RETINA IN THE DIABETIC BB-RAT: ROLE OF AN AUGMENTED POLYOL PATHWAY.

by

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A thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirement for the degree of Doctor of Philosophy.

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Dedicated to my parents

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ABSTRACT:

Increased polyol pathway activity is thought to be an important pathogenetic mechanism in the development of diabetic complications. To imply this pathogenetic mechanism in any diabetic complication, the responsible enzyme, aldose reductase, must be demonstrated in the target organs of diabetic complications, and the diabetic changes in the target organs must be prevented by inhibiting the elevated enzyme activity. In the present study aldose reductase was purified from the testis of the non-diabetic BB-rat and antibody against this enzyme was raised in rabbits. The antibody was used for light and ultrastructural immunocytochemical localization of the enzyme in the retina. The enzyme immunoreactivity was demonstrated in the ganglion cells, Müller cells, retinal pigment epithelium and endothelial cells and pericytes of the retinal capillaries. Quantification of the enzyme immunoreactivity in the retinal pigment epithelium at the ultrastructural level showed a significant increase in the immunorectivity in diabetic animals which was associated with abnormalities of basal plasmalemmal infoldings in the pigment epithelium. The retinal localization of aldose reductase may be helpful in

explaining the abnormalities of affected cellular constituents in diabetes, such as retinal pigment epitheliopathy and degenerative changes in pericytes and in endothelial cells.

In the second part of this study the effects of aldose reductase inhibition on the diabetic retinal microvascular changes were studied and were compared with that of good blood glucose control accomplished by vigourous insulin treatment. A heterogeneic response of diabetic retinal capillary basement membrane thickening was noted when diabetic BB-rats were subjected to treatment with insulin and the aldose reductase inhibitor Statil. Insulin treatmant prevented diabetic retinal capillary basement membrane thickening both in the superficial and in the deep capillary beds, whereas treatment with Statil prevented basement membrane thickening in the deep capillary bed only. This finding suggests that biochemical events involved in the pathogenesis of capillary basement membrane thickening in diabetes may be modified by the topographical and physiologic differences in the two capillary beds.

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LIST OF ABBREVIATIONS:

ATP -- Adenosine triphosphate.

BB-rat -- Bio-breeding rat.

BM -- Basement membrane.

BRB -- Blood retinal barrier.

CNBr -- Cyanogen bromide.

DEAE -- Diethylaminoethyl.

ERG -- Electroretinogram.

HLA -- Human leukocyte antigen

ICA -- Islet cell antibody.

IDDM -- Insulin dependent diabetes mellitus.

IGF I -- Insulin like growth factor I.

IRMA -- Intraretinal microvascular abnormalities.

MHC -- Major histocompatibility complex.

NAD -- Nicotinamide adenine dinucleotide.

NADPH -- Nicotinamide adenine dinucleotide phosphate (reduced form)

NIDDM -- Non-insulin dependent diabetes mellitus.

PBS -- Phosphate buffer saline.

PZI -- Protamine zinc insulin.

RDGF -- Retina derived growth factors.

SDS-PAGE -- Sodium dodecyl sulphate polyacrylamide gel.

VIII-VWF -- Factor VII related Von-Willebrand factor.

1. INTRODUCTION

I. Background:

Diabetes mellitus is a disease of antiquity. Hindu physicians were probably the first to recognize diabetes, and they recorded it in the ancient religious "Vedas", which appeared from 1500 to 600 BC. They were subsequently formulated into medical textbooks between the years 1000 BC and 700 AD (Frank, 1957). The recorded history of diabetic retinopathy does not go back that far. The first case was described by Jager in 1855, a few years after the introduction of the ophthalmoscope.

The classic works of Banting and Best and the introduction of insulin marked the beginning of the modern era of diabetes treatment (1921). However till today diabetes mellitus remains an ever increasing, common and chronic disease. Statistical data shows that the diabetic population doubles every 15 years, (Report of the National Commission of Diabetes to the Congress of the United States, 1975). Together with the developed countries, in recent years there has also been a tremendous increase in the incidence of diabetes in the developing countries (Ahuja, 1983). Due to advances in medical care of diabetes, patients can anticipate a longer life span. With this increased longevity, chronic

complications of diabetes such as retinopathy, neuropathy, nephropathy and atherosclerosis are becoming more common.

The ocular apparatus suffers a number of diabetic complications, of which diabetic retinopathy is the most important. Diabetes is the most important systemic disease causing blindness (Caird et al, 1969). The average prevalence of retinopathy in diabetics is 52% (L'Esperance and James, 1981). Diabetic retinopathy is the most common cause of blindness among diabetics accounting for more than 70% and is the leading cause of new blindness in the 20 to 74 year age group (Amos, 1974 and Caird et al., 1969). Retinopathy accounts for a 25-fold increase in the incidence of blindness in patients with type-I diabetes and a 2 to 3 fold increase in type 2 diabetes compared with an age-matched non-diabetic population (Report of the Second National Diabetic Research Conference, 1984).

The rationale for the treatment of diabetic complications is twofold i) to treat the underlying disease, in order to prevent the development of complications and ii) treatment directed to the specific complications if and when they become established. The former mode of treatment is preferable, since it has as its goal achievement of physiological insulin regulation.

If a normal glucose homeostasis can be established, the development and/or progress of secondary complications affecting the eye, kidney and the nervous system may be prevented or halted (Sutherland, 1983). In recent years augmented polyol pathway activity and/or increased non-enzymatic glycation have emerged as two potentially important pathogenetic mechanisms in the development of diabetic complications. In order to develop specific treatment modalities, a better understanding of these pathogenetic mechanisms is essential.

In the following a short description of diabetes mellitus and its complications will be given, together with a discussion of suggested pathogenesis and pathological features of diabetic retinopathy with particular emphasis on the possible role of an augmented polyol pathway. Experimental models of diabetic retinopathy will be reviewed and the structure and ultrastructure of the retina and its capillaries will be briefly discussed.

II. Diabetes mellitus and secondary complications:

Diabetes mellitus is defined as an inappropriate elevation of fasting or post-prandial plasma glucose levels. Several types of diabetes mellitus can be defined which differ in their pathogenesis, clinical appearance and treatment (National Diabetes Data Group, 1979).

Type 1 diabetes mellitus, also referred to as insulin dependent diabetes mellitus (IDDM) usually occurs before 40 years of age. Although predominant in the young, it can occur in all ages. Its predisposition is genetically determined and is associated with histocompatibility loci HLA-DR3 or DR4 (Report of Second Diabetic Research Conference, 1984). The loss of insulin secretion in IDDM appears to result from an autoimmune destructive process which is directed against the insulin producing β-cells of the pancreas. This process may be triggered by environmental factors such as viruses or toxins (Kahn, 1985). The list of viruses involved in the development of IDDM include coxsackie-B, rubella and various encephalitis viruses. Antibody [islet cell antibody (ICA)] is produced against the virus or toxin induced degraded islet tissue. ICA may lead to β-cell destruction by complement mediated lysis. There is also an invasion of islets by I_a positive cytotoxic T-

lymphocytes leading to their destruction. Superimposed physical or emotional stresses, infections or trauma may trigger off β-cell destruction, leading to the clinical picture of insulinopenia and hyperglycemia. In a rare subgroup of type 1 diabetes autoantibodies are also produced against other endocrine organs leading to a multi-endocrine deficiency syndrome (Forsham, 1983).

Type 2 diabetes is also known as non-insulin dependent diabetes (NIDDM). This type of diabetes is most common in older subjects, usually but not always obese. Genetic and probably environmental factors interact in the production of this condition (Report of the Second National Diabetes Research Conference, 1984). The possibility that NIDDM may be due to overeating leading to increased insulin secretion, insulin receptor downregulation, insulin resistance and glucoose intolerance has been put forward. No relationship between the HLA loci and NIDDM has been established. Inhibition of insulin action may occur at the pre-receptor, receptor or at the post-receptor level (Forsham, 1983). NIDDM is associated with obesity in 80% of patients. In non-obese patients the primary lesion may be at the target cells with some form of post-receptor defect leading to hyperglycemia, hyperinsulinemia and secondary changes in the insulin receptor leading to deterioration of the

disease. In both obese and non-obese patients abnormalities in the B-cells may be present impairing the insulin secretion (Kahn, 1985).

Chronic complications occur in all types of diabetes mellitus. In IDDM, prior to the discovery of insulin, death invariably occured due to ketoacidotic coma. Today chronic complications are the major cause of mortality and morbidity in both IDDM and NIDDM. The important chronic complications include large vessel disease, nephropathy, neuropathy and retinopathy.

Large vessel disease or macroangiopathy is essentially an accelerated form of atherosclerosis, which accounts for the fact that myocardial infarction is three times as common in the diabetic population as in non-diabetic individuals. Similarly the incidence of cerebrovascular accidents and peripheral gangrenes are 5 to 50 times more common in diabetics compared to non-diabetic controls (Colwell et al, 1979).

Glomerular basement membrane thickening and mesanglial expansion are two characteristic features of diabetic nephropathy (D'Elia et al, 1985). Progressive diabetic nephropathy manifests itself as albuminuria, and its occurance can be predicted by preceeding microalbuminuria (The Oslo Study, 1987). Eventually hypoalbuminemia and nephrotic syndrome develop which may

lead to uremia. Uremia is the dominant clinical complication of long term IDDM. The incidence of renal complications in diabetic patients surviving more than 20 years is 50% (Halter and Porte, 1987). Presently 25% of new dialysis patients in the United States are diabetic and diabetic nephropathy is the major indication for renal transplantation (Report of the Second National Diabetic Research Conference, 1984).

Diabetic neuropathy can be classified broadly into symmetric distal somatosensory polyneuropathy, autonomic polyneuropathy and mononeuropathy multiplex. Hyperglycemia and insulin deficiency are thought to be major pathogenetic factors in the production of diabetic neuropathy (Report of the Second National Diabetic Research Conference, 1984). Distal symmetric polyneuropathy is the most common form of diabetic neuropathy affecting about 2.5 million people in the United States (Sima, 1985). Clinically sensory loss in a "glove and stocking" distribution and motor weakness are the main features. These findings are sometimes associated with pain and a burning sensation in the limbs. Autonomic neuropathy may be manifested as bowel, bladder or sexual dysfunction with symtoms such as nocturnal diarrhea, impotence and retrograde ejaculation. Postural hypotension, syncope and loss of cardiovascular

reflexes are other common autonomic dysfunctions in diabetic subjects (Lockey and Tarsy, 1985).

Diabetic retinopathy is most likely a manifestation of small vessel disease. A detailed discussion of this complication is outlined below.

The current state of knowledge suggests that all chronic hyperglycemic states regardless of their etiological background, confer risks for the same group of complications (Barbosa and Saner, 1984; Finegold et al, 1986; Winegrad, 1987). Some factors common to all types of diabetes mellitus apparently contribute to the secondary complications in succeptible tissues. Although diabetes may act through unique mechanisms to increase risk for progressive disease, the specific disease process(es) that it can evoke in the individual susceptible tissue is modified by the distinctive structural and biological features of the tissue (Winegrad, 1987).

III. Diabetic Retinopathy in Human:

A. PATHOLOGY:

Clinically, diabetic retinopathy can be divided into background or non-proliferative retinopathy and proliferative retinopathy. Background retinopathy consists of capillary microangiopathy, venous abnormalities, intraretinal haemorrhages, hard exudates, nerve fiber layer infarctions (soft exudates) and macular changes. Venous dilatation is thought to be the earliest clinical manifestation of diabetic retinopathy (Buzney and Weiter, 1984). Howeyer studies with vitreous fluorophotometry suggest that an increase in vitreous fluoroscence, due to breakdown of the blood-retinal-barrier (BRB) at the level of retinal pigment epithelium and capillary endothelial cells may be an even earlier manifestation of diabetic retinopathy, occuring before the development of any visible fundus lesions. BRB-breakdown may be preceded by an alteration of the retinal pigment epithelium or the retinal neuronal tissue; a stage which has been termed preretinopathy (Cunha-Vaz, 1983). An increase in retinal blood flow, detected by laser dopler velocimetry, is another early dynamic change in diabetic retinopathy prior to visible lesions of the fundus (Feke et al, 1983). The features of capillary

microangiopathy include loss of pericytes, basement membrane (BM) thickening, microvascular obstruction and permeability changes, capillary non-perfusion, microaneurysms and intraretinal microvascular abnormalities (IRMA). The IRMA's include dilated, telangiectatic, leaky capillaries, shunting blood in the microcirculation surrounding areas of non-perfusion.

The venous abnormalities consist of dilatation, duplication and beading of retinal veins and occlusion of central or branch retinal veins. The hard exudate is a collection of lipids and lipoproteins from leaking capillaries, usually in the outer plexiform layer of the retina. Focal areas of retinal capillary non-perfusion may damage axons in the nerve fiber layer causing interruption of axonal transport and axonal swelling. Histologically, focal areas of infarcted neurons appear as cytoid bodies (Murphy and Patz, 1983). The macular changes consist of macular edema with progression to macular retinoschisis, macular hole or macular ischemia due to retinal vascular occlusions. IRMA, venous occlusion, nerve fiber layer infarction, profuse retinal hemorrages and microaneurysms have been grouped under the term preproliferative retinopathy (Frank, 1986). It is believed that retinal hypoxia, a fundamental pathogenetic factor in the progression of retinopathy to the

proliferative stage, develops as a consequence of these changes and other rheological abnormalities in diabetes (Little, 1983). The hypoxic retina liberates retina derived growth factors which stimulates neovascularization (Patz, 1984).

Proliferative retinopathy consists of neovascularization and fibrous tissue proliferation which can be intraretinal, located on the retinal surface, on the optic disc or manifested as new retinal vessels in the vitreous. Proliferative retinopathy may lead to visual loss by means of preretinal or vitreous haemorrhages and tractional retinal detachment.

B. PATHOGENESIS:

A variety of factors compromising blood supply, oxygen transport and delivery are probably involved in the pathogenesis of diabetic retinopathy (Ashton, 1983). The exact mechanisms responsible for diabetic retinopathy are poorly understood. Hyperglycemia appears to be the primary pathogenetic agent in the development of early lesions in the diabetic retina (Frank, 1984). Rheological and physiological abnormalities of the blood are also thought to play a major role in the pathogenesis of retinopathy (Little, 1981). The rheological factors

include (i) a reduced level of 2,3-diphosphoglycerate in red blood cells in early and poorly controlled diabetes secondary to a decreased level of plasma inorganic phosphate. The oxygen releasing potential of 2,3-diphosphoglycerate deficient red blood cells is reduced and a supra-normal oxygen binding power impairs oxygen release and causes hypoxia in the target tissue.

(ii) An increase in hemoglobin A_1C is due to non-enzymatic glycation of hemoglobin, whereby glucose binds to the terminal valine residue of the β-chain of hemoglobin and blocks the action of the already decreased 2,3-diphosphoglycerate causing a further impairment of oxygen release.

(iii) The retinal microcirculation may be affected by the aggregation of red blood cells, increased plasma protein and impaired fibrinolytic activity. An increased aggregability of platelets has been demonstrated, which may be secondary to an elevated synthesis of platelet thromboxane A_2 and protaglandin-E (Haluska et al., 1982). Hyperaggregability of diabetic platelets in the presence of ADP and epinephrine is associated with an elevated level of factor VIII-related Von Willebrand factor (VIII-VWF) and anti-hemophilic globulin factor (Colwell et al., 1976, 1979). VIII-VWF appears to be influenced by growth hormone, the role of

which was first drawn to attention by Hansen (1971). Since growth hormone increases protein synthesis through a secondary factor named somatomedin or insulin growth factor I (IGF-I), the plasma protein levels, including those of fibrinogen and α_2 macroglobulin increase (Hall and Luft, 1974). Increased concentration of plasma proteins raises the blood viscosity and the aggregation of red blood cells, which may result in focal obstruction of the microcirculation leading to areas of non-perfusion and retinal hypoxia (Little, 1976).

In addition to rheological disturbances, altered glucose metabolism is an important factor in the development of retinopathy. As a result of insulin deficiency and hyperglycemia, excessive glucose is metabolized via an activated polyol pathway. The rate limiting enzyme in this pathway is aldose reductase. The end products of the polyol pathway, sorbitol and fructose, accumulate intracellularly resulting in increased cellular osmotic pressure and osmotic decompensation of the cell. Alternatively augmented polyol pathway activity may adversly affect myo-inositol metabolism (NIH Conference, 1984; Frank, 1984, Winegrad, 1986). This series of events may lead to capillary cellular swelling, resulting in an increase of the diffusion distance. Aldose reductase has been localized

to Muller cells, ganglion cells and to pericytes of the human retina (Akagi et al., 1983, 1984). The same enzyme has also been demonstrated in Muller cells, ganglion cells, pigment epithelium and in the capillary pericytes and endothelial cells (Chakrabarti et al, 1987; Ludvigson and Sorenson, 1980) and in cultured microvessels of the rat (Kennedy et al., 1983). Pericyte degeneration and loss in the diabetic retina may be due to the osmotic effect of sorbitol. Loss of structural support due to pericyte loss may subsequently lead to the development of microaneurysms (Kinoshita 1986). Human retinal endothelial cells cultured in a medium with a high glucose concentration exhibited cytotoxic features with the accumulation of PAS-positive granules, cytoplasmic vacuolisation and cell degeneration (Tripathy and Tripathy, 1982). Augmented polyol pathway activity may also be responsible for BM thickening, since aldose reductase inhibitors prevent BM thickening in both diabetic and galactosemic animals (Frank et al, 1983; Robison et al, 1983; Chandler et al, 1984). A prevention of the breakdown of BRB in galactosemic rats has been achieved by the treatment with an aldose reductase inhibitor (Lightman et al, 1987). Various aldose reductase inhibitors are now being investigated clinically and experimentally as to their potential

effect in prevention or treatment of diabetic retinopathy. In a clinical trial by Cunha-Vaz et al. (1985) Sudinlac, an aldose reductase inhibitor which is also a prostaglandin synthetase inhibitor, has been shown to prevent breakdown of the BRB.

Thickening of capillary BM has been accepted as the characteristic morphologic feature of diabetic microangiopathy. Possible biochemical mechanisms include enzymatic glycation of hydroxylysine residues of collagens by glucose and/or galactose via the -OH groups of the amino acid (Spiro and Spiro, 1971) and non-enzymatic glycation of lysine residues by the same sugars at the exposed e-NH_2 groups (Cohen et al., 1980; Urbanowski et al., 1982; Cerami et al, 1987). Other possibilities that have been suggested include increased synthesis of collagen or other BM proteins such as laminin or fibronectin, either as a primary event or as a compensatory mechanism secondary to decreased production of BM proteoglycan (Szarfman et al., 1982). Increased polyol pathway activity may play a role in capillary BM thickening, as it has been shown that both galactosemia and diabetes induced capillary BM thickening can be prevented by aldose reductase inhibitors (Robison et al., 1983; Frank et al., 1983; Chandler et al,1984). However, it is still not clear how aldose reductase affects the

above mentioned activities.

The thickening of capillary BM is probably associated with endothelial cell damage and plasma leakage (Ashton, 1974). Thickening of capillary BM in diabetes has been considered as a proliferative response to injury by the cellular constituents of the capillary (Williamson and Kilo, 1983). Repeated endothelial cell death and regeneration, with retention of old and the addition of new layers of BM, may be a factor in the production of BM duplication and thickening (Vracko and Benediti, 1970). Deposition of pericyte debris, due to inadequate removal, within basement membrane may be another factor in the production of basement membrane thickening (Tilton et al,1981). McMillan (1983) suggested that modified viscous properties of the red blood cell leading to reduced membrane deformibility, may cause these cells to exert a greater mechanical force on the capillary wall which might be responsible for BM thickening; this hypothesis remains to be substantiated (Williamson and Kilo, 1983).

Areas of endothelial cell degeneration and proliferation have been noted in diabetic retinopathy (Bloodworth, 1982), and endothelial cell damage is thought to be a factor in the breakdown of the BRB (Cunha-Vaz, 1983).

The loss of capillary pericytes is one of the early features of the disease (Cogan et al., 1961). The normal ratio of pericytes to endothelial cells in healthy young individuals is 1:1. Normal aging is associated with a decrease in the number of endothelial cells but not in that of pericytes (Apple, 1981). In diabetic retinopathy this ratio is altered with a progressive loss of retinal pericytes. According to Cogan et al. (1961) this change in the ratio is the key pathological feature of diabetic retinopathy. Pericyte loss may impair blood flow regulation, as it has been shown that pericytes possess contractile properties (Das et al, 1987). Hyperglycemia, in addition to insulin deficiency have also been implicated as being directly responsible for pericyte loss (Frank, 1984; King et al, 1986). Chuna-Vaz (1983) is of the opinion that the pericyte damage is secondary to endothelial cell damage. While damaged endothelial cells can easily be replaced by proliferation and sliding of neighbouring cells, the pericytes cannot be replaced in the same way, due to their special location where they remain trapped in the vessel wall. The weakness created in the vessel wall by the loss of pericytes may explain the formation of microaneurysms and retinal hemorrhages in diabetic retinopathy.

Microaneurysms are seen near areas of capillary non-

perfusion and pericyte loss, yet no correlation has been established between microaneurysms and pericyte loss (Oliviera, 1966). However microaneurysms are not pathognomonic of diabetic retinopathy as they are also seen in other retinal diseases such as branch retinal vein occlusion, dysproteinemic retinopathy and Coat's disease.

Neovascularization is the most severe problem in diabetic retinopathy. If untreated it causes haemorrhages, glaucoma and traction retinal detachment leading to blindness. Neovascularization is triggered by retinal ischemia in an attempt to revascularize the non-perfused areas. The existence of a possible angiogenic factor, to explain retinal neovascularization, was first proposed by Michelson in 1948. It has been postulated that areas of non-perfused retina liberate an angiogenic substance (Patz, 1984). Angiogenic activity has been demonstrated in retinal extracts from various species including human (Chen and Chen, 1980). These extracts promote thymidine uptake by vascular endothelial cells and initiate vascularization in cultured chorioallantoic membranes (Glasser et al., 1980). As to the characterization of the angiogenic factor(s), an active proteinaceous macromolecule has been isolated from bovine retinal extract (D'Amore et al., 1981). A low molecular

weight angiogenic factor, which induces neovascularization in the chick chorioallantoic membrane, has been isolated from healthy adult cats (Kissun et al., 1982). Autologous retinal implants into the rabbit corneal pockets has also been shown to stimulate angiogenesis (Felton et al., 1980). The presence of a low molecular weight substance which inhibits aortic endothelial cell proliferation and thymidine incorporation has been shown in normal as well as in diabetic vitreous (Reymond and Jacobson, 1982; Jackobson et al., 1984).

The possible pathogenetic mechanisms responsible for diabetic retinopathy discussed here are summerized in Figure 1 (Chakrabarti et al., 1985).

IV. Diabetic Retinopathy in Experimental Animals:

Various animal models have been examined to find a suitable model for diabetic retinopathy in which the retinopathic changes can be produced and studied in a longitudinal fashion. Animal models used for the study of diabetic retinopathy can be divided into diabetic and non-diabetic animal models. Among non-diabetic animals, galactosemic animals need special considerations in the light of an augmented polyol pathway as a potentially important initial mechanism in the development of the various lesions seen in the diabetic retina.

A. DIABETIC ANIMAL MODELS:

Animal models, with either spontaneous or induced diabetes have been investigated with regard to diabetic retinopathic lesions. Diabetic dogs are unique in that they reproducibly develop microaneurysms and other advanced lesions of diabetic retinopathy, regardless of the method used to produce diabetes (Engerman and Bloodworth, 1965; Patz et al, 1965). Diabetes-like retinopathy has been produced in galactosemic dogs, strengthening the suggested pathogenetic role of an augmented polyol pathway activity in diabetic retinopathy (Engerman and Kern, 1984). Although, immunocytochemical

localization of aldose reductase in the dog retinal microvasculature has produced negative results in paraffin embedded sections (Kern and Engerman, 1984), isolated microvessels, have been demonstrated to possess hexitol producing activity (Kern and Engerman, 1985). Recently Akagi et al (1986) have demonstrated immunohistochemically the presence of aldose reductase in capillary pericytes in trypsin-digested retinal preparations in the dog model. Diabetic dogs exhibit increased permeability of retinal blood vessels and intraretinal neovascularization (Engerman and Bloodworth, 1965, Engerman et al 1971, Wallow and Engerman, 1977). Good blood glucose control initiated early after the onset of diabetes has an inhibitory effect on the development of retinopathy (Engerman et al 1977), but not so when good blood glucose control is initiated after a period of poorly controlled diabetes(Engerman and Kern 1987). The only disadvantage of the dog model, is the lengthy time necessary for the development of these lesions.

Retinal lesions similar to those seen in human diabetic retinopathy, such as microaneurysms, nerve fiber layer infarctions and exudates have been reported in monkeys, after long term chemical diabetes (Gibbs et al, 1969; Bresnick et al, 1976; Tso et al, 1986).

Retinopathic lesions have been produced in diabetic rodents, including the rabbit, following chemically induced or spontaneous diabetes. The lesions developing in these animal models are of the early microangiopathic type, consisting of pericyte loss, acellular capillaries and BM thickening. BM thickening has been shown to occur reproducibly in diabetic rodents. Although some investigators have reported findings such as microaneurysms and neovascularization in murine diabetes (Musacchio et al, 1964; Toussaint, 1966; Heath and Rutter 1966; Agarwal et al, 1966; Kuczourwski et al, 1970; Papachristodoulou et al, 1976), others have failed to find such changes (Engerman et al, 1964; Pometta et al, 1966; Morri et al, 1974; Naeser and Argen, 1978).

The spontaneously diabetic Chinese hamster shows BM thickening and degenerative changes in capillary pericytes, pericyte "ghosts", glycogen deposits in the Müller cells as well as aneurysm-like lesions (Ghosh et al, 1970; Federman and Gerritsen, 1970; Soret et al, 1974). Chinese hamsters with streptozotocin induced diabetes have also been shown to produce aneurysm-like lesions (Sibay et al, 1971).

The streptozotocin-diabetic rat is a widely studied model of diabetic microangiopathy. A series of early angiopathic changes have been demonstrated in this model.

The list of findings include endothelial cell proliferation, vascular dilatation, microaneurysms, BM changes, loss of capillary pericytes, vessel atrophy and electrondense deposits in Müller cells (Sosula et al, 1972; Babel et al, 1974; Papachristodoulou et al, 1976; Struder et al, 1976; Hori and Mukai, 1979 Sharma et al, 1985; Chakrabarti et al, 1986, 1987). Retinal pigment epitheliopathy, manifested by abnormalities of basal plasmalemmal infoldings and increased permeability of horseradish peroxidase, has been demonstrated in this model (Grimes and Laties, 1980). Breakdown of the BRB at the level of endothelial cells (Ishibashi et al, 1980) and BM thickening adjacent to the perivascular glia has also been reported in this model (Fischer and Gartner, 1983). Tilton et al (1986), described BM thickening and an increase in the capillary circumference covered by pericytes, but failed to find any evidence of pericyte loss. Janes (1959), and Von Sallaman et al (1972) also noted an absence of microaneurysms and degenerative changes in the retinal capillaries of streptozotocin-diabetic rats.

An increase in vitreous fluorescence, a marker of BRB breakdown, has been demonstrated as a reversible change in streptozotocin diabetic rats, since it can be ameliorated by vigourous insulin therapy (Kernell and

Arnqvist, 1983) and syngeneic islet cell transplantation (Krupin et al, 1979). Prevention of capillary pericyte degeneration and loss and BM thickening has been reported following pancreatic islet cell allotransplantation (Chakrabarti et al, 1986, 1987).

In a normal rat electroretinogram (ERG) a,b and c waves are demonstrable, among which the c-wave is generated by the retinal pigment epithelium. A progressive reduction of the c-wave amplitude in the ERG, has been observed in diabetes. This change is preventable by myo-inositol supplementation and an aldose reductase inhibitor therapy (MacGregor and Matschinsky, 1985). Polsum et al (1983) however failed to find any protective effects of an aldose reductase inhibitor on the development of retinopathy in this model, whereas other investigators demonstrated a prevention of BM thickening by an aldose reductase inhibitor (Chandler et al, 1984).

Alloxan induced diabetic rats show BM changes, microaneurysms and new vessels (Toussaint, 1966; Kojima, 1971; Orloff et al, 1975). Heath and Rutter (1966) have reported that iminodipropidnitrile, when injected in alloxan diabetic rats as well as in normal rats, induces a rapid development of pericyte loss, endothelial cell proliferation and microaneurysms. Microaneurysms have been reported in rats made diabetic by pancreatectomy

(Musacchio et al, 1964) and in rats made diabetic by cortisone and growth hormone(Agarwal et al, 1966). However substantial controversy exists as to whether the advanced retinopathic such as microaneurysms and neovascularization can be produced in rodent diabetes (Engerman et al, 1982)

The spontaneously diabetic Bio-Breeding (BB) rat is probably the most authentic model of human IDDM. A detailed discussion of this model is outlined in section V.

Congenitally diabetic KK-mice develop microaneurysms and acellular capillaries as a consequence of diabetes (Kuczourwski, 1970; Duhalt et al, 1973). Streptozotocin diabetic mice have been shown to develop pericyte loss (Argen et al, 1979), BM thickening and endothelial cell proliferation; the two latter changes are prevented by pancreatic islet transplantation (Cuthbertson and Mandel, 1987).

Alloxan diabetic rabbits exhibit increased retinal sorbitol levels, decreased myo-inositol and Na^+-K^+-ATPase activity as well as an elevated Na^+-content in the pigment epithelium of the retina, suggesting that these defects may underly the breakdown of BRB (MacGregor et al, 1986; MacGregor and Matchinsky, 1986).

Diabetic carp have also been shown to exhibit

vasodilatation and degenerative changes in the retinal vessel wall (Yokote, 1973).

B. NON-DIABETIC ANIMALS:

Among non-diabetic animal models galactosemic animals need special mention in light of the probable role of an augmented polyol pathway in the production of diabetic retinopathy, since galactose is readily converted to galactitol, an equivalent of sorbitol. As mentioned earlier galactosemic dogs develop a retinopathy which is structurally similar to the retinopathy seen in human diabetes (Engerman and Kern, 1984). An increase in the BM thickness and increased permeability of the BRB both of which are preventable by the treatment with an aldose reductase inhibitor have been demonstrated in galactosemic rats (Frank et al, 1983; Robison et al, 1983; Robison et al, 1986; Lightman et al, 1987). Sucrose fed rats also develop endothelial cell proliferation, pericyte loss, microaneurysms and acellular vessels together with photoreceptor degeneration (Papachristodoulou, 1976; Cohen et al, 1972; Yanko et al, 1972). The the pathogenetic role of an augmented polyol pathway activity may vary topographically with regard to pericyte damage, since isolated microvessels from both

the retina and brain show polyol producing activity (Kennedy et al, 1983), changes such as pericyte loss are seen only in the retina and not in the cerebral capillaries (DeOlivera, 1966). Rats on a selenium deficient diet have been reported to develop pericyte loss (Lockwood and Eckhert, 1987).

Since there are few established diabetic animal models which demonstrate proliferative diabetic retinopathy, other animal models which show proliferative retinopathies have been used for the study of vasoproliferation. Angiogenic activity has been found in retinal and vitreal extracts from various species (Chen and Chen, 1980). Glaser et al (1980) isolated a factor from bovine retina which stimulates proliferation of endothelial cells and neovascularization of the chick chorioallantoic membrane. Various researchers have been able to identify several factors, which may collectively be termed retina derived growth factors (RDGF) (Sebag and McMeel, 1986). Aortic endothelial cells, in the presence of RDGF, were found to produce increased amounts of protease and collagenase leading to basement membrane degradation and endothelial cell proliferation (Gross et al, 1983). RDGF were found to promote an increase in cell movement (Herman and D'Amore, 1984), endothelial cell proliferation (Glaser et al, 1980), increased DNA

synthesis in endothelial cells (D'Amore et al, 1981), and directional guidance of the new vessels (Thompson et al, 1982). Retinal pigment epithelium was found to produce a growth factor, which stimulates fibroblast and glial proliferation (Bryan and Campochiaro, 1986) and acts as an inhibitor of neovascularization (Glaser et al, 1985).

In animal models neovascularization can be produced by retinal venous occlusion (Virdi and Hayreh, 1982), autologous retinal grafts in the corneal pocket (Federmen et al, 1980), implantation of homologous tumors or fibroblasts in the vitreous, intravitreal injection of lactic acid (Tano et al, 1980; Finkelstein et al, Gerke et al, 1976). Intravitreal injection of insulin produces a chronic immunogenic vasculitis followed by a proliferative retinal response and iris neovascularization. An immunopathogenetic mechanism has hence been proposed for the development of diabetic retinopathy following this observation (Shabo et al, 1983). This has further been supported by the demonstration of an antigenic sharing by pericytes and pancreatic β-cells (Nayak et al, 1987). Experimental retrolental fibroplasia in different species produced by the administration of oxygen has many similarities to proliferative diabetic retinopathy and has been used for the study of angiogenesis (Patz et al, 1978).

Intraretinal neovascularization has also been produced following irradiation to the eye (Irvine and Wood, 1987).

V. The BB-rat as a model of type 1 diabetes and secondary complications:

The spontaneously diabetic BB-rat develops a insulin dependent diabetes mellitus subsequent to a selective destruction of pancreatic β-cells resulting from an insulitis (Marliss et al, 1981). A genetic factor with an altered immune mechanism is responsible for the development of insulitis (Marliss et al,1981). The occurance of diabetes appears to be limited to the major histocompatibility locus, with an autosomal recessive inheritence with variable penetrance (Colle et al, 1981, 1982). Susceptibility of diabetes is linked to the $RT1^u$ of major histocompatibility complex (MHC). Class II subregion of the $RT1^u$ complex appears to be necessary for disease expression. It appears that $RT1^u$ class II antigen of rat MHC is the MHC restricting antigen, and plays a role in presenting the antigen to autoreactive lymphocytes (Colle et al, 1986; Prud'homme et al, 1987). Lymphopenia, virtual absence of helper and cytotoxic T cells, presence of lymphocytic insulitis, prevention of diabetes by immunosuppressive agents, neonatal thymectomy and passive transfer of diabetes by lymphocytes points to the involvement of cell-mediated immunity in the destruction of the β-cell and development of the diabetic

syndrome (Like and Rossini, 1984; Like et al, 1979; Nakhoda et al, 1981, Poussier et al, 1983). Together with cell-mediated events, humoral immunity may also play a role (Prud'homme et al, 1984). The development of diabetes is manifested by profound hyperglycemia, hypoinsulinemia, relative glucagon excess, hyperketonemia, polydipsia, polyuria, glycosuria, ketonuria, dehydration and weight loss (Marliss et al, 1982).

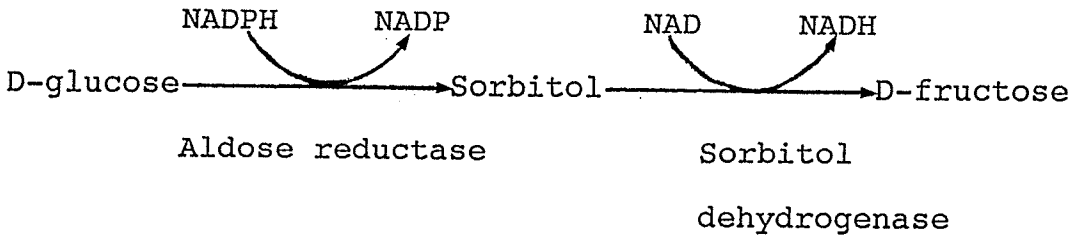
Sensory and autonomic neuropathy are two well characterized diabetic complications in the BB-rat and the spectrum of changes seen in human diabetic neuropathy has been produced in this model (Sima et al, 1986). BB-rat has also been advantageously used for the study of nephropathy (Brown et al, 1982; Cohen et al, 1987). In the retina these animals develop pericyte degeneration and loss, endothelial cell degeneration, BM thickening and microthrombi (Sima et al, 1985). Retinal pigment epitheliopathy has been demonstrated in this animal model (Blair et al, 1984). Recently Williamson et al (1987) reported an increased retinal vascular permeability in the BB-rat and in the streptozotocin diabetic rat as well as an increased retinal polyol accumulation in the latter, which were prevented by both aldose reductase inhibitor therapy and castration. BM thickening in the

sexually mature BB-rat is partially prevented by an aldose reductase inhibitor and completely so by vigorous insulin therapy (Chakrabarti et al, 1987), suggesting the important role of hyperglycemia as well as an increased polyol pathway activity in the development of diabetic BM abnormalities. Aldose reductase has been localized immunohistochemically in retinal capillary pericytes, endothelial cells, pigment epithelium, ganglion cells as well as in the Müller cells of the BB-rat (Chakrabarti et al, 1987).

VI. Polyol pathway and the complications of diabetes.

Hyperglycemia has been suggested as the main culprit in the pathogenesis of chronic diabetic complications such as nephropathy, neuropathy and retinopathy (Winegrad, 1987). Glucose levels in these target tissues reflect that of extracellular fluids, since glucose entry is neither regulated by insulin nor is it rate limiting for the metabolism of these tissues (Winegrad et al, 1972; Kador and Kinoshita, 1985). Metabolic events related to abnormal glucose metabolism in these organs, such as non-enzymatic glycation of protein (Brownlee et al, 1984) and increased polyol pathway activity (Kador and Kinoshita, 1985), may therefore play important roles in the development of chronic complications.

The first class of enzymes in the polyol pathway, termed aldose reductases, are NADPH-requiring aldehyde reductases which convert glucose and other aldosugars to carbohydrate derived polyols. The second enzyme, sorbitol dehydrogenese, is an NAD requiring dehydrogenase, which converts polyols to their corresponding keto-sugars (Winegrad et al, 1972).



Increased polyol pathway activity, subsequent to hyperglycemia, has been suggested as an important pathogenetic factor in the development of several diabetic complications (Kador and Kinoshita, 1985). An augmented polyol pathway activity in the affected organs of diabetic complications may not only be due to increased synthesis of the enzyme aldose reductase, but also may be due to activation of the enzyme following glycation of its peptide (Tsai and Waite, 1983; Das and Srivastava, 1985). Sorbitol, the product of the polyol pathway, penetrates biological membranes poorly, and once accumulated intracellularly, it creates a hypertonic condition (NIH Conference on Aldose Reductase, 1984). The osmotic effect of accumulated polyol is probably an important factor in the formation of diabetic cataract (Winegrad et al, 1972; NIH conference, 1984; Frank, 1984), whereas an osmotic basis for tissue damage is less well established in other affected tissues. A further and probably more important consequence of increased polyol pathway activity is tissue depletion of myo-inositol, since aldose reductase inhibitors completely

prevent the decrease in tissue myo-inositol in the diabetic kidney (Beyer-Meyers and Cohen, 1984), peripheral nerve (Greene et al 1985) and blood vessel (Morrison 1984). Myo-inositol depletion results in secondary changes in the phosphoinositide metabolism interfering with Na^+-K^+-ATP-ase activation by a protein kinase C dependent mechanism. In the diabetic peripheral nerve, the inactivation of Na^+-K^+-ATPase leads to 4-5 fold increase in intra-axonal Na^+ concentration, inactivation of Na channels and a selective conduction block of large myelinated fibers (Sima and Brismar, 1985; Greene et al, 1987). The increase in intracellular Na^+ reduces the transmembrane gradient driving Na-dependent myo-inositol uptake; further depleting myo-inositol and completing a self reinforcing metabolic defect (Greene and Lattimer, 1982; Greene et al, 1984).

In peripheral nerve the reduced Na^+-K^+-ATPase function is directly related to nerve conduction slowing that occurs in animal and human diabetes (Brismar and Sima, 1981; Greene et al, 1984; Sima and Brismar, 1985) and to impaired protein synthesis in diabetic spinal ganglion cells (Thomas, 1984). The polyol and myo-inositol related Na^+-K^+-ATPase defect is also implicated in the early structural changes, such as axo-glial dysjunction, in rat peripheral nerve (Sima and Brismar,

1985; Greene et al, 1987). A similar mechanism may be involved in the breakdown of the BRB in diabetes as it has been demonstrated that in the diabetic retina an increase in glucose and sorbitol levels and reduced myo-inositol content occur in all retinal layers. These changes are associated with a reduction in the Na^+-K^+-ATPase and an increase in the Na^+ concentration in retinal pigment epithelium and in the outer nuclear and outer plexiform layers of the retina (MacGregor et al, 1986; MacGregor and Matchinsky, 1986). In the galactosemic animal models it has been shown that the breakdown of BRB can be prevented by treatment with an aldose reductase inhibitor (Lightman et al,1987). Glomeruli isolated from acute streptozotocin diabetic rats also show an increase in hexitol, reduction in myo-inositol content and Na^+-K^+-ATPase activity (Cohen, 1986).

The importance of an increased polyol pathway activity in diabetes lies in the fact that this pathway can be blocked by various aldose reductase inhibitors.

VII. Structure of the Retina:

The retina in man and rat consists of the following layers from without inwards: (Prince, 1956) (Figure 2)

1) Pigment epithelial layer.
2) Layer of photoreceptors.
3) External limiting membrane.
4) Outer nuclear layer.
5) Outer plexiform layer.
6) Inner nuclear layer.
7) Inner plexiform layer.
8) Ganglion cell layer.
9) Nerve fiber layer.
10) Internal limiting membrane.

The pigment epithelium is formed by a single layer of cuboidal cells resting on a basement membrane that forms part of Bruch's membrane. The basal plasmalemma of these cells consists of regular infoldings of about 1 um in length. Along the inner surface of the cells there are numerous finger-like cytoplasmic processes which extend between the outer segments of the photoreceptors. The presence of pigment granules is a characteristic feature of the cells in this layer.

The layer of photoreceptors (rods and cones in human, rods only in the rat) is made up by the outer

segment of the photoreceptors. A connecting cilium joins them with the inner segment.

The external limiting membrane appears as a deeply stained punctate line external to the outer nuclear layer. This is not a true membrane but formed by the adjoining ends of the Müller cell processes. The nuclei of the photoreceptors compose the outer nuclear layer; the rod nuclei being in the inner aspect and the cone nuclei in the outer aspect. The area where the photoreceptors are connected to the dendrites of the bipolar cells is called the outer plexiform layer. The fibers of horizontal, amacrine and Müller cells are also present in this layer. The cell bodies of bipolar, horizontal, amacrine and Müller cells lie in the inner nuclear layer. The Müller cell processes extend from the outer limiting membrane to the inner limiting membrane and have supportive and nutritive functions. The inner plexiform layer contains the synapses of the bipolar and amacrine cells with the ganglion cells. The ganglion cell layer is composed of a single layer of cells except at the edge of the area centralis, where two layers can be found. The axons of the ganglion cells make up the nerve fiber layer. Neuroglial cell and Müller cell processes also form part of this layer. The inner limiting membrane is a thin membrane that forms the inner

limit of the retina.

The retina in man and most mammals, including the rat, is vascularised by the central retinal artery and its branches in the inner layers, to the junction between the inner nuclear layer and the outer plexiform layer (Prince, 1956). The choriocapillaries supply the outer layers of the retina. The main arteries and veins are located in the nerve fiber layer and the ganglion cell layer. The arterioles arise from the main branches, either by side branching or by dichotomous branching (Hogan et al., 1971). The retinal vessels in the rat are arranged in two capillary beds, a superficial and a deep capillary bed (Bernstein and Hollenberg, 1965). The post capillary venules seem more prominent in the deeper layer while the pre-capillary arterioles are more allied to the superficial layer (Wise et al., 1971).

The trypsin digested retinal preparation (Toussaint et al., 1961) permits the identification of two types of cells within the capillaries, pericytes and endothelial cells. The normal ratio of these two cell types in a healthy adult human is 1:1 (Yanoff and Fine, 1975). However, the normal ratio in many laboratory animals appears to be lower than that in man (Engerman et al., 1982). In the normal BB-rat retina Sima et al (1986) have reported a ratio of 0.71:1. The endothelial cells are

oriented along the axis of the capillary and have long pale staining nuclei, whereas the pericytes lie outside the endothelium and have round dark staining nuclei (Hogan et al., 1971). Retracted capillaries appear as bloodless cords and are called mesodermal bridges.

VIII. Ultrastructure of the retinal capillaries:

The retinal capillaries are lined by endothelial cells resting on a basement membrane. The basement membrane is split to accomodate pericytes and their processes (Hogan et al., 1971). The cell body is non-fenestrated and thin except in places where the nucleus is located. The cell junctions near the vascular lumen are of the zonula occludens type. The chromatin is evenly distributed in the nucleus with slight aggregation along the nuclear membrane. Numerous pinocytotic vesicles are present along both borders of the cell. Smooth and rough endoplasmic reticulum and free ribosomes are scattered throughout the cytoplasm. The Golgi apparatus and the centrioles are encountered in the vicinity of the nucleus. The mitochondria are relatively small in size and few in number (Toussaint and Dustin, 1963; Ishikawa 1963; Hogan and Feeny, 1963). Ultrastructurally the presence of Weibel-Palade bodies are considered to be specific for vascular endothelial cells. These are rod-shaped cytoplasmic components, consisting of a bundle of fine tubules and enveloped by a tightly fitted membrane (Weibel and Palade, 1964). Although these bodies were identified in the endothelial cells in various species (Matsuda and Sugiura, 1970),

some investigators have failed to find these bodies (Buzney and Massicotte, 1979; Frank et al., 1978); the function of these organelles is still obscure.

The pericytes encircle the vessel wall, and wrap it with their processes. They are seen as profiles of cytoplasm in most electronmicrographs (Hogan et al., 1971). The cytoplasm contains all the organelles described in endothelial cells, except for the Weibel-Palade bodies. Pinocytotic vesicles are also present (Ishikawa, 1963).

Human capillary basement membranes show cavitations which increase in number and size with age (Hogan and Feeny, 1963; Toussaint and Dustin, 1963). Although the basement membrane of rat and man are ultrastructurally similar, no cavitations are found in the normal rat (Hogan and Feeny, 1963; Kissen and Bloodworth, 1961).

2. HYPOTHESIS

Diabetic complications are thought to be the result of tissue dysmetabolism caused by hyperglycemia. In the target organs of chronic diabetic complications, retina, peripheral nerve and kidney, glucose uptake and metabolism is not insulin dependent. Instead glucose Transfer is accomplished by facilitated uptake in these tissues and glucose concentration reflect that of the ambient glucose levels in tissue fluid and plasma. Excess tissue glucose is thought to be shunted through the polyol pathway giving rise to high tissue sorbitol levels. The conversion of glucose to sorbitol is catalyzed by the species-specific enzyme aldose reductase. Sorbitol may have a direct adverse osmotic effect or secondary effects on myo-inositol, phosphoinositide and Na^+-K^+-ATPase metabolism. The present hypothesis is that an increased polyol pathway activity may underlie the early changes in the diabetic retina. For the validity of this hypothesis the enzyme, aldose reductase, must be present in the affected tissues and possibly increased in the diabetic state. Furthermore, if this hypothesis is correct, abnormalities in the affected tissues should be prevented

by, i) maintainance of normal blood glucose, thus correcting the initiating metabolic defect, hyperglycemia and, ii) inhibiting the increased aldose reductase activity by the use of an aldose reductase inhibitor.

3. SPECIFIC AIMS

a) Purification and immunologic identification of BB-rat aldose reductase.

b) Light microscopic immunocytochemical localization of aldose reductase in the BB-rat retina.

c) Ultrastructural localization of aldose reductase and quantification of the enzyme in normal and diabetic BB-rat retinas.

d) Evaluation of the effects of aldose reductase inhibition and euglycemia on the retinal microvasculature in diabetic the BB-rat.

4. MATERIAL AND METHODS:

I. Aldose reductase purification:

Aldose reductase was purified from 80 grams of fresh testis from non-diabetic BB rats (Courtesy Dr. A.A. Like, Dept. of Pathology, Univ. of Massachusetts Medical School, Boston, Mass. U.S.A.) using a modification of the method described by Ludvigson and Sorenson (1980). All steps were carried out at 4^{o}C in the presence of 5 mmol/l mercaptoethanol. The tissues were homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada) in 5 mmol/l tris buffer, pH 7.4 in a weight to volume ratio of 1:2. The homogenate was centrifuged at 45,000 g for 30 min. and the supernatant was collected. Solid ammonium sulphate in the amount of 17.5 g/100 ml was slowly added to the supernatant under gentle stirring producing a saturation of 30%. The solution was allowed to stand for 1 hour and the precipitate was removed by centrifugation at 15,000 g for 30 min. Additional solid ammonium sulphate in the amount of 30 g/100 ml was added to the supernatant to yield a saturation of 75%. The solution was again allowed to stand for 1 hour and pellets were collected following centrifugation at 15,000 g for 30 min (Ludvigson and Sorenson, 1980). They were dissolved in 40 ml of 5

mmol/1 tris buffer, pH 7.4, containing 5 mmol/1 mercaptoethanol. This solution was dialysed against 4 changes of each 800 ml of the same buffer using Spectropore no. 2 membranes (45 mm diameter, 12,000-14,000 mol. wt. cut off).

The dialysate was applied to a gravity packed DEAE (Diethyl aminoethyl) cellulose column (2.5 x 30 cm) equilibrated with 5 mmol/1 buffer (pH 7.4). The column was washed with the same buffer and eluted with a linear NaCl gradient (5-250 mmol/1); 7.5 ml fractions were then collected.

The pooled fractions containing aldose reductase activity were concentrated by pressure dialysis using an Amicon filter with a YM 10 membrane, 10,000 mol. wt. cut off (Amicon Corp., Danvers, Mass, USA) and applied to a gravity packed hydroxylapatite column (1.5 x 30 cm) equilibrated with 5 mmol/1 Na-K phosphate buffer (pH 6.8). The column was subsequently washed with the same buffer and eluted with a linear 5-400 mmol/1 Na-K phosphate buffer gradient; 7.5 ml fractions were again collected.

The fractions containing aldose reductase activity were pooled, concentrated by pressure dialysis and applied to a Sephadex G-100 column (2.5 cm x 100 cm) equilibrated with 5 mmol/1 tris phosphate buffer (pH

7.4): 10 ml fractions were collected. The pooled fractions containing enzyme activity were rechromatographed on a Sephadex G-100 column. The purified enzyme was dialysed to remove mercaptoethanol and stored frozen (-20°C) in small aliquots. The aldose reductase peak from the 2nd Sephadex G-100 run was used for immunization of rabbits. Other fractions showing enzyme activity were pooled to be used as pre-absorption controls for future immunohistochemical studies. The molecular weight of the isolated enzyme was determined by comparing it with standard molecular weight protein markers on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE).

II. Aldose reductase assay and units:

Aldose reductase was assayed according to Gabbay and Kinoshita (1976). Enzyme activity was measured spectrophotometrically at 340 nm in glass cuvettes of 1 cm light path. Glyceraldehyde and glucoronate were routinely used as substrates. The final concentrations of the reagents in the assay were 1 mmol/1 glyceraldehyde or 10 mmol/1 glucoronate, 73 umol/1 of NADPH, 5 mmol/1 of mercaptoethanol and 67 mmol/1 of Na-K phosphate buffer

(pH 6.2). The substrates were omitted from the control cuvettes. The reaction was started by adding substrate to the test cuvette and was followed on a LKB Ultrospec 4050 spectrophotometer (LKB Biochem Ltd., Cambridge, England) at 25°C. Aldose reductase activity was measured as the loss of NADPH absorbancy at 340 nm.

One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 umol NADPH per min. The specific activity was defined as units per mg protein. Protein concentrations were measured according to Lowry et al. (1951).

III. Antibody preparation and identification:

Antibodies against isolated aldose reductase were raised according to the following procedure. One mg. of isolated enzyme in Freund's complete adjuvant (1:1, vol:vol) was injected subcutaneously in New Zealand rabbits. This was followed by the injection of two doses of 0.5 mg of the same enzyme in Freund's complete adjuvant at 2-week intervals; after 2 further weeks an intravenous booster dose of 0.5 mg of the enzyme was given. The rabbits were bled one week after the intravenous booster injection. Serum was separated and

the antibody was purified by affinity chromatography using cyanogen bromide (CNBr) activated sepharose. In this method CNBr-sepharose was allowed to swell in 1 mM HCl. $NaHCO_3$ (0.1M, pH8.3) was used as the coupling buffer. Purified aldose reductase was conjugated with sepharose in the presence of 0.2M glycine-NaOH (pH 8.0). Following incubation with antiserum, the antibody was eluted with glycine-HCl, neutralized with tris buffer and dialysed against phosphate buffer saline (PBS). Non-immune serum collected prior to the primary immunisation was used as control serum for immunohistochemical studies. The antiserum was subjected to double immunodiffusion on Ouchterlony plates against purified, semi-purified (hydroxylapatite peak), crude and heat inactivated (56^{o}C for 15 min.) aldose reductase and also against the hexose dehydrogenase peak of the DEAE-cellulose elute. The specificity of the antibody was also judged by Western blot analysis on zeta-probe membrane against purified and crude enzymes. An in vitro study for inhibition of enzyme activity was performed according to Gabbay and Cathcart (1971). Immune and non-immune serum absorbed with liver powder was used for this purpose. Ten ul of purified enzyme was incubated with increasing amounts of immune serum (10 ul to 320 ul) in a final volume of 400 ul. As a control, non-immune serum

was used. All samples contained 5 mmol/1 of mercaptoethanol. The enzyme activity was assayed using glyceraldehyde as substrate after 20 min of incubation at room temperature.

IV. Light microscopic immunocytochemical procedures:

Retinas from 5 non-diabetic BB-rats were fixed overnight in Bouin's fixative and embedded in paraffin. Following deparaffinisation the sections (15 from each retina) were treated for 30 min with methyl alcohol containing 0.05% H_2O_2 to block endogenous peroxidase activity, washed in PBS (5 min x 3) and incubated with normal serum for 2 h. Trypsin digested preparations of the retina were carried out as described below. However incubation with trypsin was limited to 10-20 min. followed by incubation with 0.1% trypsin inhibitor solution (Soybean) for 30 min to minimize destruction of enzyme by trypsin and then processed following the same method. The sections were washed with PBS and incubated with primary antibody for 24 h at 4°C. Liver powder absorbed antiserum in the dilution of 1:500 was used, since this dilution was found to produce optimum staining after trials with various dilutions ranging from 1:100 to

1:2000. Liver powder absorbed non-immune serum in the same dilution was used as control. Following rinsing in PBS (5min x 3) the sections were incubated with goat anti-rabbit IgG (1 in 10) for 2h at room temperature. The sections were then rinsed again in PBS (5min x 3) and treated with rabbit peroxidase-antiperoxidase (1 in 100) for 1 h at room temperature. The sections were again washed in PBS (5min x 3) and incubated with freshly prepared 0.05% diaminobenzidine hydrochloride solution in 0.05M tris buffer (pH 7.6) containing 0.01% H_2O_2 for 3 to 8 min. following which the sections were washed in PBS (5min x 3), dehydrated in graded alcohol and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ, USA)

Glyceraldehydre, sodium-D-glucoronate, and NADPH were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Hydroxylapatite and zeta-probe membrane were obtained from Bio-Rad Laboratories, Richmond, Calif., USA. Sephadex G-100 and DEAE-cellulose and CNBr activated sepharose were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Goat anti-rabbit IgG and rabbit peroxidase-antiperoxidase were obtained from Miles Scientific, Naperville, Il, USA.

V. Ultrastructural immunocytochemistry.

Ultrastructural immunocytochemical localization of aldose reductase was carried out using the Protein-A gold method described by Bendyan (1984). Five 6 months diabetic male animals and 5 age- and sex-matched non-diabetic control animals were used. Following anesthesia with Na-pentobarbital (50mg/Kg of body weight) they were perfused with 0.2M phosphate-buffered 1% gluteraldehyde (ph 7.4). The volume of the perfusate was 2.5ml/gm of body weight. Following perfusion, retinas were dissected out and post-fixed in the same fixative for 2 hours. Non-osmified tissues were dehydrated in a graded series of alcohol and embedded in Epon. Ultrathin sections (90-150 nm) were cut with a diamond knife on an ultramicrotome (Reichert Jung, Vinnea, Austria) and picked up on nickel grids (300 mesh) and were subsequently used for on-grid immunomarking. The grids were washed with distilled water and incubated in phosphate-buffered saline (PBS) containing 1% ovalbumin for 10 minutes. They were then incubated with primary antibody without washing (affinity purified antibody, 1ug/ml). Controls used include a) preabsorbtion control, b) incubation with unconjugated protein-A after antibody incubation followed by incubation with protein-A gold, c) incubation with the

diluent of the antibody (PBS). The third control was used for the determination of background staining by non-specific binding of protein-A gold during the quantification procedure. Following 2 hours incubation at room temperature, the grids were washed with PBS and incubated with protein-A gold (SPI Supplies, Division of Structure Probe Inc, West Chester, PA, USA) (1 in 20 dilution for 1 hr). The grids were jet washed, stained with uranyl acetate (4% solution in 70% ethanol) and Reynold's lead citrate and examined electronmicroscopically.

VI. Quantification of gold labelling.

Quantification of the presence of aldose reductase based on gold labelling was carried out in the retinal pigment epithelium, since these cells exhibit large profile areas of cytoplasm allowing for a more accurate quantification of the relatively low cytoplasmic enzyme concentration. Five male 6-months diabetic animals and 5 age- and sex-matched control animals were investigated. Following tissue preparation and staining as outlined in the previous section, ten randomly selected pigment epithelial cells from one specimen of each animal were

photographed at a magnification of 5000. The electronmicrographs were enlarged to a final magnification of 45,000. The cytoplasmic areas were measured from these micrographs and the number of gold particles counted. The amount of staining was expressed as,

No. of gold particles/um^2 in antibody treated specimens - No. of gold particles/um^2 in non-antibody treated specimens

(Bendyan, 1984)

VII. Morphologic and morphometric examination of the retina.

A. Animals:

Male prediabetic diabetes-prone BB-rats as well as non diabetes prone male BB-rats were obtained from the University of Massachussetts, Worchester, MA (courtesy Dr. A.A. Like). Three weeks after onset of diabetes, defined by the detection of glycosuria (keto-diastix, Ames Division, Miles laboratories, Etobicoke, Ontario) and hyperglycemia (Glucometer, Ames Division, Miles laboratories, Elkhart, Indiana), diabetic rats were

randomly assigned to 3 treatment groups:

a) Diabetic animals with poor diabetic control were kept insulin-deficient and maintained on small doses (0.5-3.0 U/d) of protamine zinc insulin (PZI) (Connaught Laboratories Ltd., Toronto, Ontario) designed to prevent ketonuria but to maintain blood glucose levels greater than 15 mmol/l [D].

b) A second group of diabetic animals were maintained at similar blood glucose levels received the aldose reductase inhibitor Statil, (Stuart Pharmaceuticals, a division of ICI Americas Inc, Wilmington, DE) 25mg/kg/d [DS].

c) A third group of diabetic animals were treated aggressively with PZI (3.0-6.0 U/d) to achieve and maintain euglycemia throughout the experimental period [DI].

Age-matched non-diabetes prone BB-rats served as controls and were divided into two groups;

d) one group, which received no treatment [C], and

e) one group, which received 25mg/kg/d of Statil [CS].

Statil was added to rat-chow (80g Statil per 100 kg of rat chow). All animals were maintained in individual air-filtered metabolic cages with water ad libitum. Urine was monitored daily with respect to volume, glucose and

ketones and the insulin dose was adjusted accordingly. Blood glucose levels were measured every second week and glycosylated Hb every three months. Five animals in each group were sacrificed after 4 and 6 months of treatment.

B. Perfusion procedure:

The animals were anesthetised with sodium pentobarbital (50 mg/kg of body weight) intraperitoneally. The thorax was opened and a canula was introduced into the left ventricle directed towards the aorta. They were perfused with 0.1M cacodylate buffered (pH 7.4) gluteraldehyde (2.5%). The volume of perfusate was 2.5 ml/g body weight.

C. Morphological Techniques:

i). Tissue collection:

In order to perform the study in a blinded fashion, all animals were given a code number and the animal identity was unknown to the investigator.

Both eyes were enucleated and post-fixed in the same fixative for 4 to 6 hours at $4^{o}C$. Qualitative and quantitative light and electron microscopic studies were performed using the left eye of each animal.

ii) Ultrastructural tissue preparation:

After fixation radially oriented retinal segments (1 mm^2) were dissected from the superior temporal quadrant near the optic nerve head and washed overnight in 0.1M cacodylate buffer (pH 7.4). The tissues were post-fixed in cacodylate buffered 1% osmimum-tetroxide (pH 7.4) for 2 hours, dehydrated in graded alcohols and embedded in Epon. Semithin sections (0.5 um) stained with toluidine blue were used for light microscopic orientation and for the detection of abnormalities such as exudates and hemorrhages. The same sections were also used for measuring retinal vascular density (described below).

Ultrathin sections (50-90 nm) were cut with a diamond knife on an ultramicrotome (Reichert Jung, Vienna, Austria) and picked up on copper grids (200 mesh) and stained with uranyl acetate (4% in 70% ethanol) and Reynold's lead citrate. The grids were examined in a Philips EM 200 electron microscope.

iii) Trypsin digestion preparation:

Following fixation, the retina from the nasal half of the left eye was dissected. It was washed overnight in running water and then incubated with 3% trypsin solution (1:250, Difco Laboratories, Detroit, Michigan, USA) in 0.1M tris buffer pH 7.8 for 30 min-60 min. The retina was then transferred to distilled water and the

internal limiting membrane was carefully removed. Adherent tissues were removed by gentle shaking. The preparations were mounted on glass slides, allowed to dry and stained with PAS-hematoxylin. The preparations were examined light microscopically for microaneurysms and were also used for the calculation of pericyte-endothelial cell ratio (described below).

iv) Quantitative structural studies:

All morphometric studies were performed with the aid of a Hewlett-Packard 9874A digitizer connected with a Hewlett-Packard 9825A desktop computer (Hewlett-Packard Co., Fort Collins, Colorado, USA).

a) Vascular Density:

Toluidine blue stained sections were examined under a light microscope and were projected to the digitizer tablet by means of an attached side tube. The total number of vessels in all retinal layers were counted and the retinal area (um^2) was measured. The vascular density per mm^2 was calculated as,

$$\frac{\text{Number of vessels} \times 1{,}000{,}000}{\text{Total area studied } (um^2)}$$

b) Pericyte-endothelial cell ratio:

Five randomly selected areas of the trypsin digested retinal vasculature were photographed and enlarged to a final magnification of 1600 times. The vascular area (um^2) was measured, the number of pericyte and endothelial cell nuclei were counted and their number per mm^2 of vascular area was calculated. The pericyte-endothelial cell ratio was obtained from these calculations.

c) Endothelial cell profile area (E), pericyte profile area (P), lumenal area (L), BM thickness, number of endothelial cells and pericyte processes per capillary:

Twenty randomly selected and transversely sectioned capillaries were photographed from each animal, 10 from the inner nuclear layer and outer plexiform layer (deep capillary bed) and 10 from the nerve fiber layer and ganglion cell layer (superficial capillary bed). The electronmicrographs were enlarged to a final magnification of 16,000 times. These electronmicrographs were used to measure the above mentioned parameters of retinal capillaries. The areas were expressed as a percentage of total

capillary area (T). Only transversely sectioned capillaries were used for these measurements. They were judged as being transversely cut if they displayed a sharp delineation of the BM border.

BM thickness was measured according to the method of McEwen et al (1987). BM is not an uniformly thick structure; this rapid and reproducible method takes into account the variability of BM thickness and gives an average value (McEwen et al, 1987). Basement membrane area (BMA) and Basement Membrane length (BML) were determined as,

$$BMA = T - (L + P + E)$$

and, $$BML = \frac{\text{The length of lines delimiting the BM}}{2}$$

The relative thickness of the basement membrane was expressed as area per unit length,

$$\text{BM thickness (nm)} = (BMA/BML) \times 1000.$$

v) Qualitative structural studies:

The same micrographs were used for qualitative assessments of BM abnormalities, such as localized thickening, laminated appearance, fibrillar deposits and projection of BM towards the surrounding glia. They were also examined for the presence of degenerative changes in the pericytes and endothelial cells. These lesions were

expressed as the percentage of capillaries showing such abnormalities. Trypsin digested preparations were examined for the presence of microaneurysms. The toluidine blue stained semithin sections were examined for the advanced retinopathic changes such as hemorrages, exudates or infarctions.

VII. Statistical analysis:

One way analysis of variance with linear contrast, Student's t-test and and linear regression by least squares method were used to analyse the data. A p-value of 0.05 or less was accepted as being significant (Schefler 1984).

5. RESULTS:

I. Enzyme purification:

The purification of aldose reductase was carried out from the testis of the BB-rat. Two enzyme activity peaks corresponding to two protein peaks were eluted from DEAE-cellulose column chromatography of ammonium sulphate precipitated fractions of rat testis (Fig. 3). The first peak eluted in the column wash had a glyceraldehyde/glucoronate reducing activity ratio of 1:4.2. The 2nd peak eluted after raising the NaCl gradient had a glyceraldehyde/glucoronate reducing activity ratio of 2.6:1. The first peak was identified as hexose dehydrogenase. The chromatography of the 2nd peak on hydroxylapatite column resulted in elution of two protein peaks, both of which appeared after raising the phosphate gradient. The enzyme activity was found only in the first peak with a glyceraldehyde/glucoronate reducing activity ratio of 3:1 (Fig. 4).

Repeated gel filtration chromatography on Sephadex G-100 was performed with concentrated hydroxylapatite column eluted fractions containing aldose reductase activity for further purification of the enzyme. The second Sephadex G-100 run resulted in a sharp protein

peak with high enzyme activity (Fig. 5). The fractions in this protein peak were separated and used for immunization of rabbits. The whole procedure was repeated several times to yield the desired amount of purified enzyme. The purification procedure has been summarized in table 1.

The isolated enzyme peak was subjected to SDS-PAGE electrophoresis to determine purity and molecular weight by comparing it with standard molecular weight protein markers. The enzyme showed a single sharply defined protein band with a molecular weight of 36.500 $\pm$ 1000 (Fig. 6a).

II. Identification and specificity of the antibody:

In order to identify and determine the specificity of the antibody, Ouchterlony immunodiffusion test, western blot analysis and in vitro inhibition of enzyme activity were carried out. The double immunodiffusion test on Ouchterlony plates with antiserum produced a single band of precipitation with purified enzyme. Single precipitation bands were also observed when the antibody was allowed to react with crude extract and the hydroxylapatite column eluted enzyme peak. No precipitation band was seen between the antibody and the

hexose dehydrogenase peak of DEAE elute (Fig. 6b). When the enzyme was heated to 56°C for 15 min there was no precipitation band formation indicating the thermolability of the enzyme. The specificity of the antibody was also confirmed by western blot analysis, which showed a single band with both crude and purified enzyme (Fig. 6c). Inhibition of enzyme activity was demonstrated following incubation with increasing amounts of immune serum but not following incubation with non-immune serum (Fig. 7).

III. Light microscopic immunocytochemical localization:

Light microscopic immunocytochemical studies in the paraffin embedded Bouins fixed retinal tissue using the indirect diaminobenzidine peroxidase antiperxoidase method revealed positive immunoreactive localization in various cell types of the retina. Aldose reductase immunoreactivity was found to be localized in the cytoplasm of Müller cell processes, ganglion cells, retinal pigment epithelium and in the walls of retinal capillaries. These structures were identified by their characteristic location and morphologic appearances.

Trypsin digested preparations of retinal capillaries revealed positive immunoreactive localization of aldose reductase in the cytoplasm of both pericytes and endothelial cells identified on the basis of their characteristic morphologic appearances (Fig. 8a-f).

IV. Ultrastructural immunocytochemical localization of aldose reductase and quantification of the Localization in retinal pigment epithelium:

Ultrastructural localization of aldose reductase was carried out in the non-osmified Epon-embedded retina using protein-A gold on grid immunomarking. This technique resulted in the localization of the antigen in the same cells as it was localized to using the lightmicroscopic technique, namely retinal pigment epithelium, Müller cells, ganglion cells and in the endothelial cells and pericytes of the retinal capillaries (Fig 9a-j). Quantification of the enzyme localization was performed by calculating the density of the gold particles in the pigment epithelium and the results are summarized in table 2. A 2.8 fold increase in the density of gold particles were found in the diabetic animals compared to controls, which was statistically significant ($p<0.05$) (Fig 10, Table 2).

V. Morphologic and morphometric evaluation of the retina

A. Clinical monitoring:

Diabetic animals (D) and diabetic animals with statil treatment (DS) showed hyperglycemia and significant reduction in body weight compared to the two control groups (C and CS) both after 4 and 6 months of treatment ($p<0.001$). In contrast diabetic animals treated vigorously with insulin (DI) showed euglycemia and normal body weights after 4 months and after 6 months of treatment. After 4 months of treatment the body weights of these animals were not different from that of the control animals but were significantly ($p<0.001$) greater than in the hyperglycemic animals (D,DS). Body weights in this group (DI) was 12-15% less than in the control groups after 6 months of treatment, but still 10-15% greater than in the hyperglycemic groups (D,DS) (Table 3,4). Variations in body weights and blood glucose levels and in the different experimental groups during the follow-up period are shown in figure 11 and 12a respectively. As expected, the glycated hemoglobin levels paralleled those of the blood glucose concentration. Both after 4 and 6 months of treatment the hyperglycemic animals (D,DS) showed a significantly higher ($p<0.001$)

glycated Hb levels, which was prevented by vigourous insulin therapy (Table 5). These findings suggest achievement of a relatively metabolic well being in the vigorously insulin treated diabetic animals. Variations in glycated Hb levels in the different experimental groups during the follow-up period are illustrated in figure 12b.

Diabetic untreated animals (D) and those treated with Statil (DS) showed polyuria (>30 ml/d), accompanied with glycosuria (>2 gm/dl) and occasional ketonuria. A 24 hour urine output of <5 ml without any glycosuria or ketonuria were seen in the control animals. Good blood-glucose control with insulin resulted in near normalization of these urinary abnormalities. In the diabetic animals vigorous insulin therapy (DI) resulted in an urine volume of <10 ml/d and glycosuria not exceeding 0.50 gm/dl.

B. Quantitative capillary morphometry:

BM thickness:

BM thickness varied according to the location of the capillaries. Capillaries of the superficial capillary bed were found to have an 1.4-1.8 fold increase in BM thickness over that of the deep capillary bed. The BM

thickness in the superficial capillary bed in all experimental groups was significantly thicker than the corresponding BM thickness of the deep capillary bed at both 4 and 6 months (Table 6,7). Mean BM thickness of control groups (C,CS) both in the superficial and in the deep capillaries, was thicker after 6 months than 4 months. Other experimental groups also paralleled this finding (Fig 13).

When the BM thickness of various experimental groups were compared by one way analysis of variance and linear contrast, a heterogeneic response of the preventive effects of different treatment modalities on diabetic retinal capillary BM thickening was noted. The effects of treatment modalities varied with regard to the location of the capillaries.

Four month treatment group: In the superficial capillary bed diabetic animals and diabetic animals treated with Statil (D, DS) showed a 1.4-1.6 fold statistically significant ($p<0.001$) thicker BM compared to that of the controls. This BM thickening was completely prevented in near-normoglycemic diabetic animals treated vigorously with insulin. Diabetic BM thickening in the superficial capillaries could not be prevented by Statil treatment (Fig 14, table 8).

In the deep capillary bed a different response was observed. Similar to the situation in the superficial capillary bed, diabetic animals showed a significantly (1.7 fold, $p<0.001$) thicker BM than controls. However, in contrast to the situation in the superficial capillary bed, the BM thickening was completely prevented by both statil treatment and vigorous insulin treatment (Fig 15, Table 8). Both of these groups (DS, DI) showed a BM thickness not significantly different from that of the control groups.

Regression analysis of BM thickness using blood glucose levels as independent variables showed a significant association between these two parameters both in superficial and in deep capillary beds ($p<0.005$ and $p<0.025$ respectively) (Fig 16a). A significantly dependent relationship was also noted when similar regressions were carried out using mean glycated Hb values as independent variables ($p<0.005$ for both capillary beds) (Fig 16b).

Six month treatment group: Results similar to that of the 4 month treatment group was also observed after 6 months of treatment. Both in the superficial and in the deep capillary beds, diabetic animals showed a 1.5 fold increase in BM thickness compared to that of control

animals ($p<0.001$). In the superficial capillary bed this BM thickening was completely prevented by vigorous insulin treatment (DI), but not by treatment with statil. The rats on vigorous insulin therapy (DI) showed a significantly thinner ($p<0.001$) BM than hyperglycemic animals (D, DS) (Fig 17; Table9). However in the deep capillary bed both statil treatment and good control with insulin were found to have a significant preventive effect on diabetic BM thickening (Fig 18; Table 9). Regression analysis of BM thickness using blood glucose levels and glycated Hb as independent variables revealed significant relationships (Fig 19).

Other quantitative parameters:

The number of endothelial cells and pericyte processes were counted from electronmicrographs. The mean number of endothelial cells per capillary varied from 1.7-2.0 and that of the pericyte processes from 1.8-2.9 in the various groups. Both after 4 months and 6 months of treatment, no significant differences were found in the number of endothelial cells and pericyte processes per capillary between groups either in the superficial or in the deep capillary beds. Other quantitative parameters analysed from the electronmicrographs included pericyte profile area, endothelial cell profile area and lumenal

area. No significant differences were noted in these measurements either in the superficial or in the deep capillary beds between groups after 4 and 6 months of treatment (Table 10-13). Pericyte-endothelial cell ratio, measured from trypsin digested retinal preparations, and vascular density, measured from toluidine blue stained semithin sections, did not reveal any significant differences between various groups either after 4 or after 6 months of treatment (Table 14).

C. Qualitative structural abnormalities:

Trypsin digested preparations:

These preparations revealed the presence of endothelial cells identified by elongated vesicular nuclei and pericytes with dark rounded nuclei frequently protuding outside the vessel wall (Fig 20). Retracted capillaries (mesodermal bridges) were also identified. However these preparations failed to show the presence of advanced retinopathic changes such as microaneurysms or shunted vessels in any of the groups.

Semithin sections:

Various retinal layers were identified in the

semithin toluidine blue stained sections (Fig 2). However like that of the trypsin digested preparations, advanced retinopathic changes such as hemorrages, exhudates or infarctions were not demonstrated.

Ultrastructural capillary cellular abnormalities:

These abnormalities were examined from the electronmicrographs of retinal capillaries. Ultrastructurally the nucleus and various cytoplasmic components of the endothelial cells were visible. Pinocytotic vesicles and occasional Weibel-Palade bodies were seen. The former did not appear to be increased in any of the experimental groups. In the deep capillary bed, after 4 and after 6 months of treatment, endothelial cells showed occasional abnormalities suggestive of early degenerative changes. The abnormalities included nuclear pyknosis and loss of cytoplasmic organelles. However the incidences of these changes were not statistically significant (Table 10-13, Fig 21). Nuclei, cytoplasmic organelles and pinocytotic vesicles were identified in retinal capillary pericytes of all animals. Various changes suggestive of early degeneration, such as nuclear

pyknosis and loss of organelles, were noted in all experimental groups both after 4 and 6 months of treatment. A 6-fold increase in the incidence of these changes were found in the deep capillary bed of diabetic untreated animals (D) after 6 months of treatment. However, analysis of variance failed to show any statistical significance (Table 10-13, Fig 21).

BM abnormalities:

BM abnormalities were noted in hyperglycemic diabetic animals. The type of abnormalities varied as to the location of the capillaries. Abnormalities in the superficial capillaries were less in number. Laminated appearence of the BM were seen most often in the superficial capillary bed whereas abnormalities such as localized nodular swelling, localized deposition of fibrillar material, and projection of BM material towards the surrounding glia, and cellular debris within the BM were commonly demonstrated in the deep capillary bed (Fig 22-23). The quantification of these abnormalities, as a group, is summarized in table 15 and 16. In the superficial capillary bed they did not differ significantly between groups after 4 months of follow-up. After 6 months of treatment however, a significant increase ($p<0.02$) in the

incidence of these abnormalities were seen in the diabetic animals. In the deep capillary bed, diabetic untreated animals (D) showed a 3.2-fold and 4.8-fold increase in the incidence of these abnormalities after 4 and 6 months of treatment respectively. This increase was statistically significant ($p<0.01$ and $p<0.001$). Statil or vigrous insulin treatment did not show a clearcut preventive effect on the development of these abnormalities (Table 15, 16).

Retinal pigment epithelium:

Normal nuclear and cytoplasmic components were identified in the retinal pigment epithelium. However, basal plasmalemmal infoldings of the pigment epithelium, although not quantified, appeared to be reduced in the diabetic hyperglycemic animals (D) after 6 months of diabetes. Along with the localized loss of infoldings, focal exaggerations of plasmalemmal infoldings were seen in these animals. Both insulin treatment and Statil treatment appeared to have a preventive effect on this change (Fig 24). Except for the basal plasmmalemal infolding changes retinal pigment epithelium appeared normal in all animals.

6. DISCUSSION:

The demonstration of the critical role of aldose reductase in the pathogenesis of sugar cataract formation inspired the concept that an augmented polyol pathway may be an important pathogenetic mechanism in other diabetic complications (Kinoshita, 1986; Dvornik, 1987). The target tissues of diabetic complications such as, peripheral nerve, kidney and retina, like the ocular lens, are exposed to fluctuating extremes of systemic glycemia. Glucose entry into these tissues, being non-insulin dependent, leads to an increase in intracellular glucose concentration, which may be subsequently shunted to the polyol pathway (Dvornik, 1987).

Increased polyol pathway activity with subsequent metabolic, functional and structural abnormalities have been well characterized in diabetic peripheral nerves. According to Greene et al (1985), the series of metabolic abnormalities include an increased polyol pathway activity, reduced myo-inositol concentration and impaired Na^+-K^+-ATPase activity. These metabolic abnormalities, attributible to hyperglycemia, may provide a basis for functional and structural alterations in the diabetic peripheral nerves (Sima, 1985). Evidence of similar metabolic abnormalities have been demonstrated in the

kidney, such as the presence of aldose reductase, and a high sorbitol content in the inner medulla in diabetes (Ludvigson and Sorenson, 1980; Gabbay and O'Sullivan 1968). Further evidence, such as prevention of tissue myo-inositol depletion in the diabetic kidney following treatment with an aldose reductase inhibitor, suggests that this metabolic cycle may be of pathogenetic significance in diabetic nephropathy (Beyer-Meyers and Cohen, 1984). Biochemical changes similar to that seen in peripheral nerves have also been demonstrated in the diabetic retina (MacGregor et al, 1986; MacGregor and Matchinsky, 1986).

The present study was designed to investigate the role of an increased polyol pathway activity in the pathogenesis of diabetes associated lesions in the retina of the spontaneously diabetic BB-rat. This model closely mimics human IDDM (Marliss et al, 1982). Several diabetic complications, such as diabetic neuropathy, nephropathy and retinal microangiopathy have been produced in this model (Sima, 1985; Cohen et al, 1987; Sima et al, 1985). The role of an increased polyol pathway activity and subsequent biochemical, functional and structural abnormalities in the peripheral nerve have been well established in this model (Sima, 1985). In the retina these animals develop BM thickening, pericyte and

endothelial cell degeneration and a retinal pigment epitheliopathy (Sima et al, 1985; Blair et al, 1984). An increase in the retinal vascular permeability, which is preventable by aldose reductase inhibitor therapy, have been demonstrated in this model (Williamson et al, 1987). The changes seen in the retina of diabetic BB-rat, such as capillary basement membrane thickening, pericyte degeneration and loss and involvement of retinal pigment epithelium closely mimic early changes of the human diabetic retina (Sima et al, 1985). Although presence of retinal pigment epitheliopathy has never been investigated in human diabetic retinopathy, defect in retinal pigment epithelium in fluroscence angiography and an increase in vitreous flurosence in vitreous flurophotometry, has been demonstrated (Plamberg et al, 1981; Cunha-vaz, 1983).

To imply an activated polyol pathway in the pathogenesis of any diabetic complication, aldose reductase must be present in the tissue and the lesions developing in these tissues must be prevented by aldose reductase inhibition (NIH conference on aldose reductase, 1984; Kinoshita 1986). The enzyme aldose reductase is thought to be species specific due to lack of cross immunoreactivity between various species (Flynn, 1986); hence in the first part of this study aldose reductase of

the BB-rat was purified and antibody was raised against it. The antibody was subsequently used for light and electronmicroscopic immunocytochemical studies of the retina. In the second part of the study the effects of aldose reductase inhibition on the diabetic retina was studied and compared with that of euglycemia.

I. Enzyme purification and Immunocytochemical localization.

Aldose reductase is present in the Sertoli cells and in mature spermatids of the rat testis (Ludvigson and Sorenson, 1980). In this study purification of this enzyme from BB rat testis was achieved to a degree similar to that obtained by other investigators (Ludvigson and Sorenson, 1980; Gabbay and Kinoshita, 1975). Further purification was not undertaken because of possible loss of enzyme yield and stability (Ludvigson and Sorenson, 1980) as the ultimate aim was to localize the enzyme immunohistochemically. The glyceraldehyde/glucoronate reducing ratio is a simple and useful criterion for the separation of aldose reductase from hexone dehydrogenase in the DEAE-cellulose column elute (Gabbay and Cathcart, 1971). Unlike Gabbay and Kinoshita (1975), who found separation of two isoenzymes

on hydroxylapatite column chromatography of bovine aldose reductase, no isoenzymes were separated in this study. The findings of this study are therefore similar to the observations made by Ludvigson and Sorenson (1980). Sephadex G-100 column chromatography led to an increased purification yield with the removal of unwanted protein.

Since aldose reductase probably acts upon the aldehyde form of D-glucose in vivo, the discrepancy between in vivo activity of the enzyme and the low Km reported for D-glucose in purified enzyme preparations (Cromlish and Flynn, 1983; Inagaki et al, 1982) raises the possibility of some alteration of the enzyme during purification. In the present study, however, a satisfactory purification was achieved as demonstrated by a single band on SDS-PAGE electrophoresis as opposed to previous reports of triplet bands (Ludvigson and Sorenson, 1980). The estimated molecular weight of the enzyme was 36,500 $\pm$ 1000, which is similar to that previously reported: 36,500 in rat seminal vesicle (Ludvigson and Sorenson, 1980), 39,000 in human placenta (Clements and Winegrad, 1972), 37,000 in bovine lens (Sheaff and Doughty, 1976) and 32,000 in bovine kidney (Gabbay and Cathcart, 1971).

The formation of a single immunoprecipitation band after double immunodiffusion of the antibody with

purified, semi-purified and crude enzyme, and no immunoprecipitation band with hexose dehydrogenase, demonstrate the specificity of the obtained antibody. This is further supported by non-immunoreactivity of heat inactivated enzyme, single bands on Western blot analysis with both crude and purified enzyme, and inhibition of enzyme activity by incubation with immune serum (Gabbay and Cathcart, 1971).

It is widely held that metabolic factors, such as activation of the polyol pathway conditioned by hyperglycaemia, contribute to the characteristic structural alterations in diabetic retinopathy (Frank, 1984).

The finding of aldose reductase immunoreactivity in ganglion cells and in Müller cell processes of the retina and in capillary pericytes is similar to the localization of this enzyme in the human retina (Akagi et al, 1976; 1983). Muller cells and ganglion cells of both canine retina and rat retina have been previously shown to possess aldose reductase immunoreactivity (Ludvigson and Sorenson, 1980; Kern and Engerman, 1981). In addition immunoreactivity in the retinal pigment epithelium and in endothelial cells of retinal capillaries was demonstrated for the first time. Although there is no previous report on the localization of aldose reductase in retinal

capillaries in paraffin embedded sections, enzyme immunoreactivity has been demonstrated in endothelial cells of rat aorta and muscular arteries (Ludvigson and Sorenson, 1980) and in cultured bovine retinal and cerebral microvessels (Kennedy et al, 1983). Isolated canine retinal microvessels were also found to contain hexitol producing activity (Kern and Engerman, 1985). In the present study trypsin digested preparations showed positive immunoreactivity both in endothelial cells and in pericytes, whereas similar studies in humans and in dogs showed localization of the enzyme in pericytes only (Akagi et al, 1983, 1987). The localization of the enzyme to retinal pericytes and endothelial cells in the BB rat may explain previously described hydropic and degenerative changes of these cells (Sima et al, 1985). Aldose reductase immunoreactivity in the retinal pigment epithelium of the BB-rat may be relevant in explaining the pigment epitheliopathy in the diabetic BB rat retina (Blair et al, 1984).

Since naturally occuring proteins other than aldose reductase may share homologous sequences and therefore may cross-react with antibodies raised against aldose reductase, the possibility of false positive immunostaining does exist. Alternatively, failure of immunostaining of a particular cell, however, does not

exclude the possibility of an increased polyol pathway activity in such tissues in diabetes due to the presence of other hexitol producing enzymes.

In this study quantification of enzyme immunoreactivity at the ultrastructural level on the vascular cells were not performed due to the fact that small amount of cytoplasm of these cells are usually exposed on a cross section which makes accurate quantification difficult. However, ultrastructural quantification of the enzyme immunoreactivity in the retinal pigment epithelium demonstrated a significantly increased content of aldose reductase immunoreactivity in diabetes which was associated with the occurance of a pigment epitheliopathy as evidenced by the loss and focal exaggeration of basal plasmalemmal infoldings. Light microscopic immunohistochemistry for aldose reductase in diabetic rat and human lens has also shown increased staining compared to that of controls in association with sugar cataract formation (Akagi et al, 1987). Significant increased aldose reductase immunoreactivity in the retinal pigment epithelium in diabetic animals in association with the loss of basal plasmalemmal infoldings of these cells may be helpful in relating an increased polyol pathway activity to retinal pigment epitheliopathy and subsequent breakdown of the BRB. A

similar retinal pigment epitheliopathy in the BB-rat has been observed by Blair et al (1984) in the BB-rat. Grimes and Laties (1983) have reported on pigment epitheliopathy in the streptozotocin diabetic rat, which was not associated with loss of basal plasmalemmal infoldings. Breakdown of BRB is an early feature of diabetic retinopathy (Cunha-vaz et al, 1983). Retinal pigment epithelium and endothelial cell tight junctions are thought to be the structural substrate of this barrier (Lightman et al, 1987). BRB breakdown in human diabetes has been shown to be prevented by aldose reductase inhibitor (Cunha-vaz et al, 1985). In a recent report by Lightman et al (1987) prevention of BRB breakdown has been demonstrated by the treatment with an aldose reductase inhibitor in galactosemic rats. These findings suggests that an increase in polyol pathway activity may play an important role in the pathogenesis of BRB breakdown. Previous studies in the diabetic BB-rat have shown an increased permeability of horseradish peroxidase across the retinal pigment epithelium in association with a retinal pigment epitheliopathy but not across the endothelial cells (Blair et al, 1984, Unpublished observations in this laboratory). These findings suggests that an increased accumulation of fluorescein in the diabetic vitreous may be due to its permeation across the

retinal pigment epithelium rather than across the endothelial cell tight junctions (Blair et al, 1984). Functionally in a normal rat ERG three waves are demonstrable; the a,b and c waves. Among these the c-wave is generated by the retinal pigment epithelium. In early diabetes a flattening of the c wave of the ERG has been demonstrated, which is preventable by aldose reductase inhibitor therapy (MacGregor and Matchinsky, 1985).

II. Morphologic and morphometric evaluation of the retina.

Hyperglycemia appears to be the primary pathogenetic agent for the development of chronic complications of diabetes (Winegrad, 1987). The various pathogenetic changes in the organs susceptible to diabetic complications are thought to develop as a direct consequence of hyperglycemia and its metabolic implications; such as, an augmented polyol pathway. The present study was designed to evaluate the role of good blood glucose control and to compare this with the effects of polyol pathway inhibition on the development of early microangiopathic changes in the diabetic retina.

The aldose reductase inhibitor used in this study was Statil. Historically the development of aldose reductase inhibitors focused on the prevention of the

development of diabetic cataract. These studies in turn, led to the development of diverse inhibitors. The First compound, which was found to modify the cataracteous process was tetramethylene gluteric acid. Subsequently the first orally effective compound, Alrestein (AY 22,284), was developed in 1969 (Kinoshita, 1974). Further research led to the development of Tolrestat, an aliphatic carboxylic acid, with potent aldose reductase inhibitory activity.

Another group of aldose reductase inhibitors belonging to spironohydantoin family were developed by other laboratories. Sorbinil (Pfizer Laboratories) was the first in vivo effective oral aldose reductase inhibitor entirely prevented the cataractogenic process (Kador et al, 1985).

Heterocyclic alkanoic acids have also been developed as aldose reductase inhibitors. Statil, a non-competetive inhibitor used in this study, belongs to this group. Statil has potent aldose reductase inhibitory action (Kador et al, 1985; Stribling et al,1985). It has been demonstrated that statil can produce a substantial inhibition of retinal aldose reductase activity (Polsum et al, 1987). Several other compounds like salicylic acid, sulindac have also shown to possess aldose reductase inhibitory activity (Kador et al, 1985).

Although these compounds are diverse, it appears that they all interact with aldose reductase and undergo a reversible nucleophilic interaction. The site of the interaction is stereospecific, contains a nucleophilic residue and is independent of either substrate or nucleophilic co-factor fold (Kador et al, 1985).

Clinically two simple parameters reflecting the metabolic well-being in the diabetic state are body-weight and blood glucose levels. In the present study hyperglycemia and reduced body-weight, seen in the diabetic animals with or without statil treatment was prevented in diabetic animals with good blood glucose control and they showed body weight similar to control animals. Another parameter representing the the state of metabolic control is glycated Hb levels, which in this study paralleled that of the blood-glucose levels. Loss of body-weight and hyperglycemia are two well-documented features of the diabetic rat (Marliss et al, 1982; Sima et al, 1985; Greene et al, 1987; Chakrabarti et al, 1987). Insulin replacement exogenously or by pancreatic islet allograft has been shown to increase body-weight and reduce glycemic level (Greene et al, 1987; Chakrabarti et al, 1987). Urinary abnormalities like increased volume and glucose were observed in the hyperglycemic animals. These changes were also prevented

by insulin replacement suggesting an improvement and near-normalization of the metabolic defect.

The main finding in the diabetic retina in this study is a heterogenic response of retinal capillary BM thickening to the two treatment modalities. The finding of a thicker BM in the superficial capillary bed compared to that of the deep capillary bed in all animals is in keeping with previous findings from this laboratory as well as by other investigators (Sima et al, 1985; Fischer and Gartner, 1983). These two capillary beds are anatomically different; the superficial bed is on the arterial side whereas the deep capillary bed is on the venous side of the capillary network (Wise et al, 1971). This anatomical difference may lead to a pressure difference which may be a causative factor in this regional variation (Sosula et al, 1972). Muscle capillary BM thickness has been shown to increase from head to foot, which would suggest that hydrostatic pressure differences may influence the "normal" BM thickness (Williamson et al, 1971). The increase in BM thickness between 4 and 6 months of age in controls as well as in other experimental groups may probably be explained as an age-related increase in BM thickness. An age-related increase in BM thickness has been shown in Sprague-Dawley rats and diabetic and non-diabetic BB-rats

(Nagata et al, 1986; Sima et al, 1985). The method used for measurement of BM thickness is relatively new (McEwen et al, 1987). This is a modification of the method described by Robison et al (1983), which takes into account the variability of the basement membrane and gives an average BM thickness.

Both after 4 months and after 6 months of treatment diabetic animals without any treatment showed a significant increase in BM thickness compared to controls. Good blood glucose control with insulin was found to have a preventive effect on diabetic BM thickening both in the superficial as well as in the deep capillary bed. Treatment with the ARI Statil prevented BM thickening only in the deep capillary bed.

The differential effects on BM thickening following Statil and insulin therapy would suggest that biochemical events are, to various degrees, involved in the pathogenesis of BM thickening in diabetes, and may be modified by topographical and physiological differences between the two capillary beds (Wise et al, 1971). Ambient high glucose levels appear to be the major factor in the BM thickening of the superficial arterial capillaries, probably inducing non-enzymatic glycation of structural proteins, whereas an activated polyol pathway seems to play a prominent role in the BM thickening in

the venous low pressure deep capillary system. Significant associations between blood glucose levels and glycated Hb values, the principle measures of diabetic control, with BM thickness in both capillary beds suggest that hyperglycemia is the common initiator of BM thickening, be it due to non-enzymatic glycation of structural proteins or due to an increased polyol pathway activity via one as yet unknown mechanism.

The reason for this suggested mechanistic difference in BM thickening in the diabetic retina is not known. It is possible that factors like intravascular pressure differences (Williamson et al, 1971) may account for a larger non-facilitated glucose permeation in a high pressure system allowing for a greater glucose concentration in the superficial capillary bed. This would promote non-enzymatic glycation of structural proteins, since this reaction is highly dependent on glucose concentration as well as time (Cohen 1986). Other factors such as a much higher affinity of glucose to bind non-enzymatically to deoxyhemoglobin than to oxyhemoglobin (Cohen, 1986) would conceptually leave less glucose available for permeation in venous capillaries. A similar reasoning may also account for the normal difference in BM thickness between the two capillary beds. However the topographical and physiological factors

responsible for this difference in therapeutic response in retinal capillaries needs further evaluation.

Vascular BM has been thought to serve as a filtration barrier as well as a scaffold for pathologic vasoproliferation (Frank, 1984). It is not clear how and why vascular BM thickens in diabetes. Together with the biochemical and hemodynamic factors, outlined in the introduction, other factors may be responsible. According to Little (1981), it may arise by overproduction or diminished removal of BM material or by entrapment of plasma proteins from vascular leakage or abnormal transport. It has been shown in human and experimental animals that good blood glucose control prevents the characteristic BM thickening in diabetes (Engerman, 1977; Friberg et al, 1985).

The effects of aldose reductase inhibitors on the capillary BM thickening has been previously studied in galactosemic animals, in which the measurements were performed on capillaries from the deep capillary bed and hence no differentiation between this and the superficial capillary bed was made (Robison et al, 1983; Frank et al, 1983; Akagi et al, 1985). The only report of the effect of aldose reductase inhibitor on diabetic retinal capillary BM thickness was performed in the deep capillary bed (Chandler et al, 1984).

Associated with the BM thickening, qualitative BM abnormalities were noted in this study. In the superficial capillary bed these abnormalities were found to be significant after 6 months of diabetes. Neither good blood glucose control, nor Statil treatment showed any clear cut protective effect. In the deep capillary bed these abnormalities were noted in the diabetic non-treated animals both after 4 and 6 months of treatment, without being prevented by any of the treatment modalities used.

Qualitative BM changes such as localized nodular thickening, vacuolation, deposition of fibrillar material may be caused by degenerated and entrapped pericytes debris, so called ghost cells (Tilton et al, 1981). It is to be noted that although an increase in the incidence of capillaries with degenerative changes in the pericytes were seen in the deep capillaries in the diabetic non-treated animals, this difference was not statistically significant. The finding of projections of BM material towards the surrounding glia has been noted by several investigators (Bloodworth and Molitor, 1965; Babel and Luenberger, 1974; Sima et al 1987). According to Bloodworth and Molitor (1965) this may be an early sign of exudate formation. A second mechanism which has been suggested is that this redundant basement membrane is

laid down as a result of disturbed metabolism of the Müller cells, which in itself may be a factor in the production of BM thickening (Fischer and Gartner, 1983).

Finally it is to be noted that in the present study no significant capillary pericyte loss, endothelial cell proliferations or change in pericyte profile, endothelial cell profile or lumenal areas were seen. These may be due to the relatively short duration of this study. Previous studies in the BB-rat have shown that these animals develop significant loss of retinal capillary pericytes after 8 months of diabetes. The same studies showed that significant BM thickening and degenerative changes in pericytes develop after 4 months of diabetes, whereas significant endothelial cell degeneration and retinal capillary microthrombi develop after 8 months of diabetes (Sima et al, 1985).

SUMMARY AND CONCLUSION:

In the present study aldose reductase was purified from the testis of the non-diabetic BB-rat and antibody against this enzyme was raised in rabbits. The antibody was subsequently used for light and ultrastructural immunocytochemical localization of this ennzyme in the retina. The enzyme immunoreactivity was demonstrated in the ganglion cells, Müller cells, retinal pigment epithelium and endothelial cells and pericytes of the retinal capillaries. Quantification of the enzyme immunoreactivity at the ultrastructural level showed a significant increase in the immunoreactivity in diabetic animals which was associated with abnormalities of basal plasmalemmal infoldings in the pigment epithelium. The finding of aldose reductase in the retinal capillary pericytes and endothelial cells may be helpful in explaining the abnormalities of these cells in diabetes. An increase in enzyme immunoreactivity in the retinal pigment epithelium in the diabetic retina may relate to the retinal pigment epitheliopathy and breakdown of blood retinal barrier in these animals.

A heterogenic response of diabetic retinal capillary basement membrane thickening was noted when diabetic BB-rats were subjected to treatment with insulin

and the aldose reductase inhibitor Statil. Insulin treatment prevented diabetic retinal capillary basement membrane thickening both in the superficial and in the deep capillary beds, whereas treatment with Statil prevented basement membrane thickening only in the deep capillary bed. This finding suggests that biochemical events involved in the pathogenesis of capillary basement membrane thickening in diabetes may be modified by the topographical and physiologic differences in the two capillary beds.

TABLE 2.

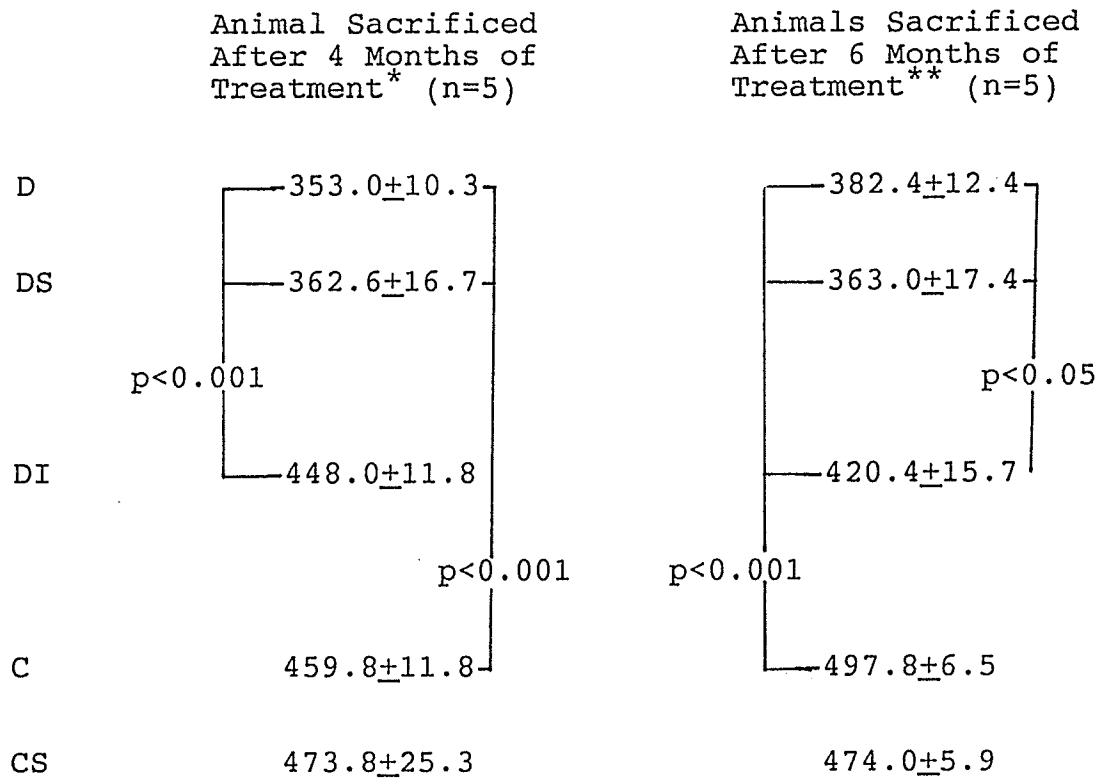
QUANTIFICATION OF PROTEIN-A GOLD IMMUNOMARKING (Particles/um^2) IN THE RETINAL PIGMENT EPITHELIUM (mean $\pm$ SE).

DIABETIC (n = 5)	3.27 $\pm$ 0.74
	$p<0.05$
CONTROL (n = 5)	1.17 $\pm$ 0.23

Significance judged by Student's two-tailed t-test.

TABLE 3

FINAL BODY WEIGHT (gms) (mean ± SE)



ANOVA F values *12.47 ($p<0.005$) and **21.26 ($p<0.005$).
Intergroup comparison done using linear contrast.
D = Diabetic, DS = Diabetic treated with Statil,
DI = Diabetic treated vigorously with insulin,
C = Control, CS = Controls treated with Statil.

TABLE 4

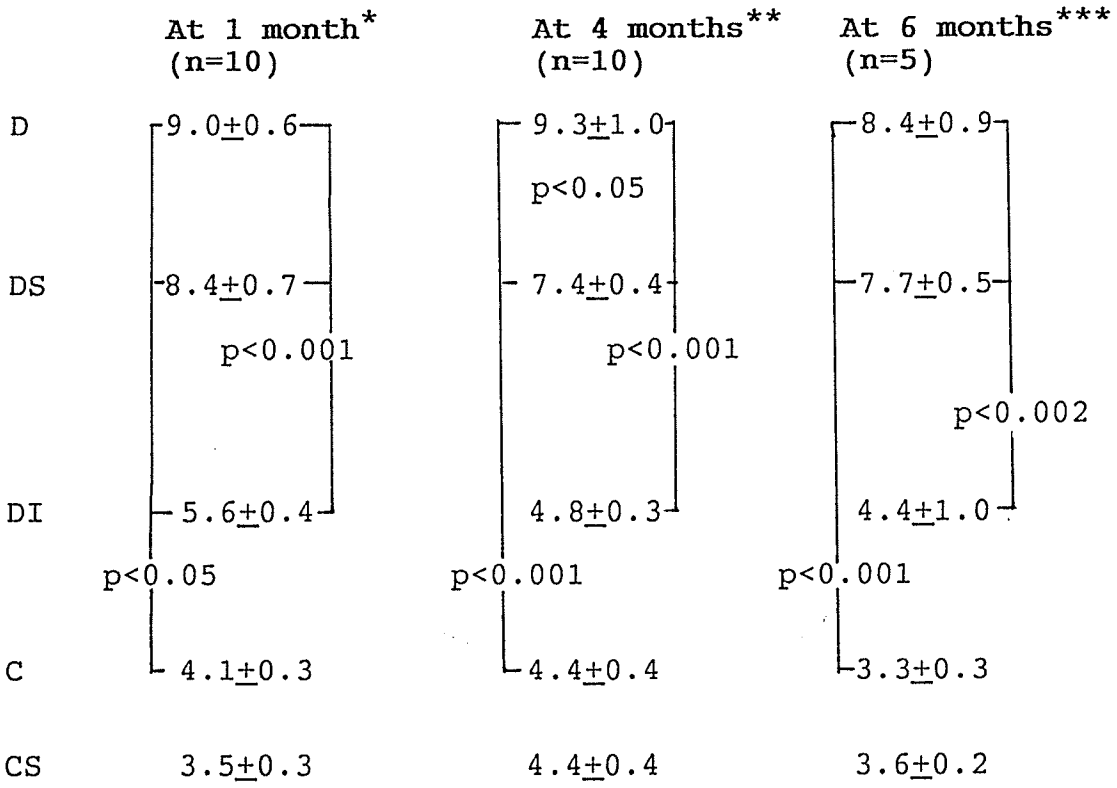
FINAL BLOOD GLUCOSE LEVELS m.mol/L (mean ± SE)

	Animals Sacrificed after 4 months of treatment (n=5)	Animals Sacrificed after 6 months of treatment (n=5)
D	17.8 ± 3.0	20.8 ± 2.1
DS	17.2 ± 1.2	24.3 ± 0.7
DI	5.9 ± 1.1	4.7 ± 0.7
C	6.4 ± 0.9	8.1 ± 1.3
CS	6.0 ± 1.3	9.0 ± 0.5

D = Diabetic, DS = Diabetic treated with Statil,
DI = Diabetic treated vigorously with insulin,
C = Control, CS = Controls treated with Statil.

TABLE 5

GLYCATED Hb VALUES (%) (mean ± SE)



ANOVA F-values *25.09 ($p<0.005$), **15.50 ($p<0.005$), and ***13.69 ($p<0.005$). Intergroup comparison done using linear contrast. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil.

TABLE 6

BASEMENT MEMBRANE THICKNESS (BMT) nm (mean ± SE)
Animals sacrificed after 4 months of treatment.
Comparison* of BMT in superficial vs deep capillary beds.

	Superficial capillary bed		Deep capillary bed
D	179.6±11.9	p<0.02	125.6±15.9
DS	152.1±12.9	p<0.002	93.4± 7.7
DI	124.1±10.5	p<0.005	85.1±10.2
C	111.3± 7.9	p<0.01	72.1± 3.3
CS	106.7± 6.4	p<0.002	68.8± 8.3

n=5 in all groups. * Significance of differences were judged by paired t-test. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil.

TABLE 7

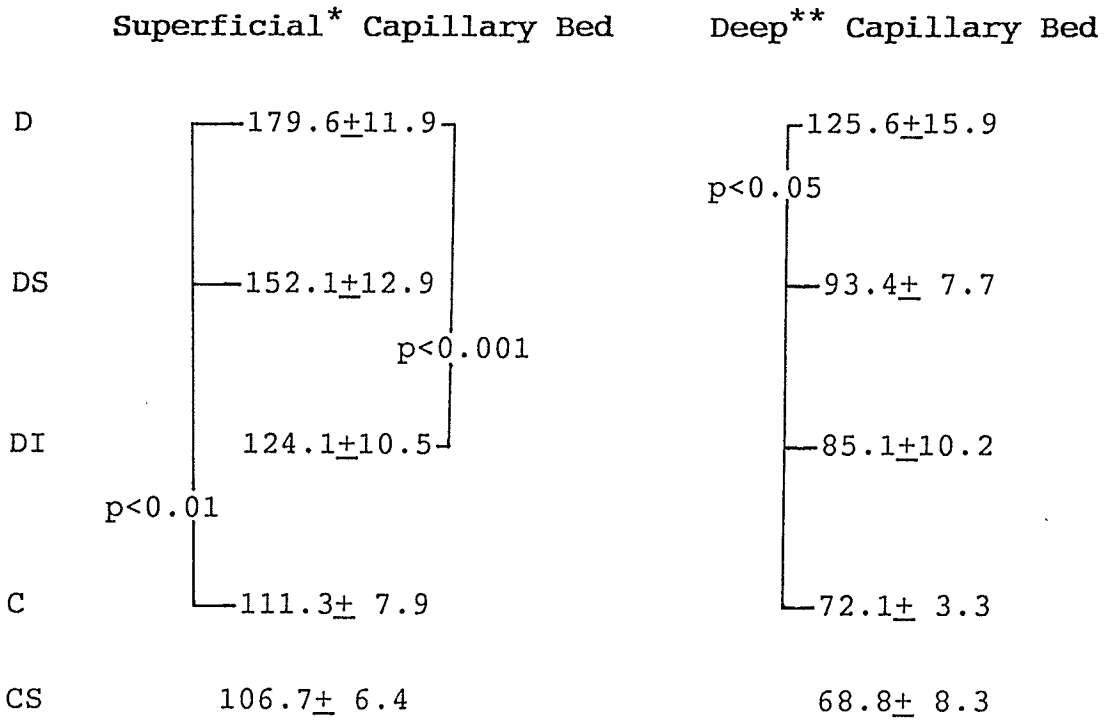
BASEMENT MEMBRANE THICKNESS (BMT) nm (mean ± SE)
Animals sacrificed after 6 months of treatment
Comparison* of BMT in superficial vs. deep capillary beds.

	Superficial capillary bed			Deep capillary bed
D	192.2±5.8	_____	p<0.001 _____	139.9±5.8
DS	175.7±4.5	_____	p<0.001 _____	99.9±4.1
DI	134.0±6.9	_____	p<0.001 _____	97.7±7.0
C	134.6±6.9	_____	p<0.002 _____	95.1±3.3
CS	127.9±6.9	_____	p<0.001 _____	82.8±8.5

n=5 in all groups. *Significance of differences were judged by paired t-test. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil.

TABLE 8

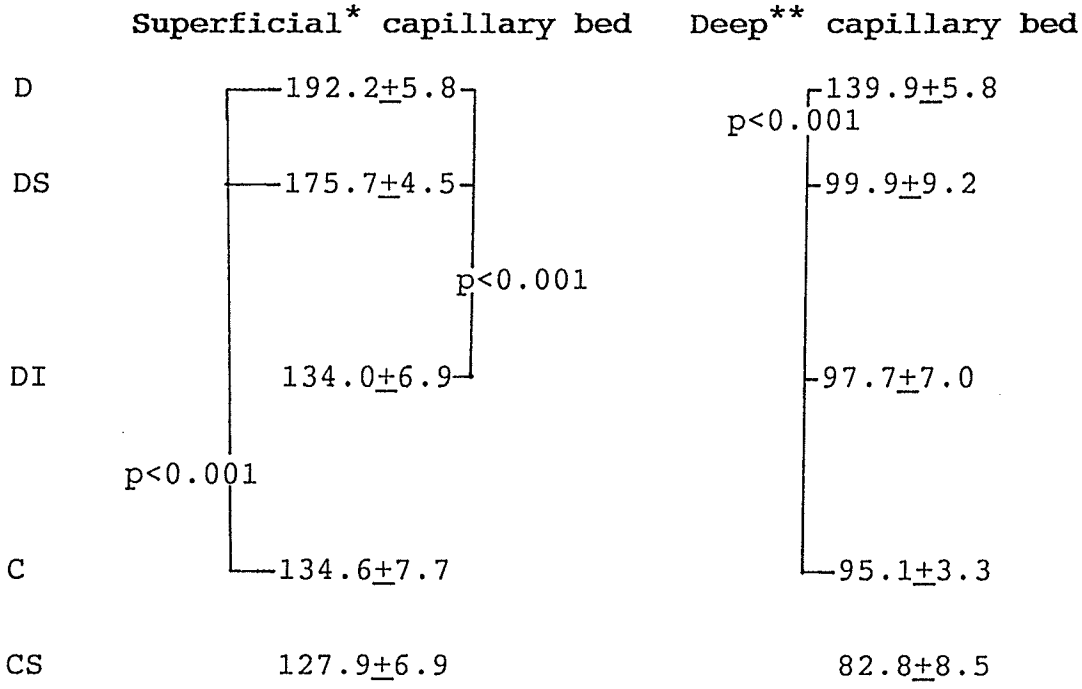
BASEMENT MEMBRANE THICKNESS nm (mean ± SE)
Animals sacrificed after 4 months of treatment.



ANOVA F values *9.02 ($p<0.005$) and **5.21 ($p<0.005$). Intergroup comparison using linear contrast. n=5 in all groups. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil.

TABLE 9

BASEMENT MEMBRANE THICKNESS nm (mean ± SE)
(Animals sacrificed after 6 months)



ANOVA F values *20.19 ($p<0.005$) and **18.57 ($p<0.005$). Intergroups comparison done using linear contrast. n=5 in all groups. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil.

TABLE 10

CAPILLARY MORPHOMETRY OF ANIMALS SACRIFICED AFTER 4 MONTHS OF TREATMENT.
SUPERFICIAL CAPILLARY BED (mean ± SE)

GROUPS	(A)	(B)	(C)	(D)	(E)	(F)	(G)
D	2.16±0.17	1.88±0.09	11.5±6.9	0	14.4±2.9	24.6±3.5	40.9±7.5
DS	2.28±0.16	1.78±0.05	7.0±2.9	0	13.3±1.8	27.0±3.9	39.1±7.9
DI	2.3±0.16	1.86±0.06	8.0±7.9	0	16.9±2.0	18.8±2.6	40.0±8.6
C	2.2±0.18	1.90±0.04	5.5±3.9	0	13.8±2.2	23.1±3.7	49.2±8.6
CS	2.58±0.21	2.00±0.05	4.0±2.4	0	15.6±3.8	24.3±2.5	47.0±8.1
F VALUE	0.82	1.66	0.29		0.30	0.84	0.31
D.F.	4,20	4,20	4,20		4,20	4,20	4,20
Significance	NIL	NIL	NIL		NIL	NIL	NIL

(A) Number of pericyte processes per capillary, (B) Number of endothelial cell per capillary, (C) Percentages of capillaries with pericyte degeneration, (D) Percentages of capillaries with endothelial cell degeneration, (E) Pericyte profile area, (F) Endothelial cell profile area, (G) Lumenal Area. Areas have been expressed as percentages of total capillary area. Comparison has been done using ANOVA. n = 5 in all groups. D=Diabetics, DS=Diabetics treated with statil, DI=Diabetics treated vigorously with insulin, C= Controls, CS= Controls treated with insulin.

TABLE 11

CAPILLARY MORPHOMETRY OF ANIMALS SACRIFICED AFTER 4 MONTHS OF TREATMENT.
DEEP CAPILLARY BED (mean ± SE)

GROUPS	(A)	(B)	(C)	(D)	(E)	(F)	(G)
D	1.9±0.16	1.8±0.05	8.2±3.8	6.0±5.4	8.6±1.6	29.1±3.8	47.6± 7.9
DS	1.9±0.16	1.9±0.06	6.9±2.8	0±0	10.8±1.7	22.1±3.7	45.2± 8.7
DI	2.02±0.20	1.9±0.13	4.0±3.9	0±0	14.8±2.6	31.7±6.9	41.9±11.3
C	2.0±0.16	2.0±0.12	5.9±3.8	3.6±3.6	8.9±1.9	24.7±4.5	56.4±8.9
CS	1.8±0.14	1.7±0.06	11.0±4.0	4.5±2.8	9.5±2.7	30.1±5.8	58.6±8.3
F Value	0.22	1.73	0.50	0.65	1.40	0.61	0.63
D.F.	4,20	4,20	4,20	4,20	4,20	4,20	4,20
Sign*	NIL	NIL	NIL	NIL	NIL	NIL	NIL

(A) Number of pericyte processes per capillary, (B) Number of endothelial cell per capillary, (C) Percentages of capillaries with pericyte degeneration, (D) Percentages of capillaries with endothelial cell degeneration, (E) Pericyte profile area, (F) Endothelial cell profile area, (G) Lumenal Area. Areas have been expressed as percentages of total capillary area. Comparison has been done using ANOVA. n = 5 in all groups. D=Diabetics, DS=Diabetics treated with statil, DI=Diabetics treated vigorously with insulin, C= Controls, CS= Controls treated with insulin.
Sign*= Significance

TABLE 12

CAPILLARY MORPHOMETRY OF ANIMALS SACRIFICED AFTER 6 MONTHS OF TREATMENT.
SUPERFICIAL CAPILLARY BED (mean ± SE)

GROUPS	(A)	(B)	(C)	(D)	(E)	(F)	(G)
D	2.5±0.3	1.8±0.10	8.0±7.9	0	13.3±1.9	22.9±4.5	31.7±7.9
DS	2.8±0.2	2.0±0.04	12.0±11.9	0	16.2±3.4	18.8±3.4	43.7±8.1
DI	2.4±0.1	2.1±0.05	0±0	0	10.6±1.6	16.4±1.1	59.7±2.9
C	2.4±0.2	1.9±0.05	0±0	0	9.8±0.9	21.4±2.9	49.1±7.9
CS	2.9±0.2	2.0±0.07	8.0±3.7	0	14.9±1.3	22.7±1.5	39.5±5.6
F-Value	1.3	1.97	0.65	0	1.82	0.86	2.38
D.F.	4,20	4,20	4,20		4,20	4,20	4,20
Significance	NIL	NIL	NIL		NIL	NIL	NIL

(A) Number of pericyte processes per capillary, (B) Number of endothelial cell per capillary, (C) Percentages of capillaries with pericyte degeneration, (D) Percentages of capillaries with endothelial cell degeneration, (E) Pericyte profile area, (F) Endothelial cell profile area, (G) Lumenal Area. Areas have been expressed as percentages of total capillary area. Comparison has been done using ANOVA. n = 5 in all groups. D=Diabetics, DS=Diabetics treated with statil, DI=Diabetics treated vigorously with insulin, C= Controls, CS= Controls treated with insulin.

TABLE 13

CAPILLARY MORPHOMETRY OF ANIMALS SACRIFICED AFTER 6 MONTHS OF TREATMENT.
DEEP CAPILLARY BED (mean ± SE)

GROUPS	(A)	(B	(C)	(D)	(E)	(F)	(G)
D	1.9±0.2	1.9±0.08	12.0±5.8	2.0±1.9	9.4±0.5	21.3±4.4	54.4±6.9
DS	2.1±0.1	1.9±0.04	2.0±1.9	0±0	9.2±0.5	23.6±5.3	54.5±7.2
DI	1.7±0.1	2.0±0.07	2.0±1.9	4.0±2.5	9.2±2.4	19.2±3.3	61.4±7.7
C	2.3±0.2	1.9±0.05	2.0±1.9	0±0	10.0±3.6	23.1±4.9	56.2±9.8
CS	2.3±0.2	1.8±0.06	0±0	0±0	10.5±2.2	21.8±3.6	56.4±6.1
F-Value	2.07	0.68	2.48	1.60	0.14	0.16	0.14
D.F.	4,20	4,20	4,20	4,20	4,20	4,20	4,20
Significance	NIL	NIL	NIL	NIL	NIL	NIL	NIL

(A) Number of pericyte processes per capillary, (B) Number of endothelial cell per capillary, (C) Percentages of capillaries with pericyte degeneration, (D) Percentages of capillaries with endothelial cell degeneration, (E) Pericyte profile area, (F) Endothelial cell profile area, (G) Lumenal Area. Areas have been expressed as percentages of total capillary area. Comparison has been done using ANOVA. n = 5 in all groups. D=Diabetics, DS=Diabetics treated with statil, DI=Diabetics treated vigorously with insulin, C= Controls, CS= Controls treated with insulin.

TABLE 14

LIGHT MICROSCOPIC STUDY OF THE RETINAL VASCULATURE (mean + SE)

GRUOUPS	PERICYTE/ENDOTHELIAL CELL RATIO		VASCULAR DENSITY /mm^2	
	4 months	6 months	4 months	6 months
D	0.58 ± 0.03	0.60 ± 0.03	143.8 ± 19.9	172.8 ± 8.0
DS	0.60 ± 0.02	0.58 ± 0.02	156.8 ± 11.6	165.8 ± 10.8
DI	0.60 ± 0.05	0.59 ± 0.02	134.3 ± 19.0	177.6 ± 17.6
C	0.66 ± 0.02	0.62 ± 0.04	153.4 ± 18.7	165.3 ± 20.3
CS	0.69 ± 0.04	0.59 ± 0.03	158.9 ± 28.9	193.5 ± 30.9
F Value	2.69	0.23	0.37	0.36
D.F.	4,20	4,20	4,20	4,20
Significance	NIL	NIL	NIL	NIL

Comparison has been done using ANOVA. n = 5 in all groups.
D=Diabetics, DS=Diabetics treated with statil, DI=Diabetics treated vigorously with insulin, C= Controls, CS= Controls treated with insulin.

TABLE 15

CAPILLARIES SHOWING BASEMENT MEMBRANE ABNORMALITIES[+] (%)
Animals sacrificed after 4 months of treatment
(mean ± SE)

	Superficial* Capillary bed	Deep** Capillary Bed
D	8.0 ± 5.8	60.5 ± 6.5
DS	2.0 ± 1.9	59.0 ± 10.8
DI	0.0 ± 0.0	46.7 ± 6.4
		p<0.05
C	2.0 ± 1.9	19.17 ± 6.0
CS	2.0 ± 1.9	23.0 ± 5.8

ANOVA F values *1.19 (Non-significant) and **7.10 ($p<0.005$). Intergroup comparison using linear contrast. n=5 in all groups. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with insulin, C = Control, CS = Controls treated with Statil. [+] Capillary basement membrane abnormalities include laminated appearence, deposition of pericyte debris within basement membrane, fibrillar deposits within basement membrane, localized nodular swelling and outwardly projection of basement membrane material.

TABLE 16

CAPILLARIES SHOWING BASEMENT MEMBRANE ABNORMALITIES (%)
Animals sacrificed after 6 months of treatment
(mean ± SE)

	Superficial* Capillary Bed	Deep** Capillary Bed
D	25.1 ± 5.9	77.3 ± 7.9
DS	26.0 ± 11.2	72.0 ± 7.4
DI	18.1 ± 5.1	82.0 ± 8.6
	p<0.02	p<0.001
C	2.0 ± 2.0	16.0 ± 8.1
CS	4.0 ± 2.4	24.0 ± 6.8

ANOVA F-values *3.32 ($p<0.05$) and **16.52 ($p<0.005$).
Intergroup comparison done using linear contrast.
n=5 in all groups. D = Diabetic, DS = Diabetic treated with Statil, DI = Diabetic treated vigorously with nsulin, C = Control, CS = Controls treated with Statil.
+ Capillary basement membrane abnormalities include laminated appearence, deposition of pericyte debris within basement membrane, fibrillar deposits within basement membrane, localized nodular swelling, and outwardly projection of basement membrane material.

Figure 1. A diagramatic representation of various suggested pathogenetic mechanisms in the development of diabetic retinopathy.

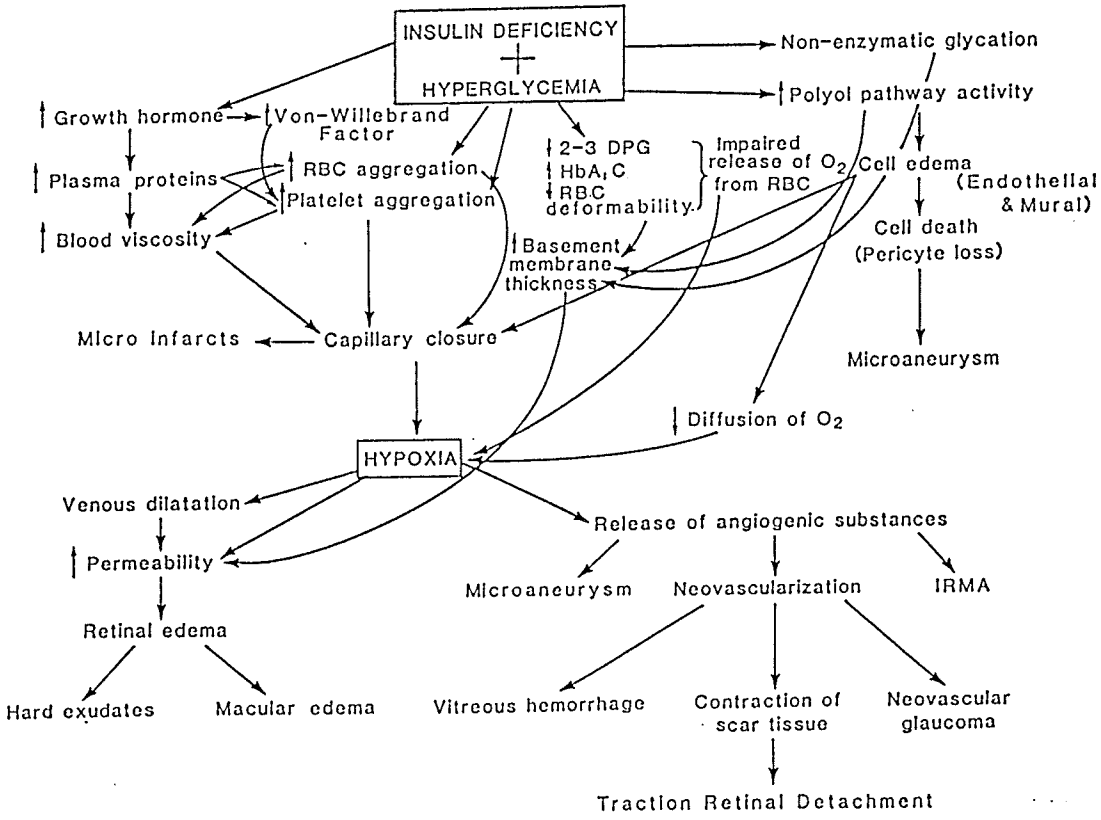

INSULIN DEFICIENCY
+
HYPERGLYCEMIA
Non-enzymatic glycation
↑ Polyol pathway activity
↑ Growth hormone
↑ Von-Willebrand Factor
↑ Plasma proteins
↑ RBC aggregation
↑ Platelet aggregation
↑ Blood viscosity
↓ 2-3 DPG
↓ HbA1C
↓ RBC deformability
Impaired release of O2 from RBC
Cell edema
(Endothelial & Mural)
Cell death
(Pericyte loss)
↑ Basement membrane thickness
Micro Infarcts
Capillary closure
Microaneurysm
↓ Diffusion of O2
HYPOXIA
Venous dilatation
↑ Permeability
Retinal edema
Hard exudates
Macular edema
Release of angiogenic substances
Microaneurysm
Neovascularization
IRMA
Vitreous hemorrhage
Contraction of scar tissue
Neovascular glaucoma
Traction Retinal Detachment

Figure 2. Toluidine blue stained semithin section of the retina showing the various layers (X 680).

Ch = Choroid.

Rpe = Retinal pigment epithelium.

Phl = Photoreceptor layer.

Elm = External limiting membrane.

Onl = Outer nuclear layer.

Opl = Outer plexiform layer.

Inl = Inner nuclear layer.

Ipl = Inner plexiform layer.

Gcl = Ganglion cell layer.

Nfl = Nerve fiber layer.

Ilm = Inner limiting membrane.

Scb = Superficial capillary bed.

Dcb = Deep capillary bed.

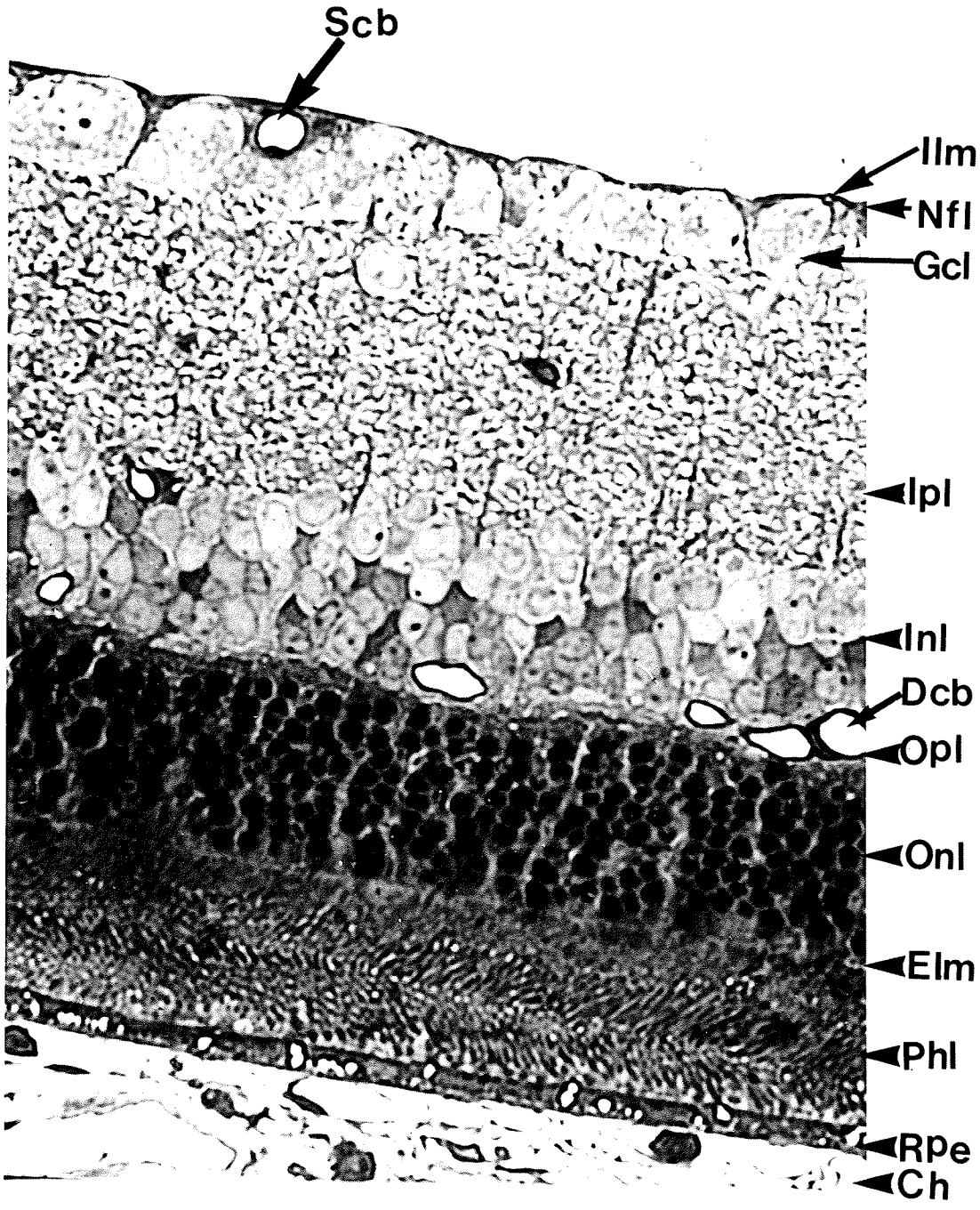
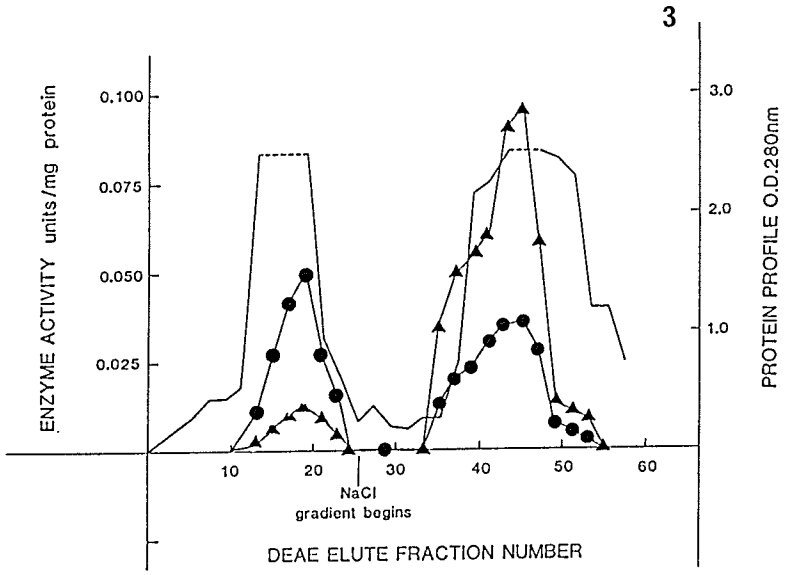
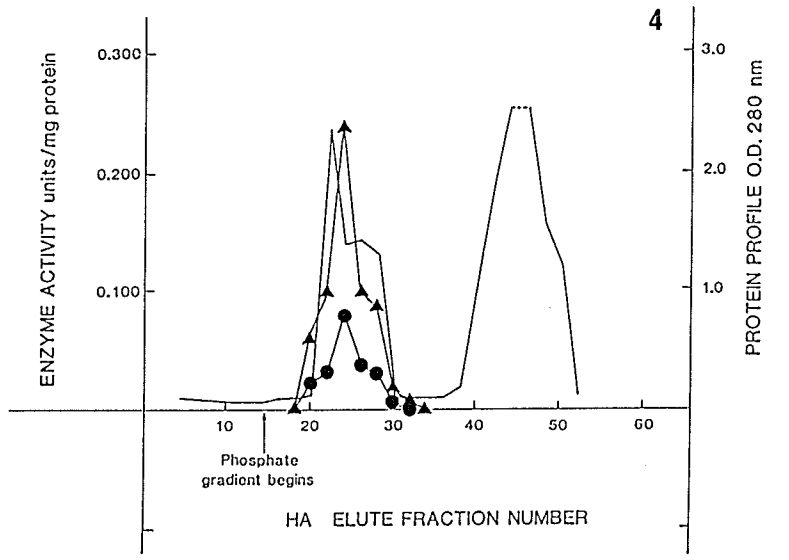

Scb
Ilm
Nfl
Gcl
Ipl
Inl
Dcb
Opl
Onl
Elm
Phl
Rpe
Ch

Figure 3. DEAE-cellulose column chromatography of dialysed amonium sulphate precipitated fraction of testis homogenate.
— Protein profile as measured spectrophotometrically at 280 nm. ●—● Enzyme activity with glucoronate as substrate.
▲—▲ Enzyme activity with glyceraldehyde as substrate. O.D. = Optical density.

Figure 4. Hydroxylapatite column chromatography of the pooled DEAE-cellulose eluted fractions containing aldose reductase activity (Legends same as figure 3).

Figure 5. Repeated Sephadex G-100 column chromatography of the pooled fractions containing aldose reductase activity from the 1st Sephadex G-100 column elute (Legends same as figure 3).


3
ENZYME ACTIVITY units/mg protein
0.100
0.075
0.050
0.025
3.0
2.0
1.0
PROTEIN PROFILE O.D.280nm
10
20
30
40
50
60
NaCl
gradient begins
DEAE ELUTE FRACTION NUMBER


4
ENZYME ACTIVITY units/mg protein
0.300
0.200
0.100
3.0
2.0
1.0
PROTEIN PROFILE O.D. 280 nm
10
20
30
40
50
60
Phosphate
gradient begins
HA ELUTE FRACTION NUMBER

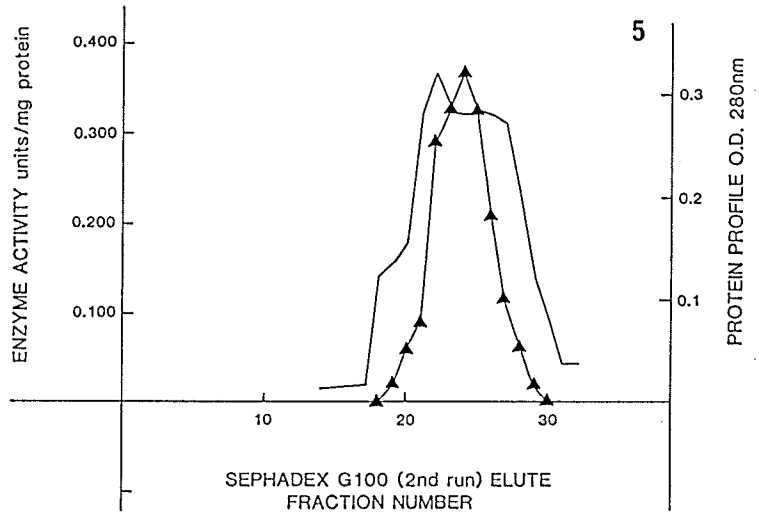

5
ENZYME ACTIVITY units/mg protein
0.400
0.300
0.200
0.100
0.3
0.2
0.1
PROTEIN PROFILE O.D. 280nm
10
20
30
SEPHADEX G100 (2nd run) ELUTE
FRACTION NUMBER

Figure 6. a) SDS-PAGE electrophoresis from aldose reductase peak of the repeated Sephadex G-100 column elute. Single band with a molecular wt. of 36,500 was seen.

b) Oucherloney plate showing immunological identity of aldose reductase. Central well(Ab) - Antibody to aldose reductase; (1) Purified aldose reductase; (2) Hydroxylapatite column eluted peak of aldose reductase; (3) Crude extract from the testis of the BB-rat; (4) Hexose dehydrogenase peak of DEAE-cellulose column elute. Single band of precipitation with 1,2 and 3 and no band formation with 4 was seen.

c) Western blot analysis showing rabbit anti-rat aldose reductase antibody. Antibody reacting with pure enzyme (left) and with crude enzyme (right), both showing single band of precipitation.

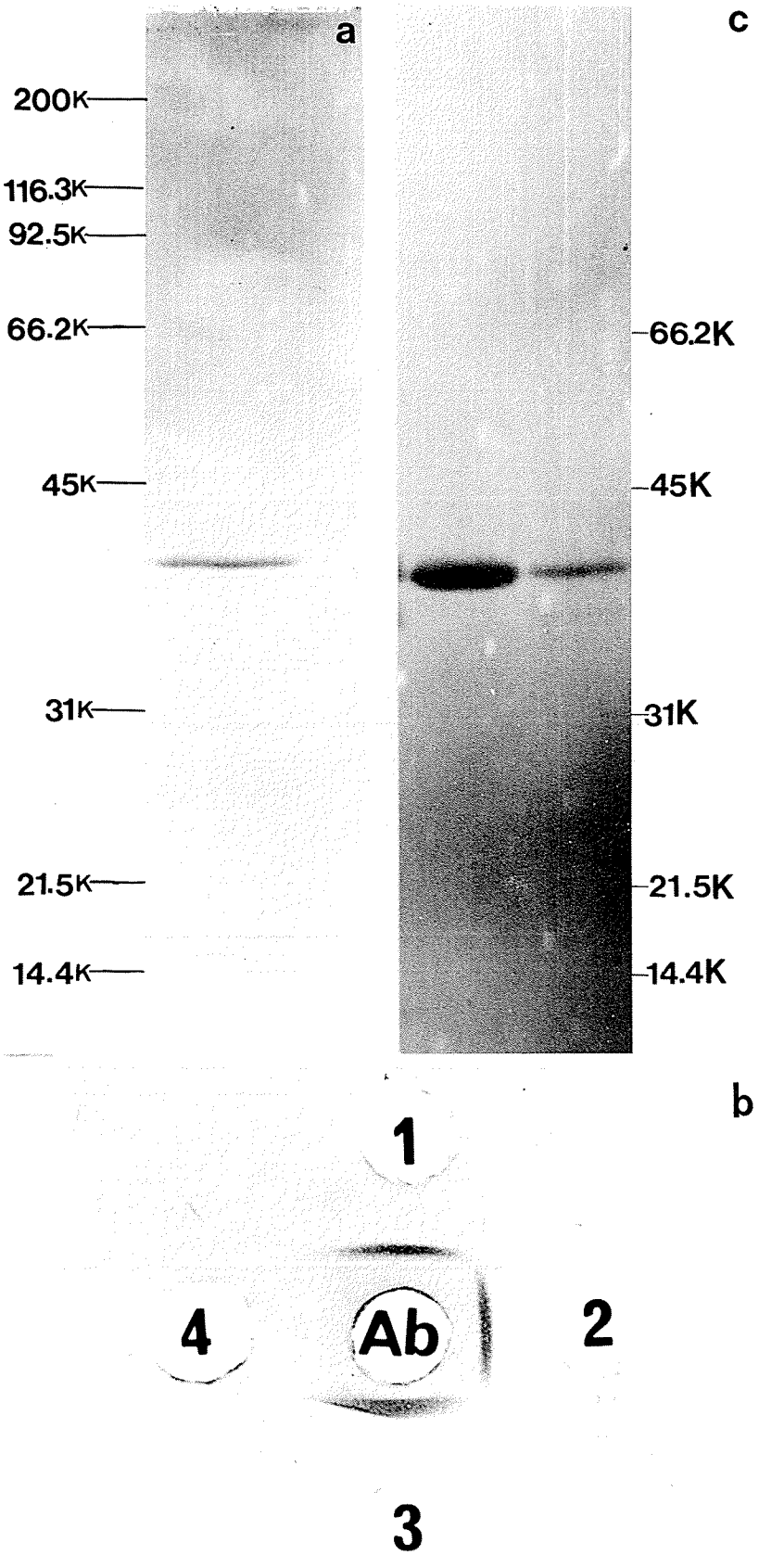

a
c
200K
116.3K
92.5K
66.2K
45K
31K
21.5K
14.4K
66.2K
45K
31K
21.5K
14.4K
b
1
4
Ab
2
3

Figure 7. Inhibition of aldose reductase activity following incubation with increasing ammount of immune serum (●——●). No inhibition of enzyme activity was noted when incubated with non-immune serum (▲——▲). In this experment 10ul of purified enzyme was incubated for 20 min. with increasing amount of immune serum in a final volume of 400ul. non-immune serum was used as controls. The enzyme activity was measured with glyceraldehyde as substrate.

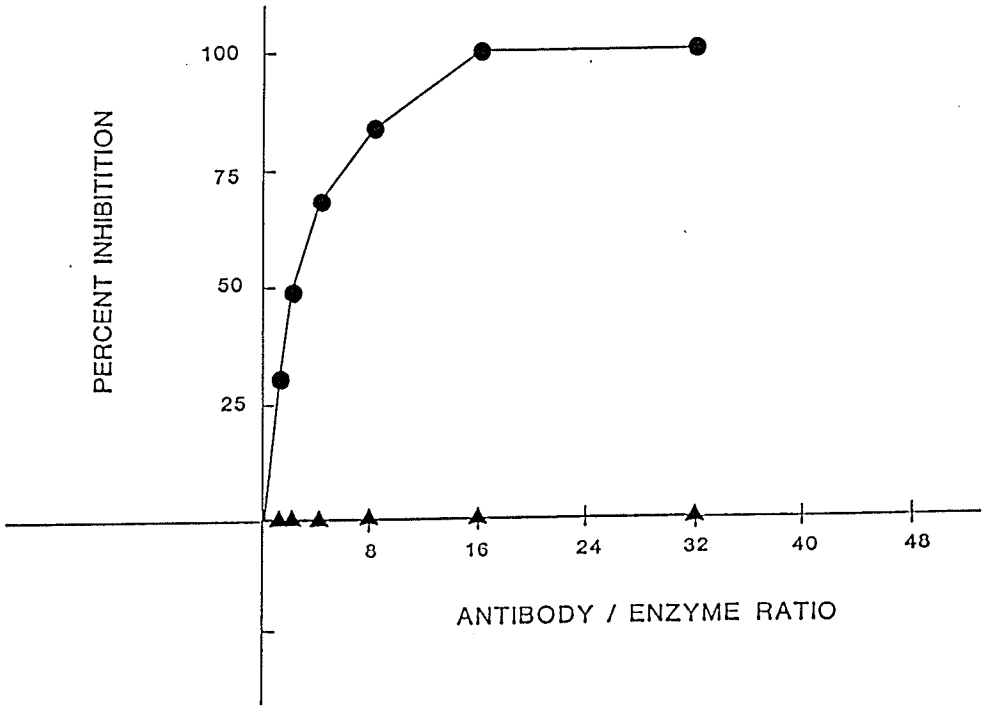

100
75
50
25
PERCENT INHIBITION
8
16
24
32
40
48
ANTIBODY / ENZYME RATIO

Figure 8. Photomicrographs of the retina from BB-rat showing immunocytochemical localization of aldose reductase. a) preabsorbtion control, b) localization of aldose reductase in the ganglion cell cytoplasm (arrowhead), cytoplasm of Müller cell processes (arrow), and capillaries (hollow arrow), c) localization of aldose reductase in the cytoplasm of retinal pigment epithelium (arrow), d) tangential section through a retinal capillary showing positive enzyme localization (arrow), e) immunocytochemical localization of aldose reductase in a trypsin digested retinal preparation showing positive immunoreactivity in an endothelial cell (E) and in a pericyte (P), f) H and E stained retinal section of the retina showing various layers; GCL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer, RPE = retinal pigment epithelium.

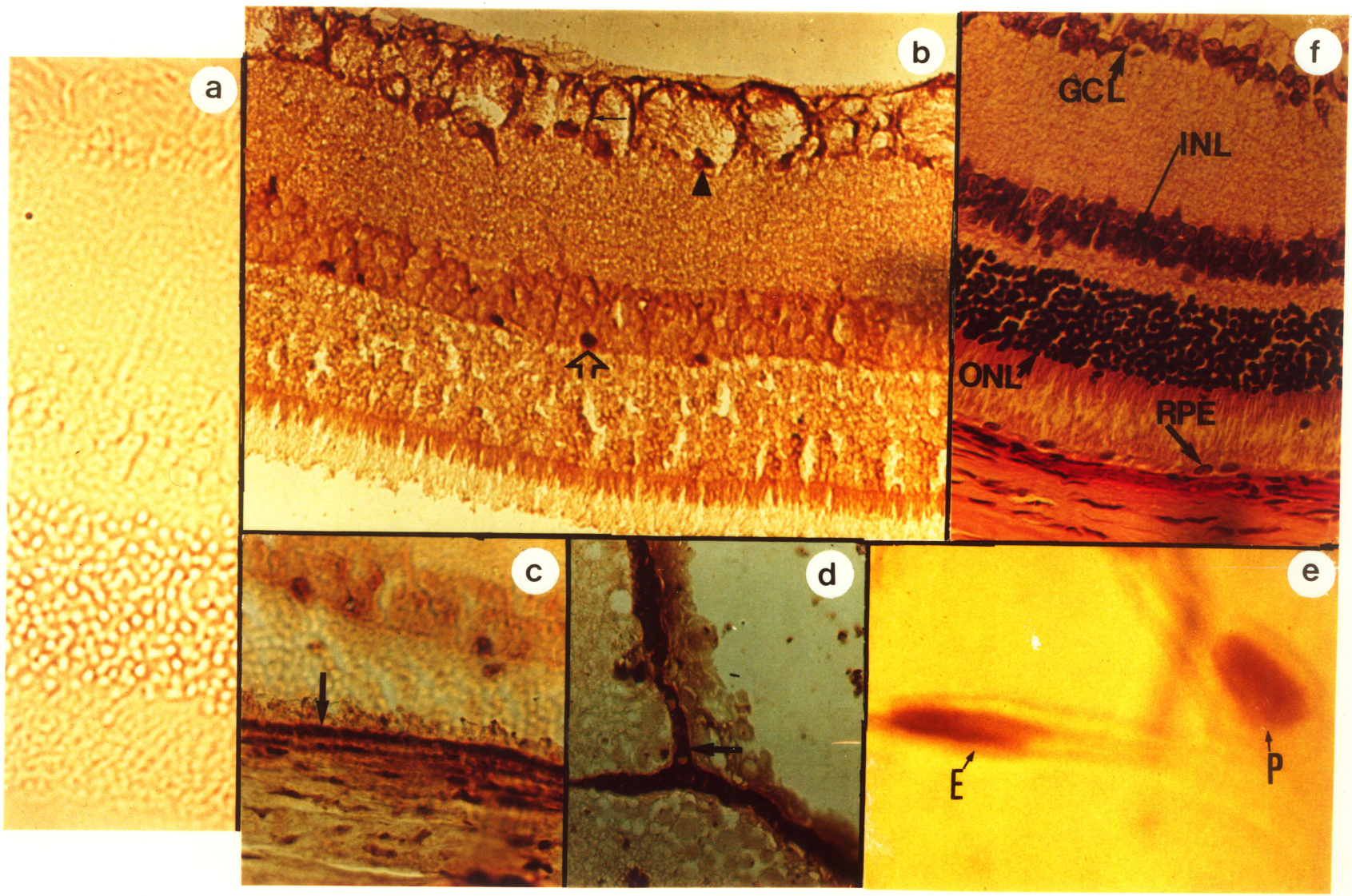
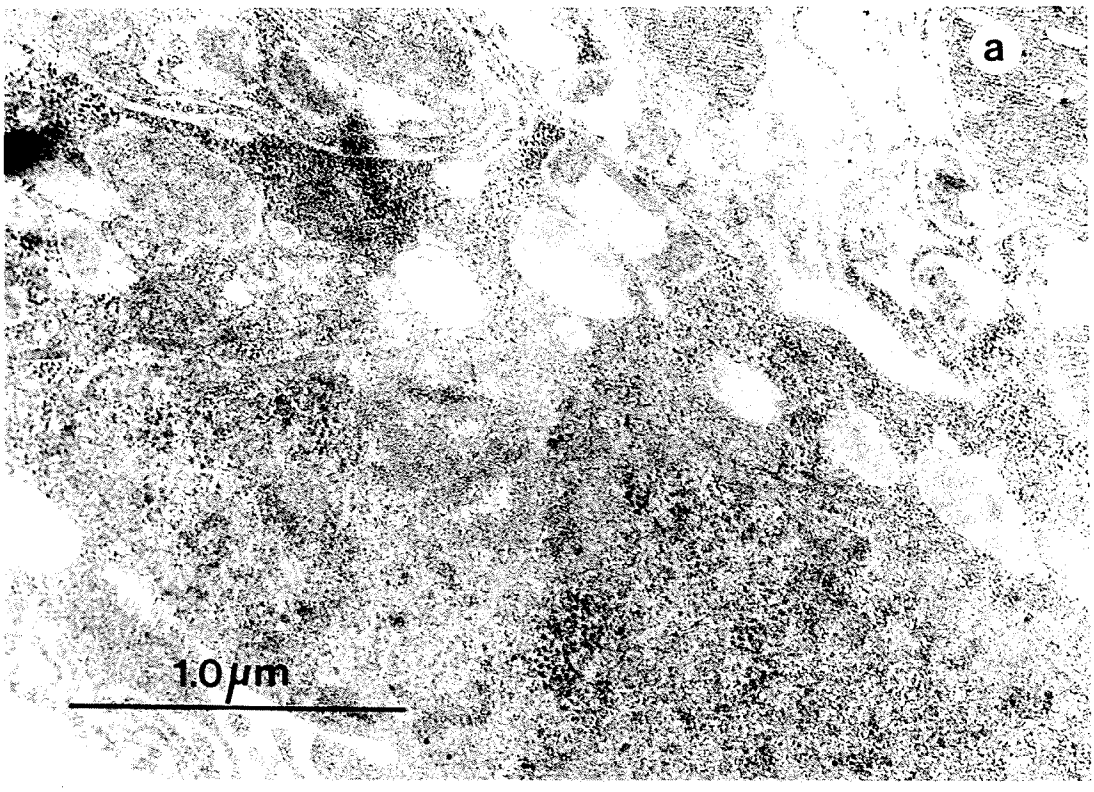
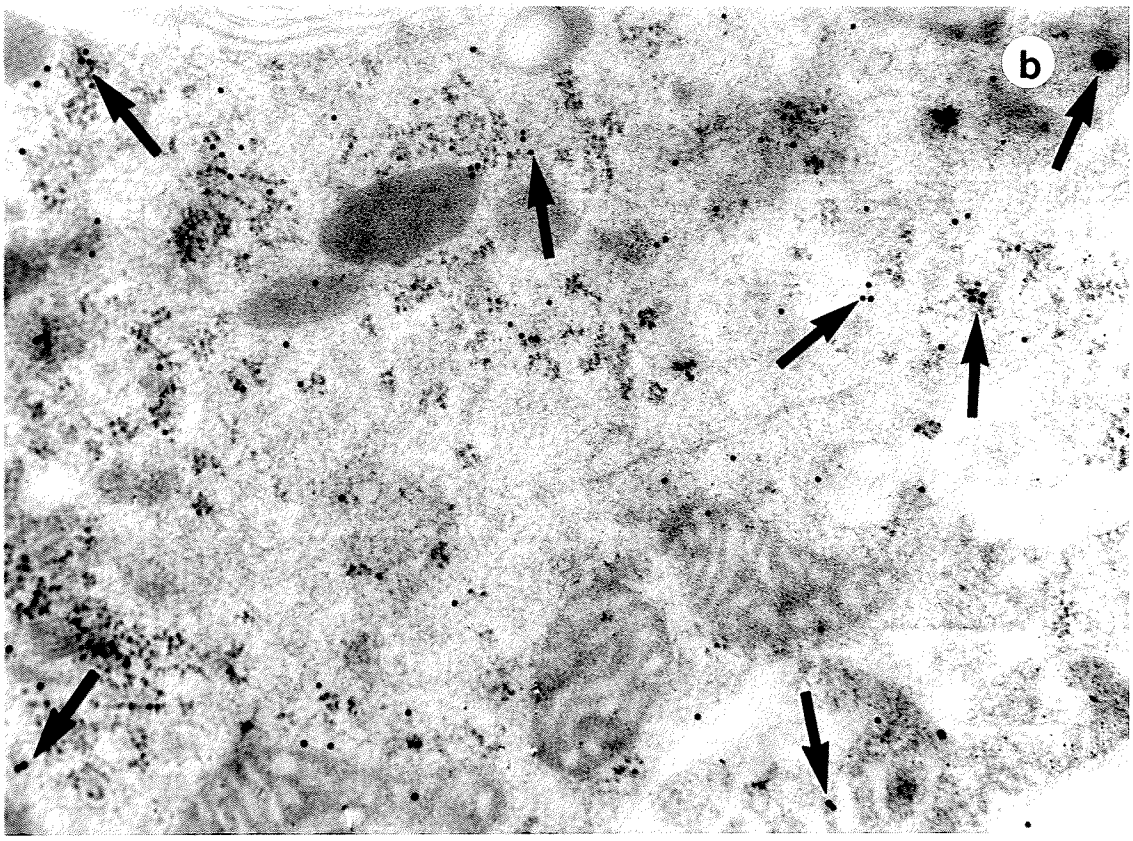
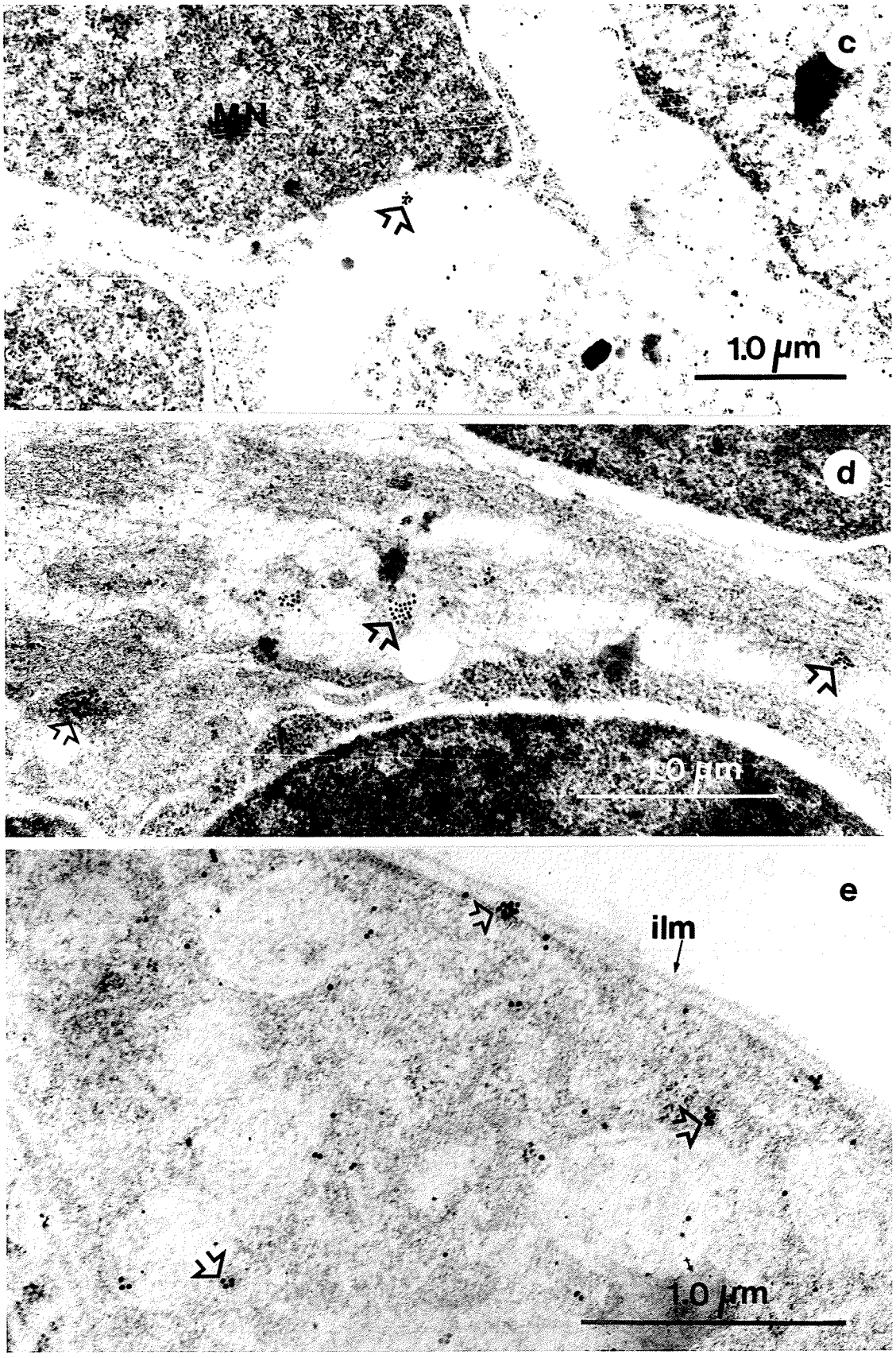

a
b
c
d
e
f
GCL
INL
ONL
RPE
E
P

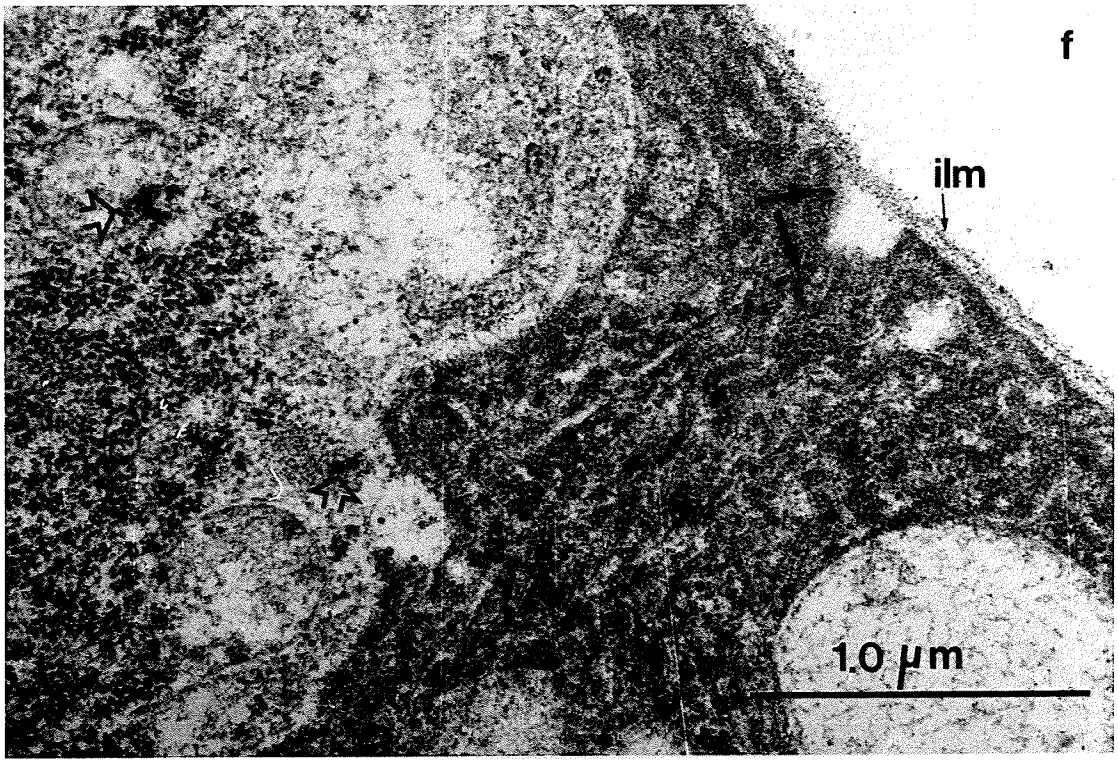
Figure 9. Ultrastructural localization of aldose reductase in the retina using the protein-A gold method.

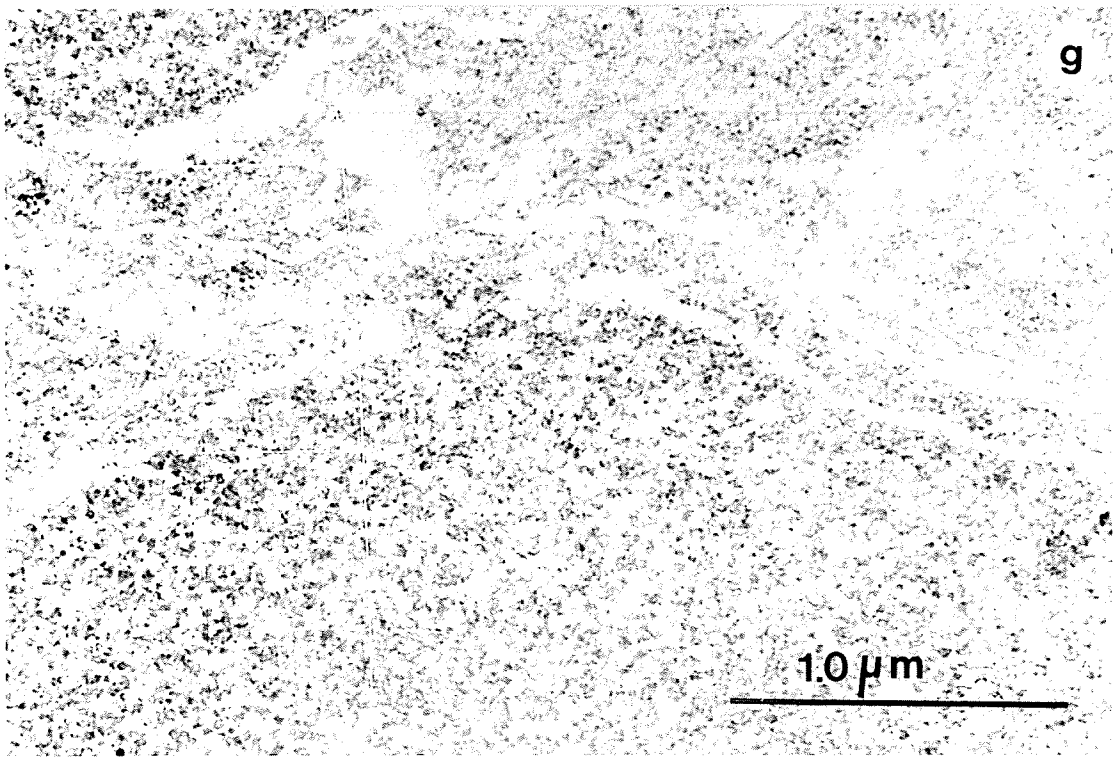
a) Pre absorbtion control staining of the retinal pigment epithelium (arrow showing background) b) positive localization (arrow) in the retinal pigment epithelium, (same magnification for a and b) c) positive localization (hollow arrow) in a Müller cell body, d) positive localization (hollow arrow) in a Müller cell process, e) positive localization (hollow arrow) at the termination of Müller cell processes at the internal limiting membrane, f) positive localization (hollow arrow) in a ganglion cell and Müller cell processes (arrow), g) preabsorbtion control staining of Müller cell processes, h) preabsorbtion control staining of a pericyte and an endothelial cell, i) positive localization in a pericyte (hollow arrow) and in the surrounding glia (arrow), j) positive localization in a endothelial cell (E, hollow arrow). MN = Müller cell nucleus, ilm = Internal limiting membrane, EN = Endothelial cell nucleus. P = Pericyte.


a
1.0 μm


b


c
MN
1.0 μm
d
0.5 μm
e
ilm
1.0 μm


f
ilm
1.0 μm


g
1.0 μm

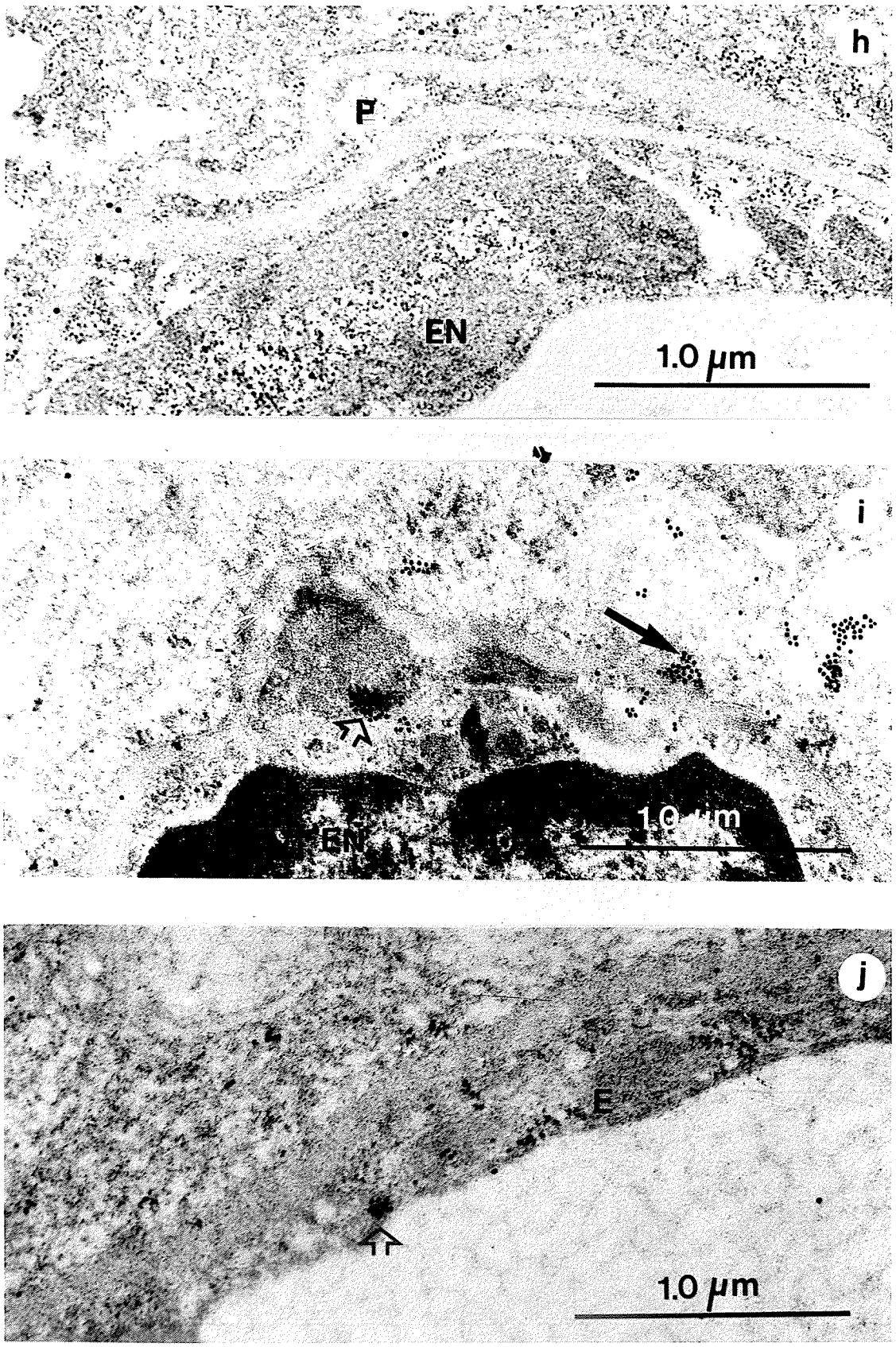
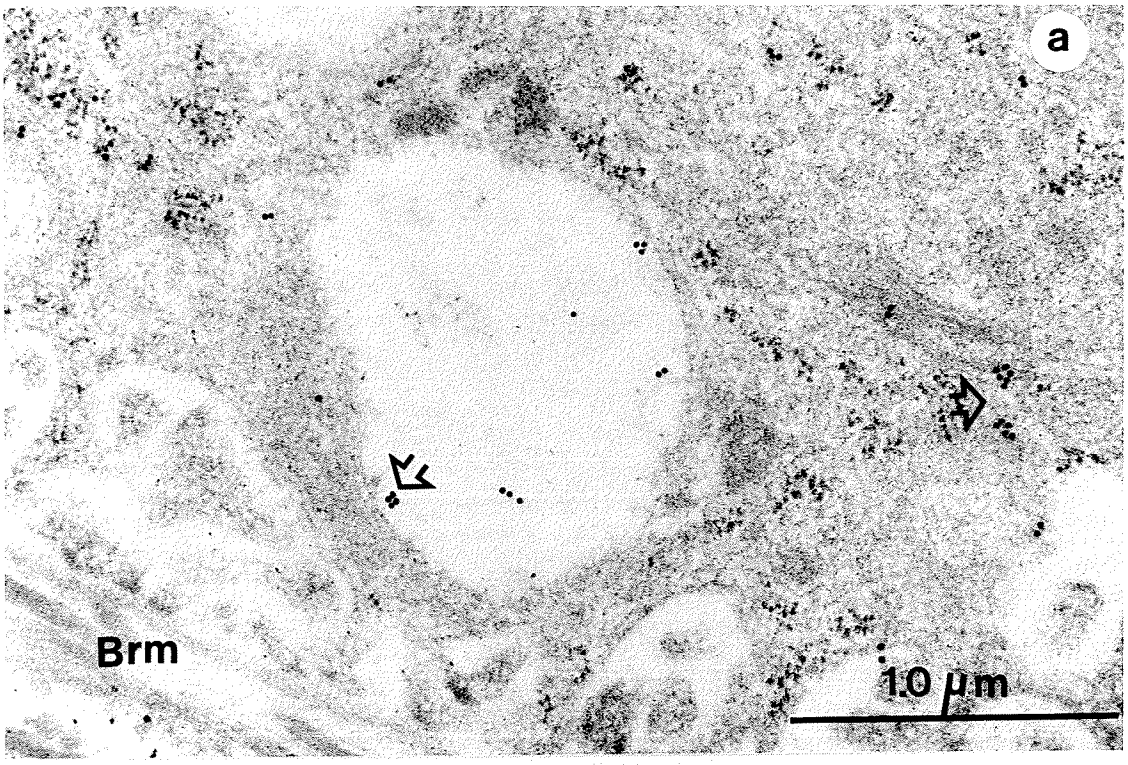

h
P
EN
1.0 μm
i
EN
1.0 μm
j
E
1.0 μm

Figure 10 Quantification of localization of aldose reductase in the retinal pigment epithelium using the protein-A gold method. a) non-diabetic control, b) diabetic. An increase in the aldose reductase immunoreactivity (hollow arrows) was seen in diabetics. Brm = Bruch's membrane.


a
Brm
1.0 μm

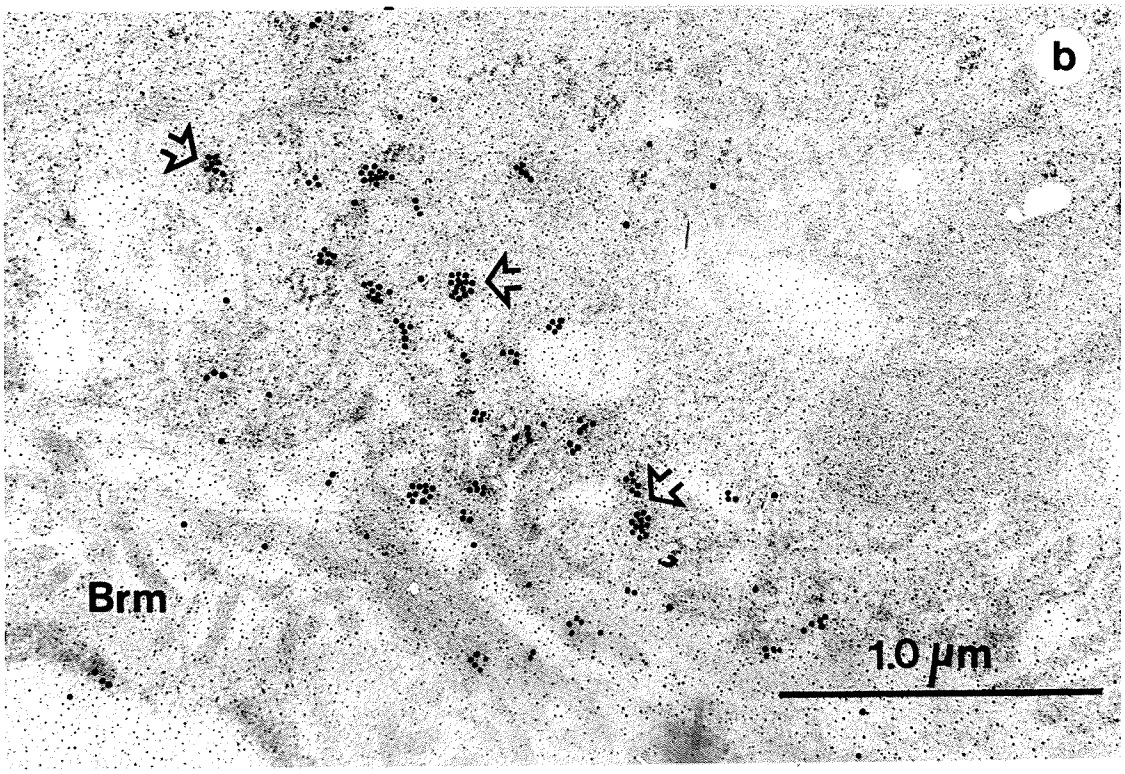

b
Brm
1.0 μm

Figure 11 Diagram showing, body weight (mean ± SE) in the various experimental groups during the follow-up period. Numbers within brackets represents the number of animals per group at the particular observation. C = Control, CS = Control on statil, D = Diabetics, DS = Diabetics on statil, DI = Diabetics on vigorous insulin therapy. C, CS and DI showed more increase in body weights compared to D and DS.

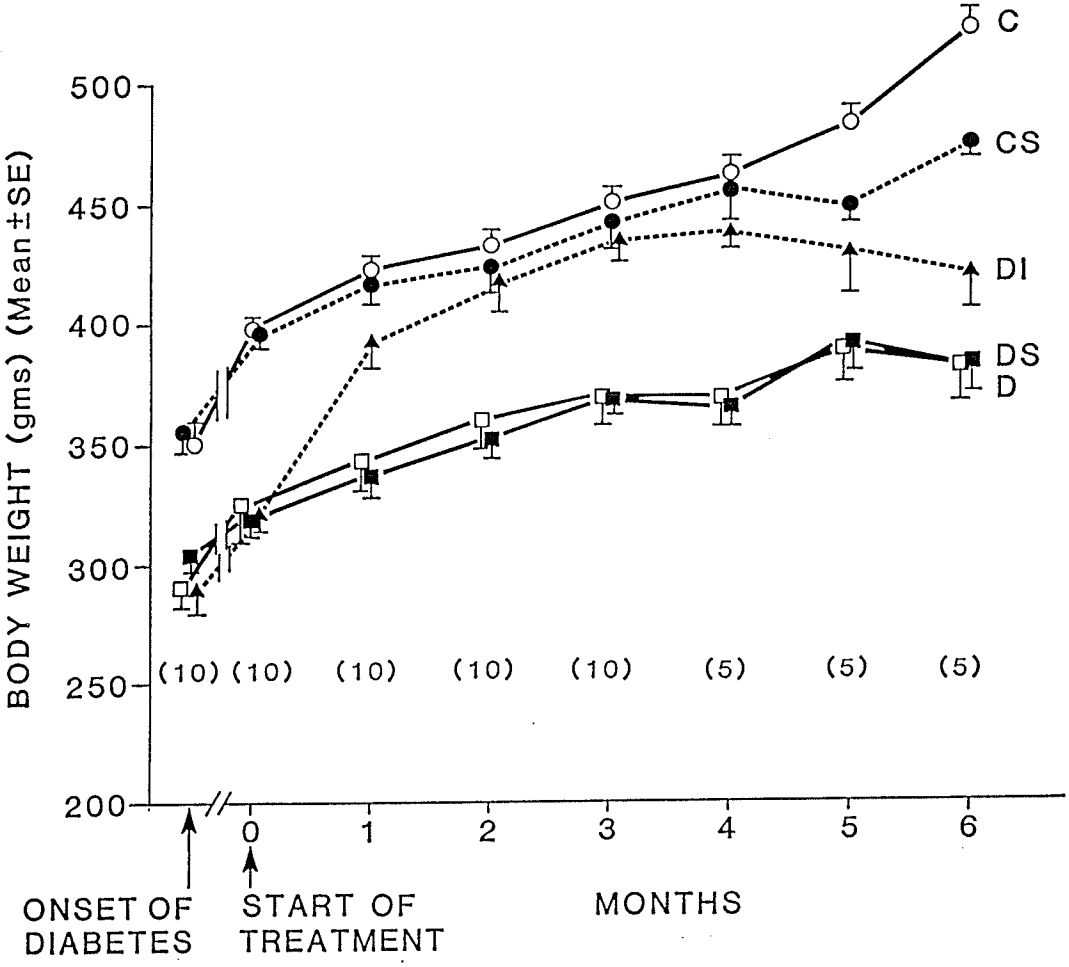
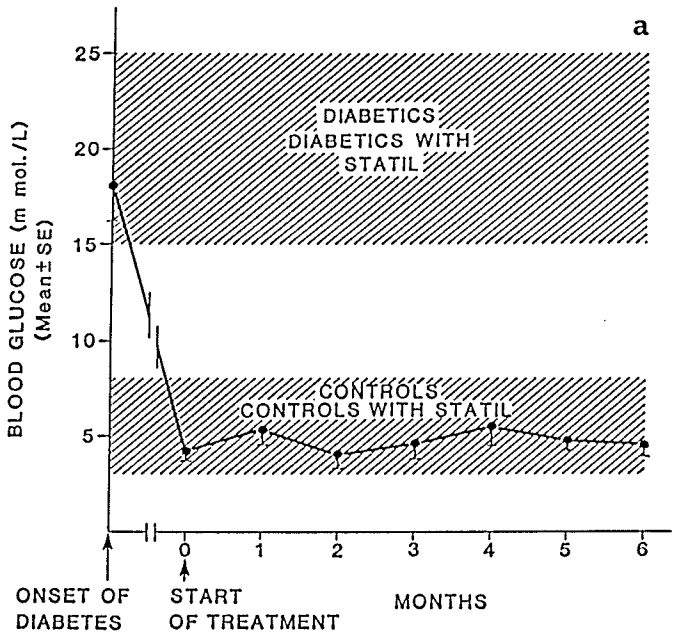

C
CS
DI
DS
D
500
450
400
350
300
250
200
BODY WEIGHT (gms) (Mean±SE)
(10) (10) (10) (10) (10) (5) (5) (5)
0
1
2
3
4
5
6
MONTHS
ONSET OF DIABETES
START OF TREATMENT

Figure 12 a) Diagram showing variations in blood glucose levels in the various experimental group during the follow-up period. Upper shaded area represents the range of variation of the diabetic hyperglycemic group and the diabetic group treated with Statil. Lower shaded area represents the variation of the control group and the control animals treated with Statil. The line represents the blood glucose (mean $\pm$ SE) levels of the group on vigorous insulin therapy. Diabetics and diabetics on Statil had a higher blood glucose level than the controls. Diabetics on vigorous insulin therapy showed a blood glucose level similar to controls.

b) Diagram showing variations in glycated Hb (%) levels (mean $\pm$ SE) in various experimental groups during the follow-up period. Number within the brackets represents the number of animals per group at the particular observation.

C = Control, CS = Control on statil, D = Diabetics, DS = Diabetics on statil, DI = Diabetics on vigorous insulin therapy. D and DS showed higher values than C,CS and DI.


a
25
20
15
10
5
BLOOD GLUCOSE (m mol./L)
(Mean± SE)
DIABETICS
DIABETICS WITH
STATIL
CONTROLS
CONTROLS WITH STATIL
0
1
2
3
4
5
6
ONSET OF
DIABETES
START
OF TREATMENT
MONTHS

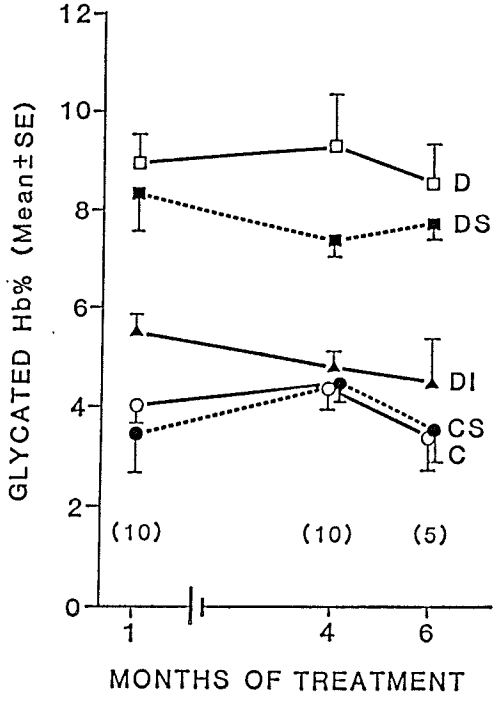

b
12
10
8
6
4
2
0
GLYCATED Hb% (Mean± SE)
D
DS
DI
CS
C
(10)
(10)
(5)
1
4
6
MONTHS OF TREATMENT

Figure 13 Mean basement membrane (BM) thickness (nm) of the superficial and deep capillary beds of all experimental groups at 4 and at 6 months. All groups showed a higher BM thickness at 6 months than that at 4 months.

C = Control, CS = Control on statil, D = Diabetics, DS = Diabetics on statil, DI = Diabetics on vigorous insulin therapy.

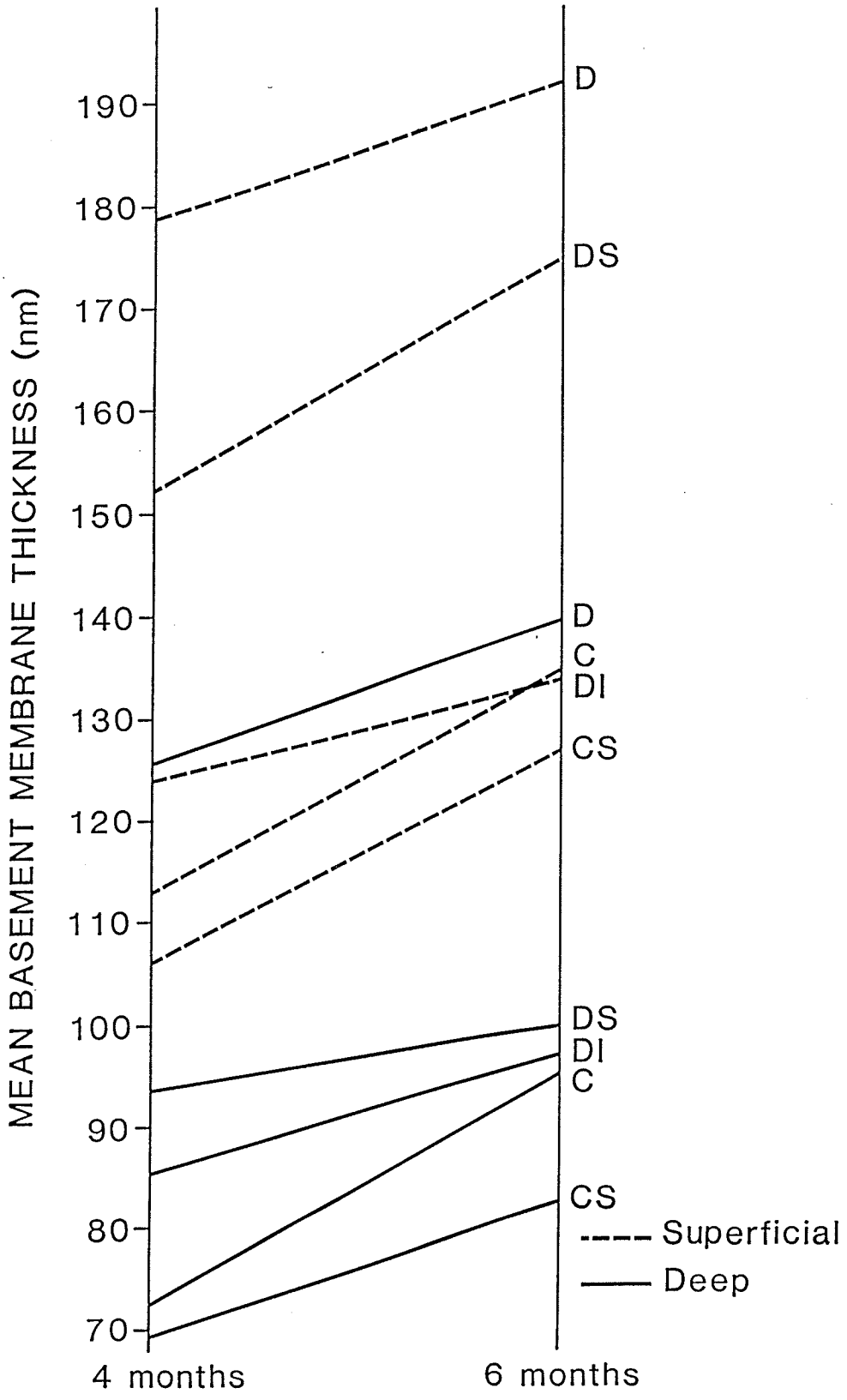
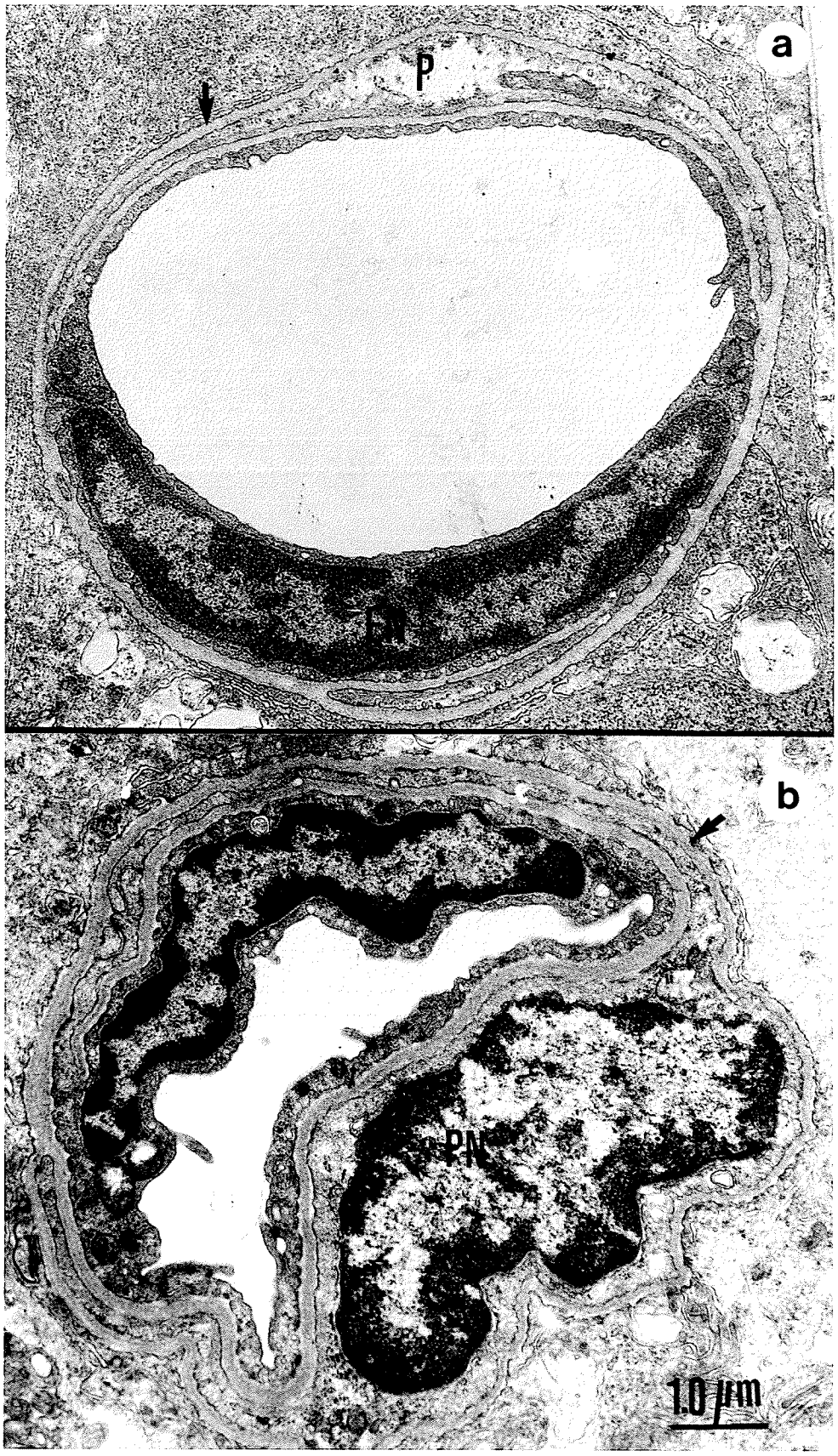

MEAN BASEMENT MEMBRANE THICKNESS (nm)
190
180
170
160
150
140
130
120
110
100
90
80
70
4 months
6 months
D
DS
D
C
DI
CS
DS
DI
C
CS
Superficial
Deep

Figure 14 Electronmicrographs of capillaries from the superficial capillary bed of the BB-rat retina showing the effects of good blood glucose control and Statil treatment on diabetic retinal capillary basement membrane thickness (BMT) after 4 months of treatment, a) Control, BMT = 92.8; b) Diabetic = 196.22; c) Diabetic on Statil = 175.83; d) Diabetics on vigorous insulin therapy = 103.51. Arrow = basement membrane, P = pericyte, PN = pericyte nuclei, EN = endothelial cell nuclei, ilm = internal limiting membrane. Same magnification from a-d. Thicker basement membrane seen in the untreated diabetic animals (b), was prevented by insulin therapy (d) but not by Statil therapy (c).


a
P
b
PM
1.0 μm

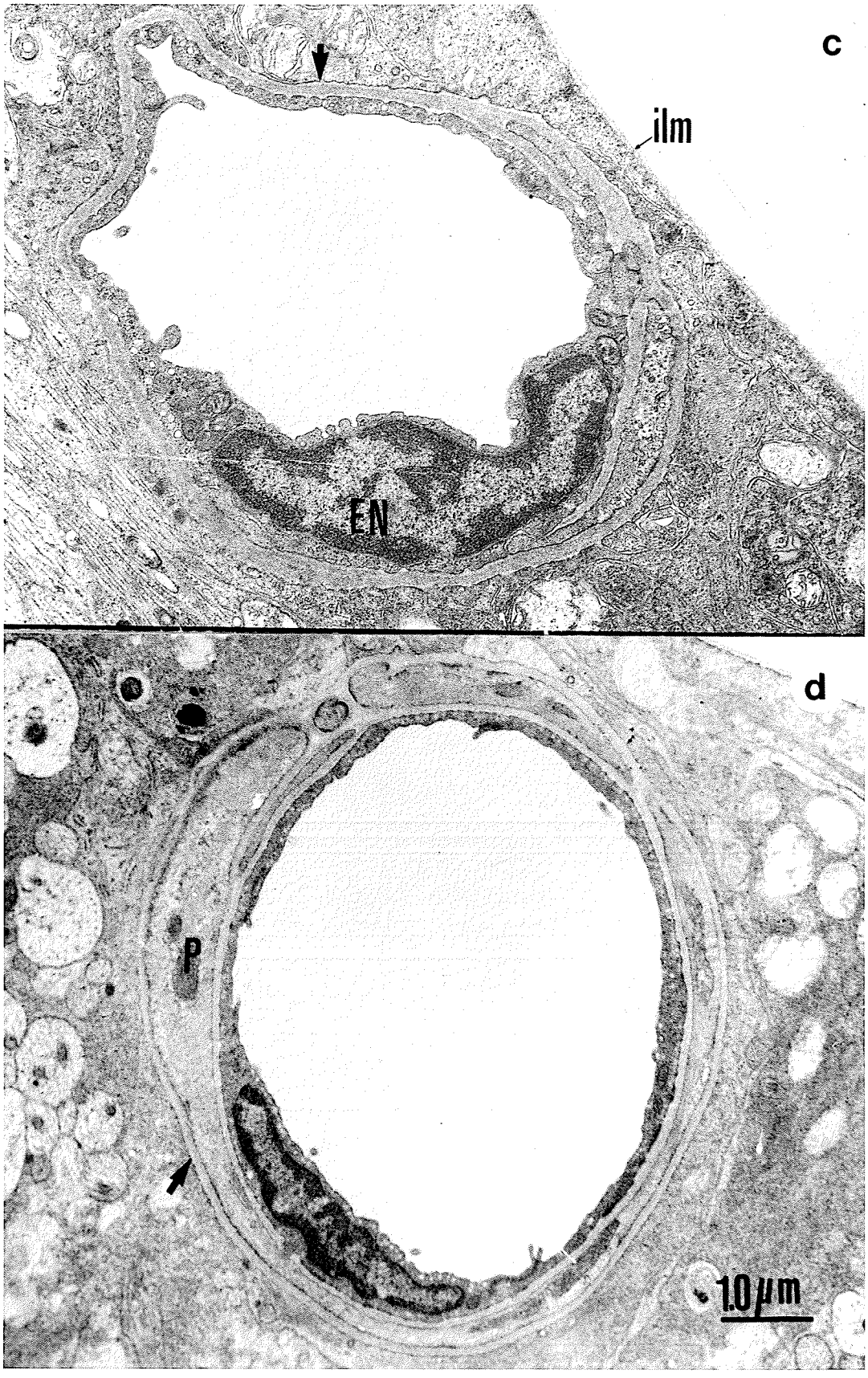
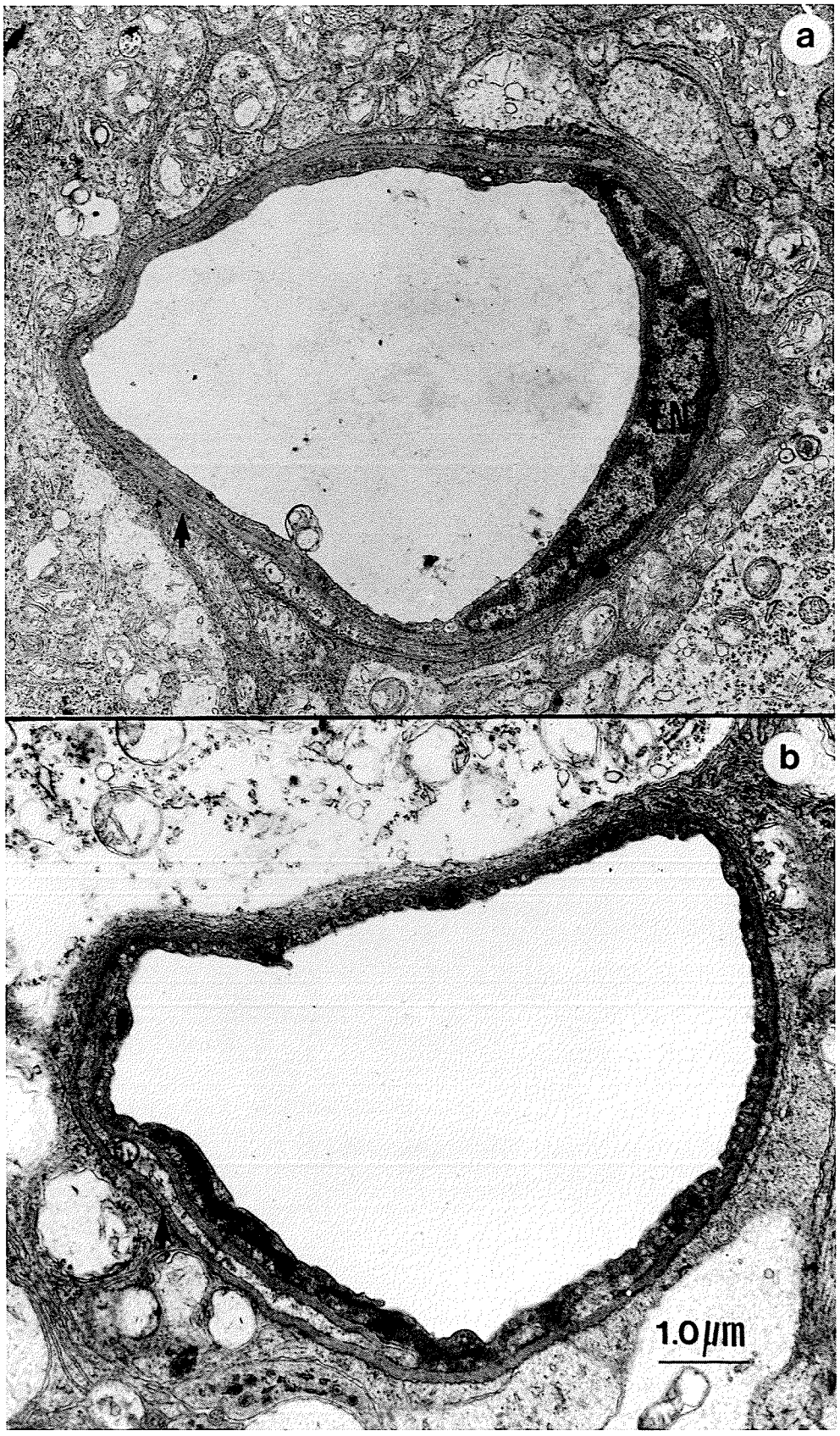

c
ilm
EN
d
P
1.0μm

Figure 15 Electronmicrographs of capillaries from the deep capillary bed of the BB-rat retina showing the effects of good blood glucose control and Statil treatment on diabetic retinal capillary basement membrane thickness (BMT) after 4 months of treatment, a) Control, BMT = 50.25; b) Diabetic = 110.59; c) Diabetic on Statil = 63.02; d) Diabetic on vigorous insulin therapy = 42.76.
Arrow = Basement membrane, P = Pericyte, E = Endothelial cell, EN = Endothelial cell nuclei. Same magnification from a-d. Thicker basement membrane in untreated diabetics (b) was prevented by both Statil and Insulin therapy (c,d).


a
b
1.0 μm

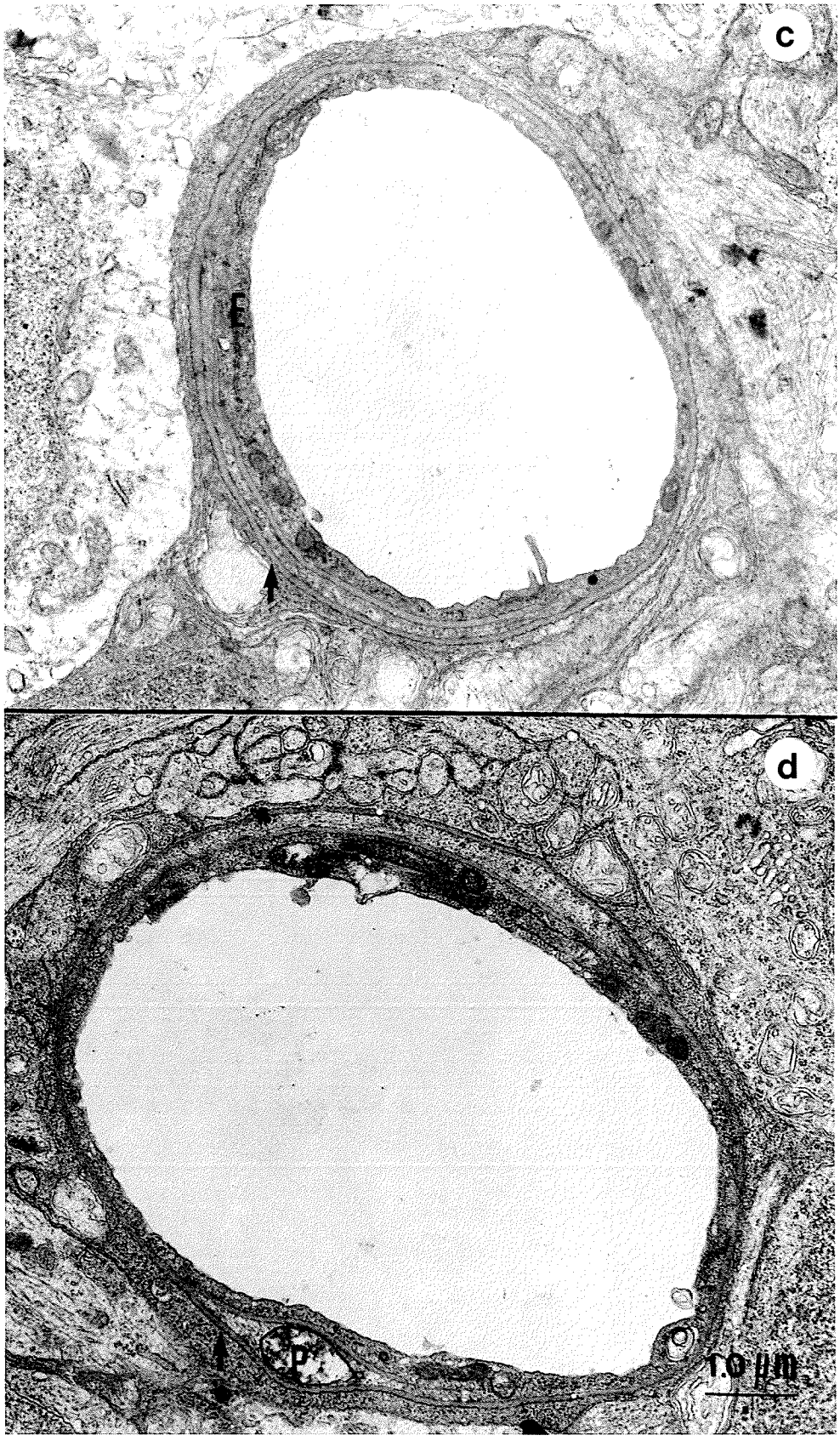

c
E
d
P
1.0 µm

Figure 16 Regression analysis of basement membrane thickness (BMT) the retinal capillaries of the superficial (——) and deep (– – –) capillary bed after 4 months of treatment in all experimental groups using a) blood glucose levels as an independent variable; a = 102.42, b = 3.05, r = 0.58, F = 11.90 ($p<0.005$) for the superficial capillary bed and a=67.47, b = 2.01, r = 0.46, F = 6.24 ($p<0.025$) for the deep capillary bed and b) using glycosylated Hb levels as an independent variable; a = 70.69, b = 11.20, r = 0.65, F = 17.24 ($p<0.005$) for the superficial capillary bed and a = 39.06, b = 8.71, r = 0.61, F = 13.80 ($p< 0.005$) for the deep capillary bed. The individual BMT's from the superficial capillary bed are represented by ▲ and those from the deep capillaries are represented by ●.

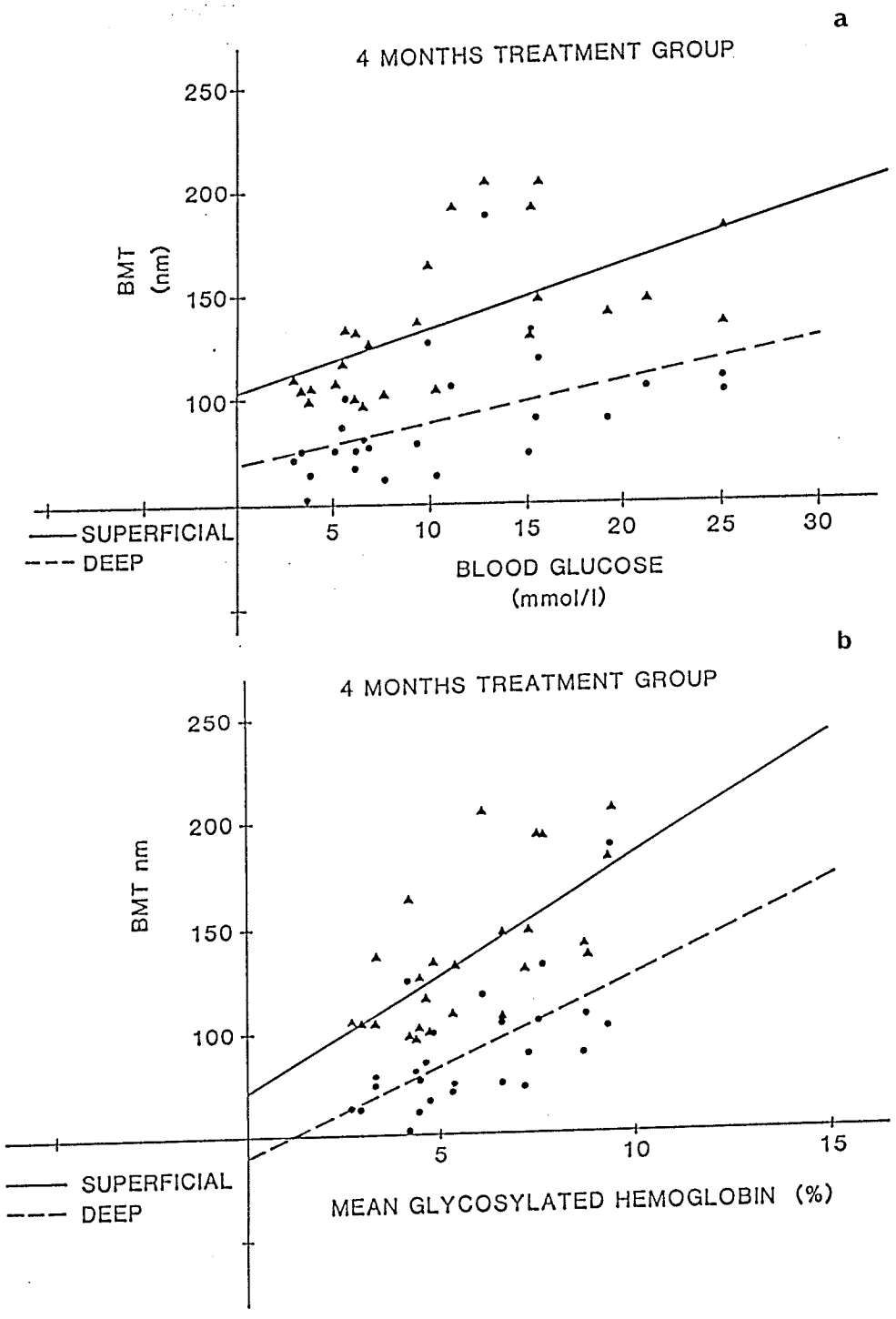
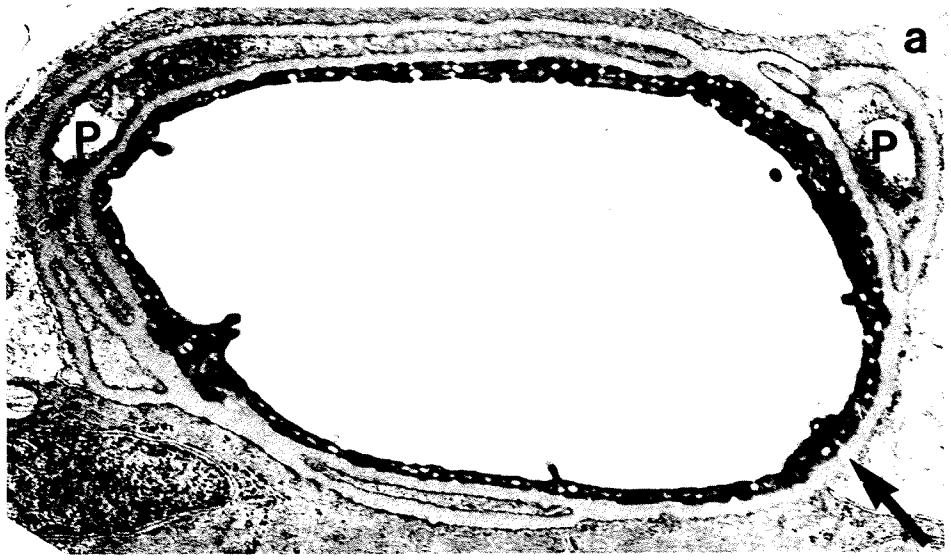
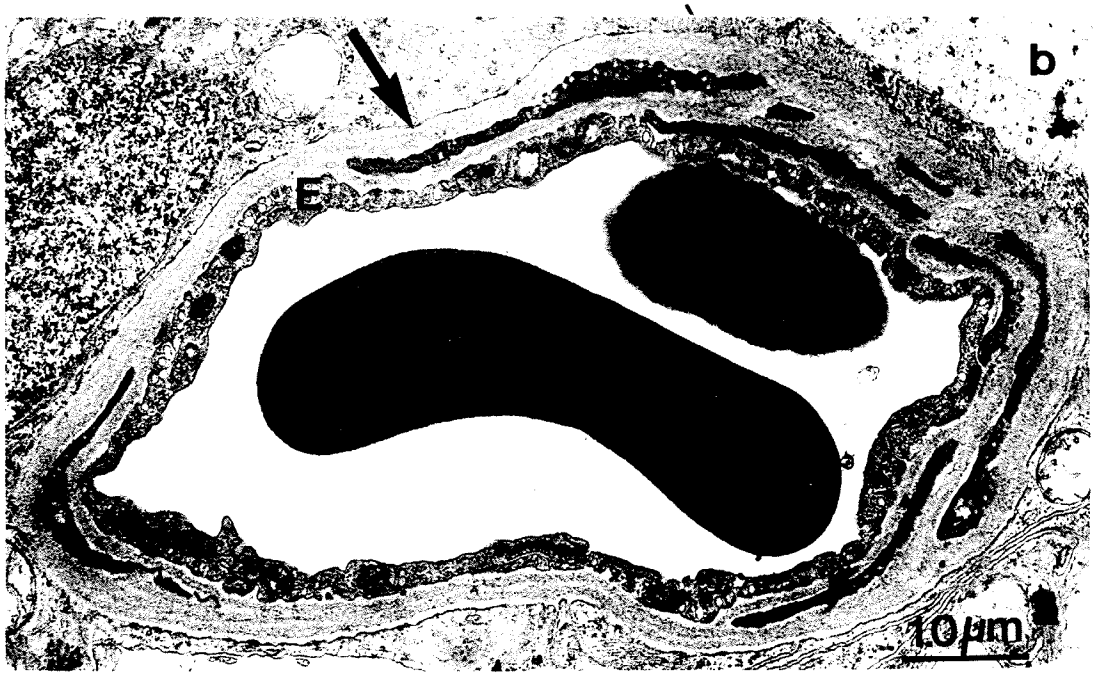
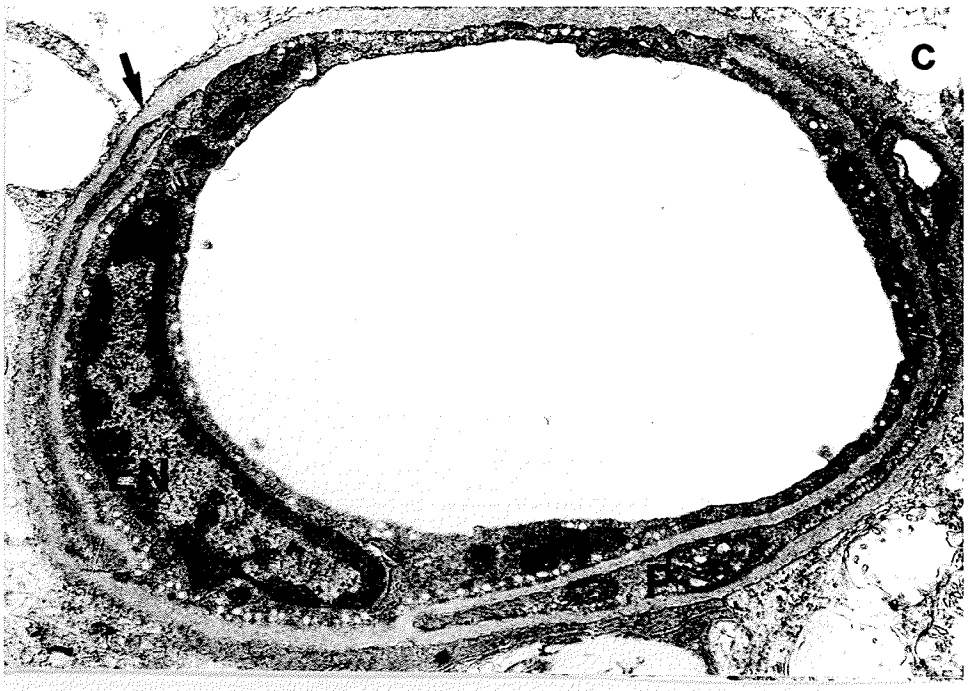

a
4 MONTHS TREATMENT GROUP
250
200
150
100
BMT
(nm)
5
10
15
20
25
30
SUPERFICIAL
DEEP
BLOOD GLUCOSE
(mmol/l)
b
4 MONTHS TREATMENT GROUP
250
200
150
100
BMT nm
5
10
15
SUPERFICIAL
DEEP
MEAN GLYCOSYLATED HEMOGLOBIN (%)

Figure 17 Electronmicrographs of capillaries from the superficial capillary bed of the BB-rat retina showing the effects of good blood glucose control and Statil treatment on diabetic retinal capillary basement membrane thickness (BMT) after 6 months of treatment, a) Control, BMT = 107.3; b) Diabetic = 220.4; c) Diabetic on vigorous insulin therapy = 89.39; d) Diabetic on Statil = 242.6. Arrow = basement membrane, P = pericyte, E = endothelial cell, EN = endothelial cell nuclei, ilm = Internal limiting membrane. Same magnification from a-d. Thicker basement membrane seen in the untreated diabetic animals (b), was prevented by insulin therapy (c) but not by Statil therapy (d).


a
P
P


b
E
1.0 μm


c

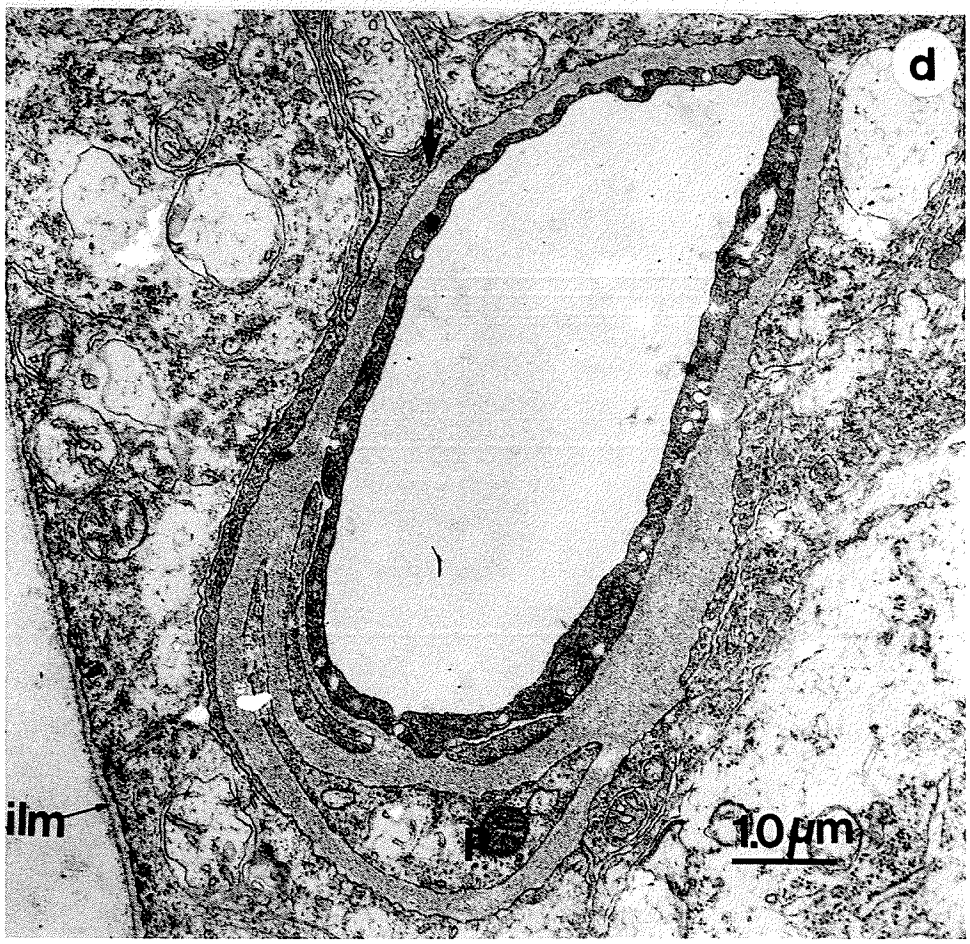
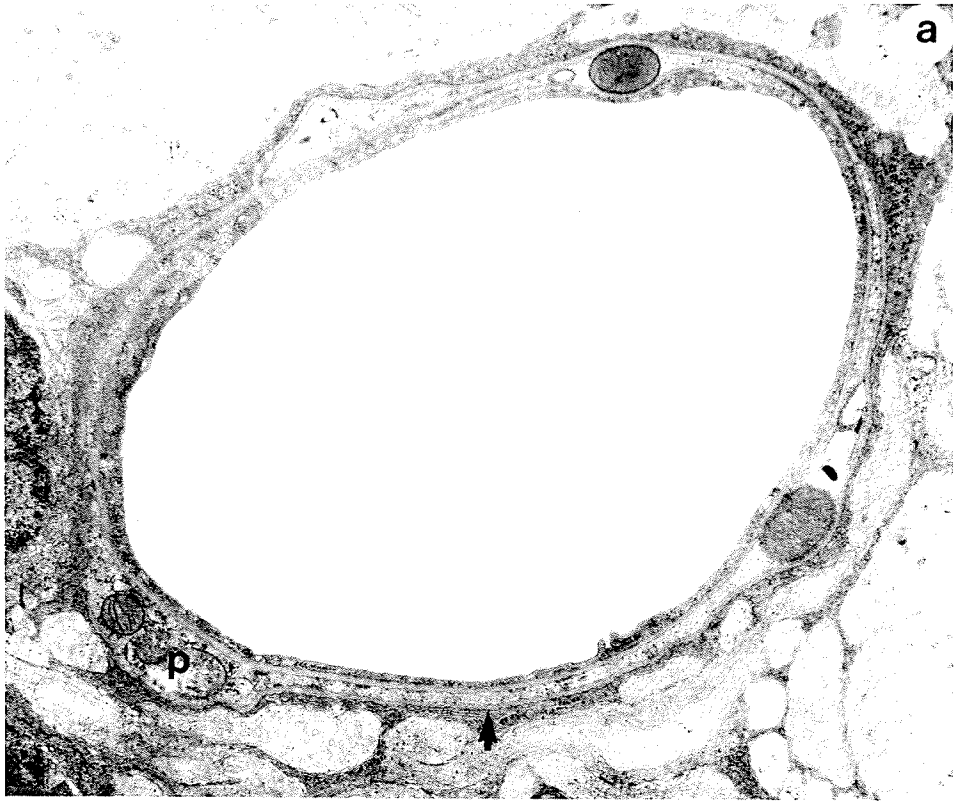
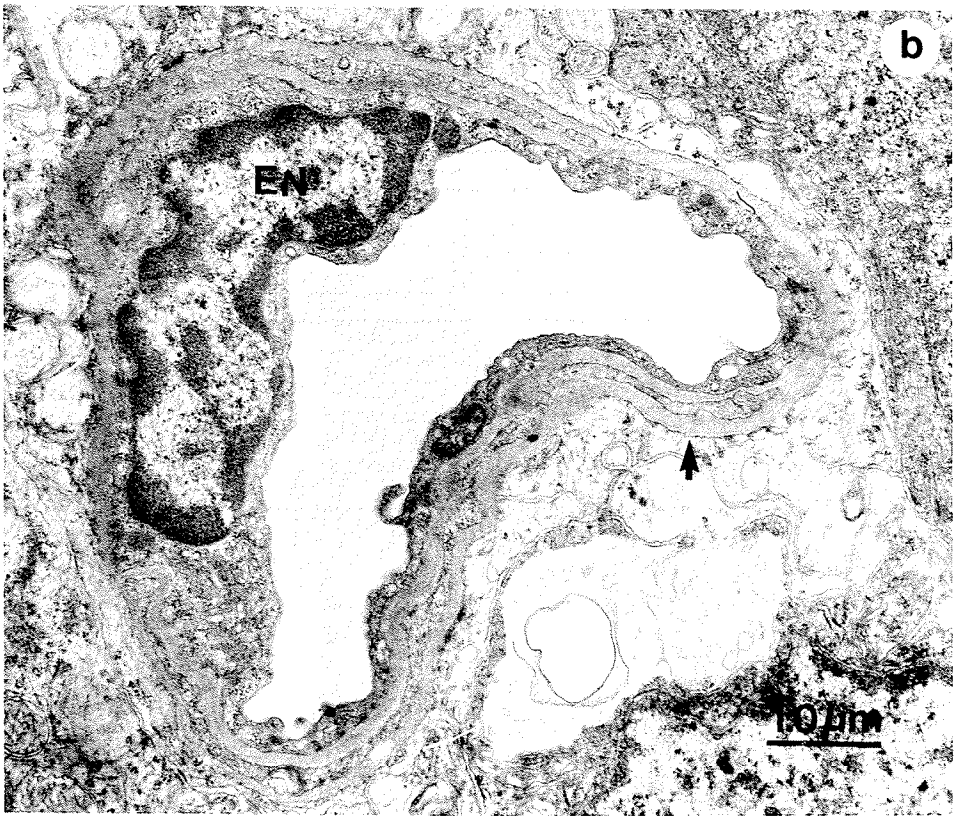
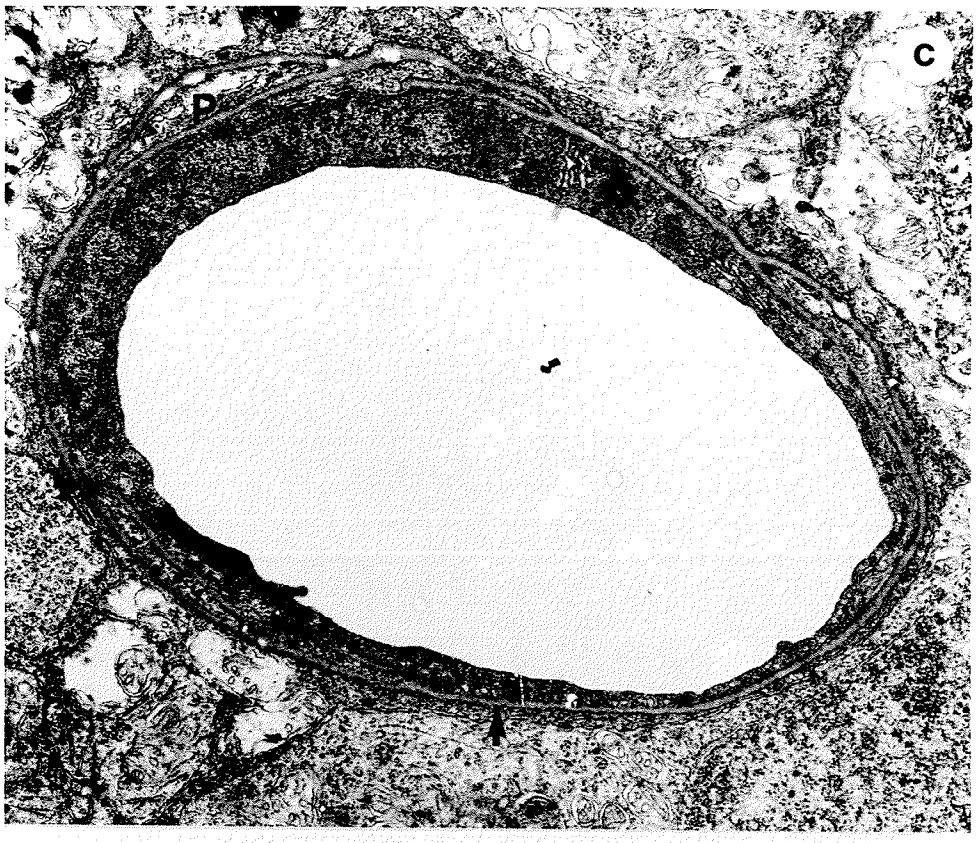

d
ilm
1.0 μm

Figure 18 Electronmicrographs of capillaries from the deep capillary bed of the BB-rat retina showing the effects of good blood glucose control and statil treatment on the retinal capillary basement membrane thickness (BMT) after 6 months of treatment, a) Control, BMT = 52.59; b) Diabetic = 140.56; c) Diabetic on vigorous insulin therapy = 85.87; d) Diabetic on statil = 62.12. Arrow = Basement membrane, P = Pericyte, EN = Endothelial cell nuclei. Same magnification from a-d. Thicker basement membrane in untreated diabetics (b) was prevented by both insulin and Statil therapy (c,d).


a
p


b
EN
1.0μm


c
P

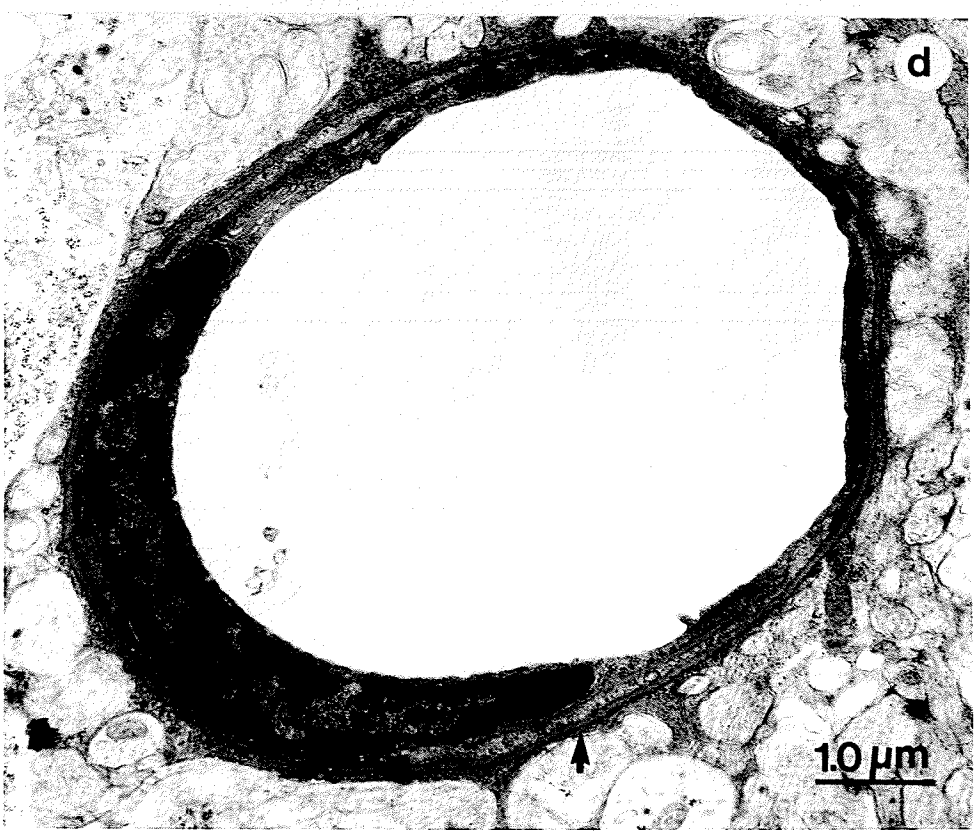

d
1.0 µm

Figure 19 Regression analysis of basement membrane thickness (BMT) in the retinal capillaries of the superficial (——) and deep (– – –) capillary bed after 6 months of treatment in all experimental groups using a) blood glucose levels as an independent variable; a = 117.38, b = 2.66, r = 0.74, F = 27.93 ($p < 0.005$) for the superficial capillary bed and a = 85.88, b = 1.29, r = 0.48, F = 7.00 ($p < 0.025$) for the deep capillary bed and b) using glycosylated Hb levels as an independent variable; a = 94.60, b = 10.06, r = 0.80, F = 39.47 ($p < 0.005$) for the superficial capillary bed and a = 59.48, b = 7.52, r = 0.79, F = 40.13 ($p < 0.005$) for deep capillary bed. The individual BMT's from the superficial capillary bed are represented by ▲ and those from the deep capillaries are represented by ●.

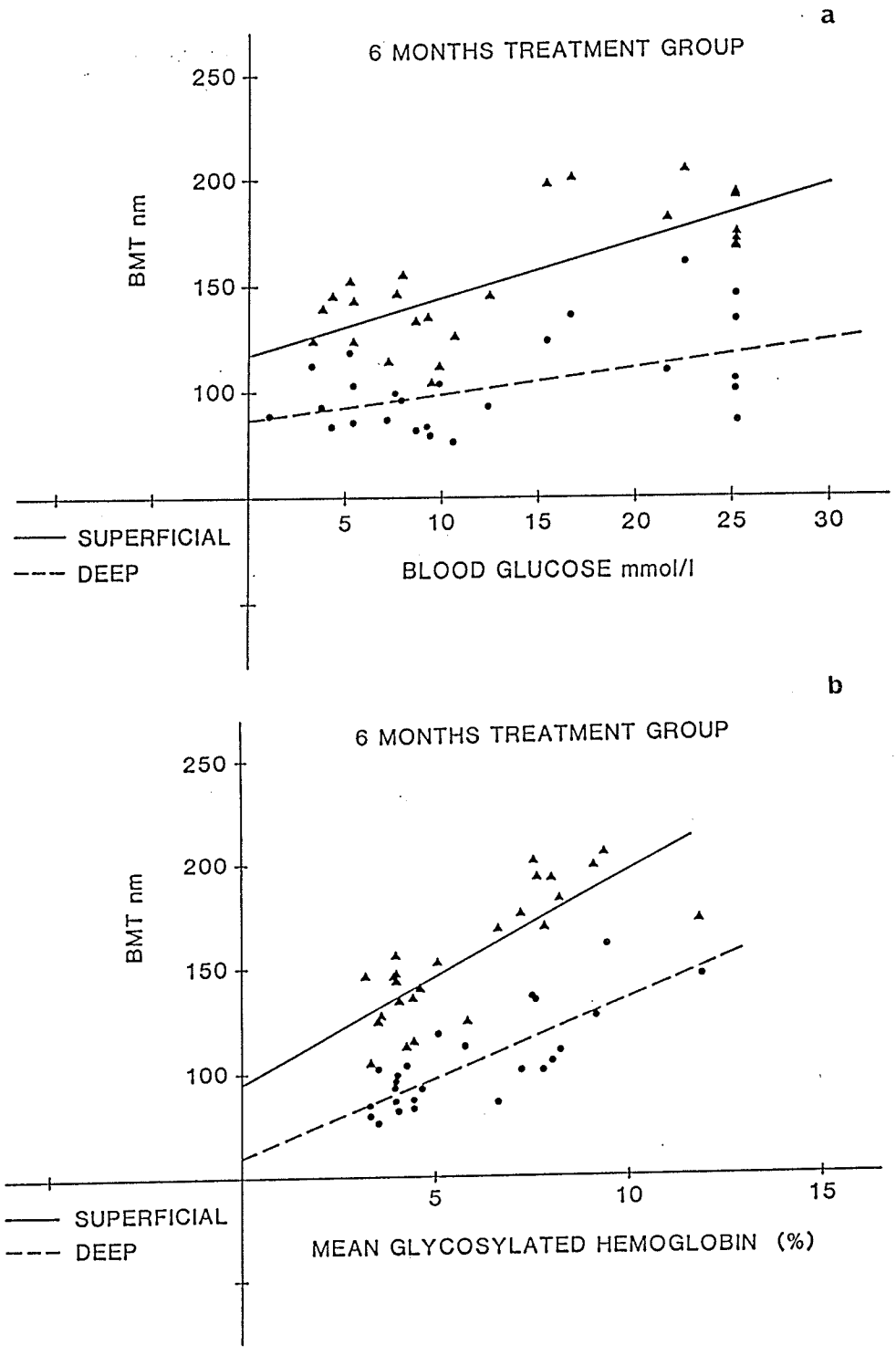

a
6 MONTHS TREATMENT GROUP
BMT nm
250
200
150
100
5
10
15
20
25
30
SUPERFICIAL
DEEP
BLOOD GLUCOSE mmol/l
b
6 MONTHS TREATMENT GROUP
BMT nm
250
200
150
100
5
10
15
SUPERFICIAL
DEEP
MEAN GLYCOSYLATED HEMOGLOBIN (%)

Figure 20 Trypsin digested preparation of the retina from a non-diabetic BB-rat showing pericyte (P), endothelial cell (E) and mesodermal bridges (MB). PAS-Hematoxylin stain, magnification X850.

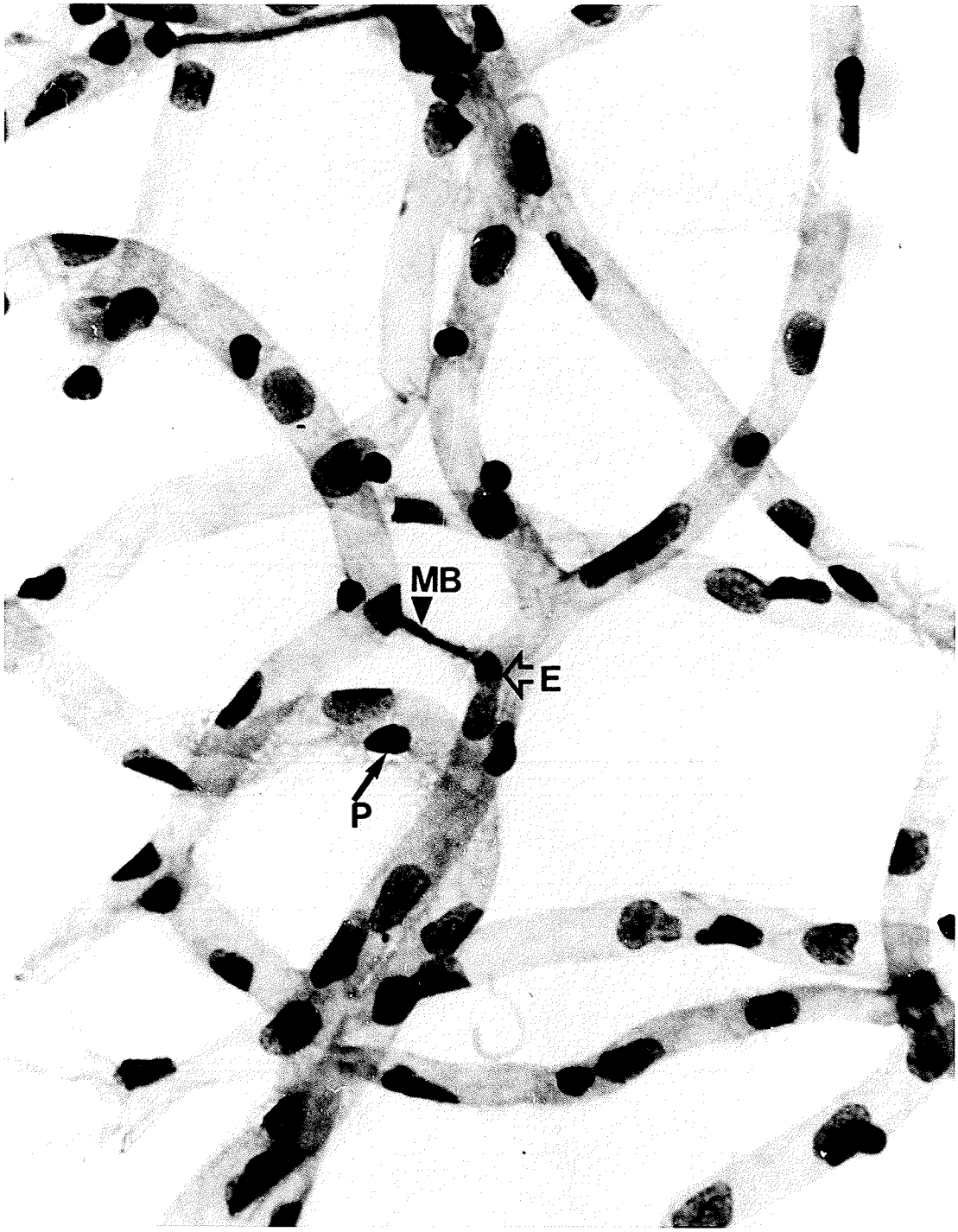

MB
E
P

Figure 21 a-c) Electronmicrographs from the capillaries of diabetic BB-rats after 6 months of treatment showing capillary pericyte abnormalities such as homogeneous cytoplasm with loss of organelles in a, b and c. Endothelial cell in c shows nuclear pyknosis (hollow arrow). P = pericyte.

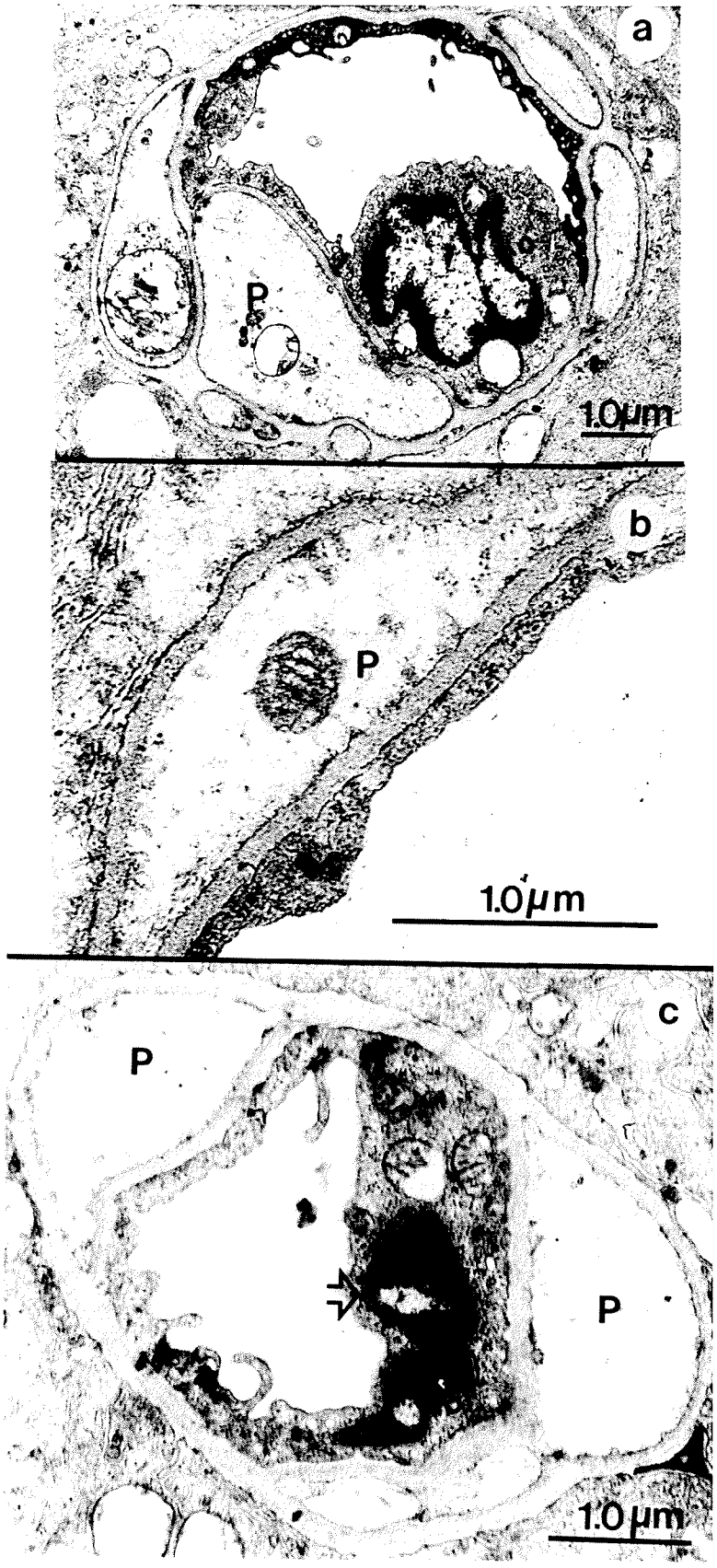

a
P
1.0μm
b
P
1.0μm
c
P
P
1.0 μm

Figure 22 Electronmicrographs from the retinal capillaries of diabetic animals after 4 months of treatment showing various basement membrane abnormalities,

a) localized nodular swelling (hollow arrow),

b) projection of basement membrane material towards the surrounding glia (arrow), and

c) fibrillar deposits within the basement membrane substance (arrow).

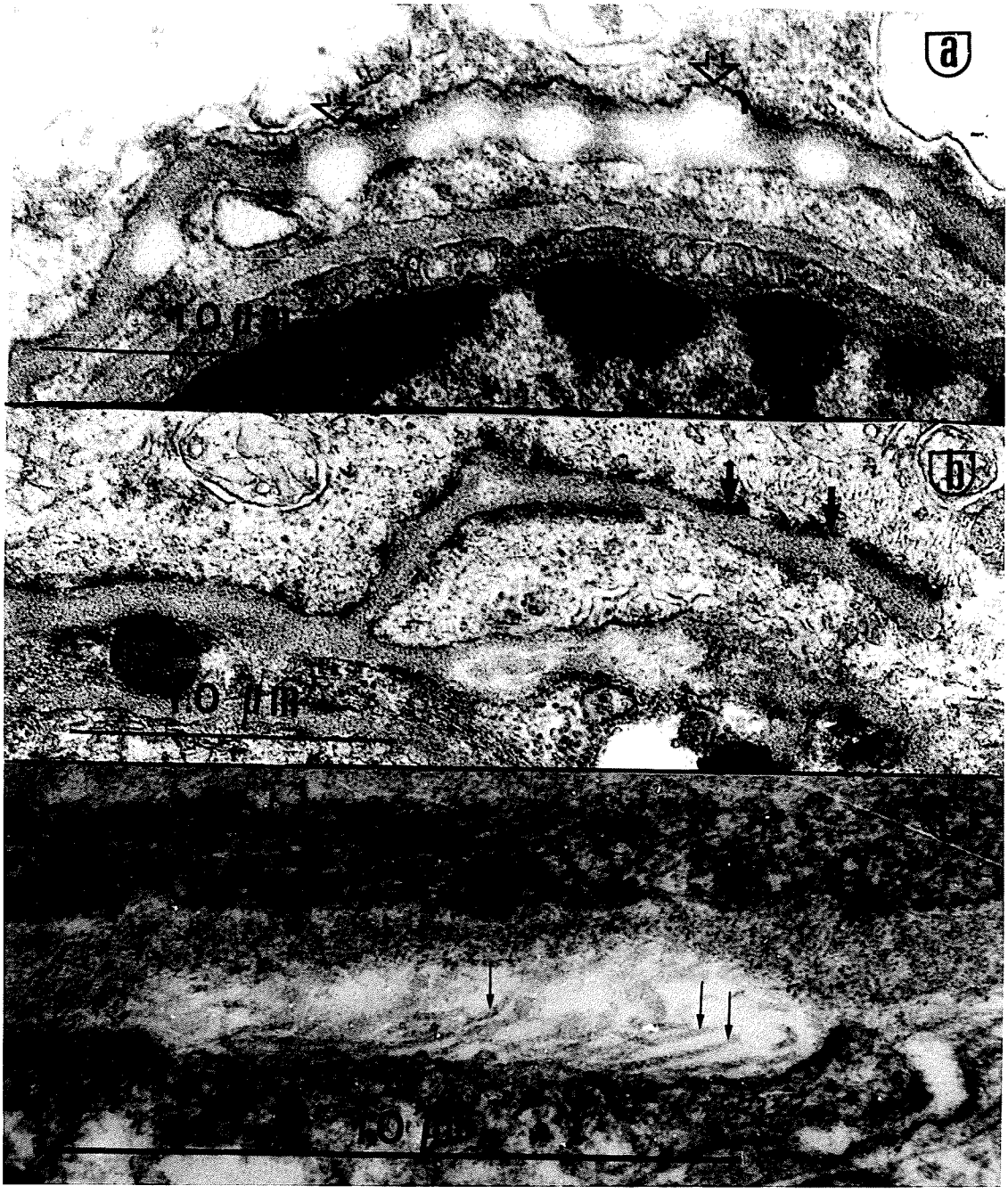
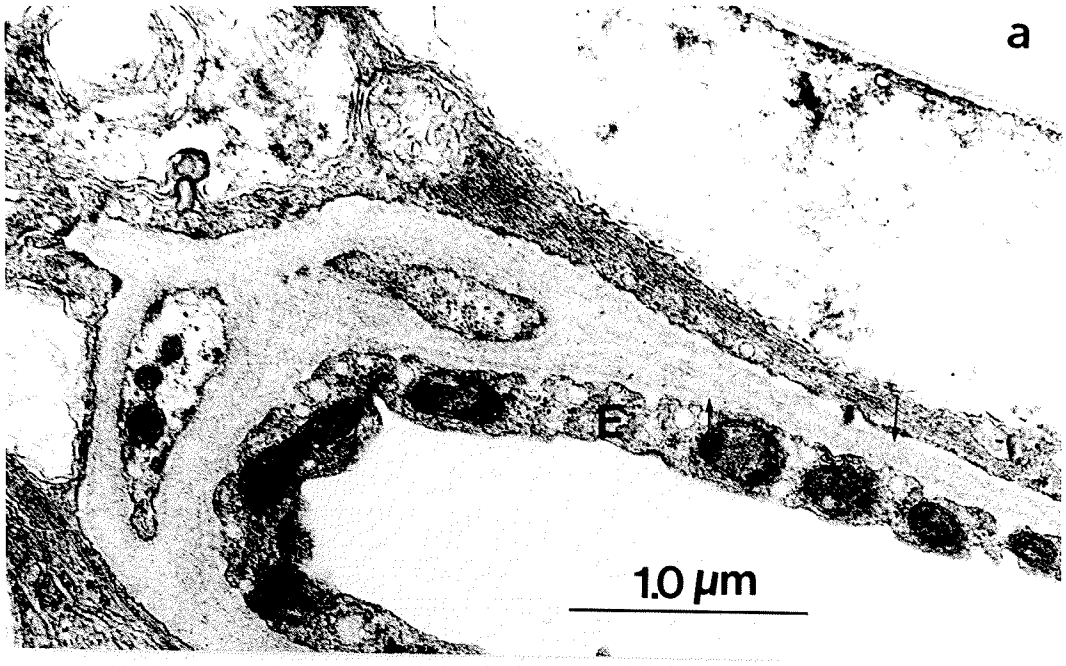

a
1.0 μm
b
1.0 μm
1.0 μm

Figure 23 Electronmicrographs from the retinal capillaries of diabetic animals after 6 months of treatment showing various basement membrane abnormalities,

a) capillary basement membrane from the superficial capillary bed showing laminated appearence (arrow).

b) capillary basement membrane showing pericyte debries within the basement membrane subatance (hollow arrow). P = pericyte, E = Endothelial cell.


a
R
E
1.0 μm

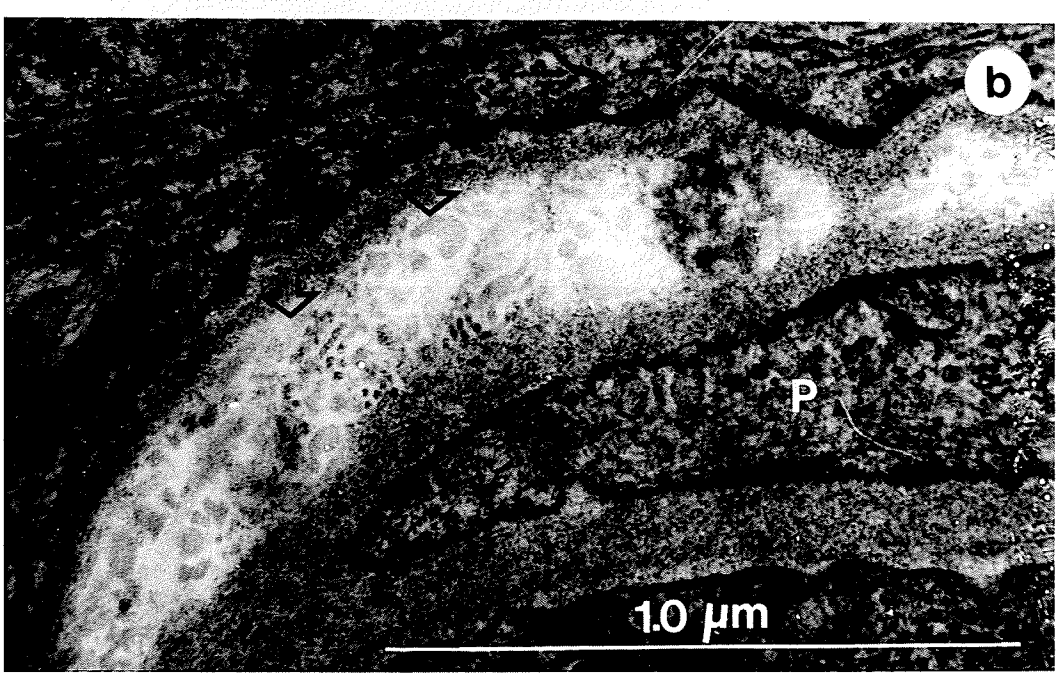

b
P
1.0 μm

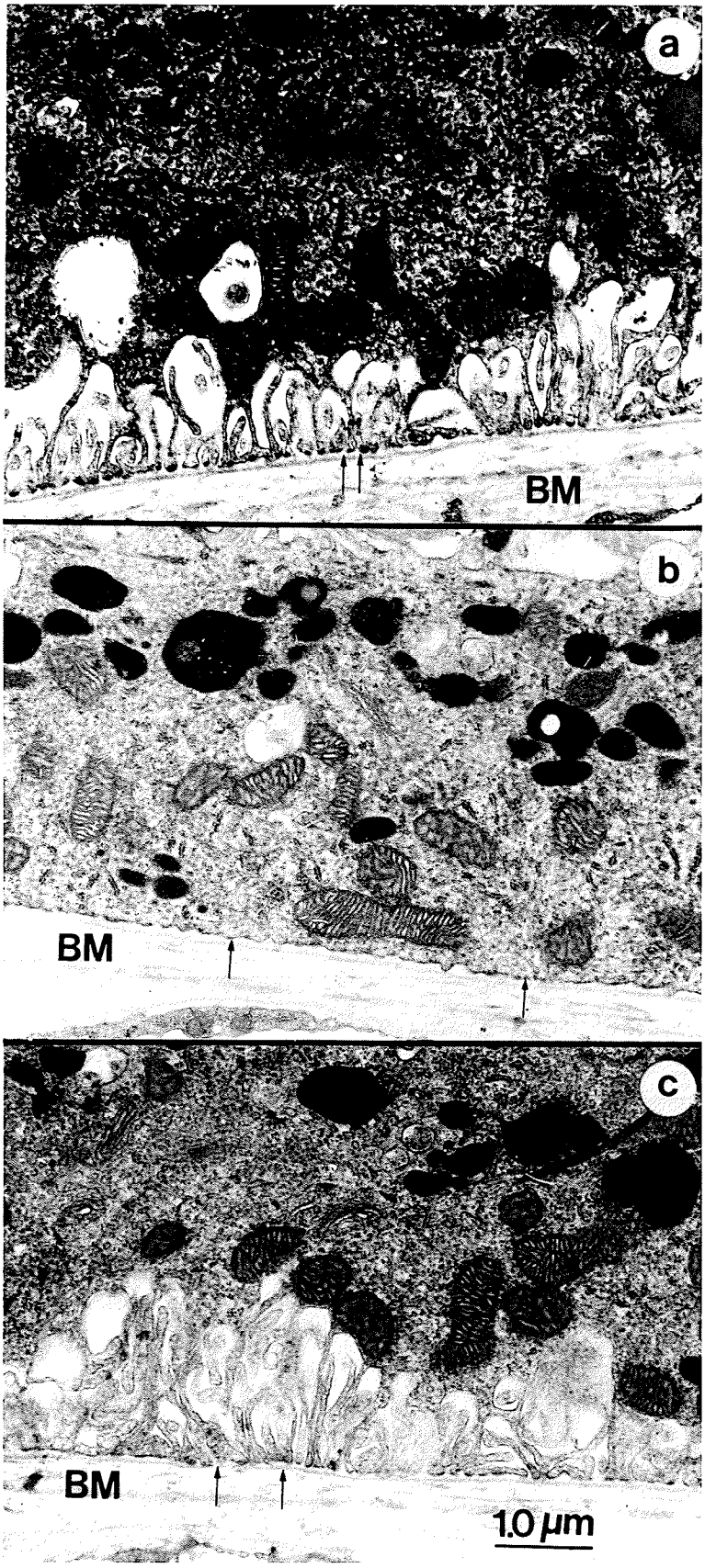
Figure 24 Electronmicrographs from the retinal pigment epithelium of BB-rat showing qualitative changes suggestive of pigment epitheliopathy after 6 months of diabetes.

a) Pigment epithelium from a normal rat showing regular basal infoldings (arrow).

b) Pigment epithelium from a diabetic rat showing loss of basal infoldings (arrow).

c) Pigment epithelium from a diabetic rat showing localized exaggeration of basal infoldings (arrow).

BM = Bruch's membrane.


a
BM
b
BM
c
BM
1.0 μm

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