THE DEVELOPMENT OF A VASCULARLY IMPLANTED PROSTHETIC ENDOCRINE PANCREAS

by

MARY ELIZABETH BROADBENT

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SUMMARY

3cm and 1.5 cm implantable prosthetic units were constructed from biocompatible fabrication materials and consisted of a polyethylene shell containing a centrally positioned acrylic copolymer hollow fibre (M.W. cut-off 100K Daltons), with a 10 cm polyethylene cannula attached at either end. Hollow fibre/cannula junctions were potted into the polyethylene shell via a disc of PVC tubing and U/V-cured dental adhesive.

The secretory capacity of prototype units was studied *in vitro* using methylene blue, ^{125}I-insulin and islets from lean and obese mice and optimal physical criteria, i.e. flow rate and perfusion pressure were established. Prototype units provided a constant rate of methylene blue release over a period of 7.5 hours at a flow rate of 0.637 ml/min. and perfusion pressure of approximately 100 mmHg. The rate of ^{125}I-insulin release from units was proportional to the concentration of labelled insulin originally inserted into the unit but independent of perfusate albumin (BSA V) concentration. Protein coating of hollow fibres by a glutaraldehyde cross-linking technique to reduce thrombogenicity, increased the lag time of prototype units and the uniformity of the protein coating could not be guaranteed.

Prototype units containing lean mouse islets released insulin at a physiological flow rate of 0.637 ml/min. and a perfusion pressure of 100 - 200 mmHg in response to an increase in perfusate glucose concentration from 5.56 mmol/l to 16.67 mmol/l. The concentration of insulin released was 1.22 ± 0.10 ng/ml and 1.68 ± 0.12 ng/ml respectively.

Prototype units containing lean mouse islets implanted into the right carotid artery of streptozotocin-diabetic rats released sufficient insulin (3.09 ± 0.40 ng/ml) to reduce hyperglycaemia and maintain the rate of normal weight gain. Implanted units remained functional for up to 12 - 15 days without change in structural integrity or trauma to adjacent tissues or organs.

Key words: implantable prosthetic endocrine pancreas
hollow fibre
biocompatible polymeric materials
insulin release
streptozotocin-induced diabetes

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For my Parents, with love ..... and in loving memory of my Grandparents: Marianne and James Bond McKenzie and Margaret and William Broadbent.

For those special times.

"If you can trust yourself when all men doubt you,
But make allowance for their doubting too;....

If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: "Hold on!"....

If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!"

Rudyard Kipling

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CHAPTER 1

INTRODUCTION

1.1. Diabetes Mellitus

Diabetes mellitus is a genetically and clinically heterogeneous group of disorders that share glucose intolerance in common (1).

Good control of blood glucose in patients with diabetes is of paramount importance in preventing the occurrence and development of secondary complications of the disease. Diabetes has been known to man for more than 3,000 years, the earliest written reference to it being found in the "Ebers papyrus" written about 1550 B.C. (2).

Since the discovery of insulin by Banting and Best (3), the life expectancy of diabetics has continued to improve, but despite intensive efforts to achieve better glycaemic control, life expectancy in diabetics is reduced by a quarter compared with non-diabetics, due to secondary complications such as neuropathy, nephropathy, retinopathy and cardiovascular disease. These secondary complications are responsible for the severe morbidity and mortality associated with diabetes mellitus today and newer and more effective approaches to treatment are required to maintain a precise regulation of carbohydrate metabolism and prevent the progression of the not inevitable secondary systemic lesions.

1.2. Classification of Diabetes Mellitus

The present classification of diabetes mellitus recommended by the National Diabetes Data Group (NDDG) and the British Diabetic Association (BDA) is based upon new information regarding genetic and immunological mechanisms in diabetes mellitus, in addition to blood glucose criteria (1).

The present classification is outlined below.

A. Primary Diabetes Mellitus

This class comprises those persons with a fasting blood glucose value $\geq$ 7.8 mmol/l (140 mg/dl) and a blood glucose value, two hours after a 75 g oral glucose tolerance test (OGTT) of $\geq$11.1 mmol/l (200 mg/dl). This class is subdivided into two groups. The Type I insulin-dependent type (IDDM) (perhaps subdivided into those with and those without autoimmune features), includes the previously used juvenile-onset category and may involve both endogenous and exogenous causes, perhaps connected with infection and/or autoimmune features such as histocompatibility antigens (HLA-DR types) and islet cell antibodies (ICA). The Type 2 non-insulin-dependent type (NIDDM), (perhaps subdivided into obese and non-obese, insulin-treated and non-insulin-treated subclasses), includes the maturity-onset type diabetes of earlier classifications.

B. Gestational Diabetes

Gestational diabetes is first recognised in pregnancy and remits afterwards. There is increased obstetric risk and foetal outcome is improved with good diabetic control. The presence of impaired glucose tolerance (IGT) in pregnancy, indicates a high risk of subsequent diabetes in later life.

C. Impaired Glucose Tolerance (IGT)

IGT is diagnosed in patients with a fasting blood glucose concentration of <7.8 mmol/l and a blood glucose concentration of between 7.8 - 11.1 mmol/l, two hours after a 75g OGTT. Whilst this patient does not generally develop visual and renal complications of the disease, there is an increased susceptibility to atherosclerotic disease, hypertension and hyperlipidaemia (1,4).

D. Previous Abnormality or Latent Diabetes Mellitus

This class applies to individuals currently with normal glucose tolerance but with previously demonstrated episodes of diabetes

mellitus or IGT occurring either spontaneously or in response to a stimulus, or as a result of a provocative test, e.g. corticosteroid-augmented glucose tolerance test, or pregnancy.

E. Potential Abnormality

This group incorporates individuals currently with normal glucose tolerance, but with increased risk for the later development of diabetes for one of a number of specified reasons (including genetic predisposition.)

F. Diabetes Mellitus or Carbohydrate Intolerance Associated with Certain Conditions and Syndromes

This group includes diabetes caused by hormonal therapy, certain drugs, pancreatic disease, genetic and chromosomal syndromes, insulin receptor abnormalities and viruses such as encephalomyocarditis (EMC) (5) and coxsachie B4.

G. Prediabetes

The term can only be applied retrospectively, and is reserved for that period of the life of a diabetic before glucose intolerance was diagnosed.

1.3. Conventional Methods of Diabetic Control and Associated Problems

Diabetic control, the maintenance of normoglycaemia within fairly narrow, well-defined limits, may be effected by dietary control, the use of oral hypoglycaemic agents and/or insulin therapy, depending on the severity and duration of the disease.

A. Dietary Control

Careful dietary management is essential for all diabetics. Whilst a strict dietary régime may serve as an adjunct to other forms of treatment in Type I diabetes, restricted carbohydrate intake alone may suffice to restore normoglycaemia in type 2 diabetes, particularly when it develops in middle age or later and is associated with obesity.

The importance of dietary fibre in the dietary treatment of diabetes should not be overlooked (6). Reduced caloric intake in subjects on a high-fibre diet, leads to weight loss (7). High-fibre diets appear to exert their effect by increasing satiety, decreasing food intake and enhancing faecal energy loss (8). The long-chain polysaccharides Guar gum and pectin form viscous gels in the intestinal tract resulting in a reduction of the gastric emptying rate (9) and a thickening of the unstirred layer adjacent to the intestinal villi. The bulking action of Guar helps to reduce energy intake by diminishing appetite. The use of Guar-containing biscuits in the diabetic diet has been found to be effective in reducing the postprandial rise in the blood glucose level and in improving glycaemic control (10,11).

Type 2 diabetes results from an excessive insulin demand rather than the cessation of insulin production and fasting or a low calorie diet reduces the metabolic demand for insulin and increases tissue sensitivity to circulating insulin levels. The concentration of endogenous insulin becomes sufficient to prevent lipolysis, hepatic gluconeogenesis and ketosis and blood glucose levels and body weight fall.

B. Oral Hypoglycaemic Agents

The use of oral hypoglycaemic agents in conjunction with dietary control to reduce blood sugar, allows a better use of endogenous insulin. Sulphonylureas are only effective where there is residual beta cell function. The use of oral antidiabetic agents began as early as 1918, when Watanabe (12) described the hypoglycaemia produced by guanidine hydrochloride in rabbits. The first treatment of a group of diabetic patients with Synthalin (decamethylenediguanidine) was described by Joslin in 1928 (13), although the hepatotoxic effects of the drug (14) resulted in its subsequent withdrawal from use.

Sulphonylureas, (derivatives of sulphonamides), were first introduced commercially for the treatment of mild diabetes in 1956. 1969 saw the arrival of the now widely used sulphonylurea, glibenclamide, for the routine treatment of Type 2 diabetes. It has a more potent hypoglycaemic action than that of the more conventional antidiabetic drugs, and is quicker acting, although chlorpropamide and tolbutamide are still widely used today.

Sulphonylureas stimulate the release of insulin from islets of Langerhans. There is also evidence that sulphonylureas influence insulin receptor binding and postreceptor pathways in insulin-sensitive target tissues. Although generally well-tolerated, there is a risk of hypoglycaemia associated with the use of sulphonylureas and an increase in patient weight.

Biguanides (Metformin) reduce blood glucose by delaying the intestinal absorption of carbohydrates and by increasing the uptake of glucose from the blood by liver, muscle and adipose tissue. They have no effect on insulin production, but like the sulphonylureas, they improve insulin sensitivity in target tissues. Metformin is the most commonly prescribed biguanide but in some people it has rather unpleasant side effects, causing lacticacidosis, nausea, queasiness in the stomach and sometimes diarrhoea. Its use is, however, nearly always associated with a decrease in body weight. Metformin is therefore prescribed for patients with mild Type 2 diabetes, associated with obesity. In certain cases, combined metformin and sulphonylurea therapy may be prescribed. Together they exert an additive hypoglycaemic action. However, the combined use of the two drugs has been reported to result in abnormalities in the blood concentrations of intermediary metabolites (15).

C. Insulin Injections

For the Type 1 insulin-dependent diabetic, a variety of insulin preparations is available, the major differences between them being the duration of action, pH, concentration, purity and source.

Most diabetics on insulin are treated with a mixture of short- and long-acting insulins twice daily (10 - 50 IU per day). Mixtures of insulins are needed because long-acting insulin alone cannot account for the sudden postprandial fluctuations in blood glucose after meals. Twice daily insulin injection affords better diabetic control than a single dose and is particularly necessary in children and young adults who find it difficult to adjust to the limitations of the diabetic way of life.

In 1956, Berson and Yalow (16) demonstrated the presence of insulin antibodies in the serum of insulin-treated diabetics. The early lente type insulins were not very "clean", i.e. they were contaminated by proinsulin, glucagon, pancreatic polypeptide and gastrointestinal hormones and patients treated with this type of insulin generated serum antibodies to these contaminants. Porcine insulin is less antigenic than bovine insulin and is still widely used today.

In recent years, the introduction of highly purified monocomponent insulin by Novo and recombinant DNA insulin by Eli Lilley has greatly reduced the levels of insulin antibodies, in treated diabetics (17). Monocomponent insulin is highly purified crystalline insulin, free from proinsulin, pancreatic polypeptide (PP), vasoactive intestinal polypeptide (VIP) and glucagon. Monocomponent insulins are more expensive to produce than conventional insulins, since their production involves purification by gel filtration and disc electrophoresis. About 10 - 15% of the total insulin is lost during purification.

All the methods of diabetic control discussed so far are, at best, a necessary inconvenience and, at worst, ineffective in either

preventing or arresting the development of the secondary sequelae of diabetes, since none is able to mimic the normal physiological minute-to-minute insulin and associated hormonal requirements necessary to prevent large scale fluctuations in blood glucose and effectively regulate glucose homeostasis.

Various alternatives to insulin injection for the insulin-requiring diabetic have been sought in recent years. Whilst some research groups have concentrated their efforts on developing techniques for the transplantation of either whole or part/segmental pancreas or even of isolated islets, others have endeavoured to develop either implantable or extracorporeal electro-mechanical devices for the delivery of insulin, the more advanced forms of which incorporate feedback control systems.

A third alternative, currently under investigation, combines the elements of both of the previous two, in that functional, isolated islets of Langerhans are transplanted from a donor to a recipient, the islets being contained within a polymeric device which presents a mechanical barrier to host antibodies, preventing rejection of the transplanted tissue.

The three alternatives are discussed more fully in the following sections.

1.4 The Electromechanical Artificial Pancreas

In recent years, the availability of various devices for the measurement of capillary blood glucose levels by diabetic patients themselves (i.e. glucose strips and -stix and glucometers) and the concept of continuous home/self monitoring, has placed emphasis on the day-to-day treatment of diabetes by self-correction (18,19). Self-monitoring has been found especially useful in elucidating problems associated with diabetic control, preventing hypoglycaemia and managing diabetic pregnancy (19). Short-term open-loop insulin

therapy has been shown to improve metabolic control and secondary complications (20).

Research into the development of sophisticated and complicated devices designed to improve insulin delivery into the diabetic patient has evolved almost exponentially over the last two decades and there are many excellent reviews on the subject (20-24).

In the early 1960's it was shown that normoglycaemia could be achieved in diabetics by linking the automatic colorimetric determination of glucose concentrations to a device that would automatically inject the required amount of either glucose or insulin. The two main problems with this instrument were its bulk and the time required for measurement of the glucose concentration (15 minutes or more). It did indicate though, that continuous monitoring of glucose concentration was possible (21).

Over the last 20 years, substantial progress has been made in the development of insulin delivery systems. A sharp division has developed amongst investigators (21) between those who argue that technological limitations will prevent great progress in the development of an artificial pancreas and therefore have developed bulky bedside machines (25-30) and those who contend that overcoming these limitations will result in the realisation of a fully implantable artificial pancreas and have developed miniaturised infusion systems (31,32).

Two categories of mechanical devices have been developed: (a) open-loop systems in which insulin is delivered in a pre-programmed manner independently of the prevailing blood glucose concentration and (b) closed-loop systems in which algorithm-controlled insulin, dextrose, or glucagon infusions are given in response to a monitored blood glucose concentration. Open-loop systems also have a manual facility

to deliver a bolus of insulin prior to meals. The open-loop systems such as the subcutaneous insulin infusion devices (33) comprise a special micropump, insulin reservoir, a power source and a mini-computer which controls the delivery of a pre-established insulin requirement subcutaneously at a constant low level. When a higher insulin concentration is required, i.e. before a meal, the pump can be made to deliver a single bolus or several additional boli of insulin. The basal insulin infusion rate is determined individually for each patient.

The major problems associated with the use of open-loop systems arise from the fact that insulin is usually delivered subcutaneously and different patients have different insulin sensitivities in their subcutaneous tissue. In some brittle diabetics, subcutaneous administration of insulin is totally unacceptable and intravenous (i.v.), intramuscular (i.m.) and intraperitoneal (i.p.) routes have to be used (20,33), each route having its own advantages and disadvantages. I.V. administration allows the rapid restoration of near normal glucose levels (34) even in brittle diabetics (35) but potential disadvantages include thromboembolic complications, septicaemia and ambulatory limitation. I.P. administration allows the rapid absorption of insulin and avoids peripheral hyperinsulinaemia. Continuous i.p. insulin infusion with an implanted remote-controlled insulin infusion device has been used to achieve and maintain good diabetic control in a Type 2 diabetic for more than 100 days. Insulin aggregation in this (Siemens) unit was prevented by using a special surface-active polymer (36).

Other limitations associated with insulin infusion devices are the small reservoir of insulin (usually seven days), fixed basal infusion rate and in some cheaper systems, the lack of a malfunction alarm.

The closed-loop insulin delivery system (25-31) e.g. the Biostator (25-28) comprises three basic components: a glucose sensor (oxygen electrode), an insulin (or glucagon) delivery pump and a computer controller that regulates the supply of insulin or glucagon in response to the prevailing hyperglycaemia using preprogrammed algorithms. An algorithm is defined as a rule of procedure needed to solve a repetitious mathematical problem (22). Algorithms provide the pattern by which a closed-loop insulin infusion system can mimic the complex process of insulin secretion by the normal beta cell (29,30). They are based solely on glucose measurements. Albisser and colleagues were the first to make a major contribution to the development of an artificial endocrine pancreas (25-28) by using an automatic system (the Biostator with algorithms), which provided insulin according to projected blood glucose levels, thus mimicking the biphasic insulin response of the normal pancreas (25-27). The ability of the artificial endocrine pancreas (AEP) to restore and maintain glucose homeostasis in human subjects given either their usual diet, a 50g OGTT or a moderate level of physical exercise has been comprehensively demonstrated (28).

Peripheral and portal routes of insulin administration using the AEP (28,37) have been compared in dogs. During computer-controlled insulin administration, normal glucose tolerance was restored using both portal and peripheral routes of insulin delivery. There were also no significant differences in glycaemic patterns, insulin infusion patterns, peripheral IRI levels and total insulin requirements between the two routes (37). Thus the peripheral route, which is more readily accessible than the portal route, has been established as the appropriate infusion site for an implantable prosthesis for controlling blood glucose concentration. A peculiarity of this route of administration is that the level of insulin delivered in this way is not subject to modulation by the liver.

The closed-loop system has a variety of clinical uses, particularly in acute emergencies such as diabetic coma, the stabilisation of diabetics during and after surgery, diabetic pregnancy and labour (38) and during kidney dialysis.

Currently under development are small, implantable, continuous insulin infusion devices and implantable glucose sensors that may in the future lead to miniaturised, implantable, glucose-controlled insulin administration systems. A totally implantable, closed-loop artificial pancreas, although some way off at present, offers a more viable long-term proposition for the maintenance of normoglycaemia in patients where it can be seen to offer advantages over intensive conventional therapy. Soeldner has developed a prototype closed-loop system, the size of a cigarette pack (23). Weak points in his system include a glucose-sensing electrode, an unreliable pumping system and the biodegradation of fabrication materials. Soeldner's prototype uses a platinum-plated electrode based on the platinum-catalysed oxidation of glucose (23). In the presence of glucose a chemical reaction occurs on the surface of the electrode by which electrons are liberated. The higher the glucose concentration, the more electrons are released. The computer then measures the electric current generated and determines the blood glucose concentrations. Components in the blood such as urea and amino acids interfere with the chemical reaction and unwanted by-products of the reaction build up on the surface of the electrode, reducing its sensitivity. Besides this fuel cell type of sensor, work is also being carried out by various groups of investigators on potentiometric and membrane-covered polarographic sensors (22,39).

A new sensor being developed at Cranfield Institute of Technology by Professors Higgins and Hill, directly measures the transfer of

electrons as glucose oxidase reacts with blood glucose. It is accurate over a wide range and has the potential for miniaturisation and implantation (40).

Relatively few pumps have been developed for insulin delivery in potentially implantable, closed-loop glucose-sensing systems. Blackshear and colleagues (41) developed such a pump for the long-term delivery of heparin into dogs. The device was based on a pump valving system that could be controlled transcutaneously after implantation. It was a bellows pump powered by pressure created from the evaporation of a liquid fluorocarbon. Various drawbacks included its large size and the need for refilling via percutaneous injection. The vapour pressure within the fluorocarbon chamber and thus the delivery rating of the pump was sensitive to changes in temperature. In current models moment-to-moment alterations in infusion rates are not possible making feedback-controlled or preprogrammed insulin infusion impossible. When attempts were made to adapt this pumping system for insulin delivery the catheter clogged with insulin after about three months' use (23,42).

The total implantation of an artificial beta cell into a diabetic dog has been described by Bessman and colleagues (31). Normoglycaemia was maintained for four days until failure of the power supply.

Maintaining the patency of the vascular access site without heparin, the use of low infusion rates and the prevention of the inactivation of insulin or glucagon in highly concentrated solutions at body temperature are just some of the problems which remain to be overcome.

At present, open-loop systems are in more widespread use than closed-loop systems. They are less expensive to develop and operate than closed-loop systems although they still require an exogenous supply of insulin. However, when the effectiveness of closed-loop i.v. insulin infusion, open-loop continuous subcutaneous insulin infusion and inten-

sified conventional insulin therapy were compared in a study involving the control of blood sugar in a group of insulin-dependent diabetics, it was found that all three methods gave good and comparable results (43).

1.5. Transplantation of the Pancreas and Islets of Langerhans

Transplantation of the pancreas and islets of Langerhans has been the subject of a number of excellent reviews (21,44-49).

The rationale for pancreas and islet transplantation is to try to mimic the normal endocrine functions of the pancreas by providing endogenous insulin and thus achieve better diabetic control than is currently available by conventional therapy.

The transplantation of insulin-secreting tissue encompasses a wide spectrum of procedures ranging from multiple organ transplantation (50) (e.g. pancreas and kidney), through whole (51) or part pancreas (52-54) transplantation to the implantation of isolated islets themselves (55-59). A brief consideration of each of these areas is given below in an attempt to indicate the main advantages and disadvantages of each procedure.

1.5.1. Pancreas and Islet Transplantation in Experimental Animals.

A. Pancreas Transplantation

Multiple organ transplants have included the pancreas in association with liver, heart and/or kidney. Using the pig as an animal model, Calne and colleagues (50) found heart and kidney allografts were rejected more aggressively than liver, whilst rejection of pancreatic allografts appeared to be slower and milder than that of kidney or heart. The data showed that liver allografts in the pig exhibited an immunosuppressive effect against themselves and other donor tissue. In fact, transplantation of the liver and pancreas together appeared to increase the immune reaction in the liver.

Whole pancreas transplantation by means of an immediately vascularised pancreatic graft remains technically difficult due to the complexity of blood vessels associated with this organ. Provision for the drainage of exocrine enzymes and prevention of anastomotic leaks and vascular thrombosis are important technical considerations in pancreas transplantation, but the limiting factor is allograft rejection, which may be obviated by the use of immunosuppressants.

When exocrine drainage is established, the pancreas may be grafted either in association with the duodenum or, more successfully and simply, as either a whole or segmental graft without the duodenum and with pancreatic duct-ureter anastomosis (51). The latter technique avoids the problems of necrosis and ulceration of the duodenum.

In the absence of provision being made for exocrine drainage, pancreatic grafts may be performed either with (52,54,60-62) or without (53) duct-ligation or duct-obliteration (63-66). Pancreatic duct-ligation is the simplest approach and involves the atrophy of the exocrine tissue leaving the endocrine portions intact. In practice, this approach suffers from several problems including pancreatitis, vascular thrombosis and both acute and chronic inflammatory reactions which may impair islet functions. Direct irradiation of the pancreas (63) and steroid administration (52) have both been employed to reduce exocrine function after duct-ligation.

Duct-ligated pancreas transplantation in isogeneically-transplanted streptozotocin-diabetic mice has been shown to restore normoglycaemia (60). Allograft recipients, however, did not recover from hyperglycaemia indicating that islets in duct-ligated pancreas cannot be successfully transplanted across a major histocompatibility barrier, unless prior irradiation and allogeneic bone marrow therapy has been carried out. The transplantation site and the amount of tissue trans-

planted are important factors to consider in duct-ligated pancreas transplantation. Duct-ligated pancreas transplants excised from streptozotocin-diabetic mice which had received isogeneic transplants subcutaneously and recovered from hyperglycaemia were found to contain both immunoreactive insulin and glucagon (61) indicating that remaining A and B cells were both capable of hormone synthesis and storage. Destruction of autotransplants of duct-ligated pancreatic segments in the pig has been shown to occur within two weeks, due to inflammation following ligation of the duct (52). Steroids were shown to have a protective effect on these grafts, preserving both their morphology and function. The addition of glucagon to the steroid treatment inhibited exocrine function and peripancreatic fluid accumulation (52). Another method of duct ligation is to inject quick-setting liquid synthetic polymer into the pancreatic duct, forming a solid cast which totally obliterates the ductal system (64-67). This process has been shown to totally eliminate exocrine secretion. Neoprene (64), acrylate glue (65), polyisoprene (66) and prolamine (67) have been used in this technique with some degree of success. Polymer injections have been shown to cause severe pancreatic fibrosis, the long-term effect of which remains to be determined.

When transplantation has been carried out without duct-ligation, the peritoneal cavity has been shown capable of absorbing pancreatic secretion without harmful effects in both dogs (53,54) and rats (68). Serum amylase concentrations increased immediately after transplantation, but returned to normal within a week as the duct gradually closed. Less inflammation and fibrosis tended to occur in these grafts than those in which immediate duct-ligation was performed. A study using dogs has shown that unligated pancreatic duct transplants via the iliac fossa do as well as transplants that receive technically satisfactory pancreatic duct anastomosis (54).

Pancreatic transplantation, if successful, has been shown to restore plasma glucose levels to normal if more than 20% of the pancreatic mass is transferred (69). Canine recipients of successful autografts have been reported to survive for months or even years with normal blood glucose and graft function (54).

B. Islet Transplantation

The number of adult islets, generally obtained by collagenase digestion of isolated pancreases of mature animals, required to completely reverse experimental diabetes depends on the integrity of the islets and the severity of the pre-existing diabetic states. Barker (70) has suggested that the amount of insulin-secreting tissue could be increased by the use of benign islet cell tumours or cultures and perhaps replicating B cells. The latent period between transplantation and the amelioration of blood glucose can be shortened and glucose tolerance improved by increasing the number or weight of islets transplanted.

Generally, greater than 1,000 islets will restore normoglycaemia in one to two days. Islets from young adult animal donors have been shown to function better than islets from older donors (71). When adequate numbers of isologous islets were transplanted, the metabolic abnormalities of diabetic rats could be promptly reversed. Islets transplanted by injection into the hepatic portal vein, lodged within the hepatic portal venules immediately afterwards but were eventually found in interstitial tissue in direct apposition to hepatocytes (48a). Neovascularisation occurred rapidly and both insulin- and glucagon-containing cells lodged in the liver could be demonstrated by immunofluorescent techniques. In a study involving the intraportal transplantation of islets into totally pancreatectomised monkeys (45), granulated B cells were subsequently found in liver parenchyma but there was an insufficient number of islets to restore normoglycaemia.

Isolated adult rodent islets have been transplanted into recipients with variable results. Intrasplenic transplantation of syngeneic and allogeneic isolated pancreatic islets from adult rats has been successfully carried out (55) and found to be almost as efficient as the intraportal injection of islets in rats and mice. Normoglycaemia was restored within a few days and maintained by the administration of a short course of antilymphocyte serum, but subsequent splenectomy resulted in a prompt return to the diabetic state. Andersson and colleagues failed to cure hyperglycaemia in obese hyperglycaemic mice (ob/ob) by intrasplenic transplantation of 500 isogeneic islets isolated from lean mice (72). Such islets did, however, restore normoglycaemia in alloxan-diabetic lean mice (72).

The liver and lung have been shown to be more favourable transplantation sites for islets in pancreatectomised and streptozotocin-diabetic rats than either subcutaneous tissue, muscle, epididymal fat tissue or the peritoneal cavity as evidenced by the longer survival time of the transplanted islets (73). This would indicate that both immunological factors and the quality of the blood supply play a role in the survival of transplanted islet tissue. The success of transplantation depends mainly on histocompatibility. Islets transplanted into inbred rats showed significantly longer survival times than when transplanted into non-inbred rats (73).

Fish are a potentially useful source of islet tissue for xenotransplantation into mammals. Xenotransplantation of piscine islets into hyperglycaemic rats has been shown to lower recipient blood glucose levels (74). The effect was prolonged both by host irradiation and enclosing the donor tissue. The transplantation of dispersed adult pancreatic tissue, either prepared from donors previously depleted of exocrine enzymes or transplanted to sites tolerating the introduction of pancreatic enzymes has been attempted, without

specific islet isolation. Kramp and colleagues (60,62) were the first to show a long term amelioration of experimental diabetes by this method. Later, Kretschmer and colleagues showed that totally pancreatectomised dogs became normoglycaemic following autotransplantation into the spleen of pancreatic fragments dispersed by collagenase digestion for 15 - 25 minutes (75). Elimination of the purification steps allowed sufficient islet tissue to be obtained from one donor for an effective transplant.

Neonatal pancreases have low exocrine enzyme content and a large proportion of islet tissue relative to other pancreatic components. Although only 4% by weight of an adult pancreas, the neonatal rat pancreas possesses about 11% of the adult islet cell mass. These properties have allowed neonatal tissue to be minced, dispersed by collagenase digestion and transplanted without specific islet isolation. Hegre and colleagues reversed alloxan diabetes in 80% of recipient rats following peritoneal isotransplantation of 200 - 500 mg of minced, four-day-cultured collagenase-dispersed, neonatal pancreas (76). The success of dispersed neonatal pancreas transplants appeared to be related to the weight of tissue transferred and the age of the donor tissue (77). Immature pancreas was considered to be the best source of material for transplantation, because it had the best growth potential and the surrounding exocrine tissue had not fully developed.

Quantitative aspects of the transplantation of dispersed neonatal pancreas have been studied by Matas and colleagues (78-80). The minimum number of pancreases required for successful transplantation was one for the intraportal route, two for the intravenous route and more than four for the intraperitoneal route. In these studies there was a latent period of several weeks but this could be reduced by increasing the number of donor pancreases used. By digesting pancreases for short periods of time with low collagenase concentration, a preparation suitable for transplantation with minimal loss of islet tissue could

be obtained (79). Neonatal pancreases prepared by this method were shown to ameliorate diabetes in 55% of recipients of a single neonatal pancreas and 77% of recipients of two or three neonatal pancreases. Mangnall and colleagues successfully reversed streptozotocin diabetes in 77% of diabetic rat recipients by intraperitoneal transplantation of collagenase-digested isogeneic neonatal rat pancreatic tissue (81). In addition, metabolic abnormalities were restored to normal. Neonatal pancreas does not have to be dispersed for successful transplantation as a free graft. Whole or part-denervated neonatal pancreas implanted under the kidney capsule of alloxan-diabetic rats has been shown to reverse the diabetes (82,83).

The reduction or even reversal of diabetic glomerular lesions in rats has been shown to occur after transplantation of isogeneic neonatal pancreatic tissue into the peritoneal cavity. Similar effects can be obtained with islets when they are dispersed throughout the liver via the portal vein (84). The liver is an advantageous site, both in regard to its modulation of circulating insulin levels and immunologically, when transplanting allogeneic islets.

Like neonatal pancreas, foetal pancreas has the same favourable ratio of high islet volume and low exocrine enzyme content, even though the total B cell mass of a single pancreas is small. Foetal rat or mouse pancreas has been transplanted intact as a free whole organ graft (85). The optimal time for the removal of foetal rat pancreas for transplantation under the kidney capsule of diabetic recipients was found to be 17 - 18 days gestation (48a). Hegre and colleagues have reversed alloxan diabetes in 67% of recipients, following isotransplantation of 40 mg of organ-cultured foetal pancreas (eight explants) beneath the recipient's kidney capsule (76). Less foetal than neonatal tissue appears to be required for the successful transplantation and reversal of diabetes (48a) and as

with neonatal tissue, there is a long latent period between transplantation of the foetal pancreatic tissue and the amelioration of diabetes. This is because of the need for growth and subsequent maturation of the endocrine component of the foetal pancreas. Purified foetal islets can be obtained by tissue culture of collagenase- or mechanically-dispersed animal pancreases (48a).

The advantages of using rat foetal pancreas for transplantation have been summarised by Brown and colleagues (86). These are that the tissue has a capacity for growth and differentiation, whilst the atrophy of the exocrine part results in the production of pure islet tissue. The small size enhances cryopreservation and allows time for serotyping, matching and preparation of the recipient. In addition, there is the possibility of diminished histocompatibility antigens, and a large pool of donor material.

The optimal time for the removal of the foetal pancreas has been shown to be after 16 - 17.5 days of gestation, when there is advanced development of the islets, but little proliferation of the exocrine tissue (86). Brown and colleagues demonstrated that the transplantation of just a single foetal rat pancreas syngeneically would completely reverse the diabetic state in recipient animals. In addition, the recipient rats exhibited normal glucose homeostasis and insulin response, before, during and after pregnancy. The increased need for insulin during pregnancy was considered to be a good opportunity to test the reserve capacity of the transplanted organ, which was found to be adequate (85). Finally, several reports in the literature have indicated that foetal rat pancreas is capable of both growth and differentiation after transplantation (87-90).

Islet transplantation can reverse diabetes characterised by B cell destruction and insulin deficiency in spontaneously occurring animal models. This observation strongly supports the hypothesis

that transplantation into human insulin-dependent diabetics will be effective if technical and immunological problems can be overcome.

In 1970, Strautz reported that islet transplantation partially reversed the obese hyperglycaemic syndrome in (ob/ob) mice and he suggested at that time that lean mouse islets supplied a satiety factor that was missing in obese mice (91). Gates and colleagues (92) have reported that the transplantation of islets from normal albino mice enclosed within millipore filter chambers was capable of normalising the blood sugar of New Zealand obese (NZO) mice (92). These animals show only mild excursions in basal blood glucose levels, but they are hyperinsulinaemic and have an impaired glucose tolerance.

1.5.2. Pancreas and Islet Transplantation in Man.

Most human recipients of pancreas transplants have had far advanced complications of diabetes at a time when the risks of immunosuppression and transplantation have been high. Ideally, pancreas transplantation should be performed early in the course of diabetes in order to prevent diabetic sequelae. However, until specific immunosuppression without side-effects is possible in man, pancreas transplantation will of necessity be restricted to patients who have already either demonstrated their propensity to develop secondary complications or who are extremely labile in their exogenous insulin requirements. The advantage of performing pancreatic transplants in diabetic patients who also require kidney transplants is that the risks associated with immunosuppressive therapy are obligatory. In most patients the two organs have been grafted simultaneously, but occasionally dysynchronous transplantation has been carried out.

Early attempts at pancreas transplantation in man were not very successful. However, despite the fact that of 128 pancreatic allotransplants carried out between December 17, 1966 and October 31,

1980, only 15 patients had functioning grafts and were insulin-independent (four for more than a year),(48b), at the end of this period, the technical problems responsible for early graft losses were becoming fewer, and although the overall success rate was not high, it was improving. The outcome of recent pancreas transplantations into patients with less severe secondary complications of diabetes has been very favourable and certain criteria for patient selection for pancreatic transplantation have now been documented (93). The most successful surgical remedy at this time appears to be segmental pancreas transplantation with total duct-occlusion and intraperitoneal (i.p.)placement to absorb residual exocrine secretions. Reasonable success has been reported with the i.p. transplantation of immediately vascularised segmental pancreatic grafts without duct-ligation (53). Pancreatic grafts injected with neoprene have been shown to maintain endocrine function in man (94).

Maitland and colleagues suggested the transplantation of human foetal pancreas as the treatment of choice for diabetic patients with no residual endogenous insulin secretory capacity (95). They proposed the forearm muscle as a potential transplantation site because of its accessibility.

The possibility of transplanting isolated islets rather than whole or part pancreas is an attractive proposition. The possible injection of islet tissue would be simpler and safer for the recipient and one pancreas should provide sufficient islet tissue for more than one diabetic recipient. In addition, islets can be subjected to a period of culture prior to transplantation in order to reduce their immunogenicity (48b). Islet transplantation in man has been largely unsuccessful and the availability of a sufficient quantity of viable human islet tissue remains a major problem. In certain countries, ethical problems arise with foetal and infant cadaver donors, whilst

in Sweden, abortion material is used. Human adult cadavers appear to be the best potential source of islet tissue for transplantation, but the isolation procedure with the fibrous human pancreas is more difficult than in animals and the yield is low. Successful islet viability depends on reducing the cold ischaemia time to a minimum. A preliminary report by Najarian and colleagues in 1977 (58) on human islet transplantation, indicated that islet transplantation in seven diabetic patients, each with a successfully established renal allograft, failed to ameliorate diabetes. However, their study did indicate that i.p. and portal vein sites of transplantation could be accomplished without complications and without inducing rejection of the previously transplanted kidney. In addition, the same group have demonstrated limited success with human islet autotransplantation following pancreatectomy (59). Largiadèr and colleagues have reported a successful long-term (10 months) functioning human pancreatic islet allotransplant obtained from a juvenile human donor (57).

The use of dispersed whole pancreas has proved disappointing in human pancreatic transplantation. Intraportal autotransplantation of the digested donor pancreas regularly produced an alarming elevation of portal blood pressure (96). The injection of separated isolated islets together with more effective methods of immunosuppression have been shown to be more effective but not a complete remedy (96,97).

1.6. Immunological Aspects of Transplantation

Immunosuppressives such as azothioprine, corticosteroids and antilymphocyte serum (ALS), have been administered to prevent pancreas allorejection (64). Cyclosporin A, used for the immunosuppressive treatment of liver and kidney transplant recipients since its first clinical trials in 1978, has been used to prolong the functional survival of pancreatic allografts in rats (98,99) and dogs (66).

Total lymphoid irradiation has been used for the prevention of pancreas allograft rejection in rats (100). Rats with long-term enhanced renal allografts will accept immediately vascularised pancreas grafts but will reject islet grafts from the same donor strain. For practical purposes, it is easier to prolong the survival of pancreas than islet allografts (101). Barker and colleagues (102) suggested that the selection of an ideal transplant site, (preferably an immunologically privileged one), minimising histoincompatibility and islet immunogenicity and immunosuppression are important factors in islet transplantation. It is well known that islets are immunogenic (103, 104). Foetal and neonatal islets are rejected just as rapidly as adult islets, although well-established foetal allografts may be less susceptible to immune destruction (105). In non-immune-suppressed recipients, foetal pancreatic islet allografts are almost always rejected before they mature sufficiently to reverse diabetes (106).

If immunosuppression is not used, fresh islet allografts are rejected very rapidly and this is particularly apparent if transplantation is across a strong histocompatibility barrier (107,108). Rejection time depends on several factors including the number of islets transplanted, the site of transplantation and the histocompatibility barrier. Immunosuppressive regimens that have been shown to prolong the survival of skin, kidney and heart allografts have been either ineffective or minimally effective in promoting the survival of islet allografts. These observations have led to the hypothesis that islet tissue transplanted as a free graft is more susceptible to immune destruction than islets transplanted as part of an immediately vascularised intact pancreatic graft (109,110). Whole pancreas, but not islet, allograft survival times have been shown to be prolonged following transplantation into adult recipients bearing immunologically-enhanced heart or kidney allografts (109).

Many different techniques have been used to try to prevent the rejection of islet allografts. Some of these have been shown to be remarkably effective. They include the use of immunosuppression by either drugs or antilymphocyte serum and the induction of specific tolerance or enhancement. In addition attempts have been made to minimise histocompatibility differences, reduce islet immunogenicity by tissue culture or donor pretreatment and employ immuno-isolation devices or mechanical barriers to prevent contact of islets with host antibodies. Azathioprine (111,112) and cyclophosphamide (112-114) have been used with conflicting results in the transplantation of both neonatal and adult rat islets.

Although corticosteroids are the backbone of most clinical immunosuppressive régimes, they are potentially diabetogenic. Prednisone has been used for islet transplantation in rats (115).

Antilymphocyte serum (ALS) or one of its derivatives has been the most effective agent in delaying the rejection of islet allografts. Marquet and Heystek reported that ALS was the immunosuppressive agent of choice for preventing the rejection of allogeneic islets (111). The pretreatment of rat islets with irradiation and silica (to reduce passenger leucocytes) was shown to more than double the survival time seen with ALS alone after transplantation across a major histocompatibility barrier (104). In addition, islets cultured for one week at 24°C were not rejected in ALS-treated animals (116). The survival time of rat islet allografts transplanted across a strong histocompatibility barrier has been increased by administering cyclosporin A for two weeks prior to transplantation (117).

Trimble and colleagues have demonstrated the long-term survival of pancreatic islets without immunosuppression when transplanted across weak histocompatibility barriers (118) and Ono and colleagues have demonstrated that the maintenance of rat islets in culture at 24°C

for one week in conjunction with a single injection of ALS, produced marked prolongation of islet allograft survival time (119).

Passenger leucocytes are thought to play a major role in sensitising a host to an allograft. The elimination of these leucocytes by donor pretreatment or by maintenance of tissue in in vitro culture before transplantation has been associated with prolonged survival of a variety of organ allografts. Mandel and Higginbotham showed that foetal mouse islets cultured for 21 days in an ambient environment were rejected within two weeks of allotransplantation (120) and Jonasson and colleagues demonstrated that monkey islets cultured for seven to nine days were rejected after allotransplantation (45). Lacy and co-workers found that the survival time of isolated rat islets transplanted into diabetic mice was prolonged markedly by maintaining the rat islets in vitro at 24°C for seven days prior to transplantation and administering to the recipient mice a single injection of antiserum to mouse and rat lymphocytes shortly before transplantation (121).

Lacy and colleagues showed that omitting the single injection of rat antilymphocyte serum considerably reduced islet xenograft survival (122).

The rejection of established rat islet xenografts can be induced by the i.v. injection of rat spleen cells and the rejection of successful allografts of rat islets has been shown to be accomplished by the i.v. injection of donor peritoneal exudate cells (123,124).

The successful transplantation of islet tissue enclosed in diffusion chambers has proved difficult to achieve. Theoretically, a semi-porous membrane should exclude cells or molecules able to mediate rejection, while allowing the flux of both insulin and glucose. The usefulness, composition, structure and diffusion characteristics of several membranes and chambers has been assessed by Theodorou and Howell (125). Gates and Lazarus reported that streptozotocin-induced diabetes in rats could be reversed by the i.p. implantation of neonatal rabbit pan-

creatic tissue enclosed in millipore chambers (126). The diabetes was re-established by the removal of the chamber. Archer and colleagues reported that both syngeneic and xenogeneic mouse islets enclosed in 50K Dalton hollow fibres restored normoglycaemia for several weeks in Chinese hamsters with mild streptozotocin-induced diabetes (127).

In contrast to these reports, other investigators have not been able to produce a significant, sustained reduction of hyperglycaemia in insulinopaenic diabetic animals transplanted with either membrane-enclosed syngeneic (128), allogeneic (129) or xenogeneic (130) islets. The inherent problems associated with the use of these chambers include poor neovascularisation, the deposition of fibrous connective tissue and the inadequate flux of both insulin and glucose between the chamber and the tissue fluid of the host.

A more sophisticated approach has been to implant a chamber within the blood stream of the diabetic animal. The contained islets were isolated on one side of a synthetic porous membrane or artificial capillary bundle (131-133). Although plasma glucose levels were lowered for a short time in streptozotocin-diabetic animals, blood-clotting and other technical problems have not allowed the devices to function for a sufficiently long enough period of time in vivo, to determine whether or not the theoretical objectives of preventing rejection and stabilising the blood sugar and thus alleviating the diabetic state, could be achieved.

If the rejection of xenogeneic islet tissue transplanted from animals to man could be prevented, the major problem of islet availability would be solved. Such a situation would allow the use of multiple donors of islet tissue. However, very quick rejection, often within a few minutes, occurs in xenografts between widely disparate species (133). For this reason, attempts have been made to contain islets in chambers made of semi-permeable membranes, so far with

very little success (126,127).

At present, suitable means for the transplantation of discordant islet xenografts for the treatment of diabetes are not available.

1.7. Preservation of Insulin-Secreting Tissue

Two basic techniques have been used for the preservation of pancreas transplants. They are cold-storage in an intracellular salt solution or hypothermic, pulsatile perfusion with plasma-like solutions (134). The latter method has proved difficult because the pancreas is a low blood-flow organ. Canine pancreases have been preserved for about 24 hours (134) and only short-term storage has been used for human pancreatic grafts (135). However, such preservation does offer the potential for histocompatibility matching and the preparation of the recipient for transplant.

The importance of islet preservation for transplantation cannot be over-emphasised in terms of building-up sufficient numbers to give the required blood glucose-lowering effect when transplanted. Short-term preservation is therefore necessary for clinical islet transplantation to be logistically practical. Three methods of *in vitro* islet preservation have been investigated, tissue culture, cold-storage and freezing or cryopreservation.

There is a great deal of evidence in the literature to suggest that foetal, neonatal and adult islet tissue, maintained in appropriate tissue culture for days, or weeks, will synthesise and secrete insulin in response to physiological stimuli and maintain their cellular integrity. In cultures of foetal and neonatal pancreas, islets have been shown to differentiate preferentially (45) and so B cells appear to be able to replicate under certain conditions (136). High oxygen levels have been shown to be detrimental to isolated islets (116), unless protected either by islet aggregation (137) or culturing at 24°C (119). At present, no pure β cell lines have been established to provide a

continuous source of sufficient insulin-producing tissue for transplantation, although the use of insulin-secreting tumours may prove beneficial, (70). Rodent islets can be maintained in culture for at least a week before transplantation without physiological deterioration. The relatively sophisticated equipment and fastidious conditions required for the maintenance of islets in tissue culture, however, make it difficult to store the quantity of isolated islets currently considered necessary for transplantation into large animal models or man. Mandel and Higginbotham, using cultured foetal mouse pancreas, have shown that islets will differentiate and proliferate in vitro over prolonged periods of culture (120). They have also demonstrated that a mild glucose stimulus potentiated insulin biosynthesis and that the islet content of less than one foetal mouse pancreas was sufficient to ameliorate the hyperglycaemia of a syngeneic diabetic mouse, even when the islets were grafted under the renal capsule.

Further evidence that human foetal β cells retain their viability in organ culture for more than one week has been presented by Ågren and colleagues (138). In addition, Andersson (139) has demonstrated that human pancreatic islets, once isolated, can be maintained in viable culture for extended periods of time up to 3 weeks. Such a culture system provides the opportunity for the provision of an islet tissue bank for subsequent transplantation (140). Human islets cultured for several weeks in vitro by this technique, have been shown to stabilise the blood sugar when transplanted into nude diabetic mice (140). Lundgren and colleagues have isolated and maintained human islets in a tissue culture system for up to three weeks (141). Special efforts were made to obtain a pure islet preparation and to demonstrate the structural and functional integrity of the islets during and after culture by monitoring insulin release. To date, free floating human islets

have been maintained in tissue culture for more than nine months. These islets have been demonstrated to preserve the ability to release insulin in response to glucose for several months *in vitro* (142).

Hellerström and colleagues demonstrated that the *in vitro* culture of minced and mildly collagenase-disaggregated foetal rat pancreas (21.5 days gestational age) resulted in the degeneration of the exocrine part, leaving islets in an isolated and viable state (143). Lambert and colleagues have studied the effects of various secretagogues on insulin release from organ-cultured foetal rat pancreas (144) and have shown the tissue to be sensitive to glucose and amino acids (144). The tissue culture of foetal rat islets has provided a useful tool for studying islet growth and development, insulin secretion and biosynthesis and glucose metabolism (145).

Cold-storage of islets is the simplest means for the preservation of islet tissue, requires the least amount of equipment and is a useful means for studying short- and long-term tissue viability.

Frankel and colleagues demonstrated that glucose-stimulated insulin release was best maintained by the storage of pancreatic islets in tissue culture medium containing a high concentration of glucose at 8°C, with short weekly periods at 37°C (146). In later studies, the same group showed that islet water formation, O_2/CO_2 consumption and $^{86}Rb^+$ uptake were reduced in cold-storage but could be restored almost to normal levels by rewarming the islets to 37°C (147). Their results emphasised the usefulness of cold-storage for the preservation of functionally intact isolated islets. Mouse, rat and guinea-pig islets have been preserved by cold-storage in Hanks' balanced salt solution (HBSS) at 4°C (148). After 15 hours of storage, the histological appearance, metabolic viability and insulin secretory ability was fully preserved but values for these parameters fell significantly to 10% of initial values by 48 hours and after 72 hours the islet tissue

had completely deteriorated (148). Several investigators have used in vitro tests to determine the optimal conditions for freezing and thawing of adult islets (149,150) and foetal rat pancreas (151). Critical factors include the type of cryopreservative used, its concentration and the rate of cooling and warming. The cryopreservative agent protects against intracellular ice crystal formation and the osmotic changes which occur during removal of the cryopreservant need to be carefully monitored. The main cryopreservatives used are dimethyl sulphoxide (10-20%), glycerol and liquid nitrogen. Standard cryopreservation techniques, appropriately modified, allow rat islets to be stored prior to transplantation (152). However, there are no reports of the successful transplantation of frozen islets obtained from large animal pancreases (48a).

Comprehensive methods for the preservation of islets at sub-zero temperatures have been described by Hellerström and colleagues (153). Cryopreservation of islet tissue was achieved without major loss of specific functions but the authors stressed that freezing and thawing techniques still needed to be refined in order to maintain complete viability of the stored islet tissue.

In the cryopreservation of mouse, rat and guinea-pig islets, slow freezing has been found not to be as effective as fast freezing combined with a 24-hour culture period after rewarming (148). The cryopreservation of foetal rat pancreas has been successfully accomplished by Brown and colleagues (86).

1.8. Novel Methods of Transplantation of Insulin-Secreting Tissue

The development of implantable electromechanical devices requires the introduction of innovative biomaterials technology before the production of a functional artificial pancreas can be realised. Pancreas and islet transplantation techniques themselves have their own implicit problems which preclude their successful use at present,

such as technical difficulties of surgery, allograft and xenograft rejection and shortage of donor tissue. There remains a real need for the development of novel methods for the transplantation of insulin-secreting tissue in such a way as to prevent immunological rejection of the implanted tissue by the host. At present, these novel methods have encompassed islet encapsulation and the use of synthetic hollow fibre devices containing islet tissue.

1.8.1. Encapsulated Islets

Gates and Lazarus (126) reversed streptozotocin-induced diabetes in rats by the i.p. implantation of neonatal rabbit pancreas encapsulated in nucleopore chambers. Subsequent removal of the implants led to a return of the hyperglycaemia and death. The pancreatic tissue removed from the implants, however, maintained its functional integrity *in vitro*, and no rejection reactions were observed. This method offered the potential for the transplantation of other endocrine tissue such as pituitary, thyroid, adrenal and ovary.

Hydrogels (water-laden polymers) have been used for the encapsulation of pancreatic tissue by Klomp and colleagues (154), who encapsulated minced, neonatal pancreatic rabbit and rat tissue in hydrogel diffusion pouches and sutured them to the parietal peritoneum of rats. A major problem encountered in this technique was the inadvertent opening of the pouches due to failure of the seals. However, the hydrogels were permeable to glucose and insulin, and minimal tissue reaction was observed.

Lim and Sun have carried out the i.p. implantation of microencapsulated Wistar rat islets into isogeneic Wistar Lewis rats made diabetic with streptozotocin (155). The microcapsular membrane was composed of cross-linked alginate, a non-toxic polysaccharide. The recipients remained normoglycaemic for almost three weeks and the microencapsulated islets remained morphologically and functionally intact through-

out long-term culture studies *in vitro* of more than 15 weeks. In tissue culture medium, microencapsulated islets continued to release insulin and remained sensitive to glucose and theophylline stimulation, responding with a typical physiological biphasic insulin release pattern for more than two months (156).

In 1981, Reach and colleagues constructed a new bioartificial pancreas consisting of rat islets contained in a millipore chamber composed of polyacrylonitrile membrane and perifused with blood ultrafiltrate (157). The system was evaluated *in vitro* and *in vivo* in diabetic rats. Ultrafiltration proved more favourable than diffusion, reducing the lag time to less than ten minutes. *In vivo* results indicated that ultrafiltration could provide satisfactory glucose-insulin kinetics for an implantable closed-loop insulin delivery system in which non-syngeneic islets were protected against immune rejection.

Islets have been enclosed in polycarbonate diffusion chambers and transplanted i.p. into syngeneic rats made diabetic with streptozotocin (128). Direct implantation of chambers containing 1100-1400 rat islets failed to reverse the diabetes over a period of 12 weeks, and viable islet tissue was not recoverable from the chambers at the end of this period.

1.8.2. Implantable Hollow Fibre Devices

Archer and colleagues placed cultured pancreatic islet cells from either mice or hamsters inside 50K Dalton hollow fibres and implanted them into adult streptozotocin-diabetic Chinese hamsters. This resulted in a return to normoglycaemia that was maintained for several weeks in both the isograft and xenograft models (127). After the reappearance of the diabetic state, a repeat transplant, containing a larger number of either hamster or mouse islets resulted in the maintenance of normoglycaemia for a significantly longer period of time.

Beta cells from neonatal rats have been cultured on bundles of artificial capillary fibres (Amicon XM-50) perfused with tissue culture medium (158). The beta cells continued to release insulin and remained responsive to changes in glucose concentration over a period of 24 days. The quantity of insulin released was similar to that produced in conventional flask cultures of beta cells. Beta cells of newborn rats, cultured on the outer surface of hollow fibres have been used to assess the blood compatibility of hollow fibre units for implantation as shunts in the vascular system (159). The results of this work suggested that careful control of fibre manufacture, pore size and unit assembly could allow continuing blood circulation, without the need for excessive systemic anticoagulation. There was evidence of clotting at the cut surfaces of the hollow fibres and biomaterial junctions were weak areas with regard to thromboresistance. Also, there was a very long lag time associated with the insulin response of the units.

Orsetti and colleagues have implanted a multiple fibre system in diabetic dogs containing isolated dog islets (160). Clotting and/or haemorrhagic problems occurred within a few hours. Sun and MacMorine demonstrated that rat and monkey islets enclosed in artificial hollow fibre devices released insulin and responded to changes in glucose concentration in vitro (161). A thousand short-term cultured rat islets, contained in an artificial hollow fibre device attached to the circulatory system of a rat made diabetic with streptozotocin, reduced the plasma glucose level from 23.3 to 7.3 mmol/l after one hour and the level remained low as long as the animal lived. Sun and colleagues subsequently repeated the experiment and extended it by implanting devices containing normal rat islets into diabetic monkeys (133). In these animals, the blood glucose levels fell from 11.67 to 5mmol/l in four hours and circulating insulin levels rose

to 93 μU/ml within half an hour. Monkey islets maintained in continuous culture *in vitro* continued to release insulin for more than eight months. In a later study, Sun and O'Shea used a looped, semi-permeable, hollow fibre in a unit (162). The system was implanted vascularly into diabetic monkeys via an arterio-venous shunt and the device restored normoglycaemia within two hours and maintained it for six days. The use of excessive anticoagulant therapy was avoided by utilising fibres which contained heparin covalently and irreversibly bonded to the luminal surface.

In 1976, Tze and colleagues restored normoglycaemia in streptozotocin-diabetic rats with the use of an implantable artificial endocrine pancreas (IAEP)containing rat islets implanted as a vascular shunt into the abdominal aorta (132). Most of the experiments were of short duration because of the excessive bleeding produced by heparinisation. In most cases, hyperglycaemia was reduced and an increase in circulating insulin level was observed in the diabetic rats after implantation of the units. Normoglycaemia was restored within 4 hours. The IAEP provided an immunologically-privileged site, because antibodies were not able to pass across the hollow fibre membrane.

Tze and Chen have also maintained rat islets in culture for at least 97 days using Amicon 3 x 50 Vitafiber® capillary culture units (163). The rat islets continued to secrete physiologically useful concentrations of insulin in response to changes in glucose concentration. In addition implantable artificial capillary units containing either about 1200 allogeneic rat islets or 3000 xenogeneic rabbit or human islets have been implanted into streptozotocin-diabetic rats (164). In the case of the allogeneic recipients, 20 survived for between 12 and 24 hours (death being due to excessive bleeding from systemic heparinisation), four survived between one

and three days, one survived for four days and one for 11 days. In xenograft models, four rabbit islet recipients survived for up to four days and the diabetic rat recipient of isolated human islets implanted into the abdominal aorta survived for eight days. In this case there was a decrease in plasma glucose leading to normoglycaemia and an increase in insulin release. The subsequent glucose and insulin response to an intravenous glucose load was corrected to near normal.

The immunological isolation of allo- and xenogeneic islet cells from the host's immune system by the barrier of the synthetic capillary has been convincingly demonstrated by Tze and co-workers(165). Their study demonstrated that the semi-permeable membrane of the synthetic capillary could prevent the recipient from being sensitised by the transplanted allogeneic or xenogeneic islet cells , and could protect the grafted allogeneic and xenogeneic cells from immunorejection.

Following a period of tissue culture in articifial capillary culture units, adult rat islets have been shown to maintain their viability and functional capacity (166). Their subsequent intraportal implantation into isogeneic rats made diabetic with streptozotocin, showed the therapeutic efficiency of the cultured islets to be equivalent to that of freshly isolated rat islets. Normal weight gain was restored and complete reversal of hyperglycaemia, glycosuria and polyuria was demonstrated in the diabetic rat recipient. The normoglycaemic state was maintained for more than 12 months.

Recently, Tze and colleagues, have constructed an IAEP containing a single coiled capillary fibre made of an acrylic copolymer (167). The IAEP contained rat islets (1000/kg body weight) and was implanted into ten streptozotocin/alloxan-diabetic dogs, as an arterio-venous shunt between the right carotid artery and jugular vein. About five

hours after implantation, plasma glucose was reduced from 19.4 to 8.3 mmol/l and after 12 hours to 5.5 mmol/l. Insulin output also increased over the period of five hours to a mean rate of 39 mU/l. This work suggested that xenogeneic rat islets contained in an IAEP could normalise glucose homeostasis in the streptozotocin-diabetic dog. The early termination of some of the experiments was due mainly to thrombus formation in the artificial capillary units. In summary, the majority of studies using hollow fibres to date, have suffered from problems of fabrication, biocompatibility, transplantation site, blood-clotting at component junctions and haemorrhage caused by excessive use of systemic anticoagulation. These problems have largely hindered studies intended to assess the response time, efficiency and beneficial effects of such units in the treatment of diabetes.

Against this background, the purpose of the work presented in this thesis was to develop and construct a prototype implantable prosthesis for *in vitro* and *in vivo* studies in rats and to use this unit to establish optimum criteria for the effective transfer of physiologically useful concentrations of insulin and glucose across the hollow fibre membrane *in vitro*. In addition, studies have been directed towards monitoring the insulin secretory response of units containing either lean or obese mouse islets *in vitro* under various experimental conditions and using the prototype unit in a small-scale animal study in order to determine the *in vivo* capacity of units containing xenogeneic mouse islets to restore normoglycaemia in streptozotocin-diabetic rats. Attempts have been made to construct units that will at least provide a sustained background release of insulin even if the presence of a significant lag time prohibits their immediate response to gross fluctuations in perfusion glucose concentration. In addition, because of the efficient immunological separation of host antibodies from transplanted tissue afforded by 100 KDalton hollow fibres, due cognizance has been given to the possibility of using constructed units

for the transplantation of islet tissue across major histo-compatibility barriers.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals and Chemicals

Animals

All animals were initially housed in an air-conditioned room at 22±2°C prior to use. Lean and obese mice from the Aston colony were allowed free access to pellet diet (Heygate & Sons, Northampton, U.K.) and tap water prior to culling by cervical dislocation and the isolation of islet tissue from the excised pancreas. Wistar rats (150 - 200 g, Bonting & Kingman, Hull, U.K.) streptozotocin-treated or otherwise were fasted for 6 hours prior to use in surgical procedures for the insertion of prosthetic units.

Chemicals

Analar grade - $CaCl_2,2H_2O$; KCl; KH_2PO_4; $MgSO_4,7H_2O$; NaCl; $NaHCO_3$; Na_2HPO_4; and glucose were purchased from BDH Chemicals Ltd., Poole, Dorset. Penicillin, 5,000 U/ml/Streptomycin 5 mg/ml was obtained from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland. Bovine serum albumin - fraction V and Collagenase (Worthington Type IV for pancreatic islets) were obtained from Miles Laboratories Ltd., Slough, England). Streptozotocin was purchased from Sigma Chemical Co.Ltd., Poole, Dorset. Insulin Binding Reagent RD12 and human insulin standard RD13 (lot K7536) were purchased from Wellcome Reagents, Beckenham, Kent, whilst ^{125}I-insulin IM38 (>50 μCi/μg) and Na^{125}I (IMS 30)were obtained from the Radiochemical Centre, Amersham, Bucks. Mouse insulin standard was obtained from NOVO Research Institute, NOVO Alle, DK-2880, Bagsvaerd, Denmark.

Fabrication Materials

Amicon HIP100 hollow fibres in cartridge form were obtained from Amicon Ltd., High Wycombe, Bucks. Portex polyethylene tubing (PP10,

PP355) was purchased from Portex Ltd., Hythe, Kent and Tygon PVC tubing was purchased from V.A. Howe & Co.Ltd., Fulham, London. Espe Visio-Bond, U/V-cured dental adhesive was supplied by Espe Division, Cottrell & Company Ltd., London.

Miscellaneous Consumables

LP3 tubes and caps were purchased from Luckham Ltd., Sussex, England and pipette tips were purchased from Anachem Ltd., Luton, Bedfordshire. 4/0 non-capillary braided nylon suture material was obtained from A.W. Showell (Surgicraft)Ltd., Redditch, Worcs.

2.2. Evaluation of Fabrication Materials

The ideal biocompatible polymer does not exist except in nature and so the choice of materials for the construction of an implantable device must be a compromise. Synthetic materials intended for long-term implantation must fulfil certain essential requirements. They must be blood and tissue compatible and resistant to changes in integrity that may be caused by contact with body fluids. Ideally, the materials should be chemically inert and non-clot-forming. They should not allow the leaching of toxic monomers, should not cause physical trauma to adjacent tissues, should be non-carcinogenic and should be capable of being engineered and assembled into the correct shape or form. They should also be capable of sterilisation (168).

2.3. Tissue Compatibility

The hydrolytic and biological environment of the body interacts unfavourably with many implanted polymers, causing rapid, irreversible degradation of their physical and biological properties. In addition, degradation by-products may interact with the surrounding tissue. The toxic properties of the alkyl cyanoacrylates used as tissue adhesives have been attributed to decomposition products

formed in the body (probably formaldehyde and cyanoacetic acid)(169).

Tissue or blood contact with the polymer may result in the leaching of small molecules out of the polymer and the transport of polymer fragments may result in symptoms which at first do not appear to be related to the implant, i.e. inflammatory reactions, oedema and antigenic reactions. More common reactions are associated with the location of the implant. Haemolysis due to abrasive surfaces, clot formation due to platelet adhesion and the necrosis of cells soon become obvious after implantation.

Tissue reactions may extend beyond the immediate environment of an implant and antigen-antibody reactions and generalised infections may be brought about by local damage and necrosis (168).

The infiltration of fibrous tissue and the subsequent encapsulation of the implant with thick scar tissue is a common tissue response. The nature, shape, mechanical strength and flexibility of the implant should match that of the surrounding tissue in instances where the implant serves as a soft-tissue substitute.

2.4. Blood Compatibility

When blood comes into contact with an incompatible material, a reduction in the rate of blood flow or the formation of thrombi may result. In addition, there may be changes in blood proteins and modifications in, or interference with, the functions of blood components. The mechanism of clotting induced by a foreign material involves an initial rapid adsorption of proteins, primarily fibrinogen, on to the exposed surface (170). After a delay of 30 - 60 seconds, platelets begin to adhere to the protein pre-coated substrate. Later, aggregates form which may interrupt the blood flow, trap red cells, or cause red cell damage and the consequent formation of thrombi (171).

2.5. Blood Compatible Materials

Three types of thromboresistant surfaces have been recognised:

a. surfaces composed of relatively inert materials,

b. surfaces with anionic or imposed negative electrical charges, and

c. heparinised surfaces.

Inert surfaces include hydrogels based on monomers such as hydroxyethyl methacrylate or acrylamide, grafts of hydrogel materials on silicone or polyurethane substrates, trifluoropropylmethylsiloxane polymers and segmented polyurethans of the Spandex type. Hydrogels (172-174) and the many other types of thromboresistant biocompatible polymers (168,175,176) have numerous biomedical applications. Much attention has been focussed on polymers with heparin-coated surfaces (177-180) and polymers with heparin chemically incorporated into the molecule (162). The surface coating of polymers such as polyethylene, Teflon film and silicone by the long-established graphite-benzalkonium-heparin (GBH) method has not been satisfactorily achieved because of the chemical inertness of these polymers. However, there is a very real need to achieve a successful bonding of heparin or other anti-coagulants to these types of material since they are currently in demand as fabrication materials for implants, artificial organs and catheters.

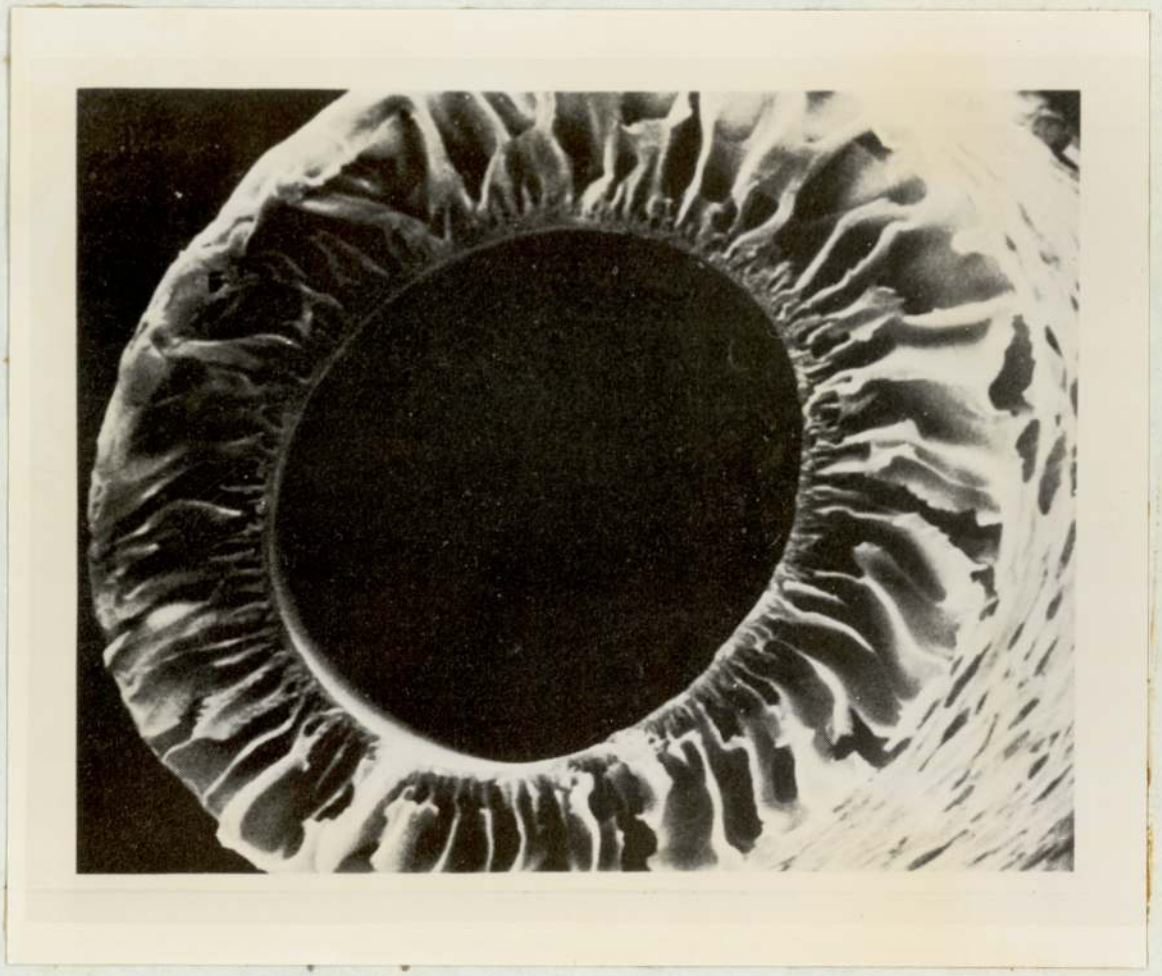
2.6. Choice of Fabrication Materials

(a) The Hollow Fibre

The most important functional feature of the implantable prosthesis is the hollow fibre. The choice of a suitable fibre depended upon the pore size at the internal or luminal surface and the bore size.

PLATE 1 shows a typical hollow fibre in cross section. The fibre was composed of acrylic copolymer with a molecular weight cut-off

PLATE 1. Cross Section of 100K Dalton Hollow Fibre.



of 100K Daltons at the luminal surface. It was chosen because it allowed the transfer of material with a molecular weight of less than 100,000 but prevented the movement of antibody molecules from the fibre lumen across the semi-permeable membrane. The smallest intact antibody, IgG, has a molecular weight of the order of 150,000.

(b) Evaluation of Cannula Tubing

The choice and composition of arterial cannulae for attachment to either end of the hollow fibre was limited by the physical dimensions of the fibre. Portex PP10 polyethylene tubing was found to have suitable flexibility and biocompatibility and could be easily inserted inside the hollow fibre. Whilst Portex PP10 was found to be the most suitable for the cannulation of rat carotid arteries, some units were assembled with PP50 cannulae in an attempt to increase blood flow to the units and reduce perfusion resistance.

Polyvinyl chloride tubing has been used extensively for short-term implantation in man. However, when used for permanent implants it has been observed to change from a flexible state to a very brittle state due to the leaching of plasticiser (168). On this basis, in the present study, it was considered unsuitable as a unit component.

(c) The Shell

Polyethylene was the material chosen for the outer shell of the unit. It is biologically inert, has good structural rigidity and could be sterilised.

(d) The Adhesive

Many cements and adhesives presently used in orthopaedic surgery are based on materials that were initially developed in orthodontics. Many of the early alkyl cyanoacrylates and amine-cured epoxy resins have been shown to be unstable in the biological environment and have toxic properties (168). In addition, some of these adhesives

have subsequently been shown to be carcinogenic.

In the present study a U/V light-cured, biocompatible, methacrylate-based liquid bonding material used extensively in the dental field, was found to be suitable for the fixing of Portex cannulae to the hollow fibre and for the final sealing of the outer polyethylene shell. In addition, small rings of biocompatible, non-toxic and non-pyrogenic Tygon PVC tubing were inserted at each end of the unit to support the mobile adhesive prior to U/V curing.

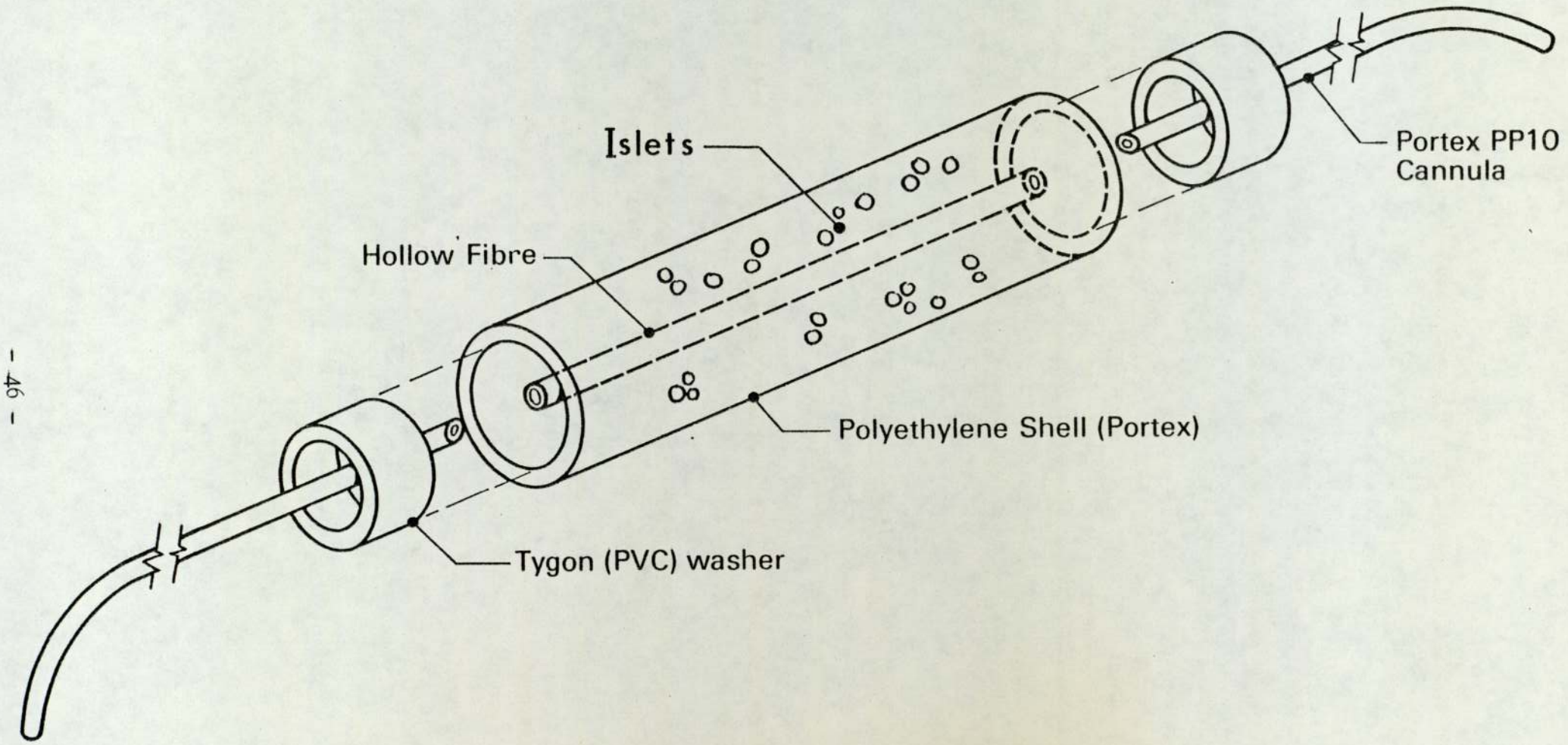
2.7. Fabrication of the Prototype Unit

(a) Construction of units for in vitro work

Hollow fibres used in the construction of prototype units were removed from a broken-down, commercially available, 20-cm. long, H1P100 filtration unit, and soaked in several changes of sterile 0.9% saline to remove all traces of glycerine. All fibres were subsequently stored at $4^{\circ}C$ in sterile, physiological saline, until required for use in the construction of units. Under these conditions, the fibres were stable, did not attract bacterial contamination and could be autoclaved.

Figure 2.1 represents an exploded three-dimensional diagram of the constructed unit. Each hollow fibre had an internal diameter of approximately 450 - 500μ and a wall thickness of approximately 150μ. A 10-cm long piece of sterilised Portex PP10 polyethylene tubing (internal diameter 0.28 mm, external diameter 0.61 mm) was inserted into each end of a 2.2 cm portion of hollow fibre and the junctions sealed with Espe Visio-Bond, a biocompatible, methacrylate-based dental sealant. The fibre with its two attached cannulae was inserted inside a 3-cm piece of polyethylene tubing (Portex PP355; internal diameter 3.50 mm, external diameter 4.50 mm), forming the outer shell (see Fig. 2.1). Each end of the outer tube was partially occluded with a small ring of flexible Tygon PVC tubing (internal

Figure 2.1. Exploded 3-dimensional View of Unit.



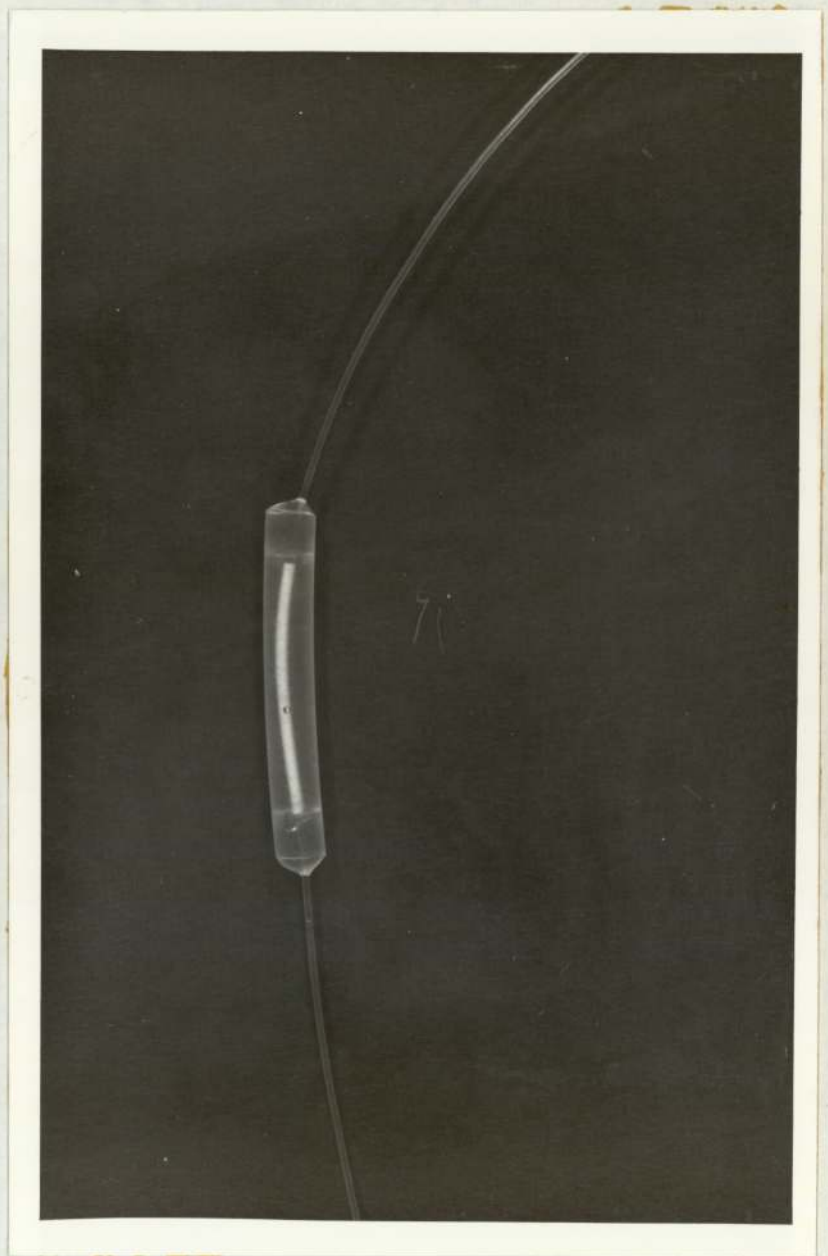
diameter 2.37 mm; external diameter 3.95 mm). One end of the unit was then sealed with a plug of the dental adhesive. This was achieved by clamping the unit vertically and placing a few drops of the liquid adhesive in the small cavity remaining between the PP10 cannula and the ring of PVC tubing. The adhesive remained in place by surface tension and was polymerised by 20 seconds' exposure to a fibre-optic U/V light source. Gentle pulling on the cannula confirmed the integrity of the bond. The cavity within the polyethylene shell was either filled with a diffusible dye (methylene blue), ^{125}I-insulin injected by means of a fine gauge (25G) needle or latterly with mouse islets suspended in oxygenated basic tissue culture medium and inserted via a finely-extruded Pasteur pipette. All air bubbles were excluded and the end sealed as described previously, rendering the unit air and watertight. Patency of the fibre-cannula junction was verified by flushing the unit with a little sterile heparinised saline from a syringe fitted with a 25G needle. PLATE 2 shows a functional assembled unit.

(b) Construction of units for in vivo work

Units were constructed as described previously but some were constructed with 3 cm. hollow fibres and others had their linear dimensions reduced by half to facilitate easier insertion into the body cavity of the animals and reduce trauma.

The total volume of the 3 cm unit was approximately 0.22 mls. The number of islets inserted into each unit depended on the source, i.e. whether from lean or obese mice. Larger numbers of islets could be obtained from obese mice than from lean mice by either collagenase digestion or microdissection. The islet content of units was expressed in terms of dry weight of islet tissue.

PLATE 2. Fully Constructed Prosthetic Unit, showing Hollow Fibre and Two Cannulae.



2.8. Sterilisation

The various components of the implantable unit were sterilised separately. Amicon hollow fibres were autoclaved (15 lbs/sq. in., 15 mins.) and stored in sterile 0.9% saline, the Tygon ring, polyethylene cannulae and shell were dipped in ethanol, rinsed in sterile physiological saline and irradiated under U/V light and the polymerisation of the adhesive with U/V light afforded a surface layer of sterilisation. The unit itself was assembled in a sterile cabinet (Hepaire) with automatic air extraction. Additionally, for *in vivo* studies, the fully constructed unit was immersed in 1% hibitane prior to surgical implantation. In *in vitro* studies, the units were perfused with freshly prepared sterile media to prevent bacterial contamination.

2.9. The *in vitro* Single Cycle Perfusion System

The Hanks' solution (Appx. 1, p.135) was autoclaved, supplemented with penicillin (100U/ml)/streptomycin (0.1 mg/ml) and stored at 4°C. (181). The stock perfusion medium was supplemented with glucose (either 5.56 mmol/l or 16.67 mmol/l) and albumin (either 0.5g/100 ml or 7.45g/100 ml), gassed with 95% O_2/5% CO_2 and the pH was adjusted to 7.4 at 37°C immediately prior to use. The perfusion system is shown in PLATE 3 and illustrated diagrammatically in Figure 2.2.

Two reservoirs of medium, one containing 5.56 mmol/l glucose and the other containing 16.67 mmol/l glucose, were maintained in a water bath at 46°C. The temperature of the water bath was set 9°C higher than physiological temperature, to allow for the temperature drop in the system. Each reservoir was connected to a 5 metre length of silastic tubing (lung) sealed in a continuously gassed (95% O_2/5% CO_2) water-filled jar at 46°C.

PLATE 3. In vitro System for the Perfusion of Units

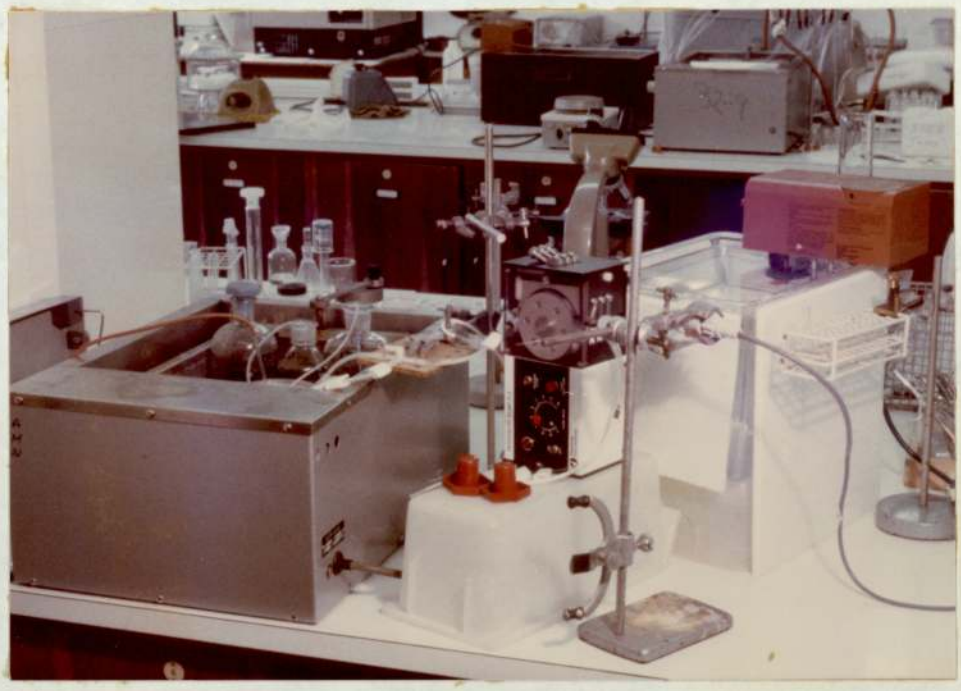
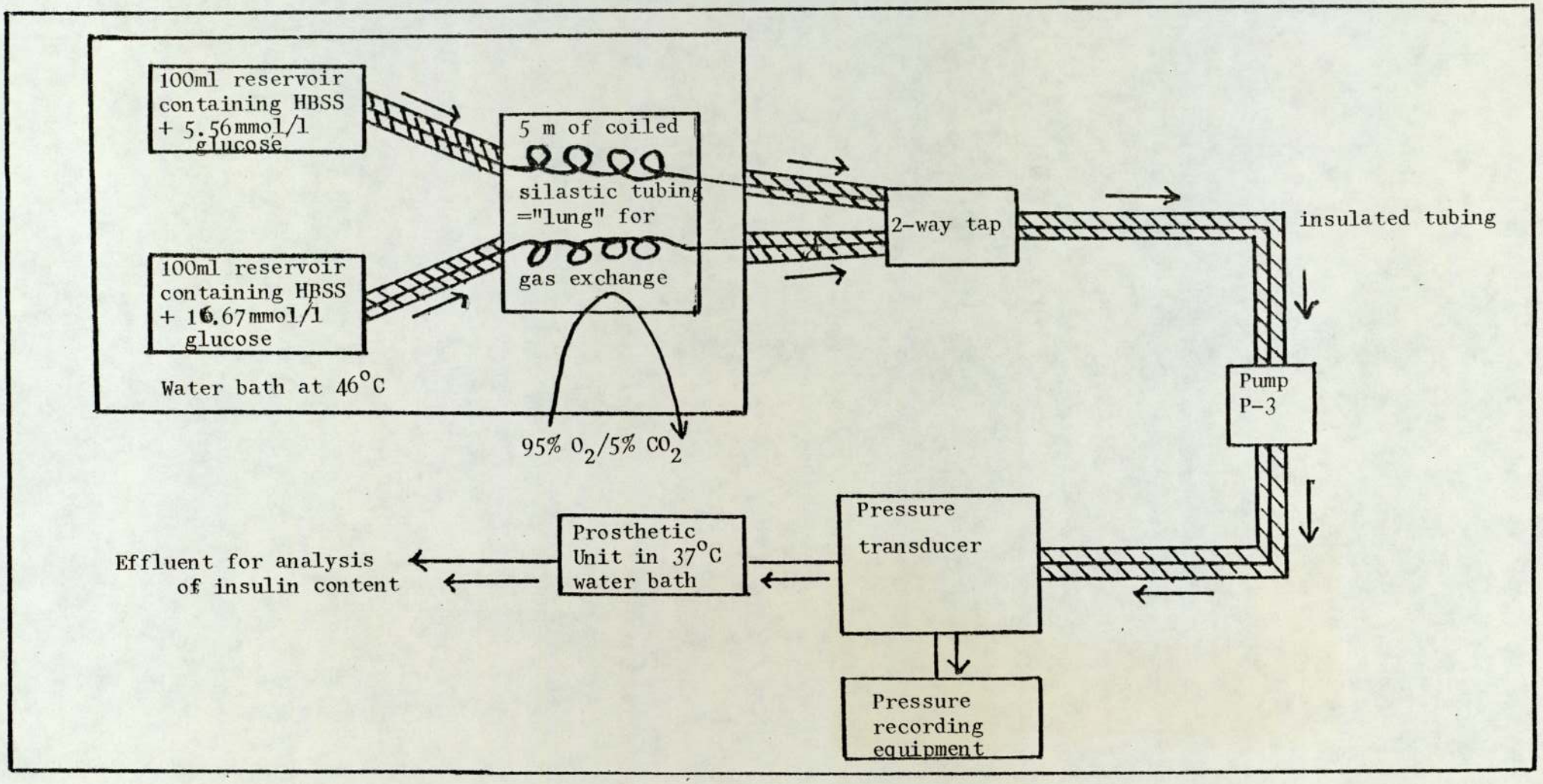


Figure 2.2. Schematic Representation of the Essential Components of the Single Cycle Perfusion System



From the silastic "lung", oxygenated buffer was directed to a 3-channel peristaltic pump (Pharmacia P-3) via a 2-way tap. By this means, medium containing either high or low glucose could be pumped continuously without generating air bubbles.

From the pump, medium was directed via insulated tubing to a physiological pressure transducer (Bell & Howell, 4-327-I) attached to a pressure recorder (Devices, M2). The transducer was connected in series with a prosthetic unit completely immersed in a covered, insulated water bath at 37°C.

The effluent from the distal end of the prosthetic unit was collected in LP3 tubes and immediately used for analysis or stored at -20°C.

The system was physiologically optimised as far as possible. Depending on the experiment, working flow rates ranged from 0.042 to 0.637 ml /min. and the pump was used at its maximum flow setting (10) for all but preliminary experiments, this flow setting provided a perfusion pressure of approximately 100 mm Hg at an albumin concentration of 0.5g/100 ml and a pressure of 125 mmHg at an albumin concentration of 7.45g/100 ml. The latter albumin concentration represented the total protein content of human serum. A typical pressure trace is shown in Appendix 2, page 136 . The dead volume in the tubing between the two-way tap and the prosthetic unit was just under 1.8 mls and represented a dead time of 2 minutes 54 seconds. The perfusion format corrected for dead time consisted of a 30 min. pre-perfusion followed by a 60 minute test perfusion.

2.10. Glucose Estimation in Serum and Perfusion Media

The measurement of glucose in serum and perfusion media (10 µl) was carried out using an automated glucose analyser (Beckman Glucose Analyser 2). The method uses the enzyme glucose oxidase (E.C.1.1.3.4) and glucose is determined by means of the oxygen rate method, employ-

ing an oxygen electrode. In the process β-D-glucose from the sample combines with dissolved oxygen from the solution according to the reaction:

$$\beta\text{-D-glucose} + O_2 \xrightarrow[H_2O]{\text{glucose oxidase (E.C.1.1.3.4)}} \text{gluconic acid} + H_2O_2$$

Oxygen is consumed at the same rate as glucose reacts to form gluconic acid. At all times during the reaction, the rate of oxygen consumption is directly proportional to the concentration of glucose then present in the reaction cup. The maximum observed rate, attained after a brief interval, required for reagent mixing and system response, is a direct measure of the concentration of glucose originally present in the sample at the time of injection. The analyser measures and holds this maximum rate and permits the digital readout to be scaled, providing a reading corresponding to the glucose concentration in mmol/l. The instrument is standardised with 8.3 mmol/l glucose. Because oxygen consumption rather than peroxide formation is measured, the only requirement for peroxide is that it must be destroyed by a path that does not lead back to oxygen. The addition of ethanol to the reagent causes peroxide to be destroyed by catalase (E.C.1.11.1.6) in the glucose oxidase, without yielding oxygen, according to the reaction:

$$H_2O_2 + \text{Ethanol} \xrightarrow{\text{catalase (E.C.1.11.1.6)}} \text{Acetaldehyde} + H_2O$$

To ensure destruction of the peroxide, iodide and molybdate are added to the enzyme reagent initiating the reaction:

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{\text{molybdate}} I_2 + 2H_2O$$

This reaction is effective even after catalase activity has diminished with storage.

The chemistry is free of interference from reducing substances and from agents used to prevent glycolysis or coagulation and the

method does not depend on optical properties of a solution. The analyser, therefore, will determine the glucose concentration even in uraemic, icteric, turbid or haemolysed specimens. A particular advantage of this instrument is that it uses 10 µl samples, a property especially useful in animal work.

2.11. Double Antibody Radioimmunoassay for Insulin

Introduction

The most commonly used, and most convenient and accurate method presently available for the measurement of serum insulin is radioimmunoassay.

The concept of radioimmunoassay was pioneered by Rosalyn Yalow and the late Solomon Berson (182). A considerable amount of literature has been written on the subject and this has been summarised in a number of excellent reviews (183 - 186).

A double antibody radioimmunoassay was used in the present study for the measurement of insulin in serum and perfusion media.

Principles of Radioimmunoassay

Radioimmunoassay is based on the principle of isotopic dilution in the presence of specific antibodies and depends on the competition between radioactively-labelled ^{125}I-insulin and unlabelled insulin for binding sites on the antibody.

The essential principle of the test is the reaction of a limited, fixed quantity of anti-insulin serum, with a mixture of the sample of insulin to be assayed and a constant amount of radioactive insulin. After the reaction has been allowed to approach completion the antibody-bound insulin is separated from free insulin by a double antibody procedure employing a pre-precipitated antibody, and the distribution of the radioactivity determined. The binding of labelled insulin to antibody is progressively inhibited by increasing amounts of unlabelled insulin, owing to com-

petition for specific binding sites on the antibody. The concentration of insulin in the plasma under test is determined by reference to a standard curve prepared at the same time.

Radioimmunoassay Components

The procedure for the double antibody radio immunoassay of insulin has been outlined below. More comprehensive details are available in Appendix 3, page 137.

1. Reagents

(a) Diluent Buffer

The assay buffer was an isotonic 0.04M phosphate buffer, pH 7.4, containing bovine serum albumin, fraction V (0.5%), thiomersalate (0.1%) and sodium chloride (0.9%). This buffer was used to dilute the radioiodinated insulin, insulin standards and, where appropriate, serum and plasma samples. It was sterilised by millipore filtration and stored at $4^{\circ}C$.

(b) Insulin Binding Reagent (pre-precipitated insulin antibody)

The freeze-dried insulin binding reagent consisted of a mixture of guinea-pig anti-insulin serum and rabbit anti-guinea-pig-globulin serum in (0.04M) phosphate buffer containing 0.02M EDTA. This pre-precipitated antibody was reconstituted by the addition of 8 ml of deionised water and the lyophilised material dissolved by gentle inversion and stored at $4^{\circ}C$ prior to use. The double antibody or immune precipitate retains the insulin binding properties of the original antiserum and was isolated together with the bound insulin by centrifugation.

(c) Radioiodinated Insulin

The labelled insulin used in the assay was highly purified porcine insulin mono iodinated by the Chloramine-T method (187,188) to a specific activity >200 $\mu Ci/\mu g$ with $Na^{125}I$ in NaOH solution

(Amersham, IMS 30) with approximately 4% damage and 60-65% incorporation. Iodination was predominantly at the A14 Tyrosine residue of insulin and labelled insulin was separated from unreacted iodide by gel filtration on Sephadex G50.

In the radioimmunoassay for insulin, the ^{125}I insulin was thawed and diluted with assay buffer containing cold porcine insulin (10^3 ng/ml) to give a count rate of 10^4 cpm/50 ul and a specific activity of approximately 50 µCi/µg.

(d) Standard Insulin Solutions

(i) Human Insulin Standard

Wellcome dried Human Insulin Standard consisted of the freeze-dried residue of 2.6-2.8 mU of insulin in 1 ml of phosphate buffer pH 7.4 containing 0.5% BSA and 0.1% sodium azide. This was dissolved in diluent buffer to give a working concentration of 0.5 mU insulin per ml. and when required further diluted to give a range of Standards containing 200, 100, 50, 25 and 12.5 µU/ml (or 8 - 0.5 ng/ml).

(ii) Mouse Insulin Standard

A stock solution of 100 ng/ml mouse insulin standard (Novo Laboratories Ltd., Denmark) was diluted to give a range of standards of 10, 5, 2.5, 1.0 and 0.5 ng/ml.

Both mouse and human insulin standards were transferred to LP3 tubes in 250 µl aliquots and stored at -20°C until required for use in radioimmunoassay.

Procedure The following protocol was used for insulin radio-immunoassay.

Table 1. Insulin RIA Protocol

	Tube Number	Initial Reactants	Insulin Binding Reagent		Working Solution of Iodinated Insulin		Buffer for Wash	
Volume		50 µl	50 µl		50 µl		500 µl	
Zero	1 - 3	Buffer	+		+		+	
	4 - 6	0.5ng/ml	+		+		+	
Mouse	7 - 9	1.0 "	+		+		+	
Insulin	10-12	2.5 "	+		+		+	
Standards	13-15	5.0 "	+		+		+	
	16-18	10.0 "	+		+		+	
Human	19-21	12.5µU/ml	+		+		+	
Insulin	22-24	25 "	+		+		+	
Standards	25-27	50 "	+		+		+	
	28-30	100 "	+		+		+	
Blank	31-33	Buffer	Buffer		+		+	
Total count	34-36	-	-		+		-	
Sample 1	37-38	+	+		+		+	
Sample 2	39-40	+	+		+		+	
Sample n	36+2n	+	+		+		+	

Vortex mix and incubate at 4°C for 6 hours

Vortex mix and incubate at 4°C for ≥ 14 hours

Vortex mix and centrifuge at 2,900 r.p.m. for 30 mins. then decant, aspirate, drain and count in γ counter

Key

+ = carry out this procedure

- = not applicable to these tubes

50 µl aliquots of zeros, standards, blanks and samples were transferred to numbered LP3 reaction tubes. 3 tubes were allocated for the total count (to which 50 µl of iodinated insulin only would be added) and left empty. The zeros (to which no unlabelled insulin had been added) and the blanks (tubes in which insulin binding reagent had been replaced with diluent buffer to serve as controls of the washing procedure) contained 50 µl of diluent buffer.

50 µl of insulin binding reagent was added to the zeros, standards and sample tubes. Then 50 µl of diluent buffer was added to each blank tube and the total count tubes were left empty. The tubes were subsequently vortex mixed and incubated at 4°C for 6 hours.

50 µl of diluted ^{125}I-insulin was added to all tubes including those allocated for total counts. All tubes (except those for total counts) were vortex mixed and incubated at 4°C for 16 hours, generally overnight. 500 µl of assay buffer was subsequently added to all tubes (except those for total counts) and the contents vortex mixed and centrifuged at room temperature for 30 minutes at 2,900 rpm (Mistral 4L, M.S.E.).

After centrifugation, the contents of the tubes (with the exception of total counts) were expelled by rapid inversion and the last drop aspirated with the aid of a Pasteur pipette. The tubes were then allowed to drain upside down on absorbent paper for 30 minutes and finally counted on a gamma counter (ICN Tracerlab gamma-set 500 spectrophotometer) for one minute (counting efficiency 57%).

All counts were corrected for background.

Zero tubes, standards, blanks and total counts were all assayed in triplicate while unknown samples were assayed in duplicate.

Calculation of Results

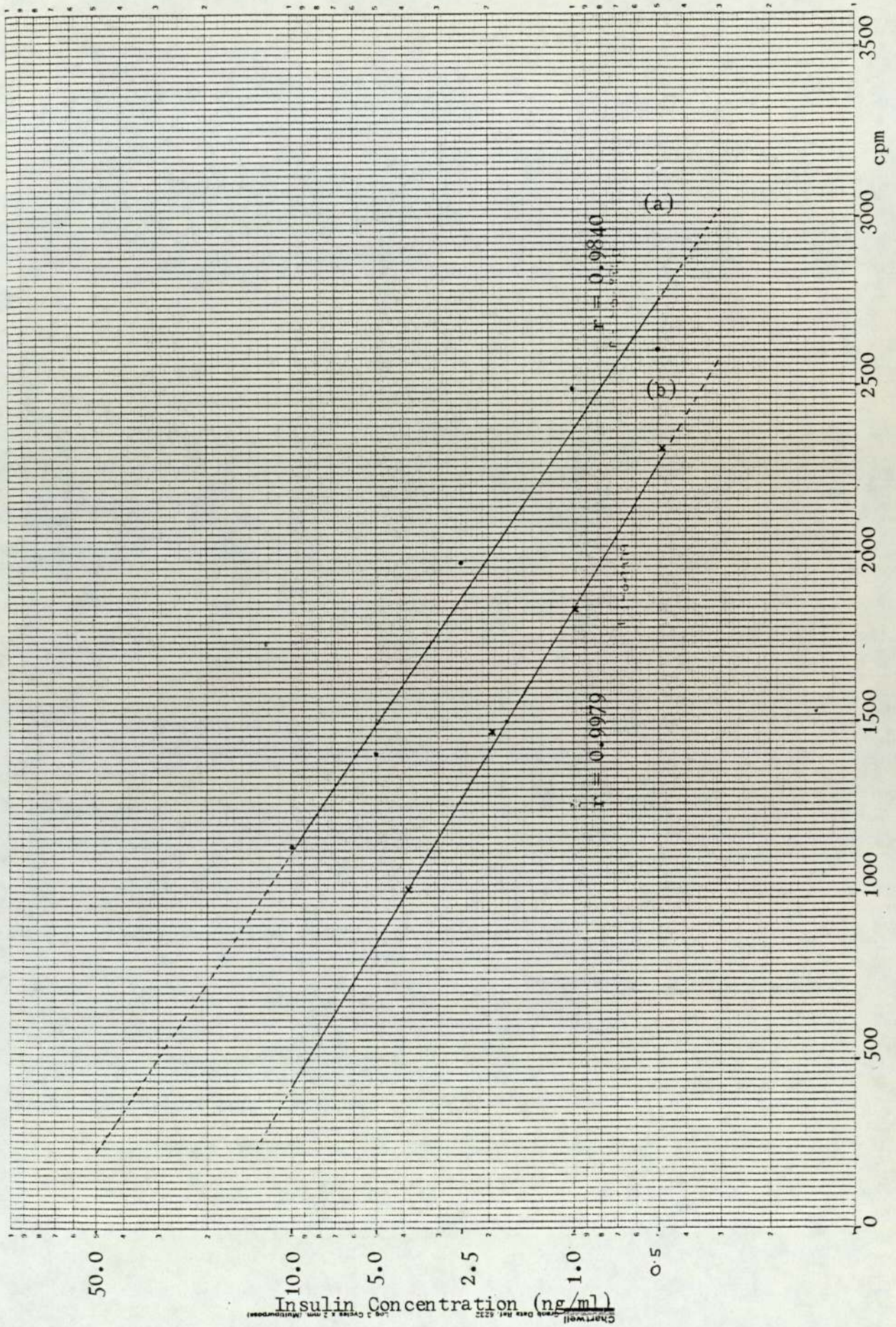
A computer programme for the calculation of results is included in Appendix 3, page 137.

The background was subtracted from each tube counted and triplicates and duplicates were averaged, any grossly aberrant values (outside 3 S.D.'s) were rejected. Correction was made for radioactive decay (1% per day) when tubes could not be counted immediately. In early assays a standard curve of $\log_{10}$ of standard insulin concentration against corrected cpm was plotted using 2-cycle "semi-log" paper. Typical standard curves for human and mouse insulin are shown in Figure 2.3. Values just outside the range of the standard curve were estimated by extrapolation and the insulin content was subsequently confirmed by radioimmunoassay after dilution. In later experiments a computer programme was utilised for the calculation of results (Appendix 3, page 137). Inter- and intra-assay coefficients of variation were found to be 10% and 7% respectively.

Quality Control (criteria for a satisfactory assay)

There was generally good agreement between replicates of standards and unknowns (cpm. generally within 2 - 3% of the mean values). Blank values were generally less than 5% of the total counts and indicated the efficiency of the washing procedure. The Blank value was a measure of the free insulin attached to the walls of the tubes after decantation and reflected the degree of separation of bound from free insulin. The zero standard count rate was always in the range 30 - 50% of the total count rate. The day to day variation in this value did not normally exceed 5%.

Figure 2.3. Typical RIA Standard Curves for (a) Mouse and (b) Human Insulin.



CHAPTER 3

A. ASSESSMENT OF THE PHYSICAL CRITERIA FOR THE OPTIMAL PERFORMANCE OF PROSTHETIC UNITS.

Introduction

In this section, studies have been carried out to assess the transfer kinetics and the performance of the unit *in vitro*.

The effect of alterations in perfusion pressure, flow rate through the unit and the permeability of the fibre has been investigated on the kinetics of methylene blue and ^{125}I-monoiodinated insulin transfer across the hollow fibre membrane. In addition, investigation has been made of the effect of prolonged perfusion (up to 450 minutes with methylene blue and 125 minutes with labelled insulin) on the rate of removal (or release) of dye and ^{125}I-insulin from prototype units.

Finally, preliminary assessment has been made of the feasibility of coating hollow fibres with protein using glutaraldehyde cross-linking (189) to reduce thrombogenicity and the effect this coating has on the transfer and release of ^{125}I-insulin from prototype units.

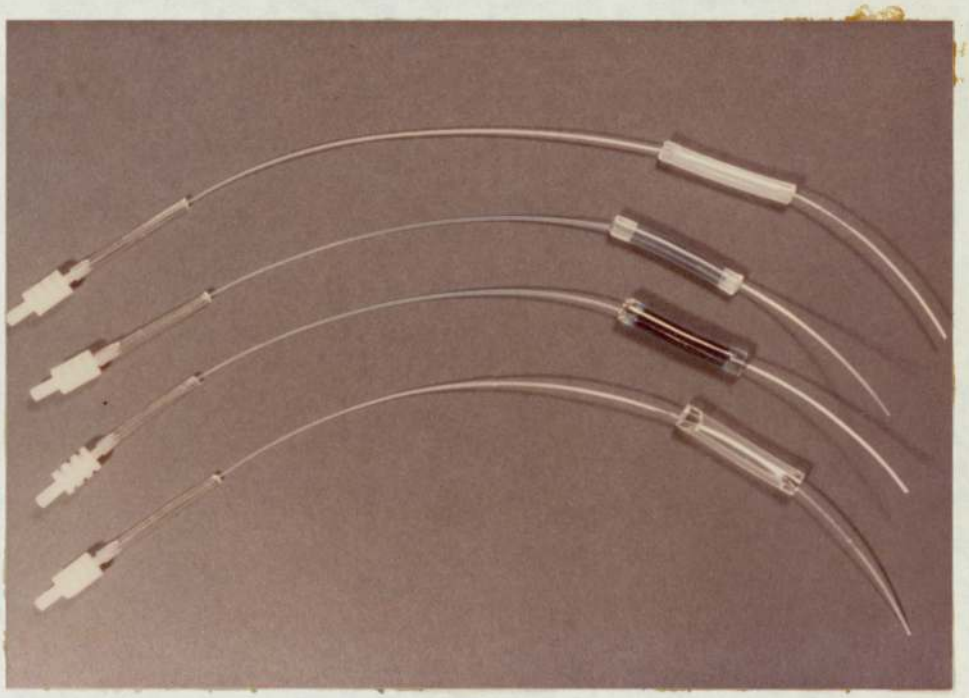
(1) The Release of Methylene Blue from Prototype Units

Materials and Methods

Units 3 cm in length were constructed as described in Section 2.7. (page 45) and their outer cavity filled with the neutral dye methylene blue (M.W.319.5) dissolved in sterile, double distilled water at a concentration of 1 mg/ml (PLATE 4).

Individual units were attached to a peristaltic pump (P-3, Pharmacia) and perfused at various flow rates with sterile, double distilled water at 20°C. 25 minute fractions were

PLATE 4. A Range of Prosthetic Units, Some Containing Methylene Blue Dye.



collected at each of the ten flow rate settings on the pump and the effect of varying the flow rate from 0.042 ml/min . to 0.637 ml/min. and hence varying the perfusion pressure (Fig.3.1) from 7.8 ±2.0 to 87.2 ±6.33 mm Hg was investigated on the release of dye from the units. The absorbance of unit effluent was measured at 665 nm (absorbance maximum of methylene blue) using matched glass micro-cuvettes. The concentration of methylene blue (µg/ml) in each fraction was estimated by reference to a standard curve of absorbance versus methylene blue concentration (Fig. 3.2) and the rate of release of dye (µg/min.) at each flow rate calculated.

The amount of methylene blue released from units over a period of 24 minutes' perfusion was determined at flow rates of 0.073 ml /min., 0.326 ml /min. and 0.537 ml /min. The amount of dye released from units over a period of extended perfusion (450 minutes) was determined at flow rates of 0.073 ml /min., 0.162 ml /min. and 0.637 ml /min.

Results

A linear relationship was established between the perfusion pressure and flow rate over the flow rate range of 0.042 ml/min. to 0.637 ml/min., (Fig.3.1). The rate of release of methylene blue from units increased with the flow rate (Fig.3.3) from 0.161 µg/min. of dye at a flow rate of 0.042 ml/min. to a maximum of 0.227 µg/min. of dye at a flow rate of 0.162 ml/min. Over the intermediate range of flow rates (0.249 ml/min. to 0.469 ml/min.) the rate of release of dye remained fairly constant, but as the flow rate increased further the rate of dye release began to fall off. The slowest rate of release, 0.126 µg/min., was observed at the maximum flow rate setting of 0.637 ml/min.

Further examination of the rate of release of methylene blue (µg/min.) and the cumulative mass removed (µg) over a period of

Figure 3.1. The Relationship between Flow Rate through the Unit and Perfusion Pressure when Perfused with Double Distilled Water at 20°C.

Mean values ± S.E.M.; n = 6 for each point.

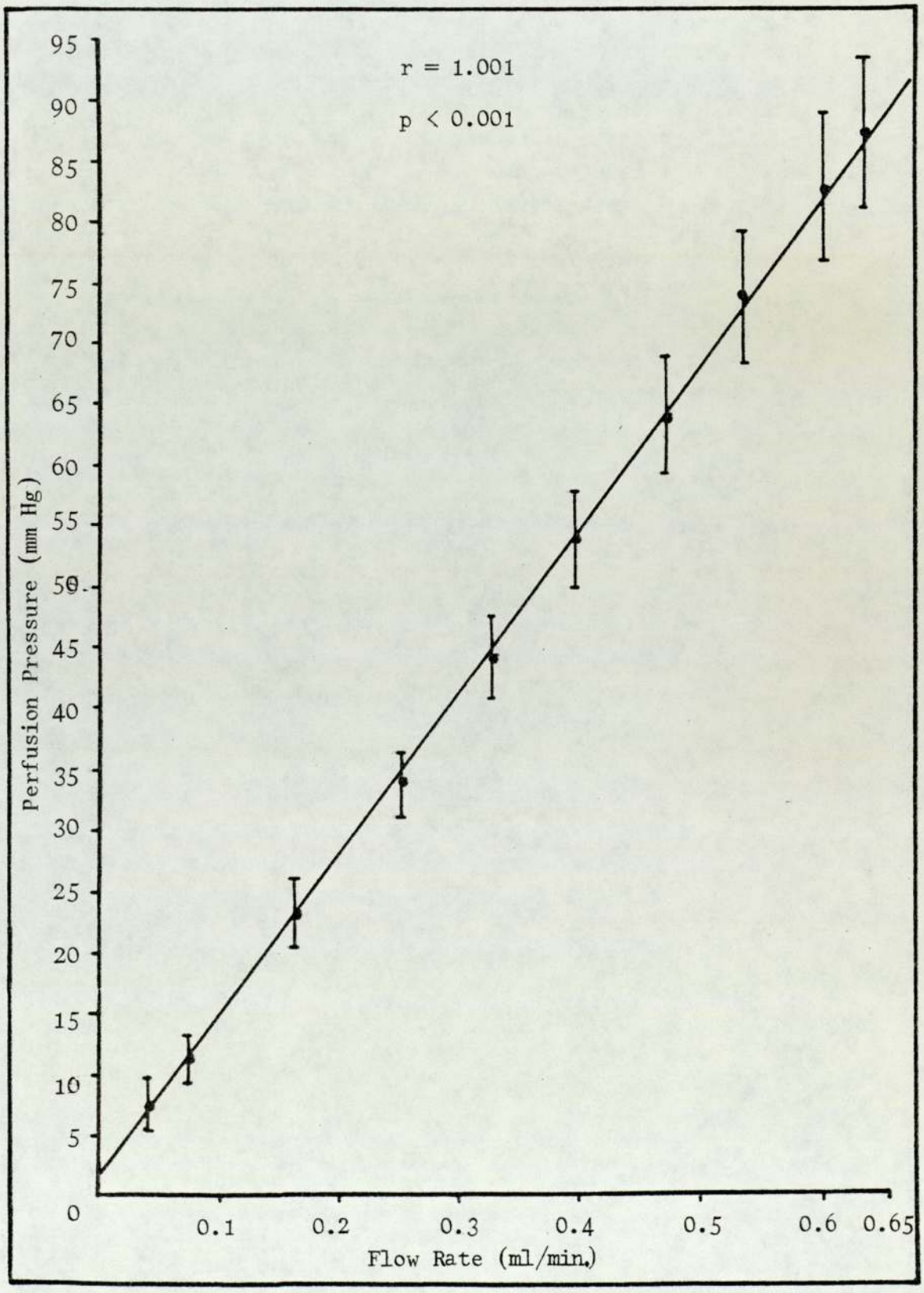


Figure 3.2. Standard Curve for the Estimation of Methylene Blue Concentration in Unit Effluent.
Mean values ± S.E.M.; n = 11 for each point.

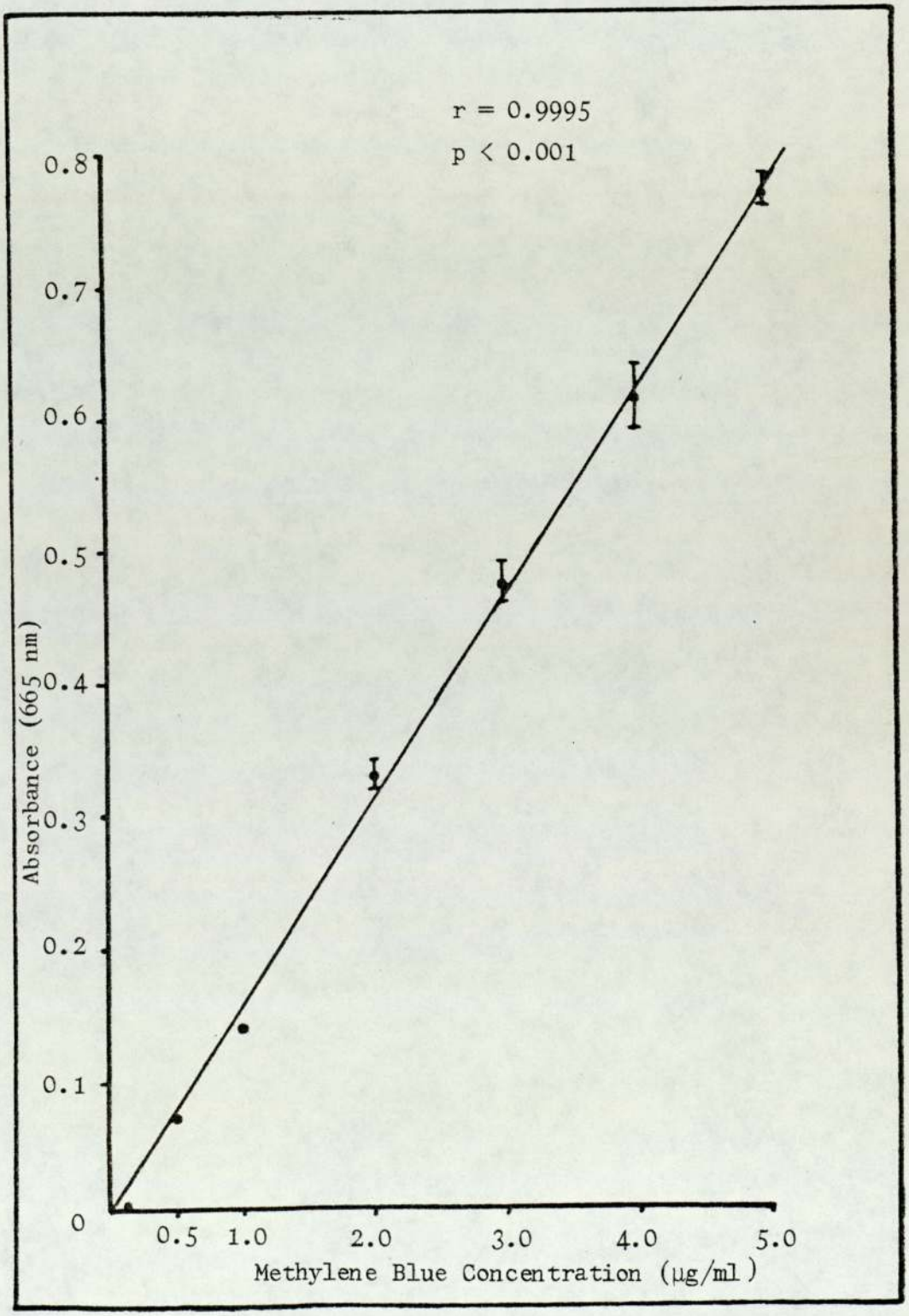
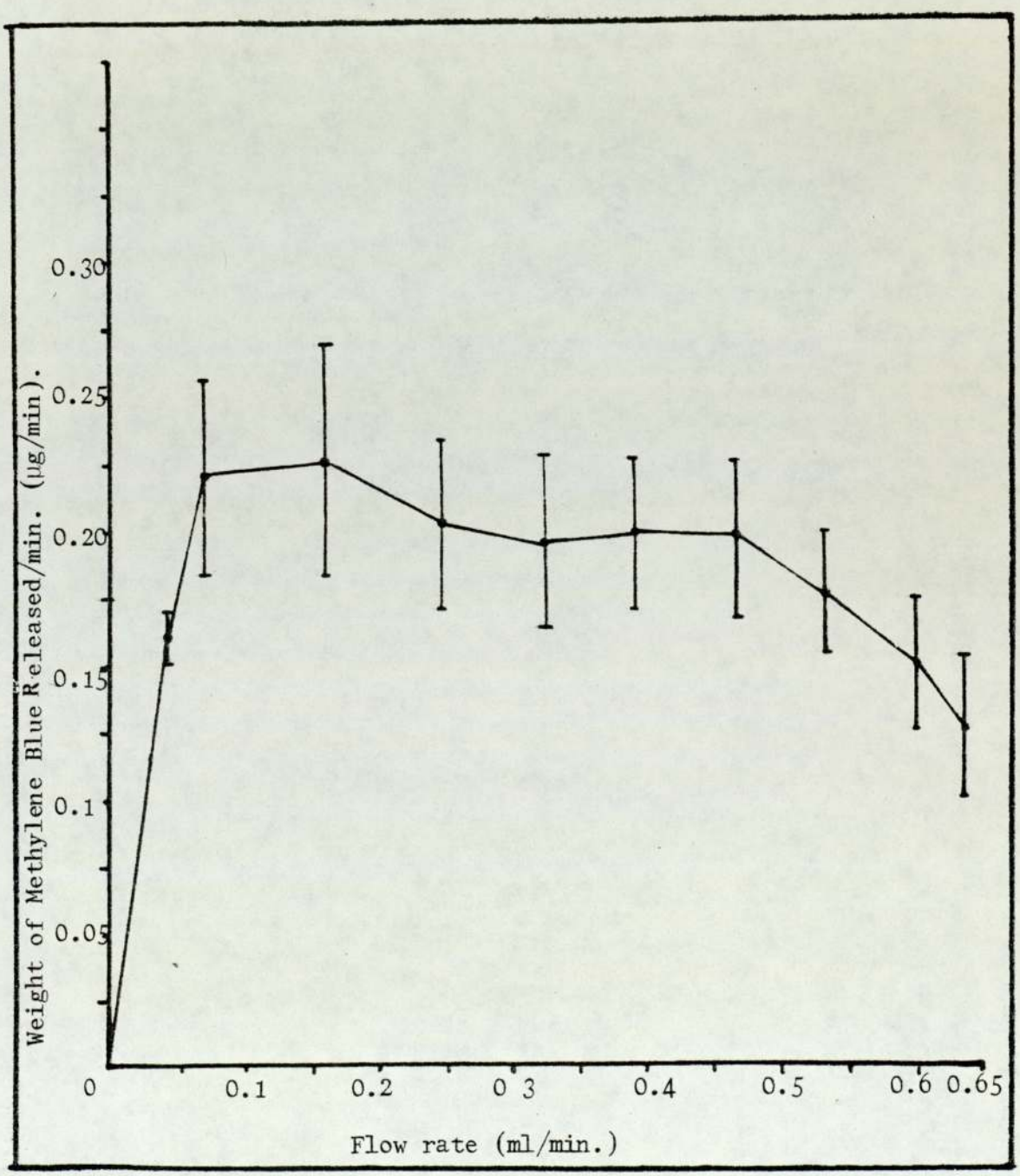


Figure 3.3. The Relationship between the Weight of Methylene Blue Released and the Flow Rate through the Unit at 20°C. Mean values ± S.E.M.; n = 6 for each point.



24 minutes' perfusion at three intermediate flow rate settings, namely 0.073 ml/min., 0.326 ml/min. and 0.537 ml/min. (Fig.3.4) confirmed that over this range of flow rates the rate of release of dye remained fairly constant, 0.363 µg/min., 0.408 µg/min., and 0.404 µg/min. respectively. Similarly, the cumulative mass of dye released at the three flow rates after 24 minutes' perfusion, did not vary significantly with flow rate and provided weights of 10.3 µg, 10.1 µg and 9.7 µg respectively (Fig.3.4).

The rates of release of methylene blue from units perfused for up to 450 minutes at two low flow rates of 0.073 ml/min. and 0.162 ml/min. and one high flow rate of 0.637 ml/min. were found to be 0.249 µg/min., 0.269 µg/min and 0.164 µg/min. respectively (Fig.3.5). The cumulative amounts of dye removed after 450 minutes' perfusion at each of these flow rates was 115.7 µg., 121.6 µg and 77.6 µg respectively.

In all the fixed flow rate perfusion experiments, the cumulative amount of dye removed was directly proportional to the perfusion time (Fig.3.5).

Discussion

These initial studies have shown that the maximum rate of release of dye from prototype units occurred over a range of relatively low flow rates, i.e. 0.15 - 0.25 ml/min. However, the perfusion pressure at these flow rates was only 20 - 30 mm Hg, considerably lower than normal blood pressure. Whilst it was desirable that the transfer of dye or other solutes across the hollow fibre membrane should be as rapid as possible, there was a need to balance this with a physiological perfusion pressure operating through the unit. On this basis, the optimal conditions for unit perfusion in the in vitro system involved a maximum flow rate of 0.637 ml/min. and a mean perfusion pressure of 87.2 mm Hg providing a rate of dye release of

Figure 3.4. Cumulative Mass of Methylene Blue Released from Units Over a Period of 24 Minutes' Perfusion at 3 Intermediate Flow Rates. □- - -□ (0.073 ml/min.); ●——● (0.326 ml/min.); x - - - x (0.537 ml/min.). Data shown represent single determinations.

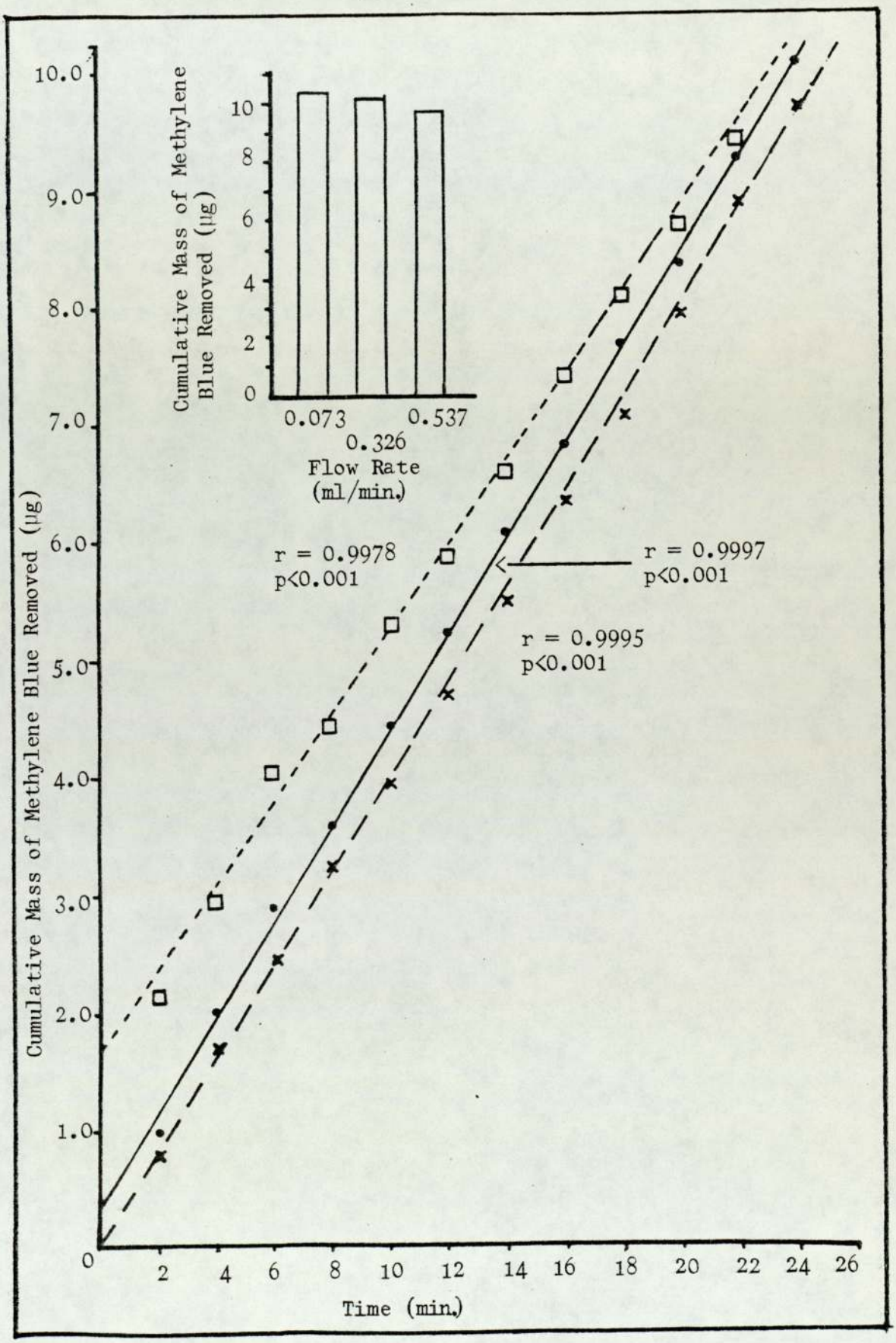
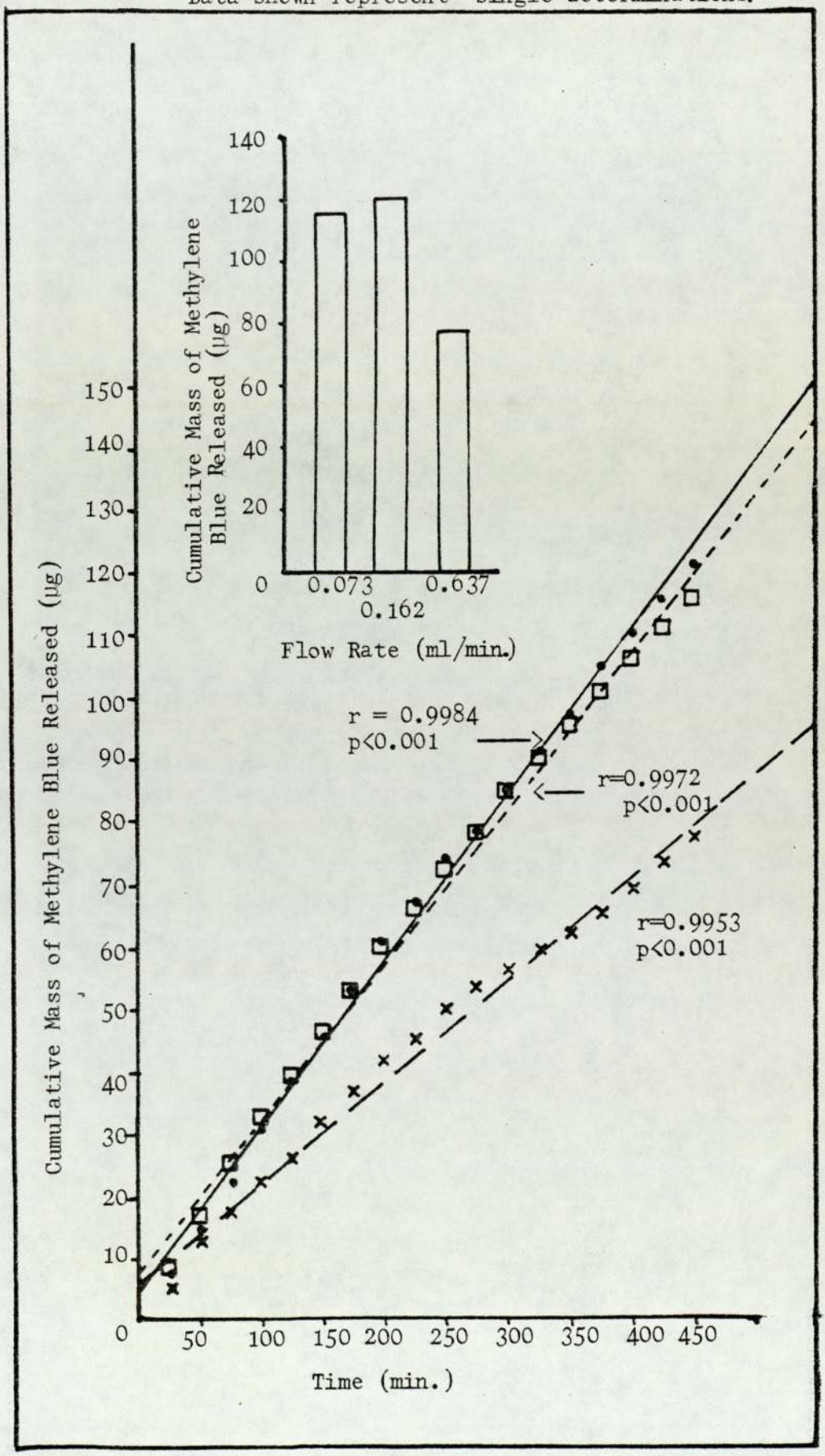


Figure 3.5. Cumulative Mass of Methylene Blue Released from Units Over a Period of 450 Minutes' Perfusion at Three Fixed Flow Rates. □ - - □ (0.073 ml/min.); ●——● (0.162 ml/min.); x - - - x (0.637 ml/min.). Data shown represent single determinations.



between 55 to 65% of maximum.

(2) The Release of ^{125}I-Insulin from Prototype Units

Materials and Methods

Units constructed as described in section 2.7 (page 45) were filled with a range of ^{125}I-monoiodinated insulin concentrations (18 - 72 ng/ml, specific activity 200-250 μCi/μg) sealed, submerged in an agitated water bath at 37°C and perfused with HBSS, gassed with 95% O_2/5% CO_2 and supplemented with BSA V at a concentration of either 0.5 g/100 ml or 7.45 g/100 ml.

The relationship between flow rate and perfusion pressure was investigated when units were perfused with HBSS containing a low concentration of BSA (0.5 g/100 ml)(Fig.3.6) and a high concentration of BSA (7.45g/100 ml)(Fig.3.7). The high concentration represented the normal total protein concentration of blood (North Birmingham Health District Data).

The effect of flow rate on the amount of ^{125}I-insulin released per minute from prototype units was determined in the presence of three different starting insulin concentrations, 72 ng/ml, 36 ng/ml and 18 ng/ml (Fig.3.8).

This was followed by measurement of the cumulative mass of ^{125}I-insulin released over a period of 125 minutes' perfusion at a rate of 0.637 ml/min. from units containing 72 ng/ml, 36 ng/ml and 18 ng/ml insulin. Perfusions were carried out with buffer containing both low (0.5 g/100 ml) and high (7.45 g/100 ml) protein concentrations (Fig.3.9).

The effect of 100 minutes' perfusion at 0.637 ml/min. on the cumulative mass of insulin released from units containing 12.5 ng/ml and 25 ng/ml ^{125}I-insulin has been evaluated with perfusion buffers containing 7.45 g/100 ml and 0.5 g/100 ml BSA respectively, (Fig.3.10).

Figure 3.6. The Relationship between Flow Rate and Perfusion Pressure through Units in the Presence of Low BSA (0.5g/100 ml). Data expressed as mean values ± S.E.M.; n = 12 for each point.

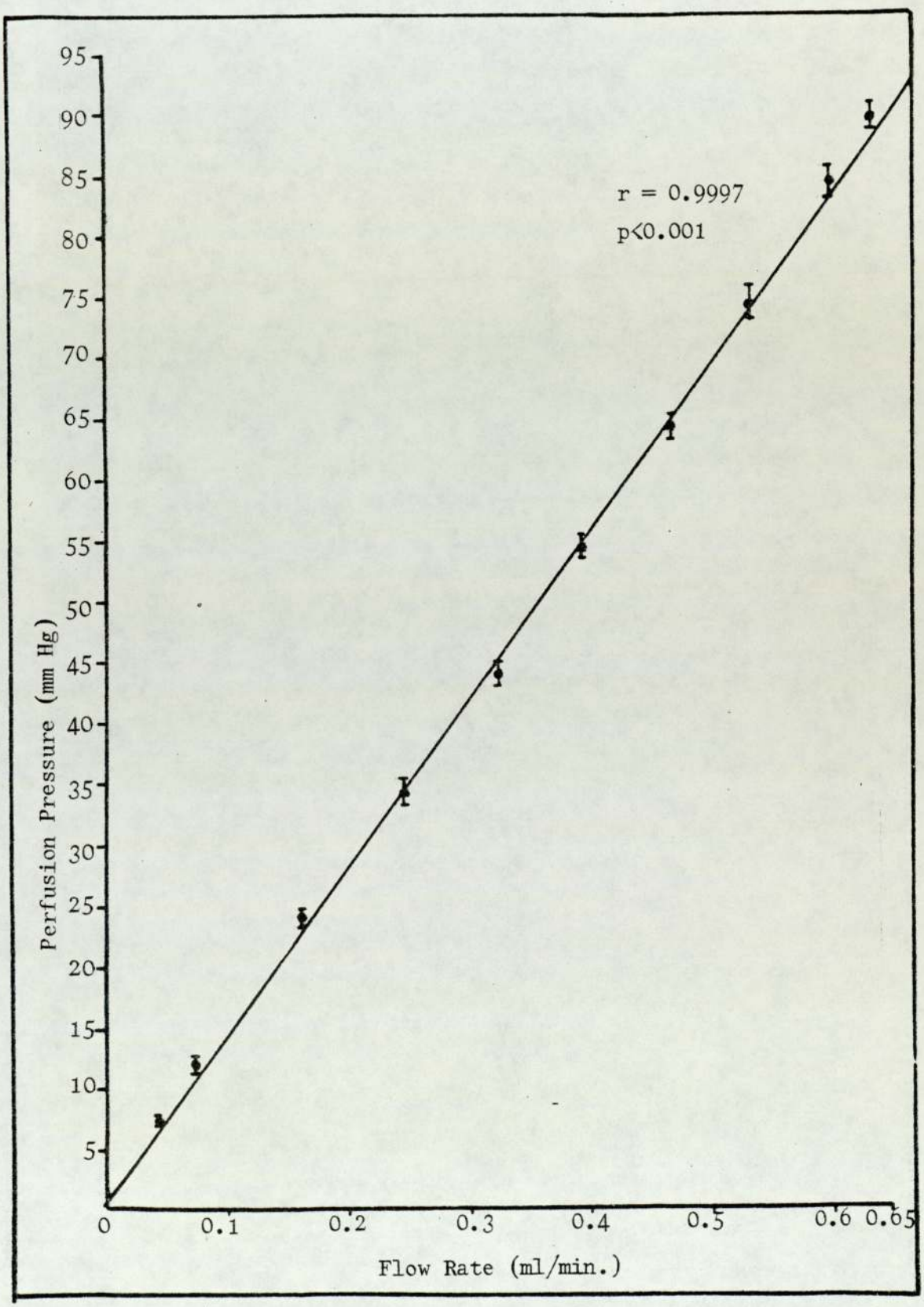


Figure 3.7. The Relationship between Flow Rate and Perfusion Pressure through Units in the Presence of Low BSA (0.5g/100 ml; ●——●) and High BSA (7.45g/100 ml; x - - x). Data are expressed as mean values ± S.E.M.; n = 6 for each point.

* $p<0.05$ compared with low BSA.

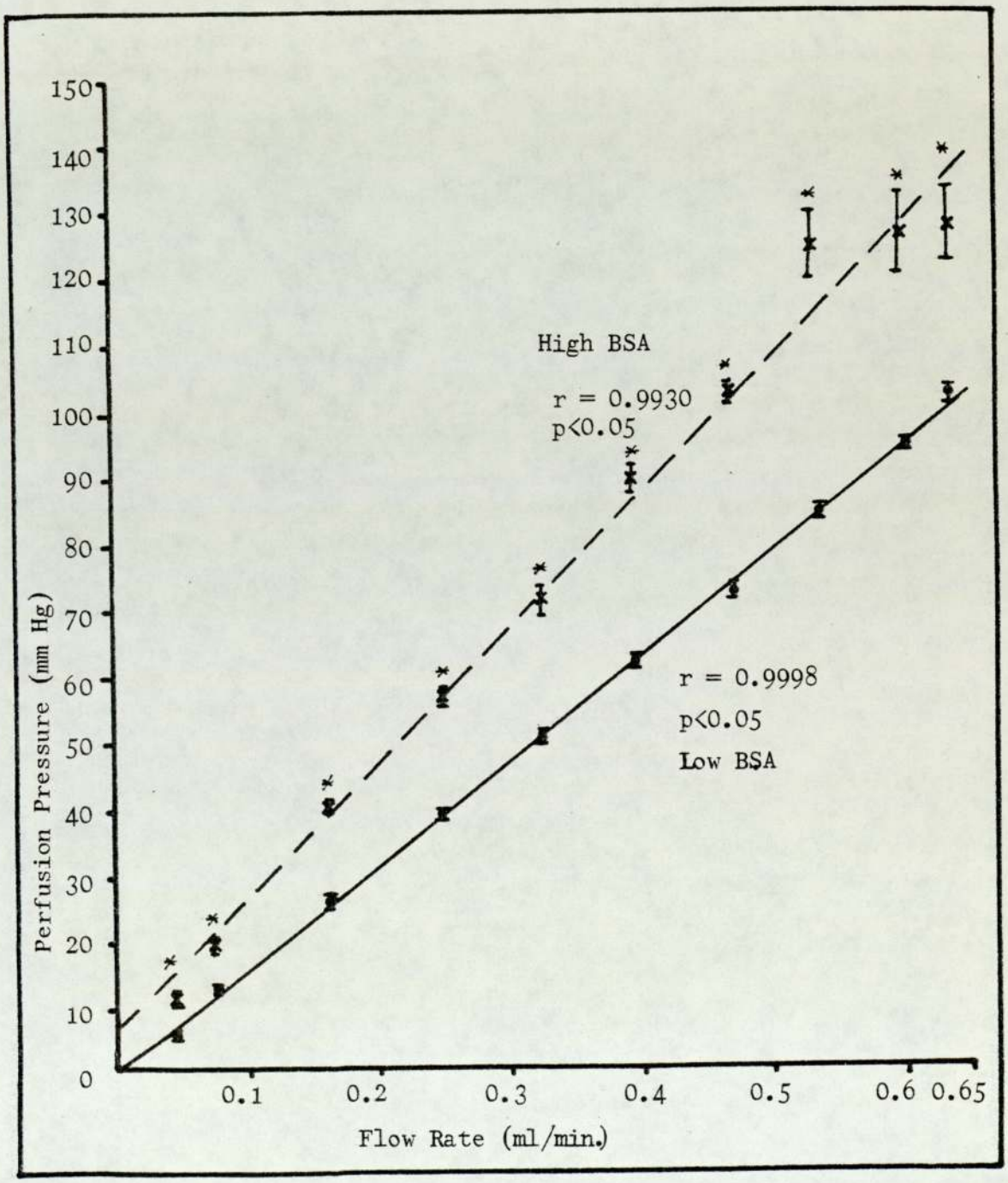


Figure 3.8. The Effect of Flow Rate on the Amount of ^{125}I-Insulin Removed from Units Perfused with HBSS Supplemented with Low BSA (0.5g/100 ml) at 37°C.

Initial concentration of ^{125}I-insulin in units: □- - □ (72 ng/ml); ●——● (36 ng/ml); x - - - x (18 ng/ml). Data are expressed as mean values ± S.E.M.; n = 4 for each point.

* $p<0.05$ compared with 72 ng/ml.

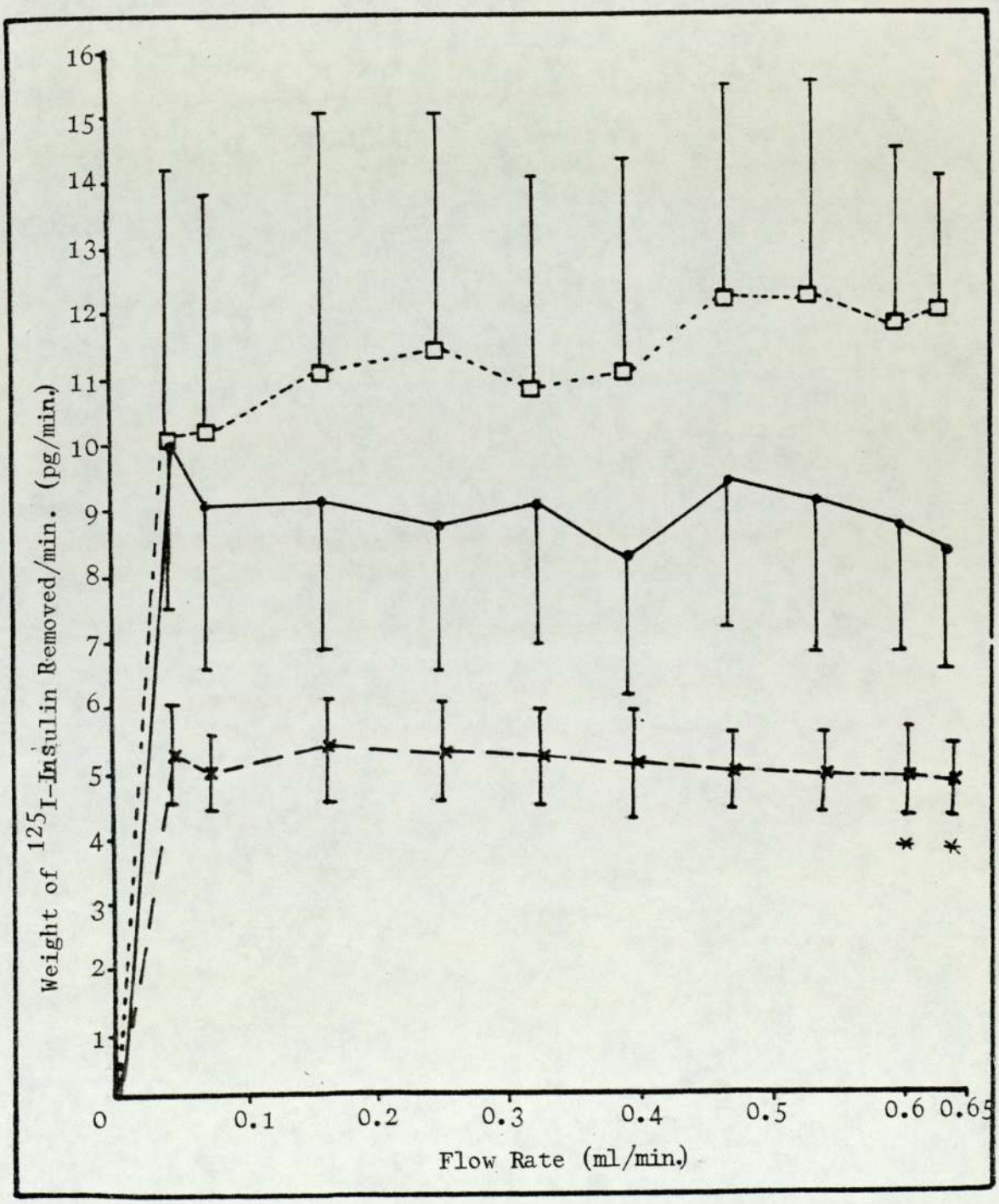


Figure 3.9. The Cumulative Mass of ^{125}I-Insulin Released from Units containing 72 (- - -), 36 (———) and 18 (— — — —) ng/ml ^{125}I-Insulin, Over a Period of 125 Minutes' Perfusion at a Flow Rate of 0.637 ml/min., in the Presence of Low (0.5g/100 ml) (□) and High (7.45g/100ml) (•) BSA. Data shown represent single determinations.

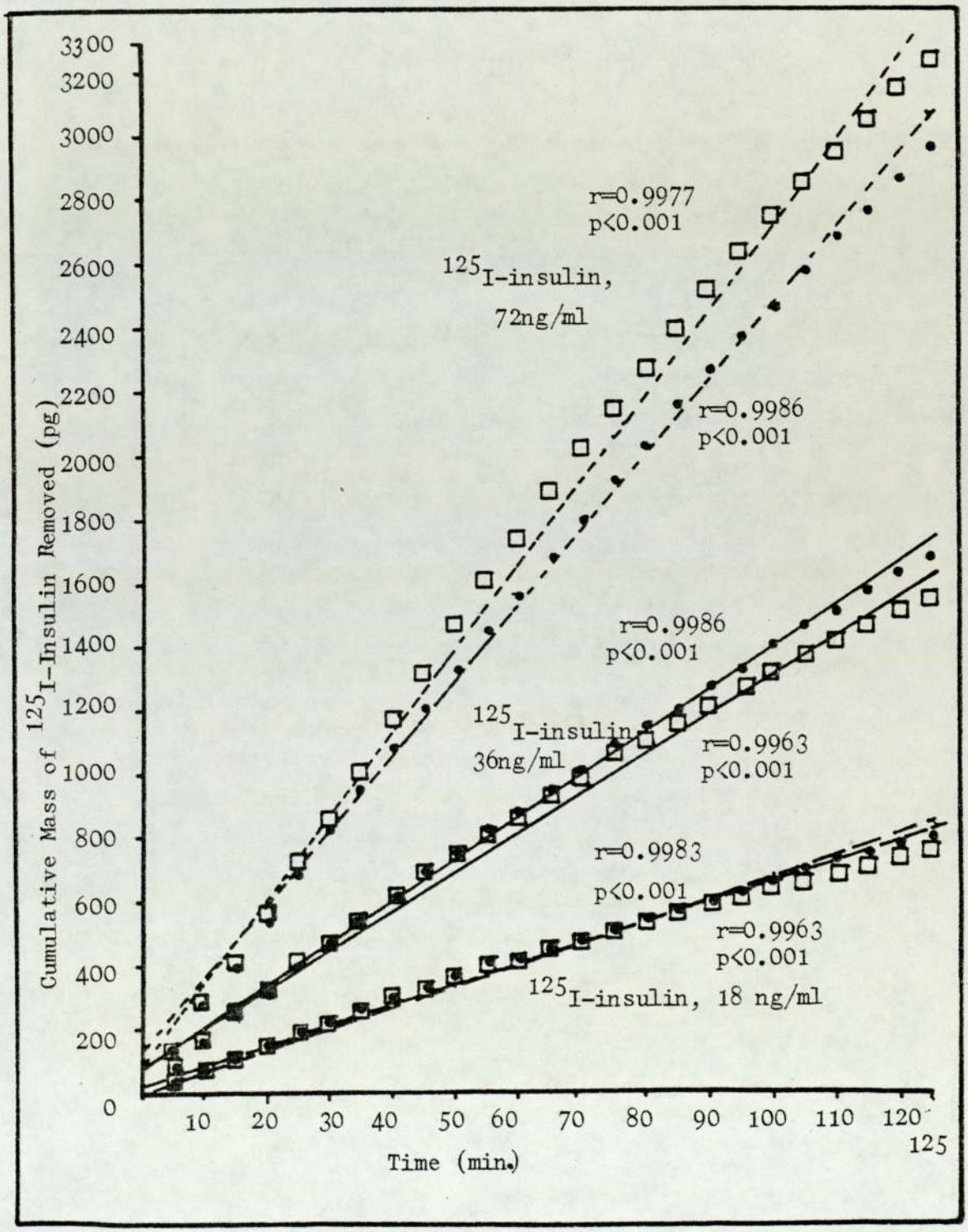
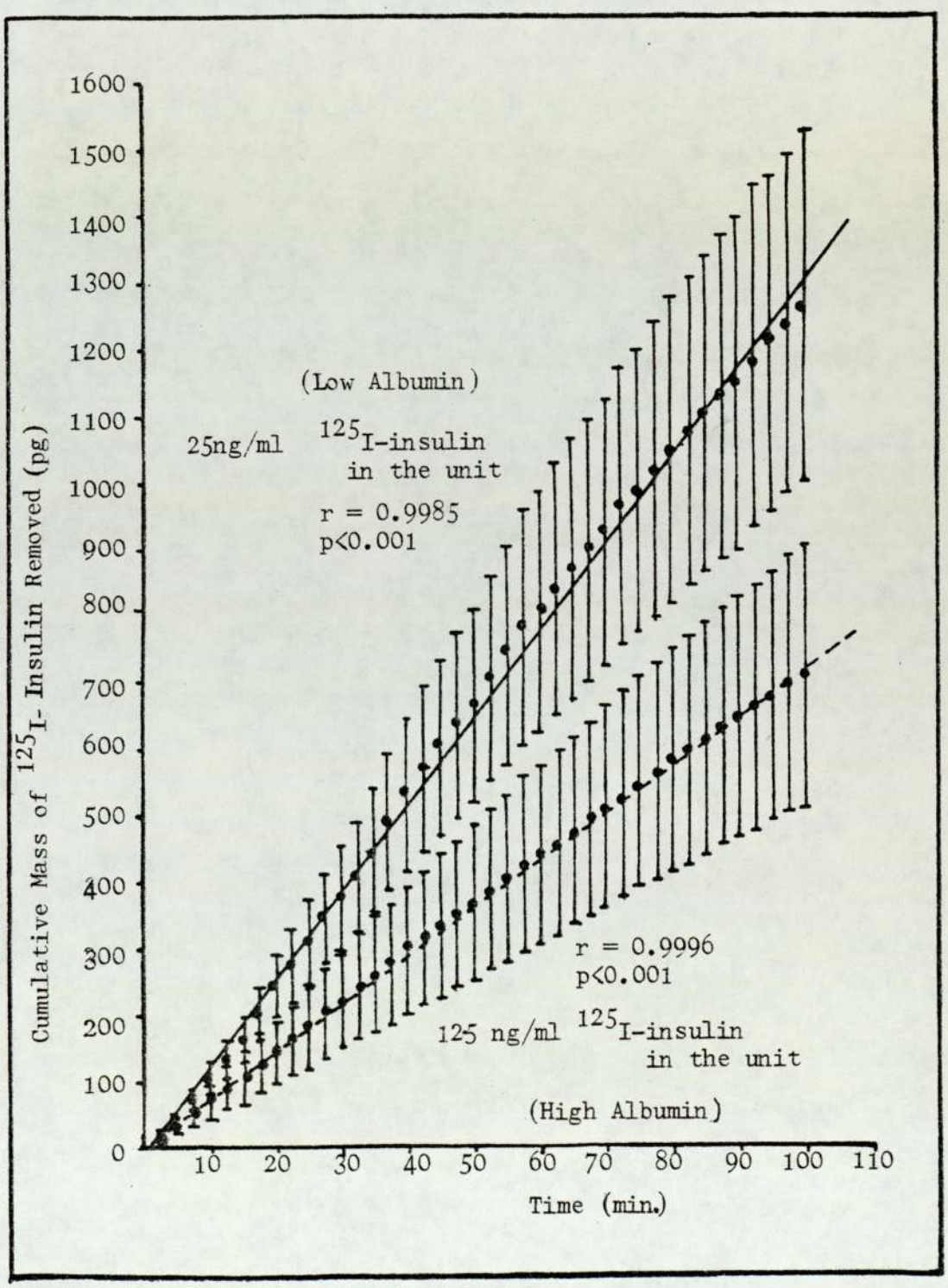


Figure 3.10 . The Cumulative Mass of ^{125}I-Insulin Released from Units Initially Containing 12.5 ng/ml Insulin (●- - -●) and 25 ng/ml Insulin (●———●) when Perfused for 100 Minutes at a Flow Rate of 0.637 ml/min. with Buffer Containing High Albumin (7.45g/100 ml) and Low Albumin (0.5g/100 ml) Respectively. Data expressed as mean values ± S.E.M.; n = 6 for each point.



Results

Increasing the flow rate produced a proportional increase in perfusion pressure when units were perfused with buffer containing either low (0.5g/100 ml) or high (7.45g/100 ml) BSA (Fig.3.7). At each flow rate the perfusion pressure was significantly higher when units were perfused with buffer containing high BSA than when perfused with buffer containing low BSA. In the presence of high BSA, the perfusion pressure at the maximum flow rate of 0.637 ml /min. was 126.50±5.84 mm Hg and consistent with the value for systolic blood pressure in man (Fig.3.7).

The rate of release of labelled insulin from units containing 18 and 36 ng/ml ^{125}I-insulin decreased marginally with increasing flow rate (Fig.3.8), while the rate of release from units containing 72 ng/ml ^{125}I-insulin was slightly increased. The rate of release of labelled insulin depended on the initial concentration of ^{125}I-insulin in the units. The rate of ^{125}I-insulin release was almost doubled when the concentration of insulin in the unit was increased from 18 ng/ml to 36 ng/ml and further increased when the concentration was raised to 72 ng/ml. However, due to the large standard errors of the mean associated with the data, the only statistically significant difference in the rate of release of labelled insulin was observed between units containing 18 ng/ml and 72 ng/ml ^{125}I-insulin at flow rates of 0.603 and 0.637 ml/min.

The proportional relationship between the rate of release and the initial unit concentration of labelled insulin can be seen more clearly in Fig.3.9. Irrespective of BSA concentration, the cumulative mass of labelled insulin released after 125 minutes was increased twofold when the initial unit insulin concentration was increased from 18 to 36 ng/ml and fourfold when the initial insulin concentration was increased to 72 ng/ml. In the presence of low BSA (0.5 g/100 ml) the

cumulative mass of labelled insulin removed over 125 minutes from units containing 18 ng/ml, 36 ng/ml and 72 ng/ml ^{125}I-insulin was 760, 1550 and 3240 pg respectively. When perfused with high BSA (7.45g/100 ml) similar units provided 800, 1680 and 2970 pg of ^{125}I-insulin respectively.

The effects of 100 minutes' perfusion at 0.637 ml/min. on the rate of release of ^{125}I-insulin from units containing initial insulin concentrations of either 25 ng/ml or 12.5 ng/ml are shown in Fig.3.10.

Again doubling the initial labelled insulin concentration of units from 12.5 to 25 ng/ml produced a two-fold increase in their secretory capacity from 707 ± 194 pg to 1262±263 pg respectively. However, because of the large standard errors of the mean associated with these data, statistical significance of the difference could not be confirmed.

In all prolonged perfusion experiments the cumulative mass of ^{125}I-insulin removed with time was linear ($p<0.05$ for all correlation coefficients). Increasing the protein content of the perfusate from 0.5 to 7.45 g/100 ml did not appear to significantly affect the secretory performance of the unit. This was critically dependent on the initial insulin concentration in the outer compartment.

Discussion

Studies with labelled insulin have shown that increasing the BSA concentration in the system from 0.5g/100 ml (a concentration chosen to prevent the adsorption of insulin to hollow fibre and cannula tubing) to 7.45g/100 ml (a concentration which represents the total protein concentration in human serum) caused a significant increase in the perfusion pressure through units. In the presence of 7.45 g/100 ml BSA, a flow rate of 0.637 ml/min. provided a perfusion pressure equivalent to the systolic blood pressure in man.

The rate of release of labelled insulin from units was not greatly influenced by the flow rate (Fig.3.8) but depended on the initial concentration of ^{125}I-insulin in the outer compartment (Fig.3.9). In prolonged perfusion experiments, doubling the ^{125}I-insulin concentration in the unit produced a twofold increase in the rate and cumulative mass of insulin released (Fig.3.10). In addition, the cumulative mass of insulin released from units was not affected by the concentration of BSA in the buffer (Fig.3.9). The initial labelled insulin studies indicate that the optimum criteria for the perfusion of 3 cm units *in vitro* involve a flow rate of 0.637ml/min. at 37°C, a buffer protein concentration of 7.45 g/100 ml. providing a perfusion pressure close to physiological of 120 - 130 mm Hg.

(3) ^{125}I-Insulin Release from Prosthetic Units Containing Protein-Coated Hollow Fibres

Materials and Methods

Hollow fibres were coated with bovine serum albumin to reduce thrombogenicity by a cross-linking method using glutaraldehyde (189). The effect this coating had on the release of ^{125}I-insulin from coated units was then investigated.

A completely assembled fibre (fibre connected to cannulae) was perfused at 0.637 ml/min. for approximately 80 minutes with 0.02M phosphate buffer, pH 6.8, containing 0.01M glutaraldehyde and 6.8 g/100 ml BSA. After allowing a period of 30 minutes for the coating to gel the fibre was perfused for 15 minutes with 0.15M sodium chloride to elute free glutaraldehyde molecules. The coated fibre was then perfused for a further 15 minutes with 0.13M glycine solution to block free glutaraldehyde functions and avoid any further chemical reaction, particularly between the coating and any exogenous proteins. Finally, the coated fibre was again perfused for 15 minutes with 0.15M sodium chloride and retained in physiological saline until required.

Attempts were also made to produce coated fibres using a more dilute solution of bovine serum albumin (68 mg/100 ml). Coating times and rinsing times were reduced to a quarter of those previously used.

Coated fibres were subsequently incorporated into prosthetic units and perfusion experiments were carried out to compare the performance of units containing coated and uncoated fibres. All units were perfused for 100 minutes with HBSS, pH 7.4, containing BSA (7.45 g/100 ml) at a flow rate of 0.637 ml/min. The initial concentration of ^{125}I-insulin in all units was adjusted to 7 ng/ml and measurement was made of the cumulative mass of labelled insulin released over a period of 100 minutes, (Fig.3.11).

The amount of bound and unbound surface bovine serum albumin released from coated units (dilute BSA coating) during perfusion was investigated by perfusing units with HBSS containing no BSA and measuring the concentration of protein eluted with time (Fig.3.12). The absorbance of perfusate was measured at 280 nm and the protein content determined by reference to a standard curve, (Fig.3.13).

The extent of protein coating on the unit hollow fibres was examined by electron microscopy. Small rings of coated hollow fibre were fixed in 0.05 M sodium cacodylate buffer, pH 7.4 containing 1% glutaraldehyde and 15% sucrose. The rings were then washed overnight with a 1:1 mixture of sodium cacodylate buffer and 15% sucrose and subsequently post fixed with 1% osmium tetroxide in 0.05M veronal acetate buffer, pH 7.4, prior to dehydration and embedding in gelatin capsules containing an epoxy-based resin. Capsules and their contents were cured for 48 h at 60°C, after which time the gelatin capsules were removed by immersion in hot water. The blocks were allowed to harden at 60°C in an oven and thin sections (grey/gold interference colour) were cut on an LKB ultra microtome using glass knives and

Figure 3.11. The Effect of Protein-Coating Hollow Fibres on the Amount of ^{125}I-Insulin Released over 100 Minutes' Perfusion at 0.637 ml/min. in the Presence of High BSA (7.45 g/100 ml). Uncoated control (□ - - - □), coated (•——— •) and dilute coated (x- - - x) fibres.

Data shown represent single determinations.

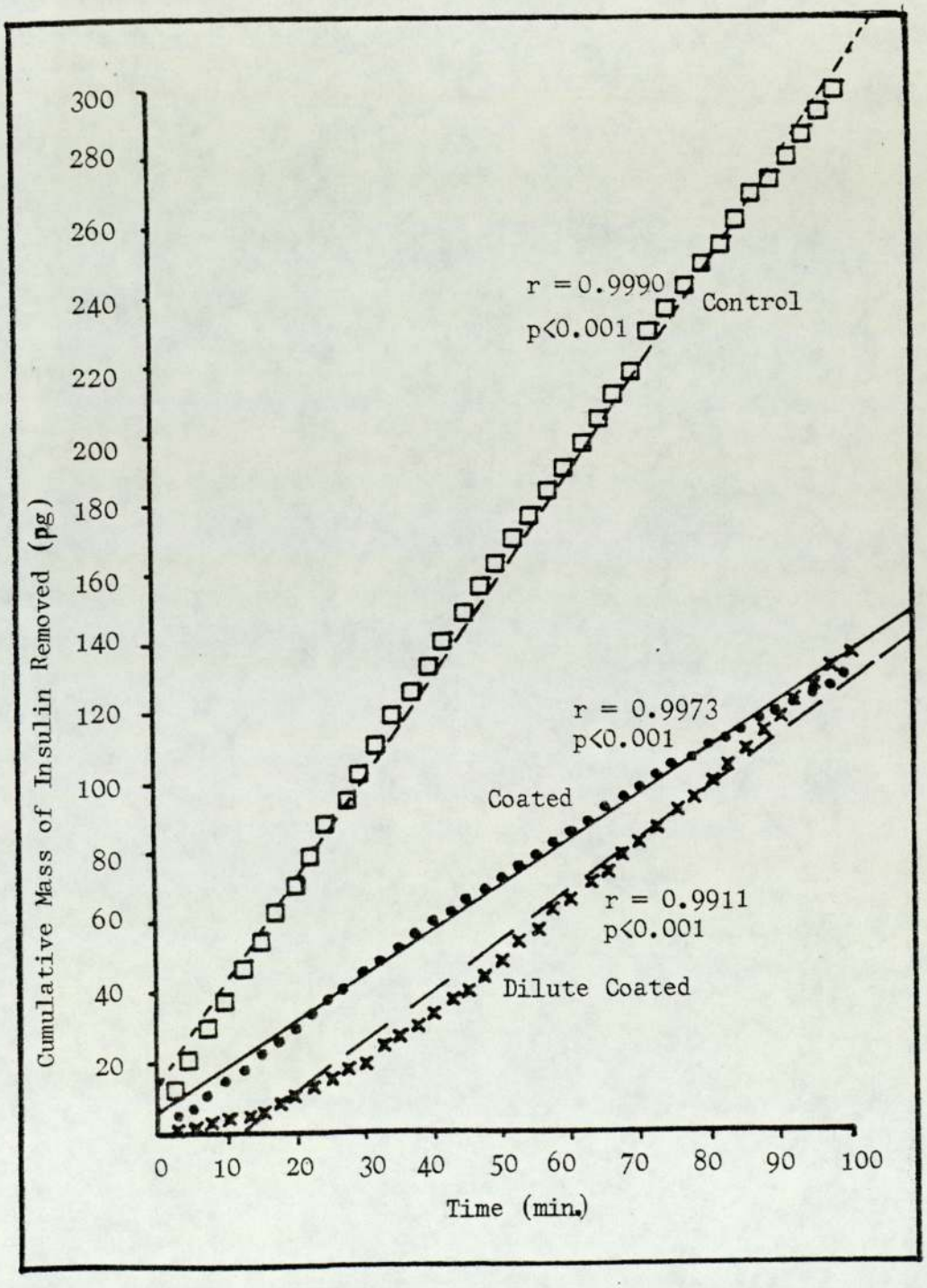


Figure 3.12. Elution of Unbound Protein from a Dilute Coated Unit with Time. Unit perfused for 100 minutes at a flow rate of 0.637 ml/min.

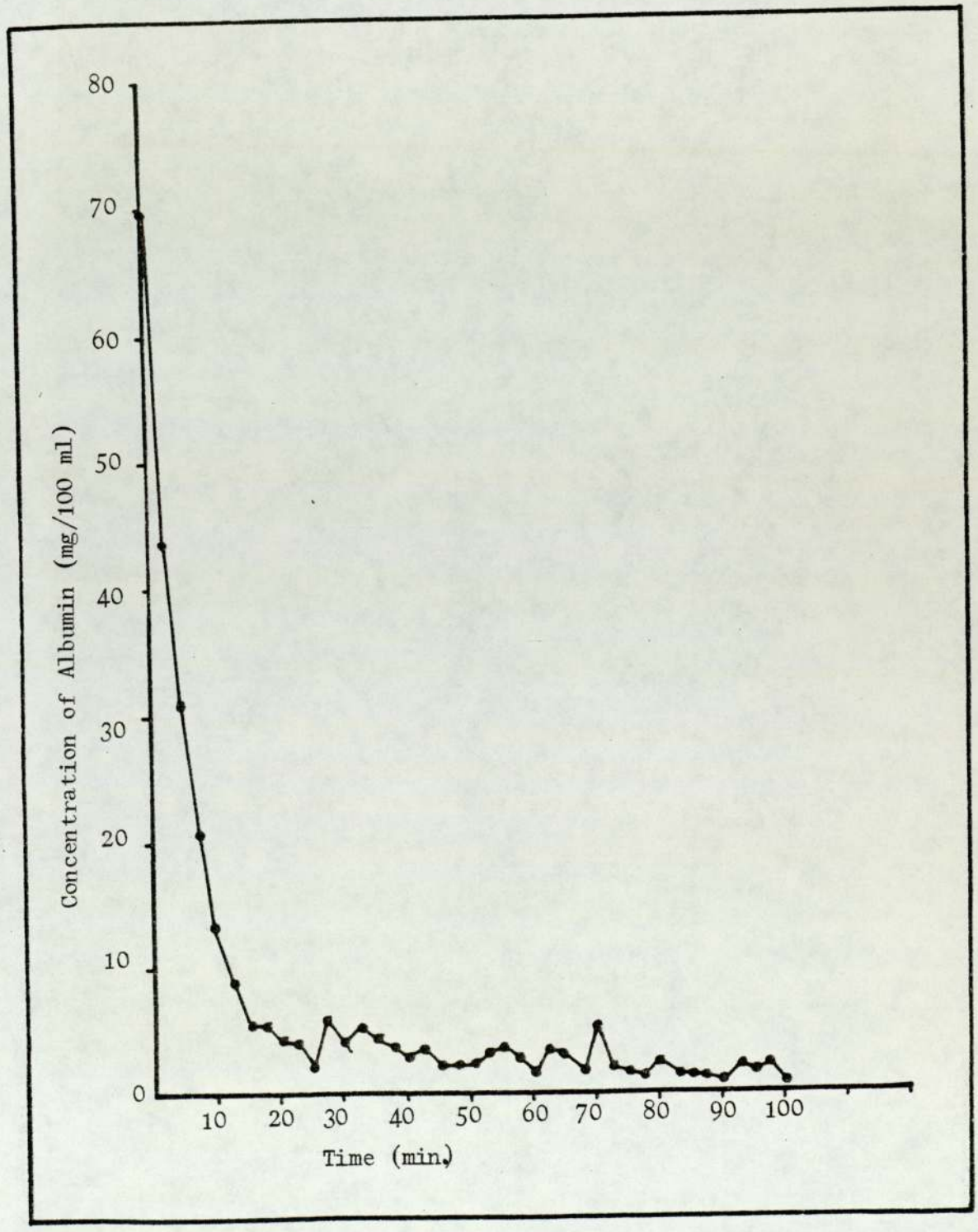
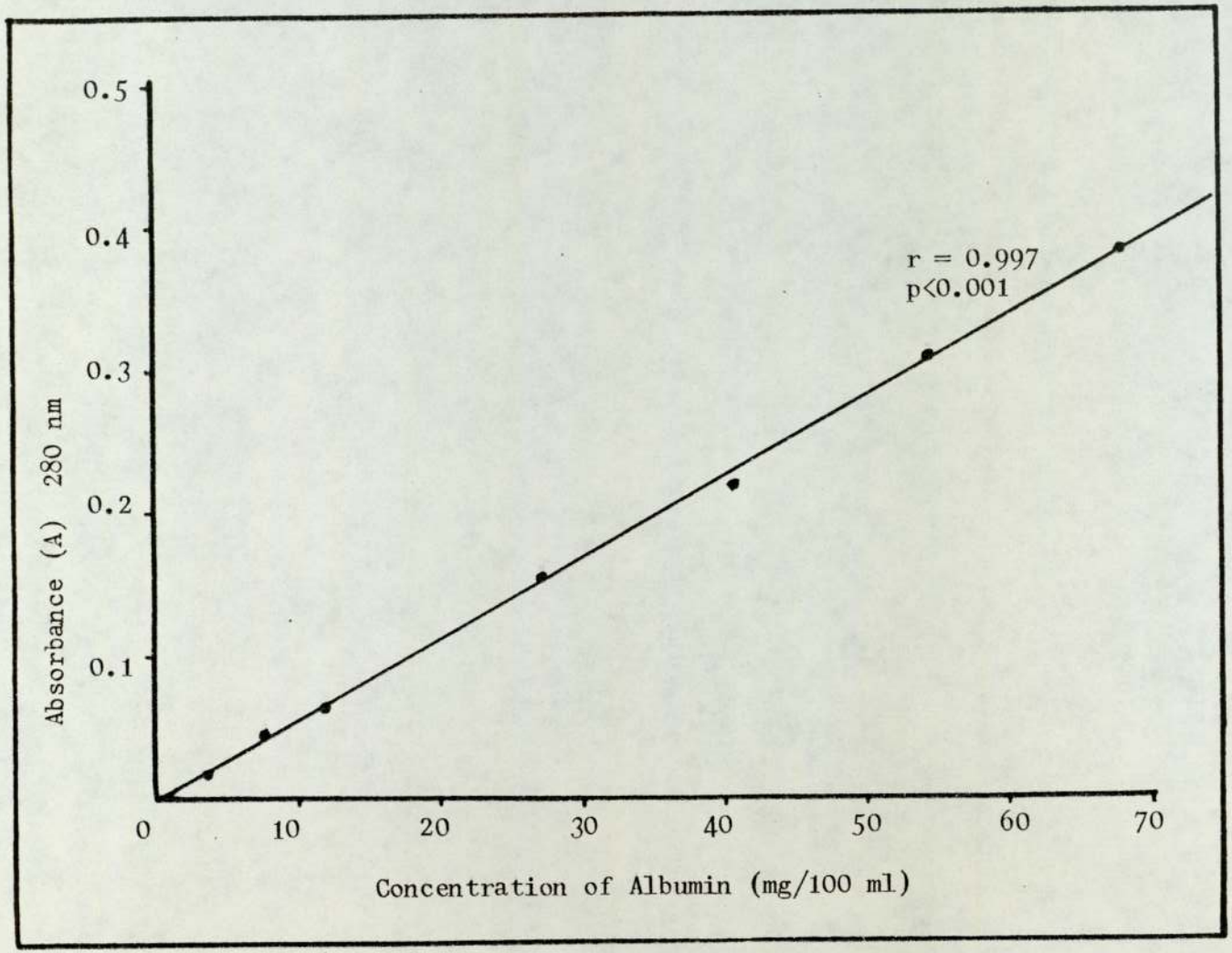


Figure 3.13. Standard Curve for the Estimation of Albumin Concentration in Unit Effluent. Data shown represent single determinations.



mounted on carbon grids. Unstained sections were examined under an AEI transmission electron microscope at 3,000 x magnification. Photomicrographs were taken of the coated luminal surface of fibres, PLATE 5.

Results

Coating hollow fibres with BSA (cross-linked with glutaraldehyde) reduced the rate of release of ^{125}I-insulin and the cumulative mass of ^{125}I-insulin released after 100 minutes' perfusion by more than 50% compared to the uncoated control irrespective of the type of coating employed. The cumulative mass released from the control unit over 100 minutes was 298.73 pg. The cumulative mass released from the coated and the dilute coated units was 129.51 pg and 136.74 pg respectively (Fig.3.11).

When a unit containing a dilute coated fibre was perfused with buffer containing no BSA there was an initial marked release of unbound protein (70 mg/ml), Fig.3.12. However, the concentration of unbound albumin fell rapidly to about 5 mg/100 ml by 15 minutes and <1 mg/100 ml at the end of 100 minutes.

Discussion

The coating of hollow fibres with the protein-glutaraldehyde cross-linking method significantly reduced the transfer of ^{125}I-insulin across the hollow fibre membrane. There was no significant difference between the effect of either dilute or concentrated coatings on the release of ^{125}I-insulin from units (Fig.3.11). The coating procedure proved to be a very difficult one to carry out, and uniform coating could not be guaranteed. The elution profile of unbound protein (Fig.3.12) indicated that after the initial purge the remaining coat was stable and remained bound to the fibre. The results of perfusion studies suggested that the coating probably blocked the majority of pores on the luminal surface, reducing the effective surface area for

PLATE 5. Electron Micrograph of Hollow Fibre Showing Luminal Surface with Protein-Glutaraldehyde Coating.

(Mag. x3,000)

insulin transfer.

Electron micrographs of coated fibres in cross section tended to support this assumption. The coated luminal surface had an almost crystalline appearance, PLATE 5. Further studies need to be carried out to fully evaluate the effect of coating methods on the thrombogenicity and efficiency of hollow fibres.

B. INSULIN RELEASE FROM PROSTHETIC UNITS CONTAINING ISOLATED MOUSE ISLETS OF LANGERHANS.

Introduction

Using the physical characteristics defined in the previous section, evaluation was made of the effects of albumin concentration, glucose concentration, prolonged perfusion time and islet weight on the release of insulin *in vitro* from units containing mouse islets enclosed within the outer unit compartment.

Materials and Methods

Units were constructed under aseptic conditions as described in Section 2.7 (page 45). Mouse islets obtained by both micro-dissection (one mouse) and collagenase digestion (two mice) were pooled and briefly maintained in sterile, oxygenated HBSS, pH 7.4 at 37°C, containing penicillin (100 U/ml) and streptomycin (0.1 mg/ml), glucose (5.56 mmol/l) and BSA V at a concentration of either 0.5 g/100 ml or 7.45g/100 ml. The isolated islets were introduced into the open end of a partially constructed unit with the aid of a finely drawn-out Pasteur pipette. All air bubbles were excluded by tapping and the end of the unit was subsequently sealed with dental adhesive. Units containing islets were stabilised for 30 minutes by perfusion with oxygenated HBSS (95% O_2/5% CO_2) at 37°C supplemented with either 0.5g/100 ml or 7.45g/100 ml BSA V and glucose (5.56 mmol/l) at a flow rate of 0.6 ml/min. and a perfusion pressure of approximately 100 mm Hg in the presence of low albumin and 200 mm Hg in the presence of high albumin. After 30 minutes' stabilisation, the glucose concentration of the perfusate was either increased to 16.67 mmol/l or maintained at 5.56 mmol/l in control experiments. Perfusion of the units was continued for a test period of either 60 minutes, or 300 minutes in prolonged perfusion experiments. The latter were

carried out to exclude any time lag required for the establishment of glucose equilibrium between the islet compartment and the hollow fibre lumen prior to the stimulation of insulin release. Insulin in the unit effluent was measured by double antibody radioimmunoassay (183) as described in the appendix, page 137 . At the end of each experiment, islets were removed from the dismembered unit, washed with saline and transferred to a small aluminium foil boat with pin holes in the base for drainage of buffer. Excess buffer was absorbed from the foil boat by placing the latter on a sheet of filter paper. Islets were dried over night at 105°C, cooled in a desiccator and subsequently weighed on an ultramicrobalance (Mettler UM.6, sensitivity 1 μg). On average, units contained 0.467 ±0.032 mg of lean mouse islets or 1.220 ± 0.139 mg of obese mouse islets.

Experiments were subsequently carried out to investigate the effect of albumin concentration on the perfusion pressure through units containing either lean or obese mouse islets and the effect of high albumin concentration, prolonged perfusion time (300 min.) and a stimulatory concentration of glucose (16.67 mmol/l) on the rate of insulin release from units containing lean mouse islets. In addition the effect of increasing the perfusate glucose concentration from 5.56 to 16.67 mmol/l was investigated on the rate of insulin release from units containing either lean (+/+) or obese (ob/ob) mouse islets and the relationship between the weight of contained islet tissue and the total amount of insulin released from units over 60 minutes was evaluated.

Results

(a) The Effect of Albumin Concentration on the Rate of Insulin Release from Units containing Lean Mouse Islets.

The effect of either low (0.5g/100 ml) or high (7.45g/100 ml)

albumin concentration in both the unit outer compartment and the perfusate on the rate of insulin release from units containing lean mouse islets has been summarised in Table 2. Increasing the unit and perfusate albumin concentration from 0.5 to 7.45 g/100 ml significantly ($p<0.05$) improved the mean rate of insulin release from units after 30 minutes stabilisation, from 0.91±0.14 to 1.65±0.28ng/min/mg. dry wt. islet and appeared to improve the rate of insulin released during 60 minutes test perfusion in the presence of 16.67 mmol/l glucose. However, because of the relatively small number of experiments and the large standard errors of the mean, these differences did not reach statistical significance. In addition, irrespective of the concentration of albumin present, increasing the perfusate glucose concentration from 5.56 to 16.67 mmol/l appeared to increase the rate of insulin release from lean mouse islets after the 60 minutes' test perfusion, but again, because of the large standard errors of the mean generated these increases were not statistically significant.

(b) <u>The Effect of Prolonged Perfusion on the Rate of Insulin Release from Units containing Lean Mouse Islets</u>.

The effects of both short-term test perfusion (60 minutes) and prolonged test perfusion (300 minutes) in the presence of 16.67 mmol/l glucose on the rate of insulin release from units containing lean mouse islets have been summarised in Table 3.

Prolonged perfusion with buffer containing 16.67 mmol/l glucose reduced the mean rate of insulin release after 300 minutes' test perfusion by about two-thirds compared to the mean rate after 60 minutes' test perfusion. In addition, the total amount of insulin released during the last hour of the prolonged perfusion experiments was reduced by 45% compared to that released in the

Table 2. The Effect of Perfusate Albumin Concentration on the Rate of Insulin Release from Units containing Lean Mouse Islets. (Mean Value ± S.E.M.)

	Rate of Insulin Release (ng/min/mg dry islet)				Total Insulin Released during 60 Minutes of Test Perfusion (ng/mg dry islet)
	n	30 minute value (stabilisation) 5.56 mmol/l glucose	n	90 minute value (test) 16.67 mmol/l glucose	
Low Albumin (0.5 g/100 ml)	12	0.91 ± 0.14	6	1.64 ± 0.48	67.30 ± 13.26
High Albumin (7.45 g/100 ml)	12	1.65 ± 0.28 *	6	3.53 ± 1.34	121.35 ± 26.79

* $p<0.05$ compared with low albumin stabilisation value

Table 3. The Effect of Prolonged Perfusion on the Rate of Insulin Release from Units containing Lean Mouse Islets.

(Perfusate contained 7.45 g/100 ml Albumin, Mean Values ± S.E.M.)

	Rate of Insulin Release (ng/min/mg dry islet)				Total Insulin Released during last 60 Minutes of Test Perfusion (ng/mg dry islet)
	n	30 minute value (stabilisation) 5.56 mmol/l glucose	n	90 minute value (test) 16.67 mmol/l glucose	
Short-term Perfusion (30 min. stabilisation + 60 min. test)	12	1.65 ± 0.28	6	3.53 ± 1.34	121.35 ± 26.79
Prolonged Perfusion (30 min. stabilisation + 300 min. test)	6	30 minute value (Stabilisation) 5.56 mmol/l glucose 1.36 ± 0.48	6	330 minute value (test) 16.67 mmol/l glucose 1.12 ± 0.21	77.15 ± 25.15

final hour of the short-term perfusion experiments, although this reduction was not statistically significant.

(c) The Effect of Increasing the Glucose Concentration on the Rate of Insulin Release from Units containing either Lean or Obese Mouse Islets.

The effect of increasing the glucose concentration on the rate of insulin release from units containing either lean or obese mouse islets has been summarised in Table 4.

The rate of insulin release from units containing either lean or obese mouse islets was increased, although not significantly, after 60 minutes' exposure to perfusate containing 16.67 mmol/l glucose. The high glucose-stimulated rate of insulin release from units containing obese mouse islets was not significantly different from the rate of release from units containing lean mouse islets (Table 4). However, the rate of insulin release during the stabilisation period was significantly higher from units containing obese mouse islets than it was from units containing lean mouse islets, ($p<0.05$). In addition, the total amount of insulin released from units containing obese mouse islets over 60 minutes' test perfusion in response to 16.67 mmol/l glucose was 70% greater than the total amount of insulin released from units containing lean mouse islets over the same period of time (Table 4), but this increase was not statistically significant.

(d) The Effect of the Weight of Contained Islet Tissue on the Release of Insulin from Units in vitro.

The total amount of insulin released over one hour in response to 16.67mmol/l glucose from units containing lean mouse islets, Fig.3.14, (but not obese mouse islets, Fig.3.15), was found to be directly

Table 4. The Effect of Increasing the Glucose Concentration on the Rate of Insulin Release from Units containing either Lean or Obese Mouse Islets.

(Perfusate contained 7.45 g/100 ml Albumin, Mean Values ± S.E.M.)

	Rate of Insulin Release (ng/min/mg dry islet)				Total Insulin Released during 60 Minutes Test Perfusion (ng/mg dry islet)
	n	30 minute value (stabilisation) 5.56 mmol/l glucose	n	90 minute value (test) 16.67 mmol/l glucose	
Units containing Lean Mouse Islets	12	1.65 ± 0.28	6	3.53 ± 1.34	121.35±26.79
Units containing Obese Mouse Islets	6	2.60 ± 0.28 *	6	3.01 ± 1.04	205.98±62.17

* $p<0.05$ compared with stabilisation period of units containing lean mouse islets

Fig.3.14. The Relationship between the Weight of Contained Lean Mouse Islets and the Release of Insulin from Units in vitro. n = 45

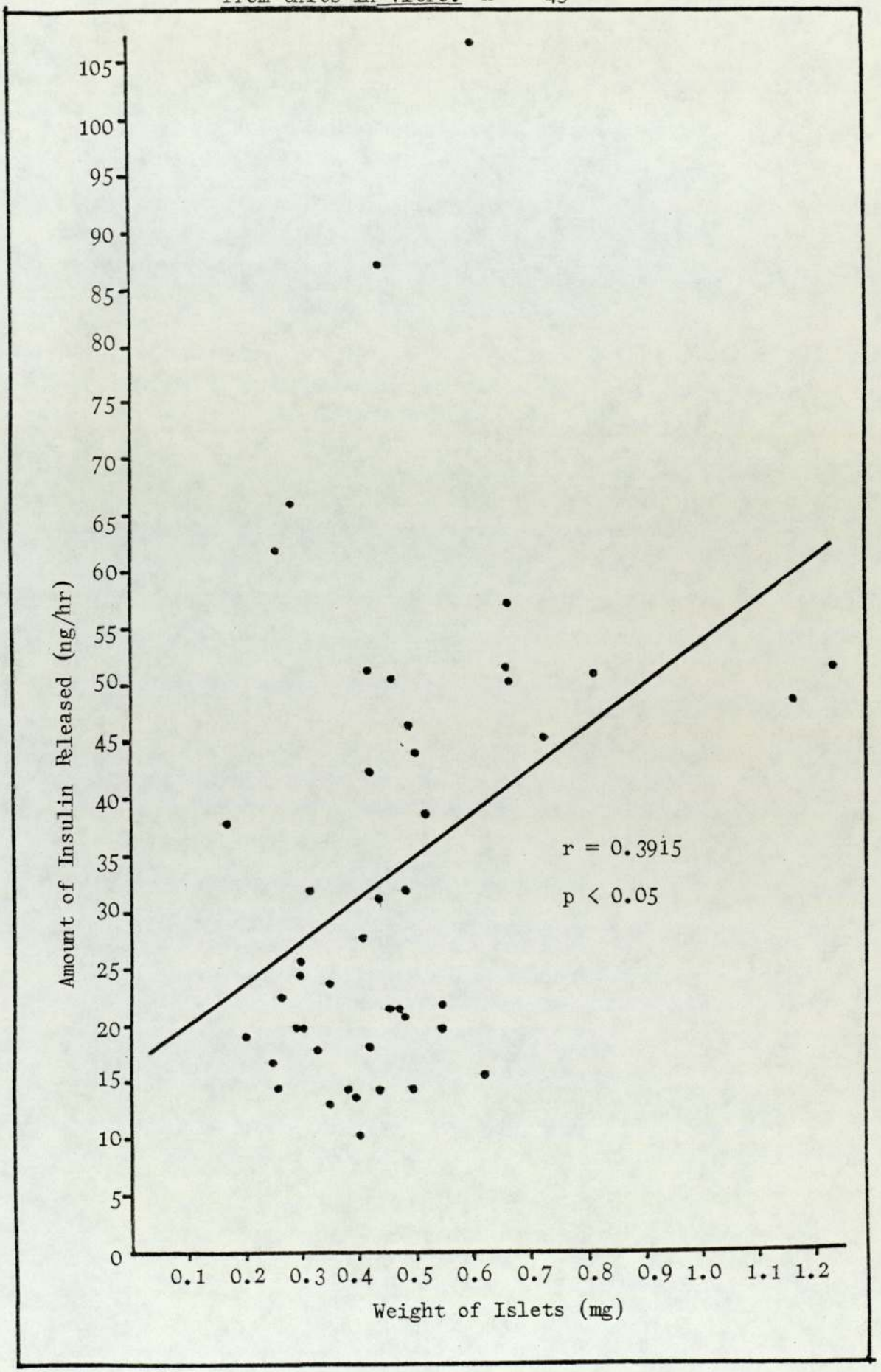
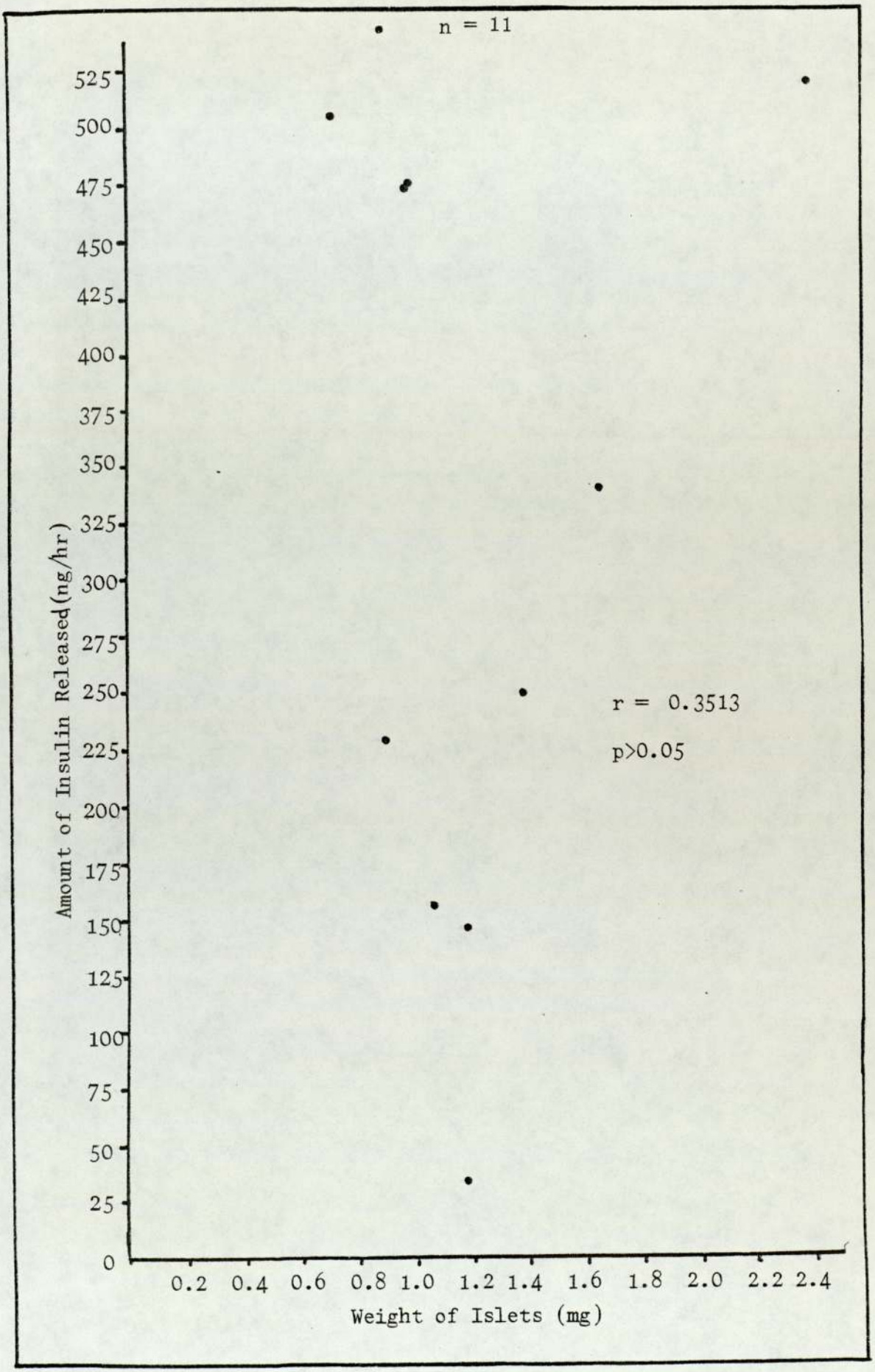


Fig.3.15. The Relationship between the Weight of Contained Obese Mouse Islets and the Release of Insulin from Units in vitro.



proportional to the dry weight of islet tissue present in the unit (correlation coefficient for linear regression, $r = 0.3915$; $p<0.05$).

(e) The Effect of Perfusate Albumin Concentration on the Perfusion Pressure Through Units Containing Either Lean or Obese Mouse Islets

Increasing the albumin concentration from 0.5g/100 ml to 7.45g/100 ml significantly increased the perfusion pressure in all experiments ($p<0.05$), irrespective of the perfusate glucose concentration or source of islet tissue (Table 5).

Table 5. The Effect of Perfusate Albumin Concentration on the Perfusion Pressure Recorded through Units containing either Lean or Obese Mouse Islets.
(Mean of pooled values for Units containing either lean or obese mouse islets ± S.E.M.)

	n	Perfusion Pressure (mm Hg)
Low Albumin (0.5g/100 ml)	27	100.63 ± 0.60
High Albumin (7.45g/100 ml)	25	181.92 ± 2.20 *

*$p<0.05$, compared with perfusion pressure in the presence of low albumin

Discussion

These studies indicate that functional hollow fibre prosthetic units containing viable mouse islets of Langerhans can be constructed which will release insulin at physiological flow rates (0.6 ml/min.) and perfusion pressures (100-200 mm Hg) in response to an increase in glucose concentration from 5.56 to 16.67 mmol/l.

Increasing the albumin concentration in the outer compartment buffer and the perfusate from 0.5 to 7.45g/100 ml significantly increased the perfusion pressure and the mean rate of insulin release from lean mouse islets after 30 minutes' stabilisation in the presence of 5.56 mmol/l glucose. In addition, increasing the albumin concentration in the perfusion medium approximately doubled both the mean rate of insulin release and the total amount of insulin released from units containing lean mouse islets during 60 minutes' test perfusion in response to 16.67 mmol/l glucose.

The rate of insulin release from units containing lean mouse islets test perfused for 300 minutes with HBSS containing 7.45g/100 ml BSA and 16.67 mmol/l glucose was considerably reduced when compared with the rate of insulin release from similar units in short-term perfusions of 60 minutes' duration. Since the glucose concentration in the unit outer compartment at the end of prolonged perfusion experiments was always found to be approximately the same as that in the perfusion medium (i.e. 16.67 mmol/l), it seemed likely that equilibrium had been established by the final hour of perfusion and that the islets were then exposed to, and thus responding to, an elevated, stimulatory glucose concentration. The decreased total insulin response over the final hour of the prolonged perfusions

compared to that over the final hour of the short-term perfusions in response to 16.67 mmol/l glucose, would suggest one of two possibilities: either the islets in prolonged perfusion studies were becoming exhausted, or, perhaps more likely, in the experiments involving short-term perfusions, insufficient time had been allowed for glucose equilibrium and islet stabilisation and the insulin response observed was thus slightly exaggerated due to an initial artifactual release of insulin from damaged islets.

Although the basal rate of insulin release from units containing obese mouse islets was significantly higher than that from units containing lean mouse islets during the stabilisation period, neither the rate of insulin release nor the total amount of insulin released over 60 minutes' test perfusion from units containing obese mouse islets in response to 16.67 mmol/l glucose was significantly different from values obtained for units containing lean mouse islets. In addition, the total amount of insulin released from units containing obese mouse islets over 60 minutes' test perfusion in response to 16.67 mmol/l glucose did not appear to be related to the weight of islet tissue present in the unit. On this basis, the relatively large islets of obese mice were considered unsuitable for use in future prosthetic units.

When units containing lean mouse islets were perfused with HBSS containing 5.56 mmol/l glucose, a mean insulin release of 1.22 ±0.10 ng/ml could be achieved. This value was roughly equivalent to the circulating insulin concentration of 12-hour fasted mice (1.20± 0.13 ng/ml) but somewhat lower than the recorded free fed circulating insulin level of 2.10±0.15 ng/ml (190). When units containing lean mouse islets were perfused with HBSS containing 16.67 mmol/l glucose

a mean insulin release of 1.68±0.12 ng/ml was achieved, a level that would usefully contribute towards the maintenance of normoglycaemia in subsequent *in vivo* studies in animals.

CHAPTER 4

IN VIVO STUDIES WITH THE PROTOTYPE IMPLANTABLE ENDOCRINE PANCREAS

4.1. Introduction

The *in vivo* behaviour of prototype implantable units has subsequently been investigated in studies involving the implantation of units containing either lean mouse islets or saline only into rats previously made diabetic with streptozotocin. The study was designed to assess the performance of units *in vivo*; their ability to reduce the elevated blood sugar to normoglycaemic levels. In addition attention has been paid to the possible traumatic effects of implantation on the rats and the biocompatibility and degradation of fabrication materials.

4.2. The Selection of Possible Implantation Sites

The selection of a suitable site for the implantation of the prosthetic unit depended on several factors. Firstly, the artery into which the unit was to be inserted had to be large enough to take the Portex PP10 cannula. Secondly, a good flow rate through the artery was necessary to reduce the possibility of thrombus formation and create the environment for the exchange of glucose and insulin. Thirdly, the implanted unit should not cause trauma to adjacent tissue or organs and thus it had to be placed in an area of the body where there was sufficient space to accommodate the relatively large size of the unit.

In the ideal situation, it would have been best to implant the unit as a venous shunt into the hepatic portal vein, since the liver modulates the release of insulin into the peripheral circulation. However, since the hepatic portal vein is relatively thin-walled and has neither sufficiently high blood pressure nor flow rate to effectively accommodate a unit without stagnation and blood clotting, this

site was considered unsuitable. The carotid arteries and abdominal aorta were considered to be the best sites for implantation because of their ability to accept the PP10 cannula, their high blood flow rates and the possibility of positioning the unit adjacent to the carotid in the cervical connective tissue or in the abdominal cavity.

4.3. Surgical Procedure

The feasibility of each of the following surgical procedures was investigated for the potential implantation of the prototype units. All surgical procedures were performed under strict aseptic conditions.

(a) Insertion of a 3 cm Unit into the Abdominal Aorta

The rat was anaesthetised with sagatal (60 mg/kg i.p.)(May & Baker Ltd., Dagenham, Essex) and placed with its ventral surface uppermost on a thermostatically-controlled rat blanket set at 37°C (CFP 8185, Homeothermic Blanket). After swabbing with hibitane (I.C.I.Ltd., Macclesfield, Cheshire), 5% concentrate diluted 1 in 5 with double distilled water, fur was removed with clippers and the abdomen opened at the mid-ventral line from the xiphisternum to the infrapubic region. The muscle layers of the abdominal wall were retracted and the alimentary canal displaced to the left allowing the location of the abdominal aorta. Displaced organs were covered with a piece of sterile gauze soaked in warm physiological saline. The abdominal aorta was exposed between the right ilio-lumbar artery and the bifurcation of the common iliac arteries. Connective tissue associated with the aorta and posterior vena cava was carefully teased away by blunt dissection.

A 2/0 non-capillary braided suture (Wrights Dental Ltd.,Birmingham) was fed under the aorta proximal to the ilio-lumbar artery and drawn forward towards the heart as far as possible. Two further

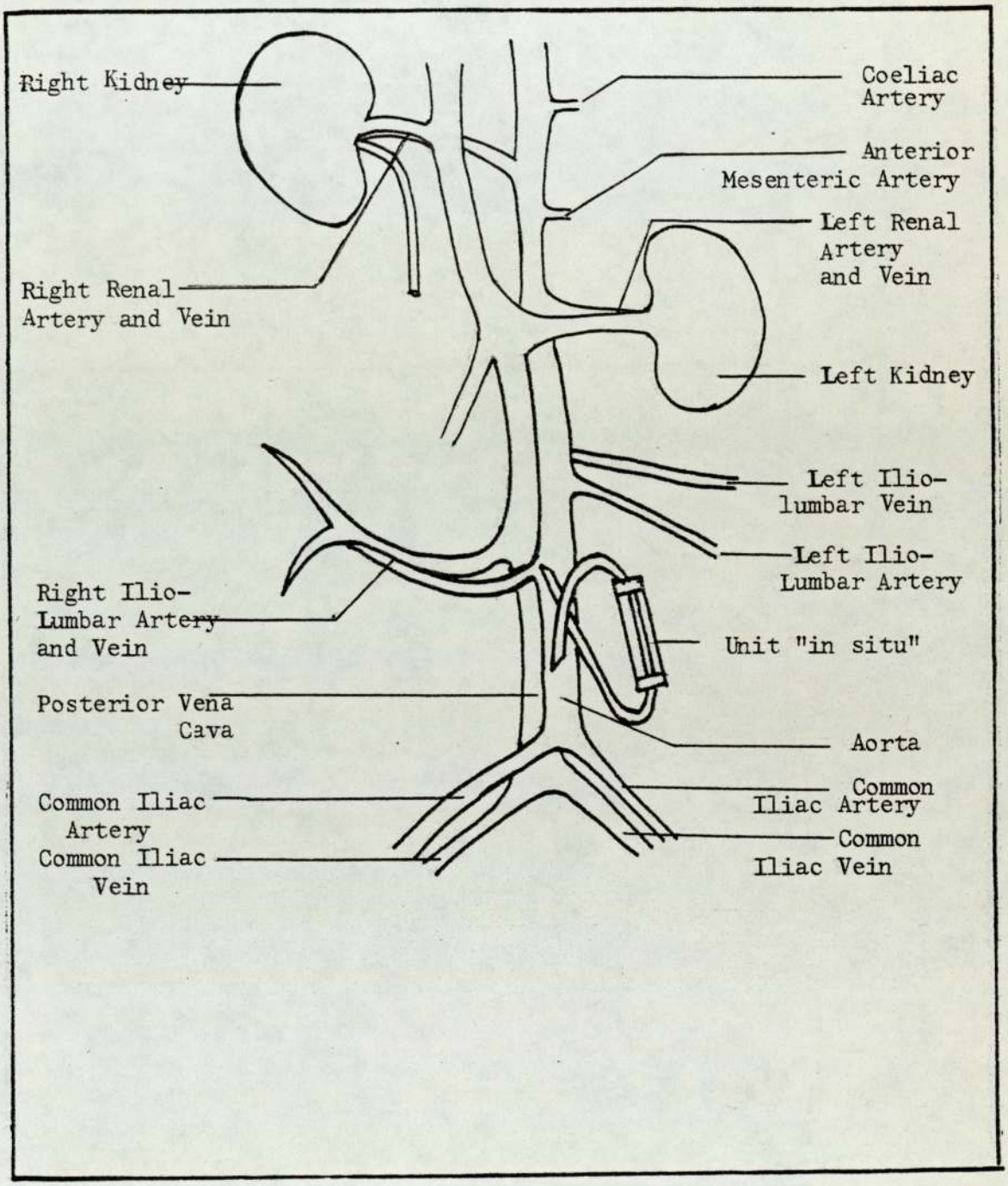
ligatures (3/0 non-capillary braided suture) were tied loosely around the aorta, one placed immediately distal to the ilio-lumbar artery, the other proximal to the iliac bifurcation. A prototype unit was flushed with sterile, heparinised saline (500 I.U/ml), dipped in hibitane to sterilise the surface and the end of the PP10 cannulae cut at an angle of 45° to allow easier insertion into the blood vessel. The 2/0 non-capillary braided suture tape proximal to the ilio-lumbar artery was gently tensioned to restrict the flow of blood through the aorta and a small dorsal incision was made in the aorta with butterfly scissors. One cannula arm of the unit was introduced 5 - 8 mm into the aorta and tied in place. A second dorsal incision was made in the abdominal aorta approximately 5 mm distally to the first incision and the other looped cannula arm inserted into the aorta and secured. The tension on the 2/0 non-capillary braided suture tape was released and blood allowed to pass through the unit. The positioning of the implanted unit is shown in Figure 4.1.

The unit was carefully positioned close to its insertion in the abdominal aorta ventral to the left kidney and the adjacent small intestine returned to its normal position. Retractors were removed and the peritoneum and muscles of the abdominal wall were drawn together and secured with a running mattress stitch using 4/0 non-capillary braided suture. The skin was then pulled together with forceps and clipped with 9 mm stainless steel autoclips (Becton & Dickinson U.K. Ltd., Oxford). The wound was cleaned and dressed with streptomycin sulphate, B.P. (Glaxo Ltd., Greenford, Middlesex) and the animal allowed to recover on the heated blanket.

(b) Insertion of Units into the Common Carotid Arteries.

Rats were anaesthetised and prepared as described in the previous section with their heads towards the operator. After removing the

Figure 4.1. Implantation of 3 cm Unit into Abdominal Aorta

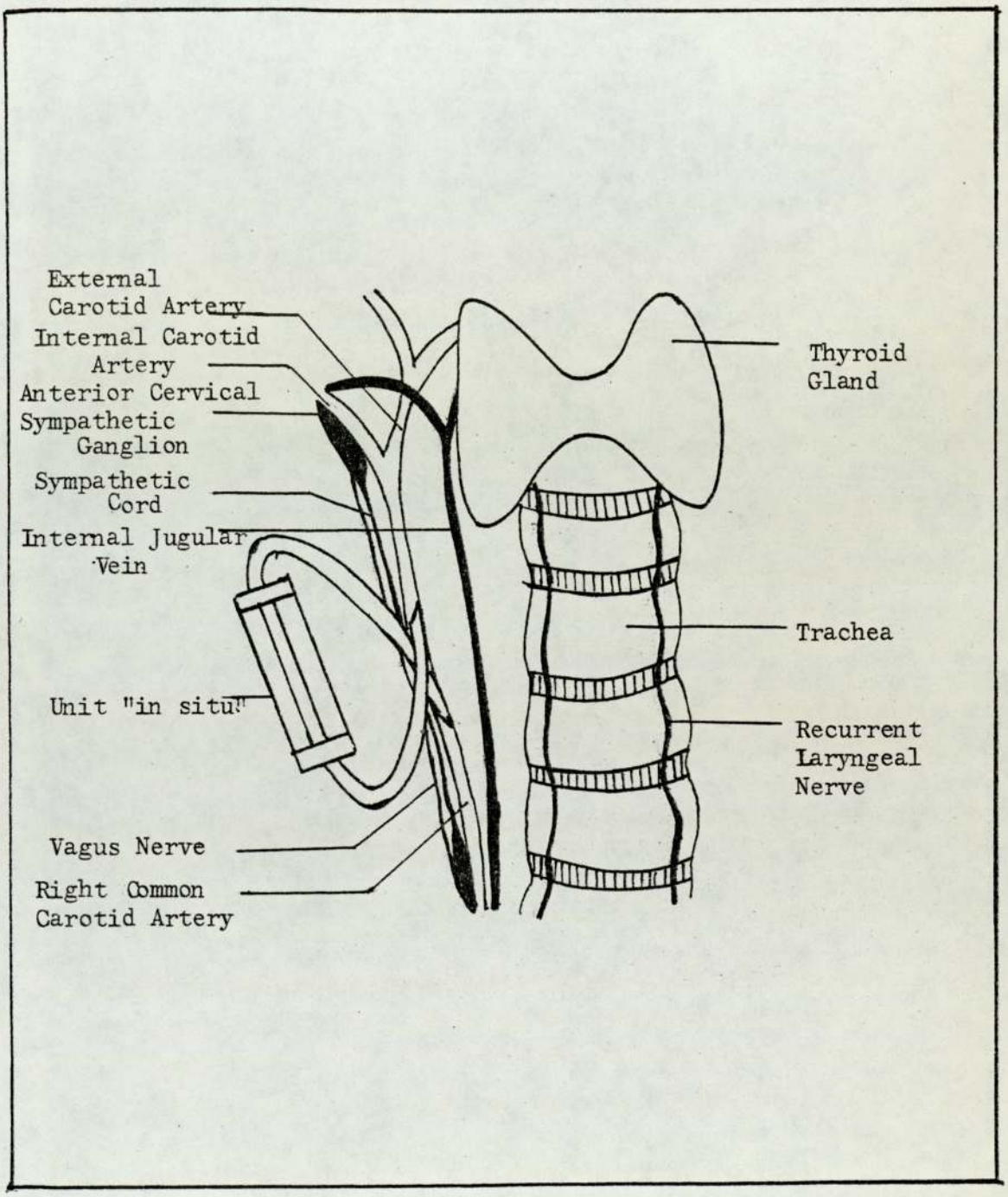


fur with clippers the neck region was swabbed with 1% hibitane and a 2 cm incision made through the skin and muscle wall and extended by blunt dissection. Capillary bleeding was suppressed by cauterisation. A vertical incision was made through the sterno-hyoid muscle covering the trachea and the muscle bands retracted to display the right common carotid artery just below its bifurcation into internal and external elements. Careful blunt dissection allowed separation of the carotid from the vagus nerve.

A 2/0 non-capillary braided suture tape was fed under the proximal end of the exposed carotid (the end furthest away from the head) and ligatures (3/0 non-capillary braided suture) were loosely tied around each end of the exposed length of carotid (Figure 4.2).

The anaesthetised animal was then turned dorsal side uppermost and a small incision made in the nape of the neck. An 11.5 cm serum needle was inserted subdermally round the right side of the neck from the ventral to dorsal surface. The PP10 cannula attached to one end of a 3 cm unit was firmly pushed on to the end of the needle and the latter was then drawn back through to the ventral surface. This procedure was then repeated on the left side of the neck such that the unit itself rested as a subdermal "saddle" across the back of the neck with its two cannulae protruding either side of the ventral opening in the neck. Light tension was applied to the 2/0 suture tape to temporarily stop the majority of the blood flow and a small incision was made in the right common carotid artery as close as possible to the heart. One cannula arm of the unit was introduced proximally and secured with a 3/0 suture. A second incision was made distally and the other cannula arm was secured in the same way. The restraining tape was loosened and blood allowed to flow through the unit.

Figure 4.2. Implantation of 1.5 cm or 3 cm Units into the Right Common Carotid Artery



After securing the ligatures firmly and removing the restraining tape, the ventral incision was closed by suturing (4/0) the divided sterno-hyoid muscle over the trachea and subsequently the muscles of the body wall. The ventral skin incision was closed with stainless steel wound clips while the dorsal incision was sutured. Both sites of incision were cleaned with hibitane and dressed with powdered streptomycin. The animal was allowed to recover on the heated rat blanket.

In subsequent operations 3 cm and 1.5 cm units were inserted into the right common carotid artery but these units were not seated as saddles, instead they were positioned ventral to the trachea in the ventral cervical subdermal tissue and secured there with a single suture. The incision was closed as before. The stages of the operative procedure are shown in PLATES 6 - 16. In addition, in several animals, 1.5 cm units were implanted into both the left and right common carotids. Survival of these animals would confirm that at least one of the units was functioning properly.

Performance of the Units in Vivo.

Animals with units inserted into either the carotid artery or abdominal aorta survived for more than eight months. When the animals were finally culled, most of the carotid units contained thrombi and were no longer functional. PLATES 17 and 18 illustrate the removal of clotted units from these animals. The units were encapsulated in fibrous connective tissue and showed good biocompatibility. Stereoscan electron microscopic examination of sections of hollow fibres from previously implanted units, PLATES 19 and 20, indicated the presence of clots on the surface and in the lumen of the hollow fibre. Hollow fibre/ cannula junctions, were areas of high risk for thrombus formation, PLATE 21.

PLATE 6. Isolation of the Right Common Carotid Artery

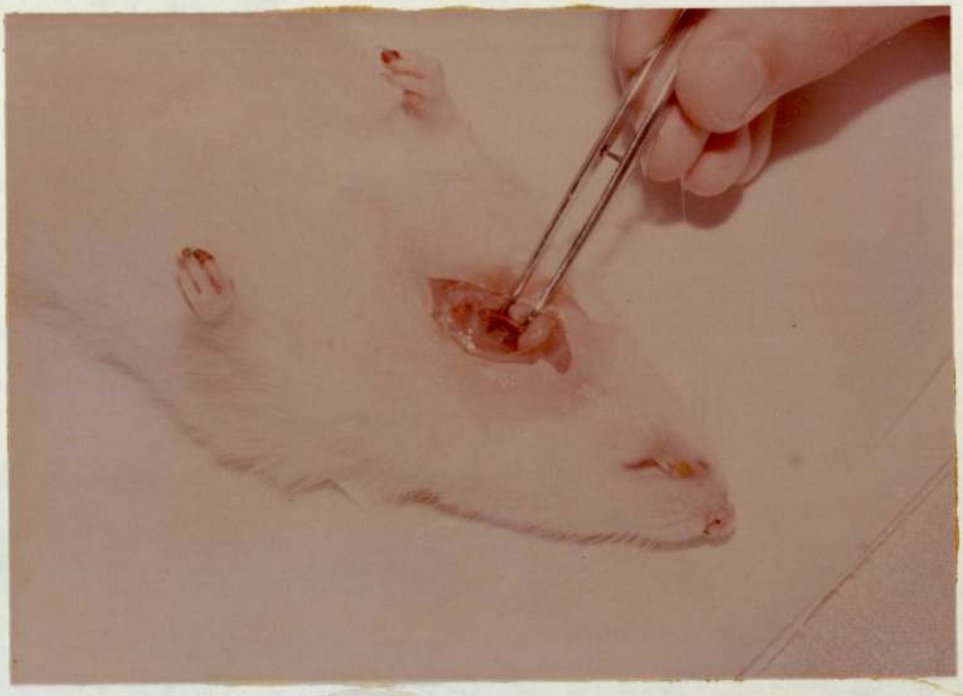


PLATE 7. Positioning of Afferent and Efferent Ligatures and 2/0 Restraining Tape

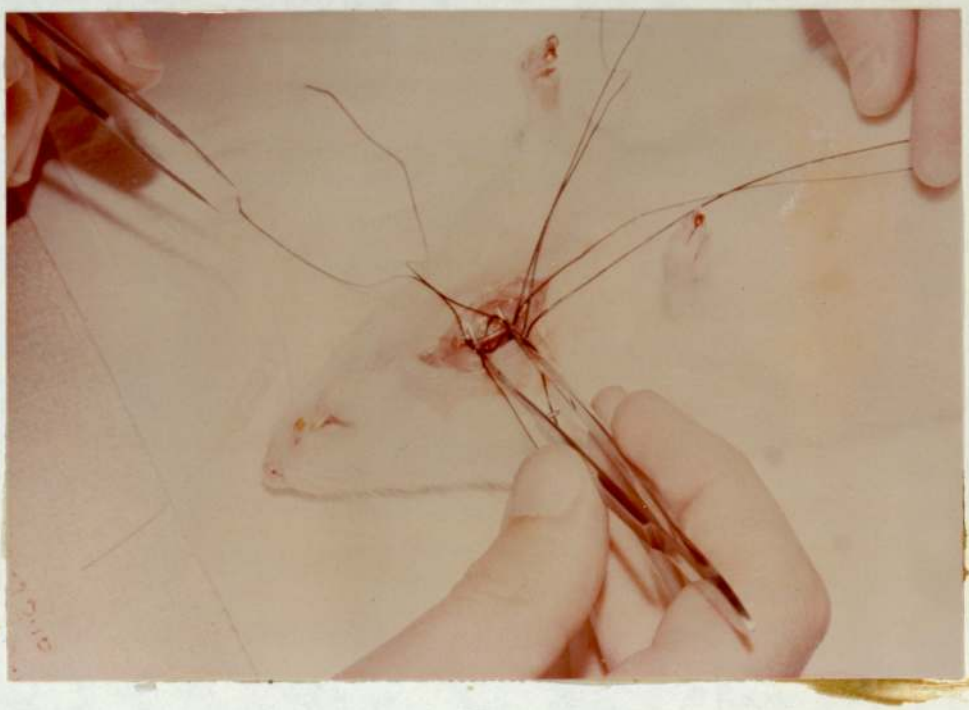


PLATE 8. Insertion of Afferent Cannula of 3 cm Unit.

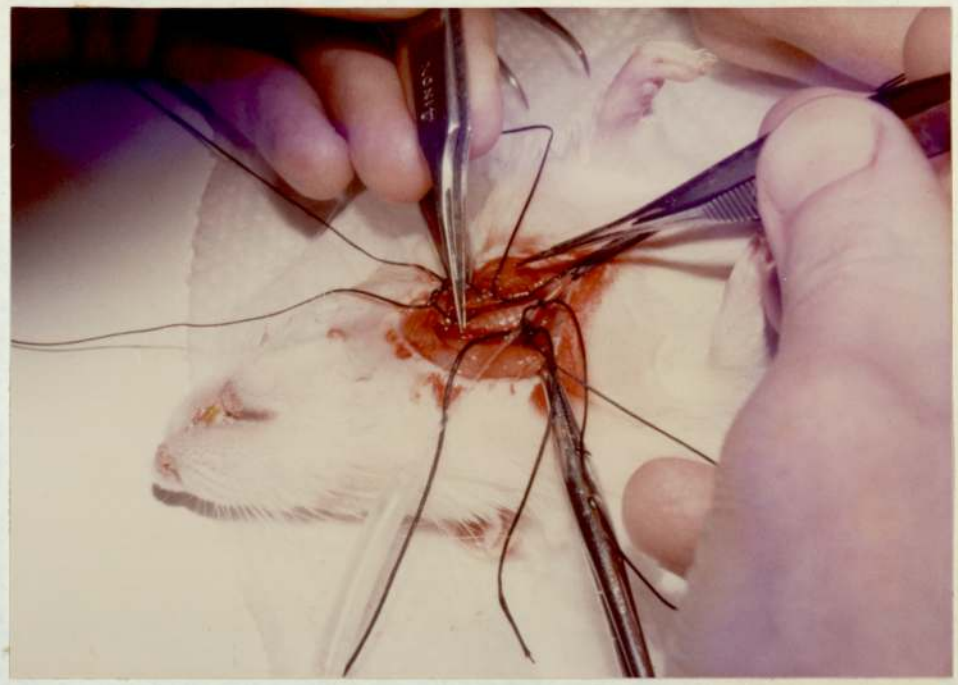


PLATE 9. Insertion of Efferent Cannula into Left Common Carotid Artery in 'Cross-over' Implantation.

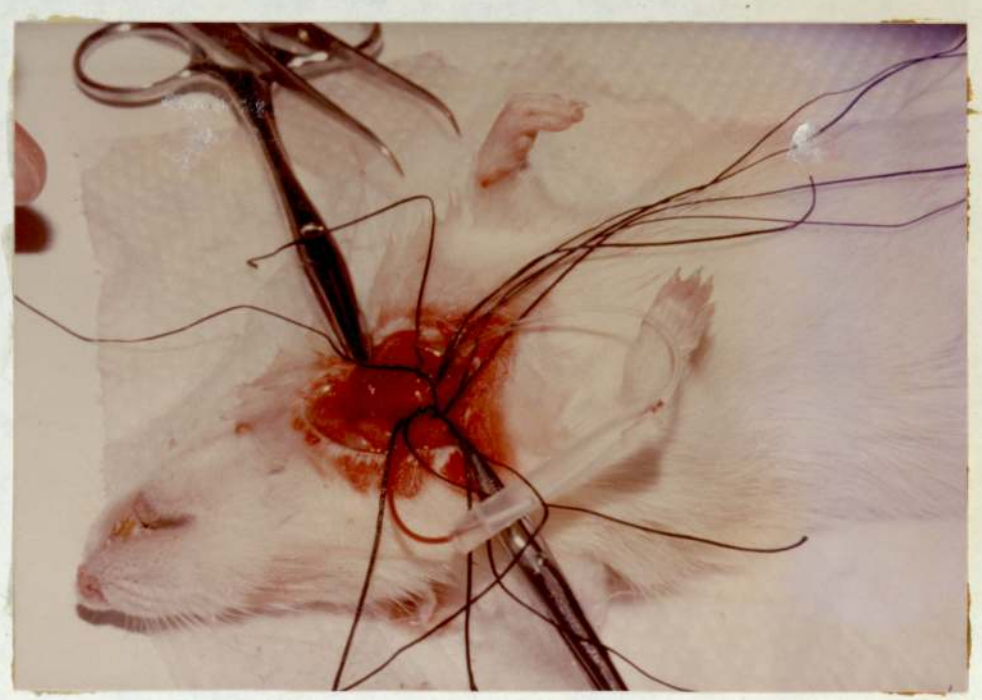


PLATE 10. 3 cm Unit "in situ". Blood Circuit Complete and Ligatures Secured.

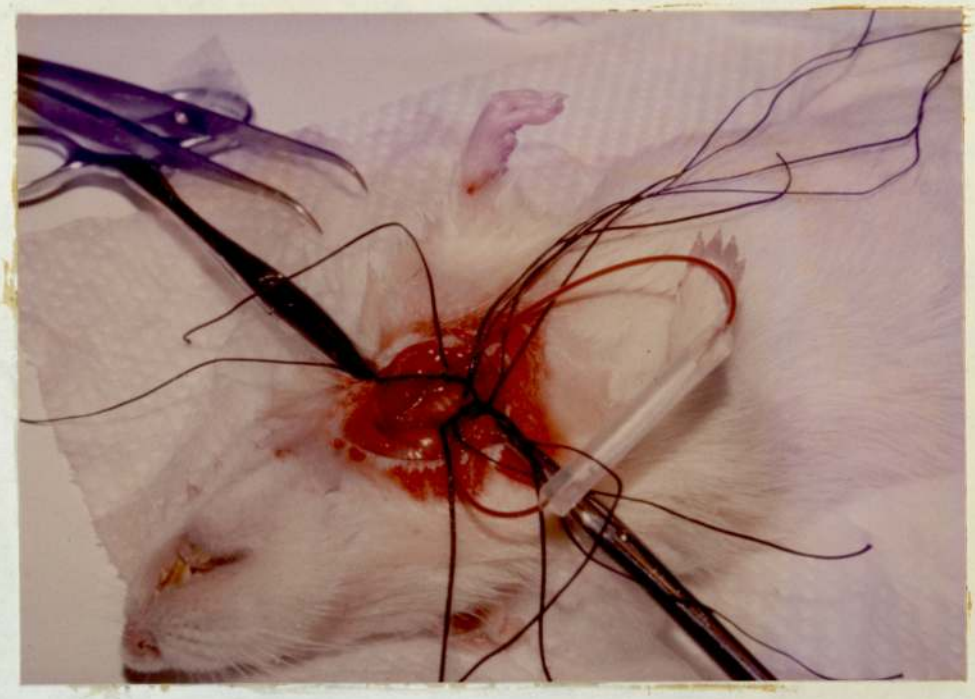


PLATE 11. Excess Ligature Trimmed and Restraining Tape Removed.

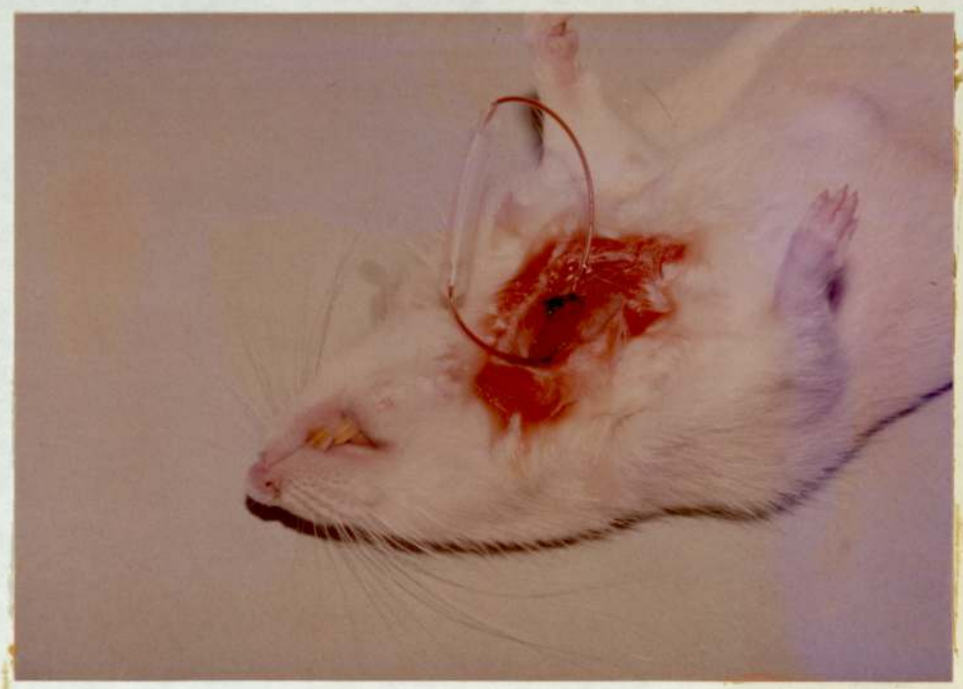


PLATE 12. Insertion of 1.5 cm Unit with Blood Flow Interrupted by the Restraining Tape.

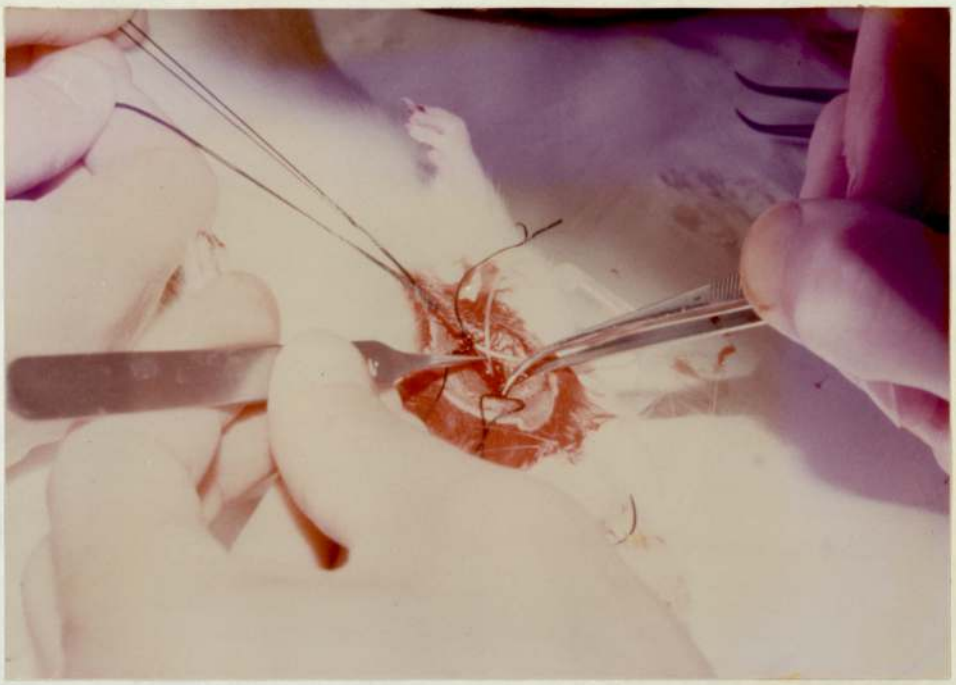


PLATE 13. 1.5 cm Unit "in situ". Blood Beginning to Flow through Cannula.

PLATE 14. Relaxation of Restraining Tape. Blood Circuit Complete.

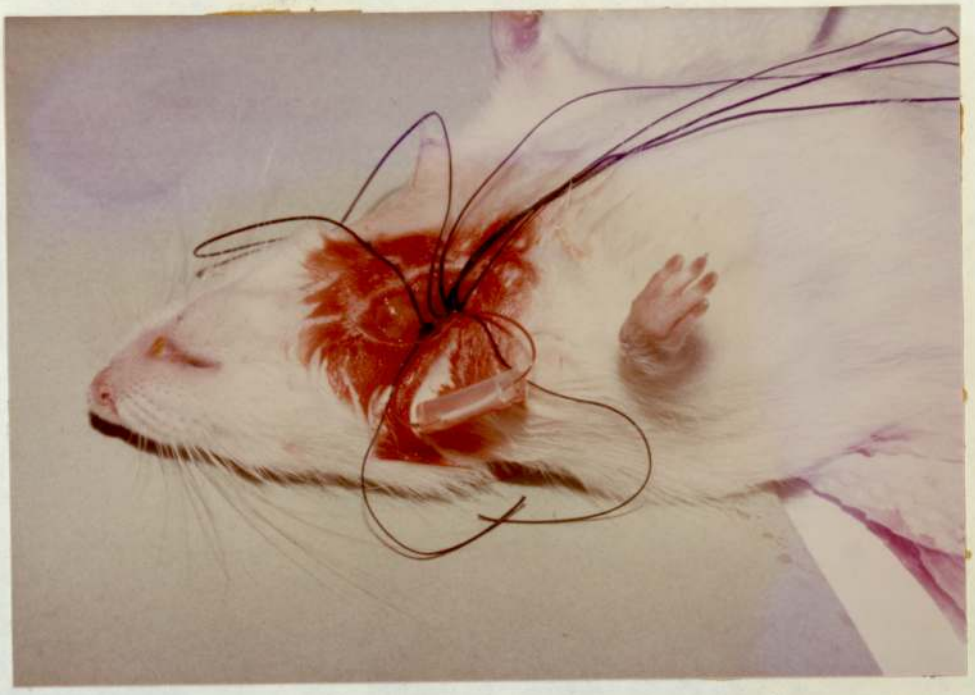


PLATE 15. Excess Ligature Trimmed and Removal of Restraining Tape.

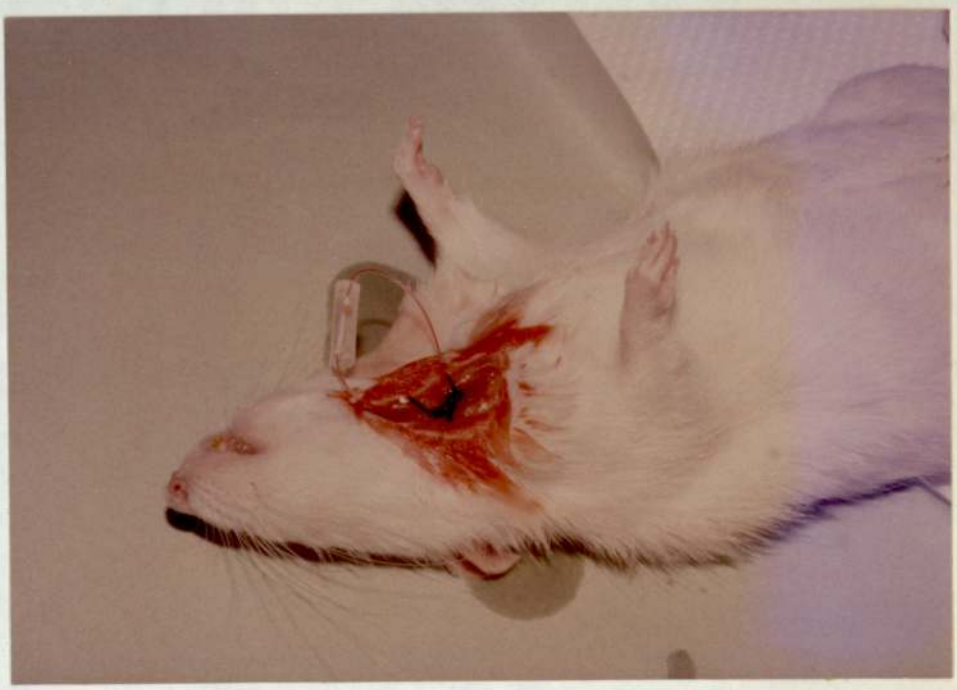


PLATE 16. Positioning of 1.5 cm Unit Under the Skin.

PLATE 17. Removal of Clotted 1.5 cm Unit from Rat with Single Implant.

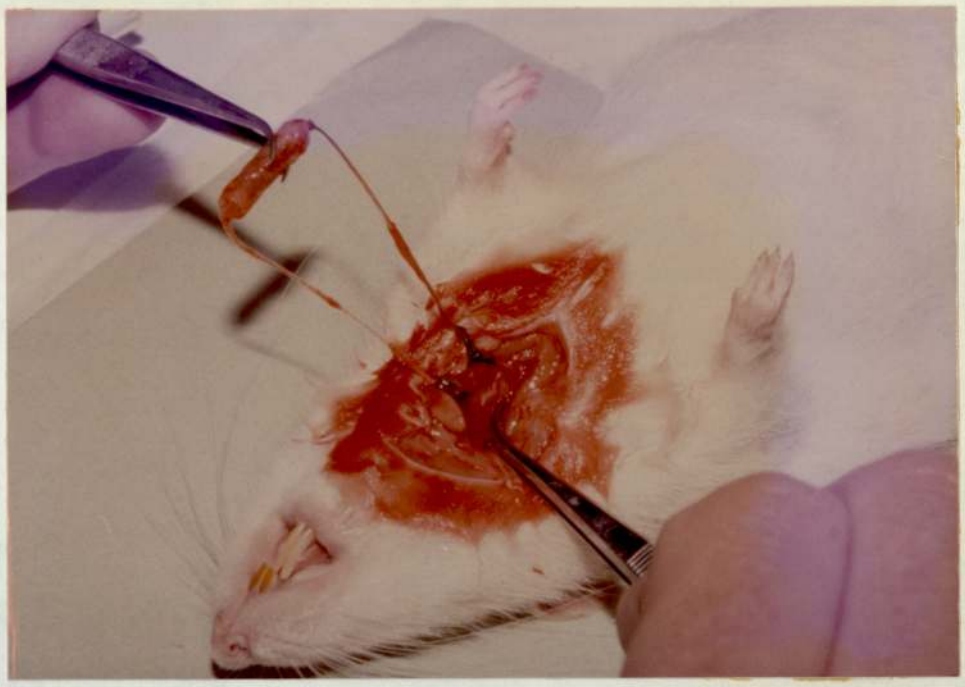


PLATE 18. Removal of One Clotted 1.5 cm Unit and One Unclotted Unit from Rat with Double Implant.

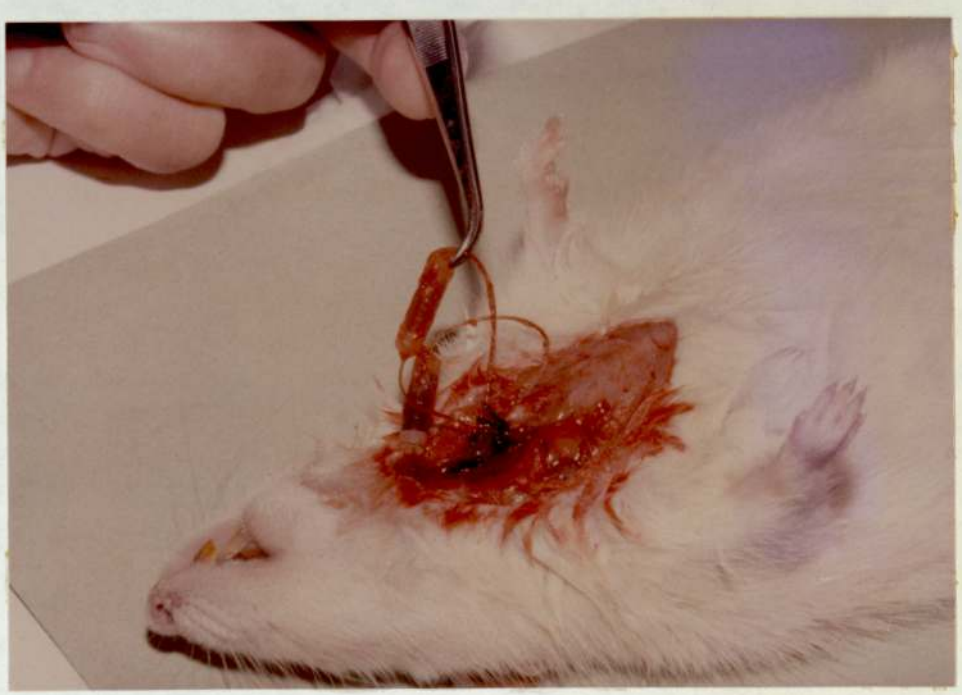


PLATE19. Thrombus Formation in Lumen of Hollow Fibre.
(S.E.M. x 3000)

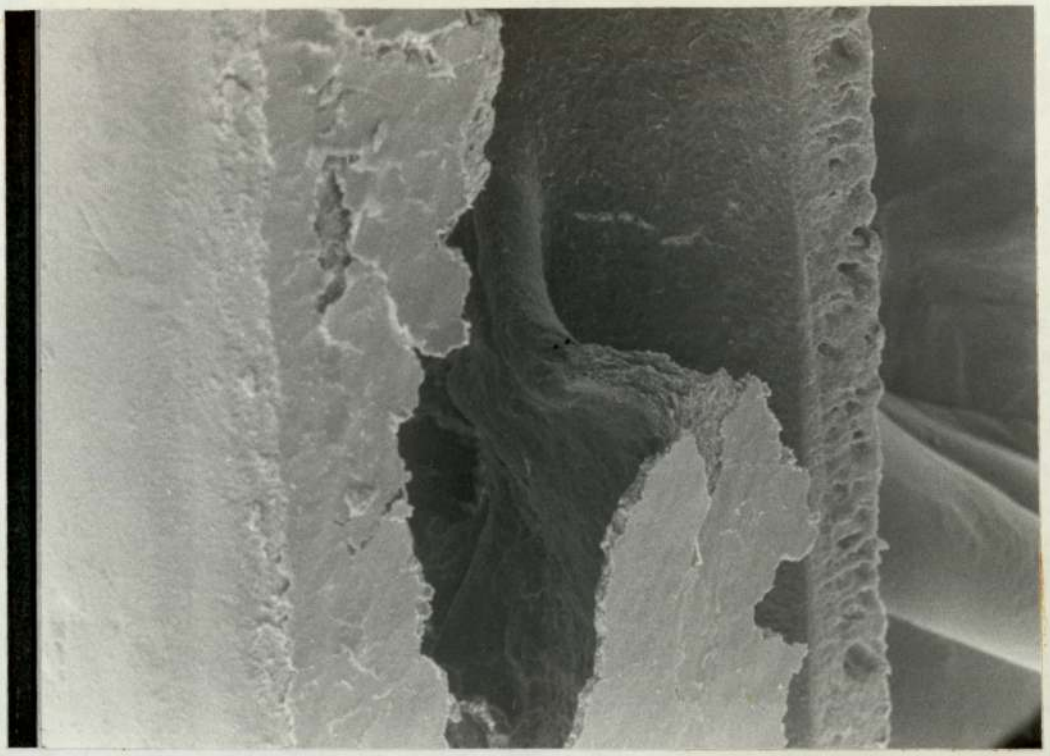


PLATE 20. Thrombus Formation in Lumen of Hollow Fibre.
(S.E.M. x 3000)

PLATE 21. Fibre-Cannula Junction. (S.E.M. x 3000)

Attempts were made to observe implanted carotid units *in situ* in the X-ray department of a local hospital. Urografin 310 M was infused directly into the carotid of host animals just below the entry of the unit and was also injected into tissue surrounding the unit. Units could just be identified in relief, PLATES 22 and 23, but these studies provided little information on the performance or acceptability of the units *in vivo*. Infusion of Urografin 310 M into the tail proved unsuccessful due to overdilution in the circulation.

4.4. Pilot Study to Evaluate the Effect of Unit Implantation on Blood Glucose, Serum Insulin and Body Weight of Streptozotocin-Diabetic Rats.

Introduction

Previous work established that healthy rats could withstand surgical manipulation, recover fully and continue to tolerate their surgical implants almost indefinitely, without showing signs of inflammatory reactions. On this basis, a preliminary study was undertaken, to determine whether rats previously made diabetic with streptozotocin and thus under stress, would survive surgery and could subsequently be used in studies designed to investigate the effect of implanted units containing lean mouse islets on host blood glucose, serum insulin and body weight.

Materials and Methods

Two rats, weighing 315 g and 415 g respectively, were made diabetic with streptozotocin (50 mg/kg) i.p. administered at 16.00 hours (191) in citrate buffer, pH 4.8 (192,193) at room temperature. Doses of 55 - 65 mg/kg streptozotocin, as recommended by several workers (132, 133, 164) resulted in uncontrollable hyperglycaemia and death. Hence in this pilot study a dose of 30 mg/kg was administered initially followed by an additional bolus of 20 mg/kg five days later, if the blood glucose had not exceeded 14 mmol/l.

PLATE 22. X-Ray Photograph of 1.5 cm Unit in Relief after Urografin 310M Injection.

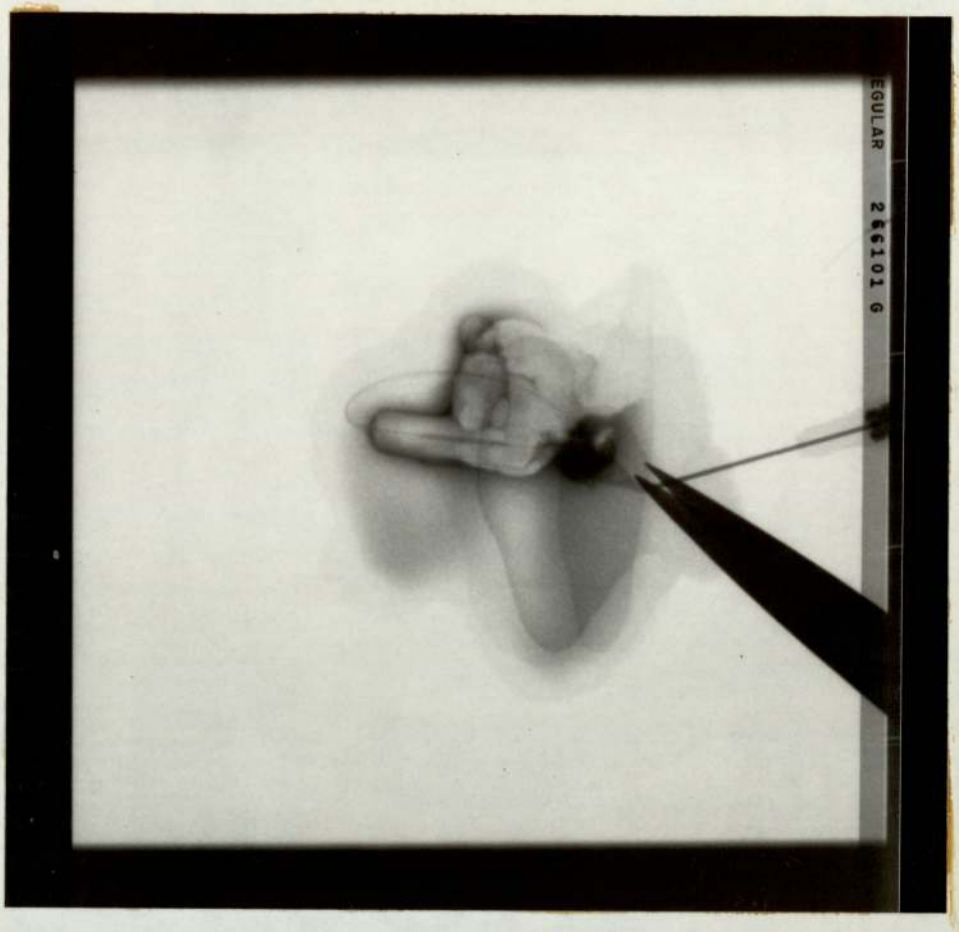
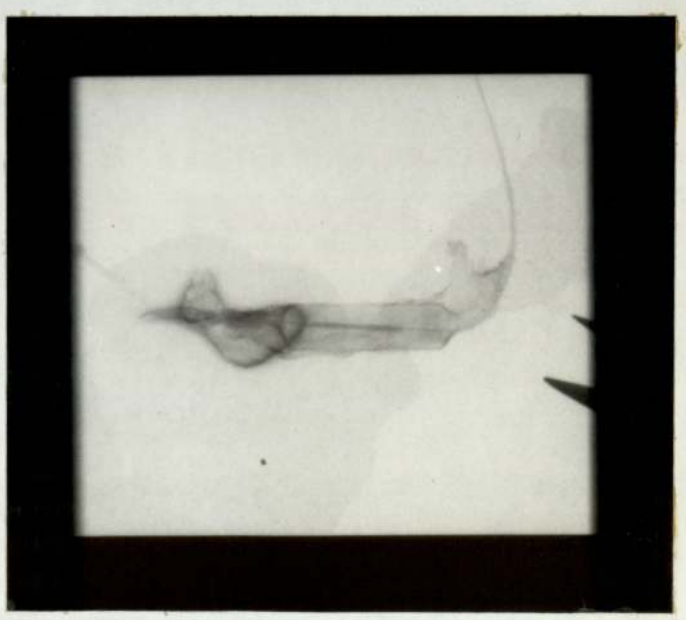


PLATE 23. X-Ray Photograph of 1.5 cm Unit in Relief after Urografin 310M Injection.



The rats were housed in an air-conditioned room at 22±2°C, maintained on a 12-hour light/dark schedule and supplied a standard pellet diet. They were allowed access to food and water ad libitum. Due to excessive urination by the diabetic animals, they were maintained on mezzanine wire grids in cages containing either sawdust or absorbent paper pads.

Hyperglycaemia was maintained at between 30 - 35 mmol/l for 10 days in rat A prior to implantation of the unit. There was no change in body weight during this time. The second animal, rat B, developed a blood glucose level of 16.3 mmol/l and there was a marginal reduction in body weight (≏ 20g) prior to implantation. Both animals received 3 cm units containing lean mouse islets. The latter were isolated by collagenase digestion first thing in the morning and transferred to sterile preconstructed units in sterile HBSS supplemented with 5.56 mmol/l glucose and 7.45 g/100 ml BSA. The units were sealed with dental adhesive in the usual way and flushed with sterile heparinised saline (500 I.U./ml). Implantation into the right carotid was performed immediately as described in section 4.3 (b), page 101.

Results

The body weight and blood glucose of both animals was monitored continuously during streptozotocin treatment, Figures 4.3 and 4.4, and implantation carried out when the blood sugar exceeded 14 mmol/l. The day after surgery, the blood glucose of rat A fell considerably to a normoglycaemic level of 8.3 mmol/l, Figure 4.3, but by day 2 had returned to a diabetic level of 17.8 mmol/l and continued to increase gradually thereafter reaching the pre-surgical level by day 11. No change in body weight was observed and the animal was culled on day 12. Implantation of a unit into rat B was associated with a marked

Figure 4.3. Pilot Study. The Effect of Unit Implantation on Blood Glucose and Body Weight of Streptozotocin-Diabetic Rat (A).

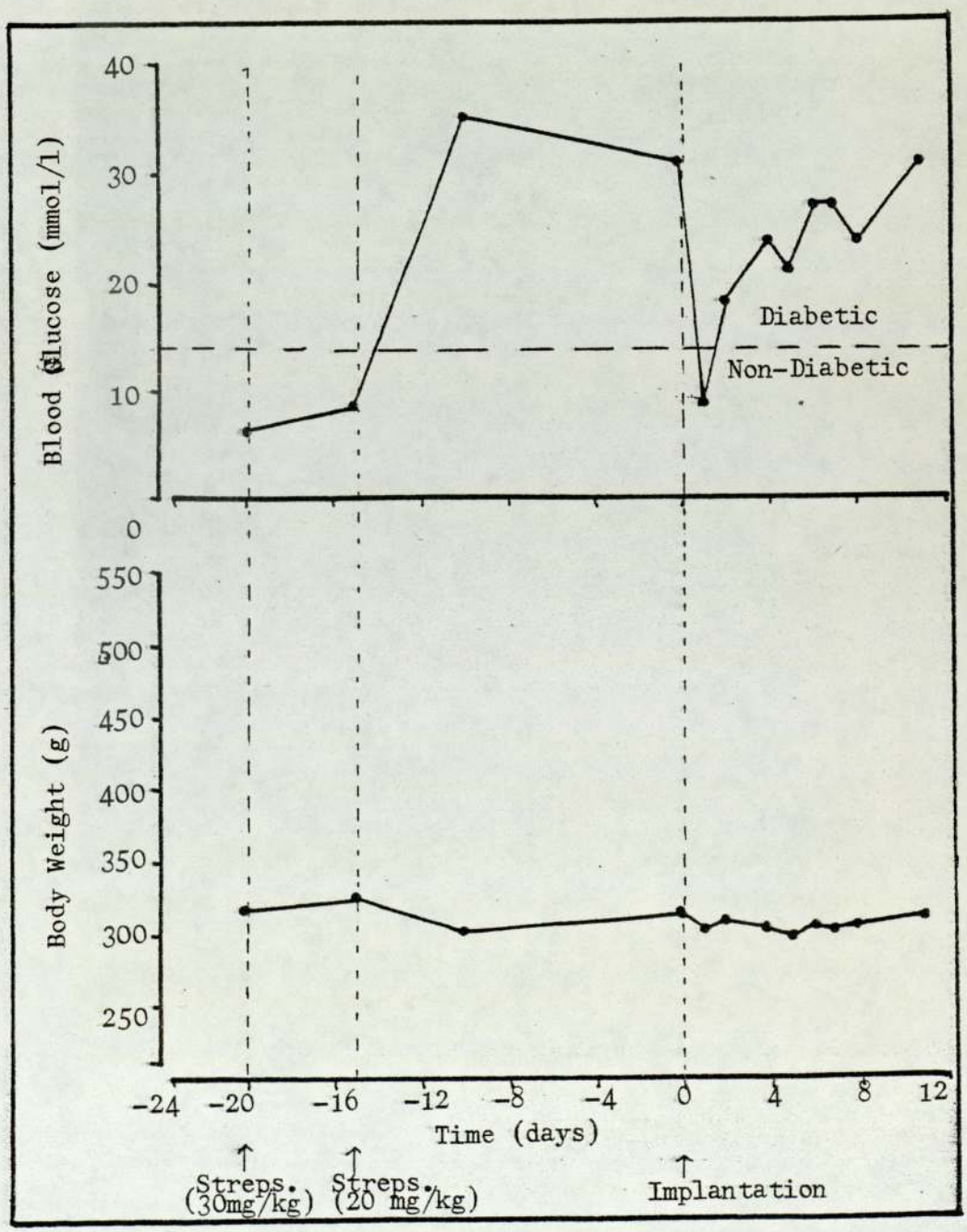
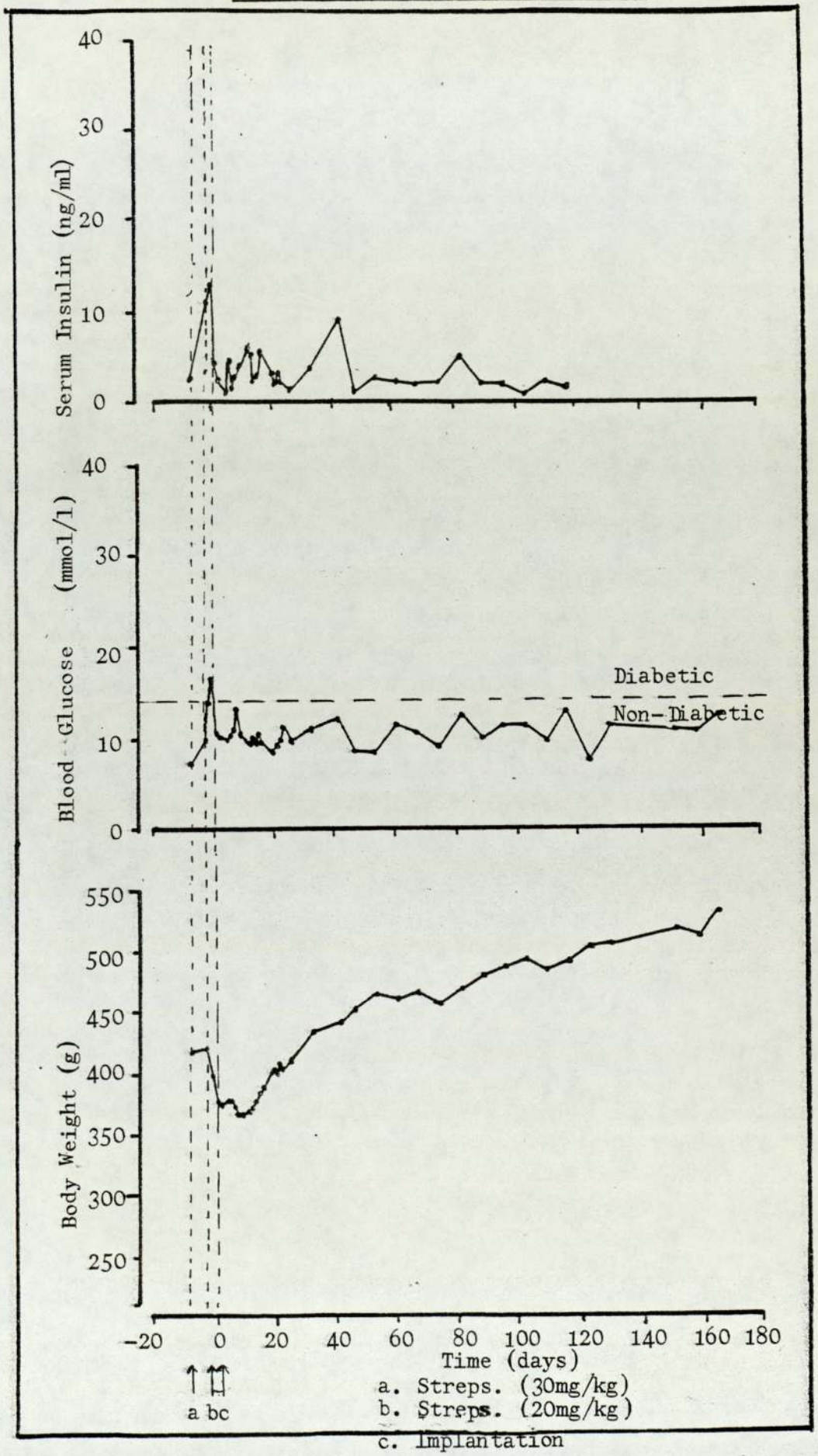


Figure 4.4 Pilot Study. The Effect of Unit Implantation on Blood Glucose, Serum Insulin and Body Weight of Streptozotocin-Diabetic Rat B.



reduction in serum insulin, blood glucose and body weight, Figure 4.4. During the first 10 days post-implantation, the body weight continued to decline, but subsequently stabilised at day 15 and thereafter continued to increase gradually over the remainder of the study. The blood glucose level was maintained marginally above normoglycaemic levels for the first 7 days post-implantation, with an average value of 10.1 mmol/l. Subsequent short-term fluctuations in blood glucose were accompanied by corresponding increases in serum insulin levels, Figure 4.4. At no time in this animal did the blood glucose level return to the pre-surgical, diabetic level and the mean serum insulin level was maintained at $\simeq$ 3 ng/ml.

Discussion

Early attempts to induce hyperglycaemia in 300g rats with streptozotocin at a concentration of 65 mg/kg were unsuccessful. This dose of streptozotocin produced great stress in the animals and was associated with uncontrollable hyperglycaemia, polyuria and a marked increase in blood viscosity prior to death. This increase in blood viscosity made the implantation of units very difficult and increased the possibility of clotting. In order to combat this, animals were made diabetic by using lower multiple doses of streptozotocin and surgery was performed as soon as the blood glucose level exceeded 14 mmol/l.

Postmortem examination of Rat A indicated that failure to restore normoglycaemia was the result of blood-clotting in the unit and one cannula of the unit had become disengaged from the carotid. In Rat B the streptozotocin-induced presurgical rise in blood glucose was accompanied by a marked rise in serum insulin. This may have been the result of a sudden destruction of β-cells by streptozotocin, producing a short-lived but substantial release of insulin from

damaged β-cells. This insulin response was substantially reduced after surgery. The unit continued to function efficiently for the duration of the study, Figure 4.4, providing sufficient insulin to maintain blood glucose values below pre-surgical diabetic levels and encouraging the normal gain in body weight. Rat B survived for more than 6 months after the termination of the study and at post-mortem the unit was free from thrombi and contained identifiable islet tissue. The unit was ensheathed in fibrous connective tissue and there was no evidence of adjacent inflammatory tissue reaction.

4.5. Full-Scale Study of the Effect of Unit Implantation on the Blood Glucose, Serum Insulin and Body Weight of Rats Made Diabetic with Streptozotocin.

Introduction

Despite reports in the literature (132, 133, 164), the initial pilot study demonstrated the difficulty of trying to achieve a stable level of hyperglycaemia in rats with streptozotocin. Hyperglycaemia induced by this means was always associated with an increased blood viscosity and this factor alone increased the difficulty of the surgical procedure and compromised the postoperative survival of the animal. However, the pilot study did show that successful implantation could be achieved and that islets transplanted in this way would remain viable and secrete sufficient insulin to reduce the blood glucose to near normoglycaemic levels.

Based on these preliminary observations, a full-scale animal study was subsequently carried out to assess the effect of units containing lean mouse islets on the blood glucose, serum insulin and body weight of streptozotocin-diabetic rats. In addition, close scrutiny was made of the effect of implantation on the integrity of unit fabrication materials and the survival of islets in implanted units.

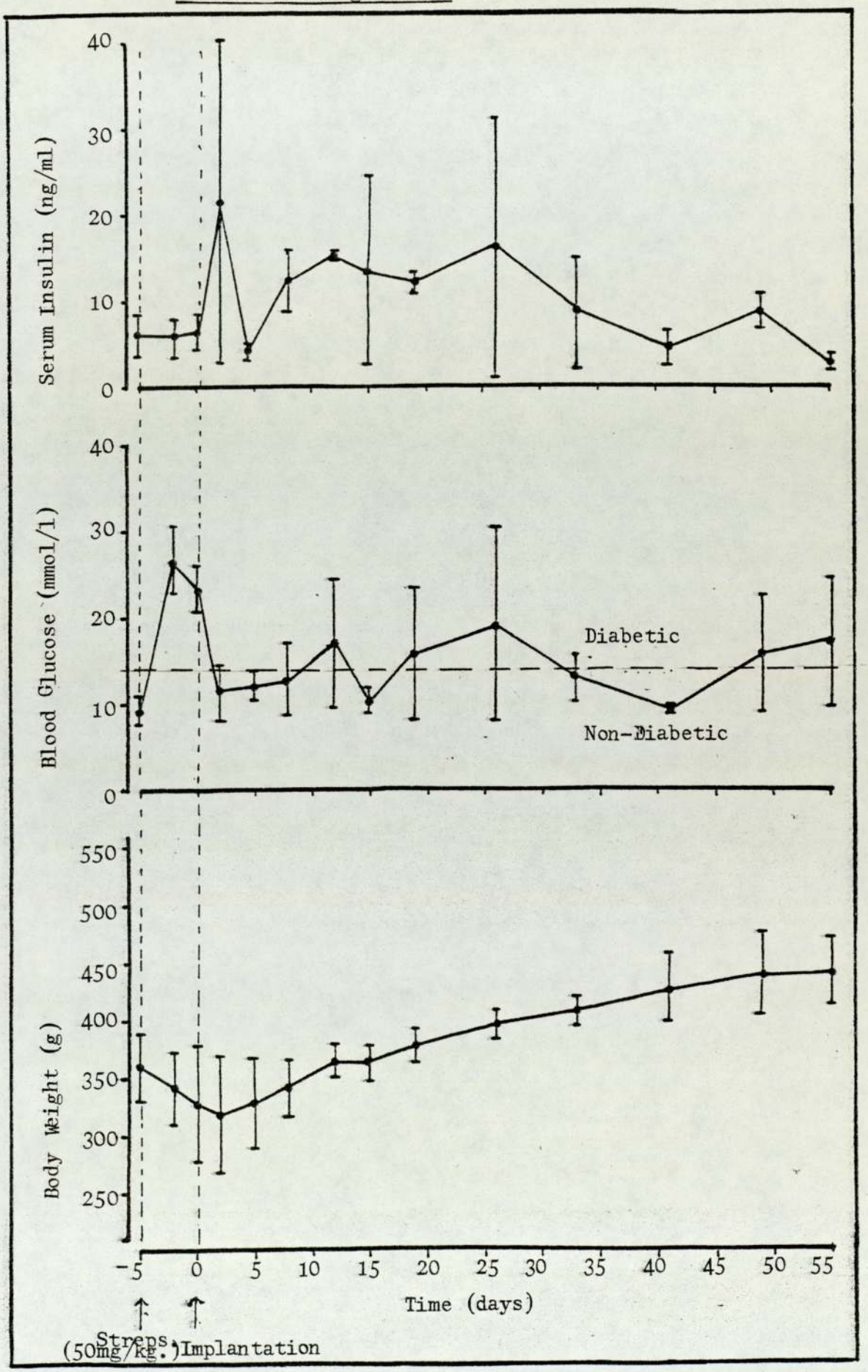
Materials and Methods

In this study, 2 groups of rats, 7 control and 8 test, mean weight 393.8 ± 10.5g, were made diabetic (blood sugar > 14 mmol/l) with either a single injection of streptozotocin (50 mg/kg, i.p. in citrate buffer, pH 4.8) or multiple injections (20-30 mg/kg) 5 days prior to unit implantation. Rats in the control group received "dummy" units containing sterile, physiological saline, the lumen of which had been flushed and filled with sterile heparinised saline (500 I.U./ml) to prevent blood clotting during surgery. 5 rats in the control group received 1.5 cm units, the other 2 received 3 cm units. Rats in the test group each received units containing pooled, lean mouse islets suspended in oxygenated HBSS (95% O_2/5% CO_2), supplemented with glucose (5.56 mmol/l) and BSA V (7.45g/100 ml). 4 of the test rats received 1.5 cm units, the remaining 4 received 3 cm units. All units were implanted into the right carotid artery over a period of two days as described in Section 4.3 (b), page 101.

Results

Three animals in the test group died during the postoperative recovery period leaving one animal with a 3 cm unit and 4 animals with 1.5 cm units. 2 out of the 7 diabetic control rats which had received "dummy" units were still able to show a substantial serum insulin response to hyperglycaemia, Figure 4.5, indicating that streptozotocin treatment had not been totally effective in these animals. Streptozotocin treatment did produce an early marked rise in blood glucose (>25 mmol/l) that was reduced substantially after implantation of the unit. A postsurgical reduction in body weight was observed which gradually recovered over the experimental period. Serum insulin levels remained high over the period of study confirming inadequate streptozotocin treatment.

Figure 4.5. Blood Glucose, Serum Insulin and Body Weight Profiles of Streptozotocin-Treated Rats Receiving "Dummy" Units Containing Saline. Values are means ± S.E.M. (n=2)



The remaining 5 rats in the control group with "dummy" implanted units showed typically diabetic profiles for blood glucose, serum insulin and body weight, Figure 4.6. Streptozotocin treatment was associated with a fall in body weight, hyperglycaemia and a temporary rise in serum insulin associated with the destruction of β cells. Immediately after implantation, there was a marginal fall in blood glucose and body weight with the serum insulin stabilising at 3.9±0.5 ng/ml. During the remainder of the study hyperglycaemia was maintained at ⩾30 mmol/l in the presence of very low serum insulin concentrations and the animals were just able to maintain their body weight, Figure 4.6.

The group of streptozotocin-diabetic test animals, like the control animals, demonstrated a significant hyperglycaemia (>30 mmol/l) and a reduction in body weight prior to implantation, Figure 4.7. Post-operatively, animals implanted with units containing lean mouse islets showed a further reduction in body weight and a concomitant reduction in the mean blood glucose level, from 34.2±1.7 to 21.1±4.2 mmol/l over the subsequent 2 days ($p<0.05$). This reduction in blood glucose was associated with a marginal increase in the serum insulin level and suggested the presence of functional units up to this time. Body weight stabilised at day 9 and increased gradually throughout the remainder of the study. From day 13 to day 27 the mean blood glucose level remained high, 28.1±0.2 mmol/l and was accompanied by a low but sustained mean serum insulin level of 3.41 ± 0.48 ng/ml. From day 27, the body weight was maintained and serum insulin remained low but the blood glucose gradually fell away to almost normoglycaemic levels (13.0 ± 4.9 mmol/l) by the end of the study. Post-mortem examination of the implanted units showed no signs of erosion of the fabrication materials. Indeed, the surface of all units was covered by a layer of fibrous connective tissue indicating good biocompatibility.

Figure 4.6. Blood Glucose, Serum Insulin and Body Weight Profiles of Streptozotocin-Treated Rats Receiving "Dummy" Units, Containing Saline. Values are means ± S.E.M. (n=5)

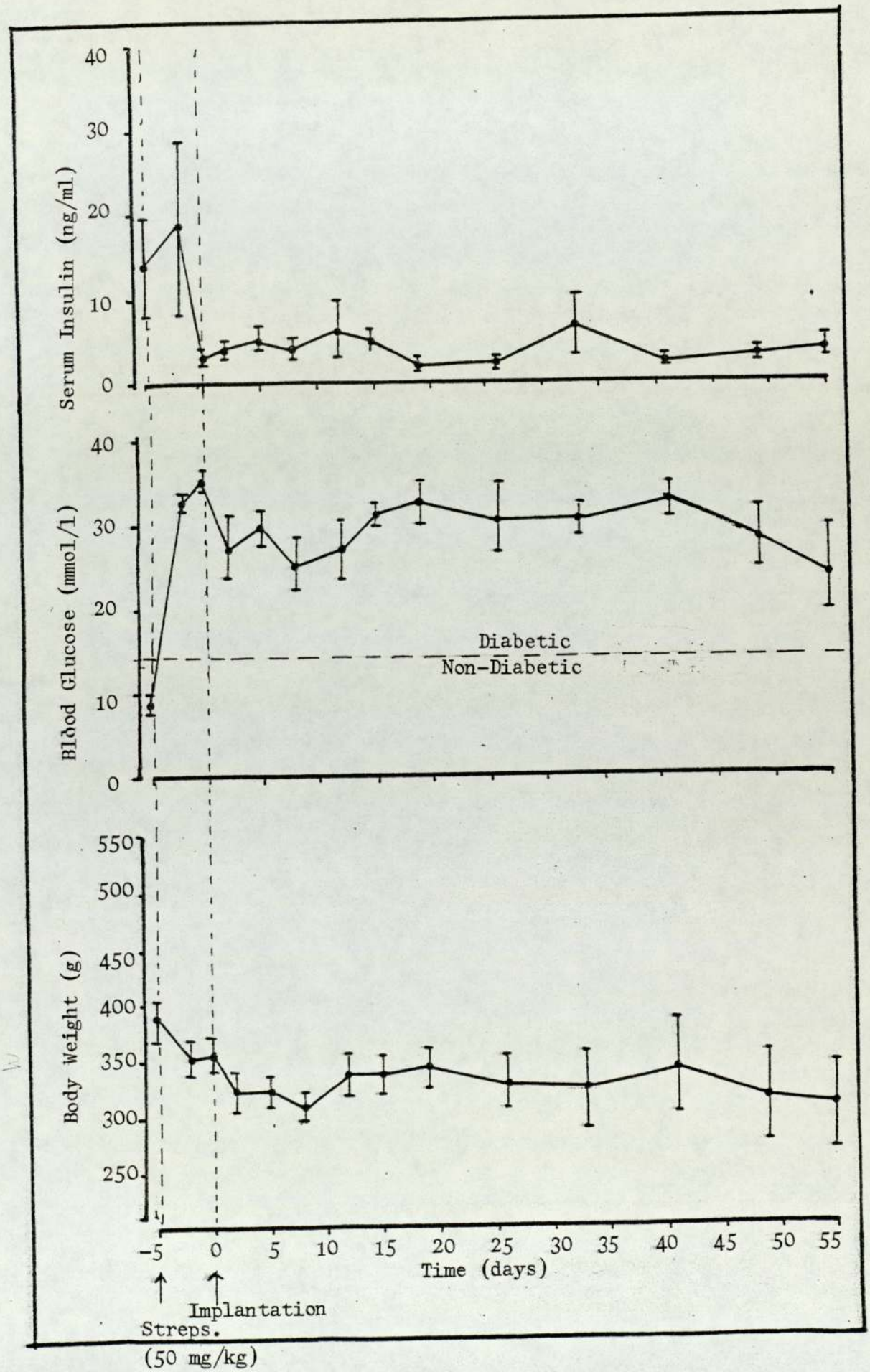
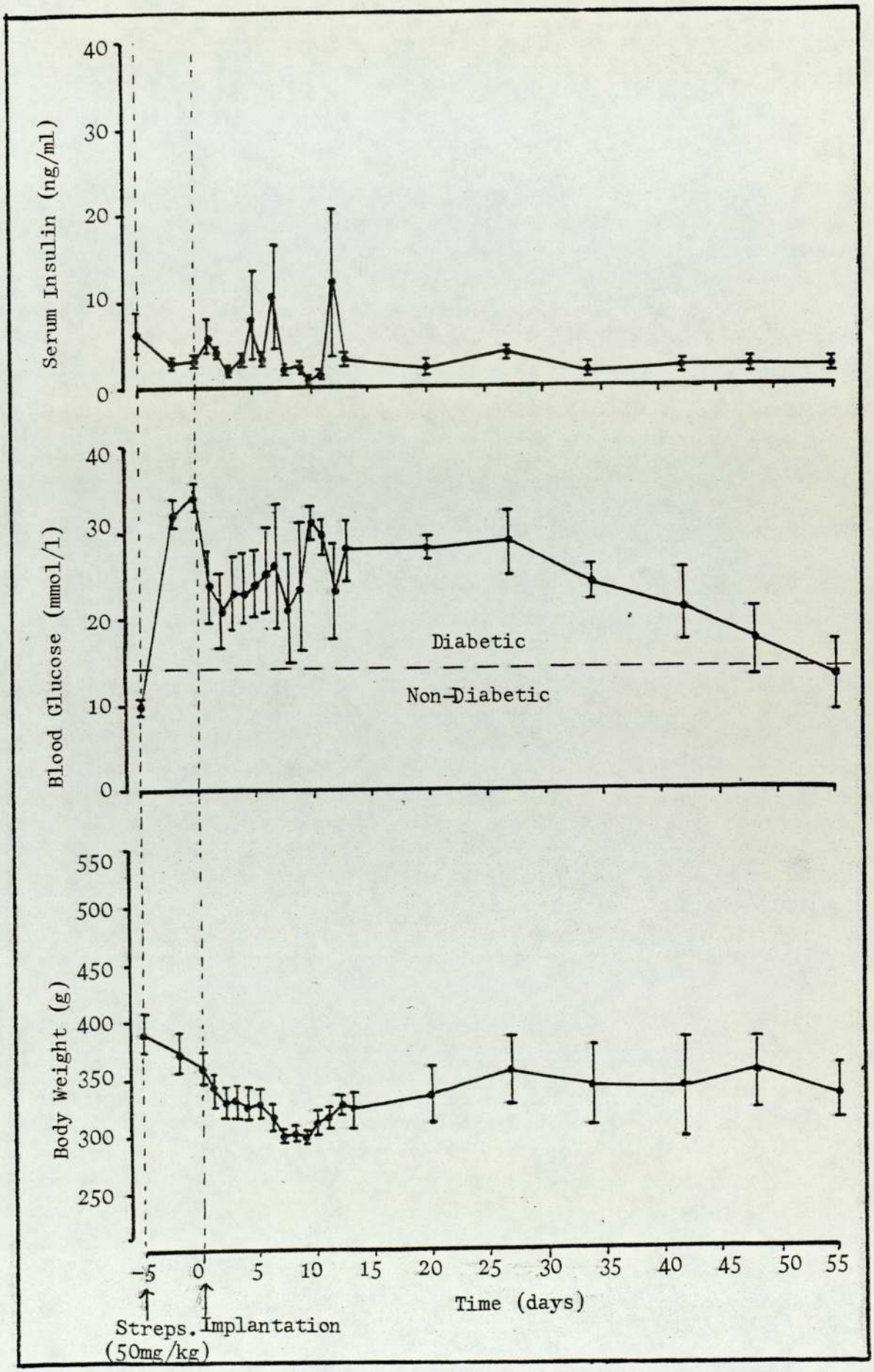


Figure 4.7. Blood Glucose, Serum Insulin and Body Weight Profiles of Streptozotocin-Treated Rats Receiving Units Containing Lean Mouse Islets.
Values are means ± S.E.M. (n=5)



Discrete islets could be identified under the light microscope although some had lost their connective tissue capsules and were smaller in size. In most of the units the medium in the outer polyethylene capsule had turned pink/red in colour probably as a result of erythrocyte haemolysis during the surgical procedure.

Discussion

Streptozotocin has been shown to cause the destruction of β cells (192) and its injection is associated with the production of damaged β-cells and the acute release of insulin into the circulation. In this situation subsequent insulin release from remaining undamaged β cells provides a low but sustained level of serum insulin in the presence of severe hyperglycaemia (usually >30 mmol/l) and a gradual fall in body weight. In the present study, implantation of saline-containing units into streptozotocin-diabetic rats confirmed these observations, Figure 4.6 and provided a low background serum insulin in conjunction with hyperglycaemia and a reduction in body weight. Of the control group receiving "dummy" units, two animals showed substantial levels of serum insulin after implantation and only a moderate hyperglycaemia, Figure 4.5. Streptozotocin treatment had clearly not been totally effective in these animals and they were subsequently excluded from the study. All rats remaining in the test group were diabetic at the time of unit implantation with body weight, blood glucose and serum insulin levels very similar to those of the control group. However, whilst blood glucose levels fell significantly in the test group after implantation and remained significantly lower than preoperative levels over the first 5 days, they never fell below a mean of 23.0±0.4 mmol/l and therefore remained diabetic. As the study proceeded however, a significant reduction in blood glucose was observed from day 27,

falling to normoglycaemic levels by day 55. Such a significant reduction was not observed for the control group, Figure 4.6. and in addition was not associated with a significantly elevated serum insulin level in test animals. Body weight was maintained and marginally elevated in test animals compared to controls, although the differences were not significant. It was clear from the profiles in Figure 4.7 that the implanted units had ceased to perform properly at around day 12 - 13, the serum insulin levels dropping to near control values. The reason for the further reduction in blood glucose after day 27 was obscure in view of the low serum insulin level but might be accounted for by an increased osmotic diuresis. The animals in the test and control studies were culled on day 60 and their units examined for deterioration. There was no evidence of the erosion of fabrication material or adhesive and all units were encapsulated in well-vascularised, fibrous connective tissue. There was no evidence of trauma to adjacent tissues or organs and there was no evidence of inflammation at the site of incision. Two of the units had clotted but the three remaining contained islets that appeared smaller but essentially normal under the light microscope. The bathing medium in the outer polyethylene shell had accumulated haemoglobin, probably derived from haemolysed erythrocytes at the time of implantation. It would appear, therefore that prototype units implanted into rats will remain functional for short periods of time (12 - 15 days), ameliorate the prevailing hyperglycaemia and maintain the body weight of rats made diabetic with streptozotocin, but more successful implantation might need to be associated with a modest anticoagulant régime.

CHAPTER 5

DISCUSSION

The present study has demonstrated that prototype functional prosthetic units can be assembled from biocompatible polymeric materials, polyvinylchloride and polyethylene, hollow fibres composed of semipermeable acrylic copolymer and biocompatible u/v-cured dental adhesive. Preliminary studies with such units containing methylene blue dye have shown that a constant rate of release could be achieved over a range of flow rates and that this release could be maintained for up to 7.5 hrs. (450 minutes). Although the maximum rate of removal of dye was obtained at a fairly low flow rate of 0.162 ml/min., a perfectly acceptable rate of removal was obtained at the higher flow rate of 0.637 ml/min. a value more consistent with physiological levels of perfusion pressure.

Data from experiments involving the measurement of insulin release from prototype units containing ^{125}I-insulin, broadly confirmed the results of methylene blue studies and indicated that the rate of insulin release was proportional to the concentration of ^{125}I-insulin originally present in the unit, but independent of the albumin (BSA V) concentration in the perfusate. A perfusate albumin concentration of 7.45 g/100 ml was chosen to reflect the physiological level in man.

The protein-coating of unit hollow fibres using a protein-glutaraldehyde cross-linking method to reduce thrombogenicity (189), decreased the rate of removal of ^{125}I-insulin and effectively increased the lag time for insulin transfer by occluding pores on the luminal surface of the fibre. In addition, the uniformity of the protein coating could not be guaranteed.

In vitro prototype units containing lean mouse islets re-

leased insulin at physiological flow rates (0.637 ml/min.) and perfusion pressures (100-200 mmHg) in response to an increase in glucose concentration from 5.56 mmol/l to 16.67 mmol/l. The rate of glucose-induced insulin release was optimal in the presence of 7.45g/100 ml albumin. Large obese mouse islets were not suitable as a source of insulin-secreting tissue, since the amount of insulin released from the islets during 60 minutes' test perfusion was not proportionately related to the weight of islet tissue present in the unit. In addition, the amount of insulin released in response to glucose challenge was not significantly different from that released by lean mouse islets. The concentration of insulin released from prototype units containing lean mouse islets in response to perfusion with HBSS supplemented with 5.56 mmol/l glucose (1.22±0.1 ng/ml) was marginally greater than the circulating insulin concentration recorded for 12-hour fasted 20 week-old lean mice (1.20 ± 0.13 ng/ml).

In addition, the concentration of insulin released in response to perfusion with HBSS containing 16.67 mmol/l glucose (1.68±0.12 ng/ml) was only marginally lower than the free fed circulating insulin level recorded for 20 week old lean mice (190). Hence the *in vitro* performance of prototype units was considered sufficient to make a useful contribution to the maintenance of normoglycaemia in subsequent *in vivo* studies in rats.

Preliminary *in vivo* studies in rats made diabetic with streptozotocin demonstrated that successful surgical implantation of functional units into the right common carotid artery could be achieved routinely and that implanted units containing lean mouse islets would secrete sufficient insulin to reduce the blood glucose and maintain the normal gradual increase in body weight. These units were shown capable of functioning for short periods of time *in vivo* (12 - 15 days) without any change in structural integrity or trauma

to adjacent tissues and organs.

Despite the modest achievements made to date with the implantable prosthesis, several problems still need to be resolved for the future. These may be broadly summarised in terms of improved performance and a reduction in the lag time (time taken for the unit to respond to glucose challenge), improved biocompatibility of components (especially the hollow fibre), the protection of xenogeneic islets from the immune response of the host, the employment of more effective sterilisation procedures, the generation of large quantities of insulin-secreting tissue and the future scaling up of the units for implantation into larger animals and man.

Perhaps the most important feature required for future implantable units will be an improved performance in terms of a reduction in the lag time required for the release of insulin in response to an increased glucose concentration in the perfusate. This will require a more rapid equilibration of glucose and insulin across the hollow fibre membrane and the appearance of significantly greater amounts of insulin in the effluent. The most obvious remedy would be to employ many fibres in parallel (133, 159, 160) or a single long coiled fibre in an attempt to increase the effective surface area for transfer (162). The possibility of using scaled-down kidney haemodialysis units might be investigated in the future. Commercially available cellulose-based multi-fibre systems have been used for several years as filtration units in kidney dialysis (194) and also for the culture <u>in vitro</u> of several cell types (158,163,166,195). The use of multi-fibre systems, however, poses the problem of finding a suitable way of sealing or embedding the end of the fibre bundle into a biocompatible potting material such as methacrylate-based sealant, epoxy resin or silicone-based resin and connecting the ends of many patent fibres to a single inlet or outlet point.

Although the materials used in the fabrication of the present prototype unit were well-tolerated, appeared to be tissue compatible and could be sterilised successfully, blood compatibility and the tendency for thrombus formation remains the second most important problem. At the present time, the successful use of prototype units in vivo will require modest systemic heparinisation, delivered via an osmotic minipump (Alzet, Alza Corp., U.S.A.) Ideally, one would wish to avoid the need for long-term systemic anticoagulant therapy and more extensive work should be directed towards the production of both heparin-(or other anticoagulant)-linked (adsorbed) polymeric materials (162) and fabrication polymers into which anticoagulant has been irreversibly covalently bound. The long-term efficiency and integrity of fibres composed of heparin-linked polymers has yet to be evaluated. The use of a single length of cellulose or acrylic copolymer fibre, semipermeable along only the middle portion of its length, might represent a viable alternative to the polyethylene cannula/fibre/cannula design currently used since polyethylene appears to be fairly thrombogenic. A single fibre composed of acrylic copolymer would seem to offer a number of advantages, notably a reduction in the number of cannula junctions and hence, potential sites of thrombus formation (159). However, such a fibre would be very prone to crimping, whereas the rigid polyethylene cannula affords a measure of flexibility and strength.

The immunological barrier afforded by the prosthesis was an important property of the design (165). The molecular weight cut-off of the hollow fibre (50 or 100 K Daltons) prevented host antibodies from reaching the enclosed islet tissue, but may not exclude antibody fragments or complement factors. Despite this, in the present study, xenogeneic islets taken from previously implanted units appeared smaller but essentially normal when examined under the light microscope.

The successful use of the unit *in vivo* required that it should be sterile. The aseptic conditions employed throughout the current *in vivo* study appeared to be sufficient to ensure that there were no problems from infection in the experimental animals. However, in the future, more comprehensive means of ensuring the sterile fabrication of units will require the use of gamma-irradiation and exposure to ethylene oxide gas.

In the present study, isolated islets were transferred to prototype units in sterile perfusion medium supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). In future studies, improved techniques must be developed for the isolation of islets under aseptic conditions. It may prove possible to use short-term cultured islets or β-cells in the implantable units. Isolated rat beta cells (158) and adult rat islets (163,166) have been cultured successfully on the surface of hollow fibres perfused with nutrient medium in commercially available capillary culture units. The ability of these islets to maintain secretory viability for at least 97 days in culture has been elegantly demonstrated (163). The setting-up of an *in vitro* capillary culture unit for the accumulation and maintenance of large numbers of viable islets or β-cells prior to transplantation would be a useful way of scaling-up and providing the large amount of tissue required for the prosthetic units of larger animals, dogs, pigs and primates.

Insulinoma represents another potential source of insulin-secreting tissue that could be usefully employed in prosthetic units. Rat insulinoma cells (196), insulin-producing cells (RIN m 5 F)(197) and transformed hamster islet cells (198) are being used by several groups for the study of the insulin secretion mechanism. However, several of these islet cell tumours do not respond to the physiol-

ogical range of glucose concentrations and after passaging in selected strains of rats (e.g. NEDH) require a period of *in vitro* culture to remove blood cells and necrotic tumour cells. In addition, these cells have a high growth rate, even *in vitro* and this may lead to stagnation, hypoxia and reduced functional activity in prosthetic units.

Finally, the *in vitro* insulin secretory performance and the ability *in vivo* of implanted biocompatible prototype prosthetic units containing xenogeneic islets to release insulin in response to streptozotocin-induced hyperglycaemia demonstrate the future potential of such units in the treatment of insulin-dependent diabetes mellitus. With future development and modification and their capacity to generate several other islet hormones (glucagon, somatostatin and pancreatic polypeptide) besides insulin, they represent the ideal physiological remedy for the present régime of multiple daily injections currently endured by the majority of insulin-requiring diabetics.

APPENDIX 1

Modified Hanks' Balanced Salt Solution (181)

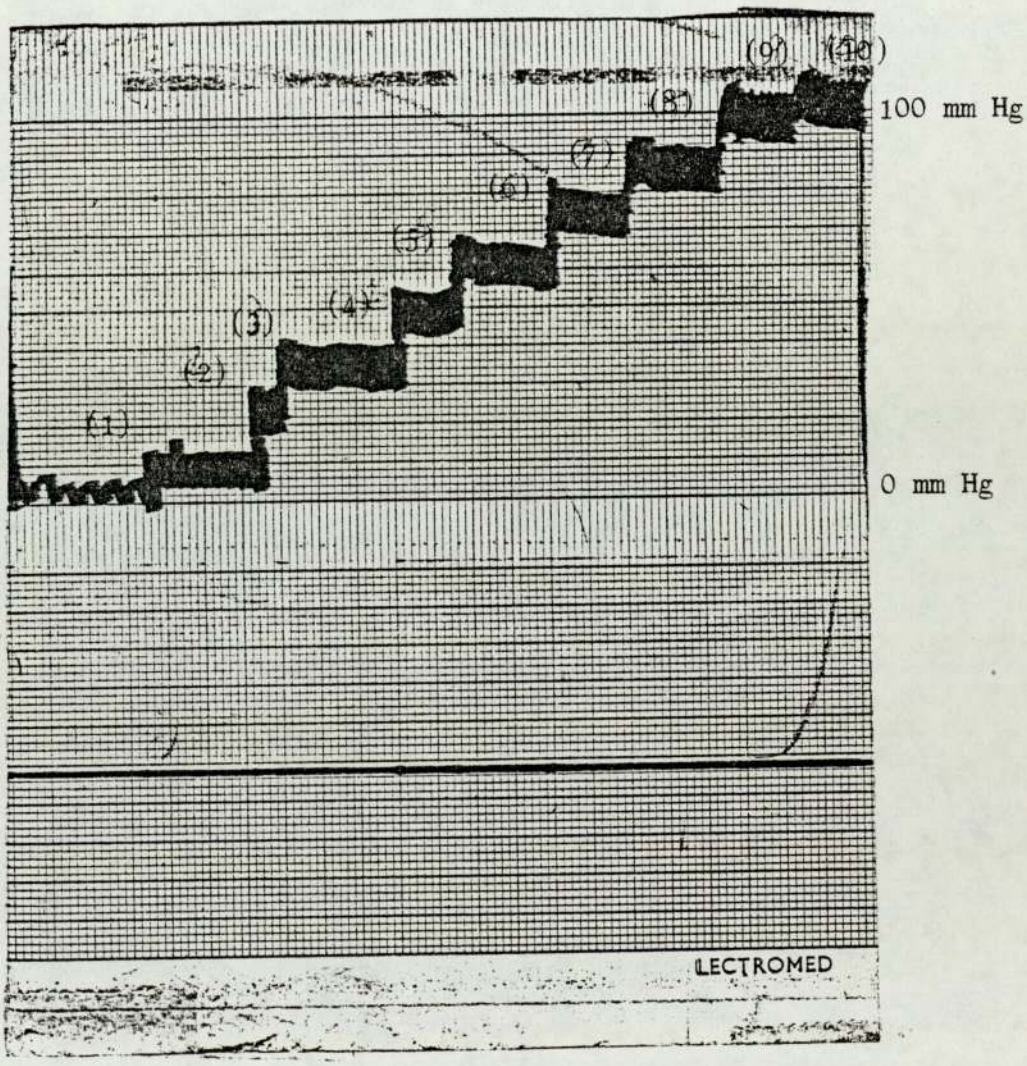
(Basic perfusion medium)

Ingredient	g/litre
$CaCl_2$, $2H_2O$	0.1855
KCl	0.400
KH_2PO_4	0.060
$MgCl_2$, $6H_2O$	-
$MgSO_4$, $7H_2O$	0.200
NaCl	8
$NaHCO_3$	0.350
NaH_2PO_4, $2H_2O$	-
Na_2HPO_4	0.0475
Supplemented with	
Penicillin/Streptomycin	100,000 U/0.1
Glucose	1 - 3
Bovine Serum Albumin V	5.0 - 74.5

APPENDIX 2.

Typical Perfusion Pressure Trace.

Flow Rate Settings in Parentheses.



Flow Rate Setting.	Flow Rate ml/min.
1	0.042 ml/min.
2	0.073
3	0.162
4	0.249
5	0.319
6	0.393
7	0.469
8	0.537
9	0.603
10	0.637

APPENDIX 3

The Double Antibody Radioimmunoassay for Insulin (183)

Assay components

(a) Diluent Buffer

Isotonic phosphate buffer, pH 7.4 composition:-

Sodium phosphate ($NaH_2PO_4, 2H_2O$)	0.04M
Sodium chloride	0.9%
Bovine albumin	0.5%
Sodium ethyl mercurithiosalicylate (thiomersalate)	0.1%

(b) Insulin Binding Reagent (pre-precipitated antibody)

Each bottle contained the equivalent of 8 ml of freeze-dried insulin binding reagent. The reagent consisted of a mixture of guinea-pig anti-insulin serum and rabbit anti-guinea-pig globulin serum in buffer of the following composition:-

Sodium phosphate	0.04M
EDTA	0.02M
Sodium azide	0.1%
Bovine Serum albumin	0.5%

pH 7.4

(c) Production of Monoiodinated Porcine Insulin

Iodination by Chloramine-T Method

The following reactants were mixed at room temperature (approximately 20°C) in the reaction vial containing $Na^{125}I$ supplied by the Radiochemical Centre.

Sodium phosphate buffer, pH 7.4 (0.04M),	10µl
Porcine Insulin (2.5 µg) in HCl (0.01M),	10 µl
$Na^{125}I$ (1mCi) (in NaOH solution),	10 µl
Chloramine-T (50 µg)	10 µl

The reaction was allowed to proceed for 10 seconds, with thorough mixing and subsequently terminated by the addition of 50 µl sodium metabisulphite (120 µg).

Mixing was continued for a further 45 seconds prior to dilution of the remaining radioactive iodide with 100 µl of potassium iodide (1 mg).

All reactants were weighed and diluted to the required concentration immediately before use.

Chloramine-T, sodium metabisulphite and potassium iodide were dissolved in freshly prepared sodium phosphate buffer, pH 7.4, 0.04M.

The iodinated insulin was purified by gel filtration on Sephadex G50. The monoiodinated product routinely had a specific activity of >200 µCi/µg with ≏ 4% damage and 60 - 65% incorporation.

(d) Standard Insulin Solutions

(i) Human Insulin Standard (Wellcome)

Stock (500 µU/ml) (ml)	Buffer (ml)	Final Volume (ml)	Final Conc. (ng/ml)	Final Conc. (µU/ml)
0.4	0.6	1.0	8	200
0.2	0.8	1.0	4	100
0.1	0.9	1.0	2	50
0.1	1.9	2.0	1	25
0.05	1.95	2.0	0.5	12.5

(ii) Mouse Insulin Standard (NOVO)

Stock Solution 100 ng/ml

Stock (ml)	Buffer (ml)	Final Volume (ml)	Final Conc. (ng/ml)
0.1	0.9	1.0	10
0.1	1.9	2.0	5
0.05	1.95	2.0	2.5
0.05	4.95	5.0	1.0
0.05	9.95	10.0	0.5

COMPUTER PROGRAM FOR THE CALCULATION OF INSULIN RIA DATA

```
  5 REM                INSULIN ASSAY
 10 REM
 15 REM
 20 REM
 25 REM  DATA statements :"Species","units",no. of standards,standa
 30 DATA "Mouse","ng/ml",5,.5,1,2.5,5,10                    concns.
 35 DATA "Human","uU/ml",5,12.5,25,50,100,200
 40 INPUT "Do you want printout ",PR$
 45 IF ASCII(CVT$$(PR$,32)) = 89 THEN 55
 50 LET PR$ = "N" : GOTO 140
 55 LET PR$ = "Y"
 60 PRINT : INPUT "File name for printout ",FL$ : IF FL$
                                                  ="INS" THEN 60
 65 ON ERROR GOTO 85
 70 GENERATE FL$
 75 ON ERROR GOTO 0
 80 ASSIGN #11 = FL$ : OPEN #11 : GOTO 115
 85 IF ERROR >< 2150 THEN DO : ON ERROR GOTO 0 : GOTO 70 :DOEND
 90 PRINT:PRINT"Area already exists-do you want to delete existing data in
 95 INPUT "( WARNING - do not delete a program area with this name
                                                  ↳",AN $
100 IF ASCII(CVT$$(AN$,32)) >< 89 THEN 60
105 ELIMINATE FL$
110 GOTO 70
115 PRINT : PRINT "Experiment title etc. for printout"
120 INPUT TI$ : PRINT #11 TI$
125 REM ****************************************************
130 REM Input standard cpm's & compute best line
135 REM ****************************************************
140 INPUT "STANDARDS : Mouse or Human or Both (M or H or B) ",MHB $
145 LET MHB$ = CVT$$(MHB$,32)
150 IF MHB$ = "M" THEN 165
155 IF MHB$ = "H" THEN 185
160 IF MHB$ >< "B" THEN 140
165 GOSUB 235
170 LET INM = IN
175 LET SLM = SL
180 IF MHB$ = "M" THEN 440
185 RESTORE
190 READ R$,R$,R%
195 FOR J% = 1 TO R%
200 READ R
205 NEXT J%
210 GOSUB 235
215 LET INH = IN
220 LET SLH = SL
225 GOTO 440
230 REM ********************* SUB
235 READ Sp$,Un$
240 PRINT : PRINT Sp$
245 PRINT "Standards","  cpm (If value not available enter "0")
250 PRINT Un$
255 IF PR$ = "N" THEN 275
260 PRINT #11 : PRINT #11 Sp$
```

```
265 PRINT #11 "Standards","  cpm"
270 PRINT #11 Un$
275 LET X,Q,Y,S,W,N% = 0
280 READ StN%
285 FOR J% = 1 TO StN%
290 READ StCo ! Standard concentration
295 PRINT StCo," ";
300 INPUT StCpm
305 IF StCpm >< 0 THEN 320
310 IF PR$ = "N" THEN 365
315 PRINT #11 USING "####.#                    -", StCo : GOTO 365
320 IF PR$ = "N" THEN 330
325 PRINT #11 USING "####.#     ############", StCo,StCpm
330 LET StCo = LOG10(StCo)
335 LET X = X+StCpm
340 LET Q = Q+StCpm^2
345 LET Y = Y+StCo
350 LET S = S+StCo^2
355 LET W = W+StCo*StCpm
360 LET N% = N%+1
365 NEXT J%
370 LET SL = (W-X*Y/N%)/(Q-X^2/N%)
375 LET IN = (Y-SL*X)/N%
380 LET CoCo =(W-X*Y/N%)/SQR((Q-X^2/N%)*(S-Y^2/N%))
385 PRINT : PRINT "Computed best line : Intercept (cpm = 0) =";IN
390 PRINT TAB(22),"Slope",TAB(46),"= ";SL
395 PRINT TAB(22),"Correlation coefficient = ";CoCo
400 IF PR$ = "N" THEN 420
405 PRINT #11 : PRINT #11 "Computed best line:Intercept (cpm=0)=";IN
410 PRINT #11,TAB(22),"Slope",TAB(46),"= ";SL
415 PRINT #11,TAB(22),"Correlation coefficient = ";CoCo
420 RETURN
425 REM ************************************************
430 REM                Print headings
435 REM ************************************************
440 PRINT : PRINT "Input sample name , or 'END' to stop program"
445 PRINT TAB(21),"or 'DIL' to change dilution factor"
450 IF PR$ = "N" THEN 460
455 PRINT TAB(21),"or 'SUBH' to insert subheading for printout"
460 PRINT "followed by cpm"
465 PRINT : PRINT "'*' indicates result outside linear region of curve
470 PRINT "    and will be incorrectly calculated by this program"
475 REM Limits of linearity set in 640,645,690,695
480 IF MHB$ = "B" THEN 545
485 PRINT : PRINT TAB(38),"Insulin concn."
490 PRINT TAB(38),"ref. ";Sp$;" standard"
495 IF PR$ = "N" THEN 510
500 PRINT #11 : PRINT #11 TAB(39),"Insulin concentration"
505 PRINT #11 TAB(27),"Dilution     ref. ";Sp$;" standard"
510 IF MHB$ = "H" THEN 530
515 PRINT "Sample","  cpm","   ng/ml","  uU/ml"
520 IF PR$ = "N" THEN 600
525 PRINT #11 "Sample","  cpm     factor      ng/ml  uU/ml" GOTO 580
530 PRINT "Sample","  cpm","   uU/ml"
535 IF PR$ = "N" THEN 600
540 PRINT #11 "Sample","  cpm     factor",TAB(45),"uU/ml" : GOTO 580
545 PRINT : PRINT TAB(38),"Insulin concentration    Insulin concn."
550 PRINT TAB(38),"ref. Mouse standard      ref. Human st."
555 PRINT "Sample","cpm"," ng/ml","uU/ml"," uU/ml"
560 IF PR$ = "N" THEN 600
```

```
565 PRINT #11:PRINT #11 TAB(38), Insulin Concentration Insulin Concentratio
570 PRINT #11 TAB(26),"Dilution  Ref. Mouse standard Human Standard
575 PRINT #11 "Sample","cpm factor ng/ml  uU/ml", TAB(69)        uU/ml"
580 PRINT #11
585 REM ************************************************
590 REM          Calculate sample concentrations
595 REM ************************************************
600 PRINT : INPUT "Dilution factor ",DF : PRINT
605 LET MX$,HX$ = "*"
610 INPUT Sam$
615 IF PR$ = "N" THEN 630
620 IF CVT$$(Sam$,32) >< "SUBH" THEN 630
625 INPUT TI$ : PRINT #11 : PRINT #11 "  ";TI$ : PRINT : GOTO 610
630 IF CVT$$(Sam$,32) = "END" THEN 780
635 IF CVT$$(Sam$,32) = "DIL" THEN 600
640 PRINT " "," "; : INPUT Cpm : PRINT Sam$,Cpm," ";
645 IF MHB$ = "H" THEN 700
650 LET CoM = INT(EXP((INM+SLM*Cpm)*LOG(10))*10+.5)/10
655 IF CoM < .5 THEN 670
660 IF CoM > 12.8 THEN 670
665 LET MX$ = " "
670 IF MHB$ = "B" THEN 700
675 PRINT USING "!####.#       !#####.#",MX$,DF*CoM,MX$,25*DF*CoM
680 IF PR$ = "N" THEN 695
685 LET FMM$ = "\   \#####          #####    !##### .#  !#####.#"
690 PRINT #11 USING FMM$, Sam$,Cpm,DF,MX$,DF*CoM,MX$,25*DF*CoM
695 IF MHB$ = "M" THEN 605
700 LET CoH = INT(EXP((INH+SLH*Cpm)*LOG(10))+.5)
705 IF CoH < 12.5 THEN 720
710 IF CoH > 320 THEN 720
715 LET HX$ = " "
720 IF MHB$ = "B" THEN 745
725 PRINT USING "!#####",HX$,DF*CoH
730 IF PR$ = "N" THEN 605
735 LET FMH$ = "\                \#####    #####          !########"
740 PRINT #11 USING FMH$, Sam$,Cpm,DF,HX$,DF*CoH : GOTO 605
745 LET FMBT$ = "!####.#       !#####.#            !######"
750 PRINT USING FMBT$, MX$,DF*CoM,MX$,25*DF*CoM,HX$,DF*CoH
755 IF PR$ = "N" THEN 605
760 LET FMB1$ = "\              \#####    #####      "
765 LET FMB$ = FMB1$+"!#####.#      !######.#          !########"
770 PRINT #11 USING FMB$, Sam$,Cpm,DF,MX$,DF*CoM,MX$,25*DF*CoM,
775 GOTO 605                                          HX$,DF*CoH
780 IF PR$ = "N" THEN STOP
785 PRINT : PRINT "File for printout : ";FL$

**********************************************************************
**********************************************************************
***************
***************    No. of pages      3    7049-TA
***************     Terminal 85. 16 FEB 84 14:34:54 Vos 2. 4.
**********************************************************************
**********************************************************************
```

APPENDIX 4

STATISTICAL ANALYSES

Significance of the difference was assessed by Students' t-test, p values < 0.05 were considered significant.

Best straight lines were constructed by the least squares method.

All calculations were performed on a programmable calculator, Olivetti Programma 101.

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APPENDIX 5

0306-3623/81/090323-04$02.00/0

PUBLISHED PAPERS NO.1

CHRONIC EFFECTS OF GLIBENCLAMIDE, CHLORPROPAMIDE AND METFORMIN ON PLASMA GLUCOSE AND INSULIN IN NON-DIABETIC RATS

CLIFFORD J. BAILEY and MARY E. BROADBENT
Department of Biological Sciences, University of Aston in Birmingham, Birmingham B4 7ET, U.K.

(*Received* 23 *March* 1981)

Abstract—1. Plasma glucose and insulin concentrations were examined in non-diabetic rats after daily intraperitoneal injections of glibenclamide (0.4 mg/kg), chlorpropamide (10 mg/kg) or metformin hydrochloride (60 mg/kg) for 8 weeks.

2. After 8 weeks the hypoglycaemic effects of glibenclamide and chlorpropamide were preserved, the insulin releasing effect of glibenclamide was preserved, but the insulin releasing effect of chlorpropamide was lost. Metformin reduced plasma insulin without a significant change in plasma glucose.

3. Glucose tolerance was impaired 24 hr after the last injection of each drug in 8 weeks treated rats. This suggests that during chronic treatment any potentially beneficial effects on glucose homeostasis were lost within 24 hr of the last injection.

INTRODUCTION

It is well recognised that in non-insulin dependent (NID) diabetics the insulin releasing effect of chlorpropamide is lost during chronic treatment, although the hypoglycaemic action may be preserved (Karam *et al.*, 1975; Lebovitz & Feinglos, 1978). Similar observations have been made during prolonged treatment with glibenclamide, but the insulin releasing effect of this agent is highly variable and may persist for many months (Feldman & Lebovitz, 1971; Owens *et al.*, 1979). It has been noted recently that chronic metformin treatment suppresses plasma insulin in NID diabetics (Nattrass *et al.*, 1979). Short-term studies in non-diabetic animals indicate that high doses of metformin raise plasma insulin, while low doses may reduce plasma insulin (Loubatieres *et al.*, 1971; Losert *et al.*, 1972; Ohnhaus *et al.*, 1978; Bobbioni *et al.*, 1979).

Previous comparisons of the chronic effects of sulphonylureas and biguanides have given little attention to plasma insulin. Furthermore, the consequence of withholding the drugs for a short period after chronic treatment has not been examined. The present study compares the acute and chronic effects of glibenclamide, chlorpropamide and metformin on plasma glucose and insulin in non-diabetic rats. In addition, glucose tolerance has been examined 24 hr after the last drug injection in the chronically treated animals.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats were maintained in an air-conditioned room at 22 ± 2°C with a regular lighting schedule of 9.5 hr light and 14.5 hr dark. A standard pellet diet and tap water were supplied *ad libitum*. Groups of rats were treated for 8 weeks with daily intraperitoneal injections of either glibenclamide (0.4 mg/kg), chlorpropamide (10 mg/kg) or metformin hydrochloride (60 mg/kg). These doses correspond to the maximum recommended clinical doses on a weight for weight basis. Controls received injections of vehicle only (buffered saline).

Food intake and body weight were monitored at weekly intervals throughout the study. No significant differences in either parameter were observed between the four groups of rats.

Acute changes in plasma glucose and insulin concentrations after injection of the drugs were examined at the beginning of treatment (day 1) and after 8 weeks of treatment. Blood samples (100 μl) were taken from the cut tip of the tail of freely fed rats immediately before and at 1 and 3 hr after injection of the drug. Glucose tolerance tests were performed on 8 weeks treated rats after a 12 hr fast and 24 hr after the last drug injection. Blood samples were taken immediately before and at 30 and 60 min after an intraperitoneal injection of glucose (2 g/kg in a 40% w/v solution).

Plasma glucose was determined by an automated glucose oxidase procedure (Stevens, 1971) using a Beckman glucose analyser (Beckman Riic Ltd., High Wycombe). Plasma insulin was measured by double antibody radioimmunoassay (Hales & Randle, 1963) using rat insulin as standard.

Groups of data were compared using Student's *t*-test. Differences were considered to be significant for $P < 0.05$.

RESULTS

Effects of drugs at day 1

Plasma glucose and insulin concentrations at 1 and 3 hr after injection of glibenclamide, chlorpropamide and metformin are shown in Table 1. At the beginning of treatment (day 1), glibenclamide produced a marked fall in plasma glucose at 1 and 3 hr, and chlorpropamide produced a small but significant decrease in plasma glucose at 1 hr. Plasma insulin concentrations were raised at 1 hr by glibenclamide, and chlorpropamide produced a smaller rise in plasma insulin at this time. Metformin did not significantly alter plasma glucose concentrations, and plasma insulin concentrations were not significantly different from the control group. However, a significant ($P < 0.01$) reduction in plasma insulin was observed at 3 hr compared with 1 hr after metformin administration.

Table 1. Plasma glucose and insulin responses to glibenclamide, chlorpropamide and metformin in non-diabetic rats at the beginning of treatment (day 1) and after 8 weeks of treatment with daily injections of the drugs

	Plasma glucose mg/100 ml				Plasma insulin ng/ml			
	0	1 hr	3 hr	Δ*	0	1 hr	3 hr	Δ*
Beginning of treatment (day 1)								
Control	134 ± 6	130 ± 6	122 ± 6	−16.0 ± 6.1	2.5 ± 0.3	2.7 ± 0.4	1.9 ± 0.3	−0.4 ± 0.4
Glibenclamide	129 ± 7	66 ± 5[1,3,4]	86 ± 8[1,3,4]	−105.2 ± 16.7[1,3,4]	2.5 ± 0.3	4.9 ± 0.6[1,4]	2.3 ± 0.5[4]	2.0 ± 0.7[1,4]
Chlorpropamide	133 ± 4	111 ± 4[1,2,4]	128 ± 4[2]	−27.0 ± 11.6[2,4]	2.4 ± 0.1	3.9 ± 0.3[1,4]	2.0 ± 0.5	0.9 ± 0.5[1,4]
Metformin	128 ± 5	126 ± 5[2,3]	130 ± 4[2]	0.3 ± 9.3[2,3]	2.3 ± 0.1	2.3 ± 0.1[2,3]	1.3 ± 0.1[2]	−0.9 ± 0.3[2,3]
8 weeks treated								
Control	131 ± 4	130 ± 2	115 ± 6	−16.7 ± 5.7	2.3 ± 0.4	1.9 ± 0.3	1.7 ± 0.2	−0.9 ± 0.6
Glibenclamide	134 ± 7	82 ± 9[1,3,4]	95 ± 6[1,3,4]	−90.7 ± 8.4[1,3,4]	2.0 ± 0.2	5.2 ± 0.8[1,3,4]	2.6 ± 0.3[1,3,4]	3.8 ± 0.9[1,3,4]
Chlorpropamide	137 ± 6	107 ± 7[1,2,4]	122 ± 9[2]	−44.5 ± 12.0[1,2]	2.5 ± 0.3	2.4 ± 0.4[2]	1.4 ± 0.3[2]	−1.2 ± 1.0[2]
Metformin	136 ± 4	125 ± 4[2,3]	123 ± 6[2]	−24.8 ± 11.4[2]	2.2 ± 0.3	2.2 ± 0.3[2]	1.0 ± 0.2[1,2]	−1.2 ± 0.4[2]

* Δ is the change in plasma glucose or insulin values calculated as the sum of the values at 1 hr and 3 hr minus twice the value at time zero.
[ ] Statistical comparisons: $P < 0.05$ compared with [1]control, [2]glibenclamide, [3]chlorpropamide, and [4]metformin.
Values are mean ± SEM of 6 rats.

Table 2. Plasma glucose and insulin responses during intraperitoneal glucose tolerance tests 24 hr after the last drug injection in non-diabetic rats treated for 8 weeks with daily injections of glibenclamide, chlorpropamide or metformin. The rats were fasted for 12 hr

	Plasma glucose mg/100 ml				Plasma insulin ng/ml			
	0	30 min	60 min	Δ*	0	30 min	60 min	Δ*
Control	99 ± 7	249 ± 10	177 ± 15	228 ± 16	1.1 ± 0.2	2.6 ± 0.4	1.6 ± 0.2	1.9 ± 0.5
Glibenclamide	111 ± 5	300 ± 20[1,4]	197 ± 7	275 ± 13[1]	1.1 ± 0.2	7.5 ± 0.3[1,3,4]	1.9 ± 0.3	7.2 ± 0.7[1,3,4]
Chlorpropamide	97 ± 10	290 ± 17[1,4]	181 ± 16	276 ± 18[1]	1.4 ± 0.3	2.9 ± 0.4[2]	1.7 ± 0.4	1.8 ± 0.7[2]
Metformin	103 ± 8	338 ± 6[1,2,3]	189 ± 22	321 ± 22[1]	1.1 ± 0.2	2.7 ± 0.6[2]	1.4 ± 0.2	1.7 ± 0.5[2]

* Δ is the change in plasma glucose or insulin values calculated as the sum of the values at 30 and 60 min minus twice the value at time zero.
[ ] statistical comparisons; $P < 0.05$ compared with [1]control, [2]glibenclamide, [3]chlorpropamide, and [4]metformin.
Values are mean ± SEM of 6 rats.

Effects of drugs after 8 weeks

In rats treated with daily drug injections for 8 weeks there were no significant changes in the fed plasma glucose and insulin concentrations 24 hr after the last injection. Administration of each drug produced a similar effect on plasma glucose to that observed at day 1: that is, glibenclamide considerably lowered, chlorpropamide lowered to a smaller extent and metformin did not significantly alter plasma glucose concentrations. Glibenclamide evoked a rise in plasma insulin at 1 hr, similar to that produced at day 1. However, in contrast to the hyperinsulinaemic effect at day 1, chlorpropamide did not evoke a rise in plasma insulin after 8 weeks. Metformin reduced plasma insulin concentrations at 3 hr in 8 weeks treated rats.

Glucose tolerance after 8 weeks, 24 hr after last drug injection

Plasma glucose and insulin concentrations during glucose tolerance tests 24 hr after the last drug injection in 8 weeks treated rats are shown in Table 2. The 12 hr fasted plasma glucose and insulin concentrations were not significantly different in the four groups of rats. However, glucose tolerance was impaired in each of the drug treated groups. The plasma insulin response to glucose was increased in the glibenclamide treated group, but the response was normal in the chlorpropamide and metformin treated groups.

DISCUSSION

The results confirm that the insulin releasing effect of chlorpropamide is lost after prolonged treatment, while the hypoglycaemic action is preserved. The chronic hypoglycaemic action of sulphonylureas in the absence of a measurable increase in insulin concentrations is attributed largely to an extrapancreatic effect. This appears to involve an increase in the number of insulin receptors and potentiation of insulin mediated metabolic events (Olefsky & Reaven, 1976; Blumenthal, 1977; Lebovitz & Feinglos, 1978; Beck-Nielson *et al.*, 1979).

Glibenclamide produced a greater hypoglycaemia and a greater hyperinsulinaemia than chlorpropamide, and retained both of these effects after 8 weeks. Glibenclamide is known to increase the sensitivity of the islet B-cells for glucose-induced insulin release (Cerasi *et al.*, 1979), although it is reported to impair insulin biosynthesis *in vitro* (Schatz *et al.*, 1977). In the present study the insulin releasing effect of glibenclamide was apparently not impeded by inadequate stores of releasable insulin. However, larger doses of glibenclamide have been shown to lose their insulin releasing effect in non-diabetic animals over the same or shorter treatment periods (Dunbar & Foa, 1974; Codina *et al.*, 1978).

A chronic extrapancreatic action is generally ascribed to the hypoglycaemic effect of biguanides, and several contributing mechanisms have been described. These include reduced intestinal absorption of glucose, reduced hepatic gluconeogenesis, enhanced peripheral glucose uptake and impaired secretion of hormones which antagonise insulin (Czyzyk, 1977; Hermann, 1979). In the present study metformin suppressed plasma insulin concentrations 3 hr after administration of the drug, although the dose given did not significantly alter plasma glucose. This supports the clinical observation that metformin can suppress insulin without significantly altering plasma glucose (Nattrass *et al.*, 1979).

Since glucose tolerance was impaired 24 hr after the last injection of the drugs in the 8 weeks treated rats, it appears that any potentially beneficial hypoglycaemic effects were lost after 24 hr. The concomitantly enhanced insulin response to glucose in the glibenclamide treated group suggests an especially reduced sensitivity to insulin in these rats. This might appear to contradict the reported increase in insulin receptors after chronic glibenclamide therapy (Beck-Nielsen *et al.*, 1979). However, it is uncertain how long this effect continues after the drug is withheld. Also, the hyperinsulinaemic effect of the drug would be expected to cause an insulin mediated receptor loss (Freychet, 1976) which might offset an increase in receptors produced by the drug.

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14. Preliminary in Vitro Evaluation of an Implantable Vascular Prosthesis Containing Mouse Islets

M. E. Broadbent and T. W. Atkins. Department of Biological Sciences, University of Aston in Birmingham, Birmingham, UK

A preliminary evaluation has been made of the insulin secretory response of hollow fibre prosthetic units (mol. wt. cut off 100 K daltons) containing lean and obese mouse islets to elevated glucose concentrations. Units were stabilised for 30 min at 37 °C by perfusing with oxygenated Hank's buffer supplemented with BSA. V (7.45 g/100 ml) and glucose (5.56 mmol/l) at a flow rate of 0.6 ml/min and perfusion pressure of 100–160 mmHg. Increasing the albumin concentration from 0.5 to 7.45 g/100 ml increased by two to threefold the insulin secretory response of lean islet units to 5.56 and 16.67 mmol/l glucose. The rate of insulin release from both lean and obese islet units was increased, although not significantly, after 60 min exposure to 16.67 mmol/l glucose. The total insulin secreted from lean islet units over 1 h was directly proportional to the weight of islet tissue present and the rate of insulin release during the stabilisation period was significantly greater ($p < 0.05$) from obese islet units (2.6 ± 0.28, ng · min^{-1} · mg^{-1}) than from lean islet units (1.65 ± 0.28 ng · min^{-1} · mg^{-1}. The significance of these data to the future use of the prosthesis in vivo will be discussed.

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