and, to a lesser extent, around each islet. In histological section separation is seen between islets, acini and lobules, presumably because of post-fixation shrinkage, but demonstrating the looseness of the tissue. The islets are round or oval, varying in size from less than 50 μ up to 500 μ (measuring the longest diameter).

The dog pancreas has lobules that are much larger than those of the rat, containing several hundred acini in cross section, and these are much more densely packed. There is definitely more fibrous tissue, particularly between lobules, around vessels and surrounding ducts. Dog islets are more numerous and more uniform in size, but are definitely smaller than those of the rat, being rarely above 150 μ diameter. Dog islets are also very irregular in shape, with interdigitations between the surrounding acini and are rarely perfectly rounded or oval.

Compared with the pancreas of the dog, human pancreas has lobules that are extremely densely packed, with little space between the acini, and are larger, with hundreds, even thousands of acini (in cross section) in each lobule. More connective tissue is present between acini and around islets, and, especially in the intralobular septa, around ducts and

vessels. The islets are rounded or oval and do not tend to interdigitate between acini. They can be very large - up to 600 μ - but vary greatly, many islets being 50 μ or smaller. The majority of the islet tissue is composed of islets between 150-250 μ diameter.

The pig pancreas exocrine tissue is similar to the human, with large lobules, densely packed, and much fibrous tissue. The islets are similar in size to human islets but much more irregular and less well defined, with a moderate degree of interdigitation between acini and a much less defined layer of connective tissue around the islet.

From the histological appearances described it would seem likely that the major differences accounting for lack of success in islet isolation (from the pancreas of species other than the rat) are the large size of the pancreatic lobules, the tight packing of the acini and the quantity of fibrous tissue between lobules and surrounding the vessels and ducts.

As described briefly in the historical review, numerous methods for islet isolation in a variety of species have been described and it is proposed to discuss these methods in detail later. First it is pertinent to discuss in general terms the ways in which these methods might have been less than satisfactory. A method for isolation of islets should ideally separate the islets from exocrine tissue leaving the islets as structurally intact as possible, maintaining full viability of the tissue, with no contamination by remnant exocrine particles. A fundamental problem with assessing previously reported methods, and thus also with the development of new methods, is deciding if these aims have been achieved. There is little point in claiming a yield of many thousands of islets, when it is not certain that the tissue fragments are actually islets, or if the tissue has been killed during the process. There is a need for a simple method, firstly, for distinguishing islets of Langerhans from exocrine tissue and, secondly, for deciding the viability of the tissue isolated. The next 2

chapters address these problems.

PART 2, CHAPTER 2.THE USE OF THE DYE NEUTRAL RED AS A SPECIFIC, NON-TOXIC,
INTRA-VITAL STAIN OF ISLETS OF LANGERHANSIntroduction

The separation and isolation of islets of Langerhans in rodents is made relatively easy, not only by the less fibrous nature of the pancreas, but also by the ease with which the islets can be distinguished from exocrine tissue using a dissecting microscope. In larger animals it is hard to distinguish islet from exocrine tissue with any degree of confidence, making the assessment of new techniques for islet isolation difficult. Often the only reliable way is by histological examination or by the measurement of insulin secretion, both techniques taking several days.

In 1911 Bensley described a technique of perfusing the pancreas of several animal species with a dilute solution of the dye neutral red. He noted that this produced dark red staining of ovoid bodies which he interpreted as islets of Langerhans. Since those observations, the technique has been used intermittently to aid in the identification of islets in the dog (Downing et al. 1980) and the rat (Bretzel et al. 1980). However, we have been unable to discover a convincing study demonstrating that the ovoid bodies described by Bensley in several species are, in fact, islets. Thus the use of neutral red to stain islets of Langerhans still rests on the original observations of Bensley.

The studies reported here confirm that neutral red given intravenously selectively stains islets in the rat, pig and dog.

The chance observation was made that rat islets stained by neutral red appeared to have lost their stain after 12 hours tissue culture and yet looked to be still viable. Experiments were performed to confirm this observation by measurement of insulin secretion and transplantation of neutral red stained islets.

Materials and methods

1. The pancreases of 6 rats, 6 dogs and 3 pigs were stained by intra-aortic injection of neutral red (with the heart still beating), excised, cooled and stored at 4° C. The pancreas of 1 rat was snap frozen in liquid nitrogen and frozen sections cut and mounted, the section then being freeze-dried and examined without further staining. Red stained areas were easily recognisable under low power objective. The position of each red stained area was plotted on a graph, using the microscope stage coordinates as X and Y axes. The section was fixed and stained with aldehyde fuchsin to reveal the islets and re-examined on the same microscope stage. The position of the islets of Langerhans were then plotted without reference to the initial graph.

The remaining pancreases of all 3 species were then chopped with scissors and dispersed with collagenase (Sigma Chemicals Ltd, Kingston upon Thames, England.) Microscopic examination of the dispersed pancreatic tissue revealed dark red stained ovoid bodies interspersed amongst large quantities of white tissue fragments. 12 of the red stained bodies were picked out using a glass micropipette, confirmed to be free of contaminating tissue, then processed for electron microscopy.

2. Islets for insulin secretion studies and transplantation were isolated by the method of Ballinger and Lacy (1972). Insulin secretion of neutral red stained islets in response to glucose challenge was compared to unstained islets, using 5 islets per tube, and 10 tubes per condition.

3. 6 diabetic LEW rats were given left renal subcapsular islet transplants using neutral red stained islets prepared from 6 neutral red injected LEW rats, uniform staining of the islets having been confirmed after isolation. Consecutive transplants (with no exclusions) underwent daily serum glucose estimations for 1 week, then twice weekly. 6 weeks after transplantation left nephrectomy was performed and serum glucose monitored to confirm return of diabetes. Histological examination of the transplanted islets was performed.

4. 3 normal Lewis rats underwent injection of 1 ml of 2% neutral red solution under anaesthesia, red staining of the pancreas being confirmed by a laparotomy incision. The animals were allowed to recover, and were monitored for weight, blood glucose and blood urea. 1 month after injection the animals were sacrificed, a thorough post-mortem carried out, and the pancreas and kidneys taken for histology.

Results

1. Injection of neutral red produced a macroscopic deep pink staining of the pancreas of the rat, dog and pig. Examination with the stereoscopic microscope revealed darkly stained ovoid bodies surrounded by light pink or white tissue (Plates 2-2-1, 2-2-2, 2-2-3). Microscopic examination of frozen sections of pancreas from rats injected with neutral red revealed red stained areas. These corresponded closely with the position of islets of Langerhans identified after aldehyde fuchsin staining of the section (Fig. 2-2-1)

Electron microscopy of red stained bodies, hand-picked from the dispersed pancreatic tissue of the rat, dog and pig, revealed that these bodies were all islets in each of the 3 species (Plates 2-2-4, 2-2-5, 2-2-6).

2. Insulin secretion in response to glucose stimulation was unaffected by neutral red staining, and was identical to that of unstained fresh islets

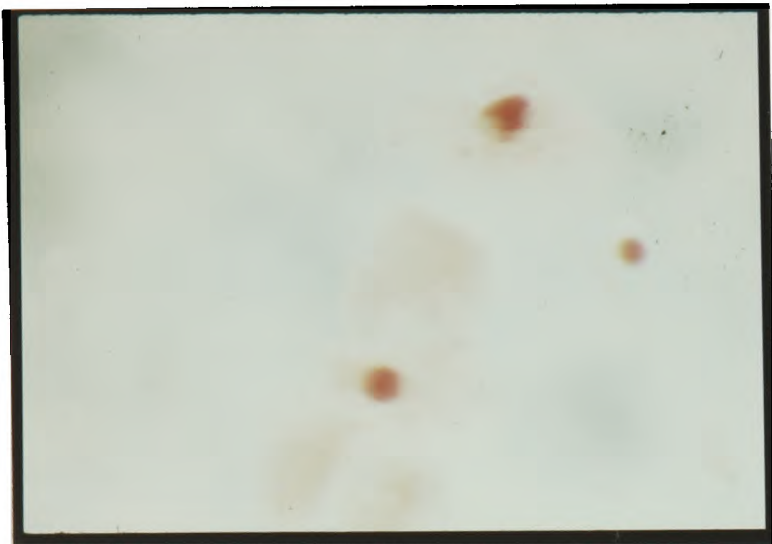


Plate 2-2-1

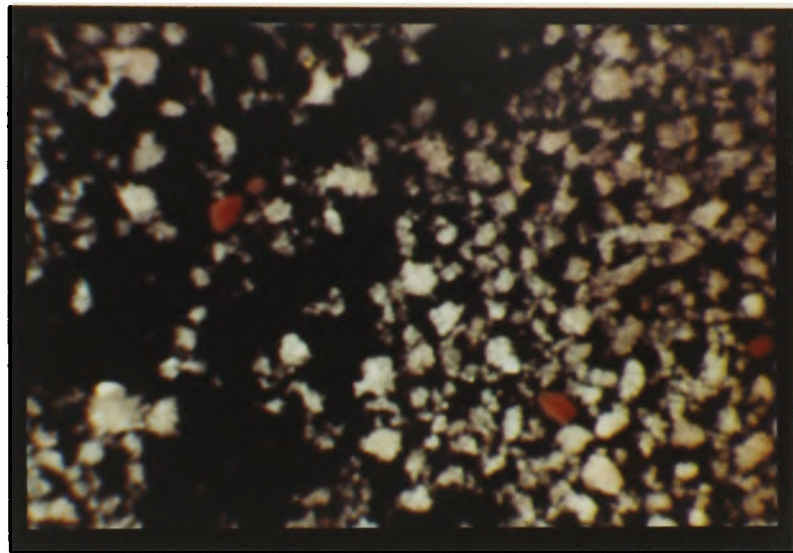


Plate 2-2-2

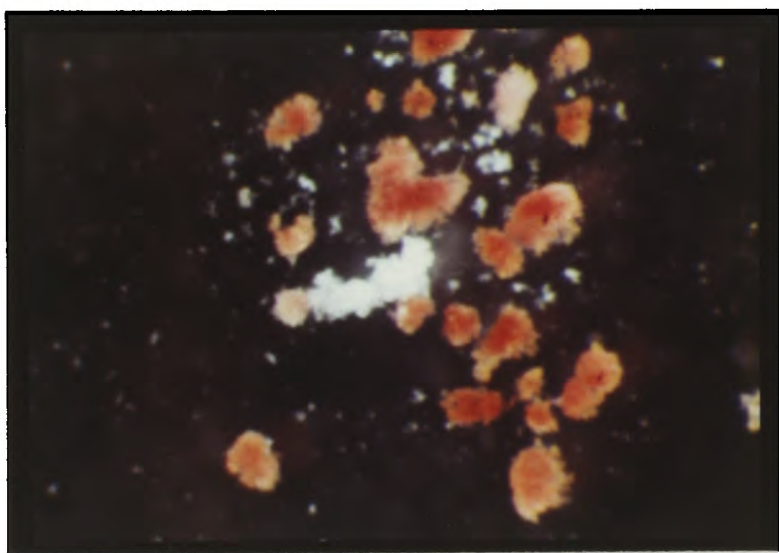


Plate 2-2-3

Plate 2-2-1 Stereoscopic microscope view of dispersed rat pancreas following neutral red injection. Dark red stained islets are seen surrounded by faint pink exocrine tissue.
Magnification x 12.5.

Plate 2-2-2. Stereoscopic microscope view of dispersed dog pancreas following neutral red injection, viewed against a black background. Dark red stained islets are easily distinguishable from white exocrine fragments.
Magnification x 25.

Plate 2-2-3. Stereoscopic microscope view of hand-picked, neutral red stained, pig islets viewed against a black background. A large fragment of unstained exocrine tissue from the same preparation is included for comparison.
Magnification x 30.

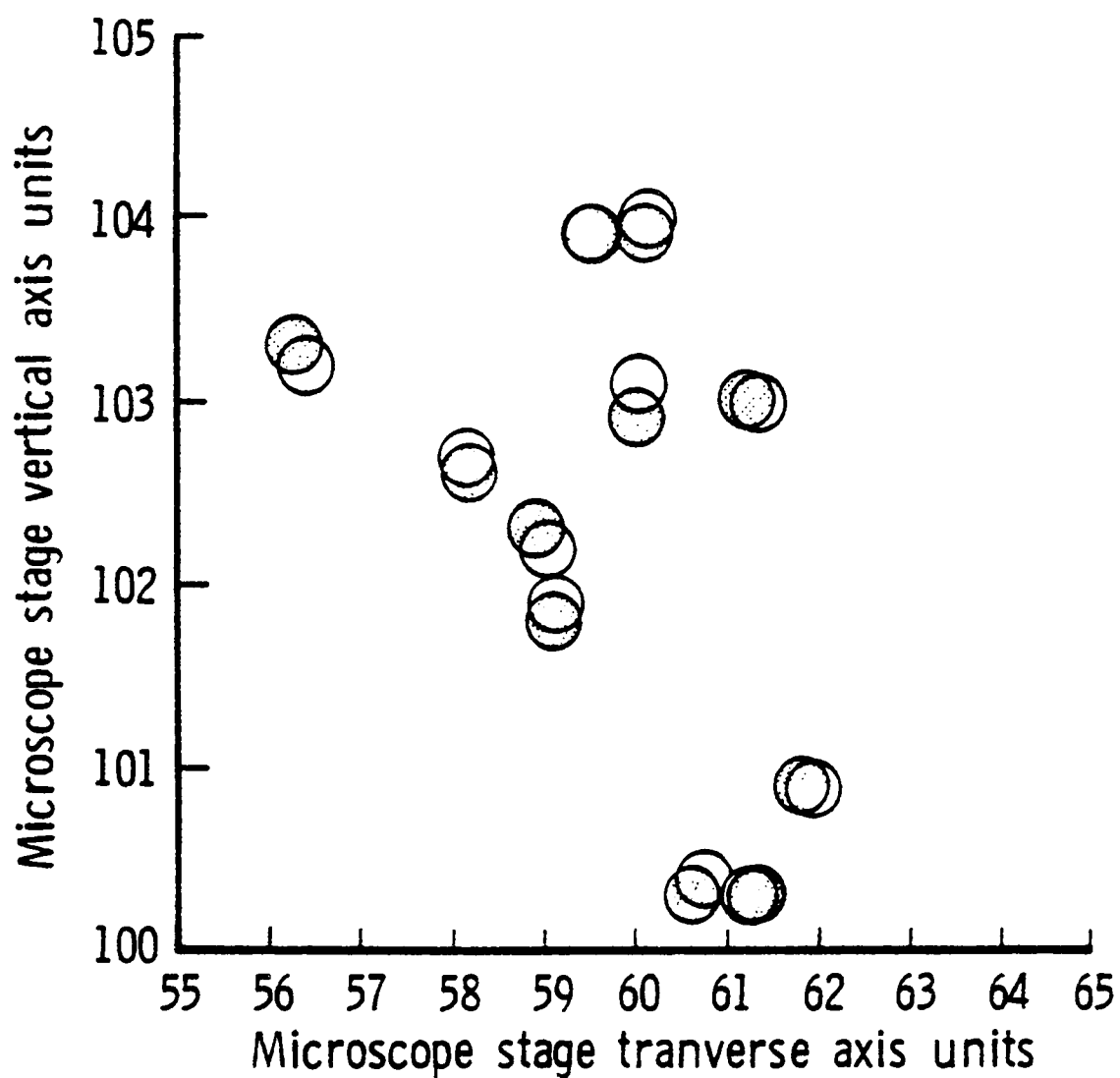


Fig. 2-2-1. Graph to show the position (in one section of rat pancreas) of neutral red stained areas (open circles) and islets of Langerhans identified after aldehyde fuchsin staining (shaded circles).

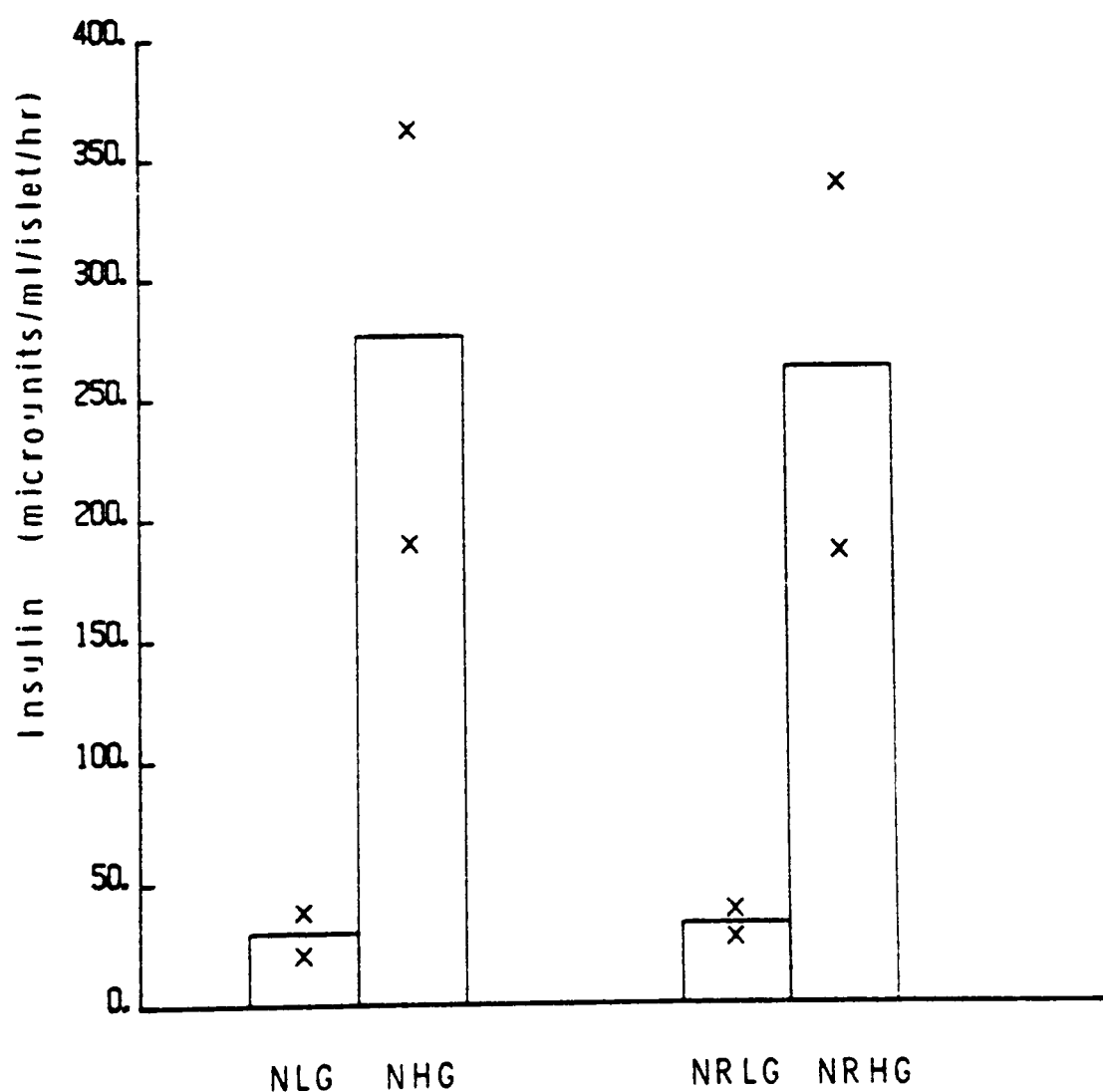


Fig. 2-2-2. Insulin secretion of normal (N) and neutral red stained (NR) rat islets in response to incubation in 2 mmol/litre (LG) and 20 mmol/litre (HG) glucose concentrations. (Mean of 10 tubes $\pm$ S.D.).

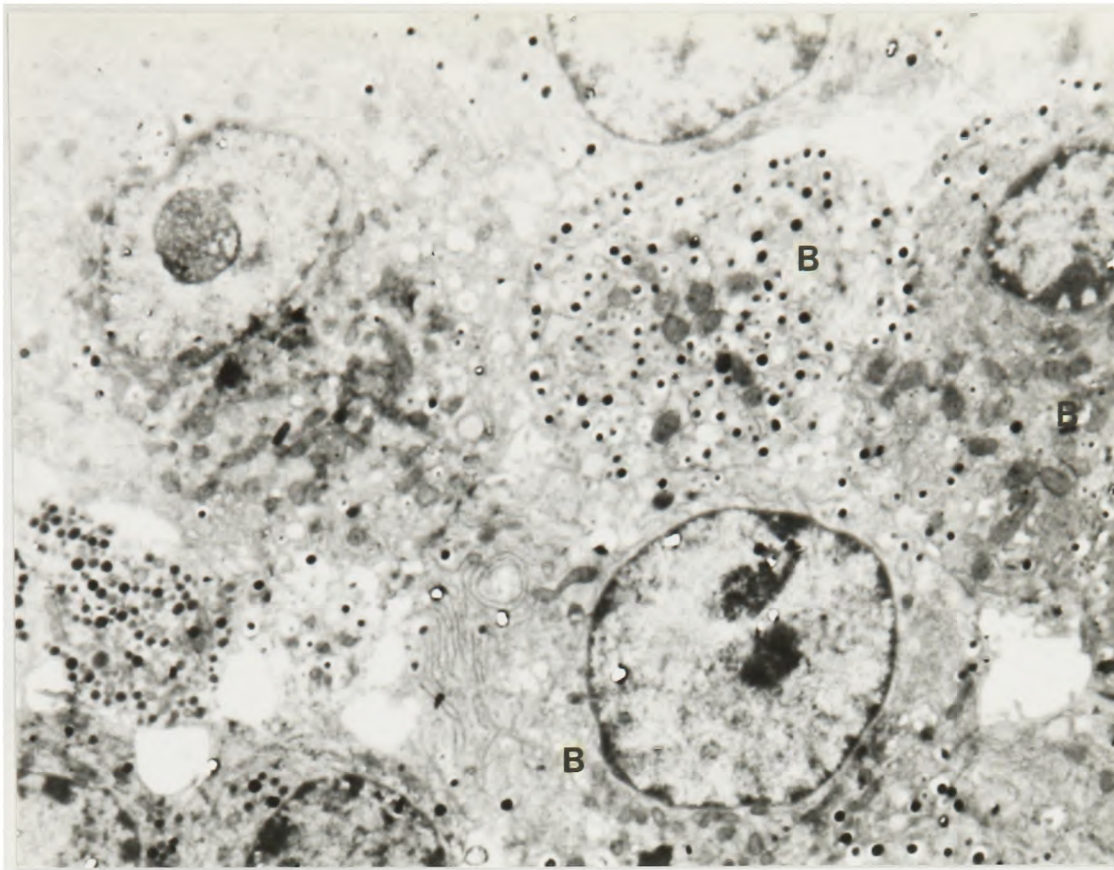


Plate 2-2-4. Electron micrograph of neutral red stained rat islet, showing B cells (B). Uranyl acetate and lead citrate. Magnification x 4,550.

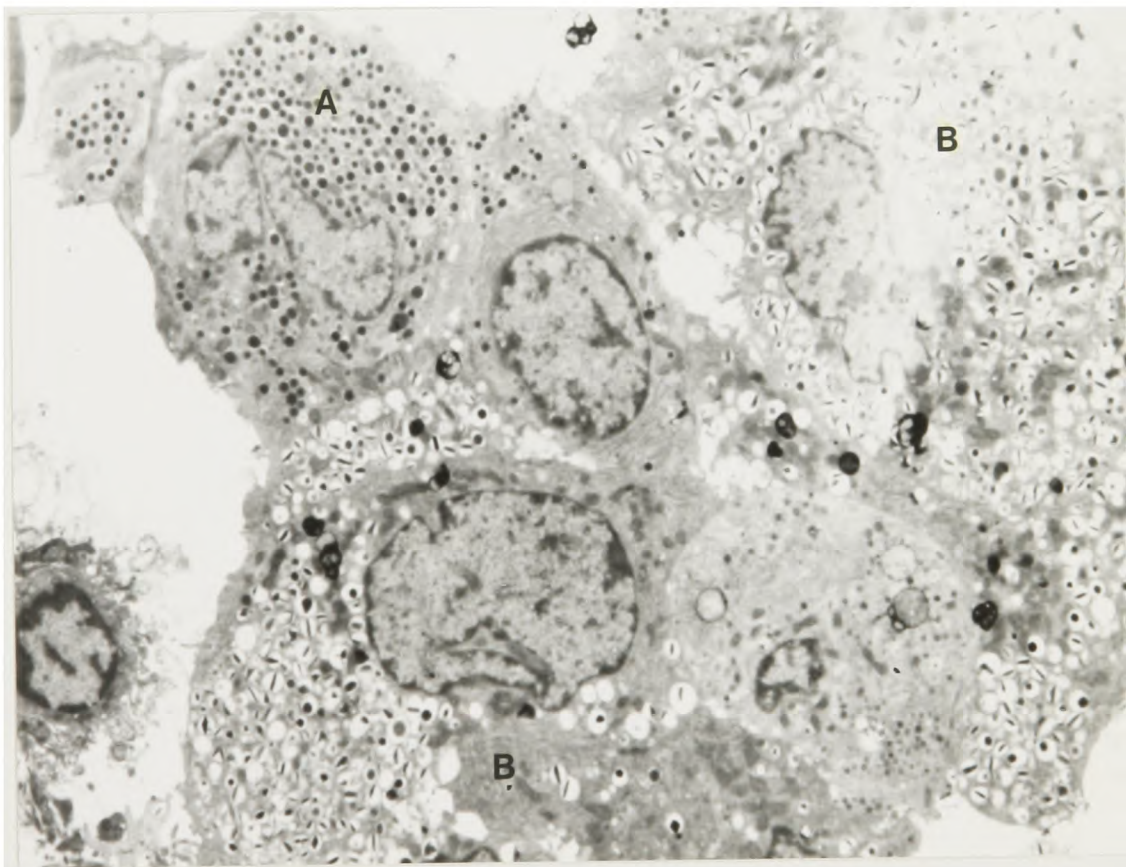


Plate 2-2-5. Electron micrograph of neutral red stained dog islet, showing B cells (B) with characteristic rod shaped granules, and A cells (A). Uranyl acetate and lead citrate. Magnification x 3 500.

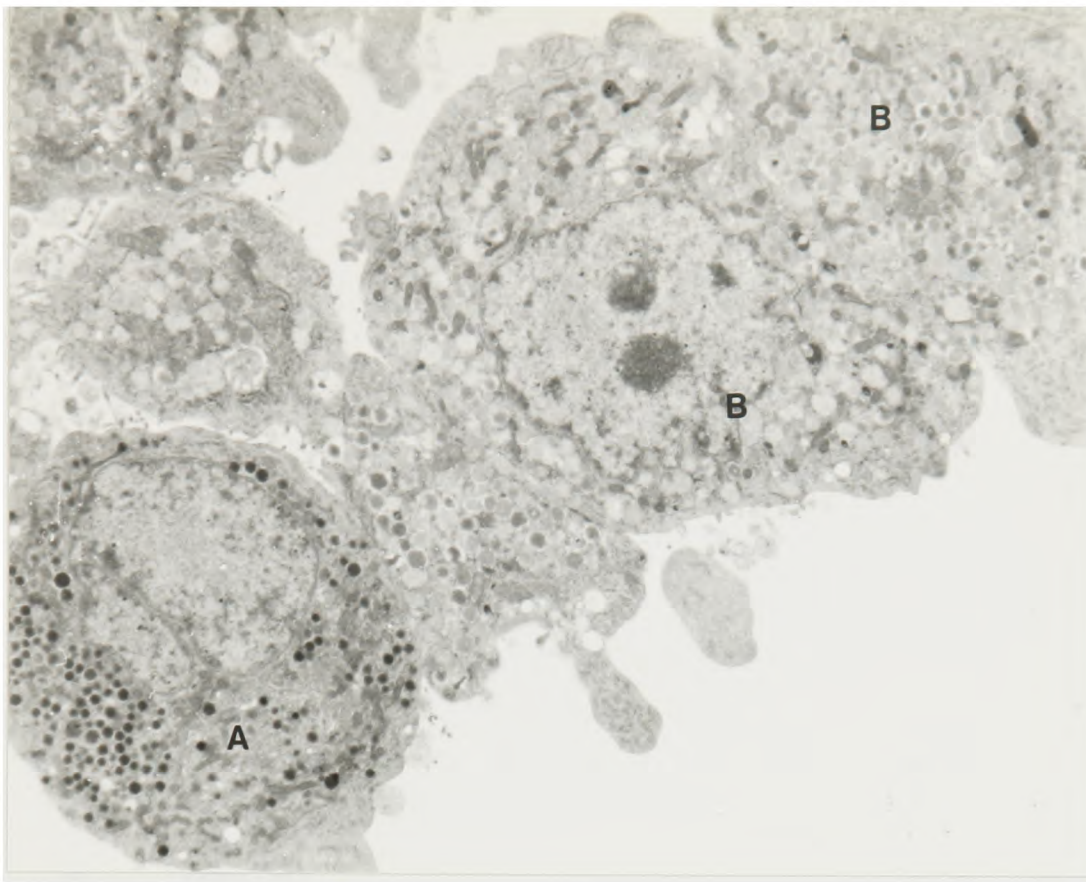


Plate 2-2-6. Electron micrograph of neutral red stained pig islet, showing B cells (B) and A cells (A). Uranyl acetate and lead citrate. Magnification x 5,200.

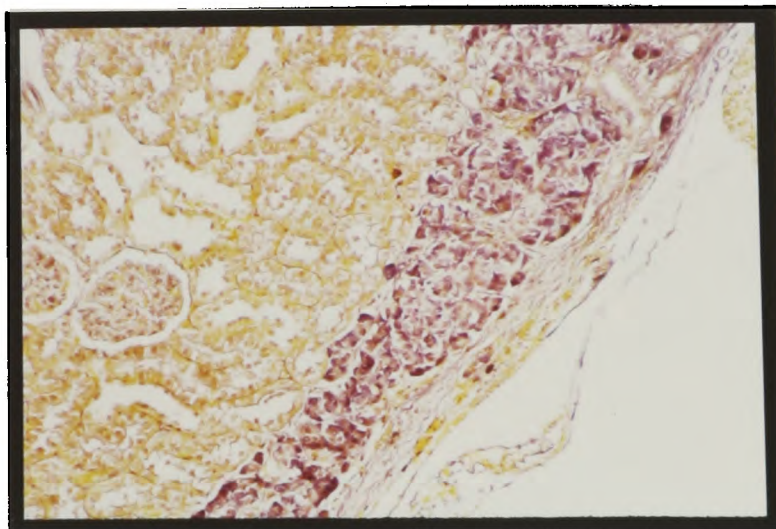


Plate 2-2-7. Photomicrograph of a section of kidney from a diabetic LEW rat given neutral red stained LEW islets under the kidney capsule 6 weeks previously. Well-preserved islet tissue is seen under the kidney capsule, with numerous B cells containing blue granules. Aldehyde fuchsin. Magnification x 150.

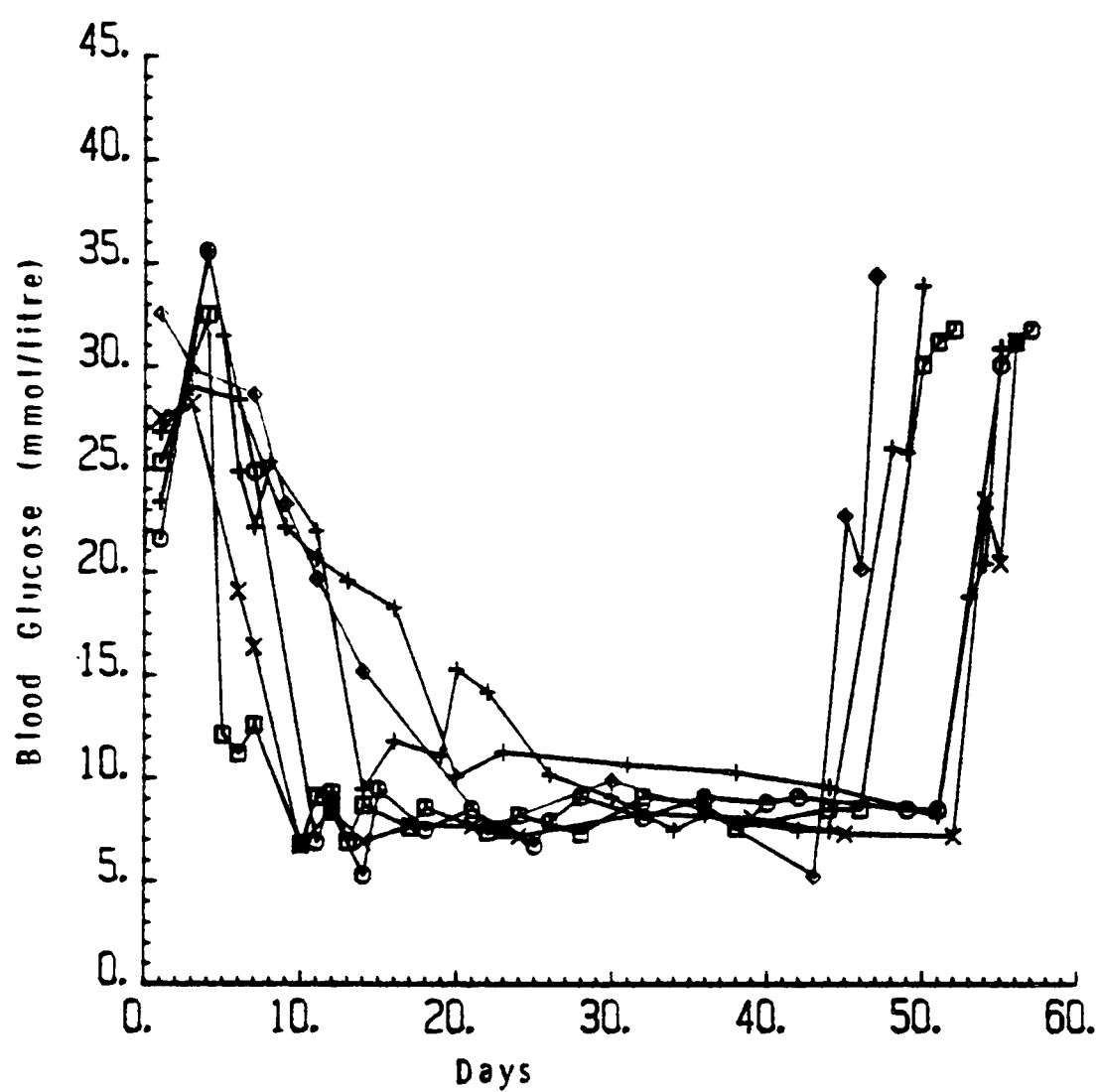


Fig. 2-2-3. Serum glucose of diabetic LEW rats given neutral red stained LEW islets under the left kidney capsule on day 0. Left nephrectomy was then performed 6-7 weeks after transplantation.

(Fig. 2-2-2).

3. Isografts of neutral red stained islets were successful in reversing diabetes in every animal transplanted. Function of the graft was confirmed by the return of diabetes after nephrectomy (Fig. 2-2-3).

Histology of the kidney showed well granulated islets under the kidney capsule of each specimen (Plate 2-2-7).

4. Normal rats injected with neutral red and allowed to recover had initial pink staining of the skin, which was lost after about a week. Weight gain was normal and the animals remained normoglycaemic and normouraemic. Post mortem revealed no abnormality, and histological examination of the exocrine and endocrine pancreas and also the kidneys was entirely normal.

Discussion

This study confirms the observations made by Bensley in 1911, that neutral red, when injected intra-arterially, stains islets of Langerhans in several mammalian species. The dye can be simply injected intravenously with the same results. The study, summarised in Fig. 2-2-1, shows that the areas stained by the dye correlate closely with the islets of Langerhans, and electron microscopy of the red stained bodies confirms that these are unequivocally islets, as shown by the characteristic granules of the A and B cells identifiable in the rat, dog and pig (Plates 2-2-4, 2-2-5, 2-2-6). Neutral red has been used previously as a non-specific stain for viable cells, often as a counterstain to such dyes as trypan blue, which stain non-viable cells (Sawicki et al. 1967, DeRenzis and Schechtman 1973, Crippen and Perrier 1974. When used in this way the dye is applied directly to the cells. There seems to be little specificity of the dye when used directly, and we could not demonstrate specificity for islets when applied directly to dispersed preparations of rat and dog

pancreas. The reason for its specificity on injection into the vascular system is unknown.

The ability of neutral red stained islets to secrete insulin and to reverse diabetes after transplantation confirms that the dye is not only lost from the islets in 12 hours, as noted in tissue culture, but also leaves the islets functionally intact. The finding that neutral red is not only specific for islets, but is also apparently non-toxic, at least for rat islets, is interesting and potentially useful. The difference in staining between exocrine and endocrine tissue is so marked that it should be possible to use the stain as a label to allow light-activated sorting of islets from exocrine tissue, possibly with greater efficiency than current separation methods. The method may be applicable to the pancreas of many species, including man.

PART 2, CHAPTER 3.THE USE OF FLUORESCCEIN DIACETATE AND ETHIDIUM BROMIDE
AS A VIABILITY STAIN FOR ISOLATED ISLETS OF LANGERHANSIntroduction

There is a need for a simple, reliable way of assessing the viability of tissue, (putative islets), following islet isolation techniques. The best method of testing viability is, undoubtedly, to transplant the fragments and show that they reverse diabetes, but this is, of course, not possible until a successful, large-scale separation technique has been perfected. Even then, it is a lengthy and expensive procedure, associated with many reasons for failure, other than the viability of the transplanted islets. Insulin secretion in response to glucose challenge has been used to assess the viability of islets (Lacy and Kostianovsky 1967), but this method has limitations as an assessment of viability, since decreased insulin production may be due to an increase in contamination, rather than loss of viability. More seriously, the technique is somewhat capricious (dead islets can release insulin), expensive and takes a considerable time before the results can be assessed. Measuring some aspect of metabolic activity is another way of showing viability, and oxygen uptake can be performed on isolated islets for this purpose (Hellerstrom 1967), but, again, the method is difficult and time-consuming. Radio-labelled amino acid incorporation can be used to measure metabolic activity in isolated islets (Ashcroft et al. 1976), but, again this is a difficult, lengthy technique and so is not often used to determine viability.

A more immediate result might be expected by using some kind of supravital staining of islets. 2 dyes have been described, anecdotally, as useful for this purpose. Trypan blue is excluded from live cells, but is taken up by dead cells, and has been used to determine islet viability (Sai

et al. 1981). Neutral red is taken up by live cells but not dead cells and has been used to determine the viability of individual mammalian cells in tissue culture, counterstaining with trypan blue (Sawicki et al. 1967), but it also has a selective staining effect for islets of Langerhans when used under certain conditions, allowing easy identification (See the previous chapter and Gray et al. 1983). Reports of the use of neutral red to stain islets seem to concentrate on identification of islets rather than on its use as a viability stain.

In preliminary studies, both trypan blue and neutral red were tested as viability stains for islets, but were not found very useful, either alone or in combination. This was because staining with 1 dye alone left unstained cells invisible, so all islets appeared to be "dead" with trypan blue and "live" with neutral red, though varying numbers of cells might, in fact, be taking up the dye. Dual staining produces an obscure picture, the result depending mainly on the relative concentrations of the 2 dyes. For these reasons, such staining methods have not been adopted for assessment of islet viability.

The fluorescent supravital stains, fluorescein diacetate (FDA) and ethidium bromide (EB), have been developed, and proved increasingly popular, for staining mammalian cells. Ethidium bromide has been used to assess the viability of dispersed islet cells, in conjunction with fluoresceinated anti-islet antibody (Dobersen and Scharff 1982), but fluorescein diacetate and ethidium bromide have not been used for assessment of viability of whole islets.

Preliminary studies

Staining of freshly prepared, whole rat islets showed uniform, bright green staining (Plate 2-3-1), whilst dead islets were easily identified by the red staining of individual cells (Plate 2-3-2). An interesting

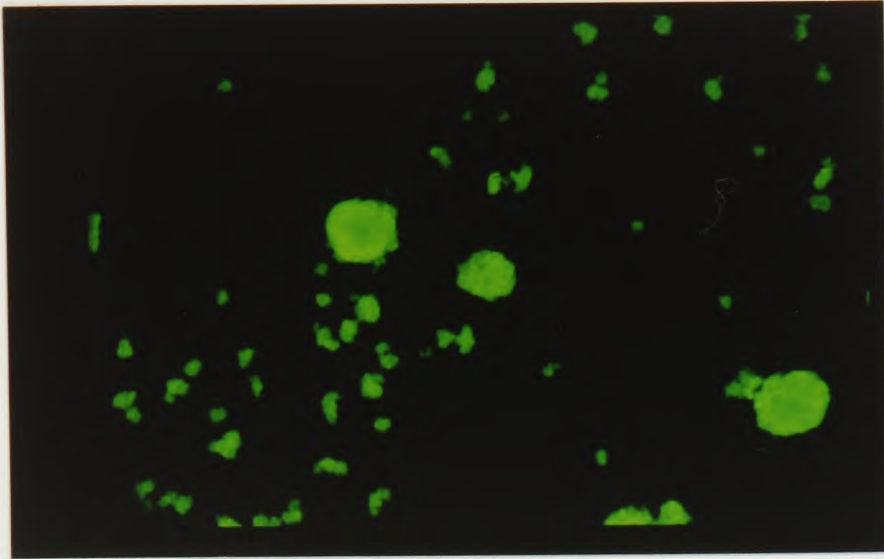


Plate 2-3-1

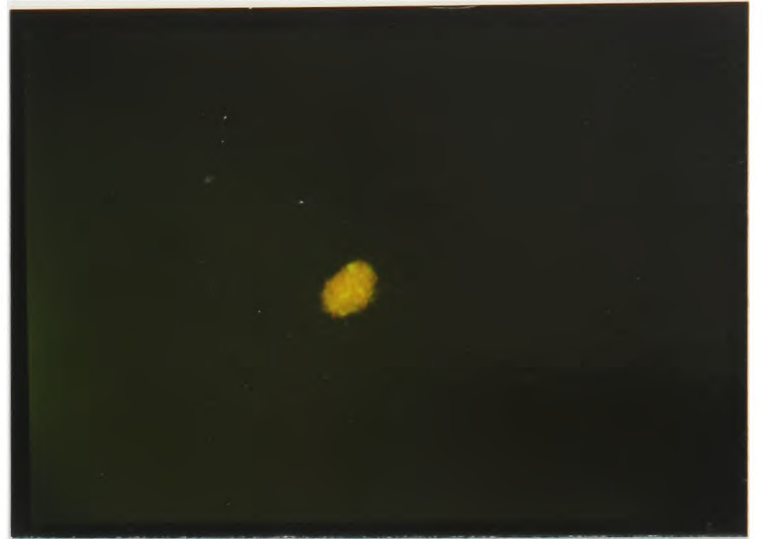


Plate 2-3-2

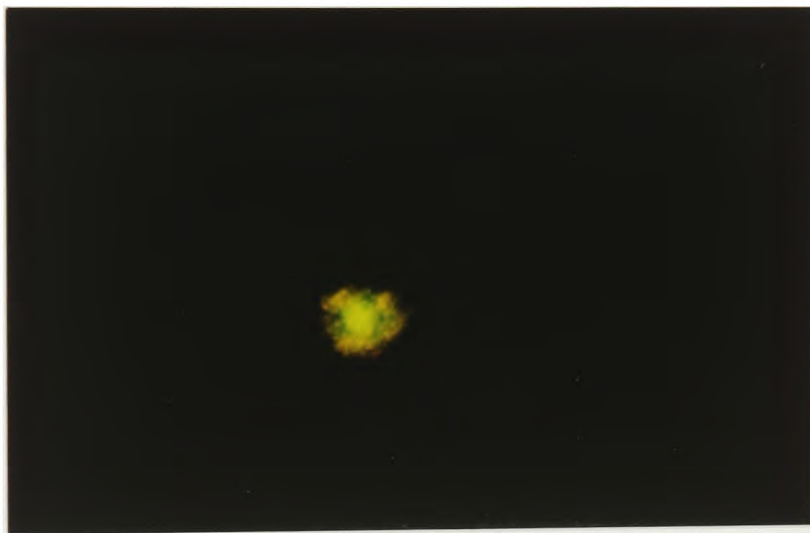


Plate 2-3-3

Plate 2-3-1. Freshly isolated rat islet preparation stained with FDA/EB and viewed through a fluorescence microscope. All tissue seen is stained uniformly bright green. The large, round bodies are islets, the smaller, irregular particles are fragments of exocrine tissue. Magnification x 25.

Plate 2-3-2. Isolated rat islet incubated in MHBSS for 4 hours at 43°C and stained with FDA/EB. Most of the cells are stained red, due to uptake of ethidium bromide, but a few scattered bright green cells can be seen. UV light. Magnification x 25.

Plate 2-3-3. Isolated dog islet, cryopreserved for 2 weeks, thawed, cultured in TCM for 24 hours, then stained with FDA/EB. There is selective death of peripheral cells (stained red), but the central cells (stained green) remain viable. UV light. Magnification x 50.

feature noted was that the stain appeared to allow discrimination of degrees of islet cell death, sometimes with characteristic patterns (Plate 2-3-3). For this reason a simple scoring system for viability was developed, where the FDA/EB stained islet was viewed as 3 concentric layers of a sphere: inner, middle and outer layers, and scored from 1-5 for each layer. This was made possible by racking the microscope focus up and down to view the whole islet. Thus, fully viable islets scored a total of 5+5+5, whilst completely dead islets scored 0+0+0. The islet shown in Plate 2-3-3 was given a score of 0+0+4.

These preliminary studies seemed to show that FDA/EB may be useful, therefore the following studies were performed to demonstrate the accuracy and reliability of the stain.

Materials and methods

FDA/EB staining of islets was performed using stock solutions of fluorescein diacetate 200 mg/ml in acetone, and ethidium bromide 200 ug/ml in HBSS. Fresh staining FDA/EB mixture was prepared for each batch of islets stained (FDA is stable for only a few minutes in aqueous solution at room temperature) by mixing 20 ul FDA stock solution into 1ml EB stock solution and adding the FDA/EB mixture (in an equal volume) to the islets, held in a drop of HBSS, on a plastic petri dish cover. The islets were then examined directly under UV light, with a Leitz fluorescence microscope, using X4 and X10 objectives. Minor variations in concentrations of FDA or EB in the final sample do not appear to affect the viability score of the islets.

The following experiments were performed:

1. Freshly prepared rat islets from 2 donors were divided in half, one half stained with FDA/EB, viewed through the fluorescence microscope, then both treated and untreated islets were tested for insulin secretion in

response to glucose (ISRG), by incubation in both 2 mmol/litre (LG), and 20 mmol/litre (HG) glucose.

2. Freshly prepared rat islets from 6 donors were divided into 4 samples and placed in 2 ml tubes, 5 islets per tube, then treated in the following manner:

- a) No further treatment. ISRG performed in both LG (5 tubes) and HG (5 tubes).
- b) 70% alcohol exposure for 5 minutes, washed twice in HBSS then ISRG performed in both LG (5 tubes) and HG (5 tubes).
- c) Exposure to heat (90° C.) for 5 minutes, then ISRG performed in both LG (5 tubes) and HG (5 tubes).
- d) No treatment initially. ISRG performed in HG with 10 μ mol/litre trifluoperazine added (5 tubes) and also in HG with 20 μ mol/litre (5 tubes).

The tubes from (a) to (d) were then renumbered by an assistant, the islets removed, stained with FDA/EB and scored for viability without knowledge of the original treatment. Insulin secretion for each tube was compared to FDA/EB score after breaking the code (insulin secretion rates above 200 or below 4 microunits/ml/islet/hour were not further subdivided).

3. Freshly prepared rat islets (from 6 donors) were placed in 2 ml tubes in HBSS, 20 islets per tube, and incubated whilst slowly increasing the incubation temperature. The first hour was at 43 °. C., the next 2 hours at 43.5 °. C. and the final hour at 44.0 °. C. The effect of this was to produce a slowly increasing unphysiological environment, expected to lead to slow death of the islet tissue. Samples of 40 islets were taken at half hourly intervals, stained with FDA/EB and scored for each islet. The stained islets were hand-picked so that there were 5 islets per tube and the ATP content was measured in each tube sample. Mean islet viability score at each interval was compared to mean islet ATP content.

4. Freshly prepared rat islets (6 donors) were stained with FDA/EB, viewed under UV light, then transplanted under the renal capsule of 3 diabetic rats. Blood sugars were recorded for 3 weeks and then the kidney, with the implanted islets beneath the renal capsule, was removed.

Results

1. Freshly prepared rat islets stained with FDA/EB appeared uniformly bright green under UV light (Plate 2-3-1). Insulin secretion in response to glucose was not significantly impaired following FDA/EB staining (Fig. 2-3-1).

2. (Table 2-3-1) Fresh islets, undergoing no other treatment, again showed appropriate secretion of insulin in response to glucose. These islets were all scored as fully viable on FDA/EB staining. Islets killed by heat showed no insulin secretion in either low or high glucose. Heat killed islets were scored as uniformly dead on FDA/EB staining. Islets killed by alcohol were scored uniformly dead on FDA/EB staining, but insulin secretion was high in both low and high glucose concentrations. Islets incubated in the presence of trifluoperazine showed diminished insulin secretion in response to glucose, and these islets were scored as being of intermediate viability.

3. Incubation of rat islets, whilst slowly increasing the temperature from 43.0 to 44° C., produced slow death of the islets, as shown by declining islet ATP content. Islet viability scores after FDA/EB staining showed good correlation with ATP content (Fig. 2-3-2).

4. 3 consecutive rats received transplants of islets stained with FDA/EB. All 3 rapidly reversed their diabetes and remained normoglycaemic for 3 weeks. During this period they gained weight and reversed their polyuria. After 3 weeks, removal of the kidney (bearing the islets) produced rapid return of diabetes (Fig. 2-3-3). Histological examination showed

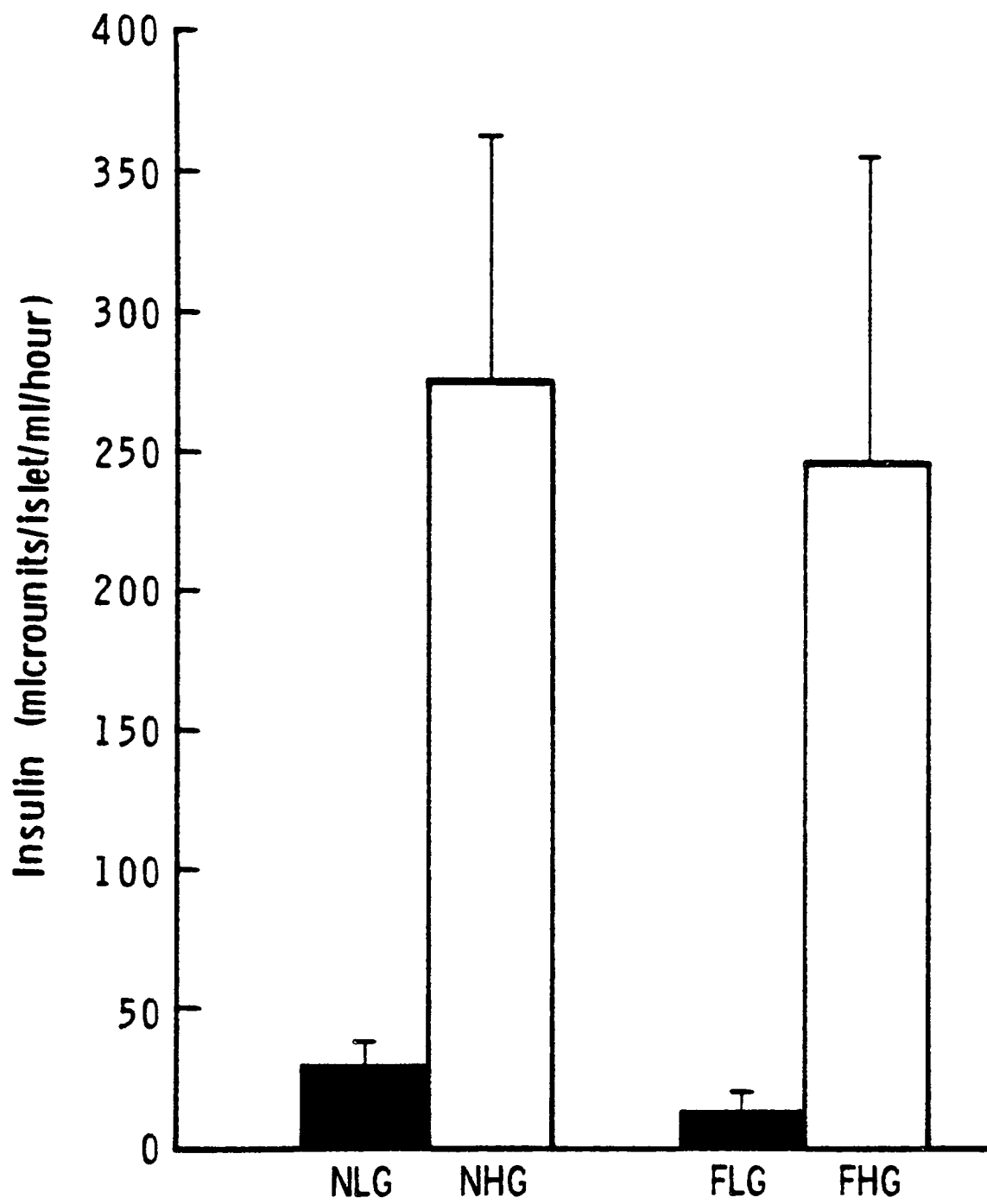


Fig. 2-3-1. Insulin secretion of normal (N) or FDA/EB stained (F) rat islets in response to incubation in either 2 mmol/litre (LG) or 20 mmol/litre (HG) glucose concentrations. (Mean of 10 tubes $\pm$ S.D.).

Table 2-3-1. INSULIN SECRETION FROM RAT ISLETS
COMPARED TO FDA/EB VIABILITY SCORE

<u>ISLET GROUP</u>	<u>GLUCOSE CONCENTRATION (MMOL/LITRE)</u>	<u>FDA/EB SCORE PER ISLET (<u>± S.E.M.</u>)</u>	<u>INSULIN SECRETION MICROUNITS/ISLET/HR (<u>± S.E.M.</u>)</u>
Normal	2	14.5 (0.1)	9 (1.25)
Normal	20	15.0 (0)	>200
Heat killed	2	0 (0)	<5
Heat killed	20	0 (0)	<5
Alcohol killed	2	0 (0)	>200
Alcohol killed	20	0 (0)	>200
TFP 25 ul/ml	20	5.8 (1.1)	12.2 (3.4)
TFP 100 ul/ml	20	3.5 (0.8)	12.2 (2.3)

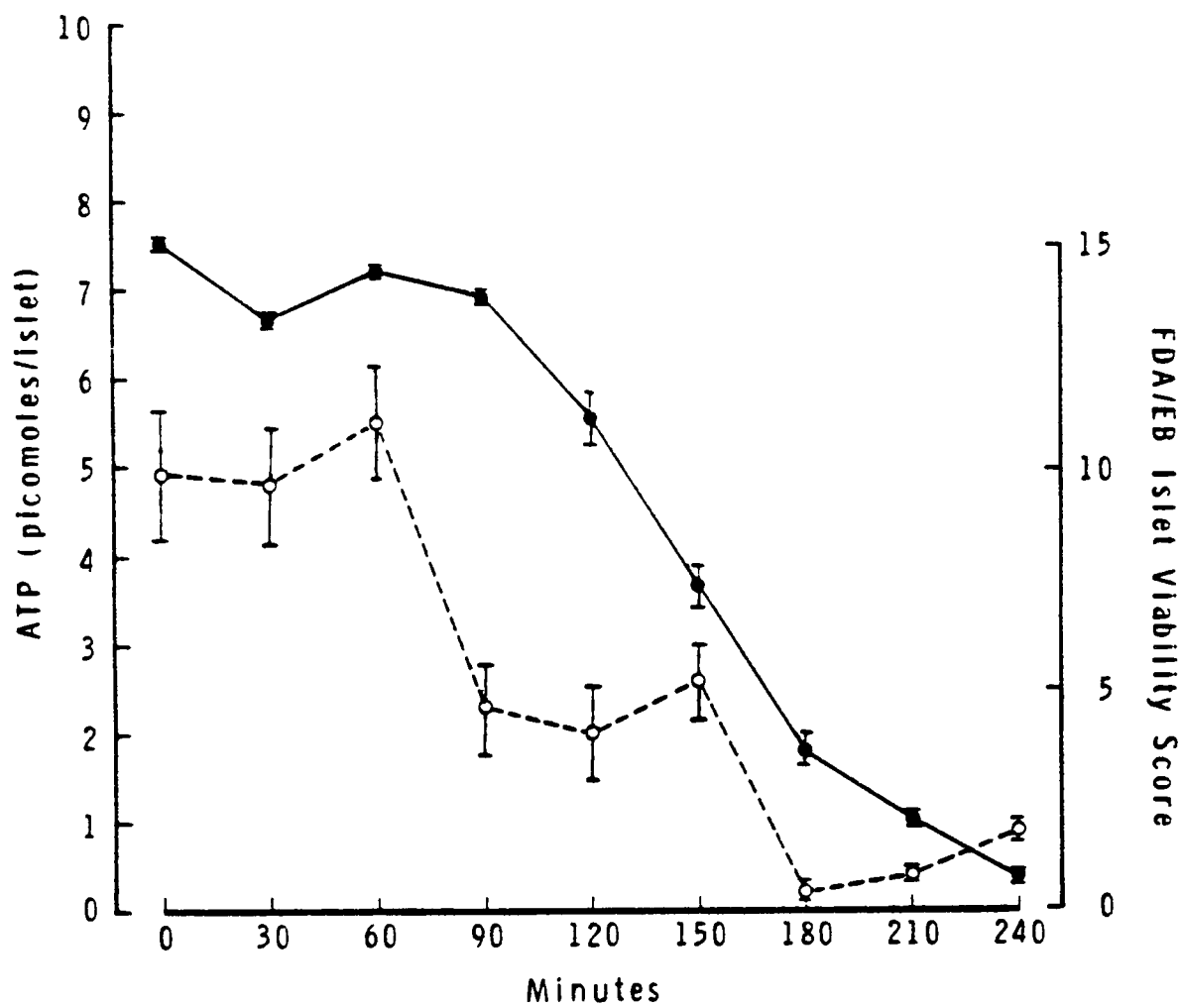


Fig. 2-3-2. Graph to show FDA/EB viability score (●) and ATP content (○) of rat islets incubated at slowly increasing (unphysiological) temperature.

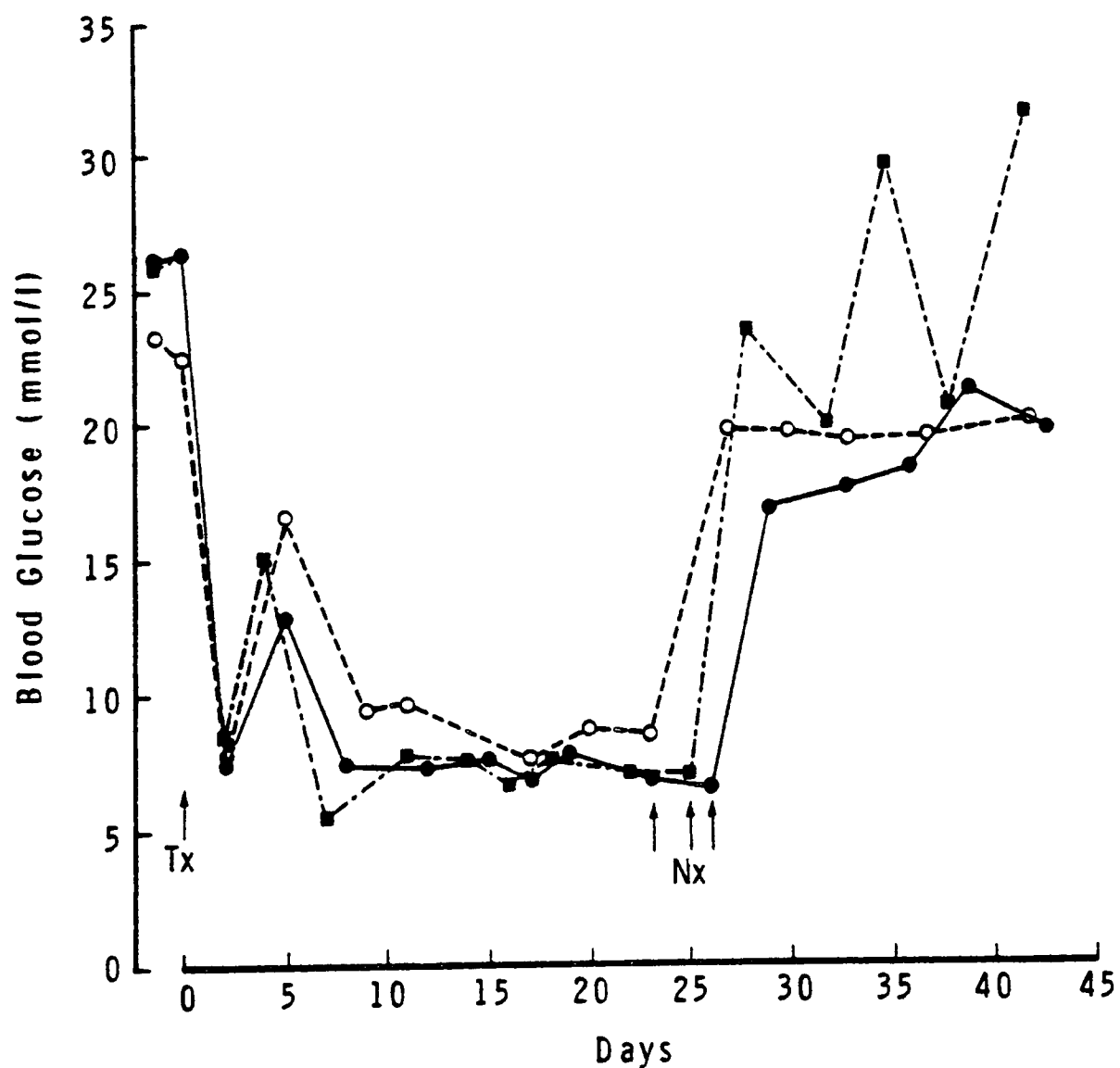


Fig. 2-3-3. Serum glucose of 3 diabetic rats given FDA/EB stained islets under the left kidney capsule at day 0. After 3 weeks the left kidney and islets were removed (Nx).

structurally intact islet tissue under the kidney capsule in every case.

Discussion

These experiments were designed to investigate the accuracy and reproducibility of FDA/EB stain for whole, isolated islets of Langerhans. Experiment 1 demonstrated that fresh islets stain uniformly green with FDA/EB, and stained islets secrete insulin in response to glucose in a manner similar to freshly isolated, unstained islets, while experiment 4 showed that stained islets function normally after transplantation to syngeneic diabetic recipients. These 2 experiments show that FDA/EB stained islets are alive, and that FDA/EB is not in itself a toxic stain, an important point, since it is unlikely that a viability stain will be accurate if it is, in itself, toxic.

Experiment 2 demonstrated that islets killed by 2 toxic agents, namely alcohol and heat, stain uniformly red, and can be differentiated from live islets with absolute accuracy, even when scored blind. Furthermore this differentiation of living from dead islets correlated with insulin secretion for live and heat-killed islets. Alcohol-killed islets produced high levels of insulin in both low and high glucose, presumably because insulin is alcohol extractable. However, all the alcohol-killed islets were scored uniformly red.

Trifluoperazine is a membrane-active agent known to reduce insulin production by islets in response to glucose, probably acting as a calmodulin inhibitor (Gagliardino et al. 1980). Islets incubated in the presence of trifluoperazine showed inhibition of insulin secretion, and this correlated with a decrease in viability score, demonstrating the ability of the stain to differentiate an intermediate degree of viability.

Experiment 3 explored further the ability of the stain to show degrees

of cell death in response to incubation of islets in non-optimal culture conditions. As the culture temperature was increased the FDA/EB score slowly decreased (Fig. 2-3-2). This decrease correlated well with decreasing ATP content in the islets, a measurement which is generally accepted to be an index of the correct functioning of islet metabolism (Ashcroft et al. 1973) and which has been shown to decrease with another noxious agent, ischaemia (Wettermark et al. 1970).

The use of the ester fluorescein diacetate (FDA) was first described in mammalian cells (Rotman and Papermaster 1966), the mode of action being thought to be dependant on the penetration of the cell wall by the non-polarised (non-fluorescent) ester, which is then converted to fluorescent fluorescein by the activity of intracellular esterases. The polarised molecule of fluorescein is then unable to pass through the intact cell membrane of living cells, and so accumulates, producing green fluorescence of the whole cell when viewed in UV light. The accumulation of fluorescein thus depends on 2 factors: the presence of intracellular esterases, and an intact cell membrane, both generally considered to be features of living cells. Ethidium bromide (EB) penetrates intact cell membranes slowly, but dead cell membranes are penetrated rapidly, with binding to nuclear DNA producing red fluorescence (Edidin 1970). Thus, when FDA/EB stained cells are viewed under UV light, living cells fluoresce bright green throughout and dead cells appear as red dots. FDA was initially used alone to demonstrate viability in human leucocytes (Bodmer et al. 1967), other mammalian cells (Sontag 1977), and mouse embryos (Mohr and Trounson 1980). Ethidium bromide has also been used for this purpose separately (Edidin 1970), but combining the 2 fluorochromes (FDA/EB) has proved most satisfactory, and their accuracy has been documented for human lymphocytes (Martel et al. 1974), granulocytes (Dankberg and Persidsky 1976), mycobacteria (Janargin and Luchsinger 1980) and mammalian tumour cells (Tarin and Price 1979).

Experiments 1 to 4 show that FDA/EB is a non-toxic, non-tissue specific stain for viability which appears to accurately assess the viability of islets of Langerhans, allowing easy and precise differentiation of living from dead islets, and, furthermore, allowing differentiation of degrees of islet injury. Sometimes characteristic patterns of islet injury are seen: for example, trauma and toxic agents tend to produce death of cells in the periphery of the islet (Plate 2-3-3), whilst lack of nutrients or oxygen tend to produce central death, especially in larger islets.

PART 2, CHAPTER 4.REVIEW OF THE METHODS USED FOR ISOLATION OF ISLETS OF LANGERHANSMethods used on the rodent pancreas

Interest in the possibility of separating islets of Langerhans from pancreatic exocrine tissue began with the report, by Hellerstrom (1964), of a method for hand dissection of islets. Using a stereo-microscope, islets were dissected from the pancreas of the mouse, rat and guinea pig. The method was slow, limiting the number of islets obtained to around 100, but this has been sufficient to perform biochemical and cryopreservation studies (Ferguson et al. 1976). However, the method is only applicable to the pancreas of small rodents, since it is not possible to distinguish the islets in the denser pancreas of larger animals.

A major advance was the discovery by Moskalewski et al. (1965) of the effect of collagenase (an enzyme preparation derived from cultures of *Clostridium histolyticum*) on the pancreas. Incubation of chopped guinea pig pancreas with the enzyme at 37° C., combined with vigorous shaking, resulted in disintegration of the tissue, with separation of the intact islets from exocrine tissue. The tissue was then placed in a petri dish and viewed through a stereo-microscope, the islets appearing as spherical or ovoid pearly-white bodies which could be picked out using a cataract knife. Although a considerable improvement on the micro-dissection method, this technique was still fairly slow and gave relatively small yields.

Lacy and Kostianovsky (1967) described several improvements, including distention of the pancreas by retrograde perfusion of the pancreatic duct with HBSS; this resulted in increased dispersion of the tissue and more effective digestion by the collagenase. Lacy and Kostianovsky also

described 2 methods for purification of the islets, the first being relatively inefficient and consisting of sedimentation for 1 minute through HBSS in a 25 cm measuring cylinder, with discard of the supernatant. The second, more efficient, method was centrifugation on a preformed sucrose density gradient, resulting in large numbers of relatively pure islets. Unfortunately the sucrose was found to produce damage to the islets, probably as a result of osmotic stresses, but this problem was circumvented by the use of ficoll, a polymer of sucrose (Ballinger and Lacy 1972). The method described by Ballinger has become the standard for preparation of islets from the guinea pig, rat and, with minor modification, mouse pancreas. The method requires some skill in judging the length of collagenase digestion, but with practice around 200-300 islets can be obtained from a rat pancreas. The purity is usually quite good - approximately 90%, the remainder consisting of small lymph nodes and fragments of ducts and vessels. These contaminants can be removed by hand-picking under a stereo-microscope, viewing the islets against a black background with side illumination. Finke et al. (1979) described a further modification using reflected green light to increase the accuracy of islet identification, and thus allowing easier removal of contaminants.

Although the method described by Ballinger and Lacy (1972) is repeatable, the yield of islets represents only around 10% of the islets in the rat pancreas. This has not prevented transplantation experiments, since it is possible to use multiple syngeneic donors, but attempts have been made to increase the islet yield. Scharp et al. (1975) introduced a digestion/filtration chamber, which allowed the release of islets as soon as they were separated from the exocrine tissue, thus preventing the further digestion and loss of islets released at an early stage. An increase in yield was claimed from 150-450 islets per rat pancreas. Buitrago et al. (1977) reported a method substituting Percoll sedimentation at unit gravity for centrifugation on ficoll, and claimed a

marginally increased yield of mouse islets. Offord and Halban (1978) replaced the ficoll centrifugation stage by filtration through 2 nylon meshes, relying on a size difference between the intact islets and smaller exocrine particles. MacDonald et al. (1980) used a mechanical tissue grinder driven at low speed to mince up rat pancreas before digestion, and claimed a 65-80% increase in islet yield. However, the success of these reported modifications remain to be proven by other investigators, and most groups still use the proven method described by Ballinger and Lacy (1972).

Methods used for isolation of islets from large mammalian pancreas

Naturally enough, the first method to be applied to the more fibrous large mammalian pancreas was that developed for the rat. Briefly, the method consisted of initial distention of the pancreas by retrograde perfusion of HBSS down the pancreatic duct, mechanical chopping of the tissue, collagenase digestion to disperse the tissue and finally separation of the islets by centrifugation on a ficoll density gradient. Initial reports of this technique used on monkey pancreas were encouraging (Ballinger and Lacy 1972) and the technique was applied in transplantation studies in the pig (Sutherland et al. 1974), monkey (Jonasson et al. 1977) and dog (Lorenz et al. 1979(a)). The results of these studies have been discussed elsewhere (pages 40-44), and it is apparent that the islet isolation technique was less than satisfactory in these experiments.

The St. Louis group have described many modifications of the basic rat technique in an attempt to improve islet yield. Scharp et al. (1980), working with the dog pancreas, introduced a digestion/filtration chamber (working on the same principle as that described in the rat) for the digestion stage and claimed an increase in islet yield. There were clearly difficulties in separating islets with ficoll using this method, since the impure dispersed pancreas was left unseparated in later

transplantation studies (Long et al. 1983). Alternative methods of pancreatic distention were also investigated by the same group (Downing et al. 1980), venous distention with HBSS being found to produce a better yield of islets than either ductal or arterial injection. Again this modification seems to have been abandoned in later studies (Long et al. 1983). More recently rather complex methods have been reported (Scharp et al. 1979) where the dispersed pancreas from the digestion/filtration stage is further dispersed to single cells and then the endocrine cells are isolated using an elutriator. The purified endocrine cells are then encouraged to re-aggregate by a system of gyro-rotational culture. The purity of the preparation derived from this method is difficult to assess, since the tissue is dissimilar to normal islets, but the yield is certainly poor.

Apparently working in parallel to the studies of Scharp et al., Lacy et al. (1982) recently described a simpler modification of the rat method, applied in this case to the beef pancreas. The only new feature is the use of the fabric velcro during the collagenase digestion phase. The chopped pancreatic tissue is stuck to the velcro, which has numerous tiny hooks on it's surface, and the digestion is interrupted frequently to remove dispersed pancreatic tissue. The advantage of the velcro is that it apparently holds on to the fibrous tissue whilst allowing separation of the parenchymal components. The islets are finally purified on a ficoll gradient. The reported histological appearance and purity of the islets is quite good, but the yield is still quite poor: 20,000 islets from a whole beef pancreas. However, this method does seem to offer an improvement over previous methods.

Horaguchi and Merrell (1981) reported a significant modification applied to dog pancreas. The duct was continuously perfused with collagenase in Ca^{++} , Mg^{++} free HBSS. After a period of between 20 to 40

minutes the gland was chopped with scissors then dispersed by vigorous shaking, filtering the tissue through a steel mesh. The tissue was further digested using trypsin for 20 to 30 minutes, shaking continuously. No further purification was undertaken. The yield of tissue seems to have been definitely improved by this method, but the purity of the preparation was probably poor, although a sixfold purification was estimated by the insulin to amylase ratio. Warnock et al. (1983) simplified the method of Horaguchi, substituting unmodified HBSS, and dispensing with the final stage of trypsin digestion. Strangely, the yield of tissue seemed to be rather less than described by Horaguchi, and the purity was very poor, the insulin/amylase ratio remaining the same as for fresh pancreas.

Noel et al. (1982) described a rather complex method applied to the dog pancreas. Again collagenase solution (in tissue culture medium) was injected down the duct, but this time as a single distention (repeated once) rather than a continuous perfusion. After incubation at 37° C. for around 20 minutes the gland was then dispersed by vigorous shaking in cold tissue culture medium. The dispersed tissue was then subjected to a complex series of culture and washing stages lasting approximately 12 hours before finally isolating the islets on a discontinuous ficoll gradient. The yield of islets was claimed to be excellent, but the method of identification of islets was not satisfactory. The purity of the preparation was not assessed.

The previously mentioned studies have all attempted to modify the islet isolation technique used on the rat pancreas. Others have blamed the poor yield on the use of enzymes, and have attempted islet isolation based on physical separation of the tissues. Hinshaw et al. (1981) reported a method claimed to be applicable to rat, rabbit and human pancreas, in which the gland is kept in cold HBSS throughout the procedure. The gland is first minced with scissors then pressed through a sieve, pore

size 200–280 μ , using gloved fingers. The tissue passing through the sieve is transferred to a 50 ml tube, shaken vigorously, then centrifuged at 60 x G for 4 minutes, the pellet then being discarded. The supernatant is then spun at 600 x G for 5 minutes, and should contain the islets, no further purification being performed. The assessment of the islet yield and purity were totally inadequate in this study.

Wise et al. (1982, 1983) described a method for pig pancreas, similar to that of Hinshaw, but using a tissue homogeniser, run at slow speed, to chop the tissue to a fine silt before passing it through a 150 μ nylon filter. The tissue passing through the filter was thought to contain the islets: no further purification being performed. Again the methods for assessing islet yield and purity were totally unsatisfactory.

An altogether different approach was reported by Matas et al. (1977), using short term (24 hour) tissue culture of chopped dog and human pancreas to produce enzyme depletion before transplantation. Evidence was presented for a rapid reduction of amylase content of cultured fragments, whilst insulin content was maintained. The viability of the tissue was not adequately assessed, however, and little purification of the islets, in terms of actual volume of tissue, seemed to occur.

Discussion

The method for islet isolation from the rodent pancreas described by Ballinger and Lacy (1972) has become the standard, and produces intact islets of proven viability and acceptable purity. The islet yield is not good, but, because of the availability of isogeneic strains, this problem is easily overcome by using more donors. The various modifications proposed to increase the yield or simplify the procedure do not seem to produce a marked advantage, which may explain why they have not become generally accepted.

Many of the methods devised for isolation of islets from the pancreas of large mammals have been used in transplantation experiments and were discussed in detail in the historical review, the conclusion reached being that no repeatable method for transplantation of isolated islets has yet been devised. A study of the methods used shows that the reason for failure in these studies probably lies in the islet isolation technique. Although, in some studies, successful isolation of large numbers of pure islets has been claimed, there appears to be an unwillingness to examine the results of these new techniques critically. Critical examination should include, as a minimum, the following:

1. An estimate of the numbers of islets isolated by a proven counting technique, using a recognised method of islet identification.
2. An estimate of the purity of the preparation, preferably confirmed by at least 1 other method.
3. Histological examination of the preparation by both light and electron microscopy to confirm that islets have been correctly identified, are structurally intact and also to show the purity of the preparation.
4. An assessment of the viability of the islets isolated by at least 2 recognised methods, such as supravital staining and insulin secretion in response to glucose stimulation.

None of the reported studies entirely fulfils all these criteria; those studies presenting the most data have tended to show that the yield has been poor and/or the purity unacceptable. The better documented studies are based on the proven method used in the rat pancreas. It would seem that the unmodified method of Ballinger and Lacy (1972) does not work well in the large mammalian pancreas. Most of the modifications described seem to have been successful in producing satisfactory dispersion of the pancreas following digestion, but have either failed to separate the islets in any degree of purity, or have such a complex method that the yield obtained is unsatisfactory. Furthermore most methods seem to have been

applied only to the dog pancreas, which is structurally rather different from that of the human. Perhaps the most encouraging study was that of Lacy et al. (1982), using beef pancreas, where a definite increase in yield of structurally intact, reasonably pure islets was demonstrated.

The studies described using entirely new methods are more difficult to evaluate, since they present very little meaningful information regarding the islets isolated. Some points of common-sense can be applied, however. Both pig and human islets are similar in size to rat islets, averaging 100 to 300 μ diameter. The large size of rat islets means that, when suspended in HBSS, they sediment rapidly at unit gravity (this fact was used in the method of Lacy and Kostianovsky (1967)). The method of Hinshaw et al. (1981) describes suspension of the dispersed pancreas in Hank's solution followed by centrifugation at 60 x G for 5 minutes. The pellet is then discarded. It is certain that any intact islets, at least from human pancreas, would be within the discarded pellet. The method of Wise et al. (1982) describes passing the dispersed pancreas preparation through a 150 μ filter, discarding the trapped tissue. This would certainly exclude more than half the intact islets, and probably more, since it is likely that some exocrine tissue would remain attached to islets smaller than 150 μ .

PART 2, CHAPTER 5.THE DEVELOPMENT OF A METHOD FOR ISOLATION OF
ISLETS FROM THE DOG AND THE HUMAN PANCREASIntroduction

A method for isolation of islets from both the dog and the human pancreas has been developed, the details of which will be presented in the following 2 chapters. The methods have considerable differences, but both were developed from studies using the rat, dog, pig and human pancreas. Numerous separate experiments were performed, and a full description of these experiments, using the customary formal presentation, would require far more space than is available in this thesis. Nevertheless, it would be incorrect and less informative to present the final techniques without some description of the ideas and experiments, often negative, that led to them. It is therefore proposed to abandon the formal approach for the purpose of describing these studies and simply present the basis of the experiments performed, with the experimental result and conclusions.

Sources of pancreatic tissue

Studies were performed using rat, pig, dog and human pancreas. Lewis rats were bred in our own colony and anaesthetised using pentobarbitone before removal of the pancreas. 17 pig pancreases were obtained (within 8 minutes of death) from a local abattoir. A further 7 pig pancreases were removed under anaesthesia from animals that were previously the subject of a urological study. 44 mongrel dog pancreases were obtained, mostly from animals that were previously the subject of non-recovery experiments to investigate the effects of anaesthetic agents. A few dog pancreases were from animals previously the subject of long-term studies on bowel diversion, the animals being anaesthetised for removal of the pancreas.

Samples of human pancreas were obtained from 28 beating-heart cadaver transplant donors at the time of donor nephrectomy for renal transplantation. Pancreases from all sources were removed as rapidly as possible, cooled by immersion in ice cold Hank's isotonic salt solution and transported, surrounded by ice, to the laboratory.

Methods used to assess islet yield and viability

There are 2 possible ways of assessing islet yield and purity. A method used frequently in previous studies has been the tissue insulin content and the insulin to amylase ratio, but there are several criticisms of this technique. The method is relatively complex and an answer is only made available several days later, thus making the performance of several experiments on 1 sample of pancreas less efficient, as it is not possible to adjust the technique according to the result of the previous experiment. Furthermore, there are many sources of error, as both amylase and insulin are unstable proteins and require different extraction techniques, a fact not appreciated in some studies. The amylase activity of pancreatic tissue decreases rapidly when placed in tissue culture medium at 37° C. (Matas et al. 1977) therefore incubation with collagenase at 37° C., particularly for prolonged periods, may lower the amylase content and thus erroneously raise the insulin/amylase ratio. Conversely, insulin may be degraded by the action of proteolytic enzymes in the pancreas. Lastly the insulin/amylase ratio does not reflect the structural integrity of the tissue. For the above reasons, insulin/amylase ratios are not used in this study.

The alternative way of estimating the yield and purity is by counting the number of islets in the final preparation. To obtain meaningful results it is vital to be able to identify islets accurately. This is not difficult with rat islets, which have a characteristic appearance, but pig

and dog islets are very difficult to distinguish from fragments of exocrine tissue. For this reason intra-arterial injection of neutral red was used to identify the islets while the isolation method was being developed. Later, when the method was established, it was possible to abandon neutral red injection and identify islets with reasonable confidence. Fortunately, human islets appear similar to rat islets, and are easily identified. The accuracy of islet identification was also checked by histological examination of all islet preparations, using haematoxylin/eosin and aldehyde fuchsin stains, with immunoperoxidase stain for insulin in some cases. Electron microscopy of individual islets was also performed in selected cases.

Counting the islets directly is only possible for small numbers: for larger yields it was necessary to employ a sampling method. A simple technique was developed, based on subdilution of the islet preparation. The complete islet yield was suspended in HBSS in a 10 ml syringe (without a needle) and a 1 ml sample taken, using a 1 ml syringe and a 19G needle inserted into the hub of the 10 ml syringe, whilst gently agitating the islets to maintain a homogenous suspension. The 1 ml sample could be further subdiluted into another 10 ml syringe and this repeated as necessary. 5-10 samples of 0.1 ml were taken from the final dilution by the same technique and the islets counted by direct observation through a stereo-microscope. The mean number of islets per sample was calculated, and the total yield estimated by multiplying by the dilution factor. An estimate of the purity of the preparation was also made by comparing the number of islets to the number of exocrine particles, with later confirmation by histological examination.

The tissue viability was assessed using fluorescein diacetate and ethidium bromide (FDA/EB) stain, as described previously. In later experiments glucose-stimulated insulin release was also used as

confirmation.

Experiments performed

i) Application of the standard rat isolation method

The standard method for isolation of islets from the rat pancreas (Ballinger and Lacy 1972) was assessed using pig, dog, and human pancreas. Using pig pancreas the technique failed totally, producing only a gelatinous mass of tissue from which no free islets could be separated, and altering the duration of digestion had no effect. The pig tissue also tended to form "pseudoislets" (described in detail on page 141). The dog pancreas was slightly more encouraging, since it did not form the same gelatinous mass as pig pancreas, but the quantity of dispersed tissue obtained following digestion was very small, despite varying the duration of digestion, and no separation of islets could be obtained on a ficoll density gradient. The human pancreas behaved similarly to dog pancreas, producing some fragmentation, but a very poor yield and no separation of islets on ficoll.

ii) Non-enzymic methods

In view of the failure of the standard islet isolation method, a study of the reported non-enzymic techniques was carried out, using pig pancreas. Initially, the effect of hand-chopping the tissue with scissors was compared to mincing with a modified tissue homogeniser (Polytron), as described by MacDonald (1980). Hand-chopped tissue remained generally viable, though with some tissue destruction confined to the periphery of the particles (Plate 2-5-1). Mincing the tissue in the Polytron resulted in smaller particles, with the size diminishing the longer the tissue was processed. Unfortunately, the smaller the particles derived, the poorer the viability of the tissue, and particles chopped to a size that would

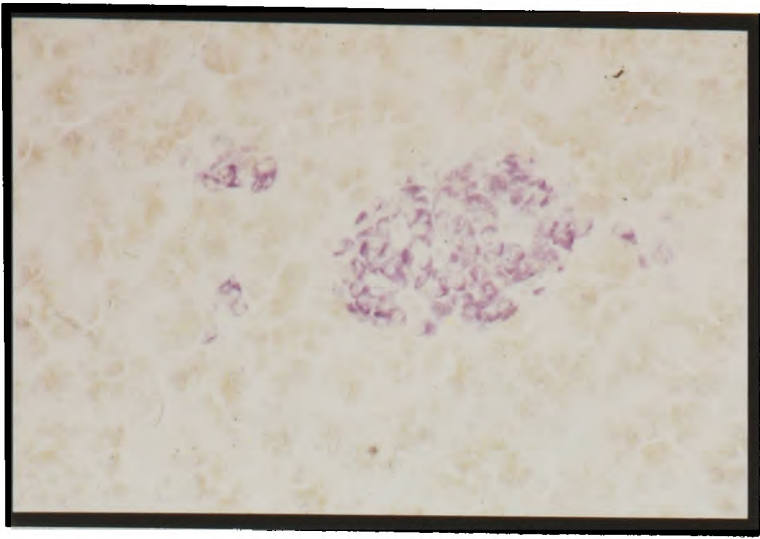


Plate 2-5-1

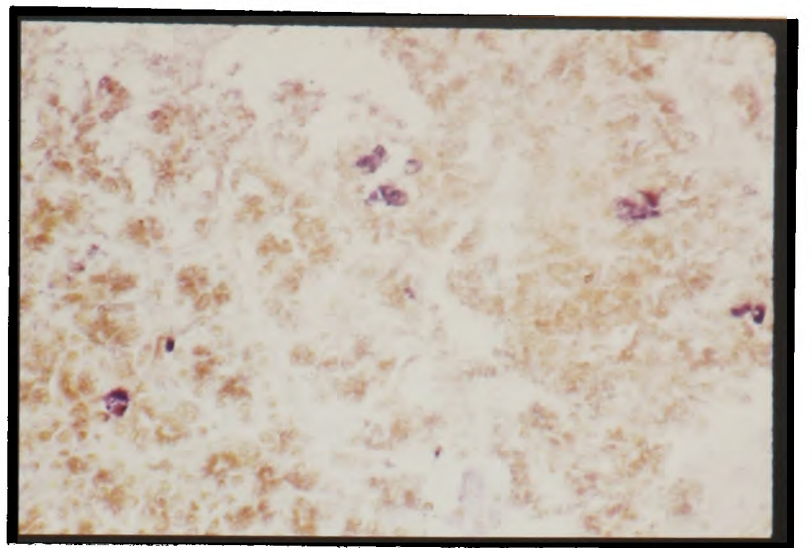


Plate 2-5-2

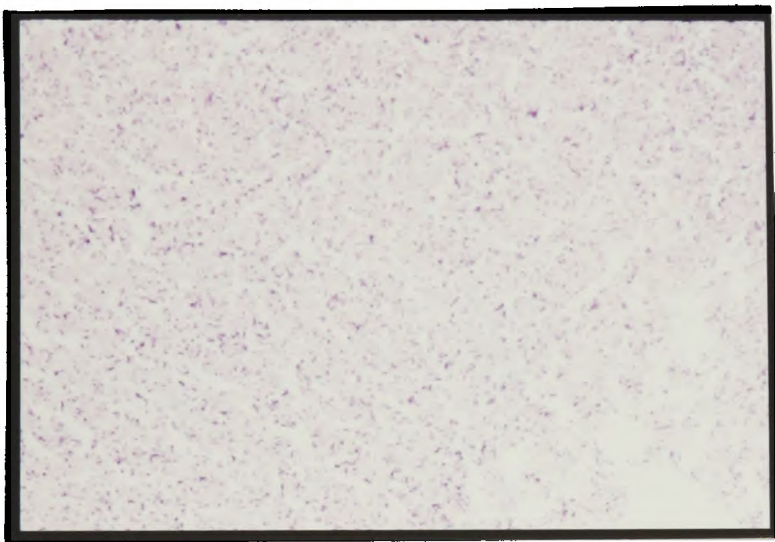


Plate 2-5-3

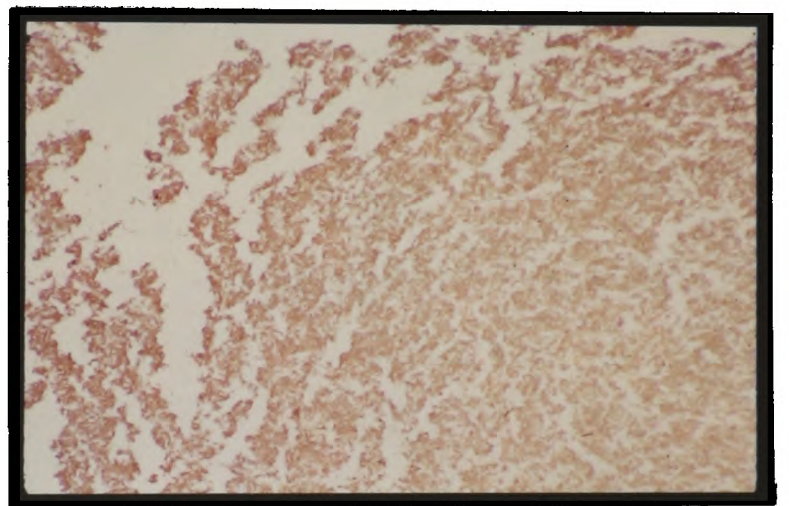


Plate 2-5-4

Plate 2-5-1. Photomicrograph of pig pancreas, following hand-mincing with scissors. The tissue remains structurally intact, with an islet easily identifiable. Aldehyde fuchsin. Magnification x 150.

Plate 2-5-2. Photomicrograph of pig pancreas following mincing in a polytron tissue homogenizer. There is extensive distortion of the tissue, with fragmentation of the islets. Aldehyde fuchsin. Magnification x 150.

Plate 2-5-3. Photomicrograph of tissue prepared from pig pancreas by the method of Hinshaw et al. (1981). The tissue is composed of an homogenous accumulation of cellular debris, with many nuclei seen to lie outside aggregates of acellular cytoplasm. Haematoxylin and eosin. Magnification x 150.

Plate 2-5-4. Photomicrograph of tissue prepared from pig pancreas by the method of Hinshaw et al. (1981). There is no identifiable islet tissue. Aldehyde fuchsin. Magnification x 150. (adjacent section to Plate 2-5-3).

just pass through a 150 u filter, as described by Wise et al. (1982, 1983), were composed of tissue that was completely dead, with histological evidence of severe tissue destruction (Plate 2-5-2).

Pressing tissue through a sieve, as described by Hinshaw et al. (1981) also resulted in death of the tissue. Furthermore, as suspected (page 135), the few histologically recognisable fragments of islet tissue present in the sieved tissue are discarded in the centrifuged pellet, the remaining "islets" being apparently composed of a suspension of dead cells (Plates 2-5-3, 2-5-4).

An interesting phenomenon was noted in the above experiments (and to a lesser extent in other experiments, usually with the pig pancreas). Examination of the islet preparation revealed numerous rounded or spherical bodies, which were more symmetrical than usual for islets, and also smaller than would be expected. These spherical bodies were strongly stained by direct application of neutral red solution, appeared to be non-viable by FDA/EB staining, and glucose stimulation experiments failed to detect any insulin secretion. Hand-picking of a number of these bodies followed by histological processing, showed them to be composed of an amorphous substance with no cellular structure. The nature of these spherical bodies is uncertain, but they may represent aggregations of degenerate cytoplasm and lipid. In view of their similarity to islets they have been termed "pseudoislets" in this thesis.

A report by Davies and Parrott (1981) that the epithelium of murine gut could be separated from the lamina propria by removal of Ca^{++} and Mg^{++} with E.D.T.A., prompted investigations to see if islets could be made to separate from exocrine tissue in a similar fashion. Several concentrations of E.D.T.A. were tested on chopped pig pancreas, with no discernible effect.

The technique of tissue culture of hand-minced pancreatic fragments described by Matas et al. (1977) was investigated using rat, dog and pig pancreas. The method was found to be impractical for dealing with the large quantities of tissue derived from the whole pig and dog pancreas, because of the number of culture vessels required. This problem apart, it was found that only the smallest fragments of tissue remained viable for more than a day, the larger fragments dying rapidly. This poor outcome was particularly marked in the pig pancreas, with the formation of numerous "pseudoislets", which were again shown, by histological examination, to be composed of amorphous acellular material. The few small fragments of tissue that appeared intact in culture, showed no evidence, on histological examination, of selective survival of islet tissue.

The disappointing outcome of these investigations into non-enzymic methods of islet isolation led to the decision to concentrate on modifications of the collagenase digestion technique, and, owing to the confusing tendency to form "pseudoislets", the use of pig pancreas was abandoned.

Modifications of the collagenase digestion technique

Grant et al. (1980) described experiments on human pancreas where up to 500 islets have been obtained for biochemical investigations, using a collagenase digestion technique. The technique is not fully described in the publication quoted, but personal observation showed that the technique is similar to the standard rat method, the major difference being a longer digestion time and much more vigorous shaking to disrupt the tissue. No attempt is made to separate the islets on ficoll, the islets required being retrieved by hand-picking. The technique was investigated using several samples of human pancreas, with definitely more success than the standard rat method. Recognisable islets were retrieved, which were confirmed, by

histological examination, as islets with good structural preservation. The tissue viability was shown to be good by FDA/EB staining and insulin release in response to glucose stimulation. However, no more than 200 islets could be retrieved from approximately 10 grams of pancreas, and the technique was extremely laborious. Attempts to apply the technique to the dog pancreas produced no recognisable islets.

The method of Lacy et al. (1982) was investigated using dog pancreas. In this method the chopped pancreas is stuck to velcro fabric during the digestion phase. A moderately improved yield of dispersed pancreatic tissue was obtained with good viability of the tissue fragments, but it was not possible to purify the islets using a ficoll density gradient.

The results of the last 2 experiments seemed to indicate that viable islets of Langerhans could be isolated from human pancreas using the collagenase, but that relatively minor modifications of the standard rat technique would not produce a dramatic increase in islet yield.

Techniques employing intraductal injection of collagenase

Noel et al. (1982) described a method using dog pancreas which is a major departure from the standard rat technique, particularly because collagenase is injected into the pancreatic duct, allowing the pancreas to be dispersed without mechanical chopping. The rest of the technique involves a complex series of culture stages, with islets being finally separated on a ficoll gradient. The technique was investigated, also using dog pancreas, and was found to be both complex and laborious. Despite careful attention to every detail, and repeated attempts, the tissue obtained from the final ficoll separation contained few islets recognisable by histological examination (Plates 2-5-5, 2-5-6). Although this result was discouraging, one feature of the method was very

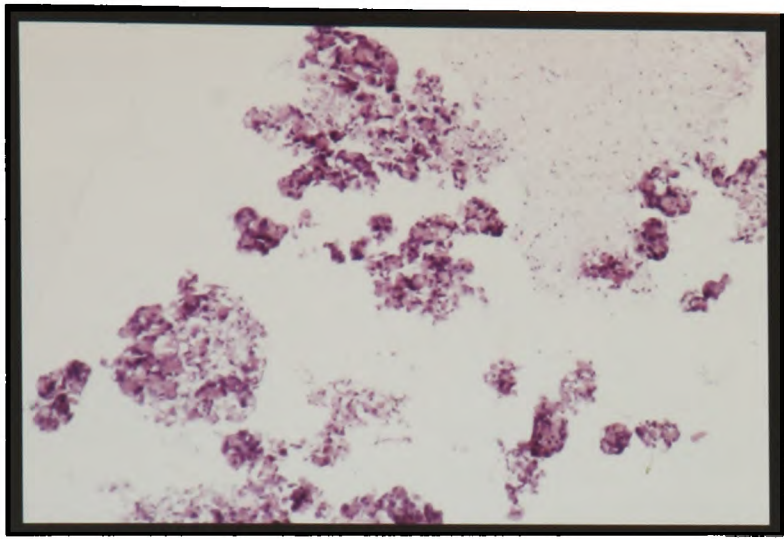


Plate 2-5-5

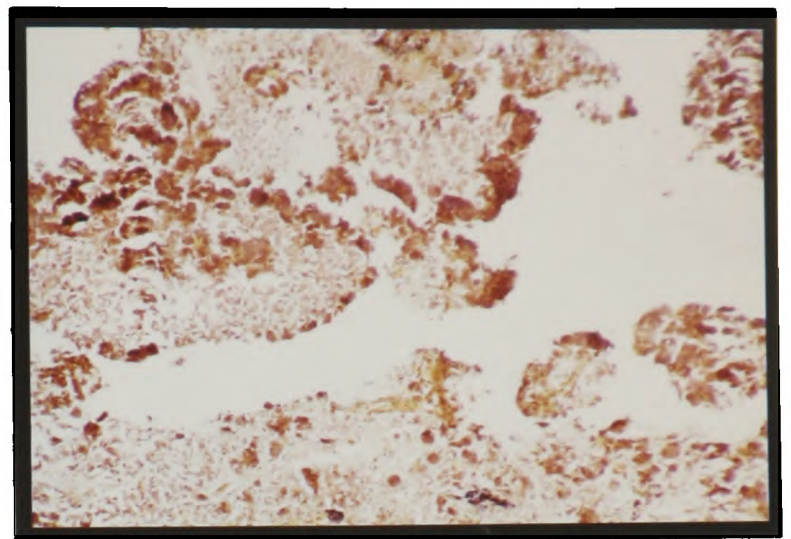


Plate 2-5-6

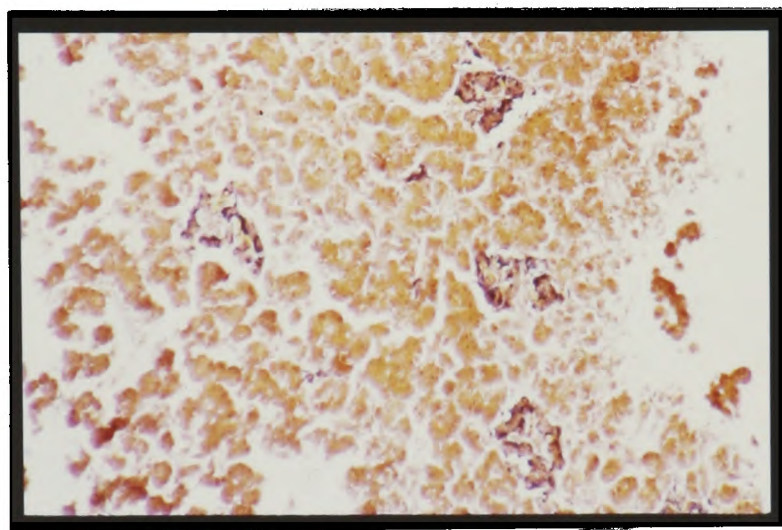


Plate 2-5-7

Plate 2-5-5. Photomicrograph of tissue prepared from dog pancreas by the method of Noel et al (1982). There is considerable necrosis of tissue, with accumulations of cellular debris. Most of the intact tissue has features suggestive of exocrine origin. Haematoxylin and eosin. Magnification x 150.

Plate 2-5-6. Photomicrograph of tissue prepared from dog pancreas by the method of Noel et al. (1982). A single, distorted, islet with dark blue stained B cells is recognisable (lower centre). Aldehyde fuchsin. Magnification x 150.

Plate 2-5-7. Photomicrograph of tissue prepared from dog pancreas by the method of Noel et al. (1982), and removed after the initial digest phase. 4 structurally intact islets with B cells containing blue granules are recognisable, and show excellent structural preservation, with evidence of separation from the surrounding exocrine tissue. Aldehyde fuchsin. Magnification x 150.

impressive, this being the initial dispersion stage.

The technique involved a double injection of collagenase solution into the pancreatic duct, followed by incubation at 37° C. for approximately 20 minutes. It was then possible to disperse the tissue by gentle shaking only, leaving behind a skeleton of the fibrous duct system. Furthermore, the yield of dispersed tissue obtained was excellent, usually over 50% (in terms of the weight of the original pancreas). The tissue could be further dispersed by gentle aspiration through a wide bore needle. The viability of the tissue was excellent, and histological examination showed the islets to be structurally intact (Plate 2-5-7), and yet there was clean separation of the islets from the exocrine tissue.

These encouraging findings led to a series of investigations into ways in which this method of pancreatic dispersion could be simplified, and the yield of tissue maximised. It may be useful to describe in detail the initial stages of the method of Noel et al. (1982).

The pancreatic duct is cannulated at laparotomy, the pancreas excised, cooled rapidly and transported in cold tissue culture medium (TCM). The gland is distended by injecting the pancreatic duct with enzyme solution containing 600-1100 units/ml collagenase (Sigma type V) and 10 ug/ml DNA'ase (Sigma) in TCM. Initially, the volume injected is 1.2 ml enzyme solution per gram of pancreas, the gland placed in a beaker and surrounded by a further 2.8 ml enzyme solution per gram of pancreas. The beaker is placed in a water bath and incubated at 37° C. for 14 minutes then rapidly cooled, washed with TCM, redistended with 0.6 ml enzyme solution and a further 1.4 ml enzyme solution added to the beaker, followed by a further incubation at 37° C. for 10 to 12 minutes. The tissue is then rapidly cooled and dispersed by repeated agitation in cold TCM. The fragmented pancreas is then washed, and further dispersed by repeated aspiration through a 14G needle. Two further washes in TCM are performed before

proceeding to the rest of the technique, not described here.

A number of modifications to the above technique were found to simplify the procedure. The use of a 2-stage distention technique was found to be unnecessary: 1 injection seemed to be quicker and just as efficient. HBSS (much cheaper) was substituted for TCM without loss of efficiency or viability of the tissue. The concentration of collagenase was varied, and a concentration of 3 mg/ml found to be optimal, this in fact being similar to the concentration recommended; however, the DNA'ase was omitted without noticeable difference. The enzyme solution surrounding the pancreas during the incubation seemed to serve no purpose and was also omitted, substituting HBSS, without affecting the result. Great care was found to be necessary when aspirating the tissue, otherwise the fragile islets easily fragmented.

Eventually a simple, repeatable technique for dispersing the dog pancreas was evolved, consisting of excision and rapid cooling of the pancreas, duct cannulation and then distention with collagenase solution. The gland was then placed in HBSS, incubated at 37° C. for 14 to 16 minutes, dispersed by agitation, washed repeatedly then finally aspirated through a needle to further disperse the tissue.

Isolation of islets from the dispersed dog pancreas

The dispersed pancreas preparation obtained by the above method was shown to contain large numbers of viable, structurally intact islets, but an even larger quantity of exocrine particles of varying sizes: little, if any, purification of the islets was noted at this stage. Repeated attempts to separate the islets on a ficoll gradient failed, until it was realised that the problem seemed to lie in the presence of a small number of relatively large particles, possibly representing somewhat undigested fragments. The introduction of a polyester filter to remove these large

particles allowed separation of islets from exocrine tissue on a ficoll gradient. The filter pore size was investigated and a size of 150 μ chosen as it allowed through most of the dog islets (dog islets being fairly small), whilst removing all the undigested particles.

Using a 5 layer ficoll gradient of density ranging from 1.08 to 1.03 it was then possible to obtain large numbers of islets, up to 4000 islets per gram of pancreas, with a purity that was sometimes as high as 90%. Unfortunately, this apparent continuing success story ends here, for although excellent purity of 90% could sometimes be obtained, more often it was much less: 10 to 20% was a quite frequent figure. Furthermore, sometimes the ficoll failed to separate the islets at all. The reasons for this variability were examined by a series of experiments, but no constant factor could be identified, though probably the answer lay in the exact point at which the collagenase digestion was halted, the optimal point seeming to vary slightly for each pancreas. A variety of other density gradients including percoll, metrizamide, and nycodenz were tried, but were not suitable. A combination of ficoll and metrizamide (lymphoprep) worked fairly well, and had the merit of simplicity. The purity was never better than 20%, but complete failure to separate islets was rare: for this reason it was adapted as the standard method for separating dog islets in later transplant experiments. The full details of the final technique for isolation of dog islets will be presented in the next chapter.

Adaptation of the dog islet isolation method to the human pancreas

Having developed a reasonably successful method for isolation of islets from the canine pancreas, attempts were made to apply the same technique to samples of human pancreas. Disappointingly, initial studies showed that the dog method appeared to be totally ineffective on the human

pancreas, as samples of human pancreas, injected with collagenase, appeared totally unaffected when dispersion was attempted after 15, 20 or even 30 minutes incubation at 37° C. No further progress was made for a time, until, in 1 experiment, the pancreas was left for 60 minutes, at which time the parenchyma of the pancreas showed clear evidence of softening, but despite this the gland still could not be dispersed by agitation. However, it was found that by gently teasing the gland apart with fine forceps, and then repeatedly agitating (whilst immersed in HBSS), the tissue could be made to fragment in a manner similar to the dog pancreas. The viability of the tissue was poor after this long incubation, and no islets were identifiable, nevertheless this seemed to be an advance. Studies were then concentrated into ways of speeding up the digestion process. 2 factors were identified as being of importance, the first being the use of a higher concentration of collagenase (6 mg/ml). Collagenase is a calcium-dependant enzyme, and it was found that raising the calcium concentration in the injected enzyme to 7.5 mmol/litre also slightly speeded up the process. Interestingly, the most important factor discovered was that of prewarming all the solutions used during the digestion process (both injected and surrounding solutions) to 39° C. (39° C. being found to be slightly more efficient than 37° C.). This simple modification speeded up the digestion dramatically, so that tissue teased apart after 21 to 23 minutes showed evidence of softening and could then be easily dispersed by agitation with a good yield of dispersed pancreatic fragments (up to 60% by volume).

Gentle aspiration of the tissue through 2 sizes of needle resulted in further dispersion of the tissue. The dispersed tissue, when observed through a stereo-microscope against a black background with side illumination, showed the presence of numerous, pearly white, spherical or oval islets, slightly larger than rat islets (averaging 100 -300µ diameter). Interestingly, the exocrine tissue tended to fragment into

much smaller particles, less than 50 μ diameter (apart from a few very large, probably partially digested, fragments of tissue). This size difference allowed the introduction of nylon mesh filters to separate out the larger islets from the exocrine tissue. A filter of pore size 106 μ was found to be optimal for retaining the most islets with maximum purity, the retained tissue then being passed through a coarse filter, pore size 500 μ , to remove the large undigested fragments. By this means the larger islets could be retrieved with a purity varying from 10 to 40%. Furthermore, the small islets that passed through with the exocrine tissue could usually be separated on a ficoll gradient to a similar degree of purity. Histological examination of the islets isolated showed good preservation of structure. Viability was excellent as determined by both FDA/EB staining and insulin secretion in response to glucose stimulation.

The modifications to the dog islet isolation technique described above thus formed the basis for the human islet isolation technique described in Part 2, Chapter 8.

Conclusions

There were several general conclusions drawn from this study of islet isolation techniques in the rat, dog, pig and human pancreas. It is clear that the pancreatic tissue of all species examined is very susceptible to mechanical trauma, and that islets of Langerhans are delicate structures easily destroyed, particularly by shearing stresses. For this reason, entirely mechanical methods of islet isolation seem unlikely to succeed, and even limited mechanical chopping should be avoided, if possible. A successful technique for islet isolation should be as simple as possible, since tissue is lost at each stage, diminishing the yield. Before islets can be separated from exocrine tissue the pancreas must first be dispersed as efficiently as possible, preferably removing the major structural

elements such as ducts and vessels, whilst maintaining tissue viability. Ductal injection with collagenase seems to fulfil these aims reasonably well, and, since the duct leads directly to the exocrine units and not the islets, this does seem the logical way to infiltrate the whole pancreas with collagenase. The methods developed for the dog and human pancreas are very different and emphasise the major differences between the pancreas and islets of man and other species, whilst showing that experiments in the dog can still be usefully modified to the human. The pig pancreas seems to have certain peculiarities that make it unsuitable for islet isolation studies, despite its apparent close similarity to human pancreas on histological examination.

PART 2, CHAPTER 6.DESCRIPTION OF THE METHOD FOR ISOLATION OF
ISLETS OF LANGERHANS FROM THE DOG PANCREASMaterials and methods

10 mongrel dogs were anaesthetised (as part of a previous experiment on the effect of anaesthetic agents) and a midline incision performed. The left common iliac artery was cannulated, a catheter passed retrogradely, so as to place the cannula tip above the origin of the coeliac artery, and 50 ml of 2% neutral red solution injected. The pancreas was excised rapidly, no attempt being made to clamp vessels (but care was taken not to cut the pancreatic tissue), cooled by immersion in HBSS at 4° C., and transported, on ice, to the laboratory. The pancreas was then divided into 3: a right lobe segment, left lobe segment and pancreatic angle segment. The pancreatic duct of each segment was then cannulated with a 2 FG plastic cannula (ligating the other end of the duct, in the case of the angle segment). Each segment was weighed, then injected via the cannula with an enzyme solution containing 3 mg/ml collagenase (Sigma type I) in HBSS at 4° C. (1 ml per gram of pancreas). After injection, the segments were transferred to a beaker containing a further 200 ml of cold HBSS, and the beaker placed in a 37° C. waterbath, shaken gently (30 times per minute). After approximately 16 minutes (the exact time being determined by the pancreas showing signs of digestion - see later) the gland was cooled by immersion in cold Hank's solution. (All HBSS used in this stage, and from this point on contained 4 gm/litre of bovine albumen).

The gland was then dispersed by repeated agitation, holding the cannula with forceps, with frequent removal and replacement of the fluid in which the pancreas was immersed. This fluid contained numerous fragments

of tissue, which were retrieved by gentle centrifugation (50 x G) for 10 seconds, the supernatant being discarded. When no more tissue could be detached the pancreatic remnant was also discarded. The fragmented pancreatic tissue was then washed once (washing being by the suspension of the tissue in fresh HBSS, followed by centrifugation at 50 x G for 10 seconds) and further dispersed by gentle aspiration and expulsion 3 times through a size 14G needle on a 50 ml syringe. The tissue was washed twice more, and then passed through a polyester filter, pore size 150u, with frequent washing with HBSS. The trapped tissue was discarded, then the filtered tissue was placed in 50 ml centrifuge tubes and centrifuged at 350 x G for 5 seconds. The tissue pellet was then under-layered on either of the following density gradients:

1. A discontinuous ficoll gradient, specific gravities 1.08, 1.07, 1.065, 1.06, 1.03, 1.00. The gradient was then centrifuged at 750 x G at 4° C. for 10 minutes. The 1.065/1.06 interface was checked under a stereoscopic microscope and usually contained islets. (Tissue obtained from 7 pancreases was separated by this method).
2. The tissue was mixed with 20 ml of lymphoprep, and 20 ml of Hank's solution carefully layered above. The tube was then centrifuged at 750 x G for 10 minutes. The interface was checked under a stereoscopic microscope and usually contained islets. (Tissue obtained from 3 pancreatic segments (3 different pancreases) was separated by this method).

The yield of tissue from either (1) or (2) was then washed 3 times before evaluation.

Islet counts were performed by subdilution, taking 5-10 samples of 0.1 ml and counting the number of neutral red stained islets visible through a stereo-microscope. The total yield of islets was obtained by multiplying the sample mean by the dilution factor.

Islet viability was assessed by supravital staining with FDA/EB, and also insulin secretion in response to glucose stimulation, using both freshly isolated islets, and the same islets after 12 hours culture. All islet preparations were processed for histological examination, using haematoxylin/eosin and aldehyde fuchsin stains. The preparation purity was estimated by eye, from histological sections, by the proportional area of islet tissue to non-islet tissue seen in representative low power fields.

Results

The pancreas of every animal was stained red by neutral red injection. Following injection of enzyme solution into the duct, and incubation, all pancreases showed excellent dispersion, with only a few, undigested lobules remaining attached to the skeletonised pancreas remnant. Gentle aspiration and expulsion through a syringe resulted in further dispersion of the tissue, so that approximately 95% of the tissue passed through the filter.

1) Ficoll gradient

The number of islets and the purity of preparation obtained are presented in table 2-6-1. In 2 preparations there was no separation of islets. The other 5 islet preparations showed great variation, with the yield varying from 15,000 up to 160,000 islets from the whole pancreas, and the purity varying from 5 to 80%. Histological examination showed good preservation of structure of islet tissue in all preparations (Plates 2-6-1, 2-6-2, 2-6-3). FDA/EB staining demonstrated excellent viability (Plate 2-6-4), confirmed by showing a good response to glucose stimulation from hand-picked islets (Fig. 2-6-1).

2. Lymphoprep gradient

All 3 preparations showed good separation of islets (Table 2-6-1), the

TABLE 2-6-1. THE NUMBER AND PURITY OF ISLETS ISOLATED
FROM THE PANCREAS OF 10 DOGS

<u>WEIGHT OF</u> <u>PANCREAS (GM)</u>	<u>FICOLL OR</u> <u>LYMPHOPREP</u>	<u>TOTAL NO</u> <u>OF ISLETS</u>	<u>ISLETS PER GM</u> <u>OF PANCREAS^a</u>	<u>HISTOLOGICAL</u> <u>PURITY (%)</u>
Whole pancreas ^b	F	15,000	461	5
" "	"	40,000	1230	80
" "	"	48,000	1476	60
" "	"	160,000	4920	30
" "	"	50,000	1538	20
" "	"	0	0	0
" "	"	0	0	0
15	L	23,000	1533	60
5	"	28,000	5,600	30
5	"	11,000	2,200	30

KEY: a = assuming average weight of 32.5 grams.

b = actual weight unknown (data lost).

(average dog pancreas = 32.5 grams
range 24 - 45 gms).

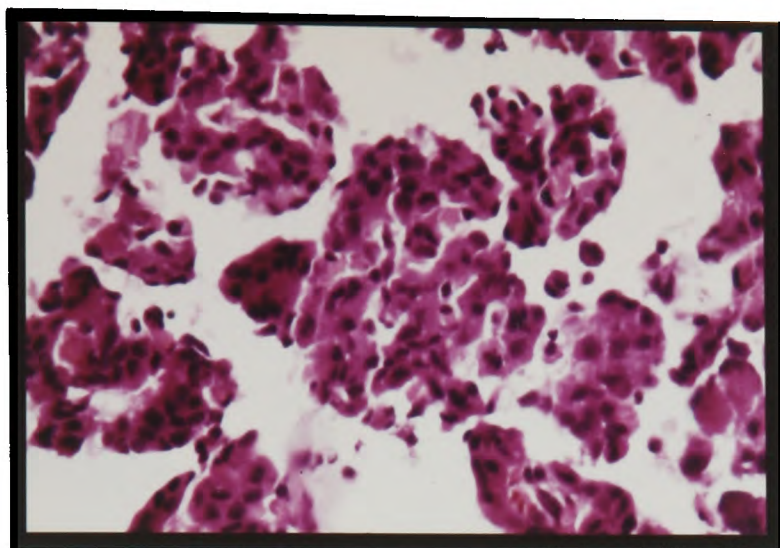


Plate 2-6-1

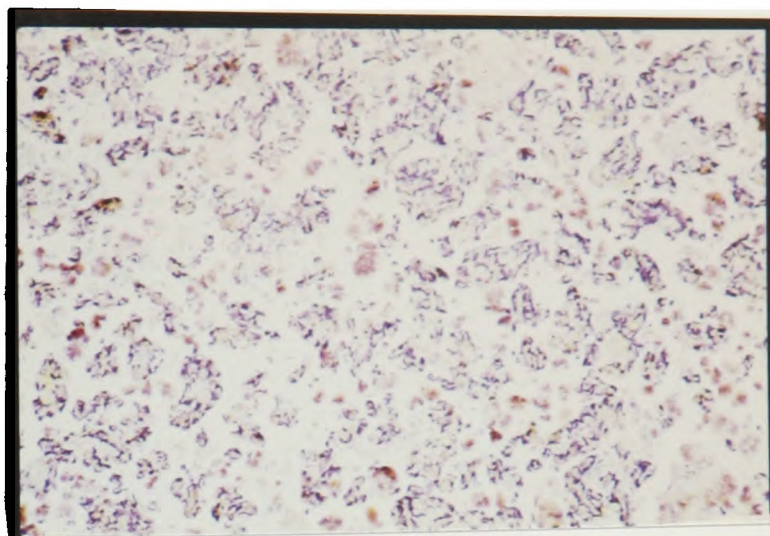


Plate 2-6-2

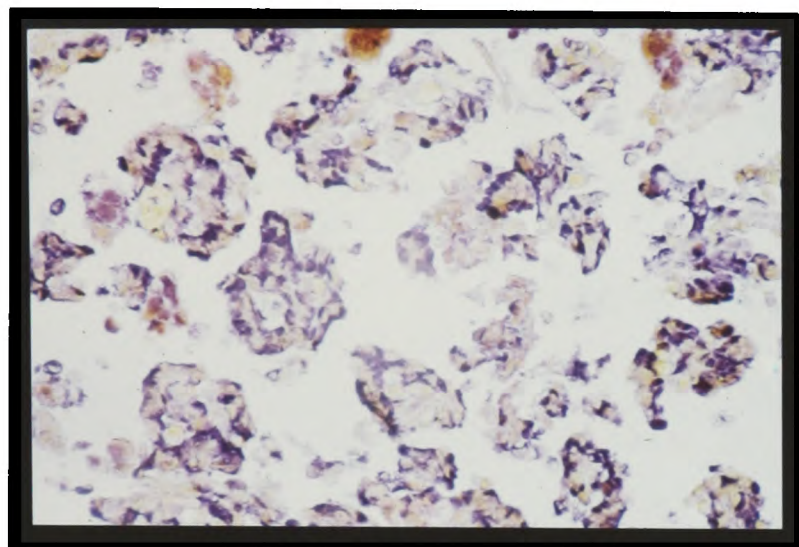


Plate 2-6-3

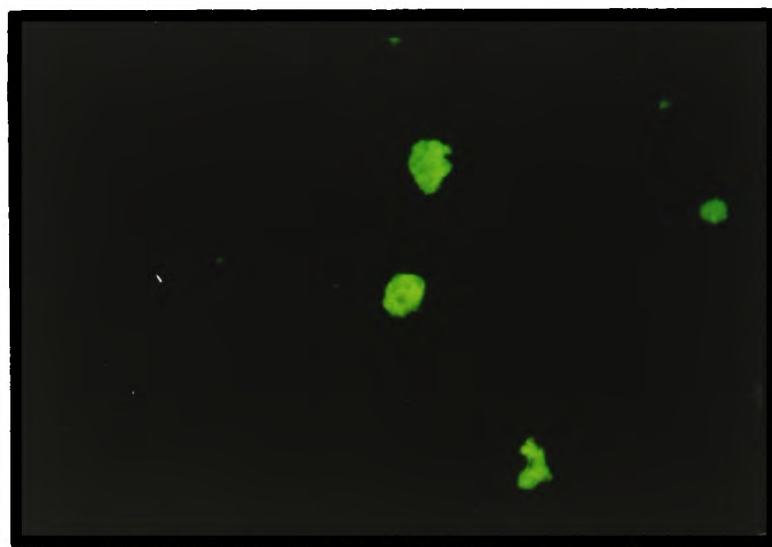


Plate 2-6-4

Plate 2-6-1. Photomicrograph of tissue prepared from dog pancreas by the method described in chapter 2-6. Numerous, discrete, islets are seen with good preservation of structure. The preparation is approximately 80% pure. Haematoxylin and eosin. Magnification x 60.

Plate 2-6-2. Photomicrograph of tissue prepared from dog pancreas by the method described in chapter 2-6. Numerous islets can be identified by their content of B cells with dark blue-staining granules. Exocrine tissue is stained uniformly yellow or brown. The preparation is approximately 80% pure. Aldehyde fuchsin. Magnification x 60. (Same preparation as plate 2-6-1).

Plate 2-6-3. Photomicrograph of tissue prepared from dog pancreas by the method described in chapter 2-6. Numerous islets are present, containing B cells with dark blue-stained granules, interspersed with other, non-stained endocrine cells and delicate capillaries. The irregular shape of normal dog islets is maintained. Aldehyde fuchsin. Magnification x 300. (Same preparation as plate 2-6-1).

Plate 2-6-4. Photomicrograph of dog islets prepared by the method described in chapter 2-6, cultured for 24 hours, stained with FDA/EB and viewed by UV light through a fluorescence microscope. The islets appear uniformly bright green, indicating good viability. Magnification x 50.

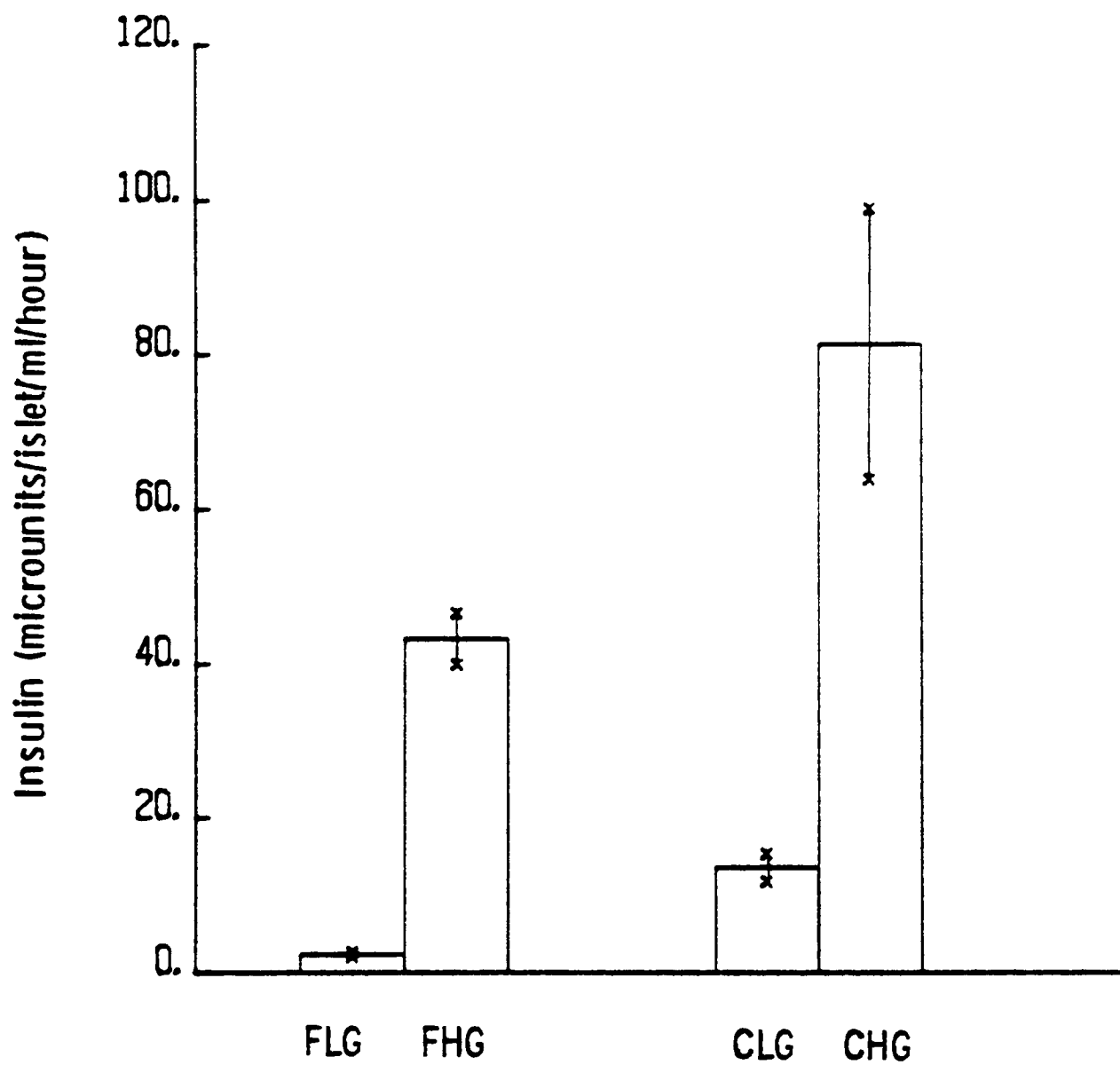


Fig. 2-6-1. Insulin secretion from dog islets prepared by the method described in chapter 2-6, using ficoll separation. Freshly prepared islets were incubated in either 2 mmol/litre (FLG) or 20 mmol/litre (FHG) glucose concentration. Values shown are the mean of 20 tubes $\pm$ S.E.M. The same islets were then placed in tissue culture for 12 hours, and then reincubated in either 2 mmol/litre (CLG) or 20 mmol/litre (CHG) glucose concentration. Values shown for cultured islets are the mean of 5 tubes $\pm$ S.E.M.

yield ranging from 1533 to 5,600 islets per gram of pancreas, with the purity varying from 30 to 60%. Again, excellent viability was demonstrated by both FDA/EB staining and insulin response to glucose stimulation (Fig. 2-6-2).

Discussion

The results presented show that the method described enables the isolation of up to 160,000 viable, structurally intact, islets from a single dog pancreas, and it is possible to obtain a preparation purity of 80% islet tissue. The dispersion of the pancreas following collagenase digestion is efficient and repeatable, but, unfortunately, the separation of islets from contaminating exocrine tissue, although sometimes very efficient, seems very variable and, sometimes, (especially when ficoll separation is used), fails completely. The reasons for this variability have been investigated but it has not been possible to distinguish any constant factor. The most likely candidate is the point at which the collagenase digestion is stopped, since this seems to vary with each pancreas. Although an assessment of the digestion process can be made from the appearance of the pancreas and the tendency for small fragments to detach on shaking, it has, so far, proven impossible to determine any characteristic that reliably marks the "end point". However, some other, as yet unsuspected, factor may be of importance.

The number of islets isolated here is less than reported by Horaguchi and Merrell (1981) or Noel et al. (1982), but, as discussed earlier, in those studies the numbers of islets were calculated by indirect methods of questionable accuracy. The purity of some of the preparations in the present report probably exceeds that of previous methods, and is certainly better documented than in previous reports.

The total number of islets present in the normal canine pancreas is

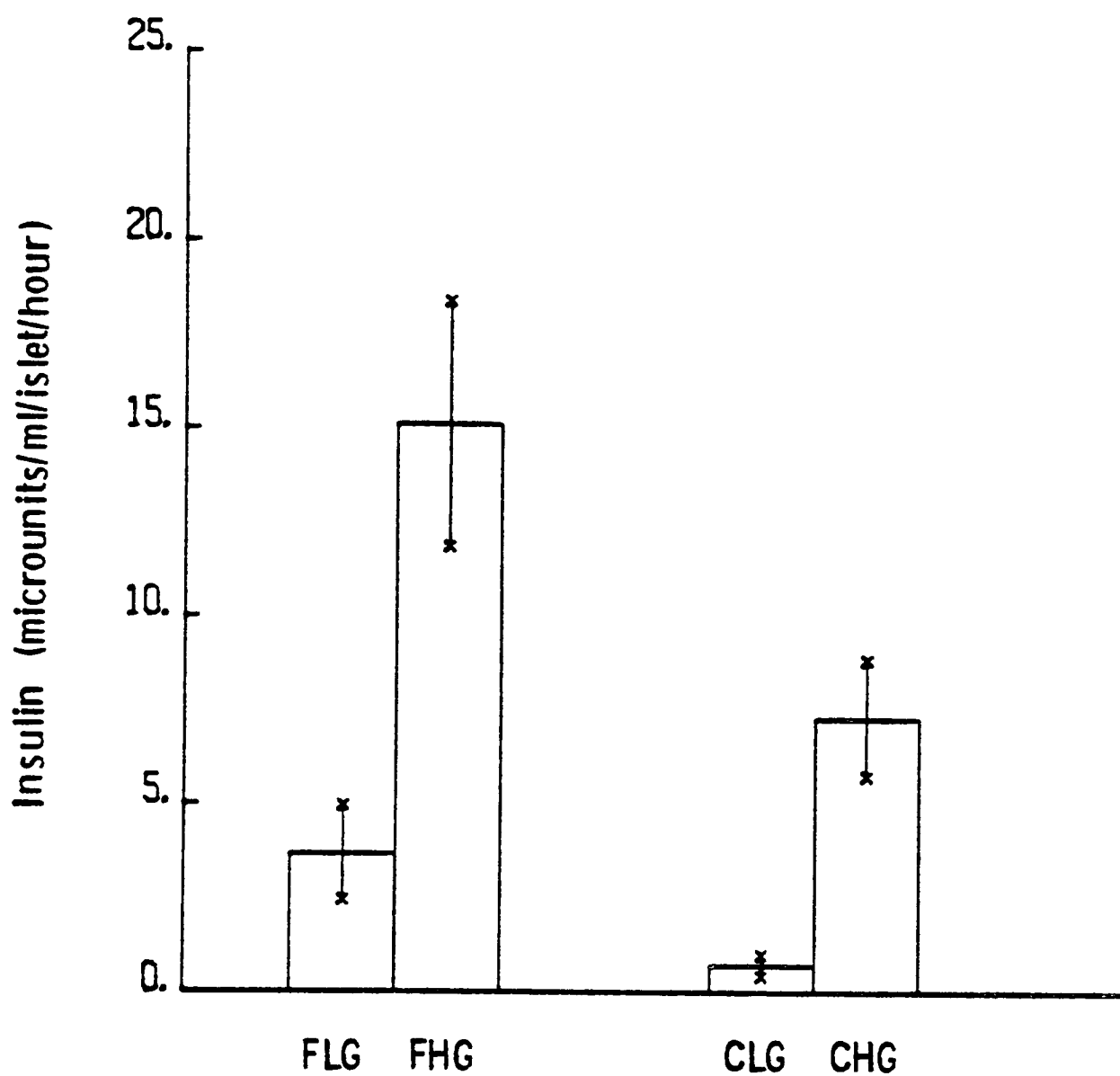


Fig. 2-6-2. Insulin secretion from dog islets prepared by the method described in chapter 2-6, using lymphoprep separation. Freshly prepared islets were incubated in either 2 mmol/litre (FLG) or 20 mmol/litre (FHG) glucose concentration. Values shown are the mean of 10 tubes $\pm$ S.E.M. The same islets were then placed in tissue culture for 12 hours, and then reincubated in either 2 mmol/litre (CLG) or 20 mmol/litre (CHG) glucose concentration. Values shown for cultured islets are the mean of 6 tubes $\pm$ S.E.M.

uncertain. Acosta et al. (1969) studied the distribution of islets in the canine pancreas, and estimated the total islet volume as 269 per mm^3 , but did not estimate the actual number of islets. Examination of normal canine pancreas shows that the average islet diameter is about 100 μ , and the average volume of the whole pancreas is approximately 30 ml, thus, assuming a roughly spherical shape, this would be equal to 500,000 islets in the whole pancreas. This is a very rough guess, but, were this figure correct, the best yield from the present experiments would correspond to approximately 30% of the total number of islets in the canine pancreas.

PART 2, CHAPTER 7.AUTOTRANSPLANTATION OF ISOLATED ISLETS IN PANCREATECTOMISED DOGSIntroduction

Having developed an apparently successful method for isolation of islets from the dog pancreas, it is clearly necessary to test the islet preparation with regard to its ability to function in a diabetic animal. The most reliable method of producing diabetes in the dog is by total pancreatectomy, which also makes available the maximum quantity of tissue for islet isolation. The probable sensitivity of isolated islets to the rejection process would introduce further reasons for failure, if an allograft model were to be used. Thus an autograft model, which should have no such problems, is the best for testing the function of the islet preparation. Work in rodents has suggested that implantation of the islets into a site with venous drainage into the portal system is advantageous, allowing control of diabetes with the minimum number of islets. In the dog, implantation into either the spleen or portal vein is possible, but the intraportal route has the advantage of greater certainty, since islets implanted directly into the spleen may be easily washed out by bleeding (particularly when the preparation volume is small, as in these experiments).

For the above reasons, autotransplantation of isolated islets to the portal vein of totally pancreatectomised dogs was chosen as the experimental model best suited to the testing of an islet preparation produced by a new method.

Materials and methods

10 mongrel dogs were sedated with morphine and chlorpromazine, and an intravenous glucose tolerance test performed. Anaesthesia was then

induced by intravenous methohexitone, and maintained by inhalation of halothane, nitrous oxide and oxygen. The abdomen was opened through a midline incision, and a total pancreatectomy was performed, care being taken to maintain the blood supply to both the pancreas and duodenum during the dissection, by leaving the vessels unligated, but placing ligatures ready to be tied. When the dissection was complete the vessels to the pancreas were rapidly ligated and the gland removed, leaving the blood supply to the duodenum and bile duct intact.

The pancreas was rapidly cooled, and transferred to the laboratory, where islet isolation was performed in the manner described in Part 2, Chapter 6. The number of islets isolated was estimated, as before, by subdilution, and counting the islets identifiable in 5 samples. The purity of the preparation was also estimated by direct observation, and, in the case of the first 5 islet preparations, was confirmed by processing the islet yield for histological examination, using both haematoxylin/eosin and aldehyde fuchsin stains. The viability of the islet preparation from 1 sample was tested by both FDA/EB staining and insulin secretion in response to glucose stimulation.

5 dogs acted as controls and were given no further treatment after pancreatectomy. The other 5 dogs underwent injection of the islet preparation into the portal vein. Portal vein pressure was measured before, immediately following, and 10 minutes after infusion.

After recovery from the operation, no food or water was allowed for 24 hours, fluid balance being maintained during this time by intravenous infusion of saline. Fluids were introduced slowly, followed by increasing quantities of solid food, supplemented with pancreatic enzymes. Daily blood samples were drawn for estimation of serum glucose and insulin. Animals that became diabetic lost weight rapidly, and the point at which the animal lost interest in food or drink was deemed the end of the

experiment, the animal being terminated by intravenous injection of pentobarbitone. A post-mortem was performed in all cases, particular attention being paid to the presence of any residual pancreatic tissue, and samples of tissue from the transplant implantation site were taken for histological examination.

Results

All 5 dogs subjected to total pancreatectomy, without subsequent islet transplantation, became immediately diabetic (Fig. 2-7-1), with the blood glucose rising above 20 mmol/L within 48 hours, and the development of polyuria, polydipsia, polyphagia and rapid-weight loss. 4 animals died within 5 days, but one dog survived, although losing weight rapidly, for 10 days. No animal showed evidence of residual pancreatic tissue at post-mortem.

The results of islet preparation from the pancreases of these 5 animals are presented in table 2-7-1. The separation of islets from exocrine tissue failed completely in 1 instance, and a poor yield of 33,000 islets was obtained from 1 other pancreas. However, a fairly good yield of between 96,000 and 130,000 was obtained from the other 3 pancreases. The estimated purity ranged from 30-50%, and was subsequently confirmed by histological examination.

The pancreases of the 5 dogs subsequently given autotransplants were processed, and gave yields varying from 24,000 to 111,000 islets. The purity was estimated to vary between 10 and 60% (Table 2-7-1). A sample of islets from 1 preparation was tested for viability, and was shown to be fully viable by both FDA/EB staining and by insulin secretion in response to glucose stimulation. The volume of tissue transplanted varied from 0.2 ml to 1.0 ml, and injection of the tissue into the portal vein led to a small immediate rise in pressure (less than 6.5 cm water) which returned

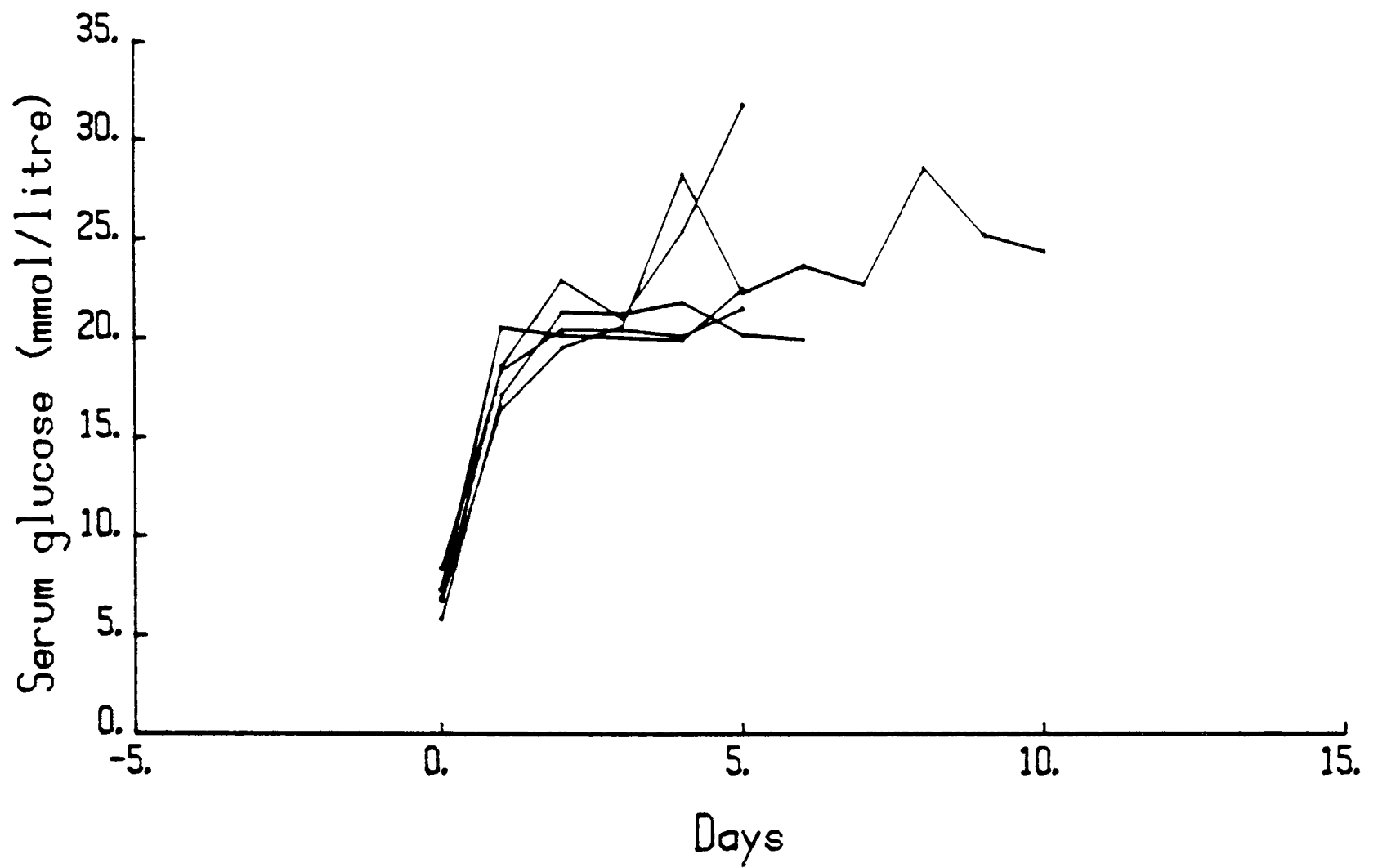


Fig. 2-7-1. Serum glucose of dogs subjected to total pancreatectomy on day 0.

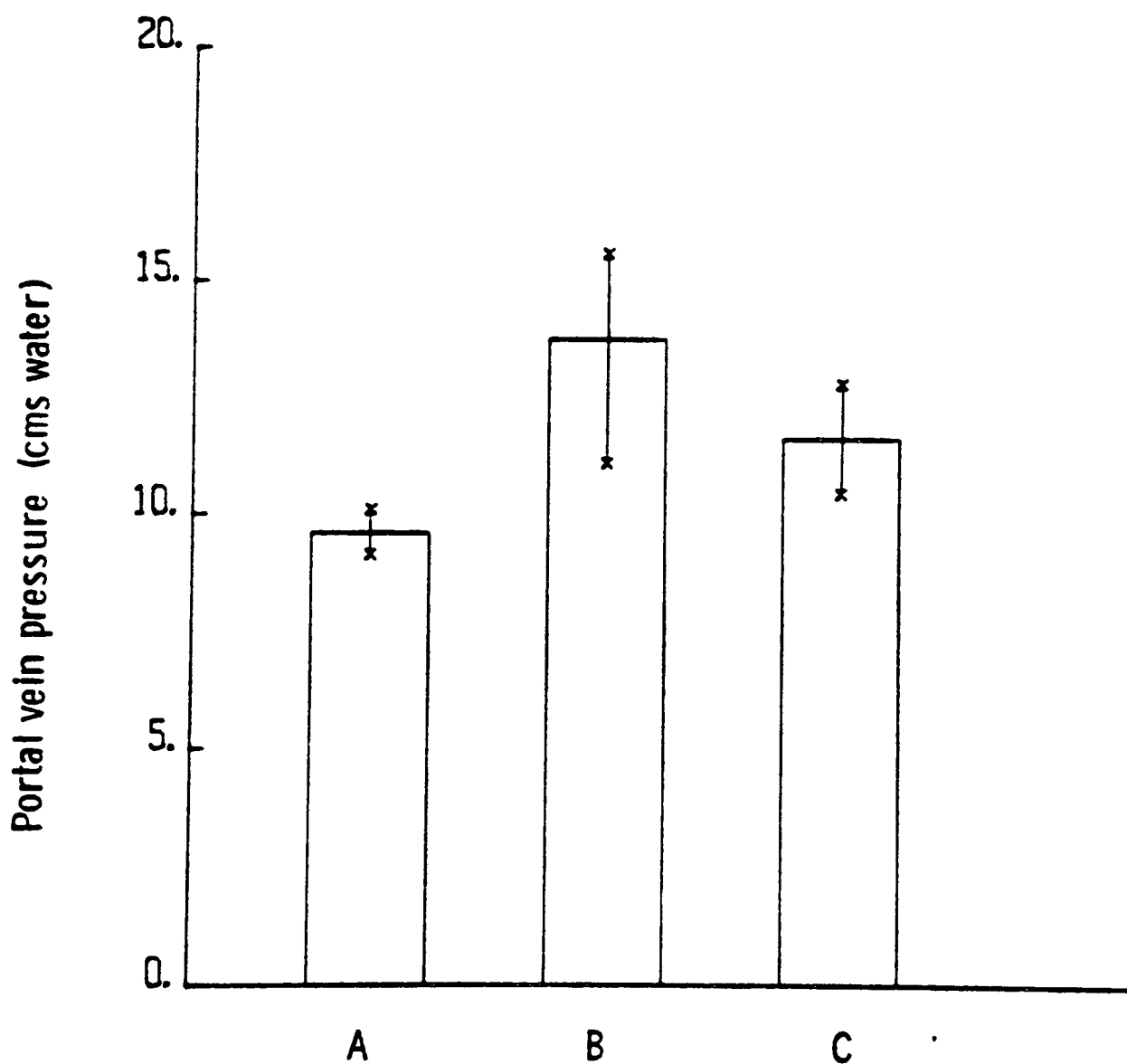


Fig. 2-7-2. Portal vein pressure of dogs given intraportal islet transplants. (mean $\pm$ S.E.M.).

A = Pressure immediately pre-transplant.

B = Pressure immediately after transplant.

C = Pressure 10 minutes after transplant.

TABLE 2-7-1. THE NUMBER AND PURITY OF ISLETS PREPARED
FROM THE PANCREAS OF 10 DOGS

<u>WEIGHT OF</u> <u>PANCREAS</u> <u>(GM)</u>	<u>NO OF ISLETS</u> <u>ISOLATED</u>	<u>PURITY</u> <u>(%)</u>	<u>TRANSPLANT</u> <u>PERFORMED</u>
35	33,000	40 ^a	No
45	120,000	60 ^a	No
30	96,000	40 ^a	No
28	0	0	No
32	130,000	40 ^a	No
25	111,600	20 ^b	Yes
24	32,000	10 ^b	Yes
35	28,000	60 ^b	Yes
39	24,000	10 ^b	Yes
32	50,400	20 ^b	Yes

KEY: ^a = Estimated by histological
examination.

^b = Estimated by direct
examination.

almost to normal after 10 minutes (Fig. 2-7-2). There were no detectable systemic effects of the islet infusion.

All transplanted dogs recovered from the operation. The results of daily serum glucose estimations are shown in Fig. 2-7-3. All the transplanted dogs eventually became diabetic, although 4 of the dogs showed a slower rise in serum glucose than the non-transplanted dogs. 1 dog remained normoglycaemic for 6 days before becoming diabetic, this being the dog that was given the most islets (111,000). The overall survival was significantly prolonged when compared with the control dogs (Table 2-7-2).

Daily serum insulin estimations reflected the serum glucose (Fig. 2-7-4), being little different from pancreatectomised, non-transplanted animals, except in the case of the dog that remained normoglycaemic for 6 days after being given 111,000 islets. In this animal the insulin level showed a slow rise, maximal on day 6, that was above the levels seen in non-transplanted dogs (although less than normal fasting levels). After day 6 the serum insulin of this animal fell, at the same time as the blood glucose rose, a finding that may suggest exhaustion of an inadequate number of functioning islets. Post-mortem examination of all animals showed no residual pancreas tissue. Histological examination of samples of tissue taken from multiple sites in the liver failed to show any islet tissue, despite examination of numerous sections.

Discussion

The results of these experiments are disappointing, and it is clear that isolated islet transplantation has failed to prevent the development of diabetes after total pancreatectomy. A transient effect of islet transplantation was seen in 4 dogs, with a slight delay in the onset of diabetes, and, in 1 dog, normoglycaemia was maintained for 6 days. There

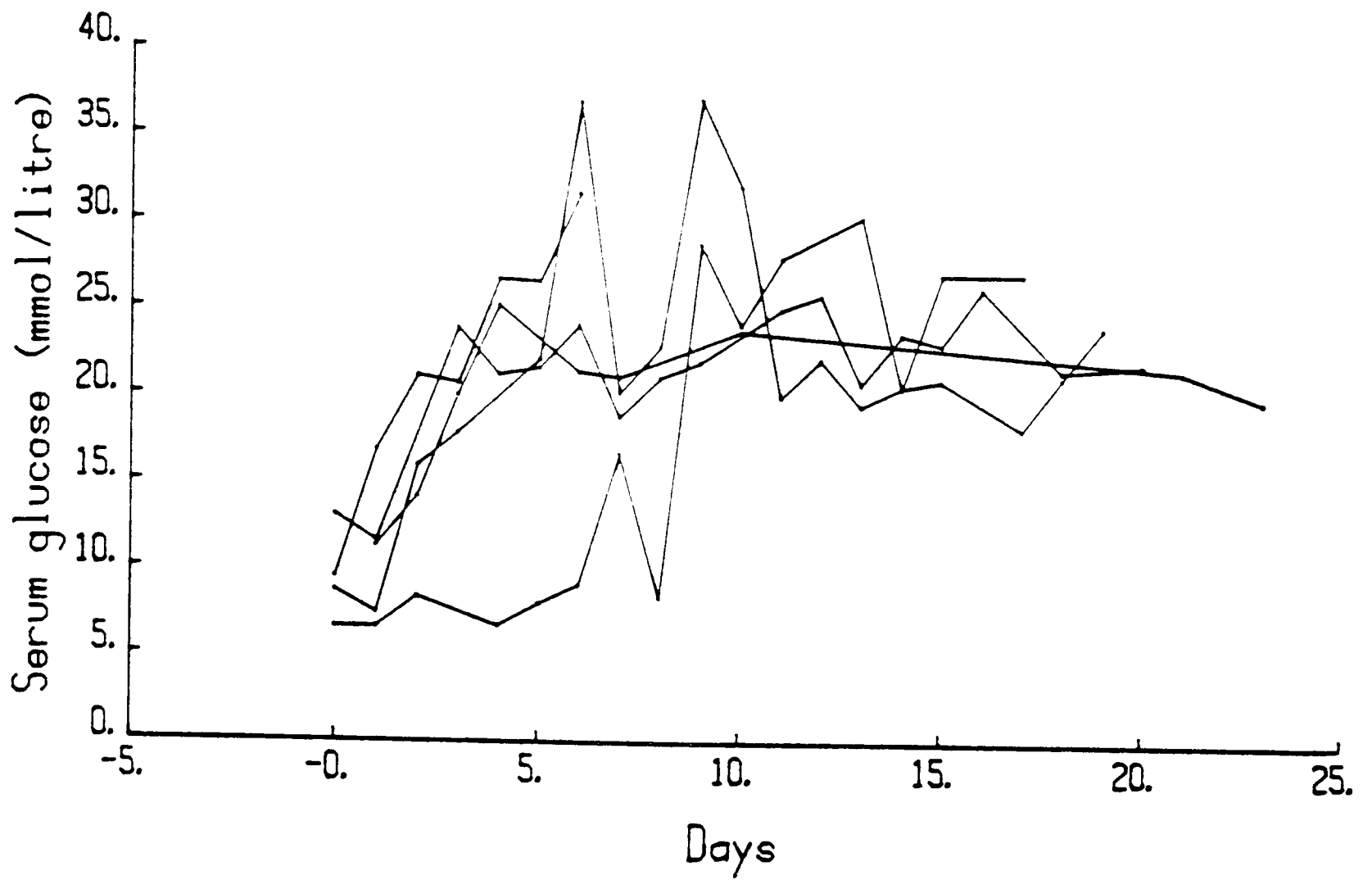


Fig. 2-7-3. Serum glucose of dogs subjected to total pancreatectomy and intraportal islet transplantation on day 0.

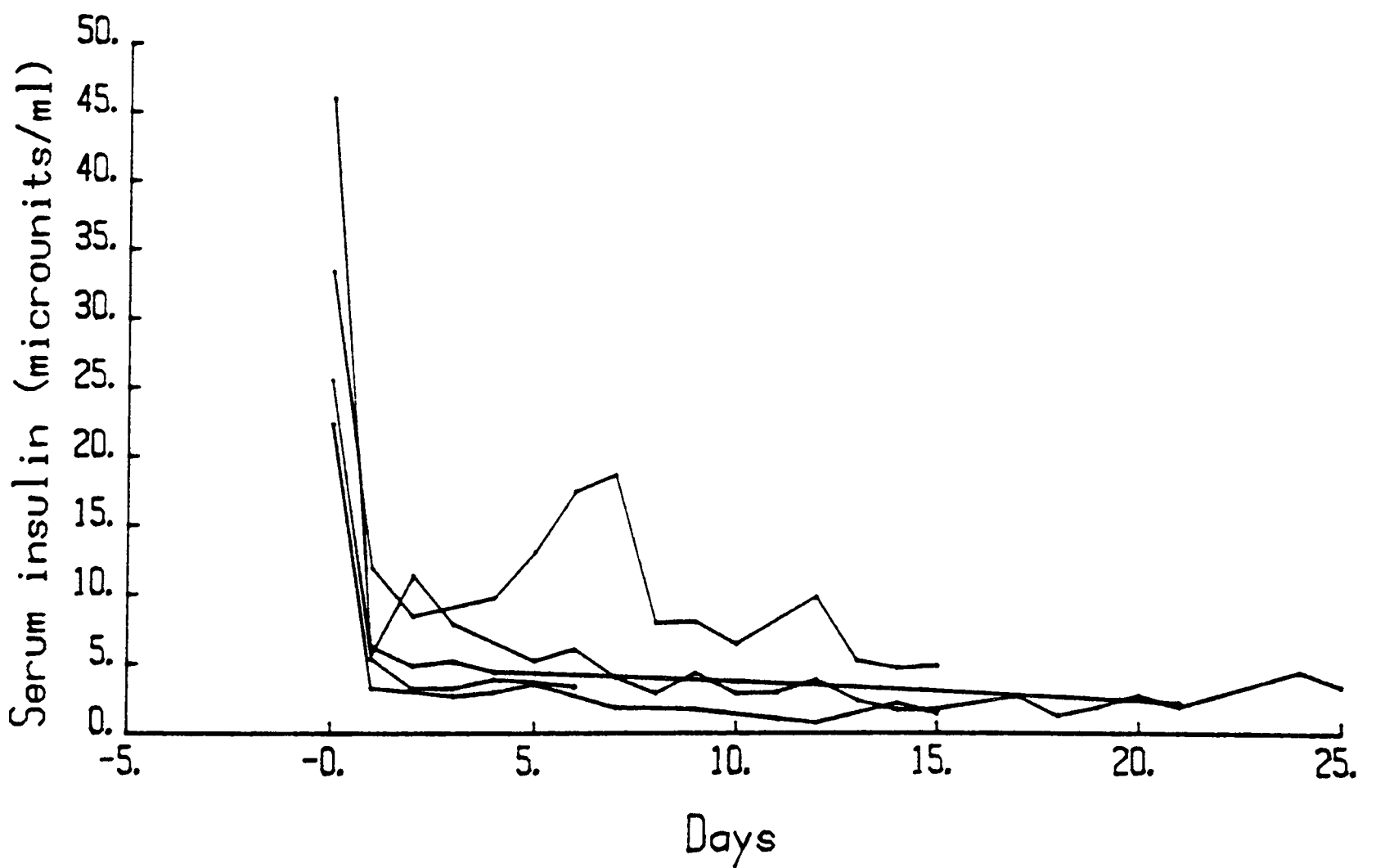


Fig. 2-7-4. Serum insulin levels of dogs subjected to total pancreatectomy and intraportal islet transplantation on day 0.

TABLE 2-7-2. THE SURVIVAL OF DOGS SUBJECTED TO PANCREATECTOMY AND GIVEN
EITHER NO TREATMENT OR INTRAPORTAL ISLET AUTOTRANSPLANTATION

	<u>N</u>	<u>SURVIVAL (DAYS)</u>
Total pancreatectomy, no further treatment	5	5, 5, 6, 7, 10
Total pancreatectomy, intraportal islet autotransplantation	5	6, 17, 19, 20, 23

The two groups are significantly different at $P < 0.05$, using the Mann Whitney U test for non-parametric data.

are several possible reasons for the failure of these experiments. The islet preparation technique did not work very well in 4 of the 5 transplanted dogs, thus the number of islets transplanted in these dogs may have been inadequate. This idea is supported by the fact that the only animal to show evidence of definite graft function received the largest number of islets (111,000), and, in this animal, the daily insulin estimations showed a pattern of increased insulin secretion, followed by exhaustion, which would be compatible with an inadequate number of functioning islets.

Another possibility is that the islets were not viable, although this is unlikely, in view of the excellent structural preservation seen on histological examination, as well as the results of viability staining and insulin secretion in response to glucose stimulation. A possibility, raised by the failure to find any islet tissue in sections of the liver of transplanted dogs, is that the islets failed to implant, either passing through the liver, or simply dying before implantation. Dog islets are quite small, so it is possible they could pass through, and end up in another, less favourable, site. From studies in the rat, it seems unlikely that islets, when trapped in the liver, would fail to implant, though, again, this is possible. However, the failure to find transplanted tissue may simply reflect the large size of the liver, relative to the quantity of tissue transplanted, especially since the islets would presumably be degranulated, and, thus, difficult to detect by histological examination.

Another reason for failure could be the extent of contamination with exocrine tissue, although the success of the Mirkovitch technique (Mirkovitch and Campiche 1977), where the islet tissue is unpurified, argues against this. Other, as yet unknown, factors may also be of importance, but the most likely reason would seem to be that the number of

islets transplanted was inadequate.

PART 2, CHAPTER 8. A METHOD FOR ISOLATION OF
ISLETS OF LANGERHANS FROM THE HUMAN PANCREAS

Materials and methods

10 human pancreases were obtained (with permission) from beating-heart cadaver kidney donors. 4 pancreases were perfused with hypertonic citrate solution via the splenic artery, whilst the others were simply stored, in saline, on ice.

The pancreas was dissected free of surrounding fat and vascular tissue, and the pancreatic duct cannulated with a 3F cannula, passing it to the distal end of the gland, and ligating the cut end of the duct firmly round the cannula. The tissue was weighed, and then distended with HBSS, containing calcium chloride 15 mmol/litre, hepes buffer 10 mmol/litre and collagenase (Sigma type I or type V), 6 mg per ml (Plate 2-8-1). The volume injected was 1 ml per gram of pancreas, and the solution was carefully prewarmed to a temperature of 39° C. Albumen was deliberately excluded from this distending solution, but all HBSS used thereafter contained 4 grams per litre bovine serum albumen. Immediately following injection the pancreas was placed in HBSS (3 ml per gram of pancreas), prewarmed to 39° C. The pancreas was then incubated for around 21 minutes at 39° C., shaking slowly (X 30 per minute) for the first 5 minutes. The exact end point of the digestion process was judged to have been reached by teasing a small area of the gland apart with fine forceps, at which point the pancreatic tissue appeared to dissolve to a fine, smooth, sand-like texture. The exact time for this stage varied from 20 to 23 minutes.

The gland was then rapidly cooled by immersion in cold (4° C.) HBSS, and at the same time distended with cold HBSS, injecting 1 ml per gram of pancreas into the duct. Once cool, the pancreas was cut into pieces of approximately 20 grams, and each piece was processed separately.



Plate 2-8-1



Plate 2-8-2



Plate 2-8-3



Plate 2-8-4

Plate 2-8-1. The tail and body of a human pancreas, following cannulation of the duct and injection of enzyme solution.

Plate 2-8-2. A segment of pancreas immediately after the digestion stage. The surface of the gland has been teased apart with fine forceps, and numerous fine fragments can be seen to have detached, forming a sediment in the surrounding solution.

Plate 2-8-3. Centrifuge tubes (50 ml) containing accumulated tissue fragments, following dispersion of the pancreas by teasing and repeated shaking in cold HBSS. The ductal remnant, with some undispersed pancreatic lobules, is seen in the stainless steel dish.

Plate 2-8-4. The simple apparatus used for passage of the tissue through a filter.

The pancreas was then gently teased apart, using fine forceps, shaking constantly in cold HBSS, with frequent removal and replacement of the fluid in which the pancreas was immersed. This fluid contained numerous fine fragments of pancreatic tissue (Plate 2-8-2). The shaking and teasing process was continued, until no more tissue was found to detach (usually around 10 to 15 minutes). The process was repeated on the next sample of pancreas. The fluid containing the dispersed tissue was centrifuged at 50 G for 10 seconds and the supernatant discarded. The pellet of tissue was re-suspended in HBSS. The fluid from each pancreatic sample was then treated similarly and then all the samples were combined (Plate 2-8-3). The tissue was washed twice more in cold HBSS, then further dispersed by gentle aspiration and expulsion 3 times through a size 14G needle, followed by a further washing and, finally, a single aspiration and expulsion through a size 16G needle.

The tissue in suspension was then passed through a nylon mesh filter, pore size 106 μ (Plate 2-8-4).

Tissue trapped on the filter was carefully washed, the filter inverted, and the trapped tissue washed into a separate beaker. This tissue was then passed through a polyester mesh filter, pore size 500 μ , again with careful washing. The trapped tissue was discarded, the filtered tissue (large islets) retained. The tissue which passed through the 106 μ filter was then allowed to settle for 5 mins and the supernatant was aspirated to leave a volume of 100 ml. The sediment was transferred to two 50 ml centrifuged tubes and centrifuged at 350 G for 5 seconds. The supernatant was discarded by inversion of the tube, and the tissue pellet underlayered on a discontinuous ficoll gradient.

The gradient specific gravities were: 1.08, 1.07, 1.065, 1.06, 1.03, 1.00. The gradient was then centrifuged at 750 x G at 4° C. for 10 minutes. The 1.03/1.00 layer interface was checked under a stereoscopic microscope and usually contained separated islets (Plate 2-8-5). This

layer was removed, washed in HBSS and added to the previously separated large islets.

The tissue obtained was examined under a dissecting microscope. Islets were easily identified by their higher opacity when viewed against a black background with side illumination. That these were indeed islets was confirmed by hand-picking 10 islets with a micropipette, fixing them in 2% gluteraldehyde, then processing the tissue for electron microscopy. Islet counts were performed on 7 pancreases by suspending a tenth of the complete yield of tissue in 10 mls of Hank's, and taking 50 ul samples whilst agitating the suspension. Counts of 5-10 samples were made by direct stereo-microscopy, and the total count estimated from the sample mean. To estimate the purity of the preparation an estimate of the ratio, by volume, of islet to exocrine tissue was made, which was subsequently checked against the histological preparation of the same tissue. The histological estimate of purity was found to be always lower than the estimate of the fresh preparation, probably due to small clumps of exocrine tissue not being apparent as such under the stereo-microscope. The final estimate of purity was, therefore, taken as that determined by histological examination.

Islet viability was estimated by 3 methods: (1) supravital staining (2) insulin secretion in response to glucose challenge (3) survival after transplantation beneath the renal capsule of nude mice.

(1) Supravital staining with FDA/EB was performed as described in Section 2 (Materials and methods).

(2) Insulin secretion in response to glucose stimulation was performed on islets isolated from 3 pancreases. The islets were incubated at glucose concentrations of 0, 10, and 20 mmol/litre, with 5 tubes, each containing 5 islets, incubated at each glucose concentration for every

pancreas.

(3) Nude mice were anaesthetised with intraperitoneal 5% chloral hydrate. 0.1 ml of 50% dextrose was given by intragastric gavage tube before islet transplantation. Sterile isolated human islets were cultured for 1 to 3 days in supplemented RPMI 1640 medium, and approximately 2000 human islets were then implanted beneath the left renal capsule. The mice were allowed to recover, and kept in isolation for 2 weeks. At this time a laparotomy was performed, and the kidney removed for histological examination.

Histological examination of both islet preparations and transplanted islets was performed, using haematoxylin/eosin and aldehyde fuchsin stains, with immunoperoxidase stain for insulin on selected sections.

Results

After the initial digestion and teasing of the pancreatic tissue, the volume of fine digest retrieved after centrifugation was, approximately, a third to half the volume of the original pancreas, assuming the specific gravity of normal pancreas is approximately 1.0 (mean 46%, range 18-83%). The pancreatic remnant consisted of ductal tissue with areas of undigested pancreatic tissue. The undigested pancreatic tissue was always where the injected collagenase had not distended the gland (mostly around the cannulated cut end) (Plate 2-8-6).

Microscopic examination of the digest, after dispersion, showed numerous, easily identifiable, free islets amongst a great excess of exocrine fragments, with most of the exocrine fragments being less than 50 μ diameter (Plate 2-8-7). Following passage through the 106 μ filter, the trapped large islets were washed off the filter, along with some larger, slightly underdigested tissue. Following passage through the 500 μ filter

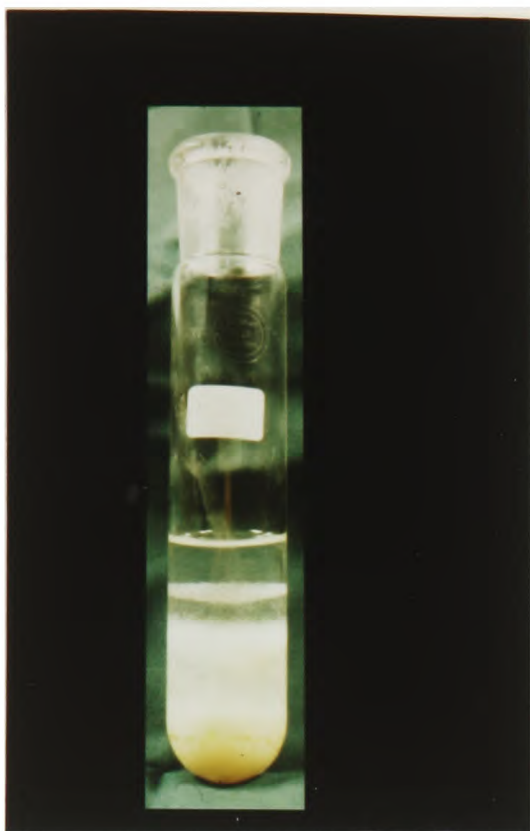


Plate 2-8-5



Plate 2-8-6

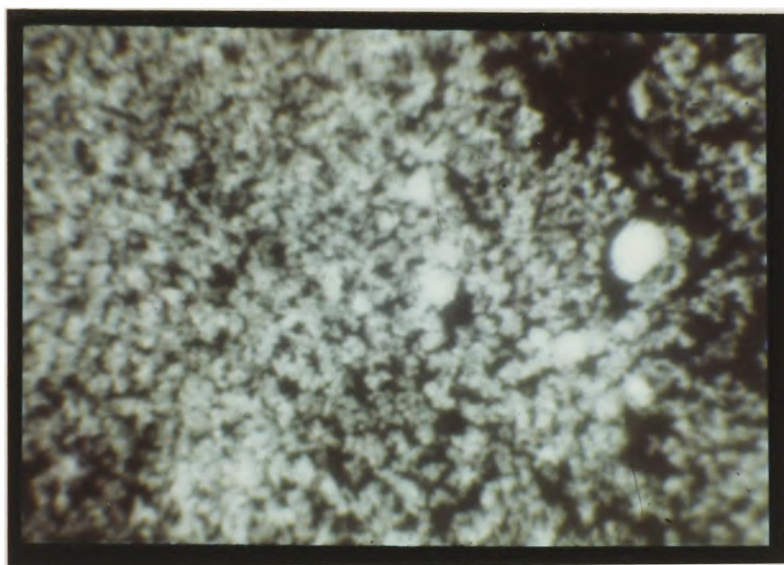


Plate 2-8-7

Plate 2-8-5. Separation of the smaller islets after centrifugation on a discontinuous ficoll density gradient. The uppermost interface contains purified islets.

Plate 2-8-6. Human pancreas sample after digestion and dispersion of the loosened tissue. The remaining tissue is composed of numerous, skeletonised, ducts, with a number of undispersed, intact lobules still attached.

Plate 2-8-7. Dispersed human pancreas (before purification) placed in a petri dish and viewed, against a black background, through a stereomicroscope. Numerous fine fragments of exocrine tissue are seen, with 1 large and 4 medium sized islets, distinguishable by their rounded appearance and increased opacity. Magnification x 10.

almost all the islets passed through, but most of the larger, undigested particles did not. The islets retrieved at this stage appeared to be around 20 - 40% pure, as estimated by the relative proportions of islet tissue to non-islet tissue. The fine tissue that passed through the 106 μ filter was found to contain numerous small islets, as well as much exocrine tissue. Following separation of the tissue on a ficoll gradient, the islets separated off fairly cleanly, with purity varying from an estimated 20 to 60%. The actual total counts of islets (both small and large) retrieved are listed in table 2-8-1. The islet yield can be expressed as the number of islets retrieved from 1 gram of pancreas. The mean number was 1101 islets per gram, S.D. 450, range 752 to 2111.

Electron microscopic examination of the tissue identified as islets confirmed them as such, and showed excellent preservation of the cellular ultrastructure (Plate 2-8-8). Sections stained with haematoxylin/eosin and examined by light microscopy again showed excellent preservation of cellular structure, and most of the islets appeared to be intact (Plates 2-8-9, 2-8-10), though a few showed signs of fragmentation. Aldehyde fuchsin staining and immunoperoxidase labelling of insulin confirmed the presence of numerous intact islets (Plates 2-8-11, 2-8-12, 2-8-13) with the purity of the preparation varying from 10% to 40%. Insulin secretion in response to glucose challenge showed increasing insulin release with rising glucose concentration (Fig. 2-8-1). Supravital staining with fluorescein diacetate and ethidium bromide showed the islets to be glowing uniformly bright green under UV light, indicating good viability (Plate 2-8-14).

Islets from 2 donors were implanted beneath the renal capsule in 15 nude mice. The first 7 mice died, some, possibly, from the effects of anaesthetic, but 4 mice died following convulsions, and a blood glucose estimation on 2 of the 4 mice showed sugars of 1.9 mmol/L and 0.9 mmol/L, suggesting that hypoglycaemia may have caused the deaths. For this

TABLE 2-8-1. THE NUMBER AND PURITY OF ISLETS ISOLATED
FROM 10 SAMPLES OF HUMAN PANCREAS

Weight of pancreas sample (gm)	Volume of digest (ml)	Ratio of digest vol. to original weight (%)	Total islet count	No of islets per gram original weight	Estimated purity (%)
8	2.75	34	NP	NP	20
22	4	18	NP	NP	30
25	10	40	18,800	752	30
22	13	59	18,600	845	10
11	3.5	28	11,350	1031	30
15	8	53	12,000	800	30
70	40	57	59,500	850	20
36	30	83	76,000	2111	40
60	30	50	80,000	1333	40
68	30	44	74,000	1088	30

NP = Not Performed

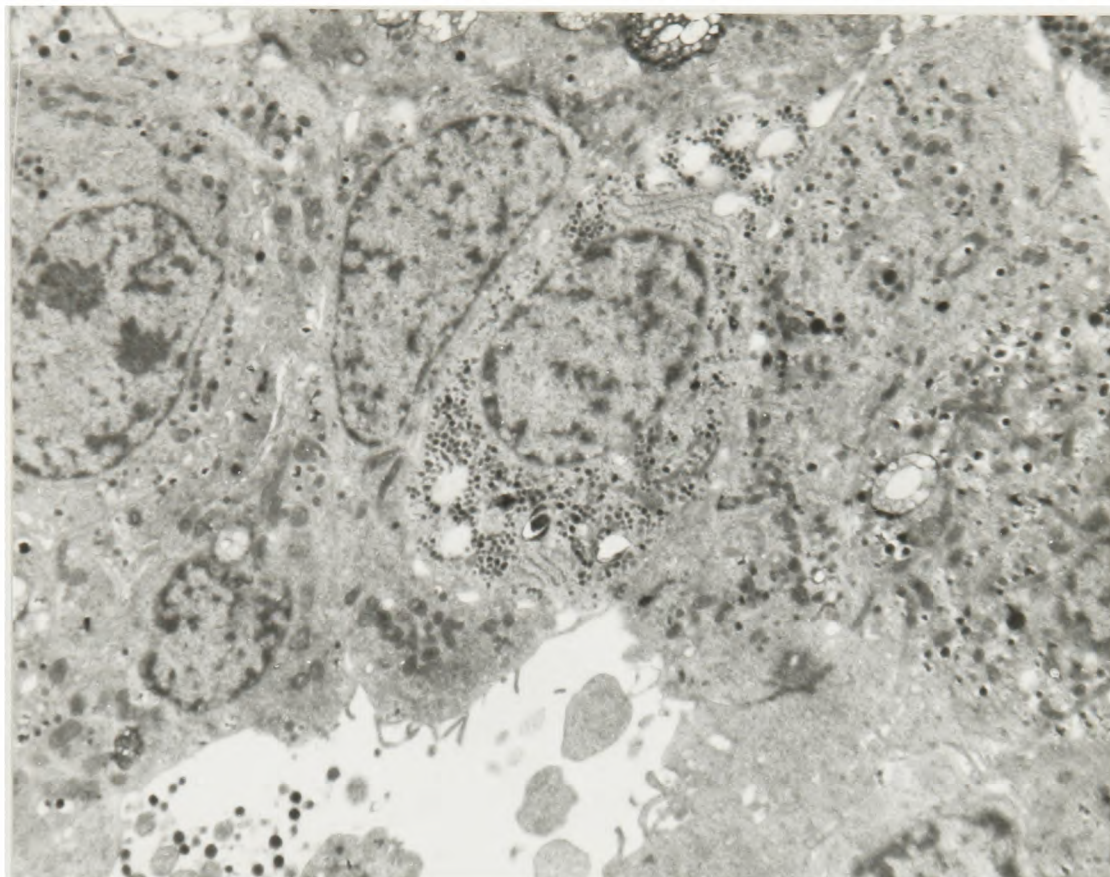


Plate 2-8-8. Electron micrograph of a human islet, hand-picked from tissue prepared by the method described in chapter 2-8. A portion of an islet is shown, with several B cells identifiable by their typical round or occasionally rod shaped granules of moderate electron density, surrounded by a relatively large space between the core and the membrane. Also visible is an A cell with more numerous, fairly large, dense granules. Magnification x 50,000.

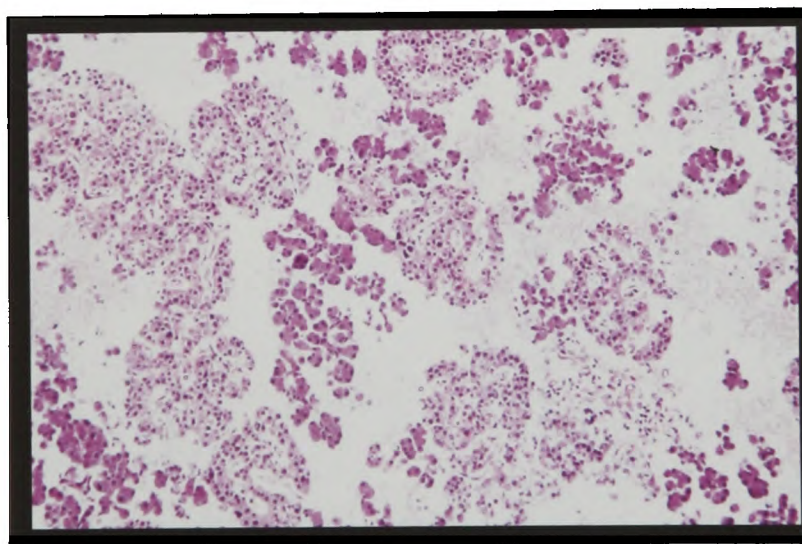


Plate 2-8-9. Photomicrograph of a representative section of tissue, prepared by the method described in chapter 2-8, showing several large islets, cleanly separated from contaminating fragments of exocrine tissue. Most islets maintain a normal structure without evidence of distortion or cell death. Haematoxylin and eosin. Magnification x 40.

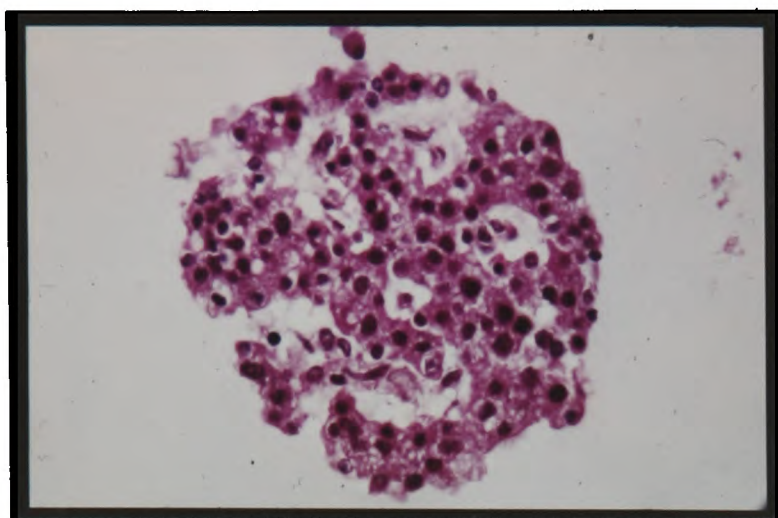


Plate 2-8-10

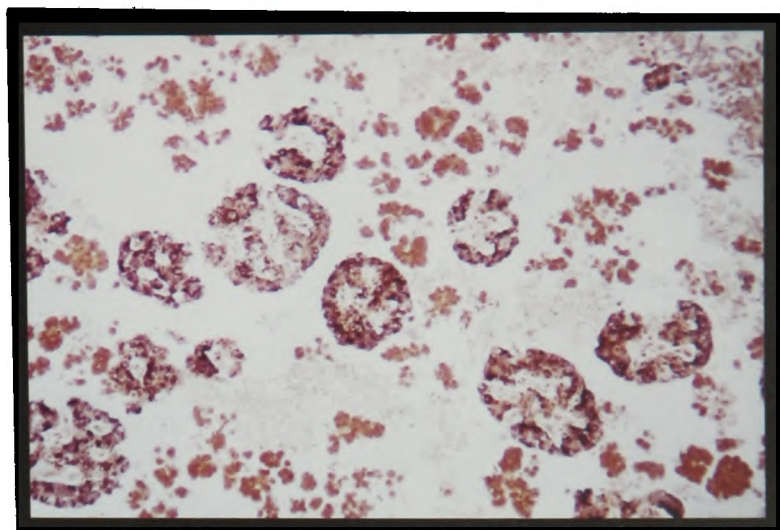


Plate 2-8-11

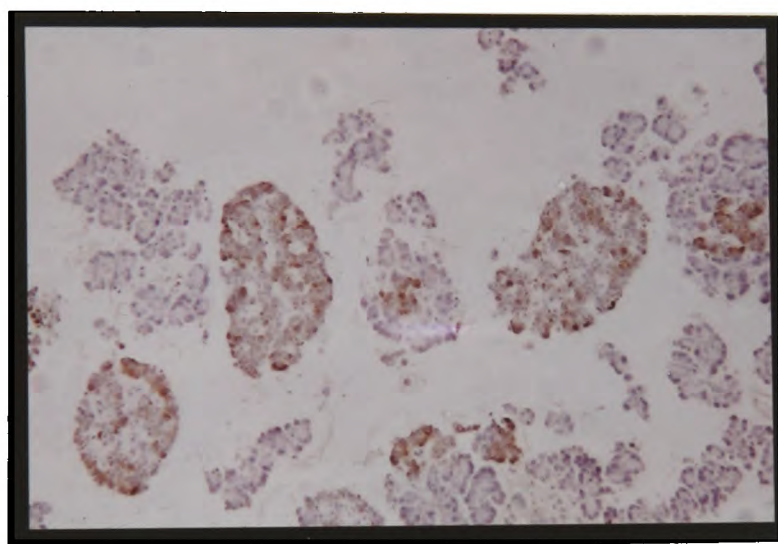


Plate 2-8-12

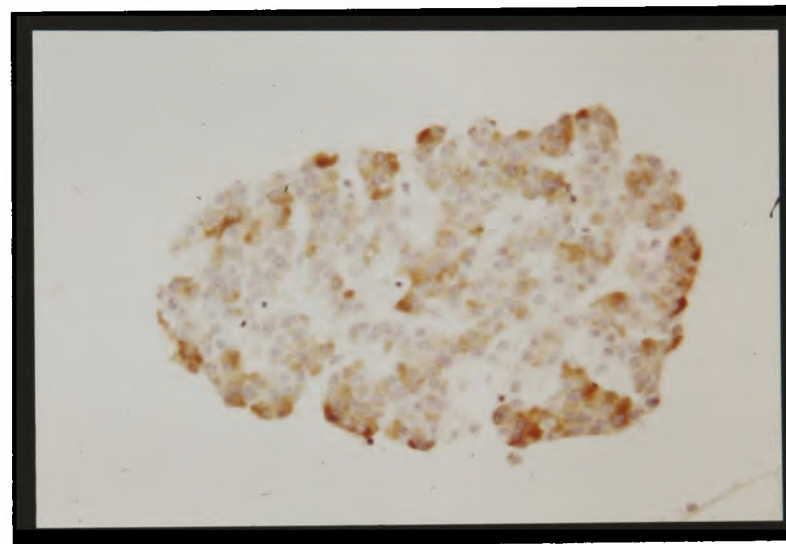


Plate 2-8-13

Plate 2-8-10. Photomicrograph of a representative islet isolated by the technique described in chapter 2-8, showing clean separation from surrounding exocrine tissue, whilst the normal delicate architecture of the islet is preserved. Haematoxylin and eosin. Magnification x 250.

Plate 2-8-11. Photomicrograph of a representative section of tissue isolated by the technique described in chapter 2-8, showing numerous, structurally intact islets, easily identifiable by their characteristic dark blue-staining B cell granules. The purity of the preparation was estimated to be 40%. Aldehyde fuchsin. Magnification x 40.

Plate 2-8-12. Photomicrograph of a representative section of tissue isolated by the method described in chapter 2-8, showing numerous structurally intact islets, with B cells easily identifiable by the brown staining of the peroxidase reaction. Immunoperoxidase stain for insulin. Magnification x 60.

Plate 2-8-13. Photomicrograph of a representative islet isolated by the technique described in chapter 2-8, showing clean separation from surrounding tissue, and B cells easily identifiable by the brown staining of the peroxidase reaction. Immunoperoxidase stain for insulin. Magnification x 200.

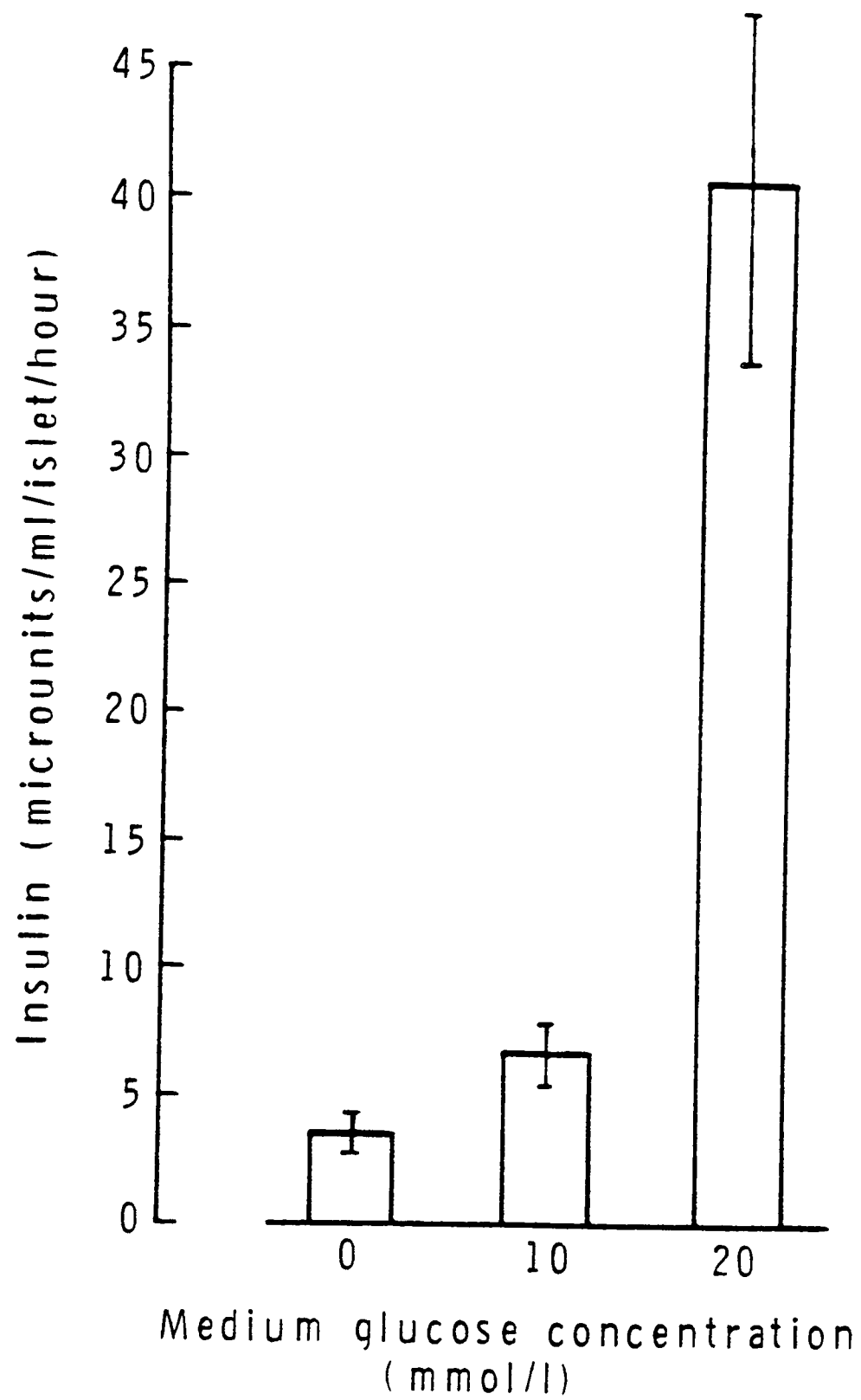


Fig. 2-8-1. Insulin secretion of human islets in response to increasing concentrations of glucose. Values shown are the mean of 15 estimations (5 from each of 3 pancreases) $\pm$ S.E.M.

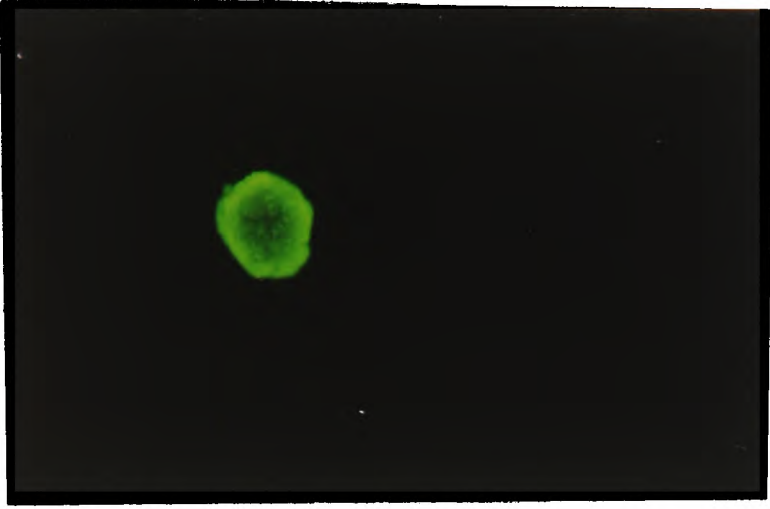


Plate 2-8-14



Plate 2-8-15

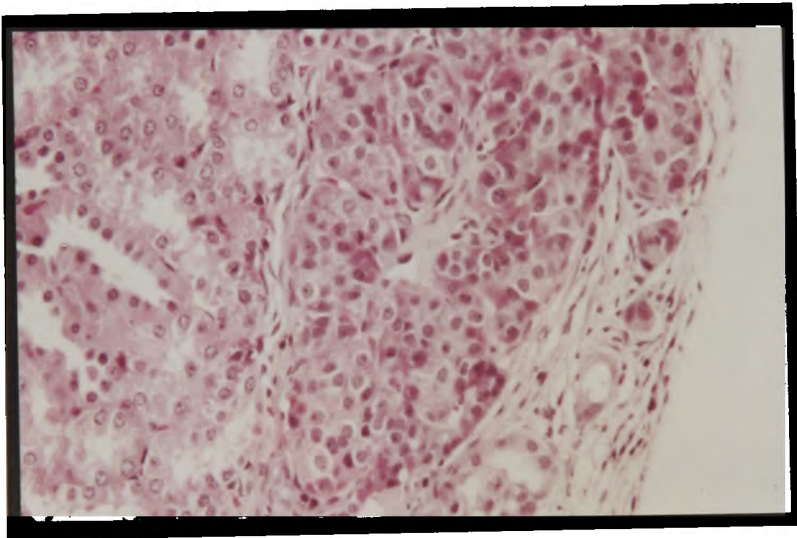


Plate 2-8-16

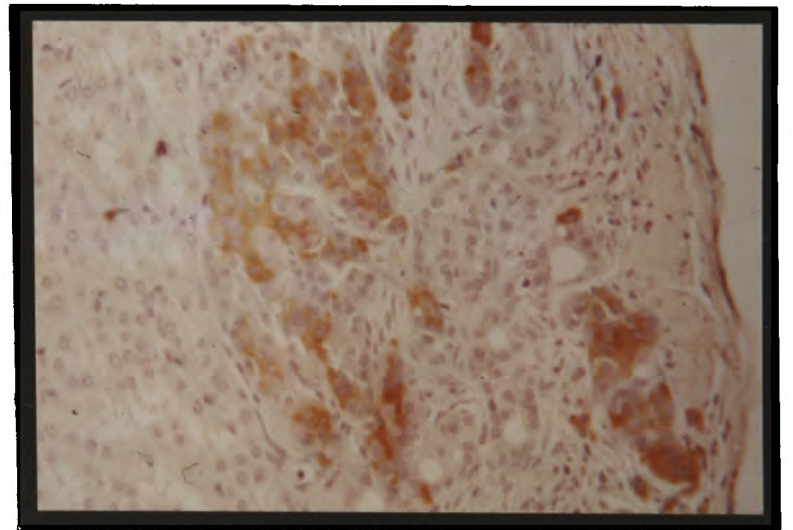


Plate 2-8-17

Plate 2-8-14. Photomicrograph of a freshly isolated human islet, stained with FDA/EB, and viewed, by UV light, through a fluorescence microscope. There is uniform bright green staining, indicating good viability. Magnification x 40.

Plate 2-8-15. Photograph of a nude mouse, given human islets under the left kidney capsule 2 weeks previously. A patch of yellowish tissue is visible beneath the left kidney capsule.

Plate 2-8-16. Photomicrograph of a section of kidney from a nude mouse, given human islets under the kidney capsule 2 weeks previously. The transplanted tissue is easily distinguishable from the kidney parenchyma, and shows vascularised, apparently healthy, endocrine tissue, with good preservation of cellular structure and no evidence of necrosis. Haematoxylin and eosin. Magnification x 150.

Plate 2-8-17. Photomicrograph of a section of kidney from a nude mouse, given human islets under the kidney capsule 2 weeks previously. The transplanted tissue is easily distinguishable from the kidney parenchyma, by the content of B cells, stained brown by the peroxidase reaction. Immunoperoxidase stain for insulin. Magnification x 100.

reason, preloading with intragastric dextrose was introduced, and the survival rate improved, although other mice died later, probably from infection. 4 mice survived for 2 weeks, and, at laparotomy, were found to have a discrete disc of vascularised, yellowish tissue under the renal capsule (Plate 2-8-15). Histological examination confirmed the presence of islet tissue, containing insulin (Plates 2-8-16, 2-8-17).

Discussion

A successful method for isolating human islets should be simple (to minimise tissue loss), quick (especially with regard to warm ischaemia of the whole organ), and should avoid any mechanical trauma (which we have found to be highly detrimental to viability). The first requirement of any method must be to disaggregate the pancreas, so that the stroma holding the islets and exocrine tissue together is removed, but leaving the stroma that maintains islet structure intact. Collagenase preparations currently available seem to be efficient for digesting connective tissue stroma. The pancreatic duct drains the pancreatic acini directly, and so seems an obvious way of delivering collagenase to the exocrine tissue without reaching the islets. The fibrous septa between pancreatic lobules and around larger ducts is much thicker than the fibrous tissue within the gland itself. Collagenase has little effect on this tissue, hence the need to tease the gland apart.

The proof of the method presented here relies on the accurate identification and counting of the number of islets retrieved from each preparation. Previously published methods that have claimed large-scale isolation of islets from the human pancreas (Sutherland et al. 1974, Hinshaw et al. 1981, Matas et al. 1977), have not reported the number of islets isolated, and have failed to provide evidence that the tissue isolated contained structurally intact, viable islets.

The human islets isolated in the experiments reported here appear very similar to rat islets, when viewed by side illumination with a black background. They have a rounded or ovoid shape, and a pearly opaque appearance, easily distinguishable from exocrine tissue. They have a similar size range, between 50 and 400 micron diameter, although some very large islets, up to approximately 600 micron diameter, are seen in the human preparation. To confirm that these islets were being correctly identified, putative islets were hand picked by micropipette and confirmed as islets by electron microscopy in every case. Further confirmation of the accuracy of identification was provided by histological examination of each preparation. The islets were counted by subdilution, sampling and then counting the number of islets per sample, the final figure being obtained by multiplying by the dilution factor. The standard error of the samples was less than 11% for each count. The accuracy of both the count and the identification of islets was checked, on 2 occasions, by a second observer with experience in the identification of both rat and human islets.

The method described above is reliable, producing around 1000 islets per gram of tissue, with apparently good structural integrity and viability. Critical points in the technique appear to be as follows. A relatively high collagenase concentration is used (either Sigma type I or type V appeared to work equally well, although care must be taken to check that each batch is satisfactory in the isolation of islets from rat pancreas). A high calcium concentration seems to speed up the digestion of human pancreas (collagenase is a calcium-dependant enzyme, and, perhaps, there is a calcium binding agent in human pancreatic tissue), and it is important that the collagenase is injected, and reaches, all the tissue: undistended areas do not digest properly. Particularly critical is the prewarming of the solutions used during the digestion process.

The last critical point is the moment chosen to stop the digestion. As stated, this is usually around 22 minutes, but does seem to vary, especially if care is not taken on the above points. There seems to be some inherent variability, however, and judging the right moment is difficult, but develops with practice. If the digestion is stopped too early the islets do not separate from the exocrine tissue satisfactorily. They are not lost, but are trapped on the 106u filter with exocrine tissue still surrounding the islet. If the digestion is allowed to proceed too far the islets themselves tend to fragment.

The number of islets retrieved by this technique seems to be an advance on previous techniques, but what percentage of the total pancreatic islet tissue is being harvested? Using crude islet numbers is a simple way of expressing the islet yield, but the reported number of islets in the pancreas varies enormously, depending on the method used to count them. Furthermore the size of each islet also varies a great deal. There are numerous small islets (less than 100u diameter), and far fewer large islets, in the human pancreas, but the larger islets make up the greater portion of the total islet tissue volume. The number of large islets may be more important in estimating the yield than simple islet numbers. The last islet yield reported in Table 2-8-1 consisted of tissue from almost a whole pancreas (68 grams), and yielded an estimated 74,000 islets. This is, at best, a small proportion of the estimated number of islets in the human pancreas, taking even the most conservative estimates of around 250,000 islets. The total volume of tissue retrieved was 1.5 ml however, and the estimated purity was around 30%. Thus perhaps 0.5 ml of this preparation was actually islet tissue. Since the estimated total volume of islet tissue is 1-2% of the gland, this may represent a higher yield than the numbers suggest, possibly due to more efficient retrieval of large islets in preference to small islets.

It may be possible to improve this method further, perhaps by ensuring more efficient distention of all lobules of the pancreas, since, as mentioned, a few lobules usually fail to distend, and these do not then digest properly. It may also be possible to improve the islet purity, for example, by culturing the islets. Whether this is necessary for clinical transplantation remains to be proven.

CONCLUDING REMARKS

There is no doubt that a way of preventing, or reversing the long-term complications of diabetes is urgently needed, and there is moderately strong evidence that strict control of glucose homeostasis would achieve that aim. The idea of transplanting insulin-secreting tissue to cure diabetes is not new, indeed, Laguesse suggested the transplantation of isolated islets in his remarkable paper (Laguesse 1893), in which the islets were first recognised as a source of an internal secretion, and were given their eponym. The development of this idea has provided a challenge that a surprisingly large number of investigators have taken up, and advances have been made, albeit in small steps.

There are a variety of ways in which insulin-secreting tissue can be transplanted. Of these, the method requiring the introduction of the least new methodology has been to transplant part, or all, of the pancreas as a vascularised organ. Despite sporadic claims to the contrary, the overall success rate for vascularised pancreatic grafts has proven disappointing. The major problems have always been related to the exocrine part of the gland, and it would seem that the transplantation of intact vascularised pancreatic tissue, 98% of which is both unwanted and troublesome, may be conceptually unsound. Furthermore, the procedure will always require a major operation, and is unlikely to be attractive to otherwise fit, young diabetics before the development of their complications.

The alternative methods for transplantation of insulin-secreting tissue all involve transplantation of tissue as a free graft. Of these methods, the most practical source of tissue would seem to be adult human pancreas, already available from kidney transplant donors. Experiments in rodents have shown the feasibility of separating islets from exocrine tissue, and diabetic animals have been transplanted successfully, but these experiments have also shown the vulnerability of islet tissue to the

rejection process. The application of isolated islet transplantation to large animals and man has been hampered by the lack of a method for isolation of pure islets in sufficient number, no satisfactory method having emerged, despite extensive investigation. Avoidance of the problems of islet isolation by transplantation of unseparated dispersed pancreas has met with some success, notably in dogs, but this method has proven ineffective and dangerous when used clinically, and it seems likely that a fairly pure preparation of isolated islets will be required, before clinical islet transplantation will be successful.

The overall conclusion of a study of the literature was that the most attractive source of tissue for transplantation of insulin-secreting cells was isolated adult islets of Langerhans, and that the major problems preventing clinical application were, firstly, how to prevent the rejection of isolated islets, and, secondly, the lack of a method for isolation of islets from the human pancreas. The studies reported here address both of these problems.

The rat is a good experimental animal in which to investigate the rejection process, since genetically defined inbred strains make interpretation of results more meaningful. The islet transplant model used in the experiments reported here (Part 1) has several advantages, particularly the ease with which the transplanted islets can be removed, with return of diabetes providing irrefutable proof of transplant function, and the transplanted tissue being easily located for histological studies.

The experiments described in Part 1 confirm the findings of Reece-Smith et al. (1982) that an animal made unresponsive to a renal allograft by cyclosporine treatment will subsequently accept islets of the same strain without further immunosuppression. This confirmation is of some importance, in view of the previous opposite findings of Nash et al. (1978), Reckard et al. (1979) and Perloff et al. (1981). The reason

for the different result is uncertain, but it should be noted that these previous studies were based on very few transplants (often just 2 rats), and also that the long-surviving allografts were produced by passive enhancement. The experiments presented here also confirmed that the effect is strain specific, using a different third party strain to that reported by Reece-Smith et al. (1982).

It was possible that the beneficial effect of a long-surviving renal allograft was due to the transplantation of islets under the transplant kidney capsule, rather than generalised unresponsiveness. This possibility was excluded by the demonstration that intraportal islets fared as well as islets placed under the transplant kidney capsule. It was important to guard against the effect being no more than an idiosyncrasy, since transplantation between certain strain combinations sometimes produces unusual results. The experiment was, therefore, repeated, using a different strain combination (chosen without knowledge of the likely outcome), and the result was the same.

Cyclosporine is a potent immunosuppressive agent in the rat, and it was possible that rats bearing long-surviving renal allografts after cyclosporine treatment developed a unique unresponsive state. This possibility was investigated by performing the same procedure in animals bearing long-surviving renal allografts induced by donor-specific blood transfusion. Again, animals bearing long-surviving renal allografts, induced by donor-specific blood transfusion, accepted subsequent islet allografts, whilst donor-specific blood transfusion alone was ineffective in preventing rejection of islets. These findings suggest that the phenomenon is not confined to the use of cyclosporine, and supports the view, held by some, that there is a common mechanism for the maintenance of allograft tolerance (at least in rats), whatever the initial agent used to induce graft acceptance.

Other, more general, implications can be drawn from the experiments described in Part 1. Since strain specificity was demonstrated, it is clear that rejection of isolated islets transplanted to the kidney capsule must be a mainly immunological phenomenon, rather than non-specific destruction. The survival of islets in animals bearing long-surviving renal allografts makes the presence of islet-specific antigens (separate from those on the kidney, and perhaps responsible for the rapid rejection of islets) rather unlikely. An intriguing possibility, investigated by a further series of experiments using auxiliary renal allografts, was that the rejection of islets might be due to their inability to maintain a state of unresponsiveness. If this were the case, removal of the renal allograft (from animals initially made unresponsive by cyclosporine treatment then given islets of the same specificity) might be expected to lead to rejection of the islets. However, the results showed that removal of the renal allograft did not affect the islets, suggesting that islets can maintain an unresponsive state on their own (although final proof of this, by a further transplant, was not obtained).

In summary, the experiments described in Part 1 have demonstrated that rats bearing a long-surviving renal allograft develop a specific unresponsive state that allows subsequent islet transplantation without rejection. This effect has potential clinical application, since the commonest indication for pancreas transplantation (at present) is diabetic renal failure, the patient requiring both a pancreas and kidney transplant. Furthermore, renal transplantation alone can be performed, even in diabetics, with low morbidity, and a fairly high percentage of grafts are accepted, using currently available immunosuppressive regimens. However, it must be remembered that the rat immune system has significant differences from that of the human, and it is uncertain if an analogous unresponsive state develops after clinical renal transplantation. Patients that have accepted their graft can often be reduced to quite low

levels of immunosuppression, but it is rarely possible to withdraw treatment altogether. Clearly, it would be desirable to confirm the effect in an animal model with an immune system closer to that of man. Other negative points to remember are that clinical application would require the development of cryopreservation techniques for human islets, and, finally, the technique is applicable only to patients needing a simultaneous renal transplant, so would not be suitable for treatment of early diabetes. Nevertheless, as a first step, it may provide a way of avoiding rejection of islets in patients with diabetic renal failure, which would be worthwhile, and might encourage further developments.

The problem of isolation of islets of Langerhans from the large mammalian pancreas, and, ultimately, the human pancreas, has been investigated in Part 2 of this thesis. At an early stage it was realised that there was a need for a simple method, both for identification of islets and also assessment of tissue viability. The use of neutral red, as described by Bensley (1911) was investigated, simplified, and its accuracy proven for the rat, dog and pig pancreas. The technique proved particularly valuable for the dog pancreas, where islets can be very difficult to distinguish from exocrine tissue, especially in impure preparations. An incidental finding, of potential value, was that neutral red stained islets remain viable, which might allow the future use of neutral red stain to further purify islets, by a sorting mechanism.

The fluorescent stains fluorescein diacetate and ethidium bromide (FDA/EB) have been introduced for the assessment of viability of a variety of cells over the last 15 years, but the use of FDA/EB for whole isolated islets has not been reported. The studies reported here showed that FDA/EB stain was simple, rapid and accurate. Increasing reliance was placed on this stain, whilst investigating various islet isolation techniques, and this allowed the rapid abandonment of several methods, even

before the results of confirmatory histological examination were available.

The use of the above 2 staining techniques, with later histological examination of tissue, allowed a large number of methods and modifications to be examined, with the conclusion that many of the previously published methods produced either non-viable tissue, or tissue that contained no islets. Mechanical methods, producing high energy shear stresses, were found to be particularly detrimental to tissue viability, whilst minor modifications of the collagenase method, as used for the rat pancreas, failed to disperse the tissue adequately.

The method described by Noel et al. (1982) provided something of a breakthrough, by demonstrating the dramatic effect of a relatively high concentration of collagenase injected into the exocrine duct, although the rest of the technique was not found to be of value. By concentrating on simplification of this technique, a reasonably effective method for isolation of dog islets was developed. Further modifications led to a method adapted to the human pancreas.

It is interesting to reflect that the key points of the method developed for the human pancreas are, in fact, quite logical, and, with hindsight, it seems remarkable that they have not been investigated (and identified) before. The exocrine duct provides direct access exclusively to the exocrine tissue, and injection of collagenase into the exocrine duct seems a logical way of obtaining selective digestion, whilst preserving the islets. Collagenase is an enzyme, and so it is likely that an initially high concentration of collagenase would be more effective than a continuously recycled low concentration (as used by other investigators), especially as continual perfusion might tend to spread the collagenase into the islet tissue, where it is unwanted. Collagenase is a calcium dependant enzyme, therefore an adequate concentration of calcium ions must be assured. The optimal temperature for collagenase activity is 37° C.,

so the need to prewarm all the solutions, to enable the enzyme to work rapidly, now seems quite obvious.

Some of the other findings of these studies on islet isolation were perhaps fortuitous, even with hindsight. The digestion process caused the exocrine tissue to disperse into fragments significantly smaller than most human islets, and this allowed purification of the larger islets by simply passing the tissue through a filter. Furthermore, the relatively uniform particle size of the tissue that passed through the filter greatly aided the separation of the smaller islets, using density difference, on a pre-formed ficoll density gradient.

The method developed for isolation of islets from the dog pancreas was moderately successful, producing up to 4000 islets per gram of pancreas, with a maximum purity of 80%. Unfortunately the method suffered from great variability, a problem that was never adequately solved. However, the structural integrity and viability of the tissue seemed excellent, as assessed by in vitro tests, and it was, therefore, very disappointing that the transplantation of islets into the portal vein failed to prevent diabetes in pancreatectomised dogs. The reasons for this failure remain uncertain, but it is possible that the number or purity of islets was inadequate, or that the intraportal site of transplantation may not have been the best choice.

The method developed for isolation of islets from the human pancreas seems, fortunately, much more reliable than the method used on the dog pancreas. The number of islets produced was around 1000 islets per gram of pancreas, with a purity of up to 40%. Furthermore, the islets isolated were much larger than those of the dog (the preparation being selectively enriched for the larger islets), and the yield probably represents a greater proportion of the islet cell mass than the numbers would suggest. The viability of the tissue has been demonstrated by in vitro tests and,

perhaps most convincingly, by transplantation into nude mice.

As must often be the case, the studies described here have not finally solved the problem investigated. Nevertheless, significant advances have been made, allowing speculation on the direction of future research. It is clear that priority should be given to the development of a model of islet transplantation in a pancreatectomised large mammal, initially as an autograft to avoid the problems of rejection. The work described here suggests that both the commonly used animals, the dog and the pig, are unsuitable for this purpose, since the structure of the pancreas of these species is significantly different from that of man, and, in the case of the dog, the islets are totally different in size and shape.

A possible alternative experimental animal would be the non-human primate, although the use of monkeys is fraught with problems and justifiable moral dilemmas. The structure of the monkey pancreas is very similar to that of the human, and preliminary studies have shown that islets of similar size and number can be obtained from the monkey pancreas, using the method developed for the human pancreas. If a model for islet autotransplantation could be developed in the pancreatectomised monkey, the effect of islet allotransplantation in animals made unresponsive to renal allografts could then be investigated. The results of such an investigation would probably have direct implications for clinical outcome, which might justify the use of monkeys in these experiments.

Previous studies of cryopreservation techniques for isolated islets have usually been confined to animal experiments, often using rat islets, since a technique for isolation of human islets in sufficient number has not been available. The technique for human islet isolation described here opens the way for studies of cryopreservation techniques using human islets. Furthermore, the development of cryopreservation techniques is a prerequisite if the phenomenon of renal allograft-induced tolerance,

described in Part 1 of this thesis, is ever to be applied clinically.

A potentially useful experimental model, worthy of further study, is the technique of transplantation of human islets to athymic nude mice, described in Part 2, Chapter 8. This could be developed further to allow both functional and histological studies of human islets after transplantation, which would be particularly useful for testing the result of procedures such as crypreservation.

In conclusion, the present studies have not solved the problem of clinical islet transplantation, but they have suggested ways in which the major obstacles may be overcome. Transplantation of isolated islets is a logical treatment for diabetes mellitus. There are now indications that, one day, clinical islet transplantation will be a reality.

SUMMARY

Two major problems preventing the clinical application of pancreatic islet transplantation were investigated. The problem of allograft rejection was studied in the rat. DA rats, made diabetic by injection of streptozotocin then given LEW islet transplants, rejected the islets promptly, whether the islets were placed under the renal capsule or injected into the portal vein.

DA rats were given orthotopic LEW renal allografts and then treated with cyclosporine 10 mg/kg for 14 days. The majority of rats accepted the renal allograft, and survived a further 60 days, at which time they were made diabetic by injection of streptozotocin. LEW islets were then transplanted under the renal capsule or injected into the portal vein, whilst 2 further groups were either left diabetic, or were given BN islets under the renal capsule. The animals given no further treatment remained diabetic, whilst animals given LEW islets, under the kidney capsule or into the portal vein reversed their diabetes and remained normoglycaemic for 100 days, in a large proportion of cases. Animals bearing LEW kidneys and given (third party) BN islets rejected the islets promptly. The results showed that once a recipient rat had accepted a renal allograft under the influence of cyclosporine treatment, it would then permanently accept an islet allograft of the same strain as the kidney. The effect was not influenced by the site of islet implantation and was specific for islets of the same strain as the renal allograft.

The possibility that this effect was a strain-specific idiosyncrasy was investigated by repeating the procedure in another strain combination. Diabetic PVG rats were shown to rapidly reject LEW islets placed under the kidney capsule. PVG rats were given orthotopic renal allografts, treated with cyclosporine, then long-survivors were made diabetic and given LEW islets under the renal capsule. All animals reversed their diabetes permanently, thus demonstrating the effect in another strain combination.

The importance of cyclosporine treatment in inducing unresponsiveness was investigated by transplanting islets into rats, made unresponsive to renal allografts by another method, namely donor-specific blood transfusion. LEW rats were given DA blood transfusions twice weekly for 6 weeks. (DA x LEW)F1 islets transplanted under the kidney capsule (after induction of diabetes) were shown to be rejected in an accelerated fashion in such animals, whilst renal allografts were accepted in a high proportion of cases. LEW rats bearing long-surviving (DA x LEW)F1 renal allografts, induced by donor-specific blood transfusion, were made diabetic, then given (DA x LEW) islets under the renal capsule. All animals reversed their diabetes permanently, demonstrating that the effect of renal allograft-induced unresponsiveness to islet transplantation is not confined to rats given cyclosporine treatment.

The possibility that islets lacked some factor responsible for maintaining a state of unresponsiveness was investigated. DA rats were given auxiliary renal allografts (without nephrectomy) and treated with cyclosporine as before. Animals that accepted their grafts were made diabetic 60 days later, then given LEW islets under the left renal capsule. All animals reversed their diabetes and remained normoglycaemic for a further 100 days. The original renal allograft was then removed, leaving the islet allograft in situ, under the recipient left renal capsule. The animals remained unaffected, normoglycaemia being maintained for a further 60 days. The findings were interpreted as showing that islets are able to maintain a state of unresponsiveness, although final proof of the point, by transplantation of a further LEW allograft, was not obtained.

In summary, these experiments on islet transplantation in the rat show that the phenomenon of renal allograft-induced unresponsiveness extends to islet transplantation. The effect is powerful, and allows transplantation of allogeneic islets without rejection, a goal rarely achieved by other

means. Furthermore, the effect has potential for clinical application.

The second major problem preventing clinical application of islet transplantation, the problem of separation of adequate numbers of viable islets from the pancreas, was studied in the rat, dog, pig and human. It was realised at an early stage that there was a need for a simple, rapid method for identifying islets and assessing their viability.

A previously described technique, employing supravital staining with neutral red, was investigated, simplified, and shown to be both reliable and accurate when applied to the rat, dog and pig pancreas. Islets stained by this technique appeared bright red and were easily distinguished from the unstained exocrine tissue. Confirmation of the selectivity of staining was obtained by use of light and electron microscopy. Stained islets were found to remain viable, as judged by insulin secretion and transplantation in rats.

Another supravital stain, fluorescein diacetate and ethidium bromide (FDA/EB), was found to be useful for assessing the viability of isolated islets. Although this stain had previously been used for assessment of the viability of single cells, there were no reports of the use of FDA/EB to determine the viability of isolated islets. Discrimination of living from dead islets was shown to be efficient, as demonstrated by a blind sorting experiment, using freshly isolated islets and islets killed by both heat and alcohol. In this experiment, viability, as determined by insulin secretion in response to glucose stimulation, correlated well with viability as determined by FDA/EB staining. Furthermore, it was found to be possible to discriminate degrees of viability, and a simple scoring system was described for this purpose, which was also shown to correlate with another index of islet viability (ATP content).

A variety of previously described islet isolation techniques were

investigated in the rat, dog, pig and human, with the aid of the above supravital stains. From this study a new technique for the separation of islets from the dog pancreas was developed. The technique used injection of collagenase into the exocrine duct, followed by a short incubation at 37° C. The gland was then dispersed without the need for mechanical chopping. Islets were separated by first passing the tissue through a 150 u filter, to remove the undigested larger tissue, then centrifugation on a preformed ficoll gradient. It was found to be possible to retrieve up to 160,000 islets from one pancreas, with a maximum purity of 80%, but the method suffered from an unpredictable variability, the cause of which was not determined.

Transplantation of islets isolated by the above technique was investigated in dogs. Dogs were subjected to total pancreatectomy and then either left untreated or given islets, isolated from the excised pancreas and injected into the portal vein. Untreated, pancreatectomised dogs became immediately diabetic and died within 10 days (mean survival 6.6 days). Dogs given intraportal islets also became diabetic, although 1 dog remained normoglycaemic for 6 days (having received the largest number of islets), and the mean survival was significantly prolonged (17 days). The reasons for this disappointing result were unclear, although the likely explanation was that the number of islets transplanted was insufficient.

The above technique was progressively adapted, using samples of fresh human pancreas obtained from cadaver renal transplant donors, until a method for isolation of islets of Langerhans from the human pancreas was developed. The method used a high concentration of collagenase, injected into the exocrine duct under pressure, followed by a short incubation at 39° C. Critical points in the technique included careful control of the temperature and calcium ion concentration during the incubation. The gland was then dispersed without the need for mechanical chopping. Islets

were separated by a 2 stage process of nylon mesh filtration and centrifugation on a preformed ficoll density gradient.

10 human pancreases were processed by the above method. The number of islets isolated was calculated by subdilution followed by counting of multiple samples, with verification of the accuracy of the counting method and islet identification by both light and electron microscopy. The average number of islets isolated was 1068 islets per gram of pancreas (S.D. 475, range 752-2111) and up to 80,000 islets were prepared from one pancreas. The purity of the preparation, as assessed by histological examination, varied from 10% to 40%. Histological examination also showed excellent preservation of islet structure. The islets were shown to be viable in 3 ways, firstly by using supravital staining with FDA/EB, secondly, by demonstrating an appropriate response to glucose in vitro, and, thirdly, by transplantation of isolated islets beneath the kidney capsule of nude mice. Histological examination of these subcapsular human islets 2 weeks after transplantation showed vascularised, healthy islet tissue, containing granulated B cells demonstrated by immunoperoxidase staining for insulin. The method was shown to be both simple and reliable.

STATEMENT OF ORIGINALITY

The studies in this thesis were carried out in the laboratories of the Nuffield Department of Surgery during the period September 1982 to June 1984, when the author was engaged in full time research, supported by the Medical Research Council of the UK. All the work described in this thesis was performed by the author apart from the specific contributions listed below:

Isolation and transplantation of islets from the rat was performed with the assistance of Mr Philip McShane, who also performed all of the radioimmunoassays for insulin.

Approximately half the rat renal allografts were performed by Mrs Joanne Gale, Mrs Jan Evins and Mr Barry Fairbrother, the rest being performed by the author.

Sectioning, processing and staining of specimens for light microscopy was performed by Mrs Elena Jeffrey, with immunoperoxidase stains by Dr Anne Clark. Processing of tissue for electron microscopy was performed by the Electron Microscopy Unit, John Radcliffe Hospital, under the guidance of Dr Peter Millard, who also interpreted the electron micrographs.

Estimation of the insulin secretion of human islets in response to glucose stimulation was performed by Dr Andrew Grant, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital.

PUBLICATIONS ARISING FROM THE WORK DESCRIBED IN THIS THESIS

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Harrison DE, Christie MR, Gray DWR, Ashcroft SJH.

Metabolic and biochemical studies on human islets of Langerhans. Diabetologia. In press. (Abstract).

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