MICROENCAPSULATION STRATEGIES FOR ISLET TRANSPLANTATION

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Summary

A variety of islet microencapsulation techniques have been investigated to establish which method provides the least occlusive barrier to net insulin release in vitro, and optimum biocompatibility for islet implantation in vivo.

NMRI mouse islets have been microencapsulated with Na^+-alginate-poly-L-lysine (PLL) / poly-L-ornithine (PLO)-alginate, Ba^{2+}-alginate and agarose gels. Both free and microencapsulated islets responded to glucose challenge in static incubation and perifusion by significantly increasing their rate of insulin release and theophylline significantly potentiated the insulin response to glucose. While little insulin was released from microencapsulated islets after short term (2 hours) static incubation, significantly greater amounts were released in response to glucose challenge after extended (8-24 hours) incubation. However, insulin release from all types of microencapsulated islets was significantly reduced compared with free islets. Na^+-alginate-PLO-alginate microencapsulated islets were significantly more responsive to elevated glucose than Na^+-alginate-PLL-alginate microencapsulated islets, due to the enhanced porosity of PLO membranes. The outer alginate layer created a significant barrier to glucose/insulin exchange and reduced the insulin responsiveness of microencapsulated islets to glucose. Ba^{2+}-alginate membrane coated islets, generated by the density gradient method, were the most responsive to glucose challenge.

Low concentrations of N^G-monomethyl L-arginine (L-NMMA) had no significant effect on glucose stimulated insulin release from either free or microencapsulated islets. However, 1.0 mmol/l L-NMMA significantly inhibited the insulin response of both free and microencapsulated islets to glucose challenge.

In vivo work designed to evaluate the extent of pericapsular fibrosis after 28 days ip.and sc. implantation of microencapsulated islets into STZ-diabetic recipients, revealed that the inclusion of islets within microcapsules increased their immunogenicity and markedly increased the extent of pericapsular fibrosis. When the outer alginate layer was omitted from microcapsules, little or no pericapsular mononuclear cell deposition was observed. The subcutaneous site was not suitable for microencapsulated islet transplantation in NMRI recipient mice. Systemic immunosuppression using cyclosporin A was effective in preventing pericapsular mononuclear cell deposition, while L-NMMA loading into microcapsules had no significant effect on pericapsular fibrosis, although it did maintain the integrity of microencapsulated islets.

Key Words : Islet microencapsulation, insulin release, microencapsulated islet transplantation, immunosuppression, pericapsular mononuclear cell deposition.

This thesis is dedicated to the memory of my brothers,

Abdul Quddus and Imtiaz Ali .

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CONTENTS

LIST OF TABLES, FIGURES AND PLATES

TABLES

FIGURES

PLATES

(H & E; x50).

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Type 1 Diabetes Mellitus

Diabetes mellitus may be defined as a state of diminished insulin action due to its decreased availability or effectiveness. The reaction of susceptible individuals to certain environmental stimuli such as β-cell cytotoxic virus and β-cell cytotoxic chemicals is abnormal, leading directly to β-cell destruction through autoimmune mechanisms or because of the lack of regeneration of β-cells after damage (1). It is thought that the histopathological hallmarks of insulin dependent diabetes mellitus (IDDM) of recent onset is insulitis, i.e. a selective destruction of β-cells occurring in concert with a lymphocytic infiltration of the islets of Langerhans (2). The extent of insulitis varies considerably between IDDM patients, and many studies have shown that between 15 - 75% of the islets are affected, most strikingly in patients who are young at onset (3,4). Since IDDM does not become clinically significant until about 70 - 90% of the β-cell mass is destroyed, islet morphology at the time of onset already represents the late stages of the pathogenesis of IDDM. In insulitis the mononuclear cell infiltrate of the islets consists of cytotoxic / suppressor and helper subsets of T-lymphocytes (5). IDDM is usually characterized by the abrupt onset of symptoms, including insulinopenia, proneness to ketosis and a dependence on exogenous insulin to sustain life (6). Chronic diabetes is also associated with a number of secondary microvascular complications including microangiopathic and atherosclerotic vascular disease, retinopathy, nephropathy, neuropathy and impaired resistance to infection. More than 60% of IDDM patients may have some impairment of vision, kidney function or peripheral blood flow, and it is these complications that are responsible for the severe

morbidity and mortality associated with the disease (7). It is generally considered that the onset of diabetic complications are secondary to disordered metabolism and that the establishment and maintenance of normoglycaemia would prevent the development or halt the progression of the lesions affecting the eye, kidney, nervous and other systems (8 - 14). Since the discovery of insulin, the longevity of diabetics has been prolonged (15). However, it has been firmly established over many years that conventional insulin therapy does not prevent the development of long-term secondary complications of the disease (16). With conventional insulin daily injection, blood insulin levels are not physiologically controlled and the blood glucose level fluctuates both above / below normal values (100mg / dL). It is believed that these fluctuations in blood glucose level are one of the main causes of the long- term complications (17). There are some suggestions, therefore, that if the normal physiological balance between blood insulin and blood glucose could be restored, then diabetic complications could be prevented, although there is now strong evidence to support this view (17).

Systems designed to administer insulin continuously may be able to respond to fluctuations in blood glucose but are unlikely to be as efficient as functioning β-cells (18). The development of new insulin delivery "open loop" techniques using mechanical pumps is an area which has received increased attention over the last 10 years (19 - 21). Despite evidence that these insulin pumps can provide an improved glycaemic control, there are a number of unresolved problems associated with their use, such as reservoir leakage, blockage of the delivery catheter, infection at the infusion site, insulin precipitation and polymerisation inside the device, lack of a reliable long-term glucose sensor and pump failure (20 - 22).

1.2 Pancreas Transplantation

The second approach towards the permanent restoration of physiologically controlled normoglycaemia in IDDM patients was whole pancreas transplantation, first performed by Kelly and Lillehei in 1966 in conjunction with a donor kidney transplant (23). Since 1966, more than 3000 pancreas transplantations have been performed in man and considerable progress has been made in the last few years (24). The best results are obtained if the pancreatic exocrine secretions are drained into the recipients bladder and if the pancreas transplant is performed at the same time as the kidney transplant (24). Clinical application of pancreas transplantation has been difficult and the procedure carries a substantial risk of technical failure. First attempts showed unequivocally that immediately vascularized donor pancreatic grafts were able to normalise plasma glucose levels and obviate the need for exogenous insulin, but technical complications were frequent and early attempts were associated with a high morbidity and mortality (25 - 28). In the majority of cases, short-term technical failure was caused either by complications associated with exocrine drainage from the graft or by vascular thrombosis (29). Management of the exocrine secretion from the grafted pancreas has been resolved by several approaches: drainage of the pancreatic duct into alimentary tract (30,31), drainage into urinary bladder (32,33), free peritoneal drainage (34 - 37), and duct obstruction by in situ injection of synthetic polymers leading to fibrosis of the exocrine tissue (36,38). Neoprene (36), acrylate glue (39), polyisoprene (40) and prolamine (41) have been used for duct obstruction with some success. When exocrine drainage is established, the pancreas may be grafted either in association with the duodenum or, more successfully and simply, either as a whole or segmented graft without the duodenum and with pancreatic duct-ureter anastomosis (42). Whole pancreas transplantation by means of an immediately vascularized pancreatic graft remains technically difficult due to the complexity of blood vessels with the organ. Segmental pancreas

transplantation is simpler than whole pancreas transplantation and avoids some of the problems related to the grafted duodenum such as necrosis and ulceration.

Several techniques have been developed that allow for exocrine drainage to be established without transplantation of the duodenum (27). Gliedman and colleagues (27) transplanted the tail of the pancreas in dogs and anastomosed the pancreatic duct to the recipient ureter. The pancreatic exocrine enzymes were inactive and injury to the urinary tract did not occur. However, many other investigators have been unable to achieve satisfactory duct to ureter anastomosis in dogs (31,43). Pancreatic grafts have also been performed either with (35,44 - 46) or without (37) duct ligation or duct obliteration (47,48). Pancreatic duct ligation is the simplest approach to pancreas transplantation. Several studies in both rats and dogs have demonstrated that adequate endocrine function could be maintained followed by duct ligation (49,50). However, many other studies have shown acute and chronic inflammatory reactions in both endocrine and exocrine pancreas after duct ligation (51 - 53). Shah and colleagues (54) noticed dense fibrosis around the islets in duct ligated grafts. In instances where pancreatic transplantation has been carried out without duct ligation, the peritoneal cavity has been shown to be capable of absorbing pancreatic secretion without harmful effects in both dogs (37,51) and rats (55). However, severe clinical pancreatitis has been observed with respiratory problems and other difficulties in some canine models (56).

The major problem associated with pancreas transplantation is allograft rejection. Patients have to undergo chronic immunosuppressive therapy usually involving a combination of three drugs, cyclosporin A, prednisone and azathioprine for the rest of their lives (57). However, immunosuppression in itself is associated with a number of complications such as nephrotoxicity and hepatotoxicity (58). Following successful pancreatic transplantation the patient will experience a normal or near normal glucose metabolism. This level of glucose

control is almost never achieved with conventional insulin therapy. If pancreatic transplantation could be justifiably performed at an early stage of the disease, it would presumably prevent the occurrence of the secondary complications of diabetes, but not reverse the progression or severity of the secondary complications once established. Before pancreatic transplantation can be widely and routinely performed the technical problems of surgery and immunosuppression in diabetics will have to be overcome.

1.3 Islet Transplantation

Islet transplantation has been studied extensively as an alternative to pancreas transplantation. Islet transplantation has a number of practical and theoretical advantages over whole pancreas transplantation. These include the fact that major surgery and its complications can be avoided by the prudent choice of transplantation site, multiple donors can be used as a source of islets, the immunogenicity of islets can be suppressed by in vitro culturing or ultraviolet-irradiation (59 - 61). Decreased immunogenicity would facilitate allograft or even xenograft acceptance without chronic immunosuppression of the recipients. The field of islet transplantation was opened by the early development of procedures, whereby intact viable islets could be isolated from the pancreata of small animals such as mice and rats. These technological developments made it possible not only to perform basic studies on the mechanisms involved in the formation, storage and release of insulin by β-cells but also to determine the effect of islet transplantation on experimentally induced diabetes in rodents. The cells of the endocrine pancreas are grouped into numerous and small clusters called islets of Langerhans (62). These clusters are scattered throughout the exocrine portion of the pancreas and together comprise only about 1-2% of the volume of the adult gland. Four distinct endocrine cell types are found within the islets: glucagon-secreting A cells, insulin-secreting β cells, somatostatin-secreting D cells and pancreatic-

polypeptide-secreting PP cells (62).

Generally, greater than 1000 islets are required to restore normoglycaemia in rodents in one to two days (63,64). Islets from young adult rat donors have been shown to function better than islets from older donors (65). Selawry and colleagues have demonstrated that collagenase isolated islets of old rats when incubated with concanavalin A, did not exhibit binding to the concanavalin A, suggesting that islets of old rats might be more damaged during the collagenase isolation procedure (65). Islet grafts have been shown to reverse diabetes in rats when the islets are transplanted by injection into the liver via the portal vein (66), the peritoneal cavity (67,68), spleen (69), subcapsular space of kidney (70 - 72), testis (73), cisterna magna (74) and thymus (75). An ideal site would be one that is safe and convenient for access, that allows complete implantation of the transplanted islets with optimal long-term metabolic function and, if possible, one that is immunologically privileged. Isolated adult rodent islets have been grafted into recipients with variable results. The best functioning islet grafts have been demonstrated following implantation into the liver, via the portal vein (76). This site requires the smallest number of islets, i.e. about 500, for the reversal of the diabetic state (76). Reckard and colleagues have found that approximately twice as many islets are required to produce normoglycaemia in diabetic rats, when the islets are transplanted into the spleen compared with when they are implanted into the liver (76). In another study, Anderson and colleagues, failed to cure hyperglycaemia in mice when the islets were transplanted intrasplenically (77). Histological survival of islets transplanted in the peritoneal cavity has been demonstrated, but this site was not found to be an effective one for the complete reversal of diabetes which seemed to require large quantities of islets i.e. > 1000 (68). Lee and colleagues have demonstrated that transplantation of freshly isolated rat islets into the cisterna magna of diabetic rats, an immunologically privileged site, can produce long-term survival of islet allograft (74). In

addition, the kidney capsule has been demonstrated to be a useful site for islet transplantation (72) suggesting that immunological factors play an important role in the functional survival of transplanted islet tissue.

1.4 The Isolation of Islets of Langerhans

A persistent and important barrier to satisfactory clinical trials of islet transplantation in man has been the inability to isolate sufficient quantities of viable islets from the human pancreas. Over the years numerous procedures for the isolation of islets from the pancreas of experimental animals and man have been described, each claiming to be an improvement on previous methods. The pig has been proposed as a donor source of islets of Langerhans that might be applicable to human transplantation in the future (78,79), mainly because of the reasonable availability of pig pancreata and also the immune problems can be avoided by using transgenic pigs. The method of choice for obtaining relatively large numbers of functional islets from a variety of animal species is to digest the pancreas with collagenase. A number of methods for islet isolation and purification based on collagenase digestion have been described (78 - 86). These methods can be subdivided into those which involve cutting the pancreas into small pieces before exposure to collagenase and those which depend upon distention of the pancreas by infusing collagenase into the pancreatic duct. The method which involves the collagenase digestion of small pieces of pancreas (80) is suitable for the isolation of islets from rat and mouse pancreas. It has not been found to be particularly successful for the isolation of islets from the pancreas of larger animals, whose organs are typically more discreet and fibrous. At present, digestion of pancreas by intraductal injection of collagenase followed by mechanical disruption has been reported to be the most efficient method for the separation of islets from the exocrine tissue (86). For islet purification, density gradient centrifugation using a variety of different media appears to

be the method of choice (81,87). Ficoll (88,89), percoll (90), albumin (81,91) and dextran (92) have been used to purify islets preparations.

1.4.1 Preservation of pancreas and islets of Langerhans

The development of a reliable method of organ preservation is the key to solving the logistical problems associated with limited tissue that hamper all transplantation efforts. Two basic techniques have been used for the preservation of whole rat pancreas transplants. They are simple cold storage (4°C) and continuous perfusion (88). The pancreas is a low flow, low pressure organ and continual perfusion can cause disruption of the vascular system, while simple cold storage does not directly cause vascular damage. Human pancreases have been preserved in University of Wisconsin or Euro-Collins solution at 4°C for about 20 hours , however, a sufficient number of viable islets cannot be isolated from a preserved pancreas (93). It is not clear whether this low yield results from failure of enzymatic digestion of the fibrous human pancreas or from injuries to the islets during preservation.

Three main methods have been investigated for the in vitro preservation of isolated islet tissue, these are tissue culture, cold-storage and cryopreservation. These methods offer several advantages for the transplantation process. Storage and accumulation in a tissue bank at low temperature provides sufficient islet material for transplantation into individual recipients. Modulation of tissue immunogenicity and subsequent purification facilitates shipment from centre to centre.

It is well documented that a significant prolongation of the survival time of rat islets transplanted beneath the kidney capsule of diabetic mice can be achieved by culturing the isolated islets in CMRL 1066 culture medium at 37°C in air and 5% CO_2 for 7 days prior to

transplantation (60). In addition, culture of the donor rat islets at low-temperature (24°C) and single injection of mouse and rat anti-lymphocyte serum would prevent rejection of the islet xenografts (94). In vitro studies have shown that both rodent (95,96) and human (97) islets maintained in culture for several days or weeks, synthesized and secreted insulin in response to elevated glucose. In cultures of fetal and neonatal pancreas, islet cells preferentially differentiate and β-cells replicate (98,99,100). It has been demonstrated that isolated human pancreatic islets can be maintained in TCM 199 culture medium for periods of time up to 3 weeks (101).

Cold storage of isolated islets is the simplest preservation method and requires the least amount of equipment. The simplicity of this method makes it highly attractive for the short-term storage of islets prior to transplantation. Mouse, rat and guinea-pig islets have been preserved by this method in Hanks balanced salt solution + glucose at 4°C (102).

A great deal of work has been carried out on islet cryopreservation in liquid nitrogen for the long-term storage of isolated islets using dimethyl sulphoxide (DMSO) supplemented Eagle's culture medium (97,103). Frozen - thawed and subsequently transplanted rat islets were able to normalise the blood sugar in streptozotocin diabetic rats (104). Human isolated islets have been shown to respond to glucose challenge in vitro on thawing after cryopreservation (97) and subsequently survive when transplanted beneath the kidney capsules of diabetic rats (105). Many workers have used in vitro tests, by measuring insulin release, to determine the optimum conditions for the freezing and thawing of isolated islets (104,106). Rajotte and colleagues (104) found that slow cooling at the rate of 0.25°C / min. to -40°C and then down to -196°C and subsequent rapid thawing from -196°C provided the highest survival rate for isolated islets, as measured by in vitro insulin release and in vivo transplantation tests.

1.5 Prevention of Graft Rejection Using Immunosuppression.

Triple immunosuppression with cyclosporin A, prednisone and azathioprine has been widely used in islet transplantation (107). The rationale for the possible use of immunotherapy in IDDM is based on the observation that T-cells play a fundamental role in the rejection of transplanted tissue and that T-cell dependent functions may be affected by different drugs with immunosuppressing actions (108 - 110). If immunosuppression is not used, fresh islet allografts, which are highly immunogenic, can be rejected very rapidly (111). The time taken for rejection, however, depends on several factors, including the site of implantation, the number of islets transplanted, the variations in the potency of immunosuppressants and the histocompatibility barrier. Cyclosporin A, an established potent immunosuppressive agent, inhibits the production of 1L-2 by human T-cell following antigen or mitogen stimulation (112). It has been widely used to prolong the functional survival of pancreatic allografts in rats (113,114), dogs (40) and in man, generally as a cocktail with other immunosuppressants (115). However, cyclosporin A, has been found to be toxic to islet function both in vitro (116,117) and in vivo (115,118), where it has shown an impairment with glucose stimulated insulin release and glucose tolerance. A recently available immunosuppressive drug, FK506, has been used in transplantation of liver (119), kidney (120), pancreas and islets (121-123). FK506 is nearly 400 times more potent than cyclosporin A in its ability to lymphocyte proliferation (124,125). FK506 like cyclosporin A, is thought to act by suppressing T-cell activation through the inhibition of interleukin-2 production and 1L-2 receptor expression on lymphocytes (124-127). It is effective in low doses (121,123) and has a synergistic effect with cyclosporin A and deoxyspergualin to prolong islet xenograft function (128,129). An inhibitory effect of FK506 on insulin release has been observed in rats (130) and dogs (131) especially with high concentration of FK506. Although corticosteroids have for many years been the backbone of most clinical

immunosuppressive regimes, they can precipitate the features of Cushing's syndrome and are potentially diabetogenic. Prednisone has been used for islet transplantation in rats (132), although a toxic effect of the steroid on islets has been reported in dogs (133).

1.6 Alteration of Pancreatic Islet Immunogenicity Using U/V Irradiation and Tissue Culture.

Various protocols have been described in rodents designed to preserve islet functional integrity while decreasing tissue immunogenicity in a bid to provide long-term allograft survival. These include ultraviolet (61,134) or gamma irradiation (135) of islets prior to transplantation with low- temperature culture (24°C) (136) and anti-Ia plus complement treatment of islets (137). Lindahl-Kiessling and Satwenberg (138) were the first to report that ultraviolet irradiation is effective in abrogating the stimulatory capacity of lymphocytes. The UV-treatment of rat and dog islets has enabled a prolonged survival of islet allografts (61,134). Brief cyclosporin A immunosuppression of recipient rats transplanted with UV-treated islets, resulted in about 100 days acceptance of islet allografts (139,140).

As far as tissue culture is concerned, Tucker and colleagues, have shown that the in vitro culture of donor rat islets will prevent their rejection when transplanted across a major histocompatibility barrier (141). In addition, culture of the donor rat islets at low temperature for seven days, plus a three-day course of cyclosporin A immunosuppression of the recipient rats, would also prevent rejection of the islet allografts (137).

1.7 Transplantation of Foetal Pancreatic Tissue

The use of foetal tissue in pancreas transplantation offers several advantages over adult

tissue. Foetal pancreatic tissue has more islet tissue proportionately than adult pancreas and the exocrine tissue is less mature and more easily disaggregated and digested. Isolated foetal cells have a greater proliferative capacity in organ culture and can provide a larger number of islets from one pancreas (142). The reversal of experimentally induced diabetes can be achieved using the islet tissue from one foetal pancreas in rat (143) and mouse (144). The initial assumptions that foetal rodent pancreas would be less immunogenic than adult rodent pancreas were proven unfounded (145). This problem of immunogenicity was circumvented in the mouse model by the prolonged culture of the foetal mouse pancreas prior to transplantation (146). This approach was found to be less successful with rat foetal pancreas (147). Foetal rodent β-cells behave differently from adult rodent β-cells, in that they release insulin poorly or not at all in response to nutrients, especially elevated glucose. This observation also applies to human foetal pancreatic tissue (148,149) as well as to that of rats (150) and mice (151). For this reason, there is a long latent period between the transplantation of foetal pancreatic tissue and the amelioration of diabetes in both man and rodents (145 - 147,152).

1.8 The Bioartificial Pancreas

A Hollow fibre bioartificial pancreatic devices

Hollow fibre bioartificial devices consist of either a bundle of semi- permeable capillary fibres or one single large diameter coiled fibre within a plastic chamber (153 - 156). The immunological isolation of allo and xenogenic islets from the hosts immune system by the wall of the synthetic capillary has been demonstrated by Tze and co-workers (157). Their study demonstrated that the macroporous semi-permeable membrane of the synthetic capillary could protect the grafted allogenic and xenogenic cells from rejection. Maki and

colleagues in 1991, reported that single devices containing dog allograft islets (6500 islets / Kg B.W.) could maintain normal blood glucose levels in pancreatectomized dogs without the need for immunosuppression (154). Devices seeded with xenogenic islets, i.e. bovine or porcine met with very little success when implanted into dogs (155). The eventual failure of these devices is the result of a loss of islet viability, collapse of the membrane and thrombosis. Van der Vliet and co-workers observed impaired islet graft function due to extensive fibrosis around the foreign materials (158).

B Microencapsulation of islets

The capsular membrane functions as a semi-permeable membrane allowing small molecular weight substances, such as nutrients and oxygen, to penetrate while excluding large macromolecules such as immunoglobulins (159 - 166). Since microencapsulated islets are excluded from both cytotoxic T-lymphocytes and immunoglobulins, rejection of microencapsulated islet grafts could be prevented. Microcapsules have been used for a variety of purposes in industry. Microcapsules are commonly found in pharmaceutical preparations such as aspirin or are used as a component of time release capsules where a coating is applied around an active ingredient, which is subsequently released by slow digestion of the microcapsules in the gastrointestinal tract. Many industrial microencapsulation processes, however, are unsuitable for living tissue due to prolonged ischemia time, toxicity of reagents and extremes of temperature and pH during fabrication. Calcium alginate gels have been used extensively to immobilize microbial cells for industrial applications (167,168). Many techniques for microencapsulating biologically active macromolecules, viable tissues and individual cells within semi-permeable membrane have been described (159,169,170). The possibility of using encapsulated erythrocytes hemolysate for organ replacement therapy was first suggested by Chang (171). Alginate is a polysaccharide extracted from brown seaweed "Macrocystis pyrifera" (kelp) which gels in

the presence of most polyvalent cations, but especially Ca^{++} (167,168).

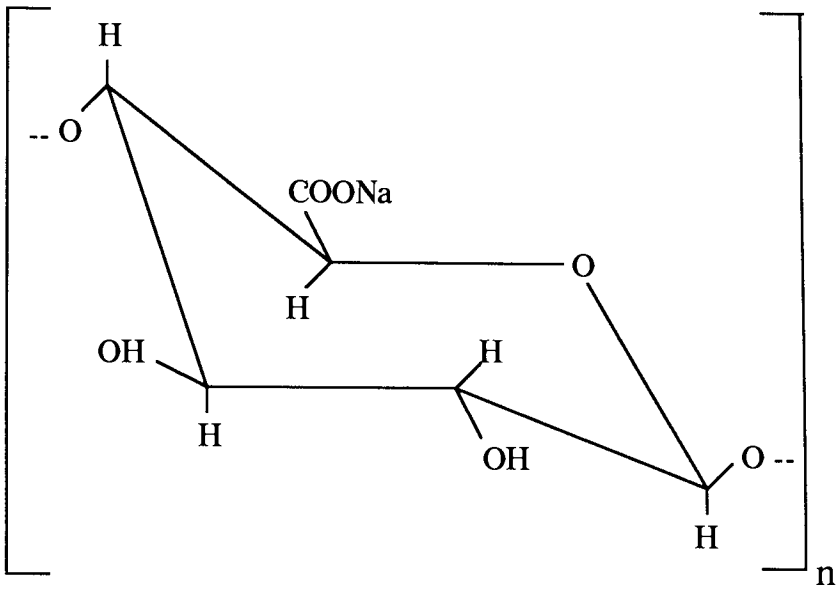


Figure 1 Na^{+} - alginate

It is composed of three types of 1-4 linked polyuronic acid blocks. These polymer blocks contain poly-L-guluronic acid segments, poly-D-mannuronic acid segments and segments of alternating L-guluronic acid and D-mannuronic acid residues, Figure 1. The proportions of these polymers in preparations of alginate determines the physical and chemical properties of the complete polymer. Alginate preparations vary in terms of molecular weight, viscosity, water regain capacity and structural strength of the gels produced when treated with polyvalent cations. Divalent cations such as calcium will reversibly cross-link the alginate chains to form a rigid gel. It is the gelling property of alginate that is of primary interest in the encapsulation process. Its instant gelling property in calcium chloride solution allows the productions of temporary gel beads around which the permanent polyamino acids, i.e. poly-L-lysine and poly-L-ornithine membrane, may be formed. The microencapsulation of islets of Langerhans with Na^{+}-alginate was initially described by Lim and Sun in 1980

(159). Studies using alginate microencapsulation showed that the shape of the alginate gel droplet was critically dependent on the sodium alginate concentration used in the first step of the microencapsulation process (161). At Na^+-alginate concentrations below 1% (w/v), the capsules were irregularly shaped and the incidence of surface irregularities and striations progressively increased with a decrease in alginate concentration (161). Concentrations between 1.2 - 2% have been shown to be suitable for spherical capsule formation (172). Poly-L-lysine or poly-L-ornithine provide a positive charge complimentary to that of the Na^+-alginate (162,163,173,174). Although several polyamino acid polymer systems have been investigated with different degrees of success in the last few years, the system of Na^+-alginate-poly-L-lysine-Na^+-alginate is still considered to be the system of choice especially for in vivo applications (163,173,175 - 177).

The first report of the successful long-term (4 months) culturing of Na^+-alginate-poly-L-lysine-polyethyleneimine microencapsulated rat islets was reported by Lim and Sun in 1980 (159). Experiments were then performed to determine whether the microencapsulated islets could survive and function in vivo. The results on intraperitoneal implantation of 3000 microencapsulated rat islets into streptozotocin diabetic rats, showed that recipients of microencapsulated islets were normoglycaemic for only 3 weeks, whereas the non-encapsulated islets survived for 6 to 8 days (159). The body weight of animals receiving microencapsulated islets transplants increased, while the body weight of the control diabetic rats did not change significantly over the same period of time (159). The rapid failure of the implanted microencapsulated islets was thought to be due to an inflammatory response induced by the polyethylenemine membrane which was subsequently found to be poorly biocompatible (178). Replacement of this outer layer of polyethyleneimine with Na^+-alginate significantly improved biocompatibility (178). A single transplant of 3000 microencapsulated islets in biocompatible Na^+-alginate-poly-L-lysine-alginate membranes

restored normoglycaemia in all diabetic rats within two days and maintained normoglycaemic for up to 10 months (178). Both in vitro (162,163,178,179,181,182,183) and in vivo (175,176,180) studies have demonstrated that rodent islets microencapsulated in Na^+-alginate-poly-L-lysine can be cultured for several weeks in vitro and will secrete insulin in response to elevated glucose with kinetics comparable with closed loop insulin delivery systems, correcting hyperglycaemia in diabetic recipients for several months. In addition, microencapsulated islets transplant recipients gained weight steadily during their normoglycaemic period and had normal urine volume, indicating good control of the diabetic state.

Both allogenic (175,176) and xenogenic (177,184) islets, microencapsulated in Na^+-alginate-poly-L-lysine membranes, have been shown to function for a prolonged period of time in streptozotocin diabetic rats and mice. Sun and O'Shea reported that single intraperitoneal allograft of 5000 microencapsulated rat islets corrected the hyperglycaemia of diabetes within 2 days and the recipients remained normoglycaemic for over a year without immunosuppression (176). The pericapsular surfaces of the recovered capsules were free of fibrosis (176). In another study, diabetes was reversed for up to 144 days, when rat microencapsulated islets were implanted into streptozotocin diabetic mice, whereas recipients receiving non-encapsulated islets remained normoglycaemic for less than 14 days (177). However, the recovered microcapsules were found to be surrounded by many layers of cellular overgrowth (177). Xenotransplantation of about 1000 microencapsulated cultured foetal rat islets has restored normoglycaemia in streptozotocin diabetic mice for up to 4 months (181). Rajab and colleagues have demonstrated that microencapsulation of islets with alginate, was superior to the use of diffusion chambers, as a means for immunoisolation of transplanted islets (185). These workers reported that rats transplanted with 3000 microencapsulated islets remained normoglycaemic for up to 6 months, whereas,

rats transplanted with 3000 islets in diffusion chambers remained normoglycaemic for only 2 weeks. However, in some studies grafted microencapsulated islets have been observed to lose their functional characteristics within a month, mainly because of pericapsular fibrosis (172,186 - 190). The capsular wall was always infiltrated by lymphocytes, fibroblasts and macrophages.

With few exceptions (173,180) the prolongation of microencapsulated islet function has not been achieved in spontaneously diabetic NOD mice (187,191,192) and BB rats (189,193) due to the production of massive fibrotic capsular overgrowth. This reaction could be delayed by both immunosuppression (194) and oxygen free radical scavenger (195) therapy of NOD recipients. Also, it has been reported that U/V irradiation and microencapsulation of donor islets is synergistic in preventing the destruction of rat islet xenografts in NOD mice (196). Without donor rat islet UV-irradiation, microencapsulated rat islet xenografts only survived for 12 days in NOD mice, whereas UV-irradiated microencapsulated rat islet xenografts functioned satisfactorily for 68 days (196).

The strain of recipient animal also appears to influence the presence of islet fibrosis and capsular overgrowth (197). Waterfall and colleagues (197) reported that intraperitoneal implantation of Na^+-alginate-poly-L-lysine-Na^+-alginate empty microcapsules elicited the specific proliferation and activation of intraperitoneal macrophages in the BB rat, which could be related to the greater degree of fibrosis observed in these animals.

Recently, it has been observed that interleukin 1β (1L-1β) can both suppress glucose stimulated insulin release and destroy islet β-cells in a dose and time dependent manner (198,199). The free nitric oxide radical has been implicated as the effector molecule

responsible for the deleterious effects of 1L-1β on β-cell function (198,200,201). The formation of nitric oxide by the islets in response to treatment with 1L-1β has been shown by electron paramagnetic resonance spectroscopy (202). The nitric oxide synthase inhibitor, N^G-monomethyl L-arginine prevents the inhibitory effects of 1L-1β on glucose-stimulated insulin secretion by isolated rat islets and can protect the islets against lysis (198,200).

Some workers have looked for alternative microencapsulating materials in place of Na^+-alginate-poly-L-lysine / poly-L-ornithine-Na^+-alginate membranes, on the basis that they are too fragile. Iwata and colleagues have utilised low-temperature (<30°C) gelling agarose to encapsulate golden hamster islets (203,204). The same low-temperature gelling agarose has been used for the microencapsulation of islets by Dupey and colleagues (205), but they were not convinced of the in vivo stability of agarose microbeads. In vivo studies by Iwata and colleagues have reported the prolonged function of agarose microencapsulated islet allografts (206), but agarose microencapsulation did not effectively protect islet xenografts from early rejection (207).

Over the last 10 years water-insoluble synthetic polyacrylate materials such as Eudragit RL (acrylic methacrylic acid ester) and poly-hydroxy-ethyl methacrylate-methyl methacrylate have been used by some groups to encapsulate islet tissue (208,209). The water insolubility of these polyacrylates is useful for maintaining the stability of microcapsules in an aqueous environment, but is a disadvantage when preparing microcapsules, since the water insolubility makes the encapsulation process more difficult and islet cells must be exposed to organic solvents and non-solvents during encapsulation. A good deal of in vivo work needs to be carried out to confirm the biocompatibility of synthetic polymer based microcapsules.

In the past there has been a general over enthusiasm by clinical investigators to transplant

large numbers of either allograft or xenograft islets directly into diabetic animal recipients in an attempt to maintain normoglycaemia without giving due consideration to the secretory competence of the islet tissue, its immunogenicity and prospects for immunoprotection, and the molecular mechanisms associated with pericapsular fibrosis and necrosis observed in transplanted islets with extended residence time. The purpose of the work reported in this thesis was not to establish the logistics associated with the amelioration of diabetes of experimental animals using large numbers of microencapsulated islets, but rather to address some of the constraints which influence the microencapsulation of islets described above in an attempt to optimise the requirements for successful microencapsulation and facilitate reliable and reproducible islet allograft / xenograft transplantation in the future. With this in mind initial in vitro work involved the characterization of the insulin secretory response to secretagogues by free and microencapsulated islets in both static and perifusion systems. Many previous studies (162,182,188,210) have confirmed that microencapsulated rat islets will secrete insulin, in vitro, in response to glucose challenge, although at a lower level than that secreted by free islets. However, a comparison of the insulin secretory capacity of differently fabricated microencapsulated islets has never been quantitatively carried out. Therefore, in the present work, various fabrication strategies will be used to microencapsulate isolated NMRI mice islets in order to establish which method of microencapsulation provides the least occlusive barrier to net insulin release. A comparison will also be made between the in vitro insulin response of free, Na^{+}-alginate poly-L-lysine, Na^{+}-alginate-poly-L-ornithine, Ba^{++}-alginate and agarose microencapsulated islets to glucose and theophylline challenge over short and extended periods of time. In addition, the effect of an additional outer-most dilute Na^{+}-alginate layer on insulin release will also be investigated, because there is evidence to suggest that the diffusion of insulin, glucose and oxygen is inversely proportional to the membrane thickness and the diffusional path distance of microcapsules (162,211).

In order to investigate the kinetics of insulin diffusion through the Na^{+}-alginate capsule wall, the rate of I^{125}-insulin release from microcapsules will be examined to provide information on microcapsule porosity and the possibility of a lag phase in the insulin release profile. This information is essential to assess the optimal potential of microencapsulated islets for transplantation. Attempts will also be made to reduce the thickness of the capsule membrane and diffusion distance by using a recently introduced Ba^{++}-alginate density gradient method of microencapsulation (211).

Previous observations have shown that activated peritoneal macrophages lyse isolated rat islets via the arginine dependent formation of nitric oxide by nitric oxide synthase (200). The nitric oxide synthase inhibitor, N^{G}-monomethyl L-arginine acetate (L-NMMA) (0.1 - 1.0 mmol/ l) has been shown to prevent the formation of nitric oxide and islet cell destruction mediated by activated peritoneal macrophages (200,201,212,213). In the second part of this study, therefore, the effect of L-NMMA (0.1 - 1.0 mmol / l) in the presence and absence of 0.2 mmol / l L-arginine will be investigated on in vitro insulin release from free and microencapsulated islets. These studies will be extended to establish whether the incorporation of L-NMMA into the alginate microcapsule surrounding the islet will in turn influence the in vitro insulin secretory response of microencapsulated islets, and possibly protect microencapsulated islets in vivo against nitric oxide production by pericapsular macrophages.

A major subject of concern associated with transplanted microencapsulated islets is the pericapsular fibrosis which develops around the implanted microcapsule. Islet morphology would be best preserved if the microcapsular membrane remained free from surrounding fibrosis. Pericapsular fibrosis almost certainly limits the diffusion of oxygen, glucose and insulin across the microcapsule membrane and contributes to the necrosis of islets. Against

this background, empty and islet containing Na^+-alginate-poly-L-lysine and Na^+-alginate-poly-L-ornithine microcapsules will be implanted intraperitoneally and subcutaneously into both normal and streptozotocin diabetic NMRI mice in order to examine the contribution that an outer alginate layer, a polyamino acid coating, the diabetic state of the animal, the presence of islets within the capsules and the location of the implantation site have on the extent of fibrosis both on and in the microcapsule membrane. Recovered microcapsules will be examined histologically after 14 and 28 days, since many previous studies (172,186,187) have shown that microencapsulated islet grafts lose their function within this time period. Studies will also be carried out to establish whether the deposition and genesis of pericapsular fibrosis can be discouraged by systemic immunosuppression using cyclosporin A and FK506 in diabetic recipients.

CHAPTER 2

2.0 GENERAL MATERIALS AND METHODS

2.1 Animals

The animals used throughout the experimental work were 10-15 week old male NMRI mice. Pure strain NMRI mice were purchased from Bantin and Kingman (Hull, U.K.). These were maintained in standard animal room facilities with a 12 hour light-dark period and fed a rat and mouse breeding diet (Pilsbury's Ltd., Birmingham) with water ad libitum.

2.2 Chemicals and their sources

Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows: KCl, NaCl, $CaCl_2.2H_2O$, KH_2PO_4, $MgSO_4.7H_2O$, $NaHCO_3$, $Na_2HPO_4.2H_2O$ and D. glucose were purchased from BDH Chemicals Ltd., Lutterworth, UK. Sodium citrate and barium chloride were purchased from Hopkins and Williams Ltd., UK. Polyethylene glycol (M.wt. 6000), bovine serum albumin (A-7888), γ-globulin (G-5009), theophylline (T-1633), L-arginine (A-3784), N^G-monomethyl L-arginine citrate (M-7033), poly-L-lysine (P-2658), poly-L-ornithine (P-3530), low-temperature gelling agarose (type 1X), DNAase 1 (bovine, DN-25), paraffin oil and dithizone (D-5130) were all obtained from the Sigma Chemicals Co., Poole, Dorset, UK. Collagenase class 4 (4188) with a specific activity of 232 U/mg was obtained from Worthington Biochemicals Co., New Jersey, USA. Sodium alginate (LMT and DJB) were obtained from Kelco international, UK. Glucose free RPMI-1640 medium, heat inactivated new born calf serum and penicillin/streptomycin solution were obtained from Gibco Ltd.,

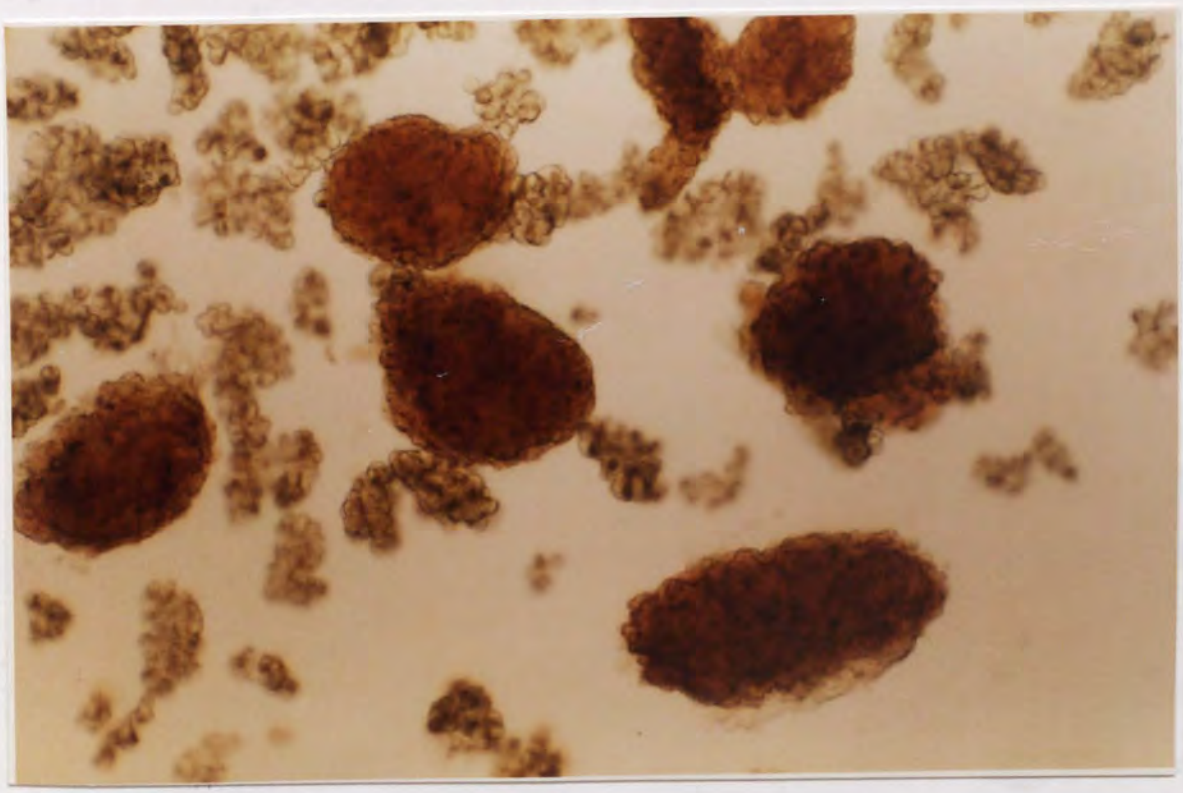
UK. Guinea pig anti-insulin serum was obtained from Wellcome (UK) and ICN immunobiologicals. Human insulin standard was obtained from Novo Nordisk, Cambridge, UK. I^{125}-insulin (IM-38 specific activity 100-125μCi/ug) was obtained from Kodac clinical diagnostics, Amersham, UK. FK-506 (04400YL) was a kind gift from Fujisawa Pharmaceuticals Co., Osaka, Japan. Cyclosporin A (M.wt. 1202.64) was obtained from Sandoz Pharmaceuticals, Leeds, UK.

2.3 Isolation of islets using collagenase digestion

The method used for the isolation of islets was a modification of the collagenase digestion procedure previously reported by Lacy and Kostianovsky (80). Mice were killed by cervical dislocation, positioned ventral side upward, swabbed with 70% alcohol and a mid-ventral incision made to display the viscera. The whole pancreas was detached from its points of contact with the spleen, stomach and duodenum using fine scissors and transferred to a petri dish containing oxygenated Hanks Balanced Salt Solution (HBSS), pH7.4 (see appendix 1). The excised pancreas was distended by injecting HBSS into the body of the tissue via a fine 26G syringe needle, trimmed of fat and chopped into very small pieces using round ended scissors. The pieces of pancreas were transferred to a round bottom test tube containing 10mg of collagenase dissolved in 5ml of HBSS. The tube was then held in the palm of the hand and wrist-shaken until the islets were free from exocrine tissue, approximately 25-30 minutes. The process was monitored by the disappearance of discreet pieces of pancreas and the appearance of cloudiness and discolouration of the solution. At this stage, the separated islets could be seen at the bottom of test tube. When digestion was complete, the digest was combined with 5ml of ice cold HBSS to arrest enzyme activity and centrifuged at 180g for 3 minutes to sediment islets. The supernatant was discarded and the pellet resuspended in HBSS and recentrifuged. The procedure was repeated to ensure

complete removal of all collagenase. The final pellet was resuspended in HBSS and poured into a glass petri-dish. 0.5ml of DNAase 1 (200μg/ml) was added to prevent clumping of the digested tissue. To aid the initial detection of islets a few drops of dithizone (DTZ) (appendix 2) was added to the contents of the petri-dish. DTZ produces a bright red staining of islets, Plate 1 (214). The contents of the petri-dish were then examined against a black background under a binocular microscope (magnification X20). The DTZ stained islets appeared as discreet, red, spherical or ovoid bodies and were separated by hand picking with a drawn out glass pasteur pipette. Islets used in experimental studies were not stained with DTZ. Approximately 200 islets were harvested from two mice and retained in a small black watch glass containing ice cold HBSS until required.

Plate 1 Dithizone (DTZ) stained mouse islets (red), DTZ staining helps to identify islets in digested exocrine tissue (X125).



2.4 Pre-incubation and test incubation of isolated islets

In order to confirm insulin secretory viability, batches of 10 islets were transferred to small capped glass vials containing 1ml of oxygenated 95%O_2 / 5%CO_2 HBSS supplemented with 5.5 mmol/l glucose, 0.1% BSA V and 2% penicillin (10000 IU/mg)/streptomycin (10000 U/ml). Islets were then subjected to 30 minutes static pre-incubation at 37°C. This pre-incubation was carried out to acclimatize the islets to the normal physiological glucose concentration and establish a non-stimulatory rate of insulin release. At the end of the pre-incubation period, the buffer was removed by aspiration and replaced with fresh buffer at 37°C containing either 5.5, 11.1 or 16.7 mmol/l glucose in the absence or presence of 5.5mmol/l theophylline, a classical well characterised insulin secretagogue. Islets were then test incubated for 1 hour at 37°C with mild agitation in a shaking water bath (80 cycles/minute). After this time a 500µl aliquot of the test medium was taken from each vial and immediately stored at -20°C for the subsequent assay of insulin. Islets test incubated in the presence of 5.5, 11.1 and 16.7 mmol/l glucose served as controls.

2.5 Na+-alginate microencapsulation of isolated islets and coating with either poly-L-lysine or poly-L-ornithine

The basic microencapsulation method used in this study was a slight modification of the original method of the Sun and O'Shea (176) subsequently reported by Fan and colleagues (180).

About 200 isolated islets were mixed with 0.5ml of either LMT or DJB sodium alginate solution (1.5% w/v). This mixture was carefully loaded into a 1ml disposable siliconised syringe fitted with a 26G needle and a 2cm length of pp10 poly-ethylene cannula (Portex

Ltd.). Maintaining constant pressure on the syringe a stream of droplets was delivered into 1.1% calcium chloride gelling solution gently stirred with a magnetic stirrer. The gel droplets (0.62 ± 0.08mm, n=10) were maintained in suspension for 3 minutes to allow them to harden. The process generated microcapsules of calcium alginate gel containing entrapped islets and the recovery of microencapsulated islets was 61.8 ± 3.3% (n=10). The droplets were collected on a 40μ mesh filter-membrane and washed once with HBSS. The microcapsules were then suspended in 0.15% poly-L-lysine (PLL) or poly-L-ornithine (PLO) solution and allowed to react for 10 minutes to form the membrane. The microcapsules were then washed with HBSS and treated in one of two ways: either, no further layers were added or a further layer of sodium alginate (0.15% w/v) was added by immersing the microcapsules for 4 minutes. The microcapsules were washed again in HBSS and finally immersed in 55mmol/l sodium citrate solution, pH7.4 for 6 minutes to liquify the calcium alginate gel within the microcapsules. The latter were finally resuspended in HBSS for use in experimental studies. Microcapsules containing one or sometimes two islets were separated from empty microcapsules under a dissecting microscope, Plate 2. The microencapsulation procedure is summarised diagrammatically in Figure 2.

2.6 Microencapsulation of isolated islets with barium-alginate

Method 1

Initial barium alginate microencapsulation of islets was carried out according to the method of Na^+-alginate microencapsulation described above. Approximately 200 isolated islets were mixed with 0.5ml of sodium alginate (LMT) solution (1.5% w/v) and loaded into a 1ml siliconised syringe as described above. Droplets were dispersed into 20 mmol/l barium

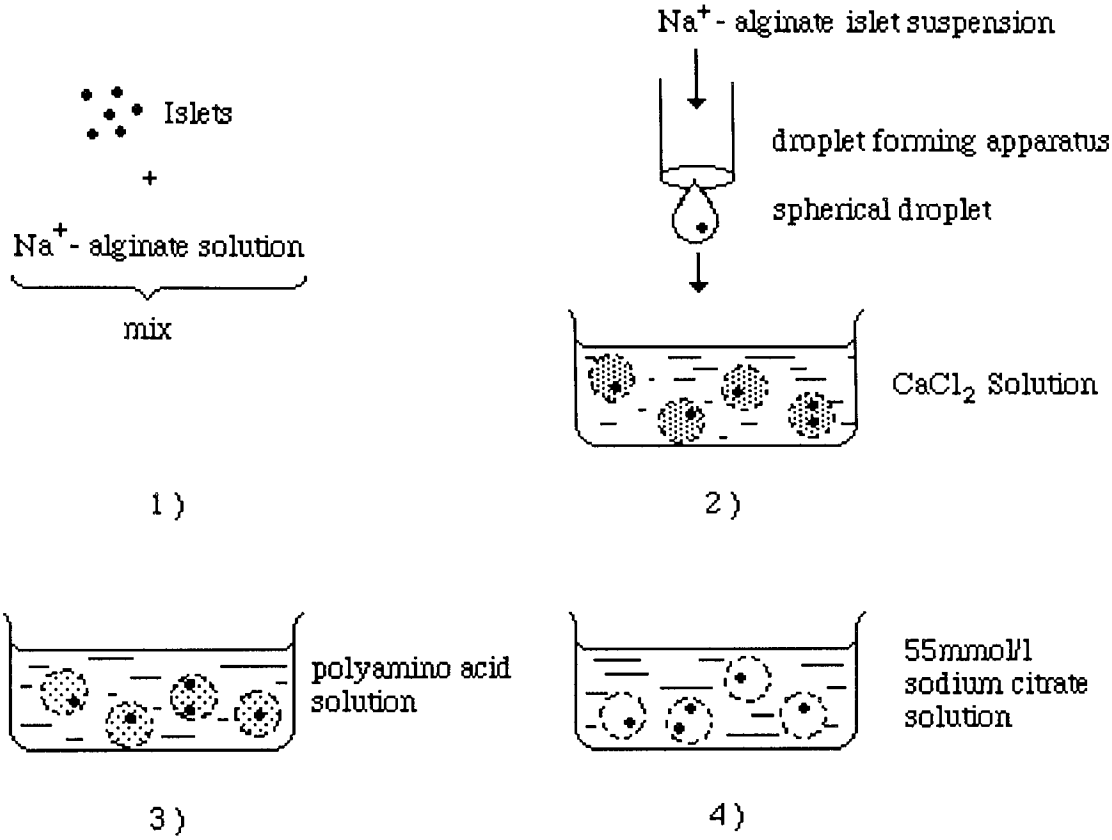


Figure 2 Diagrammatic summary of Na+-alginate - PLL or PLO microencapsulation procedure.

1) formation of islet suspension

2) gel sphere formation

3) formation of semi-permeable membrane

4) removal of intracapsular gelled alginate.

Plate 2 Na^+ - alginate - poly - L - lysine - alginate microencapsulated mouse islet (X50)

chloride solution and retained there for about 10-15 minutes to allow microcapsules to harden. Barium alginate microcapsules were prepared with and without polyamino acid coatings (PLL or PLO). Polyamino acid coating was thought to increase the structural strength of the microcapsules. The generation of barium alginate microcapsules was an irreversible process. Microcapsules containing islets were separated from empty microcapsules (encapsulation efficiency 54 ± 2.5%, n=10) by hand under the dissecting microscope and suspended in HBSS prior to secretory studies, Plate3.

Barium alginate membrane coating of islets using a ficoll density gradient technique

Method 2

Zekorn and colleagues (211) have devised a technique that generates Ba^{2+}-alginate membrane coated islets which have been shown to have a short diffusion path and an enhanced insulin response to secretagogues such as glucose. Briefly, 1% (w/v) Na^{+}-alginate (LMT) was dissolved in 0.1M HEPES buffer, pH7.4. About 200 isolated islets were suspended in 200µl of this Na^{+}-alginate solution and transferred to the top of a discontinuous gradient composed of the following four layers (top to bottom) 300µl 1% Na^{+}-alginate solution; 2500µl 8% ficoll 400 (Sigma) in HEPES buffer; 500µl $BaCl_2$-ficoll (20mmol/l $BaCl_2$, 10% ficoll 400 in HEPES buffer) and 500µl RPMI 1640 + 20% (w/v) bovine serum albumin in a 12ml centrifuge tube. After addition of the islet-alginate suspension, the gradient tube was centrifuged at 800g for 12 minutes and coated islets could be located in the bottom layer. In order to remove the upper layers, the Na^{+}-alginate on the top was precipitated by adding 0.5ml of 20mmol/l. $BaCl_2$ solution and then removed as a gel in toto. The alginate coated islets (encapsulation efficiency 22.2±1.5%,n=6) were separated using a pasteur pipette and washed in HEPES buffer before being used in secretory experiments and histological examination, Plate 4.

Plate 3 Ba^{2+}- alginate microencapsulated mouse islet, prepared using method 1 (X50)

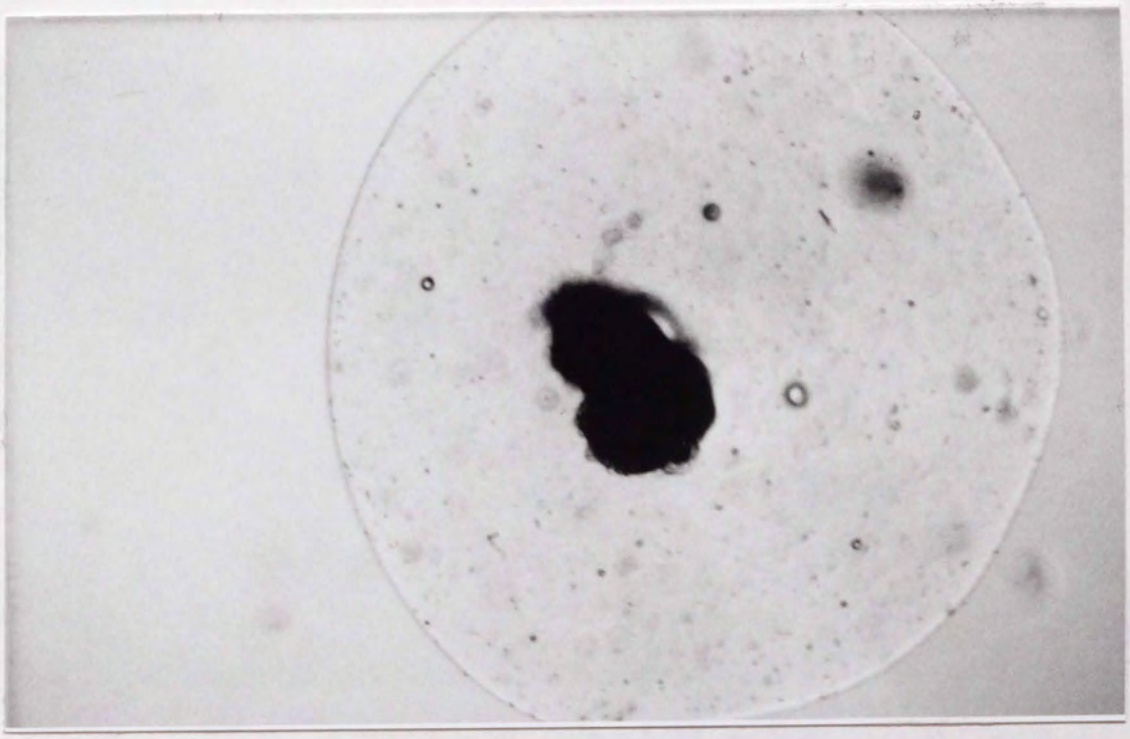
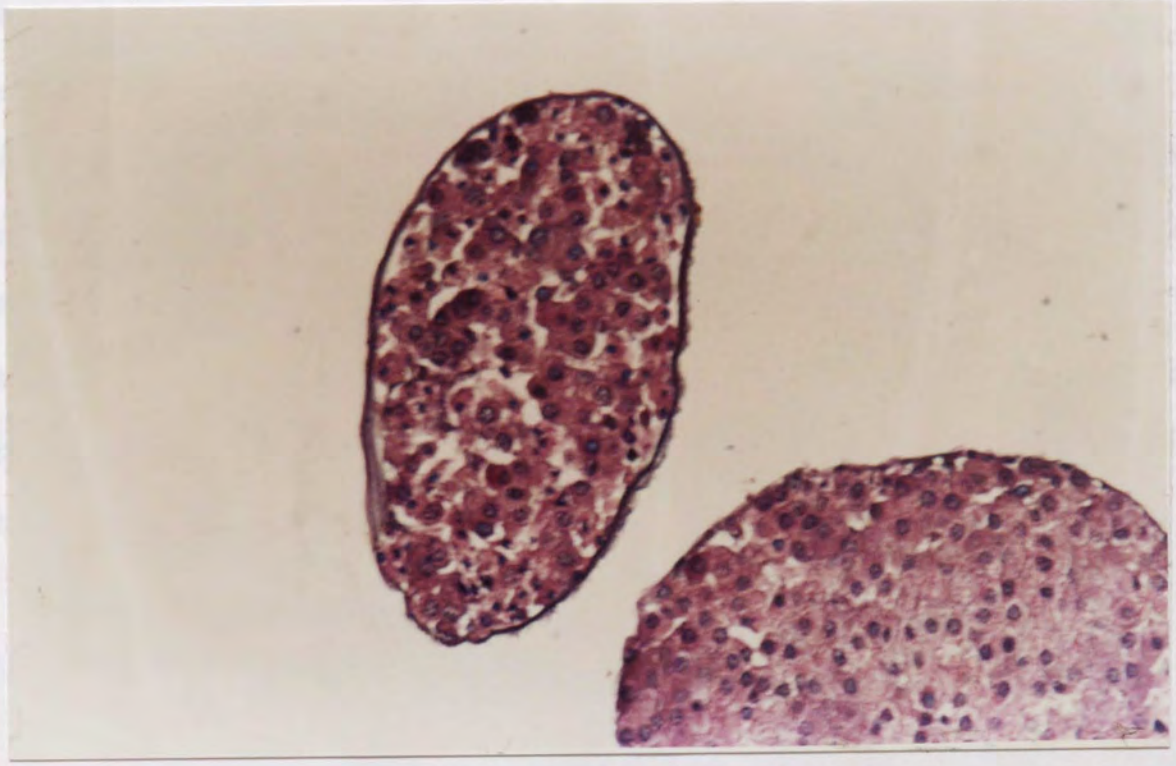


Plate 4 Histological section of Ba^{2+}- alginate coated microencapsulated islets prepared by method 2 (X400)



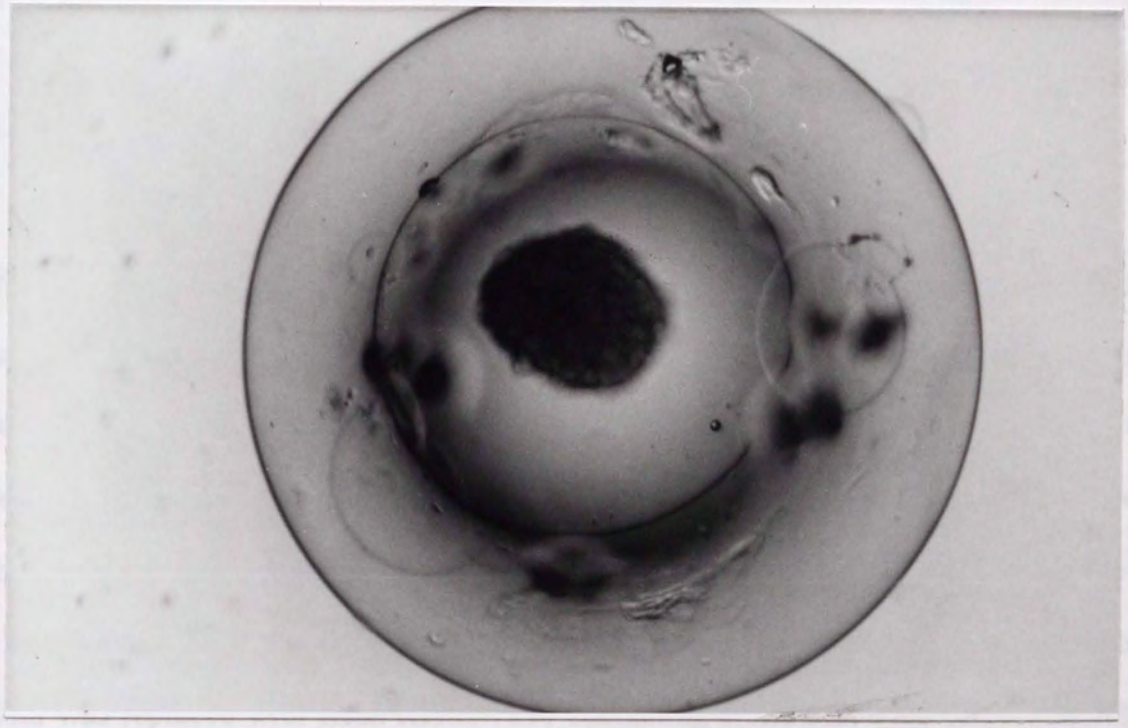
2.7 Microencapsulation of islets in agarose beads

Islets were microencapsulated in agarose according to the method of Nilson et al (215). Briefly, 1ml of 10% low-temperature (<15°C) gelling agarose in phosphate buffered saline, pH7.4 was autoclaved at the pressure of 15lbs / sq. inch at 120°C for 20 minutes, in a 50 ml round-bottom glass centrifuge tube and then cooled to 40°C. About 200 isolated islets in 1ml HBSS were mixed with the agarose solution. 20ml of light paraffin oil (mineral oil, which had also been autoclaved and cooled to 40°C) was carefully poured into the centrifuge tube. The contents were emulsified at room temperature by shaking gently by hand for about 2-3 minutes. The centrifuge tube was then immersed in an ice-bath for 10 minutes in order to gel the agarose droplets. 30ml of HBSS was then added and the tube centrifuged at 1000g for 20 minutes. The oil phase was removed by aspiration. HBSS was added again and after gentle mixing, the suspension was recentrifuged and the remaining oil separated off. The agarose microbeads were resuspended in HBSS and placed in a petri-dish. The microcapsules containing islets were separated by hand under a dissecting microscope, Plate 5. The recovery of microencapsulated islets after agarose microencapsulation was 27±4.5% (n=6).

2.8 Insulin secretion studies with microencapsulated islets in static culture

Both free and microencapsulated islets were cultured overnight in glucose free RPMI-1640 culture medium supplemented with 10% new born calf serum, 5.5 mmol/l glucose and 2% penicillin (10000 IU/ml)/streptomycin (10000 U/ml) in a humidified atmosphere of 5%CO_2/95%O_2 at 37°C prior to use in secretion studies. Overnight cultured non-microencapsulated mouse islets were served as controls. 10 microencapsulated islets were transferred to small glass vials containing 1 ml of oxygenated HBSS supplemented with

Plate 5 Agarose microencapsulated mouse islet (X50)



5.5 mmol/l glucose, 0.1% bovine serum albumin and 2% penicillin/streptomycin and pre-incubated at 37°C for 30 minutes. At the end of this pre-incubation period, the medium was aspirated and replaced with test medium at 37°C containing either 5.5, 11.1 or 16.7 mmol/l glucose in the presence or absence of 5.5 mmol/l theophylline and incubated for a further 2 hours at 37°C in a shaking water bath (80 cycles/minute) with occasional oxygenation. After this time period a 500µl aliquot of the test buffer was collected from each vial and stored at -20°C until required for insulin radioimmunoassay.

2.9 The effect of extended incubation time on the secretion of insulin from microencapsulated islets

In order to obtain an appreciable release of insulin (only small amounts of insulin were released after 2 hours) and take account of the diffusion of insulin and glucose through the microcapsule wall, the incubation time for microencapsulated islets was extended from 2 to 24 hours. As described previously, batches of 10 microencapsulated islets were pre-incubated for 30 minutes at 37°C in RPMI-1640 containing 5.5 mmol/l glucose, 10% new born calf serum and 2% penicillin/streptomycin. After 30 minutes pre-incubation, the medium was replaced with fresh RPMI-1640 containing either 5.5, 11.1 or 16.7 mmol/l glucose in the presence or absence of 5.5 mmol/l theophylline and microencapsulated islets test incubated for a further three consecutive 8-hour periods (24 hours in total) with occasional shaking and oxygenation. After each 8-hour period, a 500µl aliquot was removed from each vial and stored at -20°C until used for insulin radioimmunoassay. After each 8 hour period the medium was completely replaced with fresh medium at 37°C.

2.10 Insulin release from perifused free and microencapsulated islets

The perifusion system used in this study was a modification of the system originally described by Lacy et al (216). The perifusion apparatus consisted of a multichannel peristaltic pump, polyethylene tubing pp20, perifusion filter chamber and a fraction collector in series. The whole system was housed in a 37°C culture room. The perifusion chamber was fitted into the perifusion system as shown in Figure 3. Either 20 free or 20 microencapsulated islets were placed in the centre of a millipore filter (pore size 8μ) supported on a stainless steel mesh sandwiched between two teflon gaskets and the two component parts of a perifusion chamber (actually called a Swinnett, Plate 6). Oxygenated 95%O_2/5%CO_2 RPMI-1640 supplemented with either a low (5.5 mmol/l) or stimulatory (16.7 mmol/l) glucose concentration, 10% new born calf serum and 2% penicillin/streptomycin was drawn through the perifusion chamber via 20pp polyethylene tubing (Portex Ltd) at a constant flow rate of 1ml/minute by means of the peristaltic pump (LKB Ltd, Sweden). Free and microencapsulated islets were initially pre-perifused for 30 minutes with RPMI-1640 supplemented with 5.5 mmol/l glucose in order to establish steady state secretory dynamics. After the 30 minutes stabilization, the glucose concentration was increased to 16.7 mmol/l by flow diversion and the test perifusion was continued for a further 60 minutes. The perifusion was collected in 1ml fractions at 1 minute intervals on a programmable fraction collector (2212 HeliRac LKB Ltd, Sweden) and then stored at -20°C until required for insulin radioimmunoassay. The total dead space of the perifusion system was 1ml and a lag time of 1 minute existed between operation of the diverting valves and the test buffer actually reaching the islets in the perifusion chamber. The lag time was compensated for by diverting the flow one minute before the test perifusion was due to begin.

Figure 3 Perifusion System maintained at 37°C in culture room

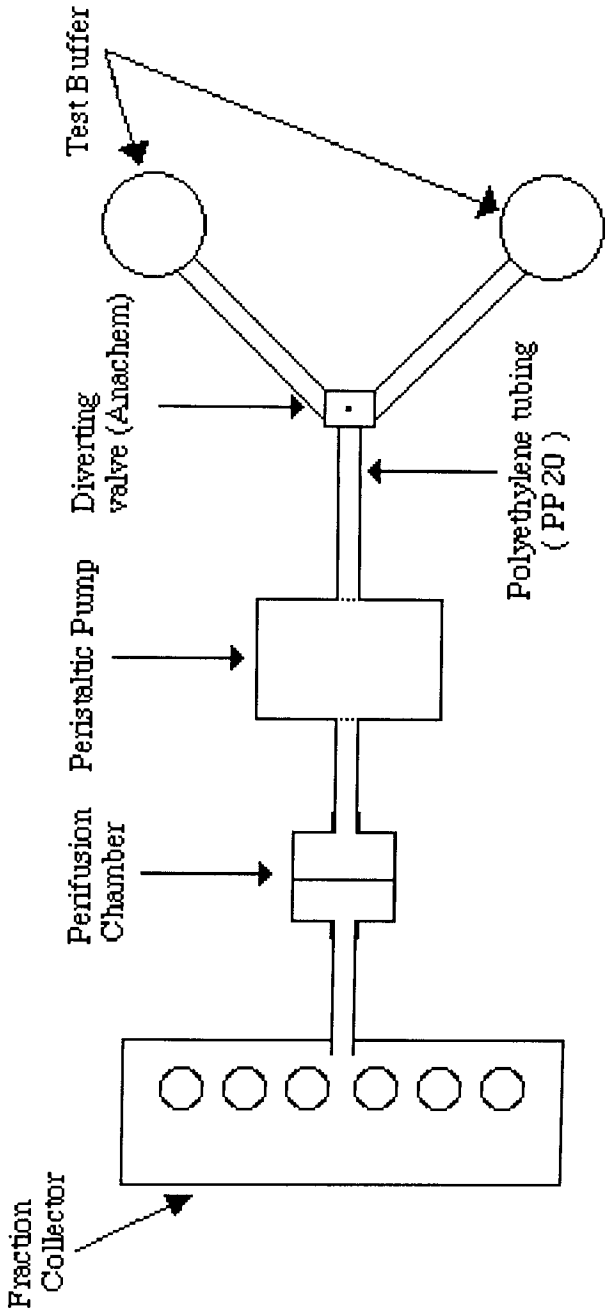
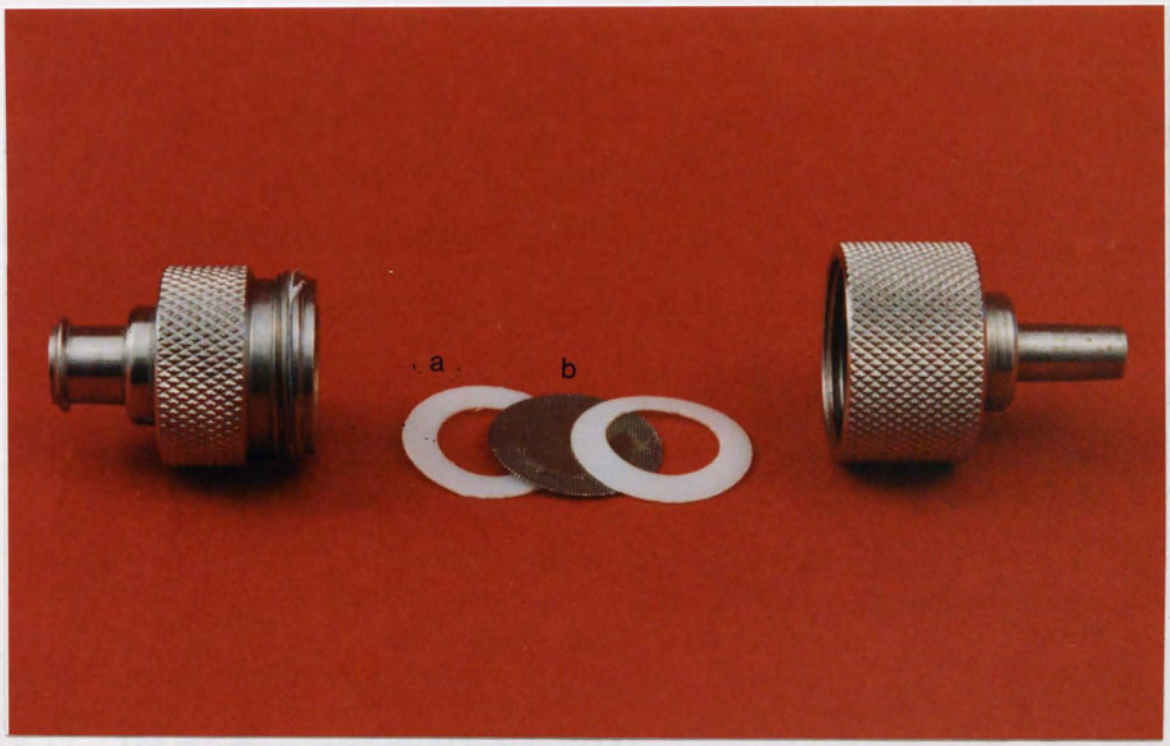


Plate 6 Islet chamber used in perifusion studies

a) teflon gaskets

b) stainless steel grid



2.11 I^{125}-insulin diffusion across the wall of empty Na^+-alginate microcapsules

This study was carried out to establish the effect that microcapsule fabrication, microcapsule diffusion distance and polyamino acid coating have on the diffusion of insulin from the interior of Na^+-alginate microcapsules. 100µl of I^{125}-insulin (specific activity 100-125µci/µg) was suspended in 400µl Na^+-alginate (1.5% w/v) solution and empty PLL or PLO - Na^+-alginate coated microcapsules prepared as described previously, see page 41. Batches of 50 empty microcapsules were inserted into small siliconised glass vials containing 1ml of either glucose free RPMI-1640 or supplemented with 16.7 mmol/l glucose. The initial radioactivity was measured using compugamma counter (1282 LKB Ltd, Sweden). Then the microcapsules were incubated at 37°C for 8 hours and the permeability of the two polyamino acid membranes was evaluated by measuring the rate of diffusion of I^{125}-insulin from the microcapsules. After incubation, the contents of the tubes were centrifuged at 1500 rpm for 5 minutes and the radioactivity measured in both the supernatant and the microcapsules fractions. The amount of I^{125}-insulin diffused from microcapsules into the test medium and the amount that entrapped in the microcapsules were determined by measuring total counts/minute using compugamma counter. The results were expressed as a percentage of the initial amount of radioactivity.

2.12 The radioimmunoassay of insulin (RIA)

The most commonly used convenient and sensitive method presently available for the determination of insulin in body fluids and culture media is radioimmunoassay. Radioimmunoassay is a very sensitive technique, enabling detection down to picogram/ml of a substance, and like most other immunological techniques has a very high specificity (217,218).

The radioimmunoassay for insulin is based on the principle of isotopic dilution in the presence of specific antibodies and depends on the competition between radioactively-labelled I^{125}-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. The antibody shows no preference in binding to insulin or labelled insulin and, since antibody and labelled insulin are both known constant values, the amount of labelled insulin-antibody formed is inversely proportional to the concentration of unknown insulin.

The important elements of a radioimmunoassay are the specificity of antibody, the species specific labelled hormone and the separation of antibody bound and free labelled hormones. In the case of insulin, most investigators have successfully used systems employing anti-porcine insulin raised in guinea pigs. This arrangement is based on the observation that guinea pig antisera reacted strongly with human insulin (219). The final step in radioimmunoassay involves the measurement of antibody bound or free labelled antigen, and this requires the physical separation of the antibody bound and the free antigen. In the case of insulin, early methods of separation employed paper electrophoresis and chromoelectrophoresis (219,220). A variety of other more versatile separation methods have since been developed. These include the adsorption of free insulin to solid phase materials such as acid washed uncoated (220) or dextran coated charcoal (221), adsorption or complexing of antibody to solid phase materials such as glass, styrene or sephadex (222) and ethanol precipitation (223). Double antibody techniques involving the precipitation of soluble antigen-antibody complexes with a second antibody anti-γ-globulin serum, usually raised in rabbit, has been widely used to separate free and antibody bound hormone in insulin RIA (224). A more recent method developed by Desbuquois and Aurbach has employed polyethylene glycol for the separation of free from antibody bound insulin and

this modification provides several advantages over the double antibody precipitation method (225). The most important being that the separation of antibody bound insulin is achieved almost instantaneously. On this basis the polyethylene glycol separation method was used for the radioimmunoassay of insulin in the present work.

Reagents used for the RIA of insulin

All reagents were prepared in double distilled water since variations in the quality of single distilled water may give rise to considerable variations in the amount of insulin bound to the antibody.

Phosphate buffer (40mmol/l), pH7.4 (appendix 3)

This buffer was used for the dilution of unknown samples, the dilution of the insulin binding reagent (antibody), the insulin standards and the I^{125}-insulin.

Insulin binding reagent (insulin antibody)

The insulin-binding reagent provided by the Wellcome Laboratories was obtained in a lyophilised form as guinea pig anti-porcine insulin and reconstituted with 19.5ml of phosphate buffer to a dilution of 1:40,000.

The insulin-binding reagent obtained from ICN Chemicals was undiluted anti-porcine insulin raised in guinea pig and diluted with phosphate buffer to provide a 1:30,000 dilution.

Human insulin standard

Human insulin was supplied in the powder form. A stock solution was prepared by dissolving 1mg of human insulin in a few drops of 1M HCl and the volume made up to 10ml with phosphate buffer. 1ml aliquots of this stock solution were dispersed into LP_3 tubes and stored at -20°C. Before each assay, one tube was thawed and diluted with phosphate buffer to give a range of working standards of 10, 5, 2.5, 1.25 and 0.625 ng/ml.

I^{125}-insulin

I^{125}-insulin (IM-38, specific activity 100-125μci/ug) was obtained from Kodac clinical diagnostics, Amersham, UK and diluted with phosphate buffer to give a total count of approximately 12,000cpm/50μl.

Radioimmunoassay Method

The assay was carried out in LP_3 tubes and all reagents were added in 50μl aliquots. Each standard was assayed in triplicate and each test sample in duplicate. The following assay tubes were set up: total counts (to which 50μl of I^{125}-insulin only had been added), a blank (tubes in which insulin antibody had been replaced with phosphate buffer to serve as controls of the washing procedure), a zero (to which no unlabelled insulin had been added) and test samples. The reaction protocol has been summarised in Table 1.

Table 1 Protocol for Insulin Radioimmunoassay (Sequence of additions)

Tube description	Tube No.	Initial reactants	Binding reagent	I^{125}-insulin
Total counts	1-3	-	-	+
Blank	4-6	phosphate buffer	phosphate buffer	+
Insulin standards ng/ml	7-9	0.62	+	+
	10-12	1.25	+	+
	13-15	2.50	+	+
	16-18	5.00	+	+
	19-21	10.00	+	+
Zero	22-24	phosphate buffer	+	+
Unknown sample	25 onward	+	+	+

Each + indicates the addition of a 50µl aliquot.

50µl aliquots of buffer, insulin standards or unknown samples were delivered directly to the bottom of the LP_3 tubes. A 50 µl aliquot of insulin-binding reagent (antibody) was added to all tubes except the total counts and blank tubes. The contents of each tube was then vortex mixed (whirlmixer) and incubated at 4°C for a period of 4 hours. This incubation period at low temperature increases the equilibrium constant for the insulin antibody reaction and allows the antibody to react with the unlabelled insulin prior to the addition of the I^{125}-insulin. A 50µl aliquot of I^{125}-insulin was then added to all tubes. The reactants were again vortex mixed and all tubes incubated for a further 16 hours, generally overnight at 4°C. After this second incubation period, 800µl of polyethylene glycol separation buffer (appendix 4) was added to all tubes except the total counts, and the contents were gently vortex mixed and subsequently centrifuged at 3000 rpm for 30 minutes. The supernatant was carefully decanted off in one continuous movement. The tubes were then inverted and allowed to dry for >1 hour after which time the pellet was counted for 1 minute on a compugamma counter (1282 LKB Ltd, Sweden) (appendix 5). A standard curve was constructed of insulin concentration (ng/ml) against counts/minute, Figure 4. In the present studies the insulin assay had an intra and interassay coefficient of variation of 3.8±0.44% and 7.6±1.12% (n=6 assays) respectively.

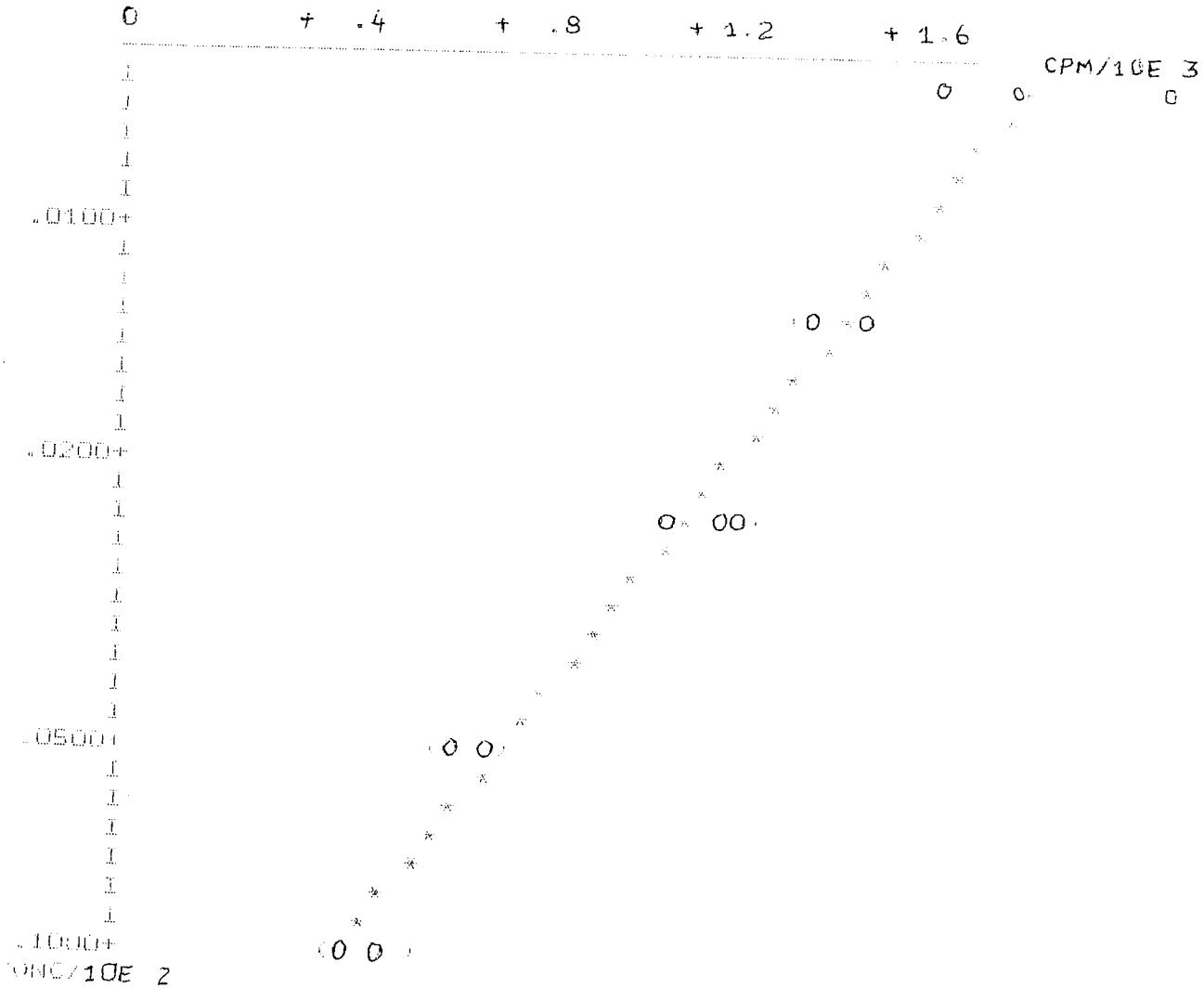
Quality control (criteria for a satisfactory assay)

There was generally good agreement between replicates of standards. Blank values were generally less than 5% of the total counts and indicated the efficiency of the washing procedure. The zero standard count rate was always in the range 30-50% of the total count rate.

Data presentation and statistical analysis

Results are expressed throughout as means ± SEM. Student's t-test was used for statistical comparisons and the differences were considered significant if $\rho < 0.05$.

Figure 4 Human insulin standard curve (with no curve editing)



CHAPTER 3

INSULIN SECRETORY CAPACITY OF FREE AND MICROENCAPSULATED MOUSE ISLETS IN VITRO

INTRODUCTION

Transplanted islets are very immunogenic and are prone to both allograft rejection (226) and destruction by autoimmunity (108,227). One method which has been proposed to immuno protect islets from host antibodies is microencapsulation of islets in Na^+-alginate (159), Ba^{2+}-alginate (211, 228) water insoluble polyacrylates (208) and agarose gels (203, 229). The microencapsulation procedure consists of entrapping purified pancreatic islets in a microcapsule with a semi-permeable membrane. Microencapsulation of islets of Langerhans has been suggested to be an effective method of combating graft rejection in both allo and xenotransplantation without the need for immunosuppression (173, 176, 206, 228, 229). Lim and Sun in 1980 proposed the use of a cross-linked Na^+-alginate membrane for the immunoprotection of transplanted islets (159). The physical and chemical properties of microencapsulating materials, such as Na^+-alginate and agarose were shown not to adversely affect the long-term in vitro morphology, metabolic integrity, viability and secretory function of islet tissue (159,176,179,203). Na^+-alginate is a macromolecule derived from kelp and composed of 1,4 linked β-D-mannuronic acid (M), ∝, L-guluronic acid (G) and alternating MG blocks, Figure 5. There is evidence to suggest that high mannuronic acid alginate is more potent in inducing cytokine production compared with high guluronic acid alginate (230). Na^+-alginate has been widely used for the microencapsulation of isolated islets of Langerhans. Na^+-alginate can be cross-linked by polyamino acid such as poly-L-lysine (PLL) and poly- L-ornithine (PLO) as well as by a number of divalent cations such as Ca^{++} and Ba^{++} (159, 176, 211, 231).

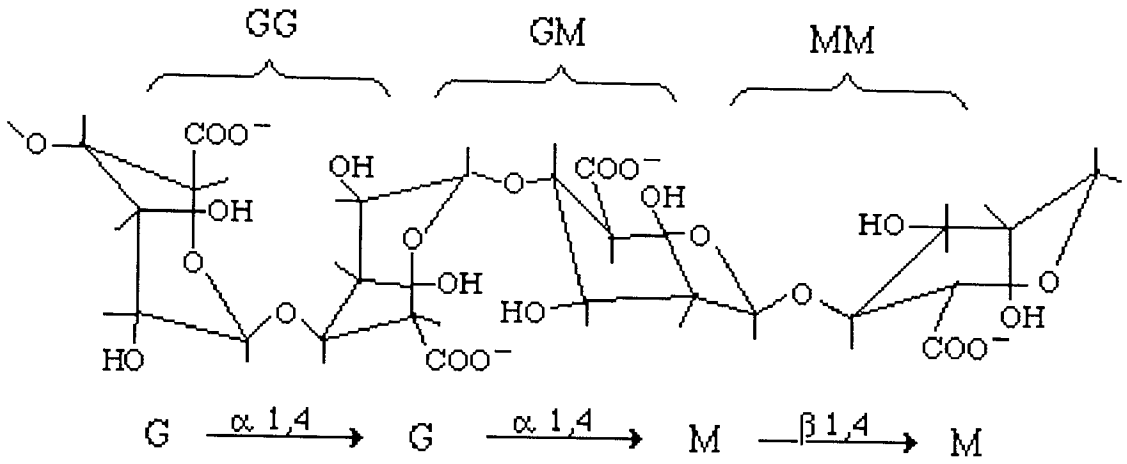


Figure 5 Structural illustration of alginate as a linear copolymer of L-guluronic acid(G) and D-mannuronic acid(M).

Agarose has also been used as a microencapsulation material for islets (203, 205, 229). Agarose like alginate is a polysaccharide and derived from sea weed. It has a long history of use in biological research techniques, such as electrophoresis and its quality is well controlled. Na^+-alginate and agarose have been shown to be suitable materials for islet microencapsulation and have no adverse effect on the islet function (176,203). Islets microencapsulated in both Na^+-alginate and agarose will remain functional for up to 100 days in tissue culture (176,203). In vitro studies have shown that Na^+-alginate / PLL, PLO-Na^+-alginate microencapsulation of rat and porcine islets can protect against the cytotoxic effects of serum from type 1 human diabetic patients (164,232). These microencapsulated islets responded rapidly to glucose stimulation by increasing their rate of insulin production (162,173,182), suggesting that the microcapsule membrane was permeable to glucose and insulin. However, the microencapsulated islets secreted insulin at a lower level than that secreted by free non-microencapsulated islets (182,210). Clearly, an adequate insulin response by the microencapsulated islets is a pre-requisite for successful islet transplantation and the amelioration of the insulin dependent diabetic state. Few studies have been carried out in which in vitro glucose-induced insulin release from

microencapsulated islets has been determined. To date, there has been no direct comparison of the in vitro insulin secretory efficiency of the more popular methods of islet microencapsulation i.e. Na^+-alginate, Ba^{2+}-alginate and agarose microencapsulation of islets of Langerhans. On this basis, in order to establish the best microencapsulation technique and optimise the choice of fabrication materials NMRI mouse islets were microencapsulated with two types of Na^+-alginate i.e. high mannuronic acid alginate (LMT) and high guluronic acid alginate (DJB), coated with either PLL, PLO and / or an outer Na^+-alginate layer, Ba^{2+}-alginate or low-temperature (<15°C) gelling agarose. The insulin secretory capacity of these differently fabricated microencapsulated islets was investigated in response to elevated glucose in the presence and absence of theophylline in both static and perifusion systems.

MATERIALS AND METHODS

Characterization of the NMRI mice

In order to characterize the NMRI mice for glucose homeostasis, overnight fasting plasma glucose and insulin levels were determined. After fasting a small cut was made on the tip of the tail of conscious mice using a scalpel blade. Blood was milked into a 200µl heparinized microfuge tube and plasma separated by high speed centrifugation. A 10µl plasma sample was used immediately for glucose determination and the remainder of the sample stored at -20°C until required for insulin determination. Fasting plasma glucose levels were determined using the glucose oxidase method on a Beckman II glucose analyser and insulin levels were determined using polyethylene glycol separation method of radioimmunoassay (225), see page 57

Isolation of NMRI mouse islets

Islets were isolated from 12-15 week old NMRI mice using collagenase digestion as described previously on page 39.

Islet microencapsulation

Islets were microencapsulated with Na^+-alginate according to the method described on page 41. The microcapsules were fabricated with the following compositions: Na^+-alginate (LMT)-PLL-Na^+-alginate; Na^+-alginate (DJB)-PLO-Na^+-alginate; Na^+-alginate (DJB)-PLL-Na^+-alginate; Na^+-alginate (LMT)-PLL (no outer Na^+-alginate layer) and Na^+-alginate (DJB)-PLO (no outer Na^+-alginate layer). Ba^{2+}-alginate microencapsulated islets were

prepared using either, a straight forward droplet technique as described previously (page 42) in which Ba^{2+}-alginate microencapsulated islets were fabricated in one step and coated with either PLL or PLO membrane, or a density gradient method which produced islets tightly coated in a Ba^{2+}-alginate membrane. Islets were also microencapsulated in low-temperature (<15°C) gelling agarose using the method of Nilson et al (215) (page 48).

Static incubation of microencapsulated islets

Since a large number of microencapsulated islets were required for a single experiment (approximately 200), it proved necessary to isolate the islets and microencapsulate them on one day and use them in secretory experiments on the next. Microencapsulated islets were maintained in an atmosphere of 95% O_2 / 5% CO_2 overnight at 37°C in sterile plastic petri dishes containing 5ml of RPMI-1640 culture medium supplemented with 5.5mmol/l glucose, 10% heat inactivated new born calf serum and 2% penicillin/streptomycin. Damaged microencapsulated islets were excluded from the study. After 30 minutes pre-incubation in the presence of 5.5mmol/l glucose, batches of 10 free non-microencapsulated or microencapsulated islets were incubated with 1ml of test medium containing either 5.5, 11.1 or 16.7 mmol/l glucose in the presence and absence of 5.5mmol/l theophylline. Free non-microencapsulated islets were incubated for 60 minutes while microencapsulated islets were incubated for 2, 8, 16 and 24 hours at 37°C in shaking water bath. After the incubation 500µl aliquots of incubation medium were removed and frozen at -20°C for the subsequent assay of insulin. Insulin release was expressed as ng / ml / 10 free or microencapsulated islets/-hour.

In perifusion work, 20 free non-microencapsulated or microencapsulated islets were inserted into the islet chamber and preperifused for 30 minutes with RPMI-1640 supplemented with

5.5mmol/l glucose. This was followed by 60 minutes test perifusion with 16.7 mmol/l glucose. 1ml aliquots of perifusate were collected for insulin RIA at 5 minute intervals. Insulin release was expressed as ng / ml / 20 free or microencapsulated islets / 3,5 minutes.

RESULTS

Characterization of the NMRI mice

The fasting plasma glucose and insulin levels of NMRI mice were found to be 5.9±0.24 mmol/l (n=14) and 0.7±0.07 ng/ml (n=17) respectively. The values are essentially normal and compare well with the fasting values of other routinely used experimental mice, such as MFI and BALB/C (233). The NMRI mouse islets were judged to be ideal for use in in vitro insulin secretory studies, especially since their islets could be routinely and more easily isolated in convenient quantities by collagenase digestion compared with standard laboratory MFI mice. The islet yield obtained using collagenase digested MFI mouse pancreas was very low (40 islets/pancreas). The digested pancreas of these mice was more fibrous and gelatinous than that of NMRI mice and most of the islets remained entrapped in this gelatinous material even in the presence of DNAase. The yield of intact islets from two NMRI mouse pancreata was usually around 200.

Insulin secretion from free NMRI mouse islets in static incubation

The effect of increasing the glucose concentration from 5.5 to 11.1 and 16.7 mmol/l in the presence and absence of 5.5 mmol/l theophylline on the release of insulin from NMRI mouse islets is shown in Table 2.

Raising the glucose concentration from 5.5 to 16.7 mmol/l caused a marked and significant increase in the rate of insulin release (143%). In the presence of 11.1 mmol/l glucose the concentration of insulin released was 104% higher than that obtained with the 5.5 mmol/l glucose but not significantly different from that obtained with 16.7 mmol/l glucose, Table 2.

5.5mmol/l theophylline alone did not stimulate the release of insulin above levels seen with 5.5mmol/l glucose. However, theophylline (5.5 mmol/l) significantly potentiated the release of insulin in response to 5.5, 11.1 and 16.7 mmol/l glucose by 63.5%, 92.7% and 236% respectively, Table 2.

Table 2

The effect of glucose in the presence and absence of theophylline on insulin release from statically incubated NMRI mouse islets (mean values ± SEM, n=6).

Treatment (mmol / l)	n	Insulin release (ng / ml / 10 islets / hr)
Glucose 5.5	6	27.80 ± 3.34
Glucose 11.1	6	56.70 ± 6.7[a]
Glucose 16.7	6	67.50 ± 6.8[a]
Theophylline 5.5	6	30.82 ± 3.8
Glucose 5.5 + theophylline 5.5	6	45.47 ± 7.3[a]
Glucose 11.1+ theophylline 5.5	6	109.3 ± 15.5[b,c]
Glucose 16.7 + theophylline 5.5	6	226.8 ± 20.4[b,c]

a , $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone.

b , $P<0.05$, significantly increased compared with 5.5 mmol/l theophylline alone.

c , $P<0.05$, significantly increased compared with the corresponding concentration of glucose alone.

The release of insulin from Na+-alginate-PLL-Na+-alginate microencapsulated NMRI mouse islets after short term (2 hrs) incubation

Pancreatic islets were successfully microencapsulated with Na^+-alginate(LMT)-PLL-Na^+-alginate. The microcapsules were generally spherical with a mean diameter of 0.62±0.08mm (n=10) and no surface irregularities or rugosity, Plate 7. An outer Na^+-alginate layer could just be differentiated at low power. The percent yield of microencapsulated islets, defined as the ratio of the number of microencapsulated islets recovered to the number of islets originally loaded into the syringe, was 61.8±3.3% (n=10). Some islets were lost by adherence to the syringe barrel surface while others became lodged in the syringe / tubing leading up to the extension nozzle.

In order to establish whether the microencapsulated islets were responsive to well characterized insulin secretagogues, microencapsulated islets were challenged with increasing concentrations of glucose in the presence and absence of 5.5mmol/l theophylline in static incubation. When batches of 10 microencapsulated islets were subjected to 2 hours static incubation in the presence of either 5.5, 11.1 or 16.7 mmol/l glucose in both the presence and absence of theophylline, the amount of insulin released in response to all treatments was significantly reduced compared with that from free non-microencapsulated mouse islets, Table 3. Microencapsulation of islets was therefore, associated with a significant reduction in insulin levels presumably due to the creation of a diffusional barrier to the influx of secretagogues and efflux of insulin. Despite this increasing the glucose concentration from 5.5 to 11.1 and 16.7 mmol/l did significantly increase, however modestly the insulin response of Na^+-alginate-PLL-Na^+-alginate microencapsulated islets in vitro by 647% and 883% respectively confirming the physiological viability of the microencapsulated islets, Table 3. The addition of 5.5 mmol/l

theophylline significantly increased the insulin response to 5.5 mmol/l glucose but had no significant effect on the insulin response of microencapsulated islets to either 11.1 or 16.7 mmol/l glucose, Table 3, presumably because of too short (2 hrs) an exposure of microencapsulated islets to theophylline / glucose challenge, or the time lag associated with the diffusion of insulin through the microcapsule wall.

Table 3

The effect of glucose in the presence and absence of theophylline on insulin release from short term (2hr) statically incubated Na^+-alginate-PLL-Na^+-alginate microencapsulated NMRI mouse islets (mean values ± SEM, n=6).

Treatment (mmol/l)	n	Insulin release (ng / ml / 10 microencapsulated islets / 2hrs)
Glucose 5.5	6	0.36 ± 0.15
Glucose 11.1	6	2.69 ± 0.33[a]
Glucose 16.7	6	3.54 ± 0.85[a]
Theophylline 5.5	6	1.40 ± 0.31
Glucose 5.5 + theophylline 5.5	6	2.45 ± 0.56
Glucose 11.1 + theophylline 5.5	6	2.49 ± 0.77
Glucose 16.7 + theophylline 5.5	6	3.4 0± 0.43

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone.

Plate 7

Na^+-alginate / poly-L-lysine / Na^+-alginate microencapsulated NMRI mice islet (X50)

The cumulative release of insulin from microencapsulated islets after long term (8, 16 and 24) hours static incubation

Since only small quantities of insulin (3.40 ± 0.43 ng/ml/10 microencapsulated islets) were secreted in response to 16.7 mmol/l glucose and theophylline after short term (2 hours) incubation, microencapsulated islets were subjected to an extended period of incubation (8, 16 and 24 hours) in order to obtain a more significant insulin response and to combat the lag phase in insulin / glucose exchange caused by the diffusion of these species through the capsule wall.

Insulin release from Na+-alginate (LMT)-PLL-Na+-alginate microencapsulated islets after extended incubation

The effect of extended incubation time on the cumulative release of insulin from Na^+-alginate(LMT)-PLL-Na^+-alginate microencapsulated islets in response to glucose and theophylline challenge is summarised in Table 4.

Compared with short term incubated microencapsulated islets, Table 3, a significantly greater release of insulin was observed from microencapsulated islets incubated for 8, 16 and 24 hours in the presence of increasing glucose concentration in both the presence and absence of theophylline, Table 4. However, insulin secretion from microencapsulated islets was always significantly lower than that shown by free non-microencapsulated islets when exposed to the same secretagogues, Table 2. Theophylline (5.5mmol/l) potentiated the insulin response to 5.5, 11.1 and 16.7 mmol/l glucose challenge after 8, 16 and 24 hours static incubation. After 24 hours incubation, 5.5, 11.1 and 16.7 mmol/l glucose induced insulin release was stimulated by some 51.9%, 25.7% and 33.7% respectively in the presence of theophylline compared with the corresponding glucose concentrations alone, Table 4.

Table 4

The effect of increasing glucose concentration, theophylline alone or in combination on the cumulative release of insulin from Na^+-alginate(LMT)-PLL-Na^+-alginate microencapsulated islets after 8, 16 and 24 hours static incubation (mean values ± SEM).

Treatment (mmol/l)	n	Cumulative insulin release (ng/ml/10 microencapsulated islets)		
		8 hours	16 hours	24 hours
Glucose 5.5	5	2.30 ± 0.41	3.4 0± 0.27	6.30 ± 0.70[a]
Glucose 11.1	6	9.40 ± 1.19[b]	14.31 ± 0.80[a,b]	21.20 ± 0.71[a,b]
Glucose 16.7	6	9.20 ± 0.89[b]	16.77 ± 0.91[a,b]	23.87 ± 1.15[a,b]
Theophylline 5.5	5	3.12 ± 0.47	7.66 ± 1.29[c]	10.56 ± 0.57[c]
Glucose 5.5 + theophylline 5.5	5	4.90 ± 1.71[d]	10.80 ± 2.14[d]	13.10 ± 0.71[d]
Glucose 11.1 + theophylline 5.5	6	13.82 ± 0.88[d,e]	22.36 ± 1.07[d,e]	28.55 ± 0.51[d,e]
Glucose 16.7 + theophylline 5.5	6	14.31 ± 0.76[d,e]	27.15 ± 1.57[d,e]	36.02 ± 1.29[d,e]

a, $P<0.05$, significantly increased compared with release after 8 hours in the presence of the same concentration of glucose.

b, $P<0.05$, significantly increased compared with 5.5mmol/l glucose after the same incubation time.

c, $P<0.05$, significantly increased compared with insulin release in response to 5.5 mmol/l theophylline after 8 hours incubation.

d, $P<0.05$, significantly increased compared with the corresponding glucose concentration alone after either 8, 16 or 24 hours incubation.

e, $P<0.05$, significantly increased compared with 5.5mmol/l theophylline alone after either 8, 16 or 24 hours incubation.

The effect of type of polyamino acid coating on insulin release from microencapsulated islets after extended incubation

Compared with poly-L-lysine, the poly-L- amino acid poly-L-ornithine (PLO) has been shown to generate membranes of superior porosity (231). On this basis microencapsulated islets have been fabricated using Na^+-alginate(DJB)-PLO-Na^+-alginate and insulin release monitored in response to glucose and theophylline after 8 hours extended static incubation, Table 5.

Microencapsulated islets containing poly-L-ornithine were spherical and had a continuous PLO coating with a mean diameter of 0.63 ± 0.09 mm (n=10), Plate 8. Only in the presence of 5.5 mmol/l glucose plus theophylline and 16.7 mmol/l glucose plus theophylline was there any significant elevation in insulin release from PLO fabricated microencapsulated islets compared with PLL microencapsulated islets, Table 6. Although the insulin response to all three glucose concentrations alone were at least 23% elevated from PLO fabricated microencapsulated islets compared with PLL, Table 6, the large standard errors of the mean incurred prevented the differences from reaching statistical significance and only in the presence of 16.7 mmol/l glucose did theophylline significantly potentiate insulin release from Na^+-alginate(DJB)-PLO-Na^+-alginate microencapsulated islets, Table 5.

Plate 8

Na^+-alginate / poly-L-ornithine / Na^+-alginate microencapsulated NMRI mouse islets showing continuous poly-L-ornithine and outer alginate coating (X50).

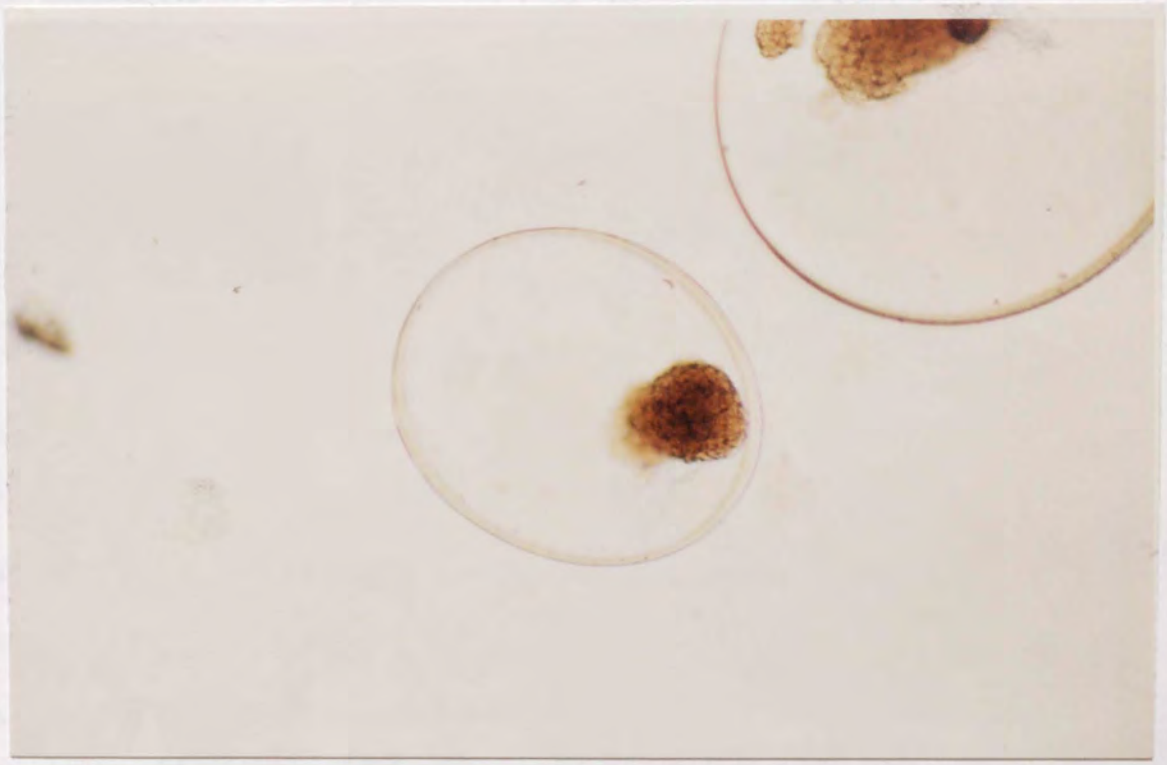


Table 5

The effect of increasing concentrations of glucose and theophylline alone or in combination on the cumulative release of insulin from Na^+-alginate(DJB)-PLO-Na^+-alginate microencapsulated islets after 8 hours static incubation (mean values ± SEM).

Treatment (mmol/l)	n	Insulin release (ng/ml/10 microencapsulated islets/8 hours)
Glucose 5.5	5	5.9 ± 3.5
Glucose 11.1	8	12.3 ± 2.6[a]
Glucose 16.7	7	13.7 ± 2.8[a]
Theophylline 5.5	6	6.2 ± 1.5
Glucose 5.5 + theophylline 5.5	6	10.4 ± 1.9[a,b,c]
Glucose 11.1 + theophylline 5.5	6	17.3 ± 4.5[b]
Glucose 16.7 + theophylline 5.5	6	24.0 ± 3.0[b,c]

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone.

b, $P<0.05$, significantly increased compared with 5.5 mmol/l theophylline alone.

c, $P<0.05$, significantly increased compared with Na^+-alginate(DJB)-PLL-Na^+-alginate microencapsulated islets exposed to the same treatment, Table 6.

The effect of type of alginate on insulin release from microencapsulated islets

It is possible that different types of Na^+-alginate i.e. alginate high in mannuronic acid (LMT) and alginate high in guluronic acid (DJB) may influence the release of insulin from microencapsulated islets. In order to investigate this possibility, mouse islets have been microencapsulated in both Na^+-alginate (LMT) and Na^+-alginate(DJB)-PLL-Na^+-alginate membranes. The insulin response of DJB Na^+- alginate microencapsulated islets to glucose and theophylline has been summarised in Table 6.

Na^+-alginate(DJB)-PLL-Na^+-alginate microencapsulated islets, Table 6 showed very similar insulin responses to increasing glucose concentrations in the presence and absence of theophylline as shown by Na^+-alginate(LMT)-PLL-Na^+-alginate microencapsulated islets, Table 4.

These observations suggest that rather than differences in the type of alginate used for microcapsule fabrication i.e. LMT or DJB, it is the type of polyamino acid membrane, either PLO or PLL that significantly influences the insulin response of microencapsulated islets to glucose / theophylline challenge. PLO coated microcapsules appear to be more responsive than PLL coated microcapsules, a property presumably based on the enhanced porosity of PLO membrane coatings.

Table 6

The effect of increasing concentrations of glucose and theophylline alone or in combination on the cumulative release of insulin from Na^+-alginate(DJB)-PLL-Na^+-alginate microencapsulated islets after 8 hours static incubation (mean values ± SEM, n=6).

Treatment (mmol/l)	n	Insulin release (ng/ml/10 microencapsulated islets/8 hours)
Glucose 5.5	6	3.4 ± 0.81
Glucose 11.1	6	8.6 ± 1.1[a]
Glucose 16.7	6	10.5 ± 1.7[a]
Theophylline 5.5	6	4.6 ± 1.26
Glucose 5.5 + theophylline 5.5	6	5.0 ± 0.93[d]
Glucose 11.1 + theophylline 5.5	6	12.8 ± 1.71[b,c]
Glucose 16.7 + theophylline 5.5	6	16.2 ± 1.39[b,c,d]

a , $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone.

b , $P<0.05$, significantly increased compared with the corresponding glucose concentration alone.

c , $P<0.05$, significantly increased compared with 5.5 mmol/l theophylline.

d , $P<0.05$, significantly decreased compared with Na^+-alginate(DJB)-PLO-Na^+-alginate microencapsulated islets exposed to the same treatment, Table 5.

The diffusion of I^{125}-insulin across the wall of empty microcapsules fabricated using either DJB or LMT alginate and PLL and PLO polyamino acids with an outer alginate layer.

In order to establish whether the type of Na^+-alginate or the type of polyamino acid used in microcapsule fabrication presented a significant diffusion barrier to the release of insulin from microencapsulated islets, empty microcapsules were fabricated from both DJB and LMT alginate containing a fixed known amount of I^{125}-insulin and coated with either PLL or PLO polyamino acids. The proportions of the I^{125}-insulin load released after 8 hours incubation into either glucose free or RPMI-1640 culture medium containing 16.7 mmol/l glucose are summarised in Table 7.

In the absence of glucose the type of alginate used in fabrication i.e. DJB or LMT had no significant effect on the passive diffusion of I^{125}-insulin from microcapsules. However, in the presence of high glucose there was some indication that LMT alginate presented a greater barrier to the release of I^{125}-insulin than DJB alginate, Table 7. Irrespective of the type of alginate used, Na^+-alginate-PLO-Na^+-alginate fabricated microcapsules showed a significantly greater passive diffusion of I^{125}-insulin than microcapsules fabricated using Na^+-alginate-PLL-Na^+-alginate after 8 hours incubation and this enhanced passive diffusion was more pronounced in the presence of 16.7 mmol/l glucose, Table 7. It may well be that the high glucose concentration increased either the number or size of pores in the PLO membrane, facilitating the increased release of I^{125}-insulin by diffusion. Whatever the mechanism this study supports the observation that PLO membranes have a higher permeability to macromolecules than PLL membranes (231).

Table 7

I^{125}-insulin permeability of Na^+-alginate microcapsules fabricated using either DJB or LMT alginate and PLL or PLO polyamino acid with an outer alginate layer after 8 hours static incubation in RPMI-1640 medium (mean value ± SEM).

Microcapsule fabrication	I^{125}-insulin release, % of total counts originally inserted.			
	n	glucose free medium	n	medium containing 16.7mmol/l glucose
Na^+-alginate(LMT)-PLL-Na^+-alginate	5	29 ± 8.0	5	30 ± 2.2
Na^+-alginate(DJB)-PLL-Na^+-alginate	5	33 ± 5.0	5	35 ± 2.9
Na^+-alginate(LMT)-PLO-Na^+-alginate	4	40 ± 3.6	5	44 ± 3.8[a]
Na^+-alginate(DJB)-PLO-Na^+-alginate	4	47 ± 5.3[b]	4	56 ± 4.5[b]

a, $P<0.05$, significantly increased compared with Na^+-alginate (LMT)-PLL-Na^+-alginate microcapsules in the presence of the corresponding glucose concentration.

b, $P<0.05$, significantly increased compared with Na^+-alginate (DJB)-PLL-Na^+-alginate microcapsules in the presence of the corresponding glucose concentration.

The effect of an additional outer alginate layer on the release of insulin from Na+-alginate microencapsulated islets

It has been reported that the diffusion of insulin, glucose and oxygen is inversely proportional to the membrane thickness and the diffusional path distance of the microcapsule (162, 211). An additional outer Na^+-alginate layer, which was first introduced to the microencapsulation procedure to strengthen the microcapsule but, may also impede insulin release. In order to establish the effect of an outer alginate layer, microencapsulated islets were fabricated using Na^+-alginate and either PLL or PLO polyamino acid coatings without an additional outer alginate (0.15% w/v) layer. The effect of low (5.5mmol/l) and high (16.7mmol/l) glucose concentration on the rate of insulin release from these microencapsulated islets after 8 hours static incubation has been summarised in Table 8.

Increasing the glucose concentration from 5.5 to 16.7 mmol/l significantly improved the rate of insulin release from microencapsulated islets fabricated without an outer alginate layer irrespective of the type of initial Na^+-alginate or polyamino acid coating used. In all cases microencapsulated islets fabricated without an outer alginate coating showed a significantly increased release of insulin compared with fabricated with an outer alginate coating, Table 8. It is clear that the outer alginate coating presents an additional diffusion barrier to the cumulative release of insulin, even though the layer may give additional structural strength to microcapsules and improved biocompatibility (178).

Table 8

The effect of omitting the outer alginate layer on glucose stimulated insulin release from Na^+-alginate(LMT)-PLL and Na^+-alginate(DJB)-PLO microencapsulated islets after 8 hours static incubation (mean values ± SEM).

Treatment (mmol/l)	Insulin release (ng/ml/10 microencapsulated islets/ 8 hours)			
	n	Na^+-alginate-(LMT)-PLL	n	Na^+-alginate(DJB)-PLO
Glucose 5.5	5	5.03 ± 0.8[a]	8	14.4 ± 1.7[c]
Glucose 16.7	6	13.70 ± 1.7[b]	5	23.1 ± 5.9[d]
Glucose 5.5 (Na^+-alginate-PLL or PLO + outer Na^+-alginate)	5	2.30 ± 0.41	5	5.9 ± 3.5
Glucose 16.7 (Na^+-alginate-PLL or PLO + outer Na^+-alginate)	6	9.20 ± 0.89	7	13.7 ± 2.8

a, $P<0.05$, significantly increased when compared with the same microencapsulated islets fabricated with an outer alginate layer in the presence of 5.5 mmol/l glucose, Table 4.

b, $P<0.05$, significantly increased when compared with the same microencapsulated islets fabricated with an outer alginate layer in the presence of 16.7 mmol/l glucose, Table 4.

c, $P<0.05$, significantly increased when compared with the same microencapsulated islets fabricated with an outer alginate layer in the presence of 5.5 mmol/l glucose, Table 5.

d, $P<0.05$, significantly increased when compared with the same microencapsulated islets fabricated with an outer alginate layer in the presence of 16.7 mmol/l glucose, Table 5.

Insulin release from Ba^{2+}-alginate (method 1) microencapsulated islets

Ba^{2+}-alginate microencapsulation (as described in method 1, page 42) had an encapsulation efficiency of 54 ± 2.5% (n=10). The microcapsules were spherical, homogeneous and gel like with a mean diameter of 0.8 ± 0.1 mm (n=10), Plate 9. However, the microcapsules were very fragile and had a tendency to fracture during handling or transfer pipetting.

The insulin secretory response of Ba^{2+}-alginate microencapsulated islets (method 1), after short term (2 hours) incubation with 5.5, 11.1 and 16.7 mmol/l glucose, has been summarised in Table 9. The insulin response of Ba^{2+}-alginate microencapsulated islets could be increased by high glucose (11.1 and 16.7 mmol/l) challenge. However, the insulin response to all concentrations of glucose was significantly reduced when compared with that of free non-microencapsulated islets, Table 2. Also, Na^{+}-alginate (LMT)-PLL-Na^{+}-alginate microencapsulated islets were significantly more responsive to 11.1 mmol/l glucose than Ba^{2+}-alginate microencapsulated islets after 2 hours incubation, Table 3. This observation suggests that the diffusion of glucose and insulin was slower in Ba^{2+}-alginate microencapsulated islets compared with Na^{+}-alginate/PLL/Na^{+}-alginate microencapsulated islets, and their relatively large size and capsule thickness would support this, Plate 9.

Ba^{2+}-alginate microencapsulated islets prepared using method 1, Table 10, showed very similar insulin responses to glucose and theophylline as Na^{+}-alginate (LMT) -PLL-Na^{+}-alginate microencapsulated islets after 8, 16 and 24 hours static incubation, Table 4. Cumulative insulin release was significantly increased from Ba^{2+}-alginate microencapsulated islets in response to increasing glucose concentration and the addition of theophylline potentiated the effect of glucose, Table 10. However, there was no significant difference between the cumulative insulin release from Ba^{2+}-alginate and Na^{+}-alginate

(LMT)-PLL-Na^{+}-alginate microencapsulated islets after 24 hours incubation in response to either 5.5 or 16.7 mmol/l glucose, Tables 4 and 10 compared.

Plate 9

Ba^{2+} -alginate microencapsulated mouse islet prepared by method 1 (X50)

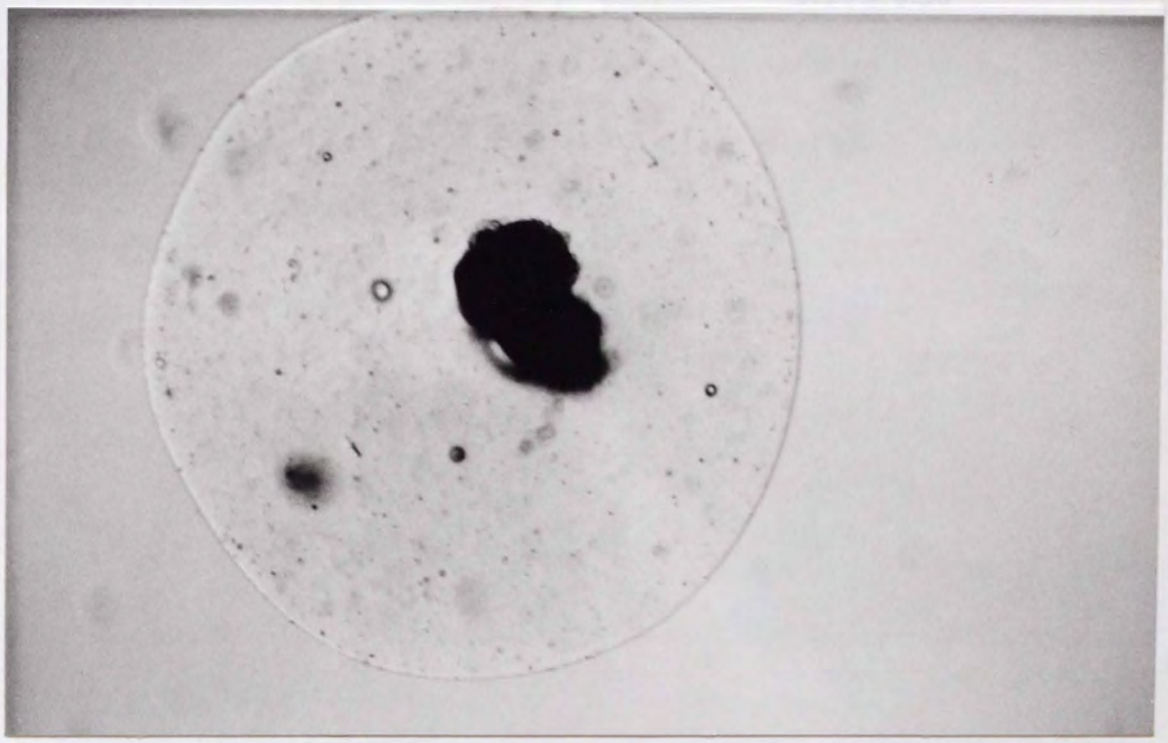


Table 9

The effect of elevated glucose on insulin release from short term (2 hours) statically incubated Ba^{2+}-alginate microencapsulated NMRI mouse islets (method 1, mean values ±SEM, n=6)

Treatment (mmol/l)	n	Insulin release (ng / ml / 10 microencapsulated islets / 2 hrs)
Glucose 5.5	6	0.54 ± 0.20
Glucose 11.1	6	1.60 ± 0.25[a,b]
Glucose 16.7	6	2.10 ± 0.56[a]
Glucose 5.5 (Na^+-alginate/PLL/ Na^+-alginate)	6	0.36 ± 0.15
Glucose 11.1 (Na^+-alginate/PLL/ Na^+-alginate)	6	2.69 ± 0.33
Glucose 16.7 (Na^+-alginate/PLL/ Na^+-alginate)	6	3.54 ± 0.85

a , $P < 0.05$, significantly increased compared with 5.5 mmol/l glucose

b, $P < 0.05$, significantly decreased compared with Na^+-alginate /PLL / Na^+-alginate microencapsulated islets in the presence of the corresponding glucose concentration after 2 hours incubation, Table 3.

Table 10 The effect of increasing glucose concentration, theophylline alone and in combination with glucose on the cumulative release of insulin from Ba^{2+}-alginate (LMT) microencapsulated islets (method 1) after extended static incubation (mean values ± SEM).

Treatment (mmol/l)	Cumulative insulin release (ng/ml/ 10 microencapsulated islets			
	n	8 hours	16 hours	24 hours
Glucose 5.5	5	3.7 0 ± 0.06	4.93 ± 0.35	6.86 ± 1.0[a]
Glucose 11.1	6	9.10 ± 1.23[b]	16.40 ± 2.53[a,b]	19.90 ± 3.33[a,b]
Glucose 16.7	6	11.12 ± 1.33[b]	18.76 ± 2.43[a,b]	23.87 ± 3.23[a,b]
Theophylline 5.5	5	3.88 ± 0.78	4.82 ± 1.05	6.82 ± 2.28[c]
Glucose 5.5 + theophylline 5.5	5	5.20 ± 1.56[d]	8.20 ± 2.42	13.80 ± 3.56[d,e]
Glucose 11.1 + theophylline 5.5	6	10.26 ± 1.06[e]	23.48 ± 2.89[d,e]	35.41 ± 4.79[d,e]
Glucose 16.7 + theophylline 5.5	6	11.44 ± 1.04[e]	22.81 ± 3.11[e]	38.86 ± 3.13[d,e]

a, $P < 0.05$, significantly increased compared with 8 hours incubation in the presence of the same concentration of glucose.

b, $P < 0.05$, significantly increased compared with 5.5 mmol/l glucose after the same incubation time.

c, $P < 0.05$, significantly increased compared with insulin release in response to 5.5 mmol/l theophylline after 8 hours incubation.

d, $P < 0.05$, significantly increased compared with the corresponding glucose concentration alone after either 8, 16 or 24 hours incubation.

e, $P<0.05$, significantly increased compared with 5.5 mmol/l theophylline alone after either 8, 16 or 24 hours incubation.

Insulin release from Ba^{2+}-alginate microencapsulated islets coated with polyamino acids PLL and PLO

Ba^{2+}-alginate microencapsulated islets were coated with the polyamino acids PLL and PLO in an attempt to increase their structural strength and the effect this manoeuvre would have on the insulin response to glucose challenge investigated. The rate of insulin release from Ba^{2+}-alginate microcapsules coated with either PLL or PLO, Table 11, was not significantly reduced after 8 hours exposure to high glucose (16.7 mmol/l) compared with the response from uncoated Ba^{2+}-alginate microencapsulated islets, Table 10. Therefore, coating Ba^{2+}-alginate microencapsulated islets with polyamino acid membranes has no significant effect on their insulin responsiveness to glucose challenge.

Table 11 The effect of elevated glucose on the cumulative release of insulin from Ba^{2+}-alginate microencapsulated islets coated with either PLL or PLO after 8 hours static incubation (mean values ± SEM, n=6).

Microcapsule fabrication	Insulin release (ng/ml/10 microencapsulated islets/8 hours)		
	n	Glucose 5.5 mmol/l	Glucose 16.7 mmol/l
Ba^{2+}-alginate-PLL	6	3.4 ± 0.70	8.2 ± 1.51[a]
Ba^{2+}-alginate-PLO	6	4.4 ± 1.10	9.7 ± 2.20[a]
Ba^{2+}-alginate (no polyamino acid coating)		3.7 ± 0.06 (n=5)	11.12 ± 1.33 (n=6)

a, $P<0.05$, significantly increased compared with 5.5mmol/l glucose.

Insulin release from Ba^{2+}-alginate coated islets prepared using the density gradient centrifugation method (method 2, page 45)

In order to reduce the diffusion distance across the microcapsule wall, Ba^{2+}-alginate membrane coated islets were generated by density gradient centrifugation method. The encapsulation efficiency of this method was rather poor, only 22.5 ± 1.5% (n=6) of the islets were microencapsulated. The majority of the islets were lost when they became embedded in the gel matrix formed at the bottom of the test tube. Microencapsulated islets generated by the density gradient centrifugation method were essentially membrane coated and markedly decreased in diameter compared with those produced by method 1, Plate 10a and b. Histological examination using haematoxylin-eosin staining, showed a distinct, continuous, closely applied coating of Ba^{2+}-alginate around the islets, Plate 10b. Some of the microencapsulated islets had a small alginate tail attached, Plate 11.

The insulin response of Ba^{2+}-alginate coated islets prepared using method 2, to low (5.5 mmol/l) and high (16.7 mmol/l) glucose after 1, 2 and 8 hours static incubation is shown in Table 12. The absolute amounts of insulin released from coated islets in response to glucose after 1, 2 and 8 hours incubation increased significantly compared with the release from microencapsulated islets generated by method 1, Tables 9 and 10. Even after 1 hour, the rate of insulin release from coated islets was considerably higher than that released from conventionally microencapsulated islets after 8 hours incubation. Indeed, the absolute amount of insulin released in response to both 5.5 and 16.7 mmol/l glucose was significantly enhanced by some 7x and 5x respectively after 8 hours incubation, compared with release from Ba^{2+}-alginate microencapsulated islets generated by method 1, Table 10. The insulin response of membrane coated islets to elevated glucose, Table 12, was not significantly different from the insulin response of free non-microencapsulated islets to glucose, Table 2.

Plate 10 a Ba^{2+}-alginate coated microencapsulated islet prepared by method 2 (x200).

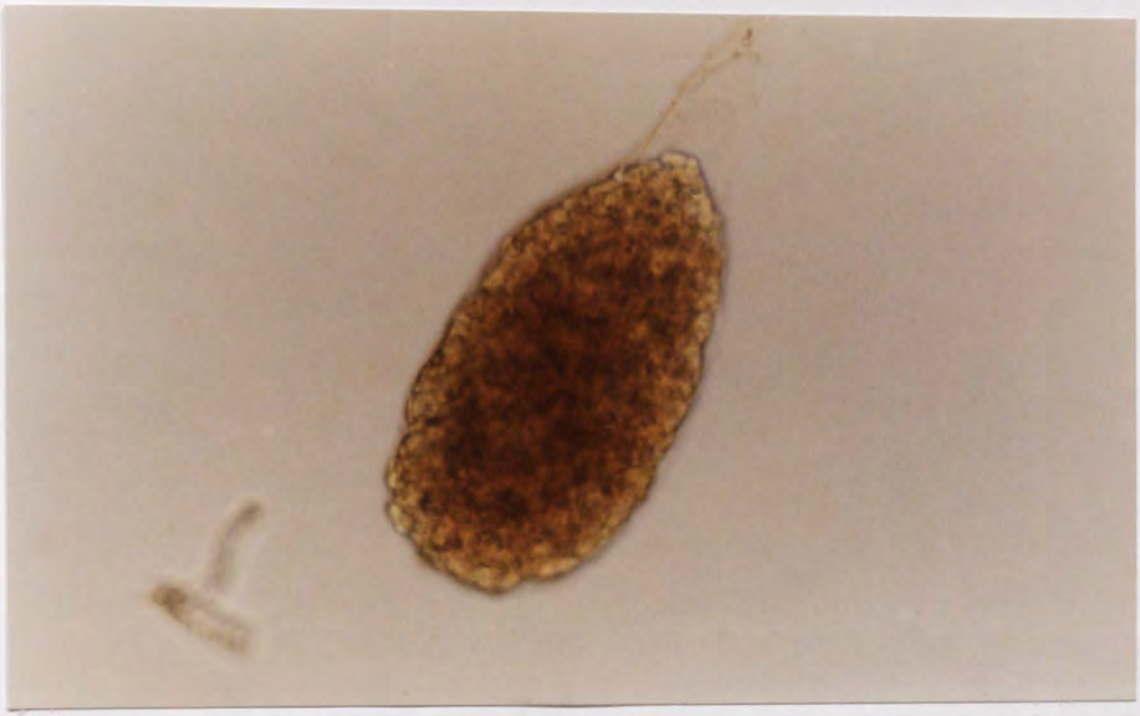


Plate 10 b Histological section of 'a' showing uniform continuous Ba^{2+}-alginate coating (H & E, X400).

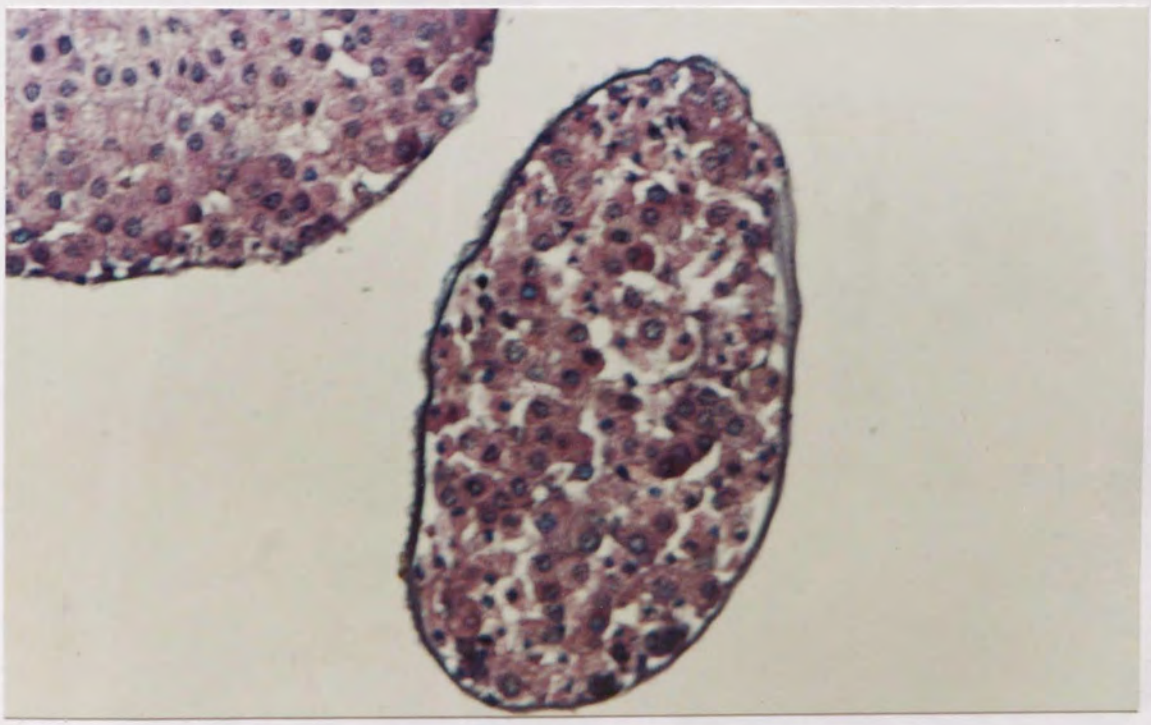


Plate 11

Ba^{2+}-alginate coated microencapsulated islets generated by method 2, showing a small alginate tail (H & E, X125).

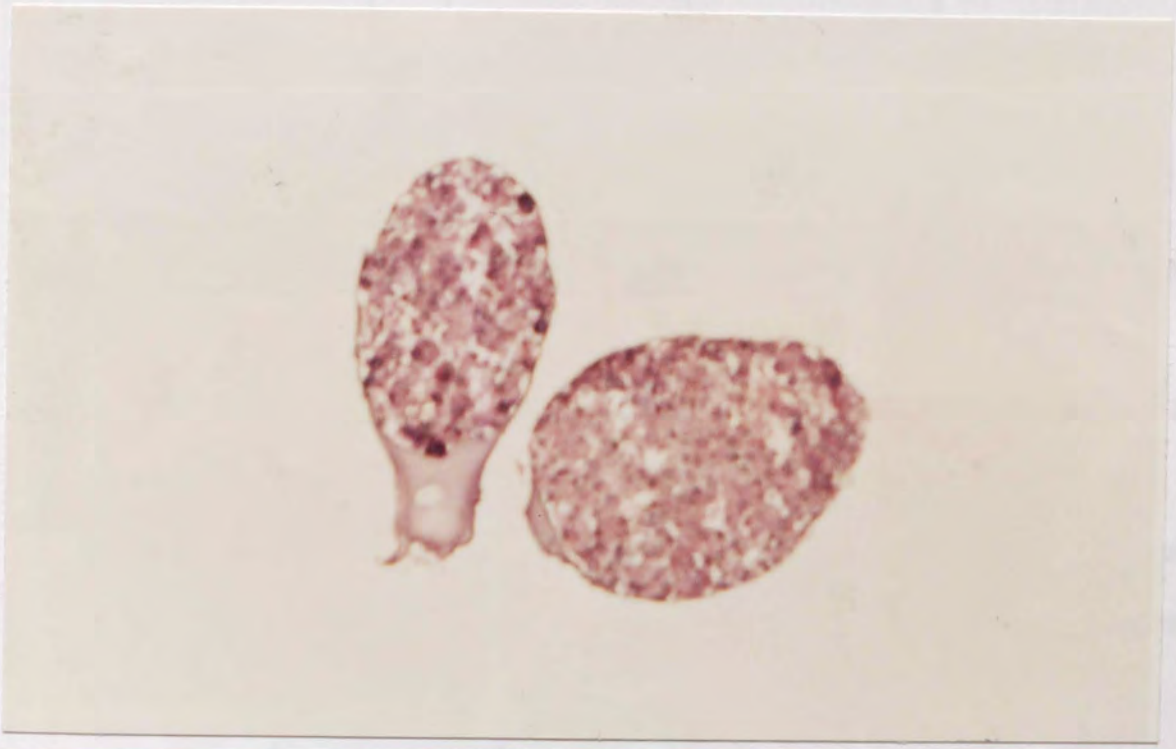


Table 12

The effect of elevated glucose on insulin release from Ba^{2+}-alginate microencapsulated islets (method 2) after 1,2 and 8 hours static incubation (mean values ± SEM, n=5).

Treatment (mmol/l)	Insulin release (ng/ml/10 microencapsulated islets)			
	n	1 hour	2 hours	8 hours
Glucose 5.5	5	13.3 ± 2.09	18.0 ± 1.50[c]	26.90 ± 2.19[b,d]
Glucose 16.7	5	23.3 ± 1.28[a]	34.0 ± 2.90[a,b,c]	55.40 ± 4.36[a,b,d]
Glucose 5.5 (method1)	5	-	0.54 ± 0.20	3.70 ± 0.06
Glucose 16.7 (method 1)	6	-	2.10 ± 0.56	11.12 ± 1.33

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose after the same incubation time.

b, $P<0.05$, significantly increased compared with insulin release after 1 hour at the corresponding glucose concentration.

c, $P<0.05$, significantly increased compared with insulin release from Ba^{2+}-alginate microencapsulated islets (method 1) after 2 hours incubation at the corresponding glucose concentration, Table 9.

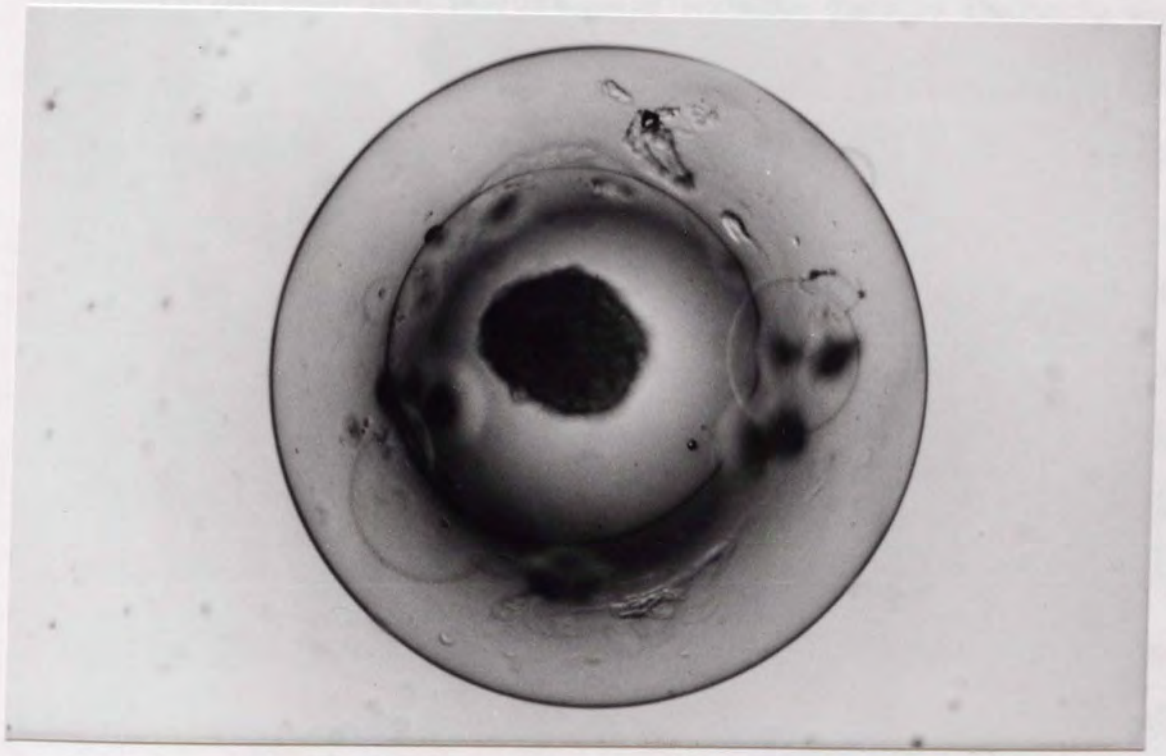
d, $P<0.05$, significantly increased compared with Ba^{2+}-alginate microencapsulated islets (method 1) after 8 hours incubation at the corresponding glucose concentration, Table 10.

Insulin release from agarose microencapsulated islets

The encapsulation efficiency of islets using agarose was only 27 ± 4.5% (n=6) and much lower than that seen with either Ba^{2+}-alginate (method 1, 54 ± 2.5%) or Na^{+}-alginate microencapsulated islets (61.8 ± 3.3%). Most of the islets remained non-encapsulated, but those that were encapsulated had a mean diameter of 0.59 ± 0.05 mm (n=10) and were smooth and spherical in shape, with a capsule within a capsule appearance, Plate 12.

Plate 12

Agarose microencapsulated mouse islet (X50).



As observed with Na^+-alginate and Ba^{2+}-alginate (method 1) microencapsulated islets, the cumulative amount of insulin released from agarose microencapsulated islets was significantly lower than that from free non-microencapsulated islets, Table 2, at all glucose concentrations, Table 13. Also, these microencapsulated islets showed a significantly reduced cumulative release of insulin in response to 11.1 and 16.7 mmol/l glucose after 2 hours incubation, compared with Na^+-alginate/PLL/Na^+-alginate microencapsulated islets, Table 3. The effect of increasing concentrations of glucose, theophylline and theophylline in the presence of glucose, on the cumulative release of insulin from agarose microencapsulated islets after extended static incubation has been summarised in Table 14. The cumulative release of insulin from agarose microencapsulated islets in response to increasing concentrations of glucose was significantly reduced after 8 hours incubation compared with Ba^{2+}-alginate (method 1) microencapsulated islets (Table 10) but had recovered to equivalent levels after 24 hours of incubation, Table 14. The insulin response of agarose microencapsulated islets to 5.5 and 16.7 mmol/l glucose was reduced by some 54% and 42% respectively, compared with Ba^{2+}-alginate (method 1) microencapsulated islets exposed to the same concentrations of glucose. In addition, after 8 hours, the rate of insulin release from agarose microencapsulated islets in response to 5.5 and 16.7 mmol/l glucose had fallen by some 26% and 30% respectively, compared with Na^+-alginate/PLL/Na^+-alginate microencapsulated islets exposed to the same concentrations of glucose, Table 4. The rather sluggish insulin response of agarose microencapsulated islets may well be the result of different diffusional properties of agarose gel compared with Na^+-alginate gel. Theophylline potentiated the effect of all concentrations of glucose on the cumulative release of insulin from agarose microencapsulated islets irrespective of the incubation time, Table 14.

Table 13

The effect of elevated glucose on insulin release from short term (2 hours) statically incubated islets of Langerhans microencapsulated in agarose (mean values ± SEM, n=6).

Treatment (mmol / l)	n	Insulin release (ng / ml / 10 microencapsulated islets / 2 hours)
Glucose 5.5	6	0.63 ± 0.23^{c}
Glucose 11.1	6	$1.11 \pm 0.19^{b,c}$
Glucose 16.7	6	$1.60 \pm 0.17^{a,b,c}$
Glucose 5.5 (Na^+-alginate/PLL/ Na^+-alginate)	6	0.36 ± 0.15
Glucose 11.1 (Na^+-alginate/PLL/ Na^+-alginate)	6	2.69 ± 0.33
Glucose 16.7 (Na^+-alginate/PLL/ Na^+-alginate)		3.54 ± 0.85

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose.

b, $P<0.05$, significantly reduced compared with Na^+-alginate/PLL/Na^+-alginate microencapsulated islets in the presence of the corresponding glucose concentration, Table 3.

c, $P<0.05$, significantly reduced compared with free non-microencapsulated islets in the presence of the corresponding glucose concentration, Table 2 (page 68).

Table 14 The effect of increasing concentrations of glucose, theophylline and theophylline in the presence of glucose on the cumulative release of insulin from agarose microencapsulated islets after extended static incubation(mean values ± SEM).

Treatment (mmol/l)	Cumulative insulin release (ng/ml/ 10 microencapsulated islets			
	n	8 hours	16 hours	24 hours
Glucose 5.5	5	1.7 0 ± 0.50	3.30 ± 0.73	4.90 ± 1.06[a]
Glucose 11.1	6	6.61 ± 1.56[b]	12.91 ± 2.73[a,b]	17.23 ± 4.14[a,b]
Glucose 16.7	6	6.43 ± 1.23[b]	14.63 ± 3.25[a,b]	20.43 ± 4.49[a,b]
Theophylline 5.5	5	2.25 ± 0.98	5.15 ± 1.43	6.37 ± 1.56[c]
Glucose 5.5 + theophylline 5.5	5	2.31 ± 0.51[d]	3.57 ± 0.96	5.99 ± 1.46
Glucose 11.1 + theophylline 5.5	6	12.04 ± 0.66[d,e]	20.80 ± 1.73[d,e]	30.50 ± 4.20[d,e]
Glucose 16.7 + theophylline 5.5	6	18.37 ± 2.05[d,e]	33.26 ± 3.81[d,e]	46.00 ± 6.50[d,e]

a, $P < 0.05$, significantly increased compared with insulin released after 8 hours incubation in the presence of the same concentration of glucose.

b, $P < 0.05$, significantly increased compared with insulin response to 5.5 mmol/l glucose after the same period of incubation.

c, $P < 0.05$, significantly increased compared with insulin release in response to 5.5 mmol/l theophylline after 8 hours incubation.

d, $P < 0.05$, significantly increased compared with the corresponding glucose concentration alone after either 8, 16 or 24 hours incubation.

e, $P<0.05$, significantly increased compared with 5.5 mmol/l theophylline alone after either 8, 16 or 24 hours incubation.

Glucose induced insulin release from perifused islets of Langerhans of NMRI mice

The dynamic release of insulin in response to glucose was monitored using a perifusion system. Either free non-microencapsulated or microencapsulated islets were pre-perifused for 30 minutes with oxygenated RPMI-1640 supplemented with 5.5 mmol/l glucose, 10% new born calf serum and 2% penicillin/streptomycin at 37°C, and then test perifused for a further 60 minutes with RPMI-1640 containing 16.7 mmol/l glucose. 1ml fractions were collected throughout and the perifusion system had a dead volume of 1ml at a constant flow rate of 1ml/minute. The rate of insulin release was expressed as ng/ml/20 free or microencapsulated islets / 3 or 5 minutes.

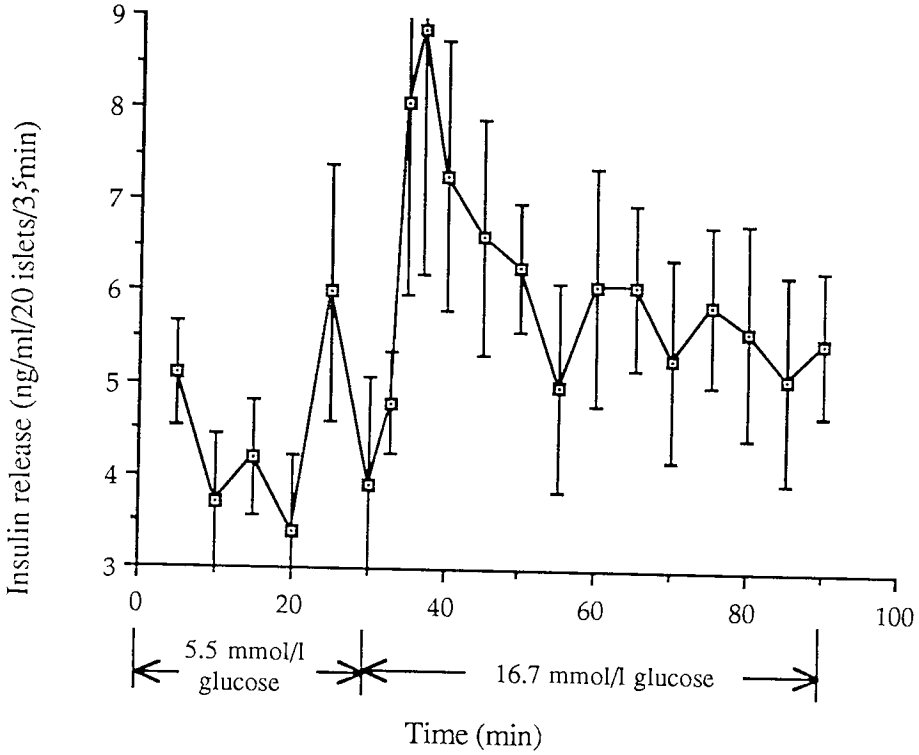
The rate of insulin release from perifused islets was increased when the glucose concentration was elevated from 5.5 to 16.7 mmol/l after 30 minutes pre-perifusion, Figure 6. This observation confirmed the viability of the perifused mouse islets and kinetic sensitivity of the perifusion system. A peak in insulin release was obtained 7 minutes after the exposure of the islets to 16.7 mmol/l glucose, Figure 6. A characteristic biphasic response was rarely obtained, and although the depressed second phase was somewhat higher than the insulin response to 5.5 mmol/l glucose observed in the pre-perifusion period, any differences were obscured by the high SEM's of the insulin secretory rate.

Glucose induced insulin release from perifused microencapsulated NMRI mouse islets

Increasing the glucose concentration from 5.5 to 16.7 mmol/l stimulated the rate of insulin release from islets microencapsulated with Na^+-alginate-PLL-Na^+-alginate, Figure 7, Na^+-alginate-PLO-Na^+-alginate, Figure 8, Ba^{2+}-alginate (method 1), Figure 9 and agarose, Figure 10. However, the peak insulin response to elevated glucose was marginally delayed

Figure 6

Glucose induced insulin release from perifused islets of Langerhans of NMRI mice (mean values ± SEM of 6 individual release profiles)



from Na^+-alginate-PLO-Na^+-alginate microencapsulated islets, Figure 8, but significantly delayed from Na^+-alginate-PLL-Na^+-alginate, Figure 7, Ba^{2+}-alginate, Figure 9 and agarose microencapsulated islets, Figure 10, compared with the insulin response shown by free non-microencapsulated islets, Figure 6. This delayed insulin response, which was most exaggerated with agarose microencapsulated islets, Figure 10, was presumably the result of a reduced diffusional exchange of glucose and insulin through the microcapsule membrane. Ba^{2+}-alginate and Na^+-alginate-PLL-Na^+-alginate microencapsulated islets generated the highest absolute release of insulin during perifusion in response to glucose challenge, Figures 9 and 7, whilst agarose microencapsulated islets produced the weakest insulin response, Figure 10.

Figure 7

Glucose induced insulin release from perifused Na^{+}-alginate-PLL-alginate micro-encapsulated NMRI mouse islets (mean values ± SEM of 5 individiual release prifiles)

* $p < 0.05$, significantly increased compared with 5.5 mmol/l glucose

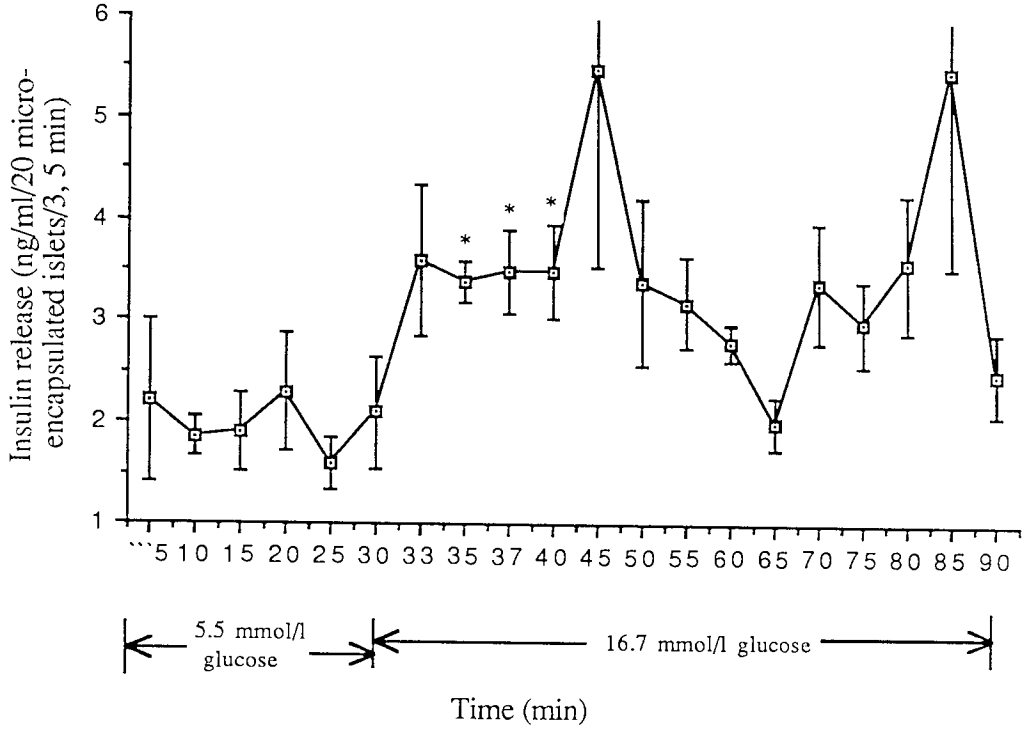
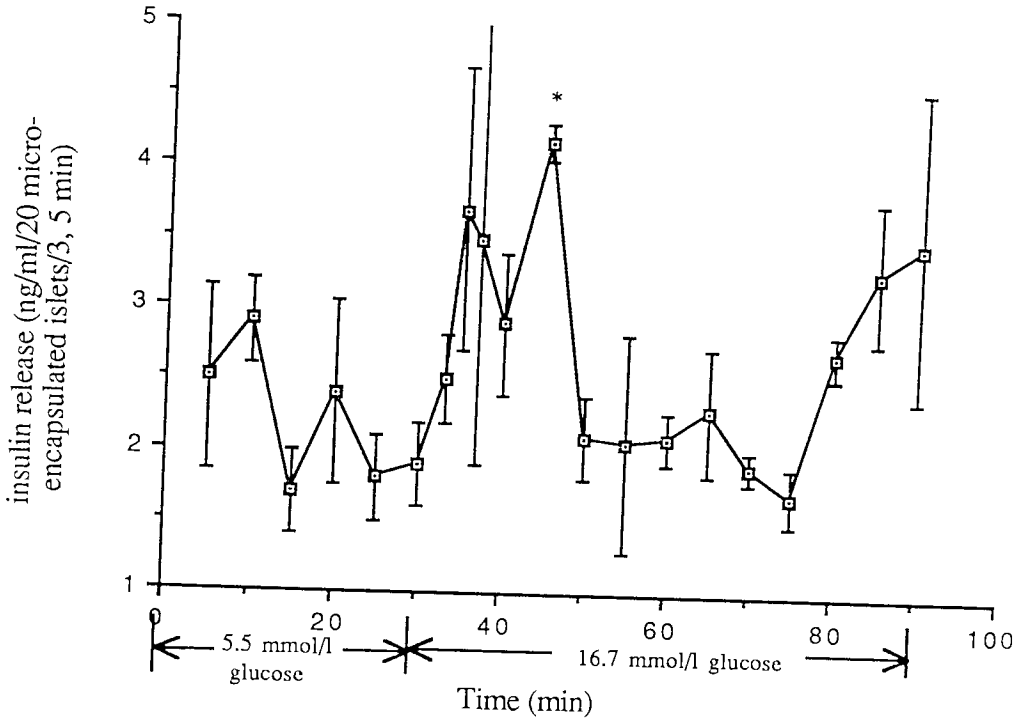


Figure 8

Glucose induced insulin release from perifused Na^{+}-alginate-PLO-alginate microencapsulated NMRI mouse islets (mean values ± SEM of 5 individual release profiles)



* $p < 0.05$, significantly increased compared with 5.5 mmol/l glucose

Figure 9

Glucose induced insulin release from perifused Ba^{2+}-alginate (method 1) micro-encapsulated mouse islets (mean values ±SEM of 5 individual release profiles)

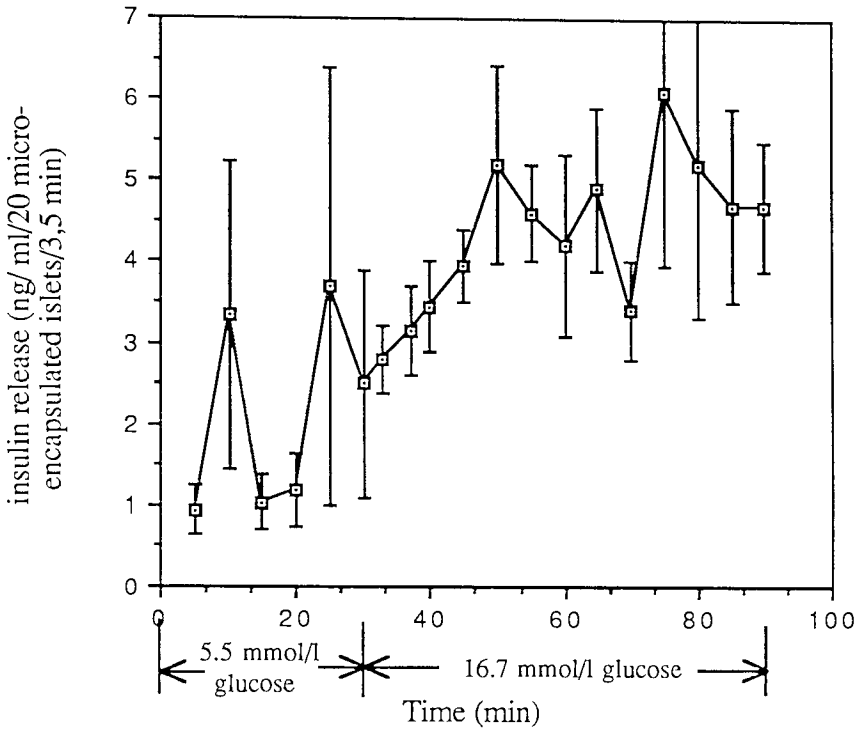
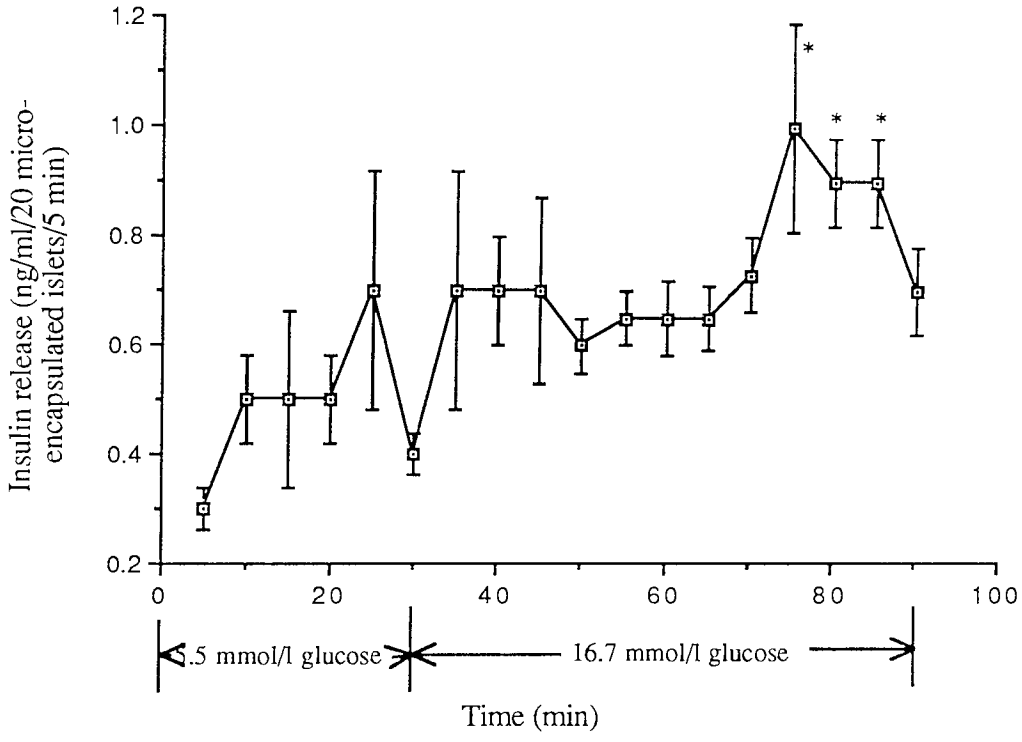


Figure 10

Glucose induced insulin release from perifused agarose microencapsulated MNRI mouse islets (mean values ± SEM of 5 individual release profiles)

* $p < 0.05$, significantly increased compared with 5.5 mmol/l glucose



DISCUSSION

Work presented so far, has confirmed the selection of NMRI mice as a source of isolated islet tissue. Their islets were easily isolated by collagenase digestion and approximately 200 clean islets could be harvested from the pancreata of two mice. Characterization of the donor mice showed that they had essentially normal fasting plasma glucose (5.8 ± 0.24 mmol/l, n=14) and insulin levels (0.8 ± 0.07 ng/ml, n=17).

Microencapsulated mouse islets were successfully fabricated using Na^+-alginate (LMT / DJB) with and without a polyamino acid coating and an outer alginate layer. Conventional microencapsulated islets were also generated using Ba^{2+}-alginate and agarose.

The viability of free non-microencapsulated and microencapsulated islets, both in static incubation and perifusion, was confirmed by their incremental insulin response to glucose challenge. In addition, theophylline potentiated the insulin response of both free and microencapsulated islets to glucose in static incubation. Theophylline is a methylxanthine, it inhibits the action of low Km cAMP phosphodiesterase and, therefore, increases the cAMP concentration in islet β-cells (234-236). It is well established, that cAMP potentiates glucose stimulated insulin release (234, 237-239). Glucose plays a dual role in pancreatic β-cells, acting both as a metabolic fuel and, at millimolar concentration, a physiological stimulus for insulin secretion and biosynthesis (239-242). Previous reports, from many workers, on the insulin responsiveness of microencapsulated islets to increased glucose, have revealed a spectrum of secretory efficacy. Some workers have observed prompt physiologically significant levels of insulin release (191, 211) while others have revealed a significant lack of sensitivity to glucose challenge and a markedly reduced release of insulin (163, 182, 243) resulting from the creation of a barrier to glucose / insulin diffusion. In the

present work, short term (2 hours) static incubation of Na^+-alginate, Ba^{2+}-alginate (method 1) and agarose microencapsulated islets, produced a very low level of insulin release in response to glucose challenge in both the absence and presence of theophylline. Clearly, the presence of the microcapsule around the islets presented a significant barrier to insulin release, when compared with the same response of free non-microencapsulated islets. A significantly enhanced cumulative release of insulin could only be obtained from microencapsulated islets when the static incubation period was extended to 24 hours over three, 8 hour periods. Little significant difference was observed between the insulin responses of Na^+-alginate-PLL-alginate, Ba^{2+}-alginate (method 1) and agarose microencapsulated islets to glucose challenge in either the presence or absence of theophylline after 24 hours incubation. In all cases, theophylline potentiated the islet response to glucose challenge. The insulin response of microencapsulated islets to elevated glucose after 8, 16 and 24 hours static incubation was considerably improved but never attained the cumulative rate of insulin release shown by free non-microencapsulated islets under similar conditions. These observations are consistent with the results of other groups, who have also shown that free islets secrete significantly more insulin in response to glucose challenge in static incubation than microencapsulated islets (163, 182, 205, 243). There is some evidence to suggest that the microencapsulation process per se can damage islet tissue. Levesque and associates observed a reduction in insulin secretion in response to glucose from previously microencapsulated free islets compared with fresh islets (210). Fritschy and colleagues have shown that CHES and sodium citrate, both materials routinely used in the microencapsulation process, significantly reduce insulin release from microencapsulated islets in response to glucose (244).

Work presented here, has also demonstrated that when Na^+-alginate microencapsulated islets were coated with poly-L-ornithine (PLO), they were more responsive to elevated

glucose and theophylline than Na^+-alginate microencapsulated islets coated with poly-L-lysine (PLL). This observation was supported by the enhanced rate of passive I^{125}-insulin diffusion from Na^+-alginate-PLO-alginate microcapsules, compared with that from Na^+-alginate microcapsules coated with PLL. The reason for this difference in insulin diffusion rate is not clear, but it is almost certainly related to differences in either porosity or some other physical properties of the PLO and PLL membranes. Indeed, Calafiore and colleagues have demonstrated, using electron microscopy and ultrastructural studies, that PLO membranes have a higher permeability than those formulated with PLL (231).

When Na^+-alginate-PLL and Na^+-alginate-PLO microencapsulated islets were fabricated without an outer 0.15% Na^+-alginate layer, they both showed a significantly greater insulin response to glucose than microencapsulated islets fabricated with an outer alginate layer. This indicated that the outer alginate layer had increased the diffusional path length for glucose / insulin exchange and was acting as a significant additional barrier to insulin release.

When the conventional Ba^{2+}-alginate microencapsulation method was replaced with a Ba^{2+}-alginate density gradient coating, method 2 (211), Ba^+-alginate coated islets showed a significantly reduced capsule thickness and hence, diffusion distance. This was reflected in their enhanced insulin response to glucose challenge compared with Ba^{2+}-alginate (method 1) microencapsulated islets. The big drawback of the density gradient Ba^{2+}-alginate method was that only 22.5 ± 1.5% (n=6) of the islets were microencapsulated. Most of the islets became embedded in an alginate matrix, which collected as a gel at the bottom of the gradient tube. The microencapsulation efficiency might be improved by modifying the densities of the layers employed and the speed and duration of the centrifugation. The insulin response of the Ba^{2+}-alginate membrane coated islets to elevated

glucose was significantly improved after 2 hours static incubation. In contrast, only very low levels of insulin release could be detected from conventional Ba^{2+}-alginate microencapsulated islets in response to glucose challenge after 2 hours incubation. Clearly, this observation suggested that the one factor which plays a critical role in the insulin response of microencapsulated islets to glucose challenge is the thickness of the microcapsule. In an in vitro study reported by Chicheportiche and Reach, the insulin secretory response to glucose of islets microencapsulated in thinner Na^{+}-alginate-PLL-alginate membranes was much faster than that of microencapsulated islets with thick capsule coatings (162). The smaller, thinner capsules showed an increased cell viability, because the microencapsulated islets had easier access to nutrients and oxygen and produced faster insulin response to fluctuations in glucose concentration because of the reduced capsule dead space. The cumulative insulin response of Ba^{2+}-alginate coated islets to elevated glucose only attained the levels observed with free non-microencapsulated islets after 8 hours incubation. Zekorn and colleagues have reported that the insulin response of Ba^{2+}-alginate coated islets to glucose challenge was not significantly different to that shown by free islets after 3 hours static incubation (211). The reason for the enhanced sensitivity of the Ba^{2+}-alginate coated islets fabricated by Zekorn and colleagues might be accounted for by differences in experimental design. The Zekorn study was based on a sequential design, in which the same microencapsulated islets were successively exposed to non-stimulatory and stimulatory concentrations of glucose for 1 and 2 hours respectively after a 4 day culture period. However, in the present study different groups of microencapsulated islets were used in experimental work involving non-stimulatory and stimulatory concentrations of glucose after an overnight culture period.

The rate of insulin release from perifused Na^{+}-alginate-PLL-alginate, Na^{+}-alginate-PLO-alginate, Ba^{2+}-alginate (method 1) and agarose microencapsulated islets was increased in

response to elevated glucose. However, a true biphasic insulin response could not be obtained with microencapsulated islets, and the peak in insulin release obtained in response to glucose challenge was always delayed compared with that shown with free non-microencapsulated islets. The flattened overall response profile was presumably the result of the time lag associated with the diffusion of insulin through the microcapsule membrane. The reduction in the second phase of insulin release from microencapsulated islets probably reflected the reduction in the metabolic capacity of the microencapsulated islets to synthesize new insulin. At best, the perifusion studies did serve to confirm the integrity and, albeit the reduced, viability of microencapsulated islets.

CHAPTER 4

THE EFFECT OF THE NITRIC OXIDE SYNTHASE INHIBITOR, N^G-MONOMETHYL L-ARGININE (L-NMMA) ON GLUCOSE STIMULATED INSULIN RELEASE FROM FREE NON-ENCAPSULATED AND MICROENCAPSULATED ISLETS IN VITRO

INTRODUCTION

The metabolism of L-arginine to nitric oxide has been shown to be important for the effector functions of many cell types, including macrophages (245). In the pancreatic islets, the amino acid L-arginine is metabolized to L-citrulline and nitric oxide via nitric oxide synthase, Figure 11, (246, 247).

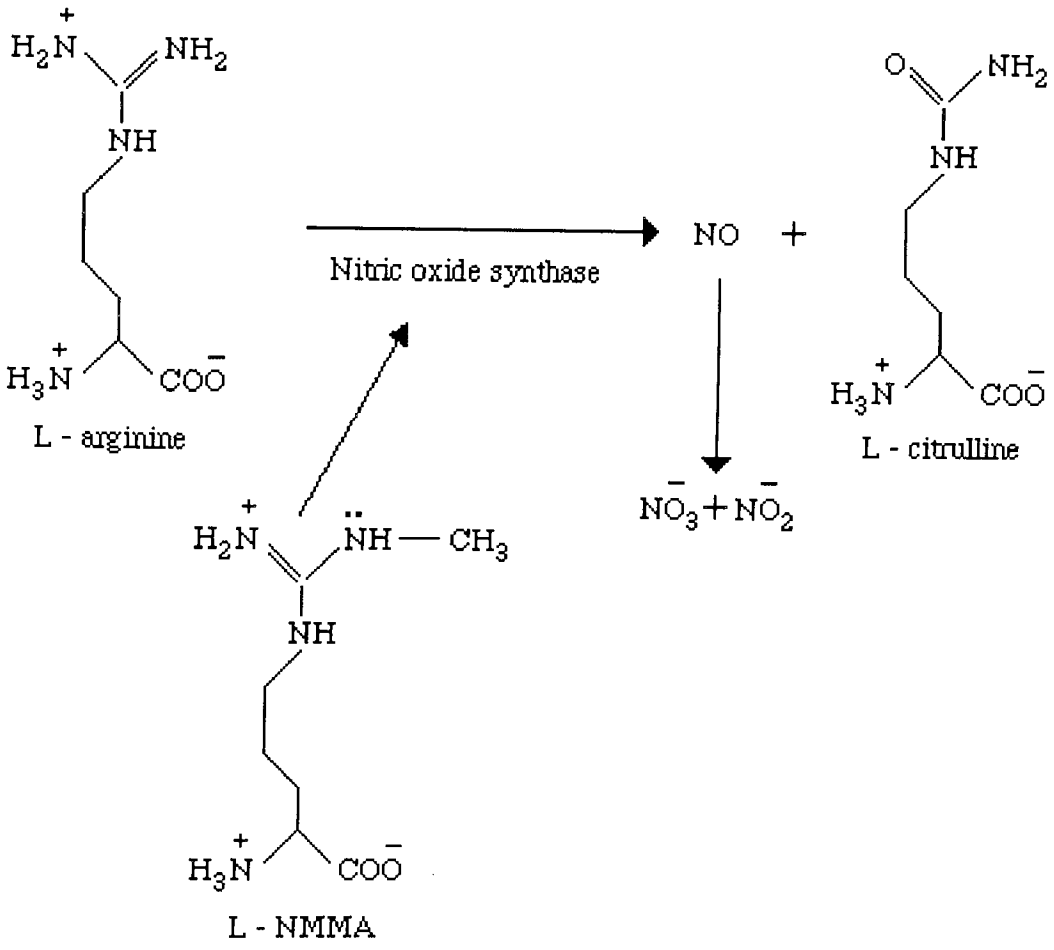


Figure 11 L-arginine-dependent nitric oxide pathway

The prevention of rat islet cell lysis by activated peritoneal macrophages in vitro (248), and the prevention of diabetes in streptozotocin diabetic mice in vivo (249) by the inhibition of nitric oxide production, suggest an important role for nitric oxide in the pathogenesis of insulin-dependent diabetes mellitus. It has been reported, that there are at least two distinct isoforms of nitric oxide synthase (EC, 1.14.23) (198, 250-252). One is constitutive, localized to the cytosol, and is Ca^{2+} and calmodulin-dependent (253). This type generates a low level of nitric oxide which functions as a cellular signaling molecule (254). The second isoform of the enzyme is inducible by cytokines and lipopolysaccharides, is Ca^{2+}-independent and was originally isolated from macrophages (248,255). This second isoform generates high levels of nitric oxide that is cytotoxic to pancreatic islets (248, 255). Both isoforms require NADPH and form citrulline as a co-product. Both isoforms of nitric oxide synthase have been found in rat islets (198, 213, 250-252) and the accumulation of nitrite in culture medium containing rat islets after stimulation with L-arginine has suggested the presence of islet nitric oxide synthase (255). Analogues of L-arginine, in which one of the guanidino nitrogen is either methylated or nitrosylated, have been shown to inhibit both isoforms of nitric oxide synthase (256). N^G-monomethyl L-arginine (L-NMMA) and N^G-nitro L-arginine methyl ester (NAME), have both been shown to block nitric oxide formation (256) by inhibiting both the inducible and constitutive isoforms of nitric oxide synthase. L-NMMA has been the most widely used inhibitor of the inducible form of nitric oxide synthase, especially in macrophages, Figure 12.

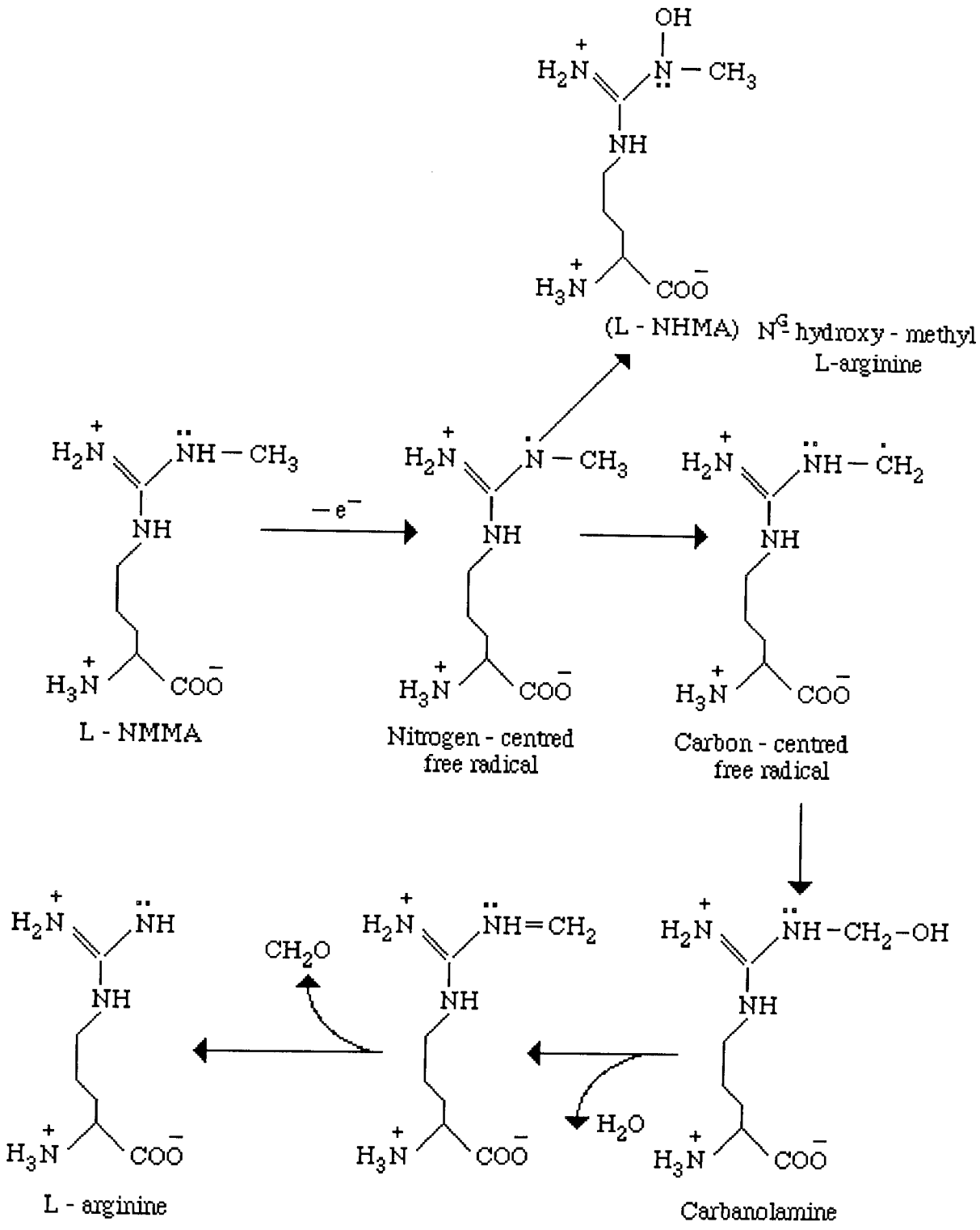


Figure 12 The mechanism for the inactivation of macrophage nitric oxide synthase by L-NMMA (257).

The nitric oxide synthase inhibitor, L-NMMA (0.1 - 1.0 mmol/l) has been shown to prevent the formation of nitric oxide and islet cell destruction mediated by activated peritoneal macrophages in vitro (248). In addition, while L-NMMA provided complete protection NAME only provided partial protection against the inhibitory effect of interleukin-1β on glucose stimulated insulin release from rat islets (251).

The destruction of β-cells is believed to be mediated by infiltrating T-lymphocytes. In BB rats model of autoimmune diabetes mellitus, macrophages have been demonstrated to be among the first inflammatory cells to infiltrate the islets (258). These macrophages secrete cytokines, including interleukin-1β and tumour necrosis factor (TNF). It has been reported, that macrophages rapidly and efficiently kill rat islet cells in vitro via an L-arginine dependent cytotoxic pathway (248). Interleukin-1β has been shown to induce the formation of nitric oxide by rat islets in vitro (198), and has been found to act synergistically with TNF to ultimately cause the functional impairment and lysis of β-cells (259-261). Pretreatment of isolated rat islets with interleukin-1β has been shown to produce a concentration and time dependent inhibition of glucose-stimulated insulin secretion, while prolonged exposure (2-6 days) of rat islets to IL-1β resulted in cell destruction (198, 199, 262). Nitric oxide, a free radical, has been implicated as the cellular effector molecule that mediates the inhibitory and cytotoxic effects of interleukin-1β on isolated rat islets (263). The nitric oxide synthase inhibitor, L-NMMA and NAME will both prevent the inhibition of glucose stimulated insulin release induced by the 18-24 hours pretreatment of isolated rat islets with IL-1β (198, 251, 252). In addition, these inhibitors have also been shown to prevent interleukin-1β-induced nitrite production, and the formation of electron paramagnetic resonance detectable iron-nitrosyl complexes by rat islets (198). The formation of these iron-dinitrosyl complexes has also been described in activated macrophages (264) and they are believed to be the result of the nitric oxide-mediated destruction of iron-sulphur centres resulting in the

loss of iron-containing enzyme activity (265). Furthermore, cycloheximide, an inhibitor of protein synthesis, has been shown to prevent both the IL-1β induced inhibition of glucose stimulated insulin release (198), and the formation of NO by islets (198). These studies provide evidence to suggest that IL-1β induces the expression of both isoforms of nitric oxide synthase by islets, and that nitric oxide may be the cellular effector molecule that mediates the IL-1β-induced impairment of islets function. Islets have been shown to be completely protected from macrophages cytotoxcity in vitro by the presence of 0.5mmol/l L-NMMA (200, 201, 248, 249).

Many groups (187, 189, 190, 266, 267) have demonstrated that previously transplanted Na^+-alginate/PLL/Na^+-alginate microencapsulated islets retrieved from the implantation site had an intense cellular overgrowth associated with the presence of macrophages and fibroblasts. The presence of a fibrotic reaction around the microencapsulated islets was potentially harmful to normal islet function, not only because it could limit the diffusion of glucose, insulin and other nutrients but also because the inflammatory cells present could secrete diffusible substances such as interleukin-1β or nitric oxide. These factors could be responsible for islet destruction and graft failure (189). It was against this background, that in vitro work was carried out to determine the effect of the nitric oxide synthase inhibitor, L-NMMA at concentrations of 0.1, 0.5 and 1.0 mmol/l on glucose-stimulated insulin release, in the absence and presence of 0.2 mmol/l L-arginine, from free non-microencapsulated and Na^+-alginate-PLL-Na^+-alginate microencapsulated islets. These concentrations of L-NMMA have previously been shown to prevent both the formation of nitric oxide and islet cell destruction mediated by peritoneal macrophages in vitro (248). The effect of incorporating L-NMMA into the Na^+-alginate microcapsules surrounding the islets was also investigated on glucose stimulated insulin release from microencapsulated islets. Providing, L-NMMA incorporation into the Na^+-alginate microcapsules did not show any adverse

effects on the insulin secretory response, then L-NMMA loaded microencapsulated islets would subsequently be used in in vivo transplantation experiments. Such a manoeuver might encourage the survival of transplanted microencapsulated islets by inhibiting the local production of nitric oxide by peritoneal macrophages.

MATERIALS AND METHODS

Isolation of NMRI mouse islets

Islets were isolated from 12-15 week old NMRI mice using collagenase digestion as described previously on page 39.

Islet microencapsulation

Isolated islets were microencapsulated with Na^+-alginate-PLL-Na^+-alginate, according to the method described on page 41.

L-NMMA incorporation during the fabrication of Na^+-alginate microencapsulated islets

Isolated islets were suspended in 1ml of 1.5%(w/v) Na^+-alginate (LMT) solution containing either 0.1 or 0.5 mmol/l L-NMMA, in a 1ml disposable siliconised syringe fitted with a 26G needle and a 2cm length of pp10 poly-ethylene canula. This mixture was delivered into 1.1% $CaCl_2$ gelling solution. The rest of the microencapsulation process was carried out as described on page 41.

The effect of L-NMMA on glucose and arginine stimulated insulin release in vitro from free and microencapsulated islets

In order to determine the effect of different concentrations of L-NMMA on insulin release, free non-microencapsulated and microencapsulated islets were cultured overnight in RPMI-1640 culture medium supplemented with 5.5mmol/l glucose, 10% heat inactivated new born

calf serum and 2% penicillin/streptomycin in a humidified atmosphere of 5%CO_2 / 95%O_2 at 37°C prior to use in secretory experiments as previously described (page 48). Batches of 10 free non-microencapsulated islets were pre-incubated in 1ml of RPMI-1640 supplemented with 10% new born calf serum, 5.5mmol/l glucose and 2% penicillin/streptomycin at 37°C for 30 minutes. After this time, the free islets were incubated for a further 60 minutes in test medium containing 0.1, 0.5 and 1.0 mmol/l L-NMMA alone, or in the presence of 5.5 or 16.7 mmol/l glucose, with and without 0.2mmol/l L-arginine. Microencapsulated islets were treated in a similar fashion except they were test incubated for a further 8, 16 and 24 hours.

Experiments were also carried out with microencapsulated islets loaded with 0.1 and 0.5 mmol/l L-NMMA in the microcapsules (1.0 mmol/l L-NMMA had been shown to have a deleterious effect on insulin release from islets in vitro, Table 15). Microencapsulated islets, so prepared, were pre-incubated in the normal way, and insulin release in response to 5.5 and 16.7 mmol/l glucose ± 0.2 mmol/l L-arginine monitored after 8, 16 and 24 hours static incubation. 500µl aliquot of the test medium were collected and stored at -20°C until required for insulin assay.

RESULTS

The effect of increasing concentrations of L-NMMA on glucose stimulated insulin release

The effect of increasing concentrations of L-NMMA on glucose-stimulated insulin release from free non-microencapsulated islets after 1 hour static incubation has been summarised in Table 15.

L-NMMA at concentrations of 0.1 and 0.5 mmol/l had no significant effect on the rate of insulin release obtained from free islets in response to either 5.5 or 16.7 mmol/l glucose. However, 1.0 mmol/l L-NMMA significantly inhibited the insulin response to both 5.5 and 16.7 mmol/l glucose, Table 15, although the inhibition by 1.0 mmol/l L-NMMA was less marked in the presence of 5.5 mmol/l glucose than in the presence of 16.7 mmol/l glucose.

The effect of L-NMMA on glucose - stimulated insulin release in the presence of 0.2 mmol/l L-arginine

Since L-arginine serves as a substrate for nitric oxide synthase activity, the effect of L-NMMA on glucose-stimulated insulin release from free non-microencapsulated islets has been evaluated in the presence of 0.2 mmol/l L-arginine. L-arginine is a potent insulinotropic agent, whose actions synergize with glucose to augment insulin release in isolated islets (268, 269), however insulin release in response to L-arginine has been shown to be concentration dependent (250).

The effect of L-NMMA on glucose stimulated insulin release in the presence of 0.2 mmol/l L-arginine from free non-microencapsulated islets is shown in Table 16.

Table 15

The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) alone or in combination with glucose on insulin release from short term (1hr) statically incubated free non-microencapsulated islets (mean values ± SEM) (concentrations are mmol/l).

n=the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	n	Insulin release (ng / ml / 10 islets / hr)
5.5 glucose alone	6	27.8 ± 3.34
16.7 glucose alone	6	67.5 ± 6.80
0.1 L-NMMA alone	6	16.08 ± 2.61
0.5 L-NMMA alone	6	19.0 ± 1.75
1.0 L-NMMA alone	6	21.6 ± 1.77
0.1 L-NMMA + 5.5 glucose	6	29.9 ± 2.57
0.1 L-NMMA + 16.7 glucose	6	53.2 ± 4.53
0.5 L-NMMA + 5.5 glucose	6	23.0 ± 2.48
0.5 L-NMMA + 16.7 glucose	6	51.6 ± 5.38
1.0 L-NMMA + 5.5 glucose	6	18.5 ± 2.28[a,b]
1.0 L-NMMA + 16.7 glucose	6	26.3 ± 2.16[a,b,c]

a, $P<0.05$, significantly decreased compared with the corresponding glucose concentration alone.

b, $P<0.05$, significantly decreased compared with the effect of 0.1 mmol/l L-NMMA in the presence of the corresponding glucose concentration.

c, $P<0.05$, significantly decreased compared with the effect of 0.5 mmol/l L-NMMA in the presence of the corresponding glucose concentration.

Table 16 The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) in combination with glucose and 0.2 mmol/l L-arginine on insulin release from short term (1hr) statically incubated free non-microencapsulated islets (mean values ± SEM) (concentrations are mmol/l).

n=the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	n	Insulin release (ng / ml / 10 islets / hr)
5.5 glucose alone	6	27.8 ± 3.34
16.7 glucose alone	6	67.5 ± 6.8
0.2 L-arginine alone	6	21.1 ± 1.42
0.2 arginine + 5.5 glucose	6	30.9 ± 2.03
0.2 arginine + 16.7 glucose	6	63.1 ± 6.73
0.1 L-NMMA + 0.2 arginine + 5.5 glucose	6	33.4 ± 3.06
0.1 L-NMMA + 0.2 arginine + 16.7 glucose	6	58.2 ± 6.0
0.5 L-NMMA + 0.2 arginine + 5.5 glucose	6	42.6 ± 4.24[a]
0.5 L-NMMA + 0.2 arginine + 16.7 glucose	6	69.5 ± 7.06
1.0 L-NMMA + 0.2 arginine + 5.5 glucose	6	24.6 ± 2.89
1.0 L-NMMA + 0.2 arginine + 16.7 glucose	6 6	32.5 ± 3.10[b]

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose + 0.2 mmol/l L-arginine alone.

b, $P<0.05$, significantly reduced compared with 16.7 mmol/l glucose + 0.2 mmol/l L-arginine alone.

The addition of 0.2 mmol/l L-arginine alone had no significant effect on glucose stimulated insulin release. 0.5 mmol/l L-NMMA significantly potentiated insulin release from free islets stimulated by 5.5 mmol/l glucose plus 0.2 mmol/l L-arginine, Table 16. However, 0.5 mmol/l L-NMMA did not significantly influence the insulin secretory response to 16.7 mmol/l glucose in the presence of L-arginine, Table 16. Only at a concentration of 1.0 mmol/l did L-NMMA significantly inhibit (48.5%) insulin secretion stimulated by 16.7 mmol/l glucose + 0.2 mmol/l L-arginine. The release of insulin produced by 0.2 mmol/l L-arginine alone was approximately equivalent to that generated by 5.5 mmol/l glucose, Table 16.

The effect of increasing concentrations of L-NMMA on glucose - stimulated insulin release from long term (8 - 24 hours) statically incubated Na^+-alginate / PLL / Na^+-alginate microencapsulated islets

The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) alone or in combination with glucose on insulin release from long term (8-24 hours) statically incubated microencapsulated islets has been summarised in Table 17.

0.1 and 0.5 mmol/l L-NMMA had no significant effect on either 5.5 or 16.7 mmol/l glucose-stimulated insulin release from microencapsulated islets even after 24 hours incubation, Table 17. As observed with free non-microencapsulated islets, Table 15, incubation of microencapsulated islets for 8, 16 and 24 hours in the presence of 1.0 mmol/l L-NMMA resulted in a significant inhibition of 16.7 mmol/l glucose-stimulated insulin release by some 54%, 50.3% and 33.5% respectively, compared with the effect of 16.7 mmol/l glucose alone, Table 17. The release of insulin in response to 0.1, 0.5 and 1.0 mmol/l L-NMMA alone was not significantly different from that obtained in response to 5.5

mmol/l glucose alone after 8, 16 and 24 hours incubation, Table 17.

Table 17

The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) alone or in combination with glucose on insulin release from long term (8-24 hours) statically incubated microencapsulated islets (mean values ± SEM) (concentrations are mmol/l).

n=the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	Insulin release (ng / ml / 10 microencapsulated islets)		
	8 hrs	16 hrs	24 hrs
5.5 glucose alone	2.3 ± 0.41	3.4 ± 0.27	6.3 ± 0.7
16.7 glucose alone	9.2 ± 0.89	16.7 ± 0.91	21.2 ± 0.71
0.1 L-NMMA alone	2.2 ± 0.69	3.6 ± 1.0	5.3 ± 1.24
0.5 L-NMMA alone	2.3 ± 0.61	3.7 ± 0.85	4.8 ± 1.07
1.0 L-NMMA alone	2.3 ± 0.36	3.5 ± 0.63	4.8 ± 0.88
0.1 L-NMMA + 5.5 glucose	3.1 ± 0.73	6.54 ± 1.38	8.01 ± 1.95
0.1 L-NMMA +16.7 glucose	10.3 ± 0.93[b]	19.0 ± 2.29[b]	26.1 ± 3.22[b]
0.5 L-NMMA + 5.5 glucose	3.2 ± 0.48	5.3 ± 0.77	8.0 ± 1.06
0.5 L-NMMA +16.7 glucose	8.4 ± 0.67[b]	14.5 ± 1.70[b]	20.2 ± 2.27[b]
1.0 L-NMMA + 5.5 glucose	2.6 ± 0.47	5.0 ± 2.04	7.4 ± 1.27
1.0 L-NMMA + 16.7 glucose	4.2 ± 1.7[a]	8.3 ± 1.15[a,b]	14.1 ± 1.5[a,b]

a, $P<0.05$, significantly decreased compared with the corresponding glucose concentration and incubation time in the absence of L-NMMA.

b, $P<0.05$, significantly increased compared with the corresponding concentration of L-NMMA alone and after the corresponding incubation time.

The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) in combination with glucose and 0.2 mmol/l L-arginine on insulin release from long term (8-24 hours) statically incubated microencapsulated islets.

The effect of increasing concentrations of L-NMMA on insulin release from Na^+-alginate / PLL / Na^+-alginate microencapsulated islets in the presence of glucose and L-arginine has been summarised in Table 18.

0.1 and 0.5 mmol/l L-NMMA did not significantly influence insulin release in response to either 5.5 or 16.7 mmol/l glucose in the presence of 0.2 mmol/l L-arginine. However, 1.0 mmol/l L-NMMA significantly decreased insulin release in response to 5.5 mmol/l glucose plus L-arginine by some 51.2% and 36% after 8 and 16 hours incubation, respectively. While the same concentration of L-NMMA significantly decreased insulin release from microencapsulated islets in response to 16.7 mmol/l glucose plus L-arginine by some 44%, 49% and 41.5% after 8, 16 and 24 hours incubation, respectively, Table 18. On this basis only 0.1 and 0.5 mmol/l concentrations of L-NMMA were used in subsequent work designed to investigate the effect of incorporating L-NMMA into the microencapsulating Na^+-alginate around microencapsulated islets on their insulin response to glucose plus L-arginine.

Table 18

The effect of L-NMMA (0.1, 0.5 and 1.0 mmol/l) in combination with glucose and 0.2 mmol/l L-arginine on insulin release from long term (8 - 24 hours) statically incubated microencapsulated islets (mean values ± SEM) concentrations in mmol/l) n = the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	Insulin release (ng / ml / 10 microencapsulated islets)		
	8 hrs	16 hrs	24 hrs
5.5 glucose alone (n=5)	2.3 ± 0.41	3.4 ± 0.27	6.3 ± 0.70
16.7 glucose alone	9.2 ± 0.89	16.7 ± 0.91	21.2 ± 0.71
0.2 arginine alone	2.7 ± 0.25	4.5 ± 0.65	6.5 ± 0.8
0.2 arginine + 5.5 glucose	4.3 ± 0.42[a]	7.8 ± 1.03[a]	9.1 ± 1.47
0.2 arginine +16.7 glucose	11.8 ± 1.06	20.1 ± 1.9	24.3 ± 3.3
0.1 L-NMMA + 5.5 glucose + 0.2 arginine	3.7 ± 0.36	6.8 ± 0.68	10.1 ± 1.12
0.1 L-NMMA + 16.7 glucose + 0.2 arginine	9.4 ± 0.78	15.6 ± 1.85	19.7 ± 2.54
0.5 L-NMMA + 5.5 glucose + 0.2 arginine	4.0 ± 0.44	7.8 ± 1.09	12.0 ± 1.82
0.5 L-NMMA + 16.7 glucose + 0.2 arginine	11.4 ± 1.34	18.6 ± 2.27	26.3 ± 2.67
1.0 L-NMMA + 5.5 glucose + 0.2 arginine	2.1 ± 0.2[b,c]	5.0 ± 0.73[b]	7.1 ± 1.05
1.0 L-NMMA +16.7 glucose + 0.2 arginine	6.6 ± 0.80[b,c,d]	10.3 ± 1.71[b,c]	14.2 ± 2.40[b,c]

Statistical significance for Table 18

a, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone after the corresponding incubation time.

b, $P<0.05$, significantly decreased compared with 0.2 mmol/l arginine + 5.5 mmol/l glucose in the absence of 1.0 mmol/l L-NMMA after the corresponding incubation time.

c, $P<0.05$, significantly decreased compared with 0.5 mmol/l L-NMMA in the presence of the corresponding glucose / arginine treatment and after the corresponding incubation time.

d, $P< 0.05$, significantly decreased compared with 0.1 mmol/l L-NMMA in the presence of the corresponding glucose / arginine treatment and after the corresponding incubation time.

The effect of incorporating L-NMMA into the microencapsulating Na^+-alginate around microencapsulated islets on their insulin response to glucose.

It was considered that the incorporation of L-NMMA into the microencapsulating Na^+-alginate around microencapsulated islets might usefully contribute towards the prevention of local nitric oxide production mediated by macrophages, in subsequent in vivo studies involving the transplantation of microencapsulated islets. However, it was first necessary to confirm that the addition of L-NMMA in this way did not significantly inhibit the response of microencapsulated islets to glucose challenge, both in the presence and absence of L-arginine. Since 1.0 mmol/l L-NMMA had significantly inhibited insulin release from microencapsulated islets in response to glucose plus L-arginine, Table 18, this concentration of L-NMMA was not used in subsequent experimental work.

The incorporation of 0.1 mmol/l L-NMMA into the microencapsulating Na^+-alginate around islets had no significant effect upon glucose-stimulated insulin release after either 8, 16 or 24 hours incubation, Table 19. However, when 0.5 mmol/l L-NMMA was loaded into the microencapsulating alginate around islets, there was a significant stimulation of insulin release in response to 5.5 mmol/l glucose after 16 hours, compared with the insulin response of unloaded microencapsulated islets to 5.5 mmol/l glucose after the same incubation period, Table 19. The reason for this increase in insulin release is not clear and was not observed after 8 hours or 24 hours incubation.

Table 19

The release of insulin from 0.1 and 0.5 mmol/l L-NMMA loaded microencapsulated islets in response to glucose (mean values ± SEM) (concentrations are mmol/l). n = the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	Insulin release (ng / ml / 10 microencapsulated islets)			
	n	8 hrs	16 hrs	24 hrs
5.5 glucose alone	5	2.3 ± 0.41	3.4 ± 0.27	6.3 ± 0.70
16.7 glucose alone	6	9.2 ± 0.89	16.7 ± 0.91	21.2 ± 0.71
0.1 L-NMMA + 5.5 glucose	6	4.1 ± 1.02	5.6 ± 1.53	6.8 ± 1.83
0.1 L-NMMA + 16.7 glucose	6	7.5 ± 0.52[a]	12.8 ± 1.79[a]	16.6 ± 3.05[a]
0.5 L-NMMA + 5.5 glucose	6	3.5 ± 0.65	6.3 ± 0.89[b]	9.0 ± 1.37
0.5 L-NMMA + 16.7 glucose	6	10.7 ± 1.18[c]	15.9 ± 2.52	20.0 ± 3.60

a, $P<0.05$, significantly decreased compared with the effect of the same concentrations of L-NMMA and glucose, when the L-NMMA was present in the incubation medium (Table 17).

b, $P<0.05$, significantly increased compared with 5.5 mmol/l glucose alone after the same incubation time.

c, $P<0.05$, significantly increased compared with the effect of 0.1 mmol/l L-NMMA in the microencapsulating alginate in the presence of the same glucose concentration and after the same incubation time.

The effect of incorporating L-NMMA into the microencapsulating Na^+-alginate around microencapsulated islets on their insulin response to glucose plus L-arginine.

The effect of L-NMMA incorporation into the microencapsulating Na^+-alginate around microencapsulated islets on their insulin response to glucose plus L-arginine has been summarised in Table 20.

The loading of 0.1 and 0.5 mmol/l L-NMMA into the islet capsule had no significant effect on insulin release induced by 5.5 mmol/l glucose in the presence of 0.2 mmol/l arginine. However, 0.1 mmol/l L-NMMA loaded microencapsulated islets significantly reduced insulin release in response to 16.7 mmol/l glucose plus 0.2 mmol/l L-arginine, by some 26% and 24% after 8 and 16 hours incubation respectively, Table 20. Certainly, the addition of 0.2 mmol/l L-arginine did not significantly influence the insulin response of microencapsulated islets to either 5.5 or 16.7 mmol/l glucose, with either 0.1 or 0.5 mmol/l L-NMMA present in the Na^+-alginate microcapsule, Tables 19 and 20 compared. In addition, 0.5 mmol/l L-NMMA loaded microencapsulated islets released significantly greater amounts of insulin in response to 16.7 mmol/l glucose plus arginine after 8 and 16 hours incubation, compared with 0.1 mmol/l L-NMMA - loaded microencapsulated islets, Table 20. Thus 0.5 mmol/l L-NMMA incorporation inside the Na^+-alginate microcapsule did not inhibit insulin release, confirming the suitability of this concentration for the local prevention of nitric oxide production in transplanted microencapsulated islets.

Table 20

The release of insulin from 0.1 and 0.5 mmol/l L-NMMA loaded microencapsulated islets in response to glucose plus 0.2 mmol/l L-arginine (mean values ± SEM) (concentrations are mmol/l). n = the number of determinations carried out on islets pooled from at least 6 mice.

Treatment (mmol/l)	Insulin release (ng / ml / 10 microencapsulated islets)			
	n	8 hrs	16 hrs	24 hrs
0.2 arginine + 5.5 l glucose	6	4.3 ± 0.42	7.8 ± 1.03	9.1 ± 1.47
0.2 arginine + 16.7 glucose	6	11.8 ± 1.06	20.1 ± 1.9	24.3 ± 3.3
0.1 L-NMMA + 0.2 arginine + 5.5 glucose	6	4.7 ± 0.44	7.5 ± 0.91	10.0 ± 1.15
0.1 L-NMMA + 0.2 arginine + 16.7 glucose	6	8.7 ± 0.57[b]	15.2 ± 0.88[b]	20.5 ± 1.24
0.5 L-NMMA + 0.2 arginine + 5.5 glucose	6	3.1 ± 0.69	5.3 ± 0.93	7.4 ± 1.28
0.5 L-NMMA + 0.2 arginine + 16.7 glucose	6	11.9 ± 0.80[a]	19.3 ± 1.58[a]	24.2 ± 1.86

a, $P<0.05$, significantly increased compared with 0.1 mmol/l L-NMMA loaded into the microencapsulating alginate in the presence of the corresponding glucose concentration, plus arginine after the same incubation time.

b, $P<0.05$, significantly reduced compared with 16.7 mmol/l glucose + arginine alone after the same incubation time.

DISCUSSION

In insulin dependent (type 1) diabetes mellitus, the loss associated with the infiltration of the pancreatic islets by macrophages and lymphocytes (258,270-272). Nitric oxide is produced by macrophages and polymorphonuclear leukocytes when stimulated by interleukin 1β *in vitro* (273). Nitric oxide has been shown to be highly toxic for islet cells (248). Islets have been shown to be protected from the effects of nitric oxide by the addition of N^G-monomethyl L-arginine (L-NMMA), an inhibitor of nitric oxide synthase, and this protection was complete with 0.5 mmol/l L-NMMA (248). The present studies were performed to investigate whether L-NMMA (0.1, 0.5 and 1.0 mmol/l), whilst exerting its protective effect, would deleteriously affect insulin secretion from both free non-microencapsulated and microencapsulated mouse islets.

0.1 and 0.5 mmol/l L-NMMA did not significantly influence glucose-induced insulin secretion, either in the presence or absence of 0.2 mmol/l L-arginine, from either free non-microencapsulated or microencapsulated islets. However, a significant decrease in glucose-induced insulin secretion was observed, when both free non-microencapsulated and microencapsulated islets were exposed to 1.0 mmol/l L-NMMA, both in the presence and absence of 0.2 mmol/l L-arginine. Laychock and colleagues have also demonstrated an inhibition of insulin release from isolated rat islets in response to high concentrations (5 mmol/l) of L-NMMA in the presence of glucose (250). In the present studies, the maximum inhibitory effect of 1.0 mmol/l L-NMMA on insulin secretion was obtained in the presence of 16.7 mmol/l glucose. The reason for this decrease in insulin release is not clear.

The present studies also confirmed that glucose induced insulin release from both free non-microencapsulated and microencapsulated islets was not responsive to 0.2 mmol/l L

arginine. This concentration of L-arginine was chosen because it was non-stimulatory, but sufficient to support nitric oxide synthase activity. It has been reported, that higher concentrations (5-15 mmol/l) of negatively charged L-arginine will depolarize the β-cell membrane and potentiate glucose - induced insulin secretion (250, 255, 274, 275). Insulin release in response to L-arginine has been shown to be concentration dependent, and L-arginine concentrations of 1 mmol/l do not significantly stimulate insulin release from isolated rat islets (250). Also, L-arginine uptake into islets is saturated at 15 mmol/l, a concentration that is 100 times higher than the normal L-arginine concentration in plasma (276). Kroncke and colleagues have demonstrated that activated peritoneal macrophages rapidly kill islet cells via L-arginine dependent nitric oxide generation (248). Nitric oxide has been shown to increase the activity of guanylate cyclase and cGMP generation in islets (198). L-arginine (10 mmol/l) also increases cGMP levels in islets and glucose potentiates this effect (250).

When microencapsulated islets were exposed to L-NMMA for an extended period of time (8-24 hours), the inhibitory effect of 1.0 mmol/l L-NMMA on glucose stimulated insulin release was evident after 8 hour incubation, and was more exaggerated after 24 hours incubation. When L-NMMA (0.1 and 0.5 mmol/l) was incorporated into the microcapsule of microencapsulated islets, 0.1 mmol/l L-NMMA significantly decreased insulin release in response to 16.7 mmol/l glucose in the presence of 0.2 mmol/l L-arginine after 8 and 16 hours incubation, compared with the same concentration of L-NMMA when present in the incubation medium. This may have been the result of reduced diffusion distance for L-NMMA to affect insulin response from 0.1 mmol/l L-NMMA-loaded microencapsulated islets. 0.5 mmol/l L-NMMA-loaded microencapsulated islets, significantly increased insulin release in response to 5.5 mmol/l glucose routinely after 16 hours incubation, but not consistently after 24 hours incubation.

CHAPTER 5

IN VIVO INFLAMMATORY RESPONSE TO Na^+-ALGINATE-POLYAMINO ACID MICROENCAPSULATED ISLETS AND THE EFFECT OF IMMUNOSUPPRESSIVE AGENTS

INTRODUCTION

Graft rejection represents one of the major barriers to successful pancreatic islet transplantation. The microencapsulation of isolated islets has been proposed as a potential method of overcoming this problem, but the biocompatibility of the capsular membranes imposes a limit on the feasibility of this approach (229, 230, 277). Islet survival and function inside the microcapsules, in-vivo, depends upon the tissue reaction of the host towards the artificial membrane. The diffusion of nutrients, oxygen, glucose and insulin across the membranes may be inhibited by an inflammatory reaction, i.e. the deposition of fibroblasts and macrophages around the microcapsules. The different microcapsule formulations used to date have largely failed to impart biocompatibility to the encapsulating material. Microencapsulated islets retrieved from the implantation site show intense pericapsular cellular overgrowth, together with active macrophage and fibroblast proliferation. Only a few groups have achieved long-term allograft success, by microencapsulating rat islets in Na^+-alginate-poly-L-lysine-alginate membranes and transplanting them into either streptozotocin diabetic or autoimmune diabetic animals (175, 178, 180). Other groups have had limited success with the same formulation, early graft failure was characterised by a marked fibrotic reaction around the capsule wall (187, 190, 194, 267, 278). These grafts failed because of poor diffusion characteristics of the capsule membranes and a lack of biocompatibility of the encapsulating material. Fritshy and

colleagues (266) found that, although microencapsulated islets allografted into rats maintained normoglycaemia, they only did so by maintaining a low level of insulin release. The microencapsulated islets did not respond to increased blood glucose levels by increasing their rate of insulin release.

There is some evidence to suggest that the pericapsular infiltrate can be inhibited by the administration of immunosuppressive agents, such as cyclosporin A and FK506 (121,267). These agents have been shown to be effective and capable of inducing long-term allograft acceptance in recipients, by blocking the activities of lymphocytes. Cyclosporin A has been widely used for immunosuppression in pancreatic transplantation, usually as a cocktail with prednisone + azathioprine (279). FK506, a new immunosuppressive agent derived from streptomyces tsukubaensis, has recently been used in islet transplantation (121-123, 131). The efficacy of FK506 in the prolongation of islet allograft survival is affected by both the dose of FK506, the duration of exposure and the site used for islet grafting (280). Cyclosporin A and FK506 are structurally unrelated (125) and bind to distinctly different receptors (281,282), but despite this they mediate immunosuppression by a similar mechanism involving the inhibition of T-cell activation by inhibiting cytokines production (125).

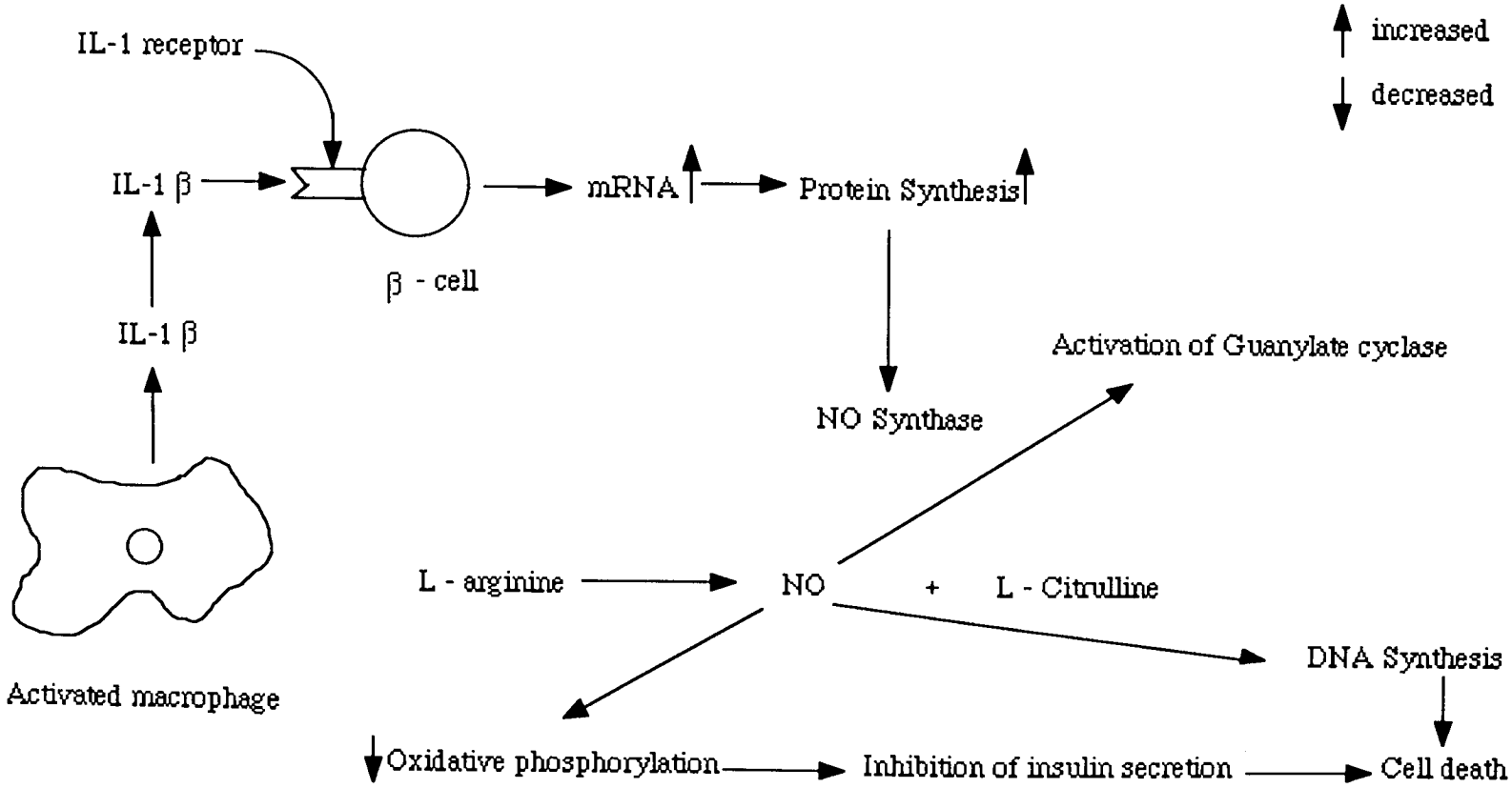
In the present work, initial in vivo studies were carried out to assess the susceptibility of differently fabricated microencapsulated islets to pericapsular fibrosis. Normal and streptozotocin diabetic NMRI mice were used as transplant recipients, and evaluation of the extent of pericapsular fibrosis after 28 days intraperitoneal and subcutaneous implantation was carried out, using conventional histological examination. Some workers have indicated that the fibrosis was largely induced by capsule composition, rather than by the islets per se (230, 283). Therefore, in the initial experiments, empty microcapsules were implanted

either ip. or sc. and retrieved after 4 weeks implantation. Work has also been directed at investigating the effects that different polyamino acid coatings (PLL or PLO), different implantation sites and the presence or absence of an outer alginate layer have on the extent of pericapsular fibrosis, and the histological viability of microencapsulated islets. It was also important to establish whether the immunosuppression of streptozotocin diabetic mice with either cyclosporin A or FK506, would effectively prevent the development of pericapsular fibrosis. Systemic immunosuppression will be confirmed using a spleen lymphocyte proliferation assay employing phytohaemagglutinin (PHA) as a mitogen (284). PHA is a plant lectin which induces the proliferation of T-lymphocytes.

Immunopathological characterisation of the pericapsular infiltrate associated with microencapsulated islets, has demonstrated a predominance of macrophages (278). In addition, the macrophage product interleukin 1β has been found to cause functional impairment and lysis of islet β-cells in vitro (200). However, interleukin 1β alone, and in combination with tumour necrosis factor (TNFα), brings about a slow lysis of islet β-cells and requires a long period of time to become effective (261, 285). On the other hand, macrophages rapidly kill islet β cells via the arginine-dependent generation of nitric oxide, and a proposed mechanism for the action of cytokines on islet beta cells is given in Figure 13.

N^G-monomethyl L-arginine (L-NMMA) as well as N-nitro L-arginine methyl ester (L-NAME) are competitive nitric oxide synthase inhibitors (256). Cultured islets can be protected from interleukin 1β-induced lysis by the addition of L-NMMA to the culture medium (248).

Figure 13. Proposed mechanism for the action of interleukin 1 β (IL1 β) on islet β -cells (263).



Based on these observations, certain novel strategies have been tested in the present work, aimed at preventing nitric oxide production from pericapsular macrophages. This was achieved by incorporating either 0.1 or 0.5 mmol/l L-NMMA into the Na^+-alginate microcapsule around microencapsulated islets. These microencapsulated islets were implanted intraperitoneally and, subsequently, retrieved after 14 and 28 days implantation, and their histological integrity evaluated. Previous work had confirmed that L-NMMA at concentrations of 0.1 and 0.5 mmol/l, did not significantly impair insulin release from L-NMMA loaded microencapsulated islets. The incorporation of L-NMMA into implanted microcapsules provided a potential means for the control of macrophage activity at the β-cell surface, without the need for systemic immunosuppression of the transplant recipient.

MATERIALS AND METHODS

Reagents

Streptozotocin (SO130 Sigma) was dissolved in 0.1 M citrate buffer, pH 4.2. Cyclosporin A was obtained from Sandoz Pharmaceuticals, Leeds, UK. 60 mg of cyclosporin A was suspended in a few drops of 96% ethanol, and then combined with arachis oil at a concentration of 6 mg/ml. FK506 was kindly supplied by Fujisawa Pharmaceuticals Co, Japan. 2 mg of FK506 was suspended in a few drops of 70% ethanol and the volume made up to 4 ml with physiological saline.

Isolation and microencapsulation of islets.

Normal male NMRI mice were used as islet donors. Islets were isolated by collagenase digestion, as described on page 39. Na^+-alginate (DJB) microencapsulated islets were generated by the standard technique described on page 41, and all solutions used in the fabrication process were either autoclaved or passed through 0.2 μm sterile filters. Microcapsules were coated with either PLL or PLO, with and without an outer coating of 0.15% Na^+-alginate.

Incorporation of L-NMMA into the microcapsules

0.1 or 0.5 mmol/l L-NMMA were incorporated into the microcapsule, as described previously, page 112.

Recipient animals and experimental design

Normal and streptozotocin (STZ) diabetic male NMRI mice were used as transplant recipients. NMRI mice, weighing 40-50 gm, were rendered diabetic by the single intraperitoneal injection of streptozotocin (140 mg/kg). Blood glucose was monitored after 7 days to ensure the onset of hyperglycaemia (26.4 ± 1.04 mmol/l, n=10), using a Beckman II glucose analyser. Blood samples for glucose and insulin determinations were obtained from the cut tail tip of all animals before transplantation and at the time of sacrifice, i.e. 14 days and 28 days post implantation. Plasma insulin levels were determined by RIA, as described previously, page 57.

The recipient mice were divided into 6 groups and treated as follows. The animal groups and their treatments have also been summarised in Table 21.

In the first group (n=40), each control non-diabetic mouse received either ip. or sc. transplants of 15-20 Na^+-alginate-PLL-alginate or Na^+-alginate-PLO-alginate empty or islet containing microcapsules.

In the second group (n=40), STZ-diabetic mice received either ip. or sc. transplants of 15-20 Na^+-alginate-PLL-alginate or Na^+-alginate-PLO-alginate empty or islet containing microcapsules.

In the third group (n=20), STZ-diabetic mice received ip. transplants of 15-20 Na^+-alginate-PLL or PLO (no outer alginate layer) empty or islet containing microcapsules.

In the fourth group (n=15), STZ-diabetic mice immunosuppressed with cyclosporin A,

received ip. transplants of 15-20 Na^+-alginate-PLL-alginate or PLO-alginate microencapsulated islets. Cyclosporin A was administered ip. or sc. in the nape of the neck in 0.1 ml of arachis oil, at a concentration of 10 mg/kg/day for 14 days. The reason for administering cyclosporin A both subcutaneously and intraperitoneally, was that serous peritoneal fluid reacted with the arachis oil and produced a white viscous emulsion. Because of this reaction, only a few microencapsulated islets could be recovered from the peritoneal fluid. The microencapsulated islets became trapped in the emulsion and had to be teased out. Most of the microcapsules appeared to be fee of cellular overgrowth.

In the fifth group (n=10), each STZ-diabetic mouse was immunosuppressed with FK506, and received an ip. transplant of 15-12 Na^+-alginate-PLL-alginate microencapsulated islets. FK506 was given intramuscularly, either over 4 days (1.0 mg/kg/day) or over 14 days (0.15 mg/kg/day), to assess the effect of different immunosuppressive regimens on the occurrence of the pericapsular fibrosis. The administration of the immunosuppressive agent started on the day of transplantation.

In the sixth group (n=20), each STZ-diabetic mouse received an ip. transplant of 15-20 either 0.1 or 0.5 mmol/l L-NMMA-loaded Na^+-alginate-PLL-alginate microencapsulated islets.

Attempts were also made to transplant Ba^{2+}-alginate membrane coated islets (prepared by the ficoll density gradient method) into STZ-diabetic recipients. Technically, it did not prove feasible to retrieve these Ba^{2+}-alginate membrane coated islets, because of their significantly smaller size and relative fragility. In order to facilitate retrieval, the Ba^{2+}-alginate coated islets were sealed in small sterilised porous cotton bags, and implanted intraperitoneally into STZ-diabetic mice. Two of the four experimental mice subsequently died on day 3 and day

5 following transplantation. After 14 days, the bags were recovered from the peritoneal cavities of the surviving recipients. The bags were heavily invested with connective tissue, and parts of the small intestine had to be separated by dissection. When the bags were opened for histological processing, no microencapsulated islets were visible. It was assumed that the membrane coated islets had been destroyed by a combination of macrophage attack, anoxia and lack of nutrients.

Surgical implantation of microencapsulated islets.

All surgical procedures were performed under aseptic conditions in a purpose built animal surgical unit, according to the Home Office regulations. Mice were anaesthetised with NO/halothane anaesthesia and, for ip. transplantation, positioned ventral side up. After swabbing the fur with hibitane (5% concentrate diluted 1 in 5 with double distilled water), a small incision was made through the abdominal wall in the left lower quadrant with a pair of scissors. Empty or islet containing microcapsules were suspended in 0.2 ml of physiological saline and delivered through the incision using a 1ml disposable syringe, minus needle. The abdominal wall was then sutured using a 16mm suture needle (Mersilk Black, UK) and 6.0 silk (ethicon). The two sides of the incision were then positioned together and clipped with stainless steel wound clips (12 mm, Michel).

For subcutaneous transplantation, the anaesthetised mouse was positioned dorsal side up and the nape of the neck swabbed with hibitane. A small incision made through the skin with scissors and a small cavity hollowed out in the connective tissue beneath the skin. Empty or islet containing microcapsules were suspended in 0.2ml of physiological saline and delivered via a 1ml disposable syringe, minus needle, into the cavity. The skin was then drawn together with forceps and the incision closed with stainless steel wound clips.

Histological examination of retrieved microencapsulated islets

Empty and islet containing microcapsules were retrieved from the abdominal cavity after 14 or 28 days post-transplantation, by repeated flushing with Hanks balanced salt solution (HBSS). Microencapsulated islets were retrieved from the subcutaneous site by gentle teasing and loosening of the attached connective tissue. The harvested microcapsules were fixed in Heidenhain's Susa fixative, (mercuric chloride) (Appendix 6), for 3-4 hours and processed by paraffin wax sectioning. After fixation, the microencapsulated islets were dehydrated in ascending concentrations of ethanol, cleaned in xylene and embedded in paraffin wax. 5μm thick sections were cut using a rotary microtome (Cambridge) and stained with either standard haematoxylin and eosin or aldehyde fuchsin.

Spleen lymphocyte activation assay for the assessment of immunosuppression.

A convenient method for quantifying the suppression of cellular proliferation is to assay DNA synthesis in cells, such as lymphocytes. The incorporation of exogenous radiolabelled ^{3}H-thymidine into lymphocytes is measured by scintillation counting. ^{3}H-thymidine (TRA 120, specific activity 185 GBq/mmol, Amersham Int. plc, UK) with low specific activity is used, both to prevent radiation damage and to ensure that sufficient exogenous tritiated thymidine is available to maintain the incorporation throughout the labelling period. In the present studies, the T-cell mitogen phytohaemagglutinin (PHA) was used to determine the proliferative responses of spleen lymphocytes.

NMRI transplant recipient mice were killed by cervical dislocation. The ventral surface of the animal was sterilised with 70% alcohol and the spleen removed under aseptic conditions. The excised spleen was placed in a sterile petri dish containing RPMI-1640 medium,

supplemented with 5.5 mmol/l glucose and 2% penicillin/streptomycin, and processed under a tissue culture hood. The spleen was cleaned of excess connective tissue, placed in fresh medium and, using sterile forceps, gently teased apart releasing the cells into the medium. After allowing the cell suspension to settle for approximately 5 minutes, a 10 ml aliquot was transferred to a centrifuge tube, taking care not to remove any of the tissue debris. The cell suspension was then centrifuged at 1200 rpm for 10 minutes. After centrifugation the supernatant was aspirated and the cell pellet resuspended in 10 ml of fresh medium. This procedure was then repeated and the cell pellet resuspended in 6 ml of fresh medium. The cell concentration was determined using a haemocytometer, and cell viability estimated using trypan blue exclusion. Cell viability was routinely greater than 95%. The final cell concentration was adjusted to 1 X 10^7 cell/ml, by appropriate dilution with medium.

Preparation of splenocyte cultures

Splenocyte cultures were set up in 96 well, flat bottom covered tissue culture plates. The total volume in each well was maintained at 100 µl and consisted of 50 µl of the splenocyte suspension, together with 20µl of PHA (10 µg/ml) and 30 µl of sterile RPMI-1640 medium. Test wells were set up in triplicate and the mean of the three wells taken as the result. Cell cultures were incubated in a humid atmosphere in a CO_2/O_2 incubator, for a period of up to 48 hours. Four hours prior to the end of the incubation period, 1 µci of tritiated thymidine, diluted to 20 µl with RPMI-1640 medium, was added to each well. At the end of 48 hours incubation the cells were harvested from each well onto individual filter mats using a Titertek cell harvester (Flow Laboratories). The filter mats were dried in an oven at 37°C for approximately half an hour. Then the individual mats were placed into scintillation vials containing 5 ml of scintillant (optiphase high safe II, biodegradable). The incorporated ^{3}H-thymidine was then counted using a liquid scintillation counter (1900TR,

Canberra Company). The mean counts per minute for the three wells were then calculated and expressed as a stimulation index and defined as:

$$\text{stimulation index} = \frac{^{3}\text{H-thymidine incorporation into treated cells}}{^{3}\text{H-thymidine incorporation into control cells}}$$

RESULTS

Following streptozotocin injection, the NMRI mice lost some 8% of their initial body weight over a period of 7 days, while control non-diabetic mice gained 6% of their body weight over the same time period, Figure 14. After 28 days, there was a net weight gain of 2.2% by the STZ-diabetic mice, but their gain in body weight was still considerably lower than that seen in control non-diabetic mice, Figure 14.

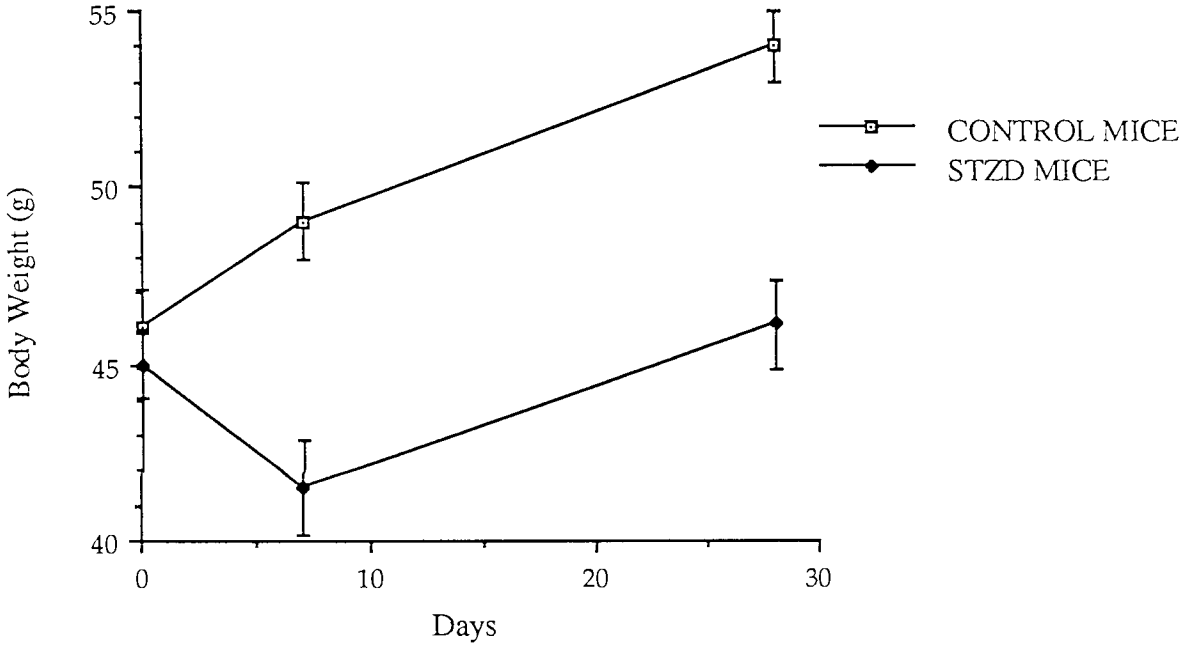
Plasma glucose levels in STZ-diabetic mice increased over a period of 7 days, from a pre-injected level of 5.8 ± 0.24 mmol/l, n=14, to a mean level of 26.4 ± 1.04 mmol/l, n=10. STZ-diabetic recipient mice did not show any significant change in these plasma glucose levels up to the time of sacrifice. Plasma insulin levels of STZ-diabetic mice were undetectable by RIA. Over the 28 day study period, all animals implanted with microencapsulated islets appeared healthy, active and unstressed.

Morphological appearance of retrieved microencapsulated islets.

Na^+-alginate-PLL-alginate microencapsulated islets recovered from the intraperitoneal cavity of STZ-diabetic mice after 28 days appeared translucent, with easily discernible islets inside. They were generally spherical and covered with a cellular overgrowth, Plate 13. Most of the Na^+-alginate-PLL-alginate microencapsulated islets were found free floating in the peritoneal cavity or adhering to the surface of the mesentery.

By contrast, Na^+-alginate-PLO-alginate microencapsulated islets were generally clumped together, and whilst they appeared to be intact, they did not appear to contain islets, Plate 14.

Figure 14 Change in body weight of control and STZ-diabetic mice over 28 day experimental period. (Each point represents the mean of 10 determinations ± SEM).



Microencapsulated islets retrieved from the subcutaneous site of control non-diabetic and STZ-diabetic mice, were packed together in a fibrous connective tissue capsule or sac, associated, and not easily separated from surrounding connective tissue, Plate 15.

Plate 13. An alginate-PLL-alginate microencapsulated islet recovered from the peritoneal cavity of STZ-diabetic NMRI mouse after 28 days transplantation (x200).

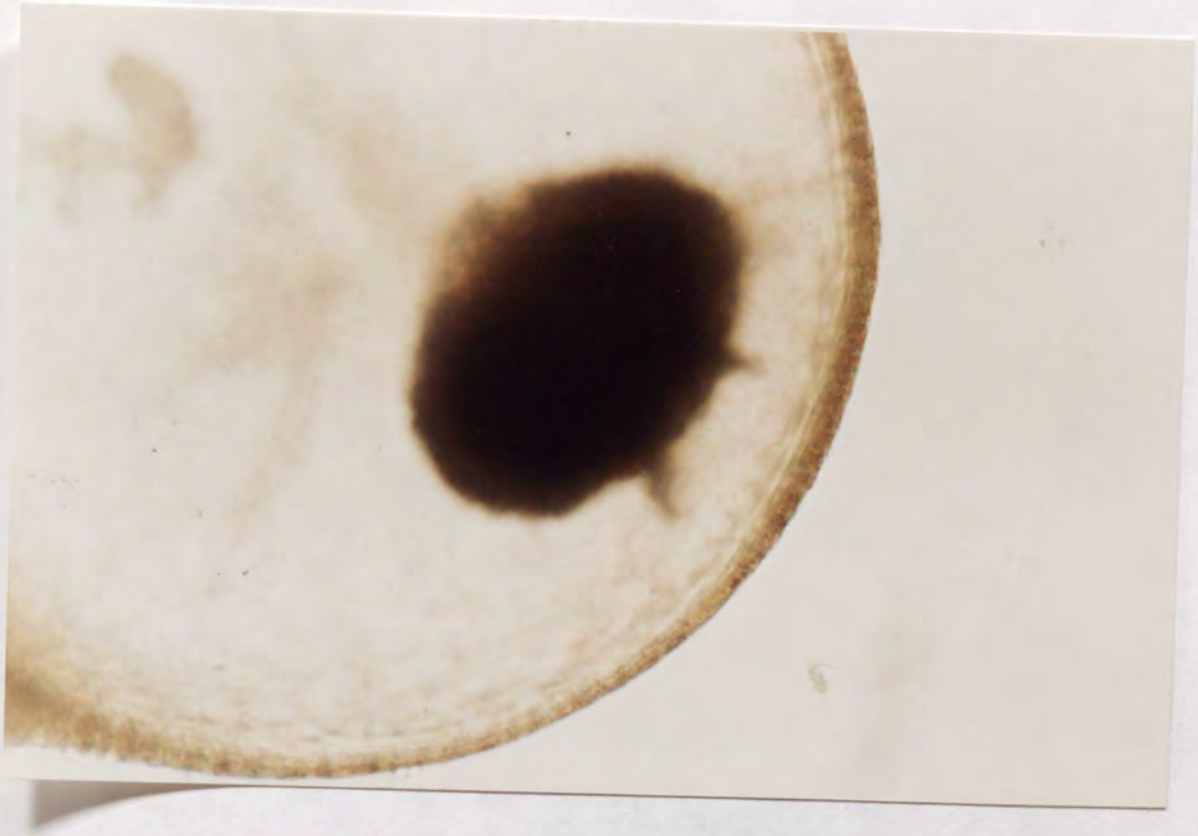


Plate 14. A clump of alginate-PLO-alginate microcapsules recovered from the peritoneal cavity of STZ-diabetic mouse after 28 days transplantation (x50). No islets were discernible.

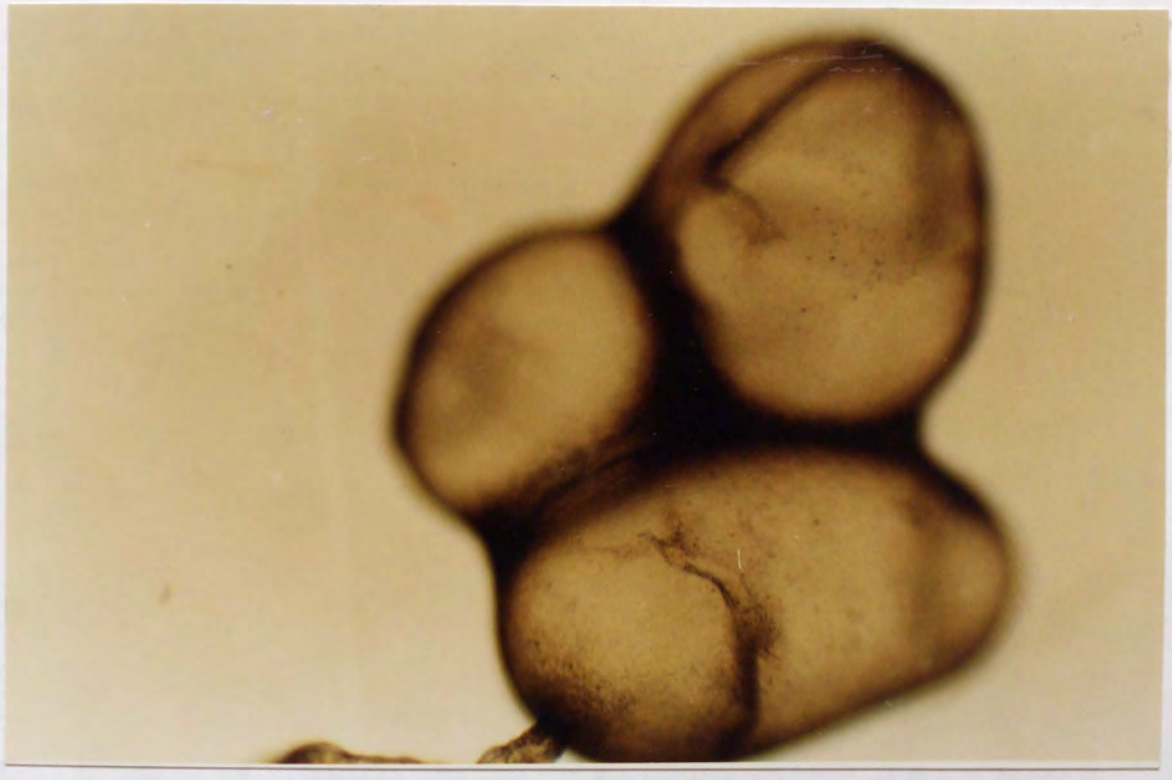


Plate 15. An alginate-PLL-alginate microcapsules recovered from the subcutaneous site of STZ-diabetic mice after 28 days implantation. The capsules were tightly packed in a sac of fibrous connective tissue (x50).

Histological examination of microencapsulated islets after retrieval.

The effect of capsule composition on pericapsular fibrosis (group 1)

In this group, control non-diabetic NMRI mice were implanted intraperitoneally and subcutaneously with empty and islet containing Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microcapsules, in order to determine the effect of capsule composition on pericapsular fibrosis.

Plate 16, shows an empty Na^+-alginate-PLL-alginate microcapsule recovered from the intraperitoneal site of control non-diabetic mice. H and E sections of the capsule revealed a rather thin pericapsular layer of cells, although the structural integrity of the microcapsule was maintained.

Empty Na^+-alginate-PLO-alginate microcapsules, implanted intraperitoneally into control non-diabetic mice for 28 days, showed a similar pericapsular deposition, Plates 17a & b, as seen with Na^+-alginate-PLL-alginate microcapsules, Plate 16. The surrounding cells were morphologically similar in their appearance and consisted of fibroblasts and macrophages. Examination of Na^+-alginate-PLL-alginate microencapsulated islets, recovered intraperitoneally from control non-diabetic mice after 28 days, were morphologically intact, although the actual capsule had shrunk due to histological processing, Plate 18. The islets appeared to be intact with little evidence of monocyte infiltration, despite the presence of pericapsular cells on the surface of the intact microcapsules.

Na^+-alginate-PLO-alginate microencapsulated islets, recovered after 28 days implantation from the peritoneal cavity of non-diabetic mice, were disrupted. The capsules showed

evidence of breaking up and the islets had been invaded by mononuclear cells, Plate 19. Surviving islets, found in some of the disrupted capsules, were vacuolated and in the process of dissolution. The capsules themselves were heavily invested with pericapsular mononuclear cells, Plate 19.

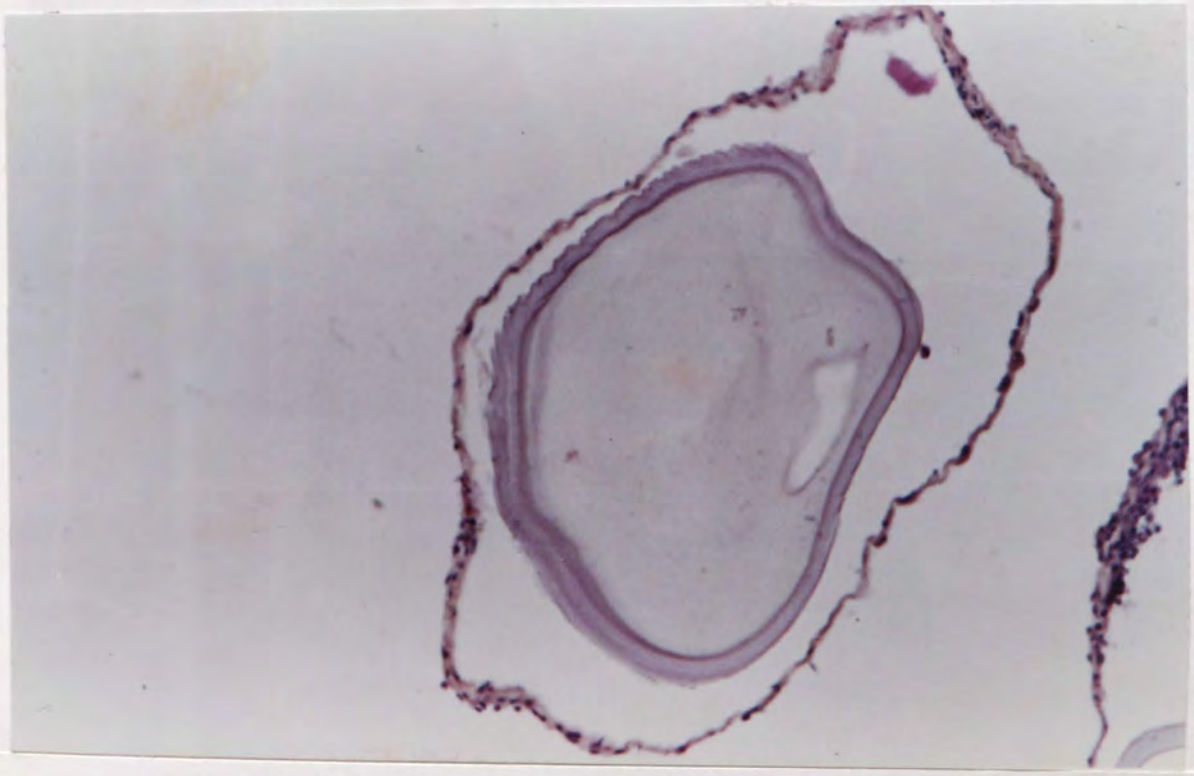
Empty Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microcapsules, implanted subcutaneously into the nape of the neck of non-diabetic NMRI mice, were found clumped together and so massively overgrown with connective tissue that they had to be retrieved by dissection, Plates 20a and b. Subcutaneous implantation of empty Na^+-alginate-PLO-alginate microcapsules was also associated with massive pericapsular fibrosis, and the accumulation of collagen, Plate 21. Even with attendant neovascularization, these microcapsules were judged to be functionally incompetent.

When Na^+-alginate-PLL-alginate microencapsulated islets were implanted subcutaneously into the nape of the neck of non-diabetic NMRI mice, only in one mouse could the microcapsules be retrieved, and even then extensive dissection was required. The microencapsulated islets were invested with an extensive and heavy accumulation of fibrous material, Plates 22a & b. Only the remnants of islets could be found within the microcapsules, yet the capsules themselves were still largely spherical and intact, Plates 22a & b. The excessive deposition of fibrous material around the microencapsulated islets might have prevented the diffusion of nutrients and encouraged anoxia, leading to the destruction of the contained islet tissue. Based on these observation, it was decided that attempts at subcutaneous implantation of microencapsulated islets should be discontinued, in favour of intraperitoneal implantation.

Table 21 Summary of the NMRI mice groups used as transplant recipient

Group of treated animals	Microcapsule fabrication	Transplantation site	Treatment	Post transplantation recovery (days)
Group 1,Control non-diabetic mice(n=40)	Empty or islet containing Na^+-alginate-PLL/PLO-alginate microcapsules	ip and sc	-	28
Group 2, STZ-diabetic mice (n = 40)	Empty or islet containing Na^+-alginate-PLL/PLO-alginate microcapsules	ip and sc.	-	14 or 28
Group 3, STZ-diabetic mice (n = 20)	Empty or islet containing Na^+-alginate-PLL or PLO (no outer alginate) microcapsules	ip	-	28
Group 4, STZ-diabetic mice (n = 15)	Na^+-alginate-PLL or PLO-alginate microencapsulated islets	ip	cyclosporin A, 10mg/kg/day ip or sc, day 0 - 13	28
Group 5, STZ-diabetic mice (n = 10)	Na^+-alginate-PLL-alginate micro-encapsulated islets	ip	FK506, 1.0mg/kg/day im, day 0-3; or FK506, 0.15mg/kg/day im, day 0-13	28
Group 6, STZ-diabetic mice (n = 20)	0.1 or 0.5 mmol/l L-NMMA loaded Na^+-alginate-PLL-alginate micro-encapsulated islets	ip	-	14 or 28

Plate 16. A section of an empty alginate-PLL-alginate microcapsule recovered from the peritoneal cavity of control non-diabetic mice (H & E; x125). Note the outer pericapsular layer of cells which is detached from the capsule during histological processing.



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 17a A section of empty alginate-PLO-alginate microcapsules from the peritoneal cavity of control non-diabetic mice (H & E; x50).

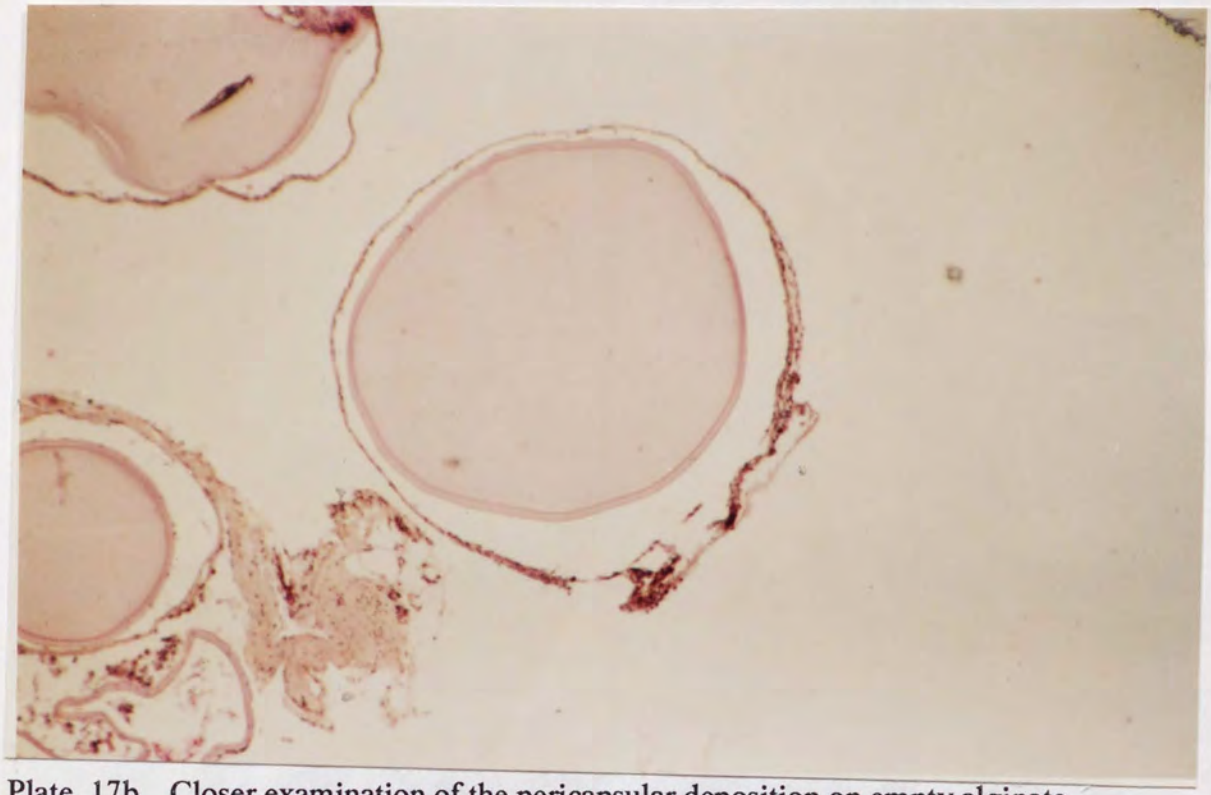
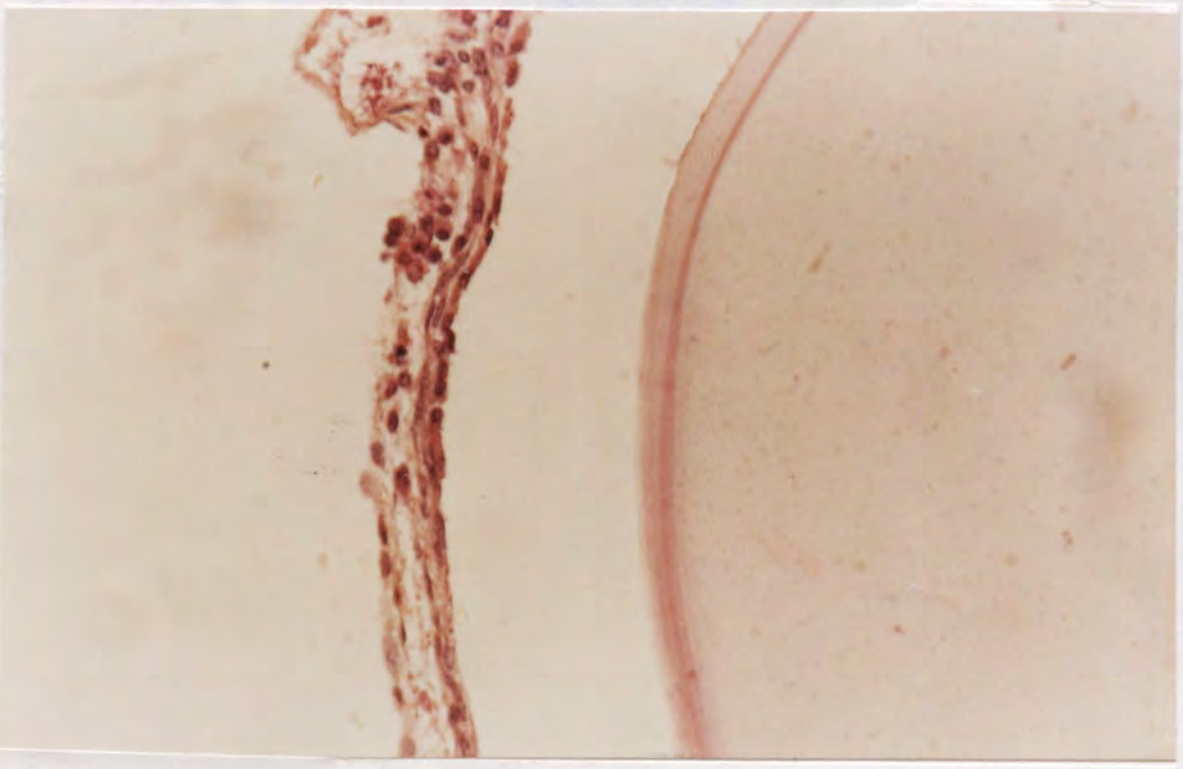
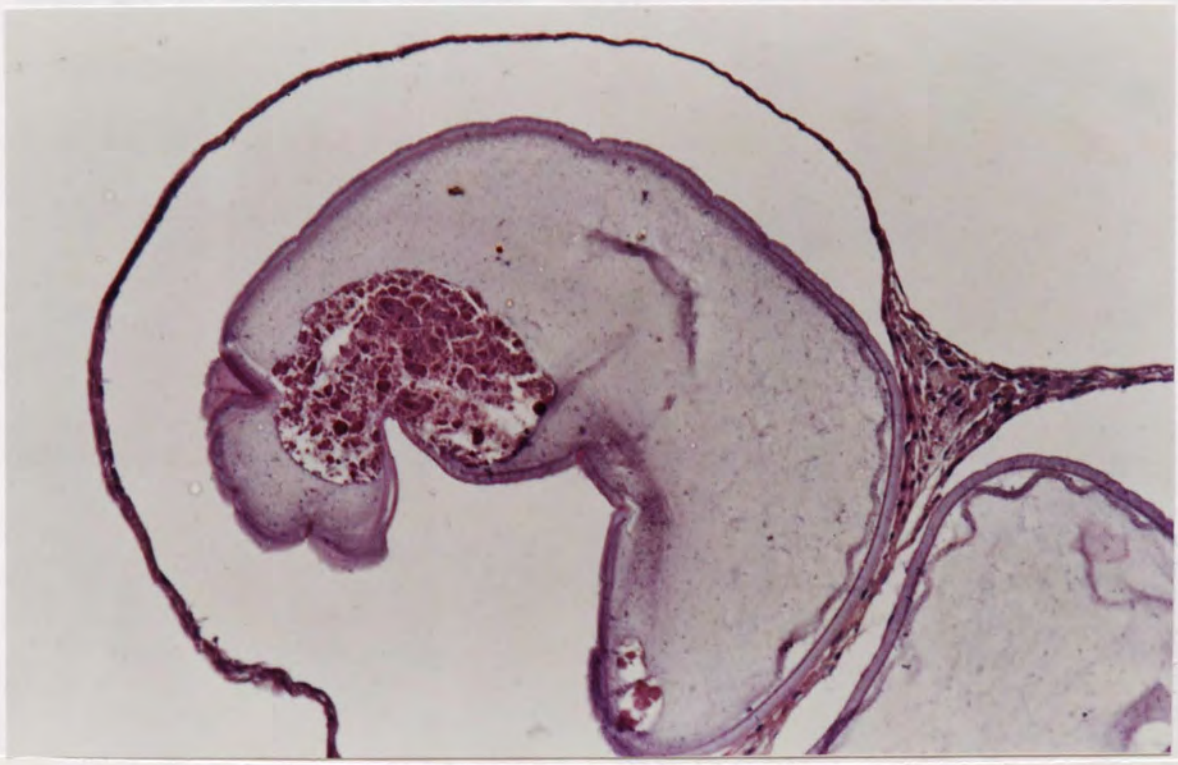


Plate 17b Closer examination of the pericapsular deposition on empty alginate-PLO-alginate microcapsule, showing fibroblasts and mononuclear cells (H & E; x200)



These plates are representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 18 An alginate-PLL-alginate microencapsulated islet recovered from the peritoneal cavity of a control non-diabetic mouse. Both the islet and the microcapsule appear to be intact, although there is significant pericapsular deposition (H & E; x125)



This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

Plate 19. An alginate-PLO-alginate microencapsulated islet from the peritoneal cavity of a control non-diabetic mouse showing a discontinuous outer capsule membrane, and the encapsulated islet breaking up with vacuolisation. The outer capsule is heavily overgrown with mononuclear cells, (H & E: x125).

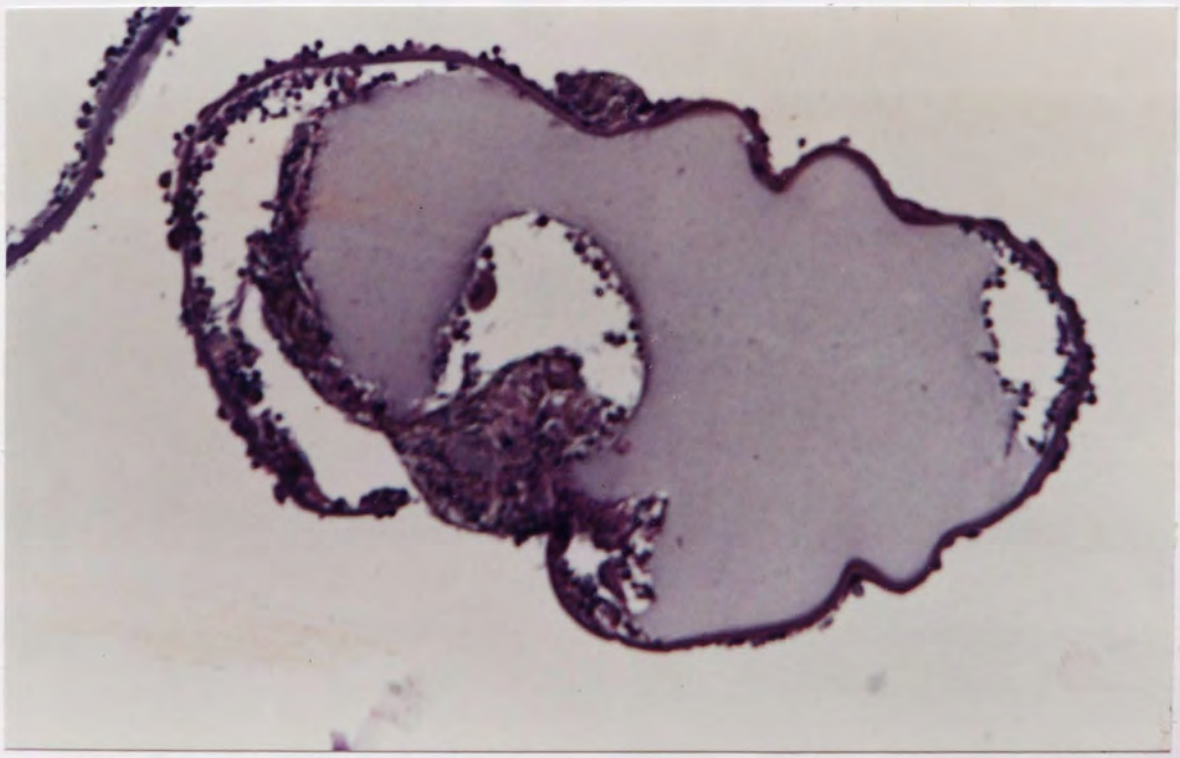
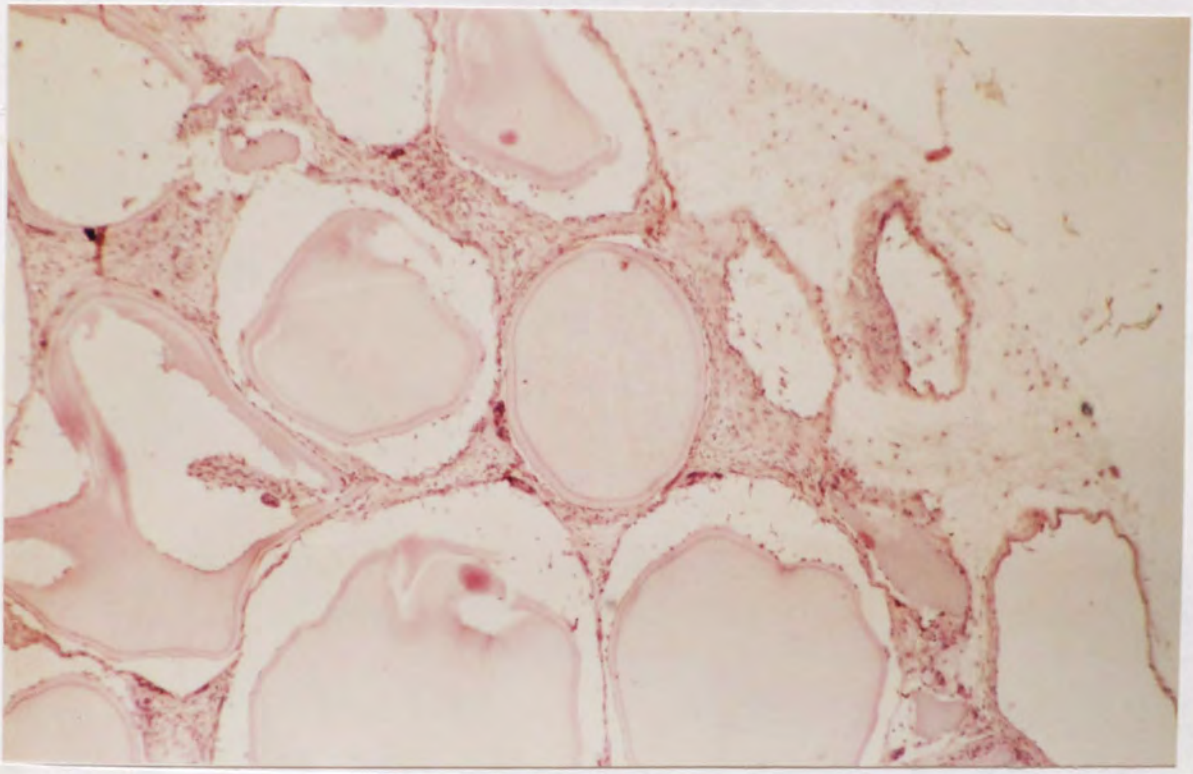


Plate 20a. Empty Na^+alginate-PLL-alginate microcapsules recovered from the subcutaneous site of non-diabetic mouse. The microcapsules were packed together and heavily overgrown with connective tissue (H & E; x50).



This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

Plate 20b Closer examination of intense fibrosis associated with subcutaneous transplantation of empty Na^+-alginate-PLL-alginate microcapsules (H & E; x200)

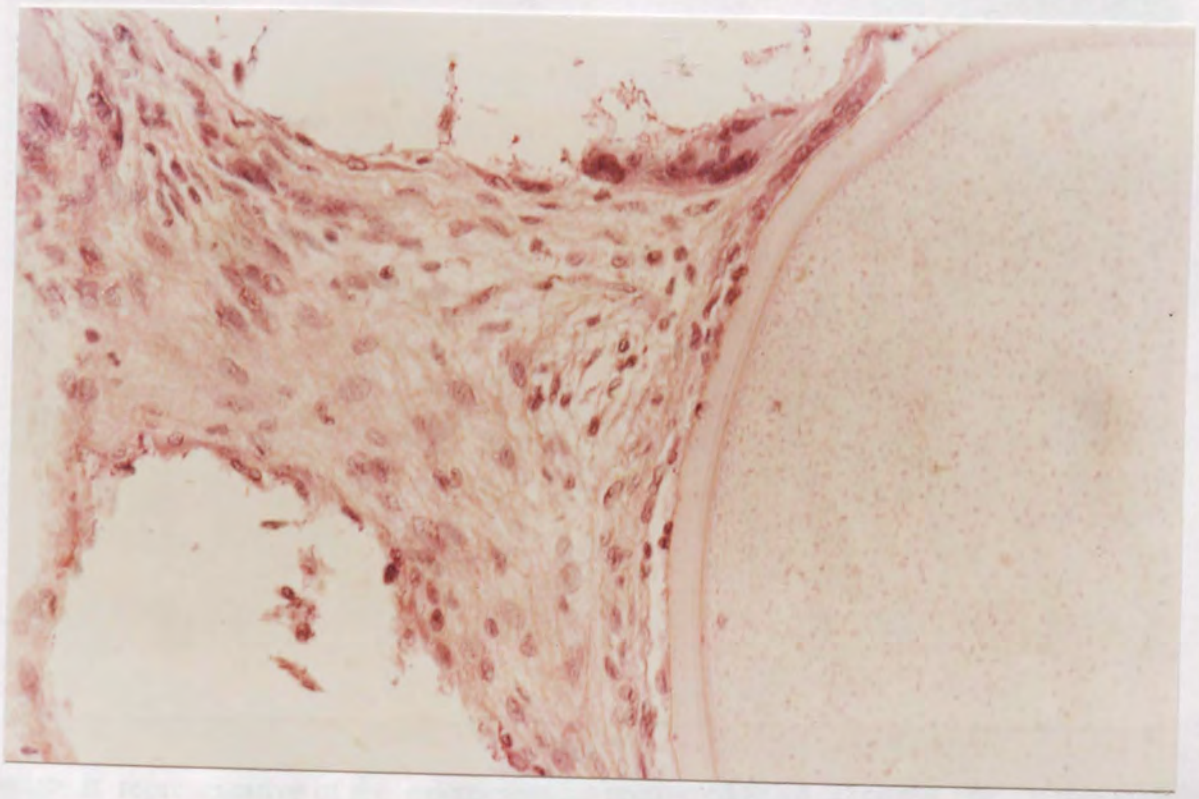
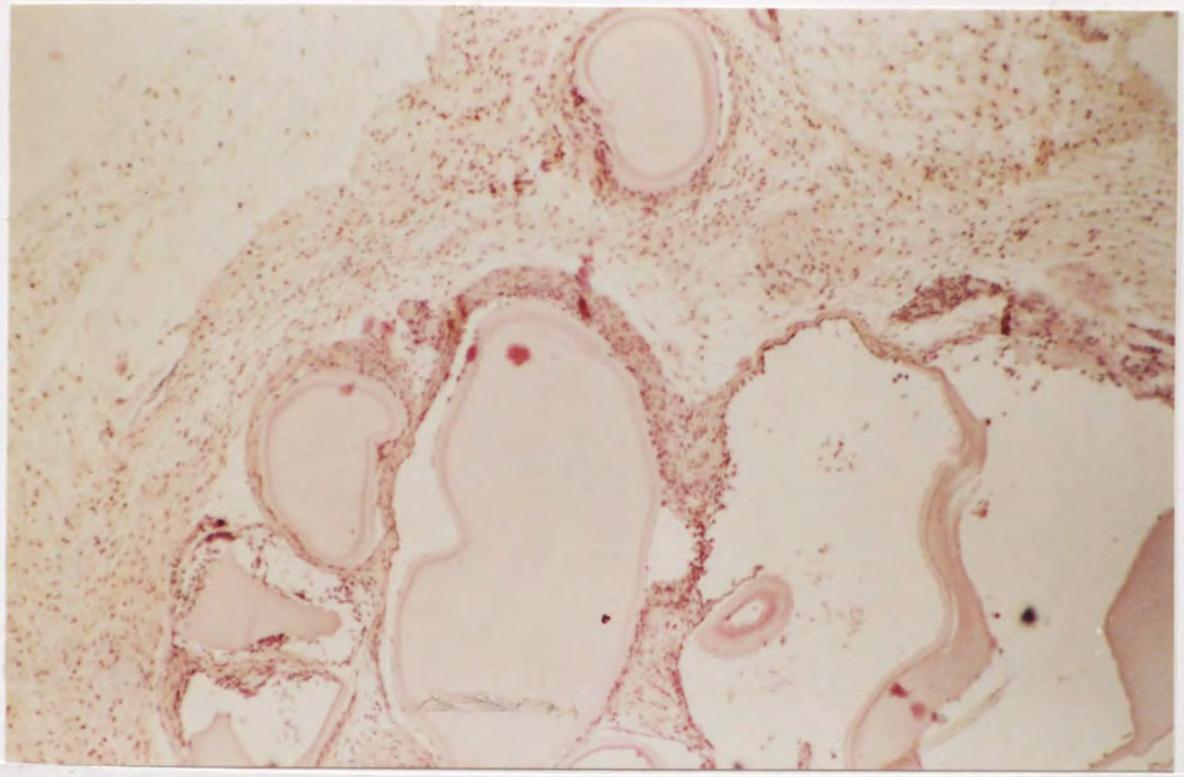


Plate 21 Empty Na^+-alginate-PLO-alginate microcapsules retrieved from the subcutaneous site of non diabetic mouse. Note the massive pericapsular fibrosis with collagen deposition, and the appearance of small capillaries (neovascularization) in the fibrotic tissue (H & E; x50)



This plate is representative of the microcapsules recovered from 2 mice given corresponding treatment

Plate 22a Na^+-alginate-PLL-alginate microencapsulated islets retrieved from subcutaneous site of non-diabetic mouse, showing remnants of islets within extensively overgrown capsules (H & E: x50)

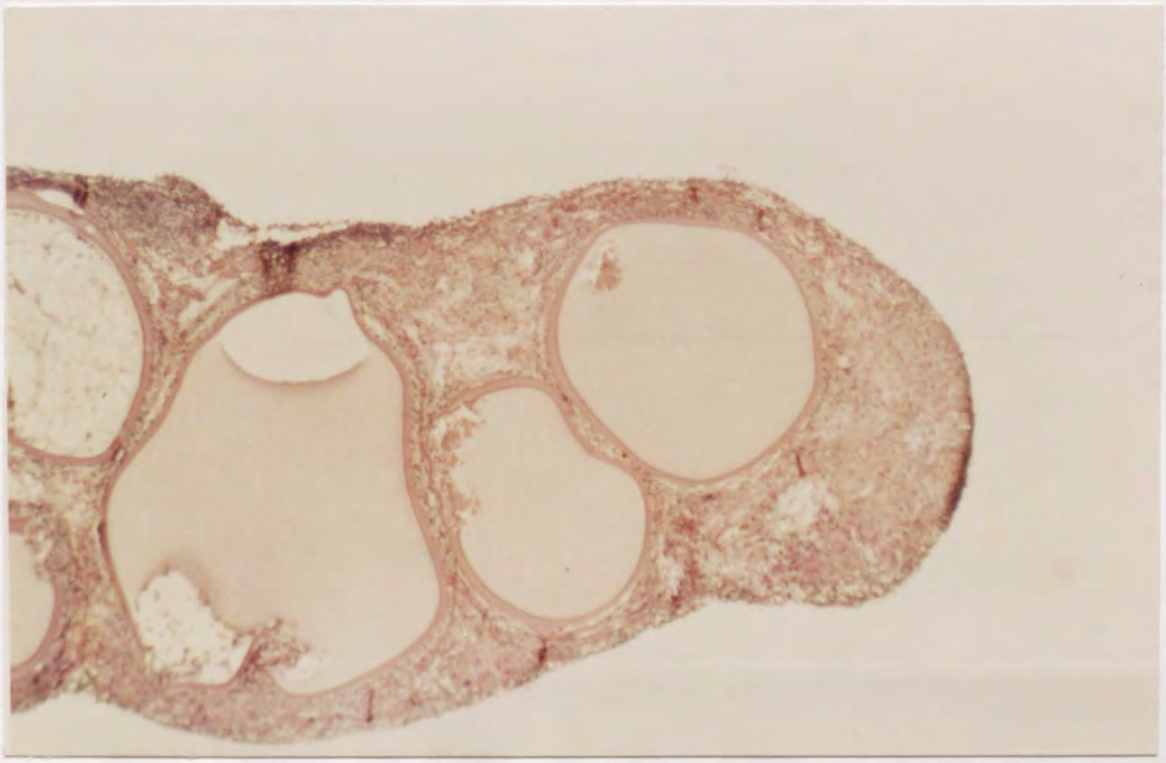
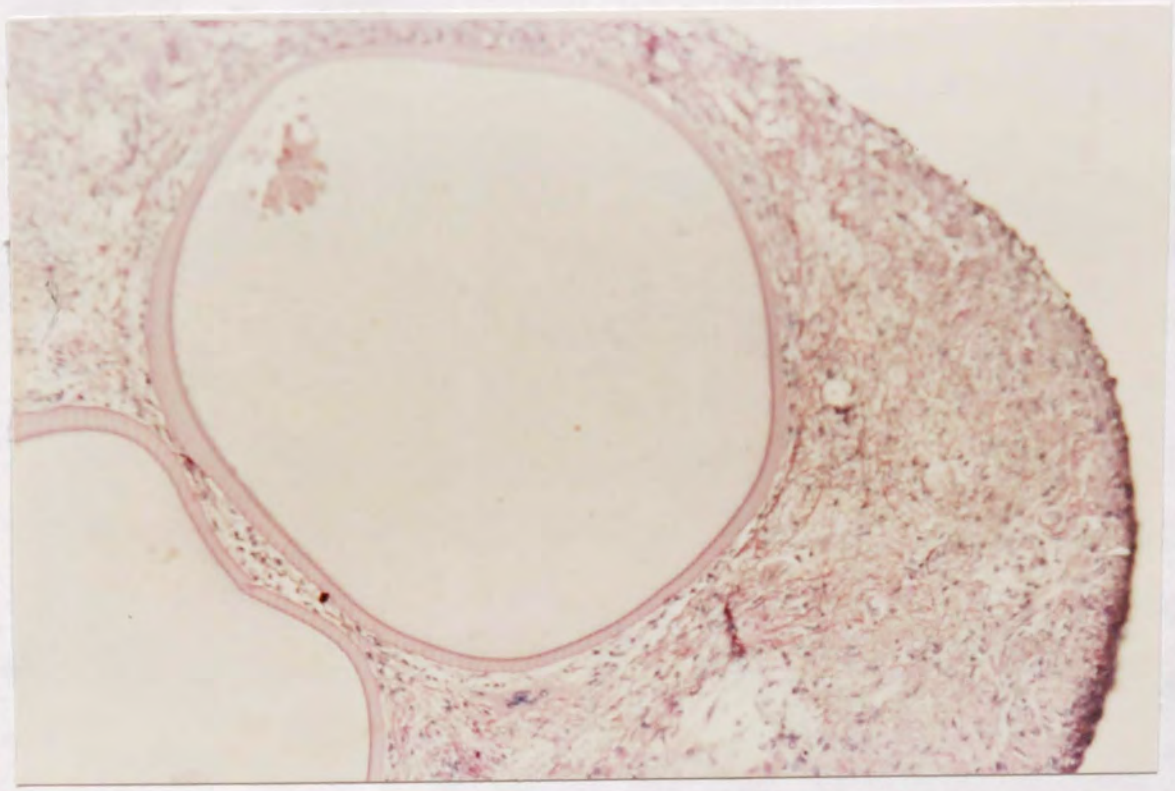


Plate 22b Closer examination of the extraordinary fibrotic reaction surrounding subcutaneously implanted microencapsulated islets. Note the deposition of collagen fibres and the appearance of neovascularization (H & E: x125)



The inflammatory response to microencapsulated islets when implanted into STZ-diabetic mice (group 2)

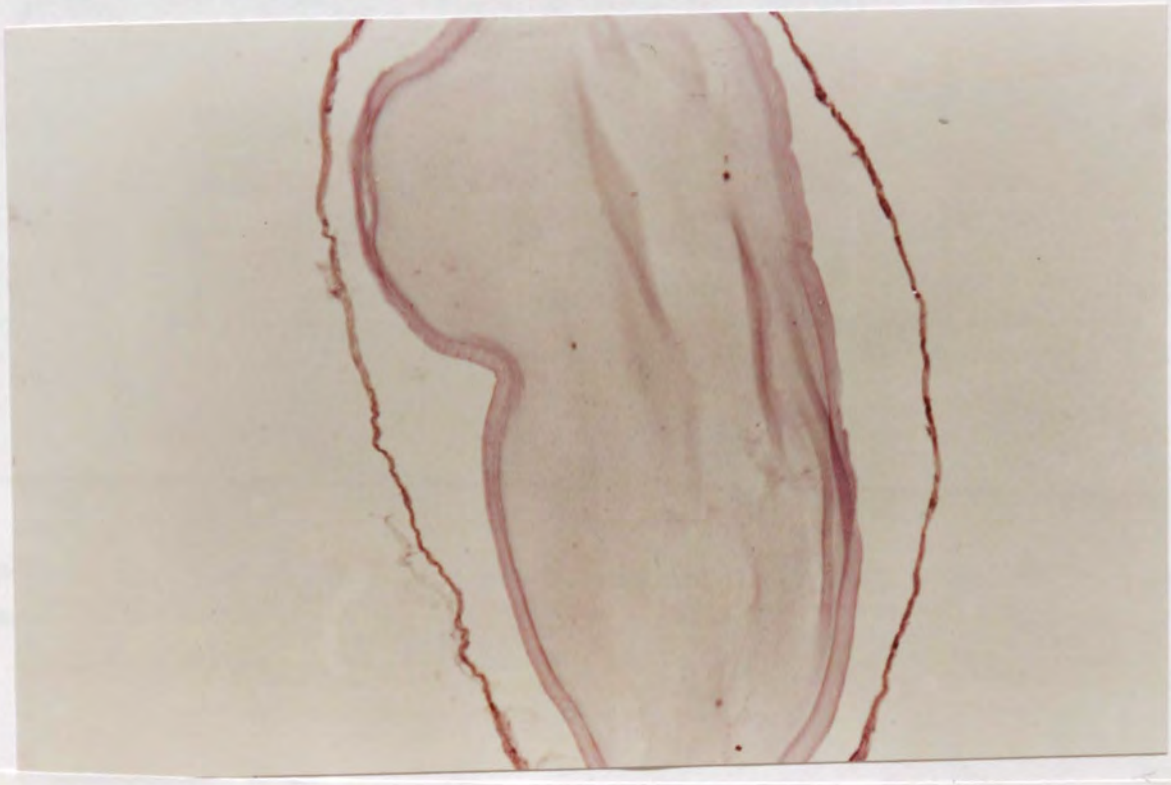
In this group,STZ-diabetic mice were implanted intraperitoneally and subcutaneously with empty and islet containing Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microcapsules. Empty Na^+-alginate-PLL-alginate and empty Na^+-alginate-PLO-alginate microcapsules, Plates 23 and 24 respectively, retrieved from the peritoneal cavity of STZ-diabetic NMRI recipient mice after 28 days, showed comparatively less pericapsular fibrosis than empty microcapsules implanted intraperitoneally into non-diabetic NMRI mice. Both PLL and PLO coated microcapsules were physically intact and had retained their generally spherical shape.

When islets were inserted into the microcapsules and implanted intraperitoneally into STZ-diabetic mice, there was a marked increase in the extent of pericapsular fibrosis around both Na^+-alginate-PLL-alginate microcapsules, Plates 25a & b, and Na^+-alginate-PLO-alginate microcapsules, Plate 26. The pericapsular response to the microencapsulated islets recovered from the diabetic mice was dense and multilayered, and comprised of both small mononuclear cells and fibroblasts. The appearance of the pericapsular cells appeared to be much quicker around microcapsules containing islets, than around empty microcapsules. These observations would suggest that a significant increase in pericapsular fibrosis is encouraged by the presence of islets in microcapsules implanted into STZ-diabetic mice, and that islet tissue is a more important inflammatory agent than the microcapsule fabrication materials. Even so, there was evidence to suggest that a greater pericapsular reaction was observed by microcapsules coated with PLO than coated with PLL, Plate 26, and that microcapsules implanted into STZ-diabetic mice received a more extensive pericapsular deposition than the same microcapsules implanted intraperitoneally into non-diabetic mice.

The islets inside PLL coated microcapsules had deteriorated significantly and their cellular integrity could not be confirmed, Plates 25a & b. In addition, the islets found inside PLO coated microcapsules were extensively degranulated, vacuolized and associated with scattered cell debris, Plate 26. The deposition of pericapsular cells appears to be a relatively early response by the host to the implantation of microencapsulated islets, and is confluent 14 days after ip. implantation, Plate 27, at a time when the contained islets are still relatively intact.

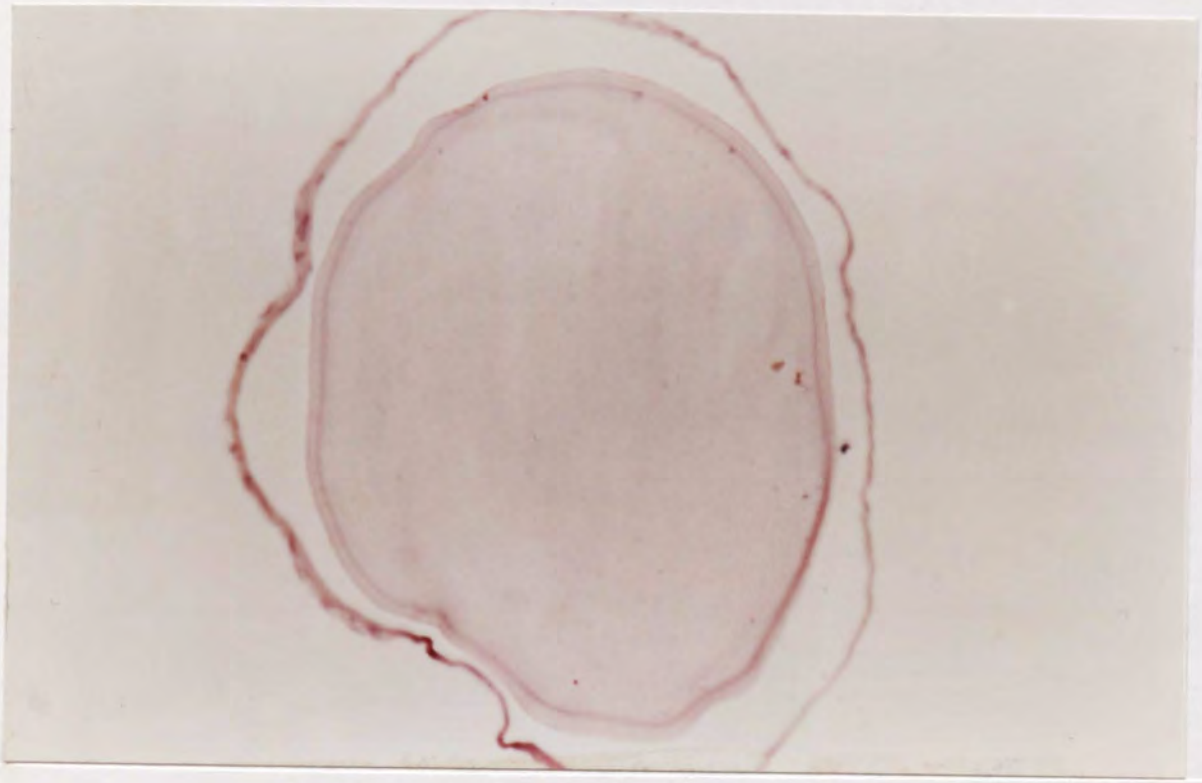
When empty Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microcapsules were implanted subcutaneously into STZ-diabetic mice, substantial pericapsular fibrosis and infiltration was evident, Plates 28 and 29. Many of the microcapsules were discontinuous and collapsed. It proved impossible to retrieve islets containing microcapsules from the subcutaneous site of STZ-diabetic mice.

Plate 23. An empty Na^+-alginate-PLL-alginate microcapsule recovered from the peritoneal cavity of a STZ-diabetic mouse. Note the light pericapsular reaction, when compared with implantation into non-diabetic NMRI mouse, Plate 16, (H & E: x125)



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 24. An empty Na^+-alginate-PLO-alginate microcapsule recovered from the peritoneal cavity of a STZ-diabetic mouse, showing relatively light pericapsular fibrosis (H & E; x125).



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 25a Na^+-alginate-PLL-alginate microencapsulated islet recovered from the peritoneal cavity of a STZ-diabetic mouse, showing increased pericapsular deposition, compared with empty microcapsules (H & E; x50).

This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 25b Closer examination of the pericapsular fibrosis on the surface of Na^+-alginate--PLL-alginate microencapsulated islets, showing the extensive deposition of small mononuclear cells (H & E: x125)

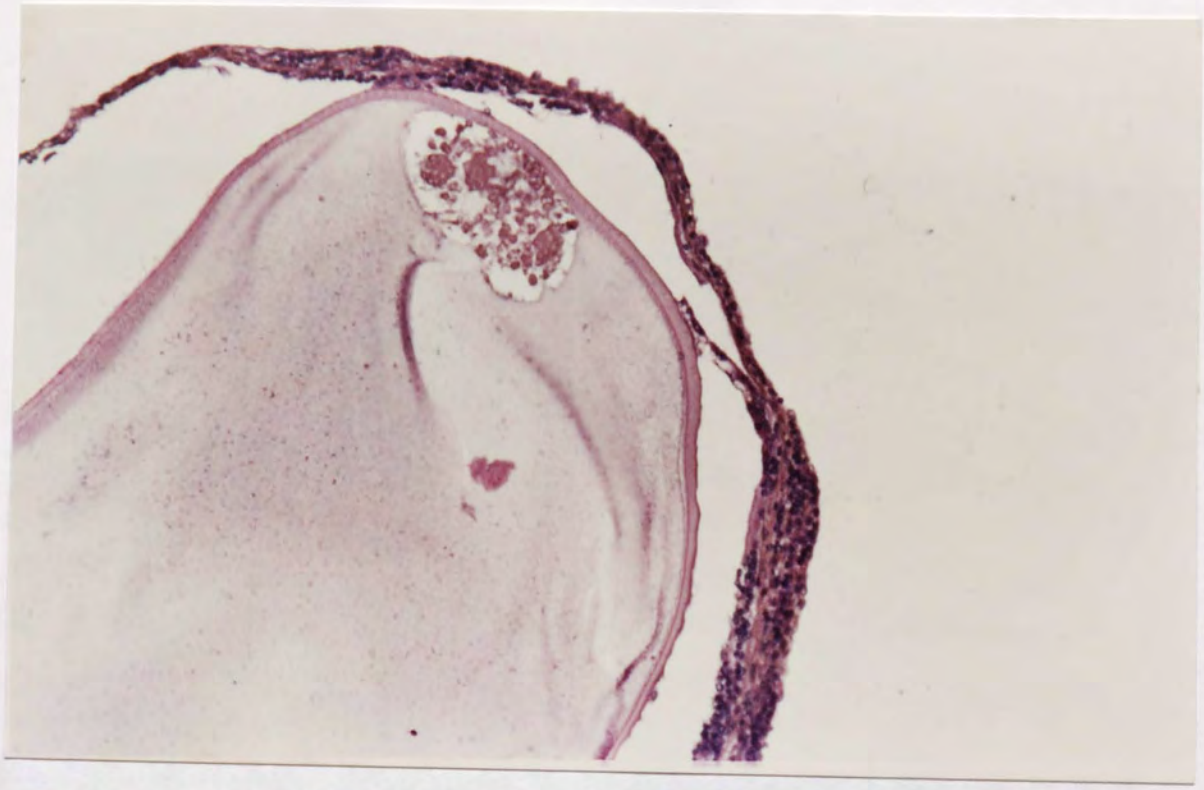
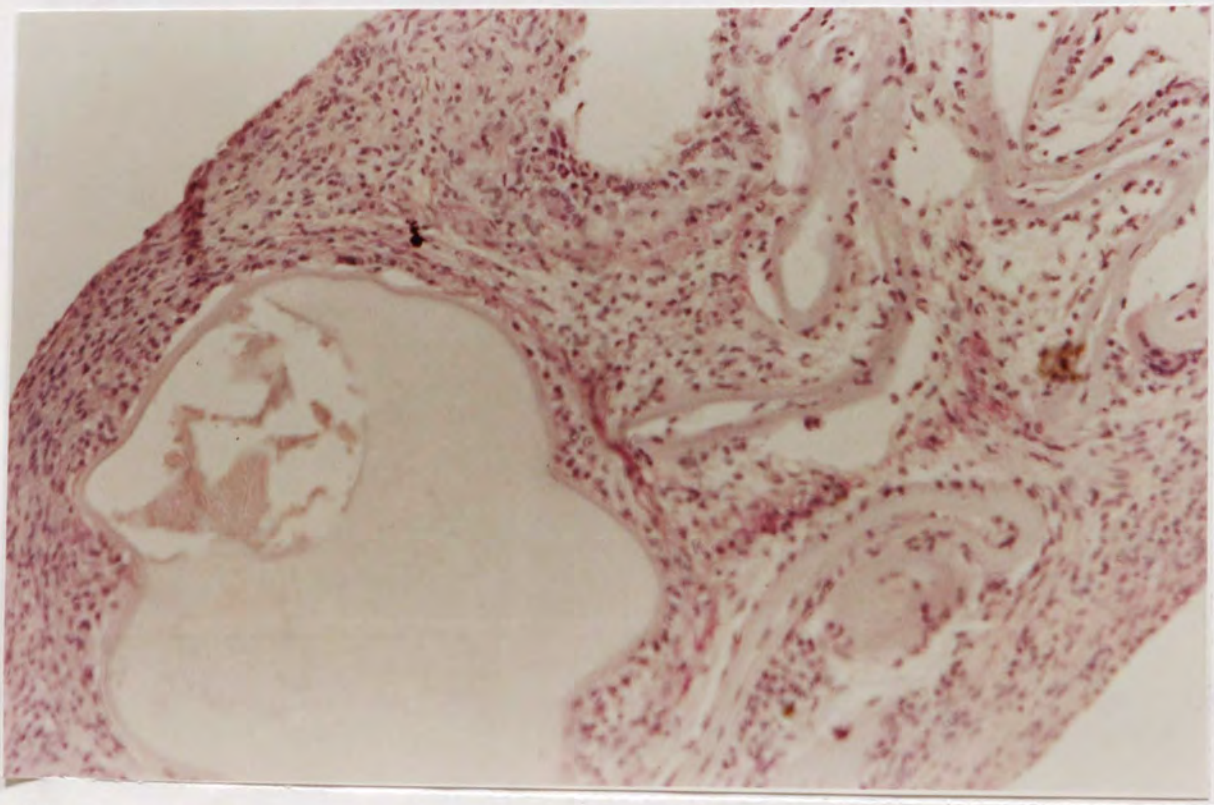
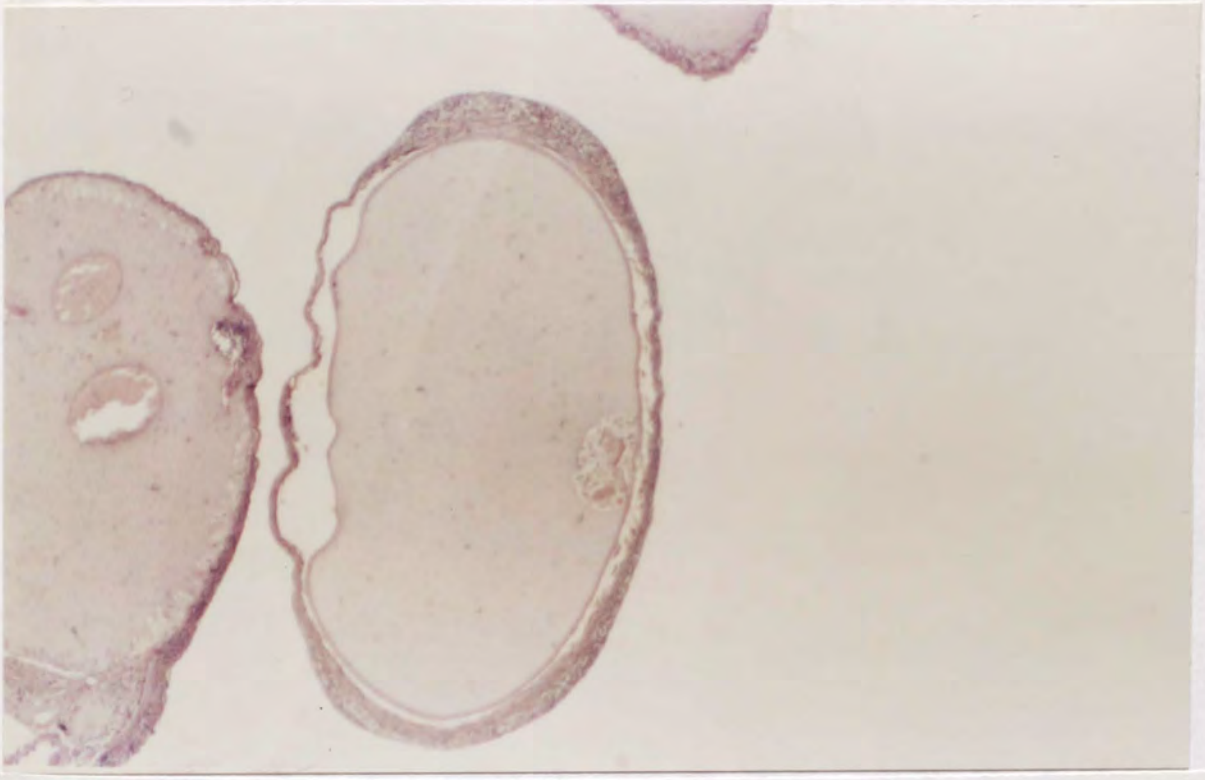


Plate 26 A Na^+-alginate--PLO-alginate microencapsulated islet recovered from the peritoneal cavity of a STZ-diabetic mouse, showing a more intense pericapsular deposition, compared with Na^+-alginate-PLL-alginate microencapsulated islet, Plates 25a & 25b, (H&E: x125)



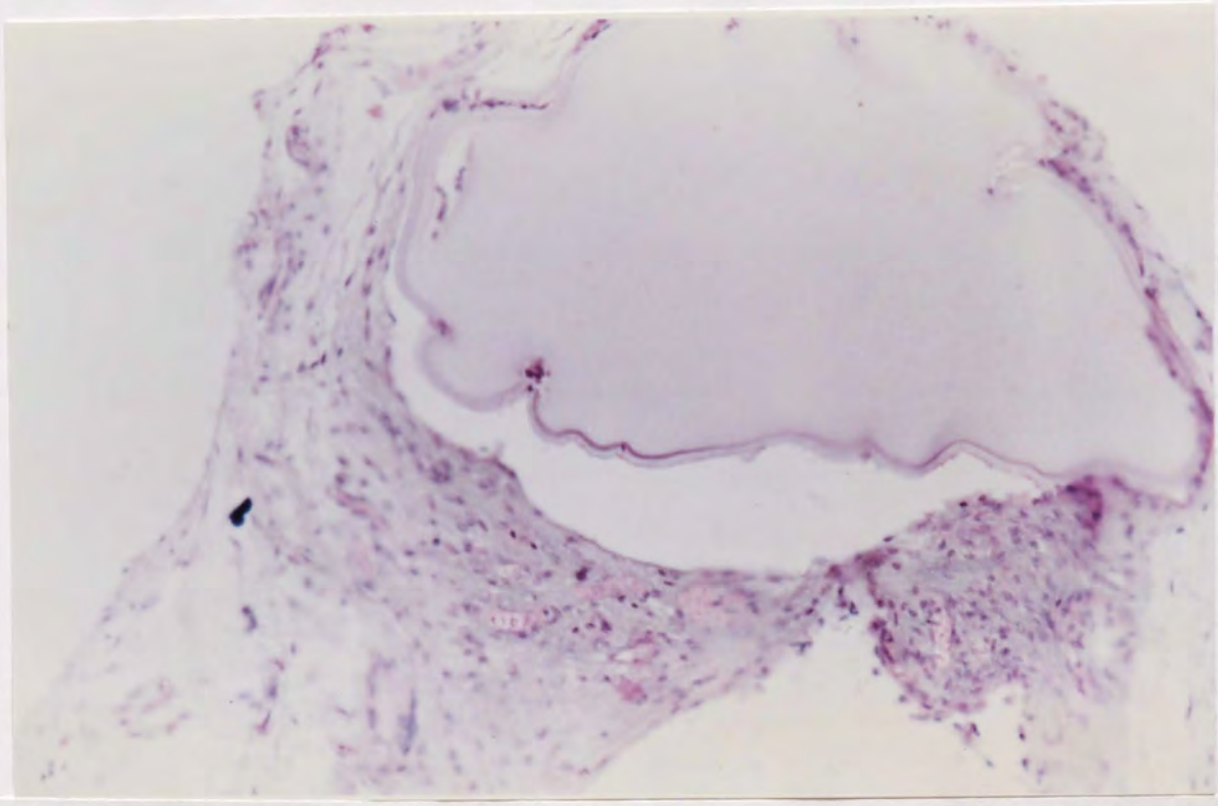
This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 27 Na^+-alginate-PLL-alginate microcapsule recovered from the peritoneal cavity of a STZ-diabetic mouse after 14 days implantation (H & E; x50).



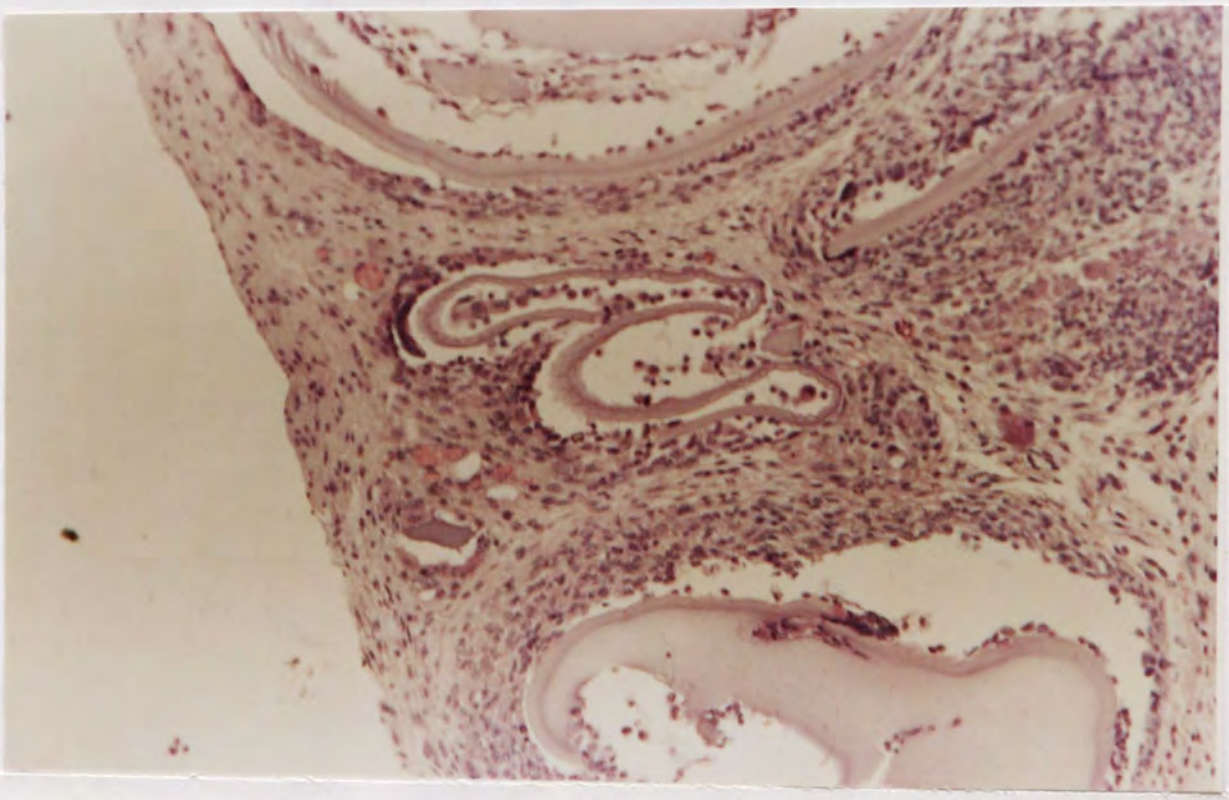
This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 28. Empty Na^+-alginate--PLL-alginate microcapsule recovered from the subcutaneous site of a STZ-diabetic mouse, showing the presence of a massive cellular overgrowth with neovascularization (H & E: x125).



This plate is representative of the microcapsules recovered from 2 mice given corresponding treatment

Plate 29 Empty Na^+-alginate-PLO microcapsules recovered from the subcutaneous site of a STZ-diabetic mouse, showing damaged, collapsed and overgrown microcapsules with massive mononuclear cell infiltration (H & E; x50).



This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

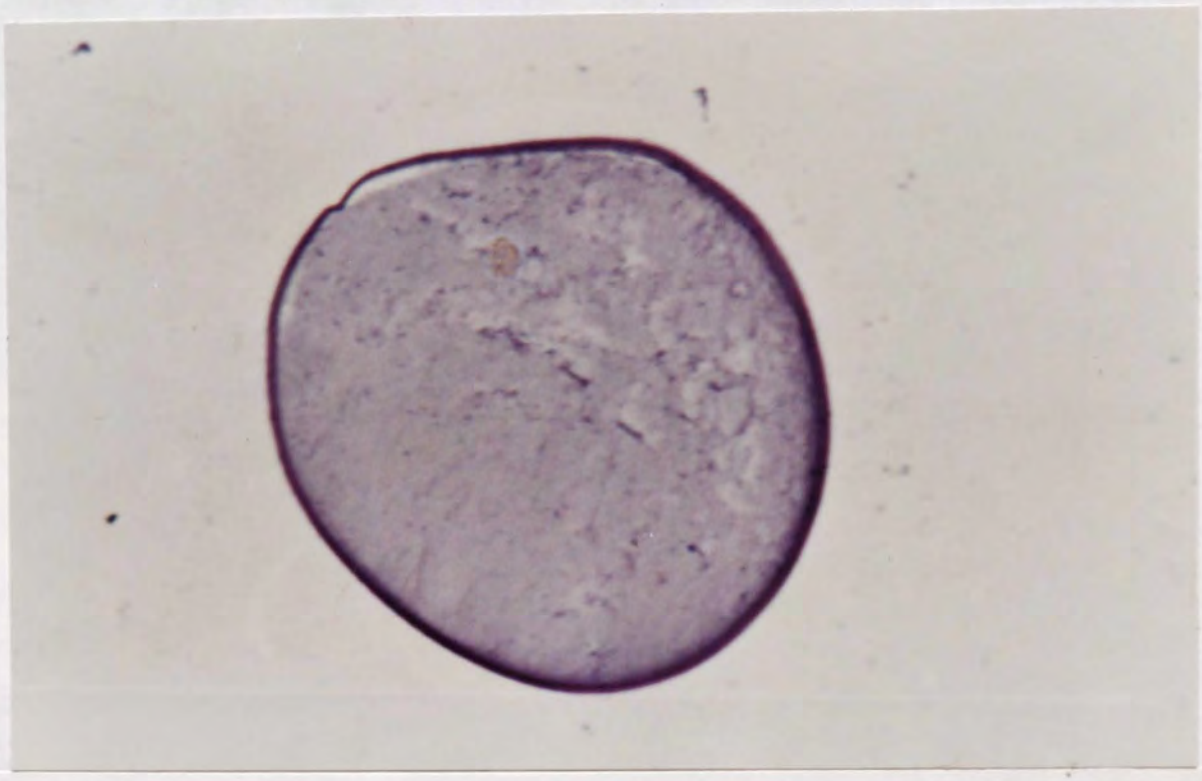
The effect of no outer alginate layer on pericapsular fibrosis (group 3).

In this group, STZ-diabetic mice were implanted intraperitoneally with empty and islet containing Na^+-alginate-PLL and Na^+-alginate-PLO (no outer alginate layer) microcapsules, in order to determine the effect of an outer Na^+-alginate coating on pericapsular fibrosis.

When an outer alginate layer was omitted from empty Na^+-alginate-PLL (Plate 30), and empty Na^+-alginate-PLO (Plates 31a & b) microcapsules, little or no pericapsular fibrosis was observed. Only Na^+-alginate-PLO microcapsules showed any evidence of surface pericapsular deposition, and the capsules themselves were intact and reasonably spherical. It was clear that the outer alginate layer tended to promote the attachment of mononuclear cells.

When islets were encapsulated within both Na^+-alginate-PLL and Na^+-alginate-PLO microcapsules, a significant pericapsular deposition was obtained, although the encapsulated islets remained largely intact (Plates 32 and 33). In addition, this observation reinforces previous findings and confirms that the presence of islets inside the microcapsule stimulates pericapsular fibrosis. The limited pericapsular deposition seen on Na^+-alginate-PLL and Na^+-alginate-PLO microcapsules without an outer alginate layer,Plates 32 and 33, contrasts with the pericapsular response associated with Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microcapsules, which was dense and multilayered, Plates 23 and 24.

Plate 30. An empty Na^+-alginate-PLL (no outer alginate layer) microcapsule recovered from the peritoneal cavity of a STZ-diabetic mouse, showing no pericapsular deposition (H & E; x125)



This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 31a Empty Na^+-alginate-PLO (no outer alginate layer) microcapsule recovered from the peritoneal cavity of a STZ-diabetic mouse, showing little pericapsular deposition (H & E; x125).

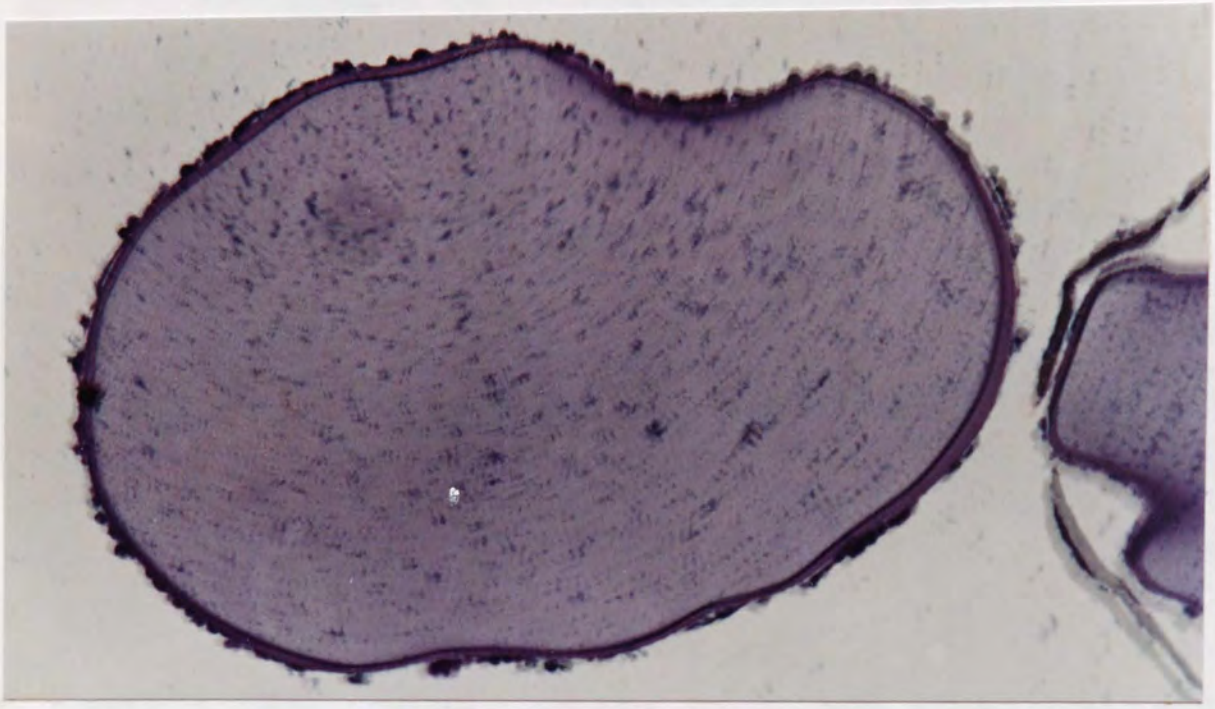
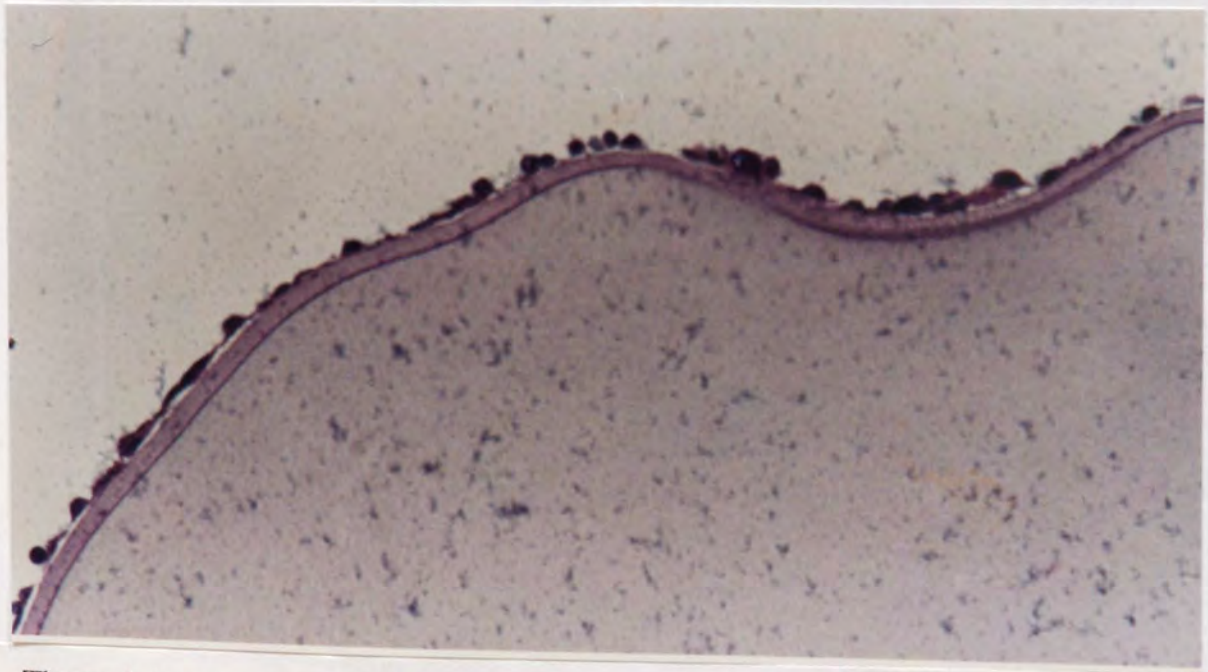
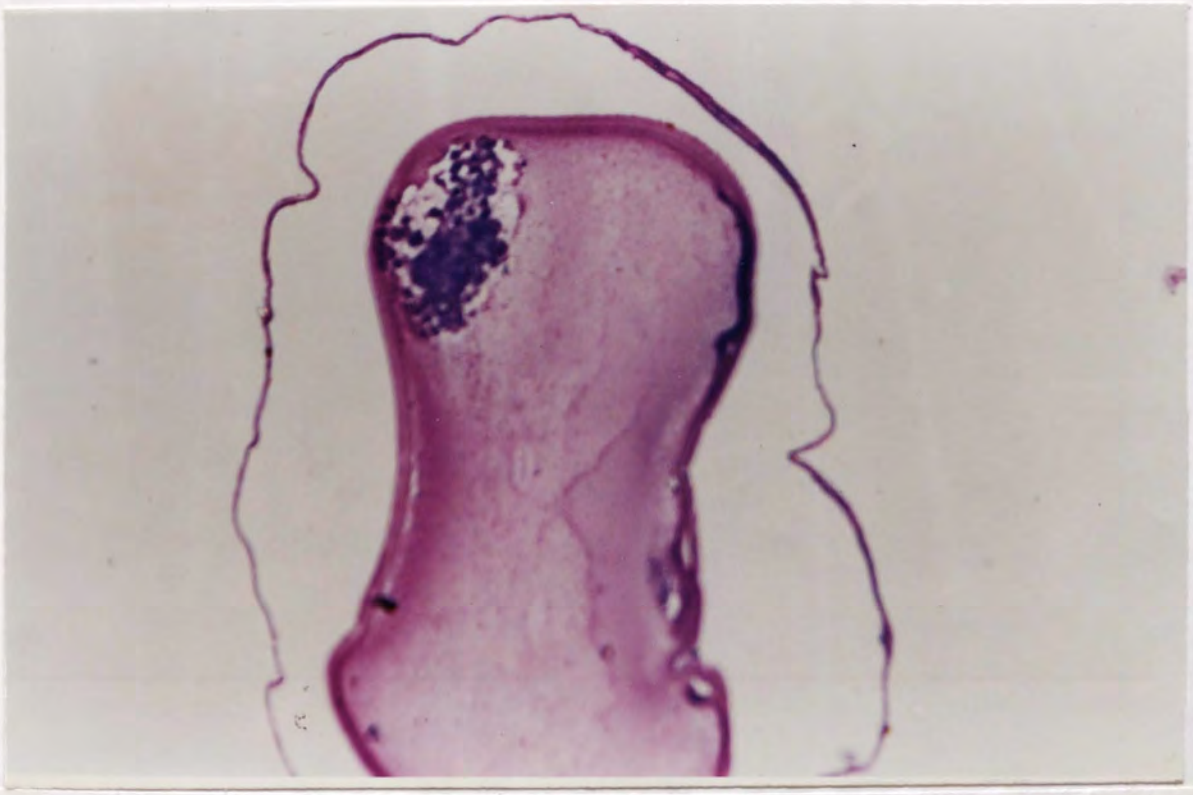


Plate 31b Closer examination of Plate 31a, showing a few rounded mononuclear cells on the surface of the capsule (H & E; x250)



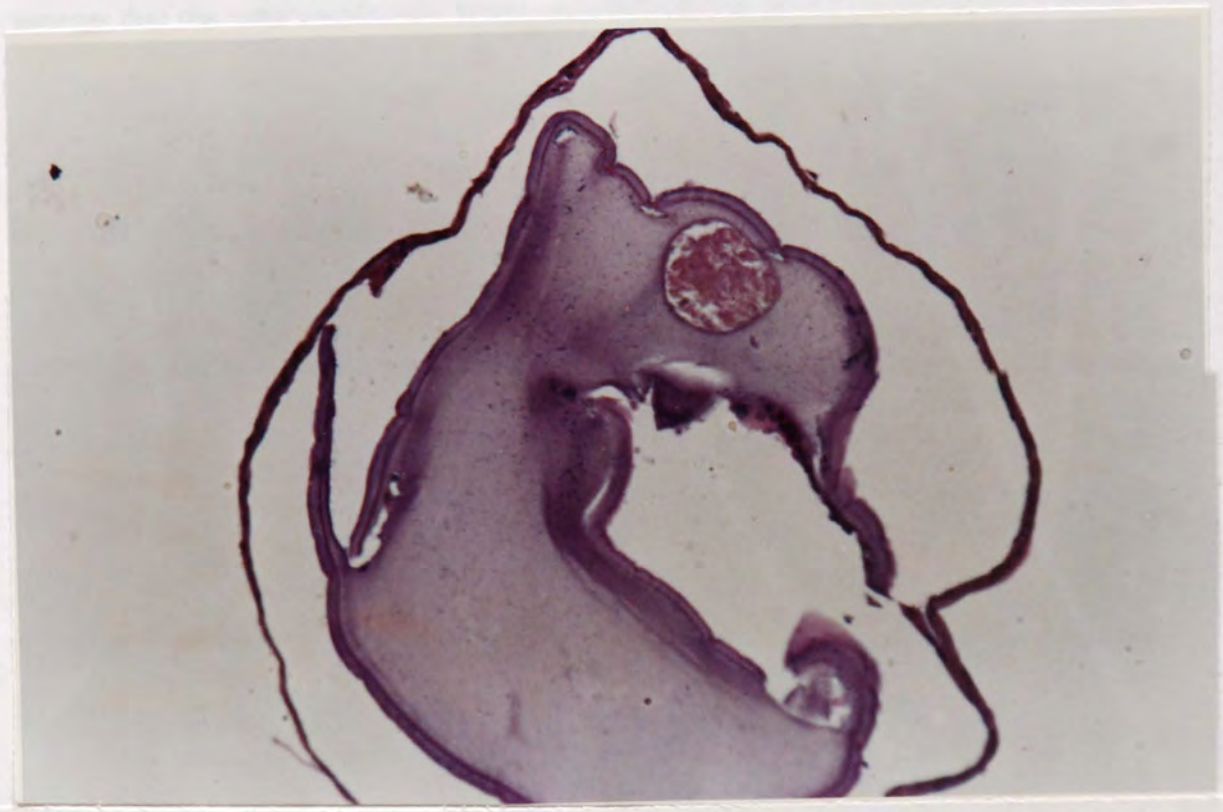
These plates are representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 32 Islet containing Na^+-alginate-PLL (no outer alginate layer) microcapsule from a STZ-diabetic mouse, showing significant pericapsular deposition, compared with empty microcapsule, Plate 30 (AF; x125)



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 33 A Na^+-alginate-PLO (no outer alginate layer) microencapsulated islet from a STZ-diabetic mouse, showing significantly less pericapsular deposition when compared with Na^+-alginate-PLO-alginate fabricated microencapsulated islets, Plate 26 (H & E; x125)



This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

The effect of cyclosporin A immunosuppression on pericapsular deposition (group 4)

In this group, microencapsulated islets were implanted intraperitoneally into STZ-diabetic mice immunosuppressed with cyclosporin A, given either intraperitoneally or subcutaneously, at a dose of 10 mg/kg/day for 14 consecutive days. Na^+-alginate-PLL-alginate and Na^+-alginate-PLO-alginate microencapsulated islets, recovered from the peritoneal cavity of cyclosporin A treated STZ-diabetic mice, showed little evidence of pericapsular reaction, and the contained islets appeared structurally intact, Plate 34. Thus it appears that the pericapsular deposition and infiltration of mononuclear cells is prevented by cyclosporin A immunosuppression. This observation was confirmed by concomitant spleen lymphocyte proliferation assay, which indicated that cyclosporin A could suppress T-lymphocyte proliferation although the difference was not statistically significant Table 22.

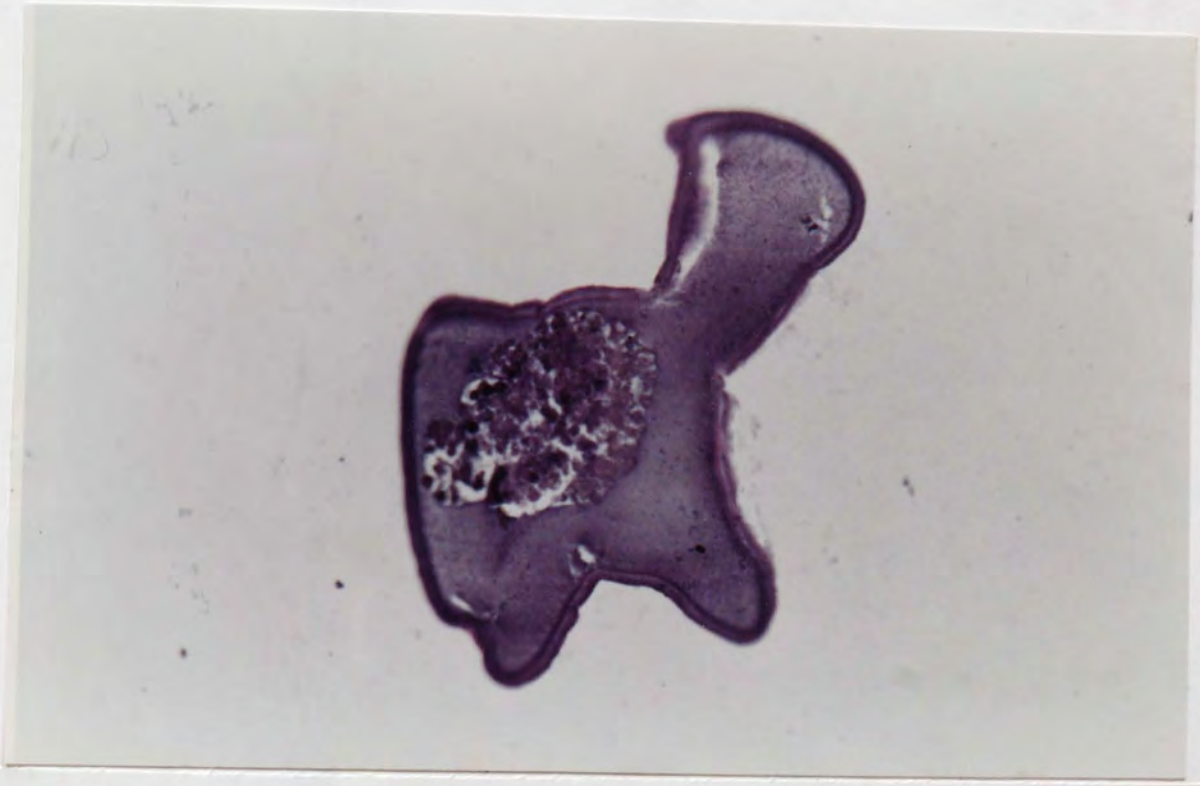
Na^+-alginate-PLO-alginate microcapsules retrieved from the peritoneal cavity of cyclosporin A immunosuppressed-STZ diabetic mice, were found to be clumped together, but associated with a reduced degree of pericapsular fibrosis compared with non-immunosuppressed STZ-diabetic mice, Plate 35. The microcapsules, although misshapen due to processing, were intact and there was no evidence of mononuclear cell infiltration into the microcapsules. By contrast, no islet containing Na^+-alginate-PLO-alginate microcapsules could be identified in any of the sections examined from non-immunosuppressed STZ-diabetic mice.

The subcutaneous administration of cyclosporin A to STZ-diabetic recipient mice, was less effective in preventing pericapsular fibrosis than with intraperitoneal administration of cyclosporin A, and some evidence of mononuclear cell deposition was observed around Na^+-alginate-PLL-alginate microcapsules, Plate 36. The lymphocyte proliferative response was only marginally suppressed by sc. administration of cyclosporin A, Table 22.

Table 22 PHA-induced spleen lymphocyte proliferation in control and immunosuppressed STZ-diabetic mice. (mean values ± SEM, n = 4)

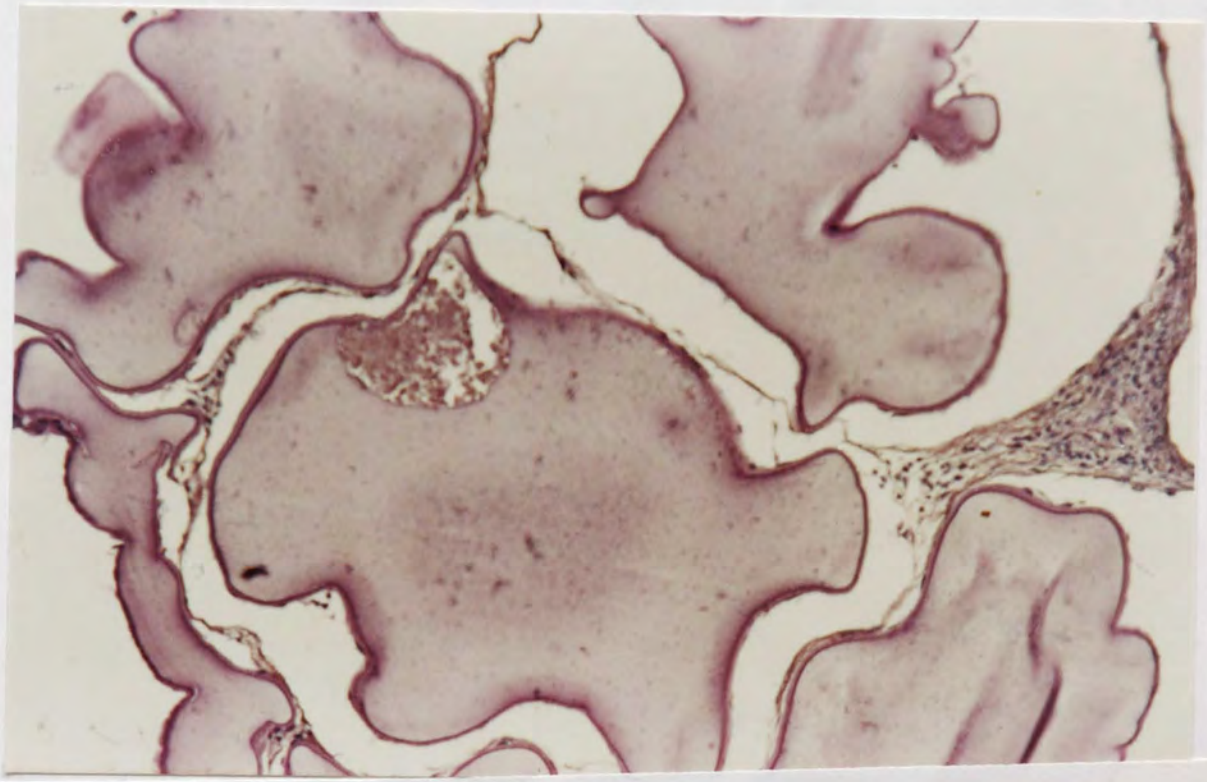
Treatment	Stimulation index	Days post-implantation
STZ-diabetic mice	2.1 ± 0.85	14
STZ-diabetic mice	2.5 ± 0.20	28
Control non-diabetic mice	2.9 ± 0.85	28
Cyclosporin A (ip) treated STZ-diabetic mice	1.6 ± 0.40	28
Cyclosporin A (sc) treated STZ-diabetic mice	1.8 ± 0.35	28
FK506 (1mg/kg/day for 4 days, short-term) treated STZ diabetic mice	2.4 ± 1.05	28
FK506 (0.15mg/kg/day for 14 days) treated STZ diabetic mice	2.1 ± 0.30	28
0.5 mmol/l L-NMMA-loaded microcapsules implanted into STZ-diabetic mice	2.8 ± 0.60	14
0.5 mmol/l L-NMMA-loaded microcapsules implanted into STZ-diabetic mice	2.6 ± 0.95	28

Plate 34 A Na^+-alginate-PLL-alginate microencapsulated islet from an interperitoneal cyclosporin A treated STZ-diabetic mouse, showing no evidence of pericapsular fibrosis. The islet appears to be intact. (H & E; x125).



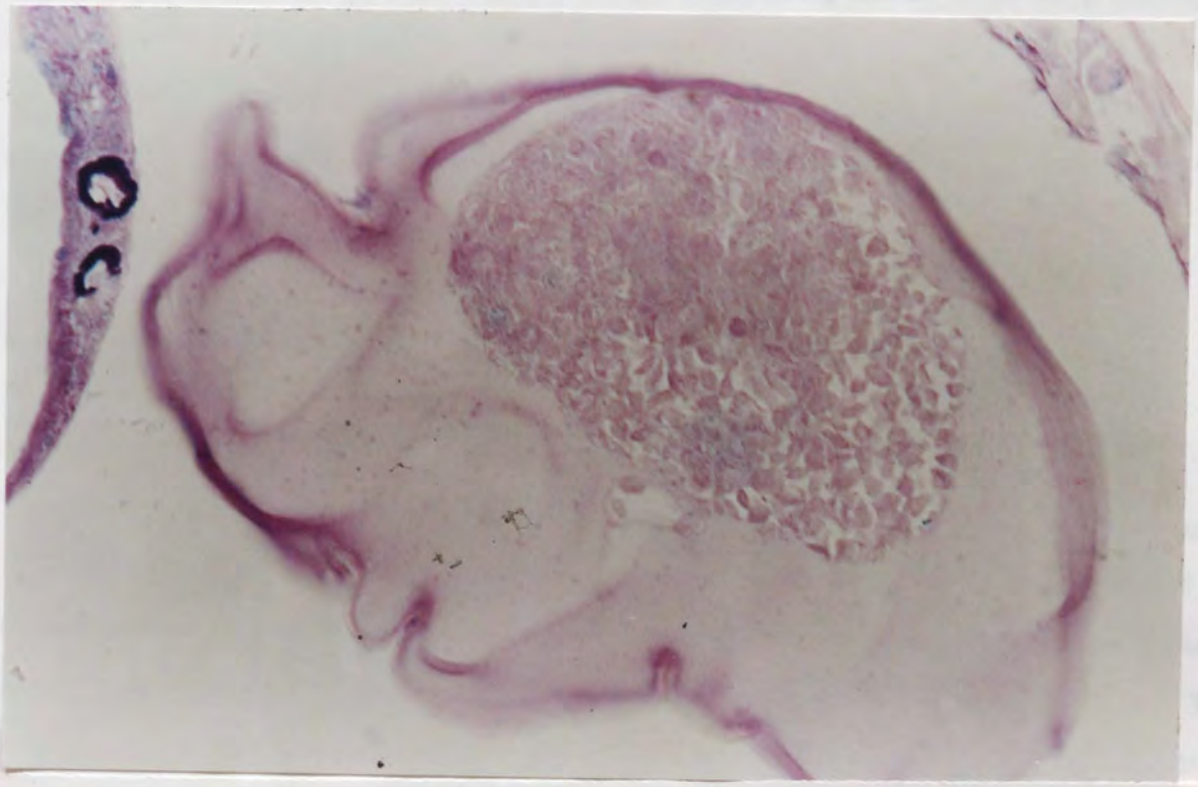
This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 35 Na^+-alginate-PLO-alginate microencapsulated islets from a cyclosporin A immunosuppressed STZ-diabetic mouse, showing little pericapsular deposition (H & E: x125).



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 36. Na^+-alginate-PLL-alginate microencapsulated islet from a subcutaneous cyclosporin A treated STZ-diabetic mouse, showing significant pericapsular fibrosis compared with Na^+-alginate-PLL-alginate microcapsules recovered from an interperitoneal cyclosporin A treated STZ-diabetic mouse, Plate 34, (AF; x250).



This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

The effect of FK506 immunosuppression on pericapsular deposition (group 5)

STZ-diabetic mice were treated with FK506 (im), either at 1.0 mg/kg/day on post operative days 0 to 3, or 0.15 mg/kg/day for 14 consecutive days. The microcapsules retrieved from 1.0 mg/kg/day short-term FK506 treated STZ-diabetic mice, were surrounded by multiple layers of inflammatory cells similar to those associated with microcapsules retrieved from non-immunosuppressed STZ-diabetic mice, Plates 37a and b. Short-term FK506 treatment appeared to have no significant effect on lymphocyte proliferation, Table 22.

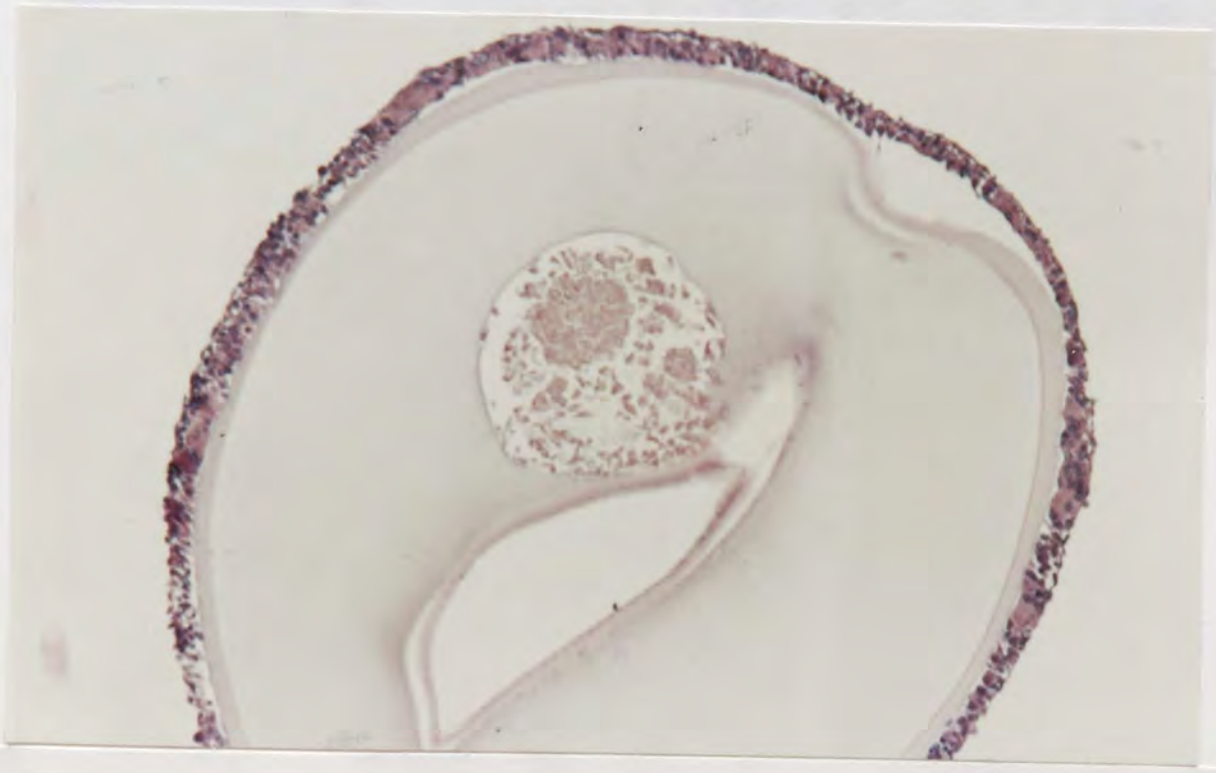
When FK506 was administered for an extended period of time, the extent of pericapsular deposition was reduced, Plates 38a & b, but the microencapsulated islets were somewhat disaggregated within the microcapsules, Plate 38a. The extended administration of FK506 had little effect on spleen lymphocyte proliferation, and presumably the extent of pericapsular deposition observed in the above studies resulted from inadequate FK506 immunosuppression.

The effect of L-NMMA loading into microencapsulated islets on pericapsular fibrosis (group 6)

In this study, 0.1 or 0.5 mmol/l L-NMMA-loaded Na^+-alginate-PLL-alginate microcapsules were implanted into STZ-diabetic mice, and the microcapsules were retrieved 14 and 28 days post implantation. 0.1 mmol/l L-NMMA-loaded microcapsules, retrieved after 14 days implantation, were enveloped with numerous layers of fibrotic tissue, Plate 39. Microencapsulated islets recovered after 28 days also showed an extensive multilayered pericapsular deposition, Plates 40a & b. The L-NMMA loaded microcapsules appeared to be more fragile than usual and had broken down in places, Plate 40b. This was probably the result of the swelling of the L-NMMA-loaded microcapsules when placed in sodium citrate buffer. The inside of the capsules also contained scattered mononuclear cells. These cells had presumably gained entrance via the break in the microcapsule membrane, Plate 40b. However, the islets present inside the microcapsules appeared to be structurally intact and still discreet. Similar results were obtained with 0.5 mmol/l L-NMMA-loaded microcapsules, and a thin pericapsular deposition was observed 14 days post implantation,

Plate 41. However, by 28 days the inflammatory response was extensive and many layers deep, Plates 42a & b. As observed with 0.1 mmol/l L-NMMA-loaded microcapsules, 0.5 mmol/l L-NMMA loaded microcapsules began to break up after 28 days, Plate 42, and infiltrating mononuclear cells could be observed inside the capsule membrane, Plate 42. Despite this, the structural integrity of the microencapsulated islet was maintained and no mononuclear cells were observed in the vicinity of the islets, Plates 42a & b. The concentration of L-NMMA used in the present study had no significant effect on lymphocyte proliferation, Table 22.

Plate 37a A Na^+-alginate-PLL-alginate microencapsulated islet from FK506 (1.0 mg/kg/day for 4 days) treated STZ-diabetic mouse. Note extensive pericapsular deposition similar to that seen on microcapsules retrieved from non-immunosuppressed STZ-diabetic mice (H & E; x125)



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 37b Closer examination of Plate 37a, showing pericapsular mononuclear cells and a disaggregated islet inside the capsule (H & E; x250).

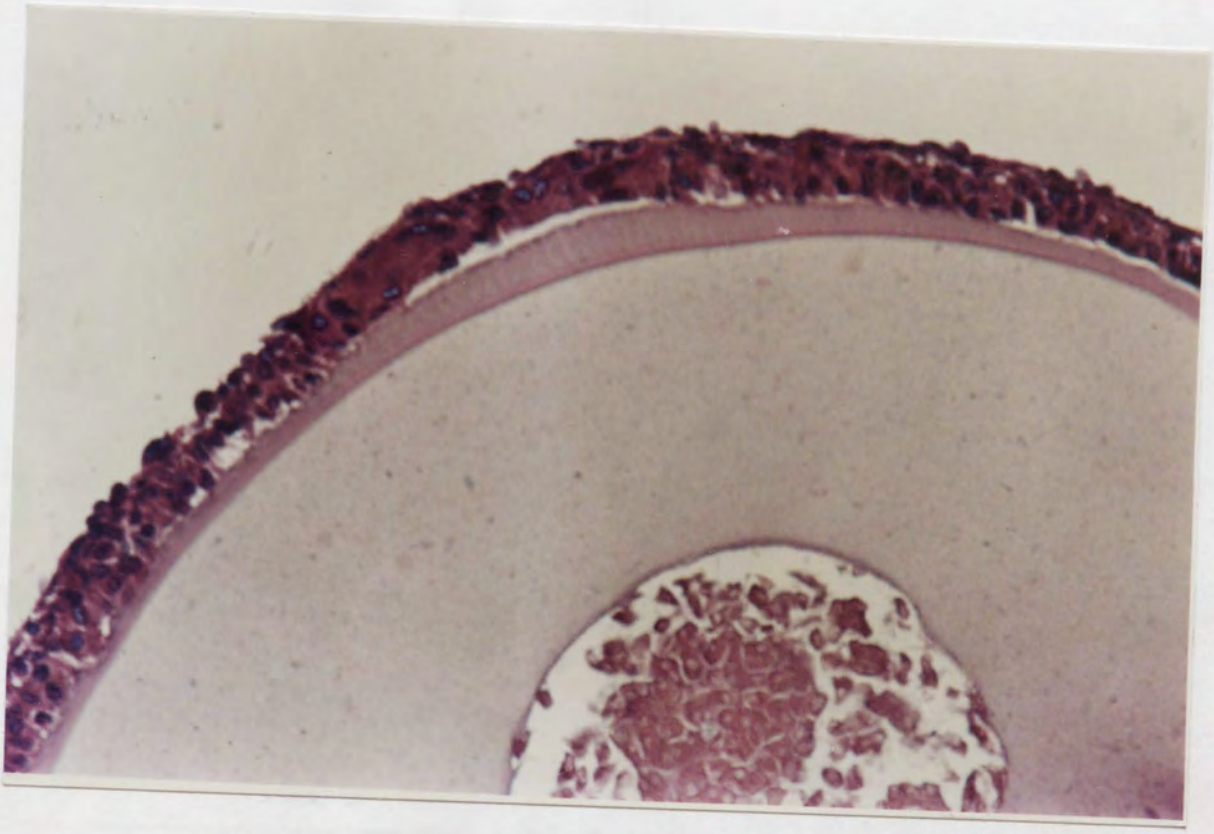
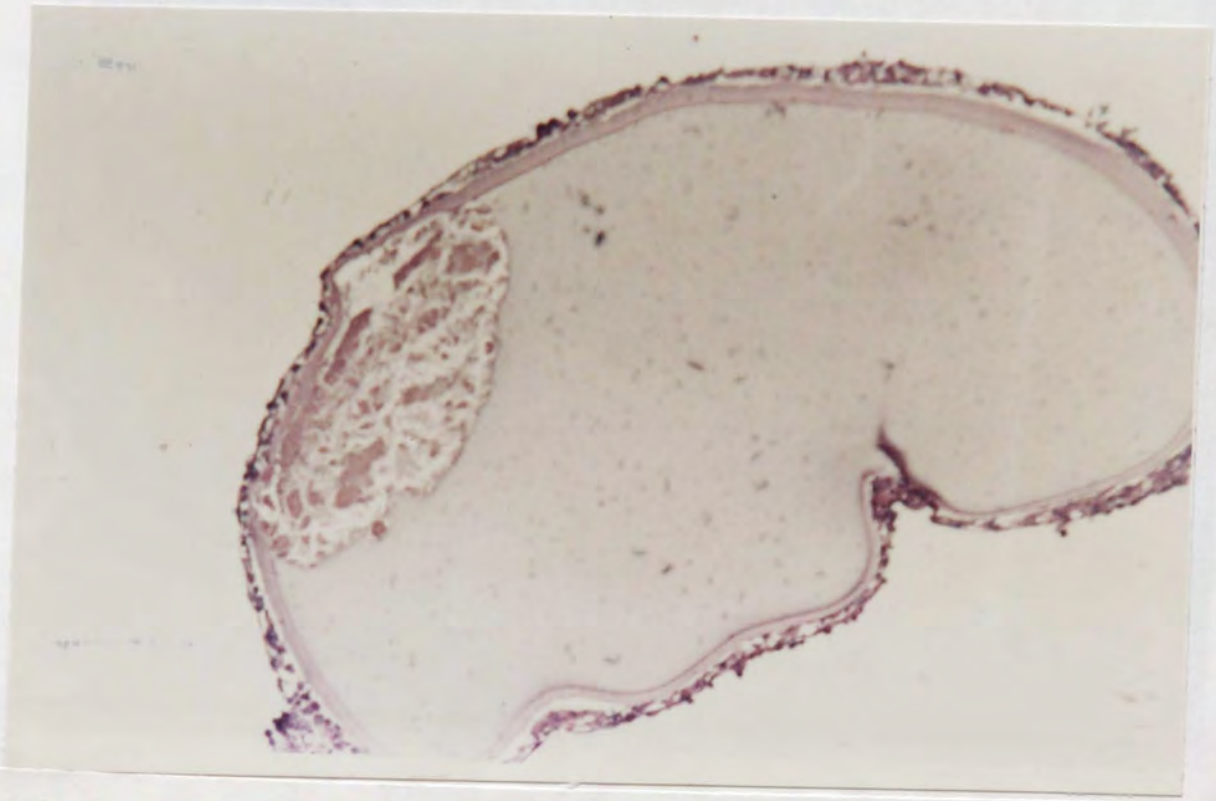


Plate 38a Na^+-alginate-PLL-alginate microencapsulated islet recovered from an FK506 (0.15 mg/kg/day for 14 days) treated STZ-diabetic mouse, showing significant pericapsular deposition (H & E; x125)



This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 38b Closer examination of Plate 38a, showing the pericapsular deposition of mononuclear cells despite FK506 treatment (H & E; x250)

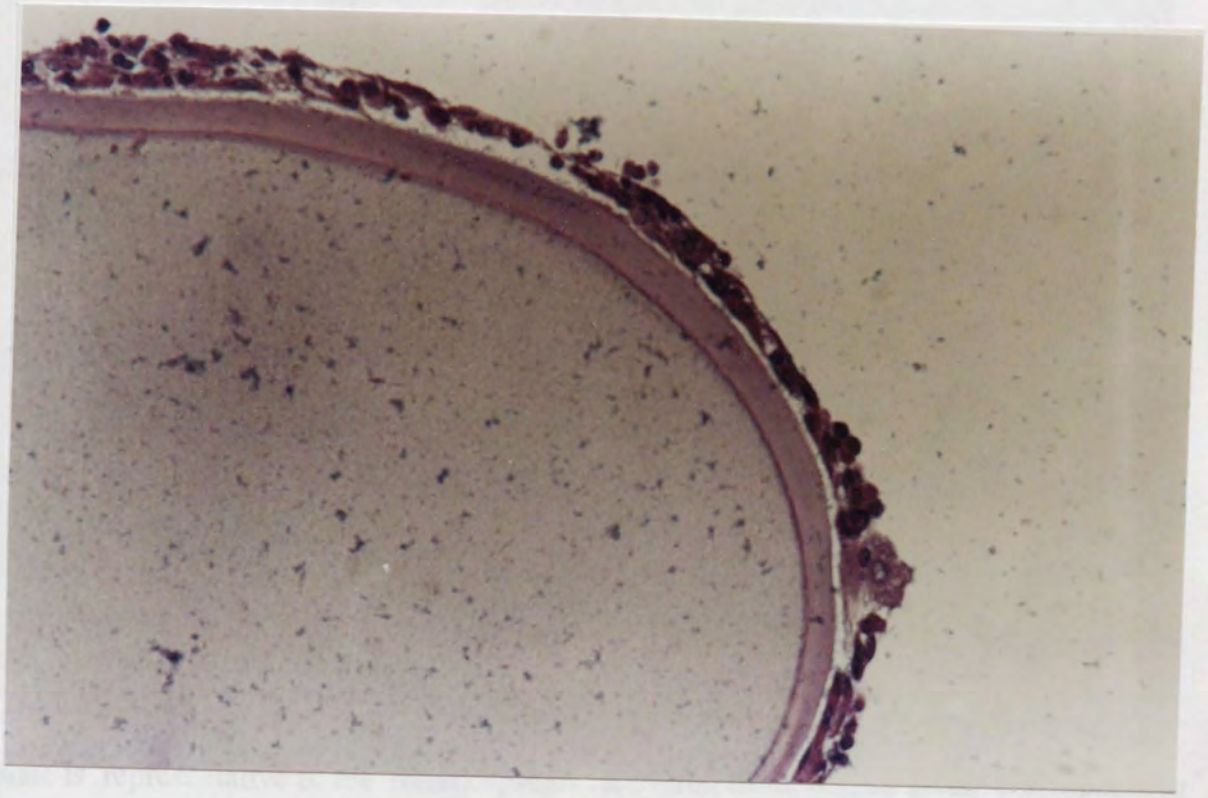
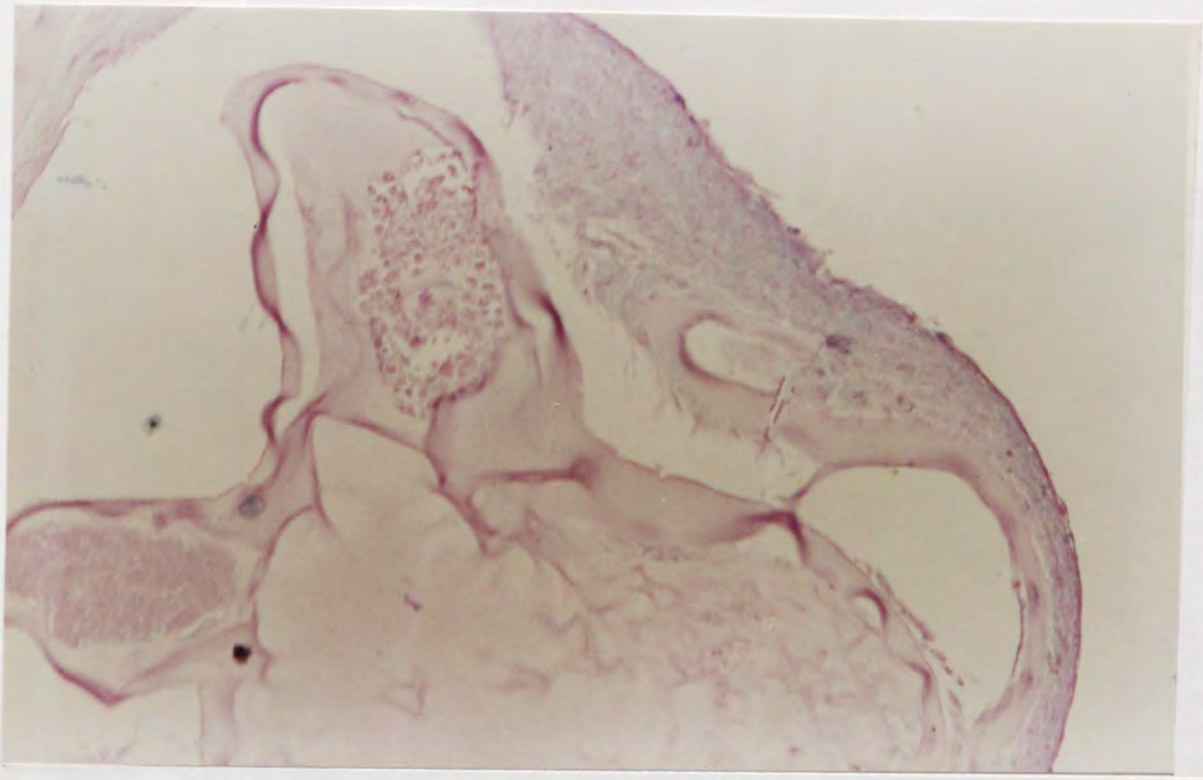
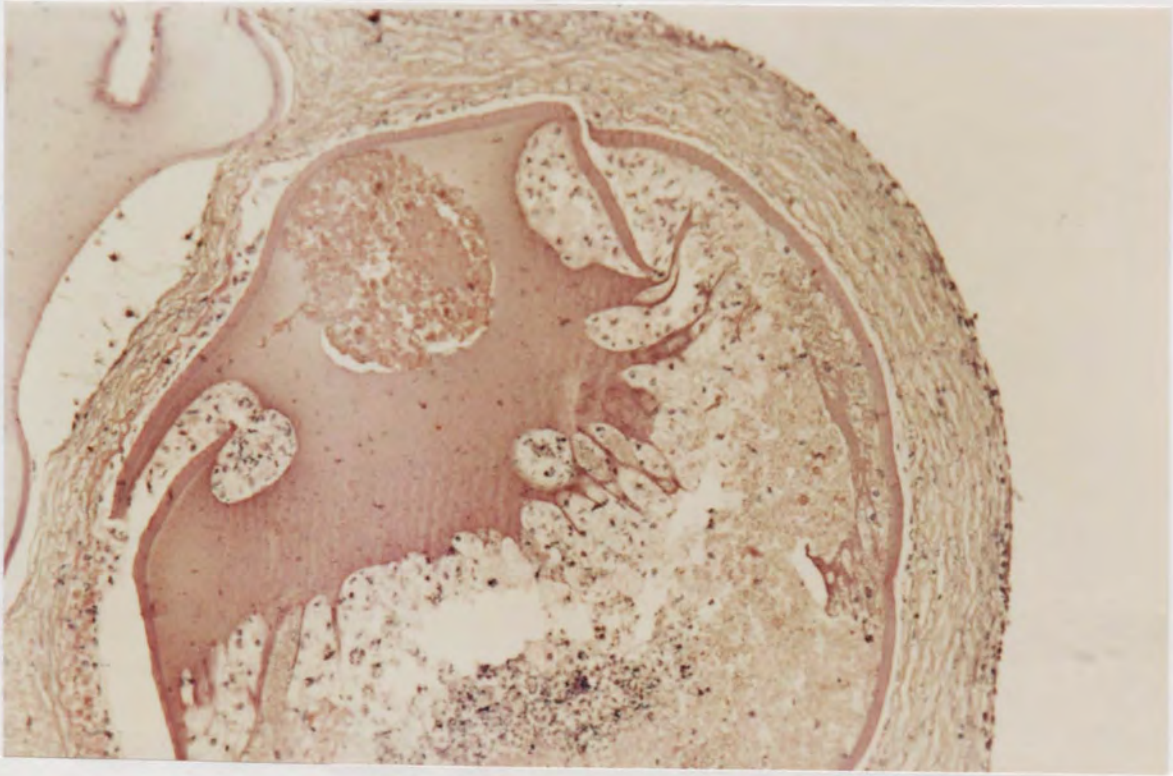


Plate 39 A 0.1 mmol/l L-NMMA loaded microencapsulated islet recovered from a STZ-diabetic mouse after 14 days implantation. The microcapsules were fragmented and were extensively overgrown with pericapsular cells (AF; x125)



This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 40a A 0.1 mmol/l L-NMMA-loaded microencapsulated islet from a STZ-diabetic mouse after 28 days implantation. L-NMMA incorporation into the encapsulating alginate did not suppress pericapsular deposition, but the structural integrity of the islet is well preserved (H & E; x125).



This plate is representative of the microcapsules recovered from 4 mice given corresponding treatment

Plate 40b Closer examination of Plate 40a, showing a discontinuity in the capsule membrane and the infiltration of mononuclear cells, although the islet appears intact (H & E; x250).

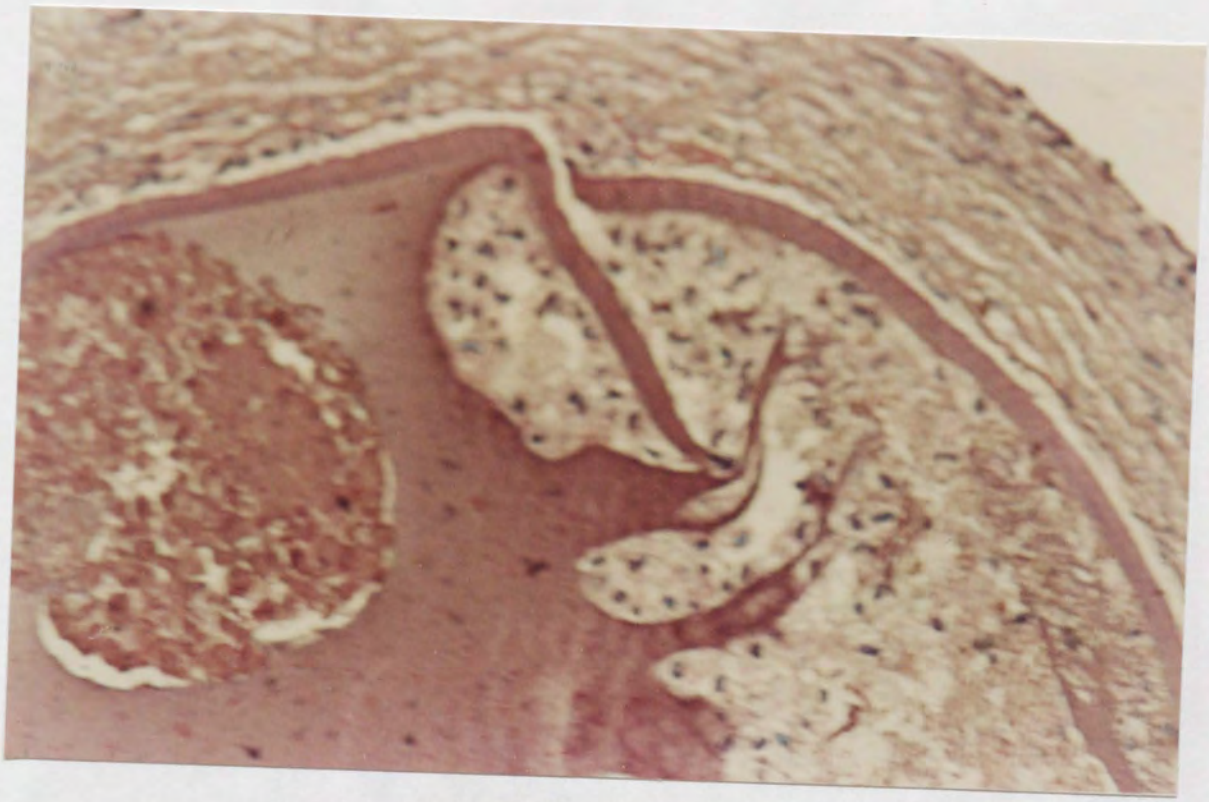
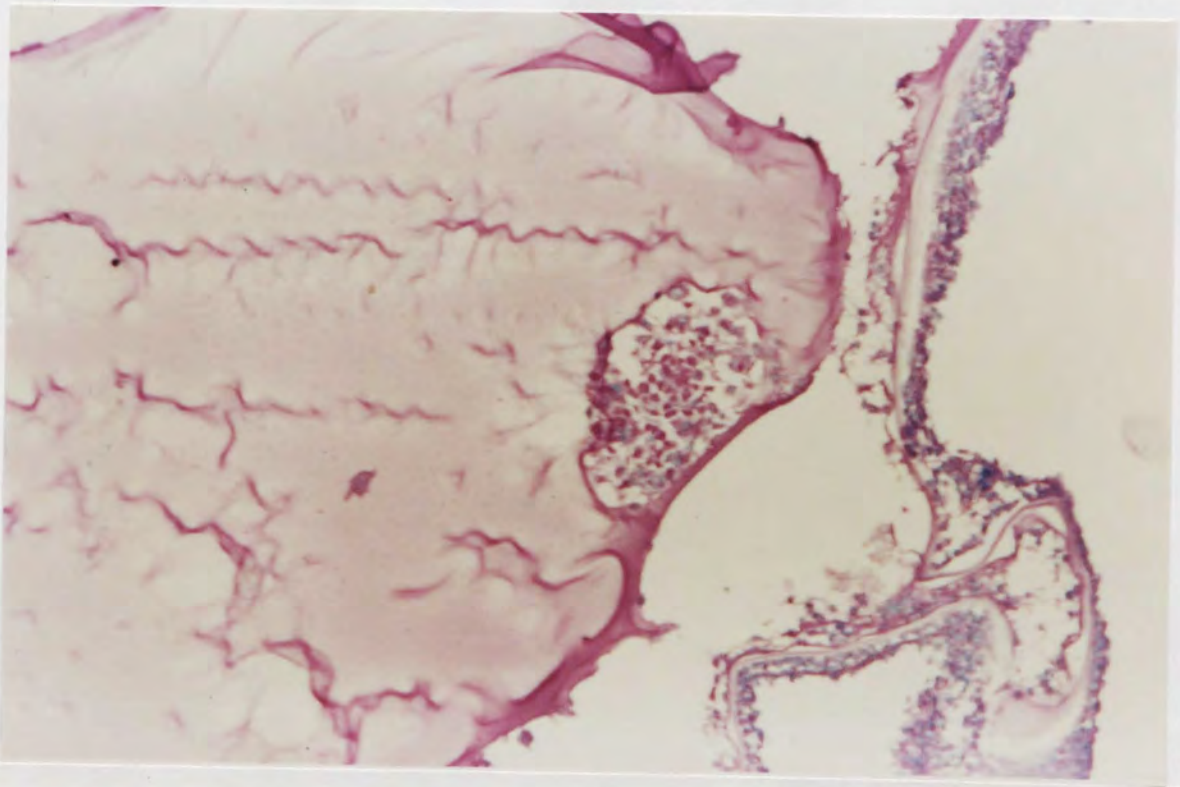
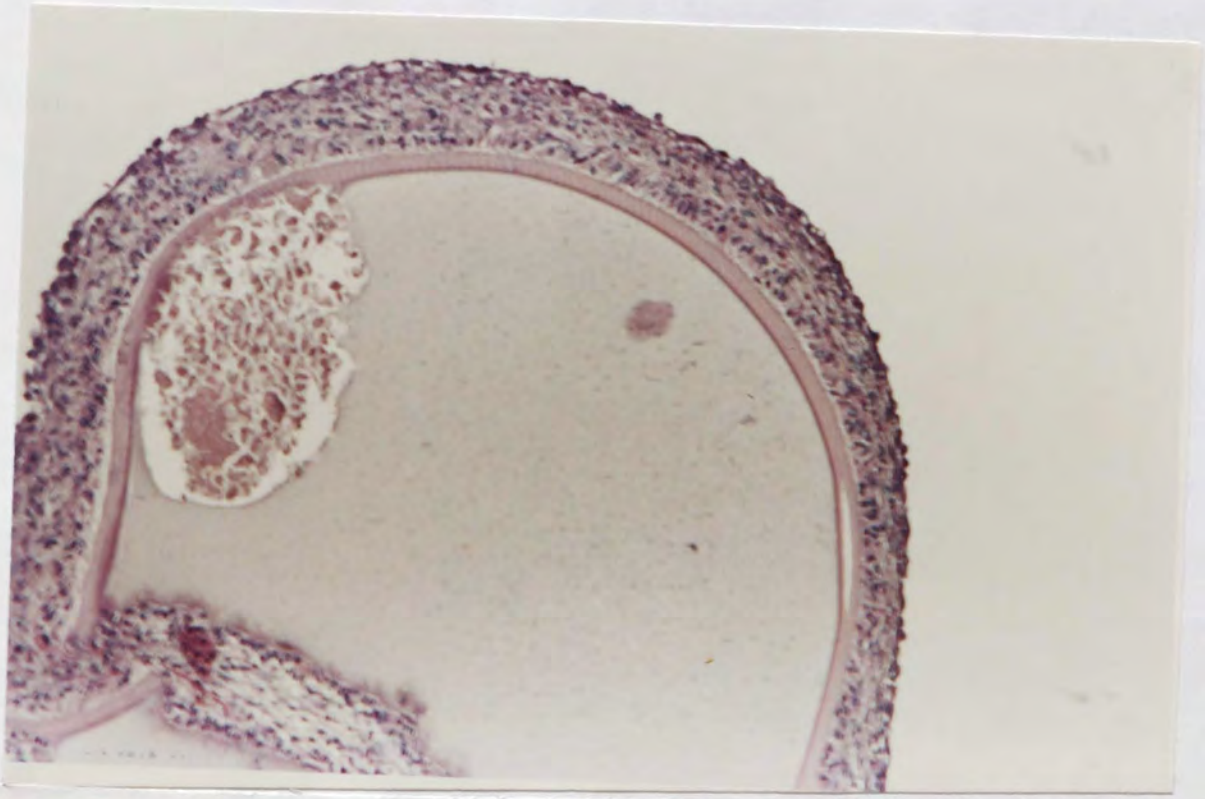


Plate 41 A 0.5 mmol/l L-NMMA-loaded microencapsulated islet recovered from a STZ-diabetic mouse after 14 days implantation. The islet is essentially intact but there is extensive pericapsular deposition (AF; x125).



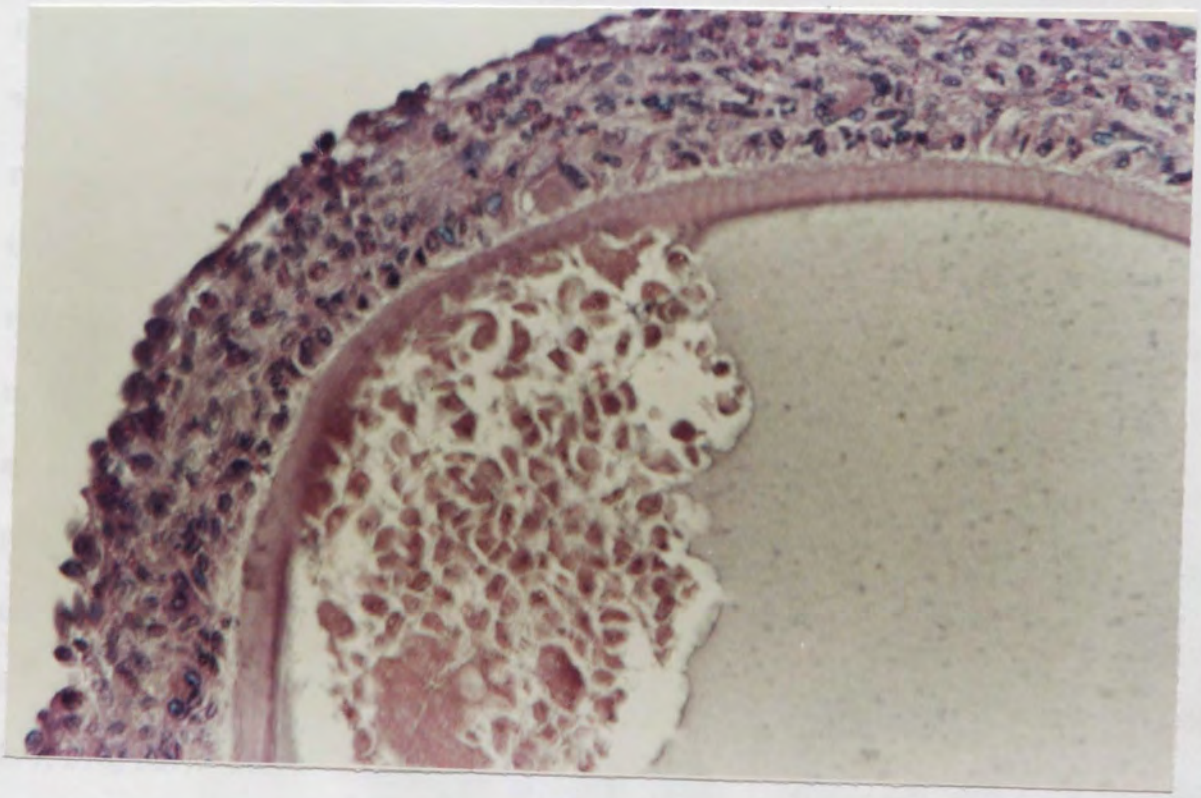
This plate is representative of the microcapsules recovered from 3 mice given corresponding treatment

Plate 42a A 0.5 mmol/l L-NMMA-loaded microencapsulated islet from a STZ-diabetic mouse after 28 days implantation. The microcapsule is ruptured and has allowed the ingress of mononuclear cells (H & E; x125).



This plate is representative of the microcapsules recovered from 5 mice given corresponding treatment

Plate 42b Closer examination of Plate 42a, showing extensive pericapsular deposition and structurally intact islet within the microcapsule (H & E; x250).



DISCUSSION

To date, only one group has succeeded in maintaining normoglycaemia in STZ-diabetic rats for up to one year with an allograft of Na^+-alginate-PLL-alginate microencapsulated islets (175, 176, 178). Most attempts have resulted in the deposition of pericapsular mononuclear cells on the surface of retrieved microcapsules irrespective of the fabrication materials used to generate the microcapsule membranes (187, 189, 190, 194, 267, 278). A pericapsular reaction was also observed in the present studies when microencapsulated islets, fabricated with Na^+-alginate (DJB)-PLL or PLO-alginate, were implanted into STZ-NMRI mouse recipients. On the other hand, empty Na^+-alginate-PLL or PLO-alginate microcapsules implanted intraperitoneally into either control non-diabetic or STZ-diabetic mice elicited little mononuclear cell overgrowth 28 days after implantation. However, the insertion of NMRI mouse islets into the microcapsules markedly increased the degree of pericapsular fibrosis around the microcapsules when implanted into STZ-diabetic mice. This suggested that the presence of islets is involved in triggering the pericapsular inflammatory response. This is consistent with the results of other investigators, who have confirmed that the target for the inflammatory response was the islets rather than the capsule fabrication material (187, 190, 194, 278, 287). Cole and colleagues have shown that when microencapsulated islets were transplanted into STZ-diabetic rats, all grafts failed within 14 days and retrieved microcapsules were surrounded by a foreign body reaction and the microencapsulated islets had started to disintegrate (189). Intact islets and soluble insulin have been confirmed as chemoattractants for macrophages (287). So the presence of islets within the microcapsules would be expected to increase the intensity of the pericapsular foreign body reaction observed in the present studies. A limited inflammatory reaction was also observed on empty microcapsules, however, the deposition of cells was relatively light compared with that on capsules containing islets, suggesting however, that it is not just the presence of

islets that will trigger pericapsular deposition. Soon Siong and co workers have suggested that it is the capsule per se that is the target for the inflammatory response rather than the encapsulated islets (288).

Na^+-alginate-PLO-alginate microencapsulated islets recovered from control non-diabetic and STZ-diabetic mice were generally clumped together and more severely overgrown than Na^+-alginate-PLL-alginate fabricated microcapsules. Certainly, PLO coated microcapsules showed surface damage on retrieval and often no islets were found inside the microcapsules. Poly-L-ornithine coated microcapsules had rather poor mechanical strength and poor biocompatibility compared with poly-L-lysine coated microcapsules. The poor biocompatibility and mechanical strength may be related to the increased porosity of PLO membranes (231). Similar observations have also been reported by other workers (177, 191, 289). Calafiore and co workers have indicated that the pericapsular inflammatory response to poly-L-ornithine coated microencapsulated islets was dense and multilayered 10 days after implantation (191) and more comprehensive than the pericapsular deposition observed on poly-L-lysine coated microcapsules.

The implantation site chosen can also significantly influence the rate and extent of pericapsular overgrowth. Microencapsulated islets elicited the strongest inflammatory response when implanted subcutaneously. Microcapsules retrieved by dissection from subcutaneous site were clumped, squashed and massively overgrown with pericapsular mononuclear cells irrespective of the microcapsule composition. The embedded microcapsules were also associated with collagen fibres and small capillaries (neovascularisation) were present in the surrounding pericapsular connective tissue. Activated macrophages and T-lymphocytes have been shown to release soluble angiogenic substances which promote neovascularisation (290). In the present work, the poor results

of subcutaneous implantation studies are in contrast to those obtained by Lacy and coworkers (291), who showed that microencapsulated rat islets implanted subcutaneously into STZ-diabetic mice were well tolerated and biocompatible. The reasons for this may have been that Lacy and colleagues enclosed Na^+-alginate immobilized microcapsules in a bio-acrylic-copolymer hollow fibre which had enhanced biocompatibility. It is well known, that the peritoneal cavity has an enhanced ability to generate a foreign body reaction. Considerable success has been achieved with the transplantation of free islets when they are lodged in the liver or implanted in immunoprivileged sites such as under the kidney capsule. However, the present fabrication technology does not allow the production of microcapsules small enough to permit implantation either under the kidney capsule or in the liver in sufficient numbers.

In the present work, when the outer alginate layer was omitted from empty microcapsules, little or no pericapsular deposition was observed. This suggested that the outer alginate layer contributed significantly to the genesis of the inflammatory reaction. It may well be that the actual composition of the alginate might be an important factor in the generation of the pericapsular fibrotic reaction and different alginate polymers have been shown to activate the complement cascade (230, 288).

Alginates are polysaccharides composed of mannuronic acid and guluronic acid interspaced with regions of mixed sequence (292). It has been reported, that the mannuronic acid and mixed sequences are potent stimulators of cytokine production. Low guluronic acid alginate has been shown to be 10 times more potent in inducing cytokine production than high guluronic acid alginate (230). Soon Siong and colleagues have shown that implanted alginate microcapsules with low mannuronic acid content were largely free of pericapsular fibrosis, while 90% of microcapsules with high mannuronic acid content showed

mononuclear cell and fibroblast overgrowth after 21 days implantation (288). However, Clayton and colleagues have demonstrated that high mannuronic acid alginate microcapsules provoke a weak inflammatory response (277). Soon Siong and co workers suggested that the appearance of an inflammatory response depended on the purity and composition of the alginate used in fabrication (288). When Clayton and co workers, used purified dialysed alginate, free of protein impurities, in fabrication the implanted microcapsules were free of capsular overgrowth (personal communication). In the present work, the presence of an outer alginate layer (high in guluronic acid) appeared to encourage pericapsular deposition as the alginate used in the present studies was not dialysed. It is clear that the purity of Na^+-alginate used in fabrication is a critical parameter in determining microcapsule biocompatibility.

Of the two immunosuppressive agents used, only cyclosporin A showed any inclination towards the generation of systemic immunosuppression in STZ-diabetic mice as determined by spleen lymphocyte proliferation assay. Neither FK506 nor L-NMMA had any significant effect on the stimulation index.

Na^+-alginate-PLL-alginate microcapsules recovered from ip. cyclosporin A immunosuppressed STZ-diabetic mice showed no signs of pericapsular fibrosis, while Na^+-alginate-PLO fabricated microcapsules were coated with a single discontinuous mononuclear cell layer. This suggested that poly-L-ornithine was some what more immunogenic than poly-L-lysine. When cyclosporin A was given subcutaneously, there was a marginal reduction in the spleen lymphocyte proliferation index, but retrieved microcapsules were coated with significant pericapsular mononuclear cells. It may well be that the concentration of cyclosporin A given subcutaneously was too low to induce significant immunosuppression.

Short term administration of FK506, even at high concentration (1mg / Kg) was unable to prevent significant pericapsular mononuclear cell deposition on the surface of microencapsulated islets retrieved from the peritoneal cavity of STZ-diabetic mice. On the other hand, microencapsulated islets retrieved from STZ-diabetic mice immunosuppressed with FK506 at a concentration of 0.15mg/Kg/d for 14 consecutive days showed little pericapsular deposition, although the islets themselves appeared disaggregated and vacuolized. There is some evidence to suggest that FK506 per se is capable of influencing β-cell function and causing morphological islet cell damage (130).

Nitric oxide generated by inflammatory macrophages is a potent toxic product (202, 248, 263). β-cell lysis caused by interleukin 1β is thought to be mediated via the formation of nitric oxide by nitric oxide synthase (248). The incorporation of the nitric oxide synthase inhibitor L-NMMA into microcapsules did not prevent excessive pericapsular mononuclear cell deposition around the microcapsules. However, the microencapsulated islets themselves appeared well preserved and structurally intact. This might have been the result of a local inhibition of nitric oxide production. In addition, L-NMMA loaded microcapsules retrieved from STZ-diabetic mice appeared to be quite fragile and broken in places allowing the infiltration of lymphocytes. There is some evidence to suggest that the loading of the capsules with L-NMMA made them swell and become more fragile. However, since the morphological integrity of the islets within microcapsules seemed to have been maintained, a combination of conventional short-term systemic immunosuppression using cyclosporin A with the loading of L-NMMA into the microencapsulating agent might be considered for islet transplantation.

CHAPTER 6

GENERAL DISCUSSION

The work presented in this thesis has demonstrated the efficacy of differently fabricated microencapsulated islets in vitro and in vivo in terms of their insulin secretory capacity and implantation biocompatibility.

Islets have been conveniently isolated from NMRI mice by collagenase digestion and identified by dithizone staining. A variety of microencapsulation techniques have been developed and modified in order to facilitate the microencapsulation of isolated islets in either Na^+-alginate-PLL/PLO-Na^+-alginate, Ba^{2+}-alginate±PLL/PLO or agarose gels. Initial work directed towards the characterisation and comparison of insulin release from both free non-microencapsulated and all types of microencapsulated islets demonstrated that islets responded significantly to glucose challenge both in the absence and presence of theophylline with a significant stimulation in insulin secretion after both short-term (2 hours) and extended (8, 16 and 24 hours) static incubation. This potentiation of insulin release confirmed that, following isolation and microencapsulation, the isolated islets remained functionally viable. Insulin secretion from microencapsulated islets was qualitatively similar to that from free non-microencapsulated islets, although the quantitative insulin response of microencapsulated islets to glucose was significantly reduced. This reduction in insulin release was almost certainly due to the creation of a diffusion barrier around the islets and an increased path length for glucose / insulin exchange from the microencapsulated islets. This observation confirmed the similar findings of many other groups (162, 182, 210, 228). Chicheportiche and Reach demonstrated a very poor in vitro insulin response by microencapsulated islets to glucose challenge compared with that from free non-encapsulated

islets (162). In addition, they demonstrated that the insulin secretory response of microencapsulated islets to secretagogues depended on the size and thickness of the microcapsule wall which acted as a diffusion barrier around the islets. These observations go some way towards explaining why the number of transplanted microencapsulated islets, required to correct diabetes and maintain normoglycaemia is much higher i.e. about 3000-5000, than the number of transplanted free non-microencapsulated islets required i.e. about 1000. Besides thickness, the type of fabrication components used to generate the capsule wall profoundly influences the insulin secretory response of the islet graft to glucose challenge. In the present work, Ba^{2+}-alginate coated islets generated by a ficoll density gradient method (211) produced a very tight membrane coated islet which greatly improved the insulin response to glucose challenge. However, the main drawback of this method was the low microencapsulation efficiency which was only about 20% and rather wasteful on isolated islets. Ba^{2+}-alginate membrane coated islets were significantly more responsive to elevated glucose, irrespective of the time of exposure, than either Na^{+}-alginate-PLL/PLO-alginate, agarose or Ba^{2+}-alginate microencapsulated islets generated by the conventional syringe droplet method and almost attained the insulin response to elevated glucose shown by free non-microencapsulated islets after 8 hours extended incubation. The Ba^{2+}-alginate membrane coating of islets provides superior and responsive microencapsulated islets for use in islet transplantation. The diffusion of insulin, glucose and oxygen through the microcapsule is proportional to the membrane surface area and there is an inverse correlation between the volume of the islet containing compartment and the diffusion distance (292a). Lum and co workers, reported that the transplantation of rat islets contained in small Na^{+}-alginate/PLL/alginate microcapsules (0.2-0.3mm) could maintain normoglycaemia in STZ-diabetic mice for about 200 days (184). They attributed this to the greater mobility of small microcapsules within the peritoneal cavity and suggested that this served to reduce the chance of microcapsules becoming deformed and damaged. An adequate oxygen supply is

essential if islets are to function satisfactorily within the microcapsule (293). Schrezenmeir and co workers (1992) have observed central necrosis in microencapsulated islets, a typical sign of hypoxic damage (293). The viability of islet and their insulin response to elevated glucose is very sensitive to hypoxia (294), which suggests that oxygen may be a limiting factor in the ability of microencapsulated islets to respond to high glucose.

In the present study, poly-L-ornithine membrane coated microcapsules facilitated a more substantial insulin response to elevated glucose than poly-L-lysine coated microencapsulated islets. Poly-L-ornithine membranes have been shown to have greater porosity than poly-L-lysine membranes and will maintain human microencapsulated islets in a viable, functional condition for an extended period of time (231).

The outer alginate layer was added to Na^+-alginate microencapsulated islets in a bid to improve biocompatibility at the expense of increasing the diffusion distance for insulin release (178). It was, therefore, no surprise that when the outer alginate layer was omitted from Na^+-alginate-PLL/PLO microencapsulated islets, their insulin response to glucose challenge significantly improved. Clearly, the outer alginate layer was acting as a significant barrier to the diffusion of glucose and insulin.

Whilst the insulin response of microencapsulated islets to glucose challenge had been well characterized <u>in vitro</u>, in static incubation, the kinetics of the insulin response from perifused microencapsulated islets <u>in vitro</u> was poorly understood. Certainly, Na^+-alginate-PLL and PLO-alginate and Ba^{2+}-alginate microencapsulated islets responded to an increased glucose concentration in the perifusion medium. However, in most cases the initial insulin response was delayed and reduced compared with perifused free non-encapsulated islets, and a well defined biphasic response was rarely observed. Similar results have been observed by other workers (159, 182). Perifused agarose microencapsulated islets produced a sluggish

response to glucose challenge, which was reduced, ill defined and probably the result of poor diffusion properties of the agarose gel capsule.

The prospect of adding L-NMMA to the microencapsulating alginate to inhibit the production of nitric oxide by pericapsular mononuclear cells and therefore protect microencapsulated islets from interleukin-1β induced lysis meant that the effect of L-NMMA had to be determined on glucose stimulated insulin release in vitro from both free and microencapsulated islets. Evidence from the literature had suggested that the effect of L-NMMA on insulin release might be dose dependent (248, 251). Subsequent investigation confirmed that 0.1 and 0.5 mmol/l L-NMMA had no significant effect on glucose stimulated insulin release from either free non-encapsulated or Na^+-alginate-PLL-alginate microencapsulated islets. However, treatment of both free and microencapsulated islets with 1.0 mmol/l L-NMMA resulted in a significant inhibition of glucose stimulated insulin release both in the presence and absence of 0.2 mmol/l L-arginine. The inhibition of insulin release from both free and microencapsulated islets by the high concentration (1.0 mmol/l) of L-NMMA was most marked in the presence of high glucose (16.7 mmol/l). It is possible that high glucose might increase the metabolism and / or synthesis of endogenous L-arginine and in turn increase nitric oxide production leading to the destruction of β-cells and elimination of insulin secretion. On the other hand, glucose induced increases in the cellular levels of Ca^{++}, free fatty acids and lipoxygenase derived arachidonic acid metabolites might modulate the activity of guanylate cyclase (295). Since glucose metabolism has been shown to enhance the production of NADPH in β-cells (239) and NADPH is required by the soluble L-arginine dependent nitric oxide synthase of vascular endothelium, macrophages and brain for activity (296), a redox mechanism may exist which might mediate the L-arginine activation of guanylate cyclase in β-cells. Studies have shown that in rats made L-arginine deficient, glucose stimulated insulin release is inhibited (297), and in islets from starved

rats, cGMP levels and the cGMP response to glucose stimulation are reduced (298) suggesting that endogenous L-arginine metabolism is important in the regulation of insulin secretion. The incorporation of either 0.1 or 0.5 mmol/l L-NMMA into the encapsulating alginate had no deleterious effect on glucose stimulated insulin release in vitro from microencapsulated islets confirming the suitability of L-NMMA loaded microcapsules for the in vivo transplantation of islets.

Over the last few years, work directed towards the viable transplantation of immunoprotected microencapsulated islets has been hampered by the relative immunogenicity of the graft and the appearance of pericapsular mononuclear cell deposition on the surface of the capsules leading to the destruction of the transplant. The intraperitoneal implantation of Na^+-alginate-PLL/PLO-alginate microencapsulated islets for 28 days, in STZ-diabetic mice leads to the near total degeneration of the microencapsulated islet and the appearance of an extensive pericapsular fibrosis. Microencapsulated islets fabricated with a coating of poly-L-ornithine were more susceptible to this fibrosis than microencapsulated islets containing poly-L-lysine. Previous studies have shown that poly-L-ornithine membranes have a higher permeability and a higher porosity than poly-L-lysine membranes (231). The poor biocompatibility of poly-L-ornithine membranes may be related to their increased porosity.

The extent and severity of microcapsule pericapsular fibrosis appears to depend on the transplant site chosen. In the present work, a massive pericapsular deposition composed of mononuclear cells, fibroblasts collagen fibres and some neovascularization was associated with microencapsulated islets implanted subcutaneously into the nape of the neck of STZ-diabetic mice. It was clear, that the site would be unsuitable for future implantation studies. Whilst immunoprivileged sites such as the testes (73), renal capsule (70), rectum (299) and

cisterna magna (74) may provide a better environment for the survival of transplanted microencapsulated islets. Only the rectum (299) could accommodate sufficient microencapsulated islets for the normalisation of diabetes in man. The use of such approaches on a routine basis in studies using mice is not feasible and the intraperitoneal site was chosen for the majority of the studies described in the present work.

The purity and chemical composition of Na^+-alginate used in microencapsulation is of great importance with regard to the biocompatibility of the final microcapsule. In recent years it has become clear that the success of the Na^+-alginate microencapsulation procedure is limited by the poor biocompatibility of the type of alginate used to form the outer layer of the microcapsules (230, 288). A high mannuronic acid content has been shown to be a significant factor with respect to the stimulation of cytokine production and the initiation of an inflammatory response (230, 288). However, Clayton and co workers, have shown that high mannuronic acid alginate capsules provoke a weak inflammatory response (277). In the present studies, although high guluronic acid alginate was used, the exact proportion of guluronic to mannuronic components was not known. It is clear, that high guluronic acid alginate, used to form the outer layer in the microencapsulation process, also attracts pericapsular deposition. When Clayton and colleagues, used purified dialysed alginate, the implanted microcapsules were largely free of pericapsular deposition (personal communication). Future microencapsulation procedures will need to employ dialysed alginate to avoid excessive pericapsular fibrosis.

No biomaterial is 100% biocompatible, it is therefore, inevitable that transplanted microcapsules will eventually attract some cellelular overgrowth. Systemic immunosuppression of the transplant recipient and/or immunomodulation of the microencapsulated islet tissue will be necessary for the successful transplantation of

microencapsulated islets. In the present work, systemic immunosuppression using cyclosporin A prevented the deposition of pericapsular mononuclear cells on Na^+-alginate-PLL-alginate microcapsules transplanted for up to 28 days. On the other hand, brief post-implant immunosuppression of STZ-diabetic recipients with FK506 (days, 0, +1-+3) and also, 14 days treatment with FK506 did not prevent pericapsular deposition. However, in both cases FK506 immunosuppression could not be confirmed with the spleen lymphocyte proliferation assay. It is clear, that a more effective immunosuppressive therapy needs to be developed for the routine transplantation of microencapsulated islets, perhaps involving the use of a cocktail of immunosuppressive agents such as azathioprine, prednisone and cyclosporin A (279) or cyclosporin A in combination with indomethacin and dexamethasone (267). Recently, two new immunosuppressive drugs have been introduced for islet transplantation. The first tenidap (Pfizer Inc. Groton,USA), has a potent inhibitory effect on interleukin-1β production by peritoneal macrophages (300, 301). The second drug, deoxyspergualin (Nippon Kayaku Co, Tokyo Japan) has been shown to be effective against lymphocytes and macrophages (302) and is a powerful anti-inflammatory drug. Of course, microencapsulation with immunosuppression is not the only means by which the immunogenicity of isolated islets can be reduced. It is well established that islet immunogenicity can be reduced by extended in vitro tissue culture (60) and ultraviolet irradiation treatment (61), although the risk of islet damage in the latter procedure is great. Brief systemic immunosuppression using cyclosporin A together with L-NMMA loading into the islet microcapsules could be usefully considered for microencapsulated islet transplantation. Aminoguanidine, a recently identified selective inhibitor of the inducible isoform of nitric oxide synthase has been shown to prevent nitrite formation by Rin-m5F cells (263). While L-NMMA is a potent inhibitor of nitric oxide synthase, it may not be suitable for clinical use because it is not selective for the inducible isoform of nitric oxide synthase and it also inhibits the constitutive isoform of the enzyme (263).

The potential of Ba^{2+}-alginate membrane coated islets for islet transplantation appears to be great, but in the present work, histological examination proved difficult and the immunoprotective capacity of the coating remains to be determined. It is possible that Ba^{2+}-alginate coated islets may be conveniently housed in biocompatible hollow fibres for subsequent ease of retrieval.

In summary, this work has evaluated the essential parameters required for the successful fabrication of microencapsulated islets of Langerhans. The clinical application of islet transplantation techniques on a broad scale, however, will almost certainly require the use of xenogeneic donor species, because of the limited availability of human pancreatic tissue. Transgeneic pigs may prove a suitable source of islets for clinical xenotransplantation in the future (79). Heald and colleagues have recently isolated large numbers of islets from transgeneic pigs using collagenase digestion (303). A multitude of factors make it difficult to interpret and compare the results of the many microencapsulated islet transplant experiments reported by different research groups. These factors include the problems associated with the use of different fabrication materials, their biocompatibility, the selection of the most suitable transplantation site, the size, number and volume of implanted microcapsules, the presence of pericapsular mononuclear cell fibrosis and islet necrosis due to attack by macrophage derived cytokines.

Based on the in vitro and in vivo work with microencapsulated islets described in this thesis, it is clearly apparent that the microencapsulation process will need to be significantly improved before it can be used confidently and effectively in vivo for islet transplantation in man.

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APPENDICES

1. Preparation of Hanks balanced salt solution (HBSS)

Ingredients	g/litre
NaCl	8.00
KCl	0.40
$MgSO_4.7H_2O$	0.20
$CaCl_2.2H_2O$	0.14
$Na_2HPO_4.2H_2O$	0.06
KH_2PO_4	0.06
$NaHCO_3$	0.35
Supplemented with Glucose	1.0
BSA∇	1.0

After gassing the buffer for 15-20 minutes with 95%O_2 : 5%CO_2, the pH was adjusted to 7.4 with either 2 mol/l NaOH or 2 mol/l HCl.

2. Dithizone (DTZ) solution

Stock solution

50mg of DTZ was dissolved in 5ml of dimethyl sulfoxide (DMSO).

Working solution

1ml of stock solution was diluted with 20ml of HBSS,and filtered before use in islet identification.

3. Preparation of phosphate buffer for insulin RIA

Ingredients	g/litre
$Na_2HPO_4.2H_2O$	6.2
BSA∇	5.0
Sodium azide	1.0

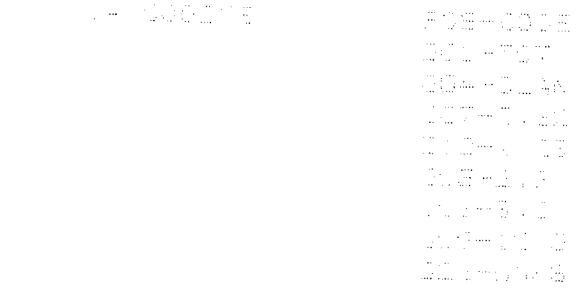
pH was adjusted to 7.4 with 1mol/l NaOH.

4. Polyethylene glycol (PEG) separation buffer

a) 200g of PEG (M.wt.6000) was dissolved in 300ml of double distilled water and the volume made up to 500ml with double distilled water.

b) 1g of γ-glubulin was dissolved in 500ml of phosphate buffer (appendix 3). Equal volumes of solutions, a and b were mixed and 0.5ml of Tween 20 was added.

5. Typical computer print out for insulin R/A



POS	CODE	TIME	COUNTS				
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002	101	60	1075				
003	101	60	1160				
	MEAN						
004	BLAN	60	547				
005	BLAN	60	535				
006	BLAN	60	521				
	MEAN						
007	0.62	60	2600				
008	0.62	60	2686				
009	0.62	60	2765				
	MEAN						
010	1.25	60	1914				
011	1.25	60	1930				
012	1.25	60	2041				
	MEAN						
013	2.5	60	1764				
014	2.5	60	1880				
015	2.5	60	1774				
	MEAN						
016	5.0	60	1216				
017	5.0	60	1221				
018	5.0	60	1274				
	MEAN						
019	10.0	60	1061				
020	10.0	60	992				
021	10.0	60	1072				
	MEAN						

6. Preparation of Heidenhain's Susa fixative

Ingredients	g/100ml
Mercuric chloride	5.4
NaCl	0.6
Trichloroacetic acid	2.4

When dissolved, added glacial acetic acid (40ml) and formaldehyde (25ml).

7. Publications arising from this thesis

Abstract 1

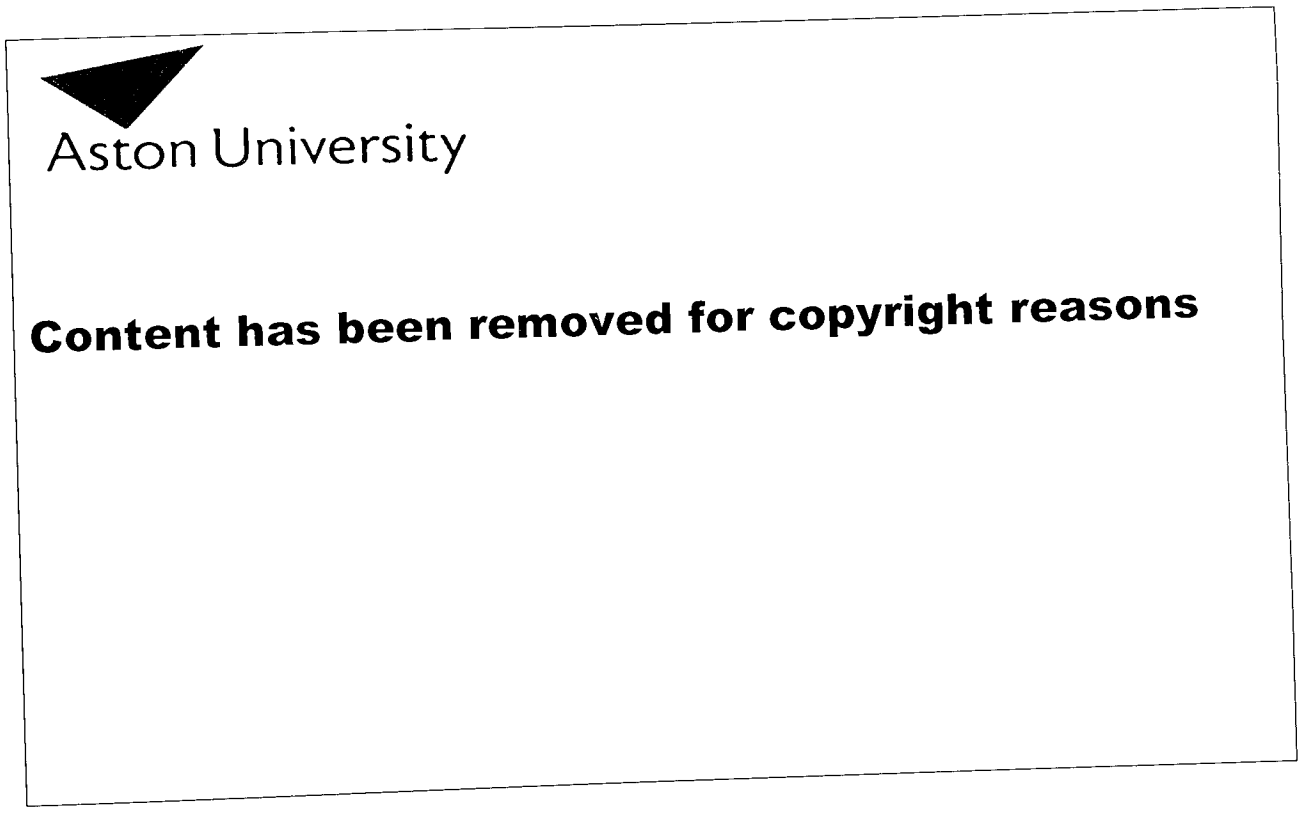
Journal of Endocrinology (1992). 135 (Suppl.):P78

P78 THE EFFECT OF DIFFERENT ISLET ENCAPSULATION STRATEGIES ON INSULIN RELEASE FROM MOUSE ISLETS IN-VITRO

Abstract 2

Diabetic Medicine (1993). 10 (Suppl.1):S48

P136. Histology of encapsulated mouse islets after implantation.



Abstract 3

Journal of Endocrinology (1993). 139 (Suppl.):P24

P24 ENHANCED INSULIN RELEASE FROM Ba^{2+}-ALGINATE MEMBRANE COATED MOUSE ISLETS

Abstract 4

Journal of Endocrinology (1993). 139 (Suppl.):P25

25 THE EFFECT OF N^GMONOMETHYL L-ARGININE ON INSULIN RELEASE AND MORPHOLOGICAL INTEGRITY OF TRANSPLANTED ENCAPSULATED ISLETS