Ultrastructure of the Retina: Effects of Age, and Dietary Protein in Normal and Diabetic Rats

by

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A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

The effects of age and dietary protein on retinal capillary structure and basement membrane thickness (BMT), retinal pigment epithelium (RPE) morphology and basal cell membrane area (BCMA), and Bruch's membrane (BrM) structure and thickness (T) were studied in normal and streptozotocin-diabetic rats. One group of animals received a reduced (8%) protein diet and a second group received a diet of standard rat chow containing 24% protein. Within each diet group, there were four treatment conditions; controls (C), streptozotocin-injected non-diabetics (STZ), untreated diabetics (DM) and insulin treated diabetics (DM+I). Six animals were sacrificed at the beginning of the experiment to serve as zero time controls (CO). After 5 months, the animals were sacrificed, the eyes removed and examined by electron microscopy. On the basis of a qualitative morphological examination, no differences were apparent between experimental groups. The morphometric analyses revealed that: i) capillary BMT was not affected by diabetes of 5 months duration, increased significantly with age ($p<0.05$), and was not affected by dietary protein, ii) RPE BCMA was unchanged with an increase in age of 5 months, and was significantly reduced in DM24% compared to C24% and DM8% ($p<0.05$), iii) BrMT increased with age, and was significantly reduced in DM24%. The physiological significance of these findings is uncertain although these data suggest that a reduced protein diet coincident with poorly controlled diabetes may preserve retinal morphology.

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LIST OF ABBREVIATIONS

BMT - basement membrane thickness

RPE - retinal pigment epithelium

BCMA - basal cell membrane area

BrMT - Bruch's membrane thickness

C8% - uninjected control on 8% protein diet

STZ8% - streptozotocin-injected non-diabetic on 8% protein diet

DM8% - untreated diabetic on 8% protein diet

DM+I8% - insulin-treated diabetic on 8% protein diet

C24% - uninjected control on 24% protein diet

STZ24% - streptozotocin-injected non-diabetic on 24% protein diet

DM24% - untreated diabetic on 24% protein diet

DM+I24% - insulin-treated diabetic on 24% protein diet

C0 - zero time control

IDDM - insulin-dependent diabetes mellitus

NIDDM - noninsulin-dependent diabetes mellitus

1. INTRODUCTION

1.0 Background Review of Diabetes

1.0.0 Definition

Diabetes (from the Greek diabētēs - a syphon) refers to a condition initially described by Aretaeus of Cappadocia in 200 A.D. as "a melting down of the flesh and limbs into urine." The term diabetes mellitus was established in 1674 by Willis who observed that the urine of diabetics tasted sweet "as if imbued with honey and sugar" (Cahill, 1985). During the 19th century, Brockman and Langerhans described the insulin-producing cells of the pancreas and Von Mering and Minkowski demonstrated that pancreatectomy resulted in diabetes in the dog (Cahill, 1985). In the past several decades, the definition of diabetes mellitus has evolved to "a lack of functional insulin" resulting in hyperglycemia (Seifter and England, 1983). This definition allows for hyperglycemia to result from too little insulin, too few insulin receptors, and/or defective insulin receptors.

Unquestionably the single largest breakthrough in diabetes research occurred in 1921 when Banting and Best prepared pancreatic extracts capable of lowering blood glucose levels in dogs (Banting and Best, 1922). Although the use of exogenous insulin has virtually eliminated death due to metabolic acidosis, the life expectancy of diabetics still remains reduced compared to the general population due to the chronic complications associated with this disease (Sherwin

and Tamborlane, 1985). Progress toward understanding the causes and the complications of this disease syndrome has been slow and at present, diabetes mellitus remains a serious metabolic disorder.

The two main forms of primary diabetes mellitus are insulin-dependent diabetes mellitus (IDDM) or type I diabetes (formerly referred to as juvenile-onset diabetes), and noninsulin-dependent diabetes mellitus (NIDDM) or type II diabet es (formerly called maturity-onset diabetes). Other types of diabetes mellitus such as gestational diabetes, maturity-onset diabetes of youth, and diabetes secondary to pancreatic disease exist, however these will not be discussed in the context of this work. Table 1 compares aspects of IDDM and NIDDM clearly illustrating that these are separate disease entities with the only feature common to both being a decrease in glucose-stimulated insulin secretion and resultant hyperglycemia.

1.0.1 Insulin-dependent Diabetes Mellitus

It is estimated that 6.8% of the North American population between ages 20-74 years have diabetes mellitus (Knowler, et al., 1985). Insulin-dependent diabetes mellitus accounts for approximately 10-25% of all diabetics (Unger, 1991). As its name implies, IDDM requires treatment with exogenous insulin for survival. IDDM stems from an autoimmune attack on the endocrine pancreas resulting in beta cell destruction and loss of insulin secretion (Kahn, 1985; Atkinson and Maclaren, 1990). The clinical symptoms of IDDM (polyuria, polydipsia, polyphagia, and ketoacidosis) (Ekoe, 1988) appear when about 80% of the beta cells have been

destroyed (Atkinson and Maclaren, 1990).

It is now apparent that the autoimmune destruction of the beta cells is evoked by a mechanism called "molecular mimicry" whereby a foreign antigen (possibly a virus) that is a twin to a native component of beta cells, evokes an immune response resulting in the destruction of the foreign antigen and also the beta cells (Kaldany, et al., 1985; Atkinson and Maclaren, 1990). The etiology of IDDM is multifactorial involving both genetic and environmental factors. IDDM results when genetically predisposed individuals experience an environmental trigger that initiates progressive destruction of beta cells. The genetic predisposition has been associated with the major histocompatibility loci DQ and DR located on the short arm of chromosome number six (Atkinson and Maclaren, 1990). The foreign antigen initiating the immune response may be a twin to a native 64 kilodalton (kD) protein specific to beta cell membranes (Atkinson and Maclaren, 1988). Auto-64kD-antibodies have been found before the onset of the clinical symptoms of IDDM in both humans and rodent models. Recently, the 64kD protein has been identified as a GABA-synthesizing enzyme decarboxylase (GAD) (Baekkeskov, et al., 1990). Ko, et al. (1991) detected, but have not identified, an anti-38kD islet cell autoantibody that precedes the development of diabetes in BB (Bio-Breeding) rats. The environmental trigger responsible for IDDM is presently not understood, although the Coxsackie and Rubella viruses have been proposed (Cudworth and Gorsuch, 1983). Guberski, et al. (1991) have recently associated the presence of Kilham's rat virus with the development of

diabetes in diabetes-resistant BB rats.

As seen in Table 1, the onset of IDDM is during childhood and young adulthood (Lebovitz, 1984). The incidence of IDDM varies greatly between races, being highest among Caucasians and uncommon or unknown to many other races including Japanese, Chinese, American Indians, and Eskimos (Ekoe, 1988). Due to beta cell destruction, no insulin is produced and some regime of treatment with exogenous insulin is necessary for survival. The concordance rate seen for IDDM in monozygotic twins illustrates the multifactorial aspects of IDDM and the impact of the environment on the development of this disease. (For an excellent review of the prediction and prevention of IDDM see Andreani, et al., 1991.)

1.0.2 Noninsulin-Dependent Diabetes Mellitus

Noninsulin-dependent diabetes mellitus (NIDDM or type II) is by far the more common of the two primary types of diabetes mellitus, accounting for 75-90% of all diabetics (Unger, 1991). Although more common than IDDM, the pathogenesis of NIDDM is less clear and more controversial. NIDDM is characterized by the loss of glucose-stimulated insulin secretion and fasting or post-prandial hyperglycemia although no morphological lesion of beta cells has been identified (Unger, 1991). NIDDM appears to be a heterogeneous syndrome developing from several possible disorders including defects involving the beta cells of the pancreas, muscle and adipose cells, and the liver (DeFronzo, et al., 1983).

A beta cell defect (possibly of genetic origin) that results in the reduction or

loss of glucose-stimulated insulin secretion is a possible cause of NIDDM. Using an animal model of NIDDM (Zucker fatty rats) Johnson, et al. (1990) demonstrat ed the loss of insulin secretion in response to 20 mM glucose was coincident with the loss of GLUT-2 transporters on beta cell membranes. The GLUT-2 transporters belong to a family of five isoforms of glucose transporters responsible for the facilitative transport of glucose (Kayano, et al., 1988; Kayano, et al., 1990). The GLUT-2 molecules have been found only associated with cells (such as pancreatic beta cells) involved in the regulation of blood glucose homeostasis and are the only high K_m glucose transporters known. Therefore, the extracellular glucose concentration governs glucose uptake by GLUT-2 into beta cells which may then trigger insulin secretion (possibly by a high K_m glucokinase) (Unger, 1991). A decrease in GLUT-2 may render the beta cells incapable of recognizing an increase in blood glucose resulting in loss of glucose-stimulated insulin secretion which then prevents correction of hyperglycemia (Johnson, et al., 1990).

Another possible cause of NIDDM is a defect of peripheral tissues (mainl y muscle and adipose tissues) that results in insensitivity or resistance to insulin. NIDDM is associated with obesity in more than 80% of patients (Kahn, 1985). In the obese noninsulin-dependent diabetic, plasma levels of biologically normal insulin are often normal or above. This suggests that the hyperglycemia is caused by something other than the loss of insulin secretion by the pancreas. The resistance to insulin in the NIDDM individual may occur at several levels in the action of insulin. The first step in the action of insulin is the binding of insulin to

its cell membrane receptor. The insulin receptor is a complex heterotetrameric integral membrane protein that responds to insulin binding by the activation of a tyrosine kinase and receptor autophosphorylation (Kahn and White, 1988). Both a decrease in the number of insulin receptors and decreased tyrosine kinase activity have been found in NIDDM (DeFronzo and Ferrannini, 1982; Takayama, et al., 1988). The decrease in receptor number is due to the down-regulation of receptors in response to hyperinsulinemia. The alteration in receptor autophosphorylation and kinase activity reduces transmembrane signalling which, in muscle and adipose cells, results in the loss of insulin-stimulated glucose uptake and prolongs hyperglycemia.

Abnormal hepatic glucose production and uptake are also associated with NIDDM. The liver of the diabetic produces glucose at normal or above normal rates even in the presence of hyperglycemia and hyperinsulinemia (which both normally inhibit hepatic glucose production) (Felig and Wahren, 1971; Felig, et al., 1974; Kolterman, et al., 1981). The liver is the primary site of uptake of ingested glucose (Bratusch-Marrain, et al., 1980). Felig, et al. (1978) and DeFronzo and Ferrannini (1982) examined glucose uptake by the liver in NIDDM patients following glucose ingestion and found uptake reduced to 50% of normal. Both hepatic resistance to insulin and enhanced glucose production contribute to the hyperglycemic state.

In summary, NIDDM is a syndrome apparently of heterogeneous origin with many factors likely contributing to the observed hyperglycemia.

As shown in Table 1 the age of onset of NIDDM is middle to old age. It is estimated that 20% of males over 65 years of age have NIDDM (Knowler, et al., 1985). Unlike IDDM, NIDDM is spread across races with the North American Pima Indians having the highest known incidence approaching 50% in persons over 35 years (Ekoe, 1988). NIDDM has a strong, but as yet unidentified, genetic comp onent with the concordance rate for NIDDM of identical twins approximately 100%. An association between overnutrition, obesity and NIDDM has been recognized for a long time. In 1921, Joslin concluded that people 6-20% above average weight were 6-12 times more likely to have NIDDM than their non-overweight counterparts. Since that time, many studies have supported a strong relationship between obesity and NIDDM.

1.0.3 Management of Diabetes Mellitus

In both IDDM and NIDDM, clinical management is aimed at the normalization of plasma glucose. For the insulin-dependent diabetic, this certainly involves receiving exogenous insulin either in a conventional manner (one or two injections/day), or by multiple daily injections, or by continuous subcutaneous insulin infusion. Noninsulin-dependent diabetics do not generally require exoge nous insulin but may be treated with sulfonylureal compounds to stimulate insulin secretion. Dietary modification and physical exercise are important in the mana gement of both IDDM and NIDDM. In NIDDM, weight reduction and physical exercise have been found to alleviate insulin resistance (DeFronzo, et al., 1983;

Reaven, 1983).

Looking toward the future, identifying individuals genetically at risk for IDDM, screening for the presence of autoantibodies against beta cells and early treatm ent by either immunosuppression or toxins specific for a select population of immunocompetent cells, may preserve beta cell function and prevent IDDM (Atkinson and Maclaren, 1990; Boitard, et al., 1991). (For a review of immunoprevention of IDDM see Boitard, et al., 1991). The approach to prevention of NIDDM will have to await further understanding of the complex pathogenesis of this disease. At present, maintenance of proper body weight is prudent advice. As Joslin stated in 1921 "Diabetes is largely a penalty of obesity, and the greater the obesity, the more likely is Nature to enforce it."

1.1 The Development of Complications Related to Diabetes Mellitus

More than seventy years have now passed since the discovery of insulin and still the development of complications due to diabetes mellitus are a major source of morbidity and mortality (Sherwin and Tamborlane, 1985). For example, in 1987 in the United States, approximately 2% of all hospital admissions were diabetes related (Jacobs, et al., 1991).

Chronic complications occur in both IDDM and NIDDM. The late complications of diabetes include hematologic abnormalities, microangiopathy {most notably affecting the kidneys (nephropathy) and the eyes (retinopathy)}, neuropathy, and macroangiopathy. A brief description of each of these

complications and the possible mechanisms contributing to their pathogenesis follows.

1.1.0 Hematologic abnormalities associated with diabetes mellitus

Numerous hematological changes contributing to relative tissue hypoxia have been associated with diabetes mellitus. Among these are decreased deformability of red blood cells (Miller, et al., 1980), increased levels of nonenzymatically glycosylated hemoglobins with increased oxygen affinities (Ditzel, et al., 1979), decreased levels of 2,3-diphosphoglycerate (2,3-DPG) reducing oxygen release (Ditzel, et al., 1978), platelet abnormalities including increased adhesiveness and aggregation (Colwell, et al., 1978), elevated levels of plasma proteins resulting in increased plasma viscosity (Lowe, et al., 1980). While the mechanisms underlying these and other hemodynamic changes are not well understood, these abnormalities appear to be related to elevated blood glucose levels as correction of hyperglycemia has been found to normalize many hemo dynamic parameters (Jonsson and Wales, 1976; Peterson, et al., 1977; Ditzel, et al., 1979). It has been proposed that these hemodynamic changes may initiate a series of events in the microcirculation resulting in the microangiopathies associ ated with diabetes mellitus (Parving, et al., 1983). (For a review of the hemostatic abnormalities in diabetes see Greaves and Preston, 1984.)

1.1.1 Diabetic Microangiopathies

The initial feature of microvascular disease associated with diabetes mellitus is an increase in the thickness of endothelial basement membranes (Williamson and Kilo, 1977). Basement membrane thickness may increase in response to increased hydrostatic pressure created as a result of the hematological changes associated with diabetes mellitus (Tooke, 1986) or as the result of increased regional blood flow (Pugliese, et al., 1989). While microvascular changes may occur in other locations they have been best characterized in the kidney and the retina. In both organs, the progression of the microvascular lesions appears related to the duration and severity of the metabolic disturbance (Mauer, et al., 1975; Engermann, et al., 1977).

i) Diabetic nephropathy

The incidence of renal failure resulting from glomerular capillary damage secondary to basement membrane thickening is increased seventeen times in diabetics compared to the non-diabetic population (Brownlee, 1985). Presently in the United States, 25% of new dialysis patients are diabetic and diabetic nephropathy is the leading cause for renal transplantation (Report of the Secon d National Diabetic Research Conference, 1984). At least 40% of diabetics will develop nephropathy after having had diabetes for more than 40 years (Raskin, 1985).

Diabetic nephropathy manifests as persistent proteinuria and decreasing glomerular filtration rate coincident with glomerular basement membrane thickening and mesangial expansion (Brownlee and Cerami, 1981; Dahl-Jorgensen, 1986).

The proteinuria associated with diabetic renal disease likely is due to altered filtration properties of the glomerular basement membrane (Brownlee, 1985). Copeland, et al. (1987) have found an increase in nonenzymatic glycosylation of glomerular basement membrane proteins in diabetes and suggested that this increase may alter the net charge of the glomerular filtration barrier. The decreasing glomerular filtration rate associated with diabetic nephropathy is a consequence of glomerular capillary occlusion due to an accumulation of basement membrane material (Brownlee, 1985). Experimental evidence presently supports increased biosynthesis of basement membrane components (as opposed to decreased degradation) as being responsible for increased basement membrane thickness (Cagliero, et al., 1988; Brownlee, 1985).

ii) Diabetic retinopathy

The incidence of insulin-dependent diabetics affected by retinopathy ranges from 17% for those with diabetes of less than five years duration, to 97.5% in those with diabetes of greater than fifteen years duration (Klein, et al., 1984; Klein, et al., 1989). Blindness is 25 times more common among diabetics than nondiabetics (Brownlee and Cerami, 1981) and diabetes represents the leading cause of new cases of blindness in people under 65 years of age (Chew, 1985).

The definitive stimulus (or stimuli) responsible for initiating diabetic retino pathy is presently not known. However, numerous structural and functional abnormalities associated with diabetic retinopathy have been described. Possibly the earliest change occurring is a breakdown of the blood-retinal barrier

demonstrated by an increase in vitreous fluorescein with vitreous fluorophotometry (Cunha-Vaz, et al., 1975; Waltman, et al., 1978; Cho, et al., 1991). This change is found before ophthalmoscopic or angiographic abnormalities are present. Structurally, evidence exists to support changes to the retinal pigment epithelium as responsible for the initial breakdown of the blood-retinal barrier (Kirber, et al., 1980; Tso, et al., 1980).

Ultrastructural changes in the retina are comparable to those of the kidney. Numerous studies report an increase in the thickness of retinal capillary basement membranes with diabetes mellitus or galactosemia (Sima, et al., 1985; Robison, et al., 1986; Tilton, et al., 1986; Robison, et al., 1988; Vinores, et al., 1988, Chakrabarti and Sima, 1989; Vinores and Campochiaro, 1989; Das, et al., 1990; Robison, et al., 1990). Concurrent with an increase in capillary basement membrane thickn ess Sharma, et al. (1985), Sima, et al. (1985), Akagi and Kador (1990) and Kador, et al. (1990) also noted a decrease in the number of retinal capillary pericytes which, according to Cogan et al. (1961), may be the key pathological feature of diabetic retinopathy. The loss of pericytes may be related to hyperglycemia as King and Buzney (1983) have found that increased levels of glucose decreases the rate of pericyte proliferation in culture. Vinores, et al. (1988) also reported a progressive increase in the thickness of the basement membrane of the retinal pigment epithelium in spontaneously diabetic BB rats.

Clinically, diabetic retinopathy can be divided into non-proliferative or background retinopathy, and proliferative retinopathy. Background or non-

proliferative retinopathy is characterized by venous dilation, microaneurysms, retinal hemorrhages, edema, and exudates (both hard exudates which are lipoprotein deposits and soft exudates which are formed in areas of ischemic infarcts) (L'Esperance and James, 1983). Hypoperfusion and retinal hypoxia resulting from these and the previously described hematologic changes are regarded as fundamental factors in the progression of retinopathy to the proliferative stage. Proliferative retinopathy is characterized by a triad of changes including retinal neovascularization, glial proliferation, and increased vitreoretinal traction leading to vitreous hemorrhage and traction retinal detachment culminating in blindness (L'Esperance and James, 1983).

Areas of neovascularization represent the hallmark of proliferative retinopathy. Neovascularization is always preceded by capillary nonperfusion and presumed to be the consequence of retinal ischemia (Patz, 1980). The new vessels appear as endothelial loops (initially without supporting connective tissue) lying preretinally along the posterior surface of the vitreous. Fluorescein angiography shows the new vessels leak profusely contributing to edema and hemorrhage (Garcia and Ruiz, 1984). The exact mechanisms resulting in neovascularization are not known. Saunders, et al. (1990) have proposed the following model for neovascularization. A quiescent capillary is subjected to injury or ischemia. In diabetic retinopathy this may be related to hemodynamic changes, increased capillary basement membrane thickness, and/or pericyte loss. Endothelial cells are then exposed to fibroblast growth factor (FGF) which

stimulates proliferation resulting in the formation of new capillaries. Prior to injury, endothelial cells are not subjected to FGF as it is sequestered in the basement membrane. Alternatively, many researchers have investigated the role of insulin-like growth factor (IGF-1) in the pathogenesis of diabetic retinopathy (Merimee, et al., 1983; Grant et al., 1987; Hyer, et al., 1989; Dills, et al., 1990). The data are discrepant and the role of IGF-1 in diabetic retinopathy remains uncertain. Glial or fibrous proliferation occurs at a later stage of neovascularization in an attempt to support the naked new vessels (Garcia and Ruiz, 1984). Areas of neovascularization often form fibrous attachments with the vitreous. Compaction of the vitreous collagen may result in traction retinal detachments, increased hemorrhagic activity and visual loss (L'Esperance and James, 1983).

1.1.2 Diabetic Neuropathy

Approximately 10% of diabetics experience neuropathies of significant severity to seek medical attention (Melton and Dyck, 1987) although changes in nerve electrophysiology can be detected in most diabetic patients even when clinical symptoms are absent (Brownlee and Cerami, 1981). Diabetic neuropathies may be classified as focal neuropathies (involving nerve trunks), distal symmetrical polyneuropathy (involving peripheral sensory and motor nerves), and autonomic neuropathy (affecting various autonomic nerves resulting in a variety of problems) (Greene and Pfeifer, 1985).

Diabetic polyneuropathy resulting in distal, bilateral sensory and motor loss,

is the most frequent form of diabetic neuropathy. The distal portions of the longest nerves are affected first resulting in a "glove and stocking" loss of sensation and motor weakness (Locke and Tarsy, 1985). Autonomic neuropathy may result in bowel, bladder, or sexual dysfunction. Autonomic dysfunctions common in diabetic patients include diarrhea, constipation, hypotonic neurogenic bladder, retrograde ejaculation, impotence, postural hypotension, and loss of cardiovascular reflexes (Locke and Tarsy, 1985).

Many morphological, functional and biochemical changes in diabetic neuropathy have been described. While the etiology of these changes is not clearly understood at present, hyperglycemia and insulin insufficiency are thought to be important factors (Report of the Second National Diabetic Research Conference, 1984). Morphological changes include endoneurial edema, segmental demyelination, axonal degeneration and decreased diameter of myelinated axons. Functional and biochemical changes associated with diabetic neuropathy include decreased nerve conduction velocity, altered myelin synthesis, decreased axoplasmic transport, and increased polyol pathway activity (Brownlee and Cerami, 1981). (For a more thorough coverage of diabetic neuropathy see Dyck, et al., 1987.)

1.1.3 Diabetic Macroangiopathy

Diabetic macroangiopathy, synonymous with atherosclerosis, is three to four times more common among diabetics than the general population (Brownlee and

Cerami, 1981) and is the leading cause of death in diabetics (Gries and Koschinsky, 1991). Diabetic macroangiopathy appears to be subject to the same risks as atherosclerosis in the general population (ie. hypertension, obesity, smoking, serum cholesterol) plus an additional unexplained diabetes-specific risk factor is also present (Gries and Koschinsky, 1991). Biochemical changes (as summarized by Gries and Koschinsky, 1991) possibly contributing to this diabetes-specific risk factor include abnormal structure and metabolism of lipoproteins, including low density lipoproteins (LDL), increased platelet activity and diabetes-specific growth factors. The diabetic condition appears to accelerate the atherosclerotic process associated with aging (Gries and Koschinsky, 1991). (For an overview of diabetic macroangiopathy see Jarrett, 1984 or Keen, 1987.)

The prevalence of all of the complications associated with diabetes mellitus tends to increase with the duration of the disease. Diabetic patients may be discordant for nephropathy, retinopathy, neuropathy, and/or macroangiopathy, and the progression of each of these complications is linked to a host of other independent variables (Greene and Pfeifer, 1985).

1.2 Mechanisms Contributing to the Pathogenesis of Complications Related to Diabetes Mellitus

Over the years, there has been considerable debate regarding the importance of blood glucose normalization and the relationship between metabolic control and the development of the long-term complications associated with

diabetes mellitus. Most experts in the field support the "metabolic hypothesis" initially proposed by Williamson and Kilo (1984) which states that the relative or absolute lack of insulin and resulting hyperglycemia impair cellular function contributing to the pathogenesis of complications related to diabetes. Extracellular glucose concentrations will affect different tissues in different ways depending on the properties of the component cells. All tissues which are prone to develop complications related to diabetes are freely permeable to glucose (ie. not requiring insulin for glucose transport) and are therefore subjected to elevated intracellular glucose concentrations when the extracellular glucose concentration is increased (Dahl-Jorgensen, 1986). Intracellular processes for the disposal of excess glucose and the consequences of these processes, have been implicated in the initiation of the pathogenic sequence. The two mechanisms currently thought to contribute to the development of complications related to hyperglycemia are nonenzymatic glycosylation of proteins and increased polyol pathway activity. As stated by Pugliese, et al. (1991) "these mechanisms are not independent of each other, but participate in a cascade of interrelated metabolic alterations which is triggered by intracellular metabolism of excess glucose and impacts on both carbohydrate and lipid metabolism; in turn, these metabolic alterations interfere with signal transduction mechanisms and energy metabolism, thus affecting cell function."

1.2.0 Nonenzymatic Glycosylation of Proteins

Many proteins undergo enzymatic glycosylation during synthesis. Nonen

zymatic glycosylation is a post-translational modification that results in the covalent linkage of carbohydrate to the nitrogen atoms of the α-amino group of the N-terminal amino acid and the ϵ-amino group of lysine (Kirschenbaum, 1984). The reaction produces an almidine or Schiff base which undergoes an Amadori rearrangement to form a ketoamine or Amadori product (Kirschenbaum, 1984; Pugliese, et al., 1991). Nonenzymatic glycosylation occurs under euglycemic conditions and is enhanced under hyperglycemic conditions (Pugliese, et al., 1991).

Many proteins have been found to undergo nonenzymatic glycosylation including the proteins in hemoglobin, red blood cell membranes, endothelial cell membranes, lens crystalline, lens capsule, myelin, tubulin, glomerular basement membranes, collagens, low and high density lipoproteins, albumin and globulins (Schnider and Kohn, 1981; Kirschenbaum, 1984; Dahl-Jorgensen, 1986). As summarized by Brownlee, et al. (1984) and Kirschenbaum (1984), nonenzymatic glycosylation may affect many structural and functional aspects of proteins including charge characteristics, solubility, stability, size, shape, viscosity, crosslinking, enzymatic activity, hormonal activity, susceptibility to proteolysis, cellular uptake, binding of regulatory molecules, and biologic half-life. Increased levels of nonenzymatically glycosylated proteins have been found in a number of diabetic tissues, however, the functional significance of their presence in vivo remains uncertain.

Numerous blood proteins have been found to have increased levels of

nonenzymatic glycosylation under diabetic conditions. Glycosylated hemoglobin is elevated in diabetics and is reflective of blood glucose levels during the previous 6-10 weeks (Miedema and Casparie, 1984). Similarly, levels of glycosylated albumin and fibrinogen are correlated with blood glucose levels in the preceding 2-3 weeks and 2-3 days respectively (Ardawi, et al., 1990). Red blood cell membrane proteins are subject to increased nonenzymatic glycosylation which may have an effect on their deformability (Miller, et al., 1980).

Nonenzymatic glycosylation of collagen may contribute to basement membrane thickening and/or changes in basement membrane function leading to microangiopathy. Glycosylation of collagen is related to decreased solubility and sensitivity to protease digestion possibly due to increased crosslinking (Schnider and Kohn, 1981). Copeland, et al. (1987) have found increased levels of nonenzymatic glycosylation of glomerular basement membrane proteins in diabetic rats without increased basement membrane thickness but with altered renal functional parameters.

Little evidence is available to determine the contribution of nonenzymatically glycosylated nerve proteins to diabetic neuropathy. Increased glycosylation of myelin has been found in both the peripheral and central nervous system (Vlass ara, et al., 1983). It has been hypothesized that this may mark the myelin for degradation by macrophages resulting in the demyelination seen in diabetic neuropathy (Kennedy and Baynes, 1984; Vlassara, et al., 1984). Glycosylation of tubulin has been observed in diabetic rat brain (Williams, et al., 1982) which may

interfere with microtubule-dependent processes.

Evidence from a number of diabetic tissues supports the presence of increased levels of glycosylated proteins, however, their functional significance in vivo has not been conclusively established.

1.2.1 Increased Polyol Pathway Activity

The polyol pathway was first described in 1956 by Hers. To date, more is understood regarding the pathophysiological role of this metabolic pathway than of its physiological significance under normal conditions.

The polyol pathway consists of two reactions. Firstly, glucose is reduced to sorbitol by aldose reductase using NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) as the hydrogen donor. Secondly, sorbitol is oxidized to fructose by sorbitol dehydrogenase using NAD^+ (nicotinamide adenine dinucl eotide, oxidized form) as the hydrogen acceptor. The activity of this pathway is low at normal glucose concentrations due to the high K_m of aldose reductase. Under hyperglycemic conditions, the activity of this pathway is increased in tissues not requiring insulin for glucose transport (Pugliese, et al., 1991).

Several hypotheses have arisen regarding the link between increased polyol pathway activity and the development of diabetes-related complications. Increa sed aldose reductase activity has been associated with the development of diabetic nephropathy, cataracts, retinopathy, and neuropathy (as summarized in Kador, 1990). A great deal of the support for the implication of aldose reductase

in the development of diabetic complications has arisen from the use of aldose reductase inhibitors that have been found to delay or prevent the onset of these changes.

One hypothesis regarding the mechanism by which increased aldose reductase activity contributes to the development of diabetic complications is based on the osmotic imbalance created due to sorbitol accumulation. Dr. J.H. Kinoshita has devoted nearly 30 years of research to the polyol pathway and was one of the first to demonstrate that diabetic cataracts were related to the accumulation of sugar alcohols and resultant hydration of lens fibres (Kador, 1990).

It is becoming increasingly apparent that the hyperosmotic effects of sugar alcohol accumulation do not only result in osmotic swelling, but rather in a cascade of biochemical changes leading to altered cell structure and function. Among the many changes described in response to increased aldose reductase activity are intracellular myo-inositol depletion, decreased phosphotide turnover, decreased protein kinase C activity and decreased Na/K-ATPase activity (Winegrad, 1987; Pugliese, et al., 1991). The mechanisms and the interactions between mechanisms by which these changes contribute to the pathogenesis of diabetic complications are less than clearly understood at this time and are being actively pursued by many researchers.

The use of aldose reductase inhibitors has been found to prevent or delay the onset of many diabetes-related pathologies. Microalbuminuria and proteinuria

in diabetic animals have been reduced by treatment with aldose reductase inhibitors (Terubayashi, et al., 1989; Tilton, et al., 1989; Pugliese, et al., 1990). In the retina, the use of aldose reductase inhibitors has prevented pericyte loss, and retinal capillary basement membrane thickening (Frank, et al., 1983; Robison, et al., 1983; Robison, et al., 1988; Robison, et al., 1989a). Decreases in motor nerve conduction velocity, axonal transport, sensory perception, and axonal dwindling associated with diabetic neuropathy have been reversed with aldose reductase inhibition (Kador, 1988; Yagihashi, et al., 1990).

These and numerous other reports clearly implicate the polyol pathway as a major player in the development of complications related to diabetes but the multiple mechanisms altered by increased sorbitol and/or fluid levels are extremely complex and await complete elucidation.

Hyperglycemia (which is unavoidable in even well-controlled diabetics) appears as the common denominator in both increased nonenzymatic glycosylation and increased polyol pathway activity. One must then question what other factors affect the development and progression of diabetic complications as not all poorly-controlled diabetics develop long-term complications, and conversely, not all well-controlled diabetics escape. Several risk factors independent of diabetes, but which interact with diabetes-induced changes, probably play an important role in the appearance and progression of complications. These diabetes-independent risk factors include hyperlipidemia, hypertension, hyperinsulinemia, and diet. For example, high protein diets have

been reported to augment the progression of diabetic nephropathy (Meyer, et al., 1983) and dietary protein restriction has been found to improve renal function (Neugarten, et al., 1983; El Nahas, et al., 1984; Remuzzi, et al., 1985; Copeland, et al., 1989; Walker, et al., 1989; Zeller, 1991).

The complications associated with diabetes mellitus are many and varied. The increase in activity of the pathways for disposal of excess glucose, combined with other risk factors (that are influenced by both genetics and the environment) all interact to determine the appearance and progression of diabetic complications.

1.3 Rationale for the Present Study

The present study was undertaken to determine the effects of age and dietary protein intake on selected aspects of retinal ultrastructure, in normal and diabetic rats. The parameters examined were retinal capillary structure and basement membrane (or basal lamina) thickness, the morphology of the retinal pigment epithelium and basal cell membrane area, and the structure and thickness of Bruch's membrane. The hypotheses were: i) that diabetes would result in alterations in retinal morphology and that the diabetes associated changes would be reduced by insulin treatment and a reduced protein diet, and ii) age associated changes in retinal morphology would occur.

The rationale for the independent variables chosen is as follows.

1.3.0 Diabetes

As explained earlier, diabetes has a significant effect on the eye. In this study, streptozotocin-induced diabetes was used to examine the effects of diabetes on the retinal structures of interest.

Streptozotocin is a diabetogenic antibiotic isolated from Streptomyces acromogenes (Junod, et al., 1967; Karunanayake, et al., 1976). Streptozotocin exerts its cytotoxic effects on pancreatic beta cells by blocking glucose uptake into these cells. The use of chemically-induced models of diabetes has been questi oned based on the possible extrapancreatic sites of the drug's action. In the present study, streptozotocin-injected animals that did not become diabetic were included to examine possible drug-induced, as opposed to diabetes-induced alterations. The streptozotocin-diabetic rat is a well used model of diabetic microangiopathy (Fischer and Gartner, 1983; Anderson, 1985; Tilton, et al., 1986; Sima, et al., 1988).

Several previous reports have demonstrated increases in retinal capillary basement membrane thickness in diabetes (Fischer and Gartner, 1983; Tilton, et al., 1986; Vinores, et al., 1988; Chakrabarti and Sima, 1989). Fewer studies have examined the role of blood glucose regulation in diabetes and retinal capillary basement membrane thickening. Chakrabarti and Sima (1989) found capillary basement membrane thickening was reduced in euglycemic diabetic animals compared to untreated diabetic animals. In this study, the effects of two levels of blood glucose concentrations (ie. hyperglycemia and euglycemia) in diabetic animals on retinal capillary basement membrane thickening were examined.

Previous studies of the effect of diabetes on retinal pigment epithelium basal cell membrane area are discrepant. Grimes and Laties (1980) and Grimes, et al., (1984) found an increase in retinal pigment epithelial basal cell membrane area whereas Chakrabarti, et al. (1990) reported no change. Blair, et al. (1984) and Vinores, et al. (1988) described focal areas of exaggerated infoldings as well as areas where infoldings were absent. In the present study, retinal pigment epithelial basal cell membrane area was quantified in control and diabetic animals with two levels of blood glucose regulation.

The effect of diabetes on Bruch's membrane thickness has not been widely examined. Caldwell, et al. (1986) and Vinores, et al. (1988) found an increase in the thickness of the retinal pigment epithelium basement membrane in diabetic animals. The basement membrane of the retinal pigment epithelium is only one component of Bruch's membrane. It was therefore of interest to examine the effect of diabetes on the thickness of Bruch's membrane and to determine if blood glucose regulation in diabetes effects Bruch's membrane thickness.

1.3.1 Age

Capillary basement membrane thickness has previously been reported to increase with age (Xi, et al., 1982; Cuthbertson and Mandel, 1986; Nagata, et al., 1986). This study was undertaken to confirm an increase in capillary basement membrane thickness with age and more importantly, examine the effect of age on the basal cell membrane area of the retinal pigment epithelium and the thickness

of Bruch's membrane which have been less thoroughly investigated.

1.3.2 Dietary Protein

As described previously, a decrease in protein intake has been found to improve diabetic nephropathy (Neugarten, et al., 1983; El Nahas, et al., 1984; Remuzzi, et al., 1985; Copeland, et al., 1989; Walker, et al., 1989; Zeller, 1991). The effect of dietary protein intake has only been examined related to the diabetic kidney. Pugliese, et al. (1990) demonstrated a significant increase in ^{131}I-BSA (bovine serum albumin) clearance in the retina comparable in magnitude to the increase in glomerular filtration rate and ^{131}I-BSA clearance in the kidney in diabetic rats. Since a decrease in protein intake has been shown to be of benefit in diabetic nephropathy, it was of interest to examine the effect of dietary protein intake on one of the other tissues affected by vascular filtration changes in diabetes, namely the retina. The effect of a low protein diet on retinal capillary basement membrane thickness, retinal pigment epithelium basal cell membrane area, and Bruch's membrane thickness have not previously been reported.

The rationale for the dependent variables examined is as follows.

1.3.3 Retinal Capillary Structure and Basement Membrane Thickness

Structural changes in retinal capillaries may be of functional significance in the course of microangiopathy in the diabetic retina. Changes in capillary base ment membrane (or basal lamina) thickness may alter the permeability properties

of the capillary contributing to hypoxia and edema. It was therefore of interest to quantify the thickness of capillary basement membranes. The retina is supplied by two separate vascular suppplies; the central artery gives rise to the superficial and deep capillary beds supplying the inner aspects of the neural retina, and the choriocapillaris supplies the outer aspects of the retina (see Figure 1). Capillaries of the outer plexiform layer (or deep capillary bed) were chosen as thin sections of retina could be cut to include the outer plexiform layer, the retinal pigment epithelium and Bruch's membrane. Previous work by Nagata, et al. (1986) and Chakrabarti and Sima (1989) demonstrated structural differences between the superficial (nerve fibre and ganglion cell layers) and deep (outer plexiform and inner nuclear layers) capillary beds, therefore care must be taken to compare only capillaries from the same region of the retina.

1.3.4 Retinal Pigment Epithelium Structure and Basal Cell Membrane Area

The retinal pigment epithelium consists of a single layer of cells forming the outermost aspect of the retina. The retinal pigment epithelium serves many important functions vital to the maintenance of proper vision. The functions of this cell layer include providing stability and proper alignment of photoreceptor outer segments (Bernstein, 1961), the storage of vitamin A precursors of visual pigments (Young and Bok, 1979), the phagocytosis and degradation of photoreceptor outer segment discs (Young, 1974, 1978), and the transport of materials between the

choriocapillaris and the photoreceptors (Steinberg and Miller, 1973). The outer neural retina relies on the choriocapillaris for oxygen and nutrients. The retinal pigment epithelium regulates the exchange of molecules between the photoreceptors and the choriocapillaris. Adjacent retinal pigment epithelial cells are joined by tight junctions ensuring that all movement between the choriocapillaris and photoreceptors is transcellular. The basal cell membrane of the retinal pigment epithelium is extensively infolded to increase the surface area for transport. Many qualitative observations regarding the extent of basal infoldings appear in the literature related to age and diabetes (Blair, et al., 1984; Vinores, et al., 1988; Vinores and Campochiaro, 1989; Chakrabarti, et al., 1990), however, few attempts have been made to quantify the basal cell membrane area (Grimes and Laties, 1980; Grimes, et al., 1984; Chakrabarti, et al., 1990). Given the critical role of this cell membrane surface in transport, this study will provide important quantitative data on the retinal pigment epithelium basal cell membrane area and the effects of age and protein intake on this highly active cell border in normal and diabetic animals.

1.3.5 Bruch's Membrane Structure and Thickness

Bruch's membrane (or complexus basalis) lies between the basal surface of the retinal pigment epithelium and the choriocapillaris. In most mammalian species examined, Bruch's membrane is a pentalaminate structure consisting of the basement membranes of the retinal pigment epithelium and endothelium of the

choriocapillaris, and two layers of collagen separated by a central, discontinuou s layer of elastic tissue. Although positioned between the retinal pigment epithelium and the choriocapillaris, Bruch's membrane is not thought to serve as a barrier to the transport of most macromolecules (Zinn and Benjamin-Henkind, 1979). Previous studies have described an increase in Bruch's membrane thickness with age (Kornzweig, 1979) and Caldwell, et al. (1986) and Vinores, et al. (1988) have reported an increase in retinal pigment epithelium and choriocapillaris endothelium basement membrane thickness with diabetes. Because the basal cell border of the retinal pigment epithelium and Bruch's membrane are intimately related (both anatomically and physiologically), both structures were examined in this study. The intent of this portion of the investigation was to determine the effects of age and protein intake on the total thickness of Bruch's membrane in normal and diabetic animals.

This study was designed and carried out in 1986 as part of a collaborative project. At that time, little work had been done related to the potential benefits of protein restriction in diabetes mellitus. In addition to the retinal parameters examined, analyses of the experimental animals in this study included kidney weight, urinary albumin excretion, creatinine clearance, nonenzymatic glycosylation of glomerular capillary basement membrane proteins, glomerular capillary basement membrane thickness, nonenzymatic glycosylation of skeletal muscle capillary basement membrane proteins, skeletal muscle capillary basement membrane thickness, and the level of alveolar bone surrounding the teeth. This

collaborative approach allowed for significant potential benefit by relating and possibly correlating, the effects of protein intake and diabetes to the whole animal.

2. MATERIALS AND METHODS

2.0 Animal Treatment and Care

The experimental protocol is outlined in Figure 2. The experiment began with 104 male Sprague-Dawley rats weighing between 250-300 grams. Six animals were sacrificed at the beginning of the experiment to serve as zero time controls (C0). The remaining 98 animals were randomly divided into two groups; 50 animals were placed on a diet containing 8% protein (ICN Biochemicals Inc., Cleveland, OH), 48 animals remained on standard rat chow containing 24% protein (Wayne F-6 Rodent Blox, Wayne Pet Food Division of Continental Grain Company, Chicago, IL). The two diet groups, although differing in protein content, were nearly isocaloric (8%- 4000 Kcal/kg, 24%- 4040 Kcal/kg). All animals were provided food and water *ad libitum*. The animals were housed in separate wire mesh cages and had an alternating 12 hour light/dark cycle with the light period beginning at 0800.

Within each diet group, 42 animals were given a single intraperitoneal injection of streptozotocin (STZ) (Sigma) (65mg/kg body weight) dissolved in citrate buffer. The remaining 8 animals on the 8% protein diet served as uninjected controls (C8%) and 6 animals on the 24% protein diet served as uninjected controls (C24%). Three days following the streptozotocin injection, blood glucose levels were quantified using Dextrosticks and read on a glucometer (Ames

Division, Miles Laboratories Ltd., Rexdale, ON). In the 8% protein diet group, 30 of the STZ-injected animals had blood glucose levels greater than 20 mmol/l and were considered diabetic. Twelve of the STZ-injected animals of the 8% protein diet group had blood glucose levels less than 10mmol/l and were maintained as a streptozotocin-injected non-diabetic group (STZ8%). In the 24% protein diet group, 33 animals became diabetic and 9 animals were not deemed non-diabetic (STZ24%) based on the same criteria as above. Within each diet group, the diabetic animals were divided into two groups; one group (DM) received very small doses of protamine zinc insulin (2-4 units daily) (Connaught Laboratories, Willowdale, ON) or no insulin to maintain hyperglycemia (blood glucose >15mmol/l). The other group (DM+I) received insulin injections (7-9 units daily) to maintain euglycemia (blood glucose <10mmol/l). All animals were tail bled weekly for blood glucose determination and insulin doses for the diabetic animals were adjusted accordingly. At two month intervals, all animals were weighed.

All animals were maintained for a period of 5 months. At the end of the experimental period, the animals were anesthetized with sodium pentobarbital (Nembutal) (50 mg/kg body weight) injected intraperitoneally. All animals were randomly assigned a code number to conceal the treatment group to which the animal belonged.

2.1 Tissue Collection

With the animals under deep anesthesia, the eyes were enucleated, slit at

the equator, and fixed by immersion in 5% glutaraldehyde in 0.1M Sorensen's buffer (pH 7.3) at 4^0C for 5 hours. The posterior half of the eyes were then removed, washed for a minimum of 2 hours in 5% sucrose in 0.1M Sorensen's buffer and cut into pieces less than 1 mm^2. The tissues were then post-fixed in 1% osmium tetroxide in 0.1M Sorensen's buffer for 2 hours and dehydrated through an ascending series of ethanol, methanol, and propylene oxide and embedded in Araldite.

Plastic embedded retinal tissues were randomly selected and subsequently reoriented to desired angles on wooden dowel rods using a wax mount. Three blocks (designated 1, 2, and 3) per animal were trimmed and thick sections (1um) were cut, dried on glass slides, and stained with 1% Toluidine blue in 1% aqueo us borax. Ultrathin sections (50-70nm) were cut on a LKB ultramicrotome and collected on uncoated 300 mesh copper grids. Two grids (a and b) were cut per block giving a total of six grids per animal (1a&b, 2a&b, and 3a&b). The tissues were stained with saturated aqueous uranyl acetate for 45 minutes and counterstained with lead citrate for 3 minutes. The sections were examined in a Philips EM 201 set at 60 Kv beam voltage. For each grid, at least two photomicrographs were made at a magnification of x2875; one of a capillary in the outer plexiform layer of the retina, and one of the basal aspect of the retinal epithelium including Bruch's membrane.

2.2 Morphometric Analysis

All negatives were enlarged to a final magnification of 20125 times. All prints considered unsuable due to poor focus or inappropriate plane of section were discarded and all remaining prints were measured. Quantitative morphometric analysis was performed on 3 structures with the use of a Summa Sketch II graphics tablet (Summagraphics Corporation, Seymour, CT) connected to a Mind 286 computer equipped with the program Sigma-Scan (Jandel Scientific, Corte Madera, CA). The accuracy and reproducibility of the measuring methods were tested by measuring drawings of known dimensions and by repeatedly measuring the same series of electron micrographs. The parameters examined were; i) capillary basement membrane thickness, ii) basal cell membrane surface area of the retinal pigment epithelium (referred to by convention as the retinal pigment epithelium (RPE) although in this species, these cells are unpigmented), and iii) the thickness of Bruch's membrane.

2.2.0 Measurement of Capillary Basement Membrane Thickness

Basement membrane thickness of capillaries from the outer plexiform layer of the retina was determined by the method of McEwen, et al. (1987) and Robis on, et al. (1983) based on Shannon, et al., (1982). The capillary basement membrane is not uniformly thick and is often split by the presence of pericytes. This method provides an average value of basement membrane thickness and takes into account all the basement membrane associated with the capillary. The boundaries of the basement membrane of transversely sectioned capillaries

(determined by clearly defined basement membranes) were carefully outlined in pen as shown in Figure 3 and secured on the digitizing tablet. Basement membrane thickness was determined as follows:

a) basement membrane area (BMA) was determined as

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

where T is the total capillary area measured by tracing around the outermost aspect of the basement membrane; E represents the area of the outermost aspect of the endothelial cell and includes the capillary lumen; and P represents the area of pericytes.

b) basement membrane length (BML) was calculated as

$$BML = \frac{\text{total length of lines delimiting basement membranes}}{2}$$

c) basement membrane thickness (BMT) was then

$$BMT = BMA/BML$$

All basement membrane thicknesses are given in nanometers (nm).

2.2.1 Measurement of the Basal Cell Membrane Area of the Retinal Pigment Epithelium

The basal cell membrane area (BCMA) of the retinal pigment epithelium (RPE) was determined by a method described by Grimes and Laties (1980) and Grimes, et al. (1984). A grid of parallel lines (1 cm apart) was placed diagonally on the micrograph so that the grid lines were at 45° and 135° to the cell base (see

Figure 4). The number of intersections between the basal cell membrane and the grid lines was counted for the 45° lines and for the 135° lines and the mean of these two counts was used in subsequent calculations. Basal cell membrane area (BCMA) (L_c) was calculated as

$$L_c = (\pi/2)\ (\#\ \text{intersections})\ (\text{distance between parallel lines})$$

The length of Bruch's membrane (L_b) underneath the test grid was fixed (12.8 cm) and the ratio of RPE cell membrane length to Bruch's membrane length was given as L_c/L_b .

2.2.2 Measurement of Bruch's Membrane Thickness

The thickness of Bruch's membrane (BrM) was determined by placing a transparency marked with six unevenly spaced lines, perpendicular to Bruch's membrane, over the micrograph (see Figure 5). The distance across Bruch's membrane from the basement membrane of the retinal pigment epithelium to the basement membrane of the choriocapillaris was measured at these six places and the mean calculated for each micrograph. All values of the thickness of Bruch's membrane are reported in nanometers (nm).

2.3 Statistical Analysis

The values used for each animal represent the mean of 3 to 12 measurements per animal. The means for each animal were used to calculate the mean of each experimental group.

Analysis of the data from this study included analysis of variance (ANOVA) (both one-way and two-way), Student's t-tests, Duncan's multiple range tests and Chi-square analysis. A p value of 0.05 or less was accepted as being significant.

3. RESULTS

3.0 Survival Rates

The number of animals in each treatment group at the beginning and end of the experiment, and the percent survival are shown in Table 2. The diabetic animals on both diets had significantly lower survival rates than the non-diabetic control groups ($p<0.001$ by Chi-square analysis).

3.1 Body Weight

At the beginning of the experiment, all animals weighed between 250-300 grams. Table 3 shows the mean body weights for each treatment group at the end of the experimental period. Two-way analysis of variance revealed that the animals on the 8% protein diet weighed significantly less than the animals that received a 24% protein diet ($F=27.04$; $df=1$; $p<0.001$). Duncan's multiple range tests confirmed that the 24% groups weighed significantly more than the comparable 8% group for all but the insulin-treated diabetics (see Table 4). On both diets, treatment condition had a significant effect on final body weight ($F=11.08$; $df=3$; $p<0.001$) with insulin-treated diabetics weighing more and untreated diabetics weighing less than the controls and STZ injected non-diabetics. Duncan's multiple range tests found untreated diabetics on both diets weighed significantly less than insulin-treated diabetics (ie. $DM8\%<DM{+}I8\%$, $p<0.01$; and $DM24\%<DM{+}I24\%$, $p<0.01$). Insulin-treated diabetics on 8% protein weighed significantly more than the three other groups on the same diet (ie. Diabetic, STZ injected non-diabetic, and

Control) ($p<0.01$). Insulin-treated diabetics on 24% protein weighed significantly more than untreated diabetics on 24% ($p<0.01$), and STZ injected non-diabetic 24% ($p<0.05$). Diabetic + Insulin 24% also weighed more than the 24% controls but the difference was not statistically significant. Within each diet group, the final body weight of the STZ injected non-diabetic animals was not significantly different from the uninjected control group.

3.2 Blood Glucose

The mean final blood glucose values (in mmol/l) for each treatment group are shown in Table 4. A two-way analysis of variance determined that diet had no effect ($F=0.39$; $df=1$; $p=0.53$) while treatment condition had a significant effect on blood glucose ($F=27.68$; $df=3$; $p<0.001$). Duncan's multiple range tests found the untreated diabetics on both diets had blood glucose values significantly higher than all other groups ($p<0.01$). There were no significant differences in blood glucose between comparable treatment groups on the two diets (eg. Control 8% was not significantly different from Control 24%). The glycosylated hemoglobin values for these animals parallel the blood glucose data (Copeland, et al., 1990).

3.3 Qualitative Structural Observations

3.3.0 Retinal Capillaries

Examples of capillaries from the outer plexiform layer of the retina are shown in Figures 3 and 6. Retinal capillaries consist of non-fenestrated endothelial

cells resting on a basement membrane. The endothelial cells are very attenuated, except in the paranuclear region. Adjacent endothelial cells are joined by zonula occludentes. Near endothelial cell junctions, short irregular villi often project into the lumen. The basement membrane surrounding the capillary is often split to accommodate pericytes and pericyte processes giving the appearance of "a cell embedded in basement membrane" (Fine and Yanoff, 1972). Glycogen-like granules are frequently present in pericyte cytoplasm. The basement membrane appeared fairly homogeneous in density and was of variable thickness around the circumference of the capillary.

There were no empirical differences in capillary appearance between experimental groups.

3.3.1 Retinal Pigment Epithelium

The retinal pigment epithelium consists of a single layer of roughly cuboi dal shaped cells resting on a thin basement membrane (Figures 4 and 7). Basally, the cell membrane is thrown into numerous, irregular microfolds. Apically, the pigment epithelial cells have many slender microvillar processes that interdigitate with the tips of photoreceptor outer segments. Laterally, adjacent cells are joined apically by junctional complexes consisting of zonulae adherentes and occludentes. The retinal pigment epithelial cells normally contain a single, large, pale staining nucleus. Internally, smooth endoplasmic reticulum is abundant while a few profiles of rough endoplasmic reticulum and numerous ribosomes are

scattered throughout the cytoplasm. Mitochondria are abundant and typically located in the basal region of the cytoplasm. Lamellar appearing phagosomes of photoreceptor outer segments are a constant feature.

No qualitative differences in the appearance of the retinal pigment epithel ium was observed between experimental groups.

3.3.2 Bruch's Membrane

Bruch's membrane (or complexus basalis) is of the same pentalaminate structure as described in other mammalian species (Rodieck, 1973; Braekevelt, 1988) (Figures 7 and 8). The five layers include the basement membranes (or basa lamina) of the retinal pigment epithelium and the endothelium of the choriocapillaris, and two collagenous layers split by a discontinuous layer of elastic connective tissue. The thickness of Bruch's membrane is quite variable due to the irregular nature of the choriocapillaris.

There were no apparent structural differences in Bruch's membrane between experimental groups.

3.4 Quantitative Observations

3.4.0 Accuracy and Reproducibility of Morphometric Techniques

In quantitative morphometric work, the accuracy and reproducibility of the chosen techniques must be established at the onset. In accordance with statistical standards (Norman and Streiner, 1986) a coefficient of variation (standard

deviation/mean) less than 5% was considered acceptable in this study. To confirm the accuracy of the digitizing system employed, a diagram of a test capillary was carefully constructed on calibrated chart recorder paper and measured repeatedly. A Student's t-test between the known and measured basement membrane thickness showed no significant difference ($p=0.969$) (data not shown). The coefficent of variation for these measures was 2.2% which was considered acceptable.

The ability of the investigator to reproduce measurements was tested in several ways. Ten prints of the same capillary were traced and measured on separate days (Table 5). The coefficient of variation was 3.8%. A series of five micrographs (designated A to E) were measured on five separate days. The results are seen in Table 6. In all cases, the coefficients of variation were less than the acceptable 5% level. Similar tests were performed on the basal cell membrane area of the retinal epithelium (Table 7) and for Bruch's membrane thickness (Table 8) and all coefficients of variation were again less than 5%.

The ability of two investigators to attain the same results was tested for the three parameters of interest; capillary basement membrane thickness, retinal epithelium basal cell membrane area, and Bruch's membrane thickness (Table 9). Measurements by both investigators (I and II) attained high levels of reproducibility (coefficient of variation <5%) however, Student t-tests revealed a significant difference between investigators with respect to capillary basement membrane thickness. In studies of this nature, it is therefore best if one person performs the

morphometric analysis to eliminate variability between investigators as a possible source of error. This also makes comparing values between studies difficult.

3.4.1 Capillary Basement Membrane Thickness

The mean capillary basement membrane thicknesses are shown in Table 10 and Figure 9.

A one-way analysis of variance of the nine experimental groups (including the zero time control group) showed a statistically significant difference ($F=3.44$; $df=8$; $p<0.01$). Duncan's multiple range tests found that capillary basement membrane thickness of the zero time control group was significantly less than all other groups ($p<0.05$). Table 13 compares capillary basement membrane thickness in the zero time control group (CO) to the mean of the comparable groups 5 months older (C and STZ24%). An unpaired Student's t-test between these means was statistically significant ($t=3.55$; $df=15$; $p<0.05$). These analyses suggest an age-related increase in capillary basement membrane thickness has occurred.

Capillary basement membrane thickness (BMT) was not affected by diet or treatment condition. Two-way analysis of variance (excluding the zero time control group) revealed no significant differences between the two diet groups ($F=0.95$; $df=1$; $p=0.33$), or between the four treatment groups ($F=0.33$; $df=3$; $p=0.80$). Also, no significant interaction effect was found ($F=0.79$; $df=3$; $p=0.51$).

3.4.2 Retinal Pigment Epithelium Basal Cell Membrane Area

The basal cell membrane area (BCMA) of the retinal pigment epithelium (RPE) (expressed as a ratio of the length of RPE cell membrane (L_c) per length of Bruch's membrane (L_b) is shown in Table 11 and Figure 10.

A one-way analysis of variance performed using all nine groups, revealed no significant difference suggesting no change in basal cell membrane area occurred with age during the five month experimental period (F=2.01; df=8; p=0.07). An unpaired Student's t-test between the zero time control group (CO) and the mean of C and STZ24% was not statistically significant (t=0.16; df=16; p=0.88) (Table 13).

A two-way analysis of variance on these data (excluding the zero time control group) found neither diet (F=2.19; df=1; p=0.15) nor treatment group (F=0.27; df=3; p=0.85) had a significant effect on basal cell membrane area, however, the interaction effect was significant (F=3.76; df=3; $p<0.05$). Duncan's multiple range testing found the basal cell membrane area of the retinal pigment epithelium of the Diabetic 24% group (DM24%) was significantly less than the Diabetic 8% (DM8%), Diabetic + Insulin 8% (DM+I8%), and Control 24% (C24%) ($p<0.05$). This suggests a reduction in retinal epithelium basal cell membrane area in the untreated diabetic group on the 24% diet which was not found in the comparable treatment group on the 8% diet.

3.4.3 Bruch's Membrane Thickness

The mean thicknesses of Bruch's membrane (in nm) are reported in Table 12 and Figure 11.

A one-way analysis of variance on the data of all nine groups was statisti cally significant ($F=2.43$; $df=8$; $p<0.05$). Duncan's multiple range tests found the Control 8% group (C8%) was significantly greater than the zero time control group (CO) ($p<0.05$), Diabetic 8% (DM8%) ($p<0.05$), Diabetic + Insulin 8% (DM+I8%) ($p<0.05$), STZ injected non-diabetic 24% (STZ24%) ($p<0.01$), and Diabetic 24% (DM24%) ($p<0.01$). Table 13, shows the results of an unpaired Student's t-test between the zero time control group (CO) and the comparable control groups five months later (C24% and STZ24%). There was no significant difference in Bruch's membrane thickness between the means of these groups ($t=0.82$; $df=16$; $p=0.42$).

A two-way analysis of variance on the diet and treatment groups (excluding the zero time control group) found diet had no significant effect ($F=2.18$; $df=1$; $p=0.15$), treatment had a significant effect ($F=3.41$; $df=3$; $p<0.05$), and there was no significant interaction of diet and treatment($F=1.10$; $df=3$; $p=0.36$). With respect to treatment group, Bruch's membrane thickness for the controls on the 8% diet was significantly greater than Diabetic 8%, Diabetic + Insulin 8%, STZ injected non-diabetic 24% ($p<0.05$) and Diabetic 24% ($p<0.01$). On both diets, the untreated diabetics had thinner Bruch's membranes compared to the controls although, the difference was not statistically significant for the 24% diet.

3.4.4 Correlation Between Parameters

To determine if the three parameters measured were related, the data were subjected to correlation and covariance analysis. No significant correlations between capillary basement membrane thickness (BMT), basal cell membrane area (BCMA) of the retinal pigment epithelium (RPE), and Bruch's membrane thickness (BrMT) were found (capillary BMT x RPE BCMA $r = 0.03$; $p=0.41$: capillary BMT x BrMT $r=0.11$; $p=0.21$: RPE BCMA x BrMT $r=0.07$; $p=0.30$).

3.5 Summary of Results

The effects of age and dietary protein intake in normal and diabetic rats on the three parameters of retinal ultrastructure evaluated in this investigation may be summarized as follows.

Capillary basement membrane thickness was not affected by diabetes of 5 months duration, increased with age, and was not affected by dietary protein intake.

The basal cell membrane area of the retinal pigment epithelium did not change with age during this study, and was unaffected by diabetes or dietary protein intake alone, but was significantly reduced in untreated diabetic animals maintained on a 24% protein diet.

Bruch's membrane thickness was greater in control animals than diabetics, tended to increase with age, and was not affected by protein intake.

4. DISCUSSION

4.0 Survival Rates

The reduced survival rates of the diabetic animals compared to the control animals may be attributable to several causes. The untreated diabetic groups (DM8% and DM24%) were maintained hyperglycemic. The relative or absolute lack of insulin likely resulted in ketoacidosis and diabetic coma for many of these animals. The insulin-treated diabetic animals (DM+I8% and DM+I24%) were given daily insulin injections to normalize blood glucose levels. In an attempt to establish euglycemia in these groups, it is very possible some of these animals were subjected to hypoglycemia and death due to insulin shock.

In no case of animal mortality was any cause of death obvious. In some instances, a clear discharge from the nose was present but no necropsies were performed to establish the cause of death.

It is difficult to compare the survival rates found in this study with other studies as these values are seldom reported. Based on the results of this study, it is essential to begin experiments of this nature with a very large number of animals in each group due to the unforseen and seemingly unpreventable loss of many animals.

4.1 Body Weight

The final body weights of the animals in this study compare to the results

of others.

The streptozotocin-injected non-diabetic groups (STZ8% and STZ24%) did not differ significantly from the uninjected control group on the same diet (ie. STZ8% did not differ from C8%; STZ24% did not differ from C24%). Anderson (1985) has previously reported similar findings. This demonstrates that the animals that received streptozotocin but did not become diabetic, were not adversely affected by the drug as they gained weight at a rate comparable to the uninjected controls.

All groups on the 8% protein diet weighed less than the comparable group on the 24% protein diet (the differences were statistically significant for all but the insulin-treated diabetics). These results are consistent with other investigations. Klahr (1989) has reported that rats fed reduced protein diets show reduced growth rates. Neugarten, et al. (1983) found rats maintained for 60 days on a 4% protein diet weighed significantly less than rats receiving a 50% protein diet, and Wang, et al. (1976) demonstrated that pair-fed rats gained more weight on a high protein diet compared to an isocaloric low protein diet.

In this study and others (Anderson, 1985; Copeland, et al., 1987) untreated diabetic animals weighed less than controls, while insulin-treated diabetic animal s weighed more than controls. Low body weight and hyperglycemia are two features characteristic of untreated diabetic rats (Marliss, et al., 1982; Sima, et al., 1985; Chakrabarti, et al., 1987; Greene, et al., 1987). Insulin replacement has been shown to reduce blood glucose concentrations and increase body weights in

diabetic animals (Chakrabarti, et al., 1987; Greene, et al., 1987).

4.2 Blood Glucose

One of the cardinal signs of diabetes mellitus is hyperglycemia. The untreated diabetic animals on both diets had blood glucose values significantly higher than all other groups. Importantly, the blood glucose levels of the insulin-treated diabetic animals were not significantly different from the control groups indicating success in establishing euglycemia in these groups.

Also of importance is the fact that diet (8% versus 24% protein) did not influence blood glucose values as there are no significant differences between comparable treatment groups on the two diets.

4.3 Retinal Capillaries

4.3.0 The Structure of Retinal Capillaries

The structure of the retinal capillaries observed in this investigation is consistent with previous descriptions (Robison, et al., 1983; Sima, et al., 1985; Carlson, 1988; Vinores, et al., 1988; Robison, et al., 1989b; Das, et al., 1990; Robison, et al., 1990). The capillaries are surrounded by a well defined basement membrane that is often split to accommodate pericytes or pericyte processes. The functional role of pericytes is uncertain. Saunders, et al. (1990) have demonstrated that contact between pericytes and endothelial cells in a coculture

system, results in inhibition of endothelial cell proliferation. Sims (1986), Carlson (1988) and Robison, et al. (1989b) have shown that although the endothelial cells and pericytes are separated over most of their length by basement membrane, frequent interruptions in the basement membrane occur allowing for direct cell/cell contact. Such junctions between endothelial cells and pericytes were frequently observed in this study. The functional significance of endothelial/pericyte junctions is speculative at present. Numerous investigators have reported the loss of pericytes in experimental diabetes and galactosemia (Sharma, et al., 1985; Sima, et al., 1985; Robison, et al., 1989a; Kador, et al., 1990). It is reasonable to suggest that retinal capillary pericyte degeneration with diabetes mellitus may precede, and allow for, endothelial cell proliferation and resultant neovascularization (Carlson, 1988).

The basement membrane surrounding retinal capillaries appears homogeneous by routine transmission electron microscopy (as employed in this investigation). Many investigations have demonstrated that basement membranes are composed of type IV collagen, laminin, heparan sulfate proteoglycan, nidog en, and fibronectin (Timpl, et al., 1984; Das, et al., 1990). Fischer and Gartner (1983) described three regions of basement membrane associated with retinal capillaries; 1) the basement membrane between the endothelium and pericyte, 2) the basement membrane between the pericyte and perivascular glial cells, and 3) the basement membrane between the endothelial cell and perivascular glial cells. They suggest that since these three regions of basement membrane are of

different origin, so too is their composition likely to be different. An elegant high-resolution scanning electron microscopy study done by Carlson (1988) demonstrated structural differences in retinal capillary basement membrane composition. Carlson describes three leaflets or components of basement membrane; 1) the subendothelial basement membrane composed of 20-30 nm granules, 2) the pericyte basement membrane and associated pericyte matrix, and 3) Müller cell (or perivascular glial cell) basement membrane composed of 40-100 nm particles. The aforementioned study provides morphological evidence that the basement membrane complex associated with retinal capillaries is not compositionally as homogeneous as it appears even with relatively high power transmission electron microscopy.

4.3.1 The Effect of Diabetes Mellitus on Retinal Capillaries

Many previous studies have reported both qualitative and quantitative changes in retinal capillary structure and basement membrane thickness with diabetes (Fischer and Gartner, 1983; Sharma, et al., 1985; Sima, et al., 1985; Tilton, et al., 1986; Vinores, et al., 1988; Chakrabarti and Sima, 1989) and in the galactosemic model of diabetes (Robison, et al., 1986; Robison, et al., 1988; Robison, et al., 1989a; Das, et al., 1990; Robison, et al., 1990). In the present study, no obvious changes in capillary structure were noted, nor was capillary basement membrane thickness increased in response to experimentally induced diabetes of 5 months duration.

The qualitative changes in retinal capillary structure occurring with diabet es that have been noted by others include pericyte loss and a change in the ratio of pericytes to endothelial cells (Sharma, et al., 1985; Agaki and Kador, 1990; Kador, et al., 1990; Robison, et al. 1990), degenerative changes of the endothelial cells and pericytes (including swollen cytoplasm and pyknotic nuclei) (Sima, et al., 1985), numerous irregularities of the basement membrane such as localized nodular swellings, lamination, the presence of pericyte debris (Chakrabarti and Sima, 1989), and the formation of vacuoles and dense inclusions (Robison, et al., 1988). Careful scrutiny of the micrographs of all experimental groups did not reveal any of these various structural abnormalities therefore, no attempt was made to quantify such findings.

Quantitative increases in capillary basement membrane thickness with diabetes have been documented by many researchers (see the references listed in Table 14). In this study, no such increases were found. There are many possible explanations for this apparent discrepancy. Many factors make comparisons between studies difficult and are important considerations when interpreting data. Among these are the type and strain of animals used, the model of diabetes used, the duration of the experimental period, and with respect to retinal capillaries, the capillary bed examined (ie. superficial or deep). In the present study, diabetes was induced in male Sprague-Dawley rats by an injection of streptozotocin, the experimental period was 5 months, and capillary basement membrane thickness was determined for the deep capillary bed. Table 14

provides a summary of the investigations reporting significant increases in retinal capillary basement membrane thickness. As can be seen in this table, few investigations have found significant increases in capillary basement membrane thickness at time periods less than 6 months. The study of Tilton, et al. (1986) is most comparable to this investigation. Tilton, et al. (1986) found no significant difference in capillary basement membrane thickness after 6 months of streptozotocin-induced diabetes, but reported a significant increase after 9 months. It is therefore very likely that the 5 month experimental period in the present investigation was simply of insufficient duration to be able to quantify any changes in capillary basement membrane thickness. It is of interest to note that neither glomerular basement membrane thickness, nor skeletal muscle capillary basement membrane thickness in these same animals was found to increase significantly (Copeland, et al., 1989; Copeland, et al., 1990).

In light of the results of others, one may question why the experimental period in this investigation was not longer. The initial intent was to proceed for a longer time course however, due to the high mortality rates in the groups of diabetic animals, the experiment was terminated at 5 months so that no group would have a sample size less than 5.

4.3.2 The Effect of Age on Retinal Capillaries

As stated in the results section, there were no qualitative differences in capillary structure observed between the zero time control group and the other

experimental groups. The retinal capillaries of the animals sacrificed at the beginning of the experiment are structurally mature and did not change overtly over the five month experimental period.

Capillary basement membrane thickness is known to increase with age in a variety of tissues (Ashworth, et al., 1960; Kilo, et al., 1972; Xi, et al., 1982; Cuthbertson and Mandel, 1986; Nagata, et al., 1986). The results of this study are in agreement with previous findings. Nagata, et al. (1986) found a linear increase in basement membrane thickness in capillaries of the outer plexiform layer (or deep capillary bed) of the rat retina from ages 4 to 32 months. In the present study, basement membrane thickness increased significantly from 74.65 nm at 2 months of age, to an average of 89.38 nm at 7 months. These results are very similar to the data of Nagata, et al. in which basement membrane thickness increased from approximately 70 nm at 2 months to 95 nm at 7 months of age. The physiological significance of retinal capillary basement membrane thickening is presently not understood. It has been proposed that both age-related and disease-related increases in retinal capillary basement membrane thickness may result in deterioration of capillary structure and function (Nagata, et al., 1986).

4.3.3 The Effect of Dietary Protein on Retinal Capillaries

No gross morphological differences were apparent between capillaries taken from animals maintained on an 8% or 24% protein diet.

Quantitatively, there was no significant difference in capillary basement

membrane thickness between the two diet groups. Analysis of glomerular base ment membrane and skeletal muscle capillary basement membrane (Copeland, et al., 1989; Copeland, et al., 1990) revealed no change in thickness related to dietary protein intake. However, in the study of Copeland, et al. (1989) diabetic animal s on the low protein diet did not have elevated urinary albumin excretion or creatinine clearance values as did diabetic animals on 24% protein. Therefore, although structurally no differences in glomerular basement membrane thickness were detectable between the 8% and 24% diabetic animals, functionally, the reduced protein diet appears to have been beneficial to kidney function. The eye is also subject to hyperperfusion and hyperfiltration with diabetes (Pugliese, et al., 1989). It is possible that the 8% protein diet may have prevented vascular changes in the eye, however, these parameters were not measured in the present study.

Based on the results of this investigation, no differences in capillary structure or capillary basement membrane thickness were apparent between the two diets. The functional parameters (such as vascular flow and albumin permeation) that may be preserved by the reduced protein diet warrant further investigation.

4.4 Retinal Pigment Epithelium

4.4.0 The Structure of the Retinal Pigment Epithelium

The morphology of the retinal pigment epithelium of the Sprague-Dawley rat is essentially similar to that described for most vertebrate species with the notable absence of pigment (Nguyen-Legros, 1978; Kuwabara, 1979; Braek evelt, 1986, 1988).

As is most often the case, the retinal pigment epithelium consists of a single layer of roughly cuboidal shaped cells (Nguyen-Legros, 1978). The presence of numerous basal infoldings is a feature common to most retinal epithelia and thought to be indicative of the transport role of this cell border in the movement of materials between the choriocapillaris and the photoreceptors (Steinberg and Miller, 1973). Slender apical processes which interdigitate with photoreceptor outer segments are abundant. Many important functions have been ascribed to the apical processes including the phagocytosis of outer segment material (Young, 1978) and the stabilization and proper alignment of photoreceptor outer segments (Enoch, 1979). The lateral cell borders are relatively smooth compared to the basal and apical surfaces. The retinal pigment epithelial cells are joined laterally by apically positioned tight junctions which form an important component of the blood-retinal barrier (Zinn and Benjamin-Henkind, 1979).

Internally, the retinal pigment epithelial cells have a large vesicular nucleu s which is characteristic of metabolically active cells (Nguyen-Legros, 1978; Braekevelt, 1986, 1988). Smooth endoplasmic reticulum is abundant reflecting the involvement of these cells in the synthesis and storage of lipid photopigment precursors (Zinn and Benjamin-Henkind, 1979). Although rough endoplasmic

reticulum is scarce, polysomes are numerous indicating that protein synthesis for internal use is an important, ongoing process in these cells. Given that one of the functions of this cell layer is the phagocytosis and degradation of shed photoreceptor outer segments (Young and Bok, 1970, 1979), it is not surprising that numerous lysosomes and phagosomes are present in the cytoplasm.

In summary, the structure of the retinal pigment epithelium in the Sprague-Dawley rat is consistent with previous descriptions of this cell layer in other species.

4.4.1 The Effect of Diabetes on the Retinal Pigment Epithelium

Many reports of the structural and functional aspects of the retinal pigme nt epithelium in diabetes appear in the literature. Loss of integrity of the blood-retinal barrier attributable to an increase in the permeability of the retinal pigment epithelium is thought to be one of the first changes to develop in diabetic retino pathy (Cunha-Vaz, et al., 1975; Waltman, et al., 1978). Tso, et al. (1980), Blair, et al. (1984), and Caldwell, et al. (1985) have demonstrated an increase in the permeability of retinal pigment epithelial cells from diabetic animals to horseradish peroxidase and lanthanum nitrate. Morphological studies describe numerous abnormalities of the retinal pigment epithelium in diabetic animals including derangements, elaborations, and focal losses of basal infoldings, dilated smooth endoplasmic reticulum, organelle degeneration, cell vacuolization, the presence of dense inclusions, shrunken nuclear membranes, necrosis, and irregularities in the

basement membrane (Grimes and Laties, 1980; Tso, et al., 1980; Blair, et al., 1984; Grimes, et al., 1984; Vinores, et al., 1988; Vinores and Campochiaro, 1989; Chakrabarti, et al., 1990).

Disregarding changes noted with respect to basal infoldings, Grimes and Laties (1980), Grimes, et al. (1984), Caldwell and Slapnick (1989) and Chakrabarti, et al. (1990) report that retinal pigment epithelial cells from diabetic and control rats appear structurally similar and found no evidence of the lesions described above. In the present investigation, no overt morphological changes in retinal pigment epithelial cells were found. Clearly, the qualitative descriptions of the effects of diabetes mellitus on the structure of the retinal pigment epithelium are disparate and inconclusive.

With respect to the effects of diabetes mellitus on the basal infoldings of the retinal pigment epithelium, reports that have attempted to quantify this cell surface range from significant increases, to no significant difference, to apparent decreases. Grimes and Laties (1980) and Grimes, et al. (1984) found increases in retinal pigment epithelial basal cell membrane length of 27-33% after 4 weeks to 6 months of diabetes mellitus. Using the same morphometric method, Caldw ell and Slapnick (1989) found no significant difference in retinal pigment epithelium basal infoldings on the spontaneously diabetic BB rat or streptozotocin-diabetic rat. Chakrabarti, et al. (1990) used a different method of quantification and reported a slight decrease (6%) in retinal pigment epithelial plasmalemmal infoldings in diabetic BB rats compared to controls, but the difference was not

statistically significant.

In the present study, there was a significant decrease in retinal pigment epithelial cell basal membrane area in the untreated diabetic animals on 24% protein diet compared to the 24% controls. The contradictory findings between reports using the same morphometric techniques (Grimes and Laties, 1980; Grimes, et al., 1984; Caldwell and Slapnick, 1989; and the present study) is puzzling. The statistically significant difference between the untreated 24% diabetics and the control 24% animals found in the present study, disappears if one compares the mean of the 24% controls and streptozotocin injected non-diabetics 24% with the 24% diabetics. It is also possible that the significance found between the 24% diabetic and 24% controls is the one in twenty times that a type 1 error occurs using a $p=0.05$.

Assuming that the significant decrease in retinal pigment epithelial cell basal membrane area of the untreated 24% diabetics is a correct finding, the explanation most likely lies in the altered metabolism of the retinal pigment epithelium in the diabetic state.

Numerous functional changes in the retinal pigment epithelium have been described with diabetes mellitus. MacGregor, et al. (1986) found increased sorbitol and decreased myoinositol levels in alloxan-induced diabetic rabbit retinal pigment epithelial cells and suggests that altered inositol lipid metabolism may be involved in the permeability changes of the retinal pigment epithelium in diabetes. The results of Caldwell, et al. (1987) suggest that alterations ofintrinsic membrane

proteins of the retinal pigment epithelial cells accompany the increase in membrane permeability in streptozotocin diabetic rats. Caldwell and Slapnick (1989) found increased cytochrome oxidase activity in the retinal pigment epithelium mitochondria of both spontaneously diabetic BB rats and streptozotocin-diabetic rats. Augmented polyol pathway activity has also been demonstrated in the retinal pigment epithelium of diabetic BB rats by increased aldose reductase immunoreactivity (Chakrabarti, et al., 1990). These findings suggest that numerous metabolic alterations occur in the retinal pigment epithelium in diabetes mellitus and may contribute not only to modifications of the basal cell membrane area, but to the development of other retinal complications.

4.4.2 The Effect of Age on the Retinal Pigment Epithelium

Numerous investigations have documented age-related changes in the retinal pigment epithelium. Friedman and Tso (1968) did one of the first studies of age-related changes in the retinal pigment epithelium examining 157 human eyes ranging in age from 4 months of fetal life to 96 years. The most notable change in retinal pigment epithelial cell structure with age described by these investigators, was a progressively increasing degree of pleomorphism in the size and shape of the cells, nuclei and pigment granules. Mishima, et al. (1978) also reported an increase in pleomorphism of human retinal pigment epithelial cell size and shape with age.

As summarized by Kornzweig (1979), ultrastructural changes occurring in

the retinal pigment epithelial cell cytoplasm with age include the formation of lipofuscin granules, increasing numbers of lysosomes and residual bodies, and degenerative changes within the mitochondria. Katz and Robison (1984) exami ned age-related morphological changes in ACI rats. They described numerous structural changes found in animals greater than 11 months of age including an increase in lipofuscin granules, changes in the morphology of the apical microvilli and a decrease in phagosome content.

In the present study, the zero time control group consisted of rats appro ximately 2 months of age. The experimental period was 5 months, therefore, the control animals examined were approximately 7 months old. No morphological changes of the retinal pigment epithelium were found between the zero time control group and the older animals. Based on the observations of others, this finding was to be expected. Senescent changes in rat retinal pigment epithelial structure are most apparent after 11 months of age (Katz and Robison, 1984).

Descriptive changes with age in the basal infoldings of the retinal pigment epithelium have been reported in several investigations. Mishima, et al. (1978) described the basal infoldings of the human retinal pigment epithelium as extended and enlarged above age 40 years. Similarly, Mishima and Hasebe (1978) found that the basal infoldings of the aged mouse (over 12 months old) were extended and enlarged, and often contained dense, amorphous material. Kornzweig (1979) described age related changes of the retinal pigment epithelium basal infoldings including an alteration in character and the deposition of a fibrillar material in the

basal infoldings. Mishima and Knodo (1981) carried out a morphological and morphometric analysis of retinal pigment epithelial basal infoldings in mice 1 to 24 months of age. They found no difference in retinal pigment epithelial basal infoldings in mice 3 to 12 months of age, followed by a progressive expansion of the apex of the infolding in mice 12 to 24 months old. They too reported the presence of an amorphous dense material in the basal infoldings of aged mice.

No change in retinal pigment epithelial basal infoldings was found betwe en the zero time control group (2 months of age) and the other experimental groups (7 months of age) in this investigation. This is consistent with the results of others described above, and also with the report of Chakrabarti, et al. (1990) in which no difference in retinal pigment epithelial basal infoldings was found in non-diabetes prone BB rats between ages 2 and 8 months. It would appear that senescent changes in retinal pigment epithelial cell basal infoldings do not occur in rats until some age greater than 8 months.

4.4.3 The Effect of Dietary Protein on the Retinal Pigment Epithelium

No morphological differences in the retinal pigment epithelium were noted in this study between the groups maintained on 8% versus 24% protein diet. As described with respect to retinal capillaries, one may have found differences in functional parameters of the retinal pigment epithelium (such as permeability) between the two protein diets, however, these measurements were not included in this study.

Dietary protein intake did not influence retinal pigment epithelial basal cell membrane area in the present investigation. The 24% protein diet was standard rat chow. The reduced (8%) protein diet did not result in any change in retinal pigment epithelial basal cell membrane area. The reduction of dietary protein was likely only to influence transporting membranes when in combination with the pressure and filtration changes associated with diabetes mellitus.

4.5 Bruch's Membrane

4.5.0 The Structure of Bruch's Membrane

Bruch's membrane (or complexus basalis) of the Sprague-Dawley rat is comparable to that described in other mammals (Kornzweig, 1979; Braekevelt, 1986, 1988). Bruch's membrane is a pentalaminate structure. From internal to external, the five layers include; the basement membrane of the retinal pigment epithelium, an inner collagenous layer, a discontinuous elastic layer, an outer collagenous layer, and the basement membrane of the endothelium of the choriocapillaris. In this species, the basement membrane of the retinal pigment epithelium appears more dense than that of the choriocapillaris endothelium. The elastic layer is well defined and most often divides the collagenous zone into approximately equal inner and outer layers. These results are consistent with the description of Bruch's membrane structure provided by Greiner and Weidman (1991).

4.5.1 The Effect of Diabetes Mellitus on Bruch's Membrane

Caldwell, et al. (1986) studied Bruch's membrane in two models of diabet es; the spontaneously diabetic BB rat and the streptozotocin-induced diabetic rat. In both models, the retinal pigment epithelium and choriocapillaris endothelial basement membranes increased in thickness in animals diabetic for 1-12 months. However, the total thickness of Bruch's membrane increased significantly only in the BB rats. In the streptozotocin-induced diabetic Long Evans rats, no statistically significant increase in Bruch's membrane thickness was found.

In the present investigation, diabetes was induced in Sprague-Dawley rats by streptozotocin and all diabetic animals were maintained for 5 months. Bruch's membrane thickness in control groups varied from 520.30 nm to 654.21 nm, and in diabetic animals, Bruch's membrane thickness ranged between 513.61 nm and 563.98 nm. In this study, the untreated diabetic animals had thinner Bruch's membranes than the control group on the same diet (DM8%=542.28 nm versus C8%=654.21 nm, and DM24%=513.61 nm versus C24%=593.00 nm) which is in contrast to the results of Caldwell, et al. (1986) in which Bruch's membrane thickness increased with diabetes. It is difficult to compare the data of Caldwell, et al. (1986) with the results of this study for several reasons. Most obviously, there appears to be a strain-related difference in Bruch's membrane thickness based on the data of Caldwell, et al (1986). The streptozotocin-diabetic Sprague-Dawley rats used in this study, may differ from both the BB and Long Evans rats. Also, in the study of Caldwell, et al. (1986), one mean is reported per experimental

group. The only information provided about the duration of diabetes is that the diabetic animals were studied from 1-12 months after the onset of diabetes with no indication as to how many animals were diabetic for exactly how many months. Based on this information, it is possible that in the group of 12 diabetic animals, 11 were examined after 12 months of diabetes and one animals was included that had been diabetic for 1 month. Although this is unlikely, the absence of information on both strains used in their investigation, makes comparisons to the present study difficult.

In addition to quantifying Bruch's membrane thickness, Caldwell, et al. (1986) examined the number of anionic binding sites in Bruch's membrane with the use of cationic tracers. They found a significant decrease in the number of anionic binding sites in Bruch's membrane in diabetes and suggest that this structural change may be accompanied by an increase in permeability similar to that found in the kidney glomeruli.

Vinores, et al. (1988) measured retinal pigment epithelial cell basement membrane thickness in BB diabetic rats with the duration of diabetes ranging from 1-12½ months. They found no significant difference to 5 months of diabetes and a significant increase in retinal pigment epithelial cell basement membrane thickness in diabetic animals with diabetes for 6 months or greater. Again comparisons of these data to the results of the present study are complicated by the use of a different strain of animal and different model of diabetes. However, the results are consistent in that no significant difference was detected in either

case at or before 5 months of diabetes.

Vinores and Campochiaro (1989) measured retinal pigment epithelial cell basement membrane thickness in galactosemic rats and found a significant increase in basement membrane thickness in rats maintained on 50% galactose for 12 months. Due to the difference in the model employed and the much longer duration of the experimental period in the investigation of Vinores and Campochiaro (1989), it is difficult to draw parallels between their data and the results of this study.

Due to the irregular nature of the choriocapillaris, Bruch's membrane thickness is quite variable, even within the same micrograph. This variability is reflected in the large standard error of the mean reported in these data. Having such a large degree of variability within groups makes detecting differences between groups difficult.

4.5.2 The Effect of Age on Bruch's Membrane

The first indications of aging in Bruch's membrane in human eyes, begins as early as 20 years and becomes extensive in elderly persons (Kornzweig, 1977, 1979). The most notable change in the structure of Bruch's membrane with age is the appearance of drusen. Drusen consists of amorphous granular material or material that is fibrillar in nature (Kornzweig, 1979). Drusen are located external to the retinal pigment epithelium basement membrane within the inner collagenous layer resulting in elevation of the retinal pigment epithelium (Hogan and Alvarado,

1967). The etiology of drusen formation is uncertain. It has been proposed that since the chemical nature of drusen resembles the contents of phagasomes found in retinal pigment epithelial cells, that drusen result from the incomplete lysosomal degradation of phagocytozed material which is then excreted by the retinal pigment epithelial cell (Kornzweig, 1979). An alternative theory of drusen formation described by Freidman, et al. (1963) proposes that drusen are extruded from the choriocapillaris and pass through Bruch's membrane coming to lie against the retinal pigment epithelium basement membrane.

In the present study, the animals were sacrificed prior to the time of the development of senescent changes in Bruch's membrane. No morphological differences were observed between the zero time control group and the 5 month older groups with respect to Bruch's membrane.

Senescent increases in the thickness of Bruch's membrane have been documented (Feeney-Burns and Ellersieck, 1985; Pauleikhoff, et al., 1990) however, early changes in Bruch's membrane thickness have not been extensively investigated. Vinores, et al. (1988) measured the thickness of the retinal pigment epithelium basement membrane (one component of Bruch's membrane) in BB rats and found no significant difference between rats ages 4 to 14 months. In the present study, the thickness of Bruch's membrane tended to increase with age although no significant difference was found between the zero time control group and the comparable control groups five months later (C24% and STZ24%). No drusen were observed in this investigation. It is possible that the appearance of

drusen and a further increase in Bruch's membrane thickness may occur in the Sprague-Dawley rat but at a more advanced age.

4.5.3 The Effect of Dietary Protein Intake on Bruch's Membrane

In this study, dietary protein intake did not affect the structure or the thickness of Bruch's membrane. As described with respect to retinal capillaries and the retinal pigment epithelium, the reduced protein diet may be beneficial to the maintenance of the permeability properties of filtration barriers in diabetes mellitus. The effect of a low protein diet on the permeability of Bruch's membrane in diabetes awaits study.

4.6 Summary of Collaborative Findings

Age was not a variable included by all investigators in this project and will therefore not be included in the following discussion.

Basement membrane thickness was examined in retinal capillaries, glome rular capillaries (Copeland, et al., 1989) and skeletal muscle capillaries (Copeland, et al., 1990). In all cases, neither diet nor treatment condition affected capillary basement membrane thickness and no significant differences between groups were reported at any location.

The untreated diabetic group on the 24% protein diet (DM24%) was significantly different from other experimental groups with respect to the following: urinary albumin excretion was increased, creatinine clearance was increased

(Copeland, et al., 1989), the level of alveolar bone surrounding the teeth was decreased (Johnson and Thliveris, 1989), the basal cell membrane area of the retinal pigment epithelium was decreased, and the thickness of Bruch's membrane was decreased. These differences were not found for the untreated diabetic group on the restricted protein diet (DM8%). These results suggest that a reduced protein diet coincident to poorly controlled diabetes mellitus is of benefit in many organs. Further investigations of this relationship are warranted.

5. CONCLUSIONS

The objectives of this investigation were to examine the effects of age and dietary protein in normal and diabetic rats, on aspects of retinal ultrastructure including capillary structure and basement membrane thickness, retinal pigment epithelium morphology and basal cell membrane area, and Bruch's membrane structure and thickness. Based on the results of this study, the following conclu sions may be made.

The structure of the retinal capillaries observed in this study is consistent with previous descriptions of these vessels. In contrast to the results of others, no increase in retinal capillary basement membrane thickness of diabetic animals was found in this study most likely due to the duration of the experimental period. Basement membranes are known to increase with age and during the 5 month duration of this experiment, a significant increase in retinal capillary basement membrane thickness was found. Dietary protein did not effect capillary structure or basement membrane thickness however, analysis of capillary permeability may have revealed differences between the diabetic groups on 8% versus 24% protein diets.

The morphology of the retinal pigment epithelium of the Sprague-Dawley rat is essentially similar to that described for most vertebrate species. No abnormalities of the retinal pigment epithelium were found although the basal cell membrane area was significantly reduced in diabetic animals on 24% protein. Age

did not alter retinal pigment epithelium morphology in this investigation.

Bruch's membrane is of the same pentalaminate structure as described previously. The structure and thickness of Bruch's membrane were not affecte d by diet. Treatment condition had a significant effect on Bruch's membrane thickness with thickness being significantly decreased in diabetics. The relationship between a decrease in Bruch's membrane thickness and the basal cell membrane area of the retinal pigment epithelium in the untreated diabetic group on 24% protein requires further study. Consistent with previous reports, the thickness of Bruch's membrane tended to increase with age in the study.

The collaborative results of this experiment suggest that kidney function, alveolar bone height and retinal morphology may be protected by a restricted protein diet in poorly controlled diabetics. These are exciting data that may be clinically of great benefit in retarding the chronic complications due to diabetes mellitus.

6. TABLES

Table 1: Comparison of IDDM and NIDDM.

	IDDM	NIDDM
Age at onset	childhood and young adulthood	middle and old age
Race	predominantly Caucasians	all races
Pancreatic insulin content	0	50% of normal
Insulin dependence	virtually always	rare
Anti-islet antibodies	85%	<5%
Primary insulin resitance	minimal	marked
HLA linkage	2½x expected frequency	same frequency as normal population
Concordance rate of identical twins	25-50%	~100%
Mechanism	autoimmune destruction	unknown
Possible initiating events	viral infection, toxins	overnutrition, obesity
First functional abnormality	decrease in glucose-stimulated insulin secretion and hyperglycemia	decrease in glucose-stimulated insulin secretion and hyperglycemia

(modified from Cudworth and Gorsuch, 1983; Lebovitz, 1984; and Unger, 1991)

Table 2: Percent survival for each treatment group.

Treatment Group	# of animals initial	final	% survival
C8%	8	7	88
STZ8%	12	10	83
DM8%	15	5	33
DM+I8%	15	9	60
C24%	6	6	100
STZ24%	9	9	100
DM24%	16	8	50
DM+I24%	17	5	29

% survival for control groups (C8%, STZ8%, C24%, and STZ24%) is significantly > diabetic groups (DM8%, DM+I8%, DM24%, and DM+I24%) determined by Chi-square analysis ($p<0.001$).

C=control, STZ=streptozotocin-injected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 3: Body weight (grams) at the end of the five month experimental period.

Intradiet Comparisons	Treatment Group	Final Body Weight (grams)	Interdiet Comparisons		
.01	C8%	519.3$\pm$35.7	*		
.01	STZ8%	525.4$\pm$20.7	*		
.01	DM8%	473.0$\pm$52.2	*		
*	DM+I8%	663.9$\pm$27.8			
	C24%	656.5$\pm$16.6	.01	.01	.01
.05	STZ24%	622.1$\pm$16.6	.01	.05	.05
.01	DM24%	598.1$\pm$20.4	.01		
*	DM+I24%	714.4$\pm$35.3	.01	.01	.01

ANOVA diet $F=27.05$ ($df=1$; $p<0.001$)
treatment $F=11.08$ ($df=3$; $p<0.001$)
interaction $F=0.96$ ($df=3$; $p=0.42$)

ANOVA $F=8.16$ ($df=7$; $p<0.001$)

Values reported are means $\pm$ standard error of the mean.
Intergroup comparisons determined by Duncan's multiple range tests.

C=control, STZ=streptozotocin-injected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 4: Final blood glucose levels (mmol/l).

Intradiet Comparisons	Treatment Groups	Final Blood Glucose	Interdiet Comparisons
.01	C8%	9.9 ± 0.2	.01
.01	STZ8%	12.6 ± 0.8	.01
*	DM8%	22.0 ± 0.34	*
.01	DM+I8%	8.2 ± 2.1	.01
.01	C24%	7.1 ± 0.8	.01
.01	STZ24%	10.5 ± 0.7	.01
*	DM24%	20.7 ± 1.0	*
.01	DM+I24%	11.7 ± 4.3	.01

ANOVA diet F=0.39 (df=1; p=0.53)
treatment F=27.68 (df=3; p<0.001)
interaction F=1.68 (df=3; p=0.18)

ANOVA F=11.99 (df=7; p<0.001)

Values reported are means ± standard error of the mean.
Intergroup comparisons determined by Duncan's multiple range tests.

C=control, STZ=streptozotocin-injuected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 5: Determination of basement membrane thickness (nm) for ten prints of the same capillary.

Print #	basement membrane thickness
1	88.8
2	96.3
3	90.4
4	90.2
5	90.3
6	93.2
7	92.8
8	91.7
9	87.0
10	98.9
$\bar{x}$	92.0
SD	3.54
CV	3.8%

Table 6: Reproducibility of capillary basement membrane thickness (nm) on five capillaries (A to E) measured on five days.

Trial	A	B	C	D	E
1	99.4	85.5	93.0	81.6	87.7
2	91.9	86.9	95.7	81.1	77.5
3	97.6	88.8	92.7	78.2	81.0
4	94.3	92.1	91.6	76.4	79.7
5	94.6	94.3	99.5	78.0	81.7
$\bar{x}$	95.6	89.5	94.5	79.1	81.5
SD	2.95	3.64	3.18	2.21	3.81
CV	3.1%	4.1%	3.4%	2.8%	4.7%

Table 7: Reproducibility of the basal cell membrane area (L_c/L_b) of five retinal epithelial cells (A to E) measured on five days.

Trial	A	B	C	D	E
1	8.4	5.6	6.3	5.5	6.8
2	7.9	5.4	6.9	5.3	7.1
3	7.9	5.3	6.7	5.2	7.2
4	7.5	5.9	6.9	5.2	6.6
5	8.3	5.8	6.7	5.1	6.6
$\bar{x}$	8.0	5.6	6.7	5.3	6.9
SD	0.36	0.26	0.25	0.15	0.28
CV	4.5%	4.6%	3.7%	2.8%	4.1%

Table 8: Reproducibility of Bruch's membrane thickness (BrMT) (nm) on five micrographs measured on five days.

Trial	A	B	C	D	E
1	535.7	538.1	562.4	513.1	579.6
2	533.2	538.0	566.9	520.6	586.4
3	525.2	542.7	560.3	517.9	585.9
4	529.2	535.7	564.7	523.8	572.8
5	531.7	525.7	568.1	519.6	574.6
$\bar{x}$	531.0	536.0	564.5	519.0	579.9
SD	4.0	6.3	3.2	3.9	6.3
CV	0.8%	0.01%	0.01%	0.01%	0.01%

Table 9: Capillary basement membrane thickness (BMT) (nm), retinal pigment epithelial (RPE) basal cell membrane area (BCMA) (Lc\Lb), and Bruch's membrane thickness (BrMT) (nm) determined by two investigators (I and II).

		capillary BMT	RPE BCMA	BrMT
Investigator I	mean	93.6	6.32	531.0
	SD	2.8	0.25	4.0
	CV	3.0%	4.0%	0.8%
Investigator II	mean	85.3	6.30	534.2
	SD	1.5	0.16	5.2
	CV	1.7%	2.6%	1.0%
Student's t-test comparing investigators		p<.001	p>.05	p>.05

Table 10: Capillary basement membrane thickness (BMT) (nm) in experimental groups.

Treatment Group	Capillary BMT (nm)	Intergroup Comparisons
C8%	92.26±2.16	.05
STZ8%	87.11±1.86	.05
DM8%	89.08±2.43	.05
DM+I8%	91.55±2.26	.05
C24%	88.53±4.37	.05
STZ24%	88.26±3.41	.05
DM24%	90.00±1.22	.05
DM+I24%	84.83±6.32	.05
ANOVA (2 way)	diet F=0.95 (df=1; p=0.33) treatment F=0.33 (df=3; p=0.80) interaction F=0.79 (df=3; p=0.51)	
ANOVA (1 way)	F=0.63 (df=7; p=0.72)	
CO	74.65±1.51	*
ANOVA (1 way)	F=3.44 (df=8; p<0.01)	

Values reported are means ± standard error of the mean.
Intergroup comparisons determined by Duncan's multiple range tests.

CO=zero time control, C=control, STZ=streptozotocin-injected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 11: Retinal pigment epithelium (RPE) basal cell membrane area (BCMA) expressed as a ratio of RPE basal cell membrane length per area of Bruch's membrane (L_c/L_b) in experimental groups.

Treatment Group	RPE BCMA (L_c/L_b)	Intergroup Comparisons
C8%	9.66±0.63	
STZ8%	11.16±0.58	
DM8%	12.09±0.85	.05
DM+I8%	11.84±0.65	.05
C24%	11.64±0.91	.05
STZ24%	10.29±0.88	
DM24%	9.15±0.55	*
DM+I24%	10.56±0.97	

ANOVA (2 way) diet $F=2.19$ ($df=1$; $p=0.15$)
treatment $F=0.27$ ($df=3$; $p=0.85$)
interaction $F=3.76$ ($df=3$; $p<0.05$)

CO	10.81±0.66	

ANOVA (1 way) $F=2.01$ ($df=8$; $p=0.07$)

Values reported are means ± standard error of the mean.
Intergroup comparisons determined by Duncan's multiple range tests.

CO=zero time control, C=control, STZ=stretptozotocin-injected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 12: Bruch's membrane thickness (BrMT) (nm) in experimental groups.

Treatment Group	BrMT (nm)	Intergroup Comparisons
C8%	654.21±31.43	*
STZ8%	594.23±33.85	
DM8%	542.28±29.00	.05
DM+I8%	532.52±27.23	.05
C24%	593.00±34.27	
STZ24%	520.30±16.32	.05
DM24%	513.61±24.82	.01
DM+I24%	563.98±50.36	
ANOVA (2 way)	diet F=2.18 (df=1; p=0.15) treatment F=3.41 (df=3; p<0.05) interaction F=1.10 (df=3; p=0.36)	
CO	528.68±22.58	.05

AVOVA (1 way) F=2.43 (df=8; p<0.05)

Values reported are means ± standard error of the mean.
Intergroup comparisons determined by Duncan's multiple range tests.

CO=zero time control, C=control, STZ=streptozotocin-injected non-diabetic, DM=untreated diabetic, DM+I=insulin treated diabetic on 8% and 24% protein diets.

Table 13: A comparison of capillary basement membrane thickness (BMT) (nm), retinal pigment epithelium (RPE) basal cell membrane area (BCMA) ($L_{c/L}b$), and Bruch's membrane thickness (BrMT) (nm) between the zero time control group (CO) and comparable groups 5 months older (C and STZ24%).

Treatment Group	Capillary BMT (nm)	RPE BCMA (L_c/L_b)	BrMT (nm)
CO	74.65±1.51	10.81±0.66	528.68±22.5
C + STZ 24%	88.41±2.71	10.97±0.64	556.65±21.1
unpaired t statistic=	3.55	0.16	0.82
p=	<0.05	0.88	0.42

Values reported are means ± standard error of the mean.

Table 14: Comparison of the type and sex of the animals used, the model of diabetes, the duration of the experimental period, and the retinal capillaries examined, in previous studies reporting significant increases in capillary basement membrane thickness.

Reference	Type of animals	Model of Diabetes	Duration of experiment	Retinal Capillaries
Das, et al., 1990	Wistar-Kyoto rats (F)	galactosemia	9 months	superficial and deep
Frank, et al., 1983	Wistar-Kyoto rats (?)	galactosemia	15-21 months	deep
Robison, et al., 1983	Sprague-Dawley rats (M)	galactosemia	6-10 months	deep
Robison, et al., 1986	Sprague-Dawley rats (M)	galactosemia	6½ months	deep
Robison, et al., 1988	Sprague-Dawley rats (M)	galactosemia	20 months	deep
Robison, et al., 1990	Sprague-Dawley rats (F)	galactosemia	28 months	deep
Vinores and Campochiaro, 1989	Sprague-Dawley rats (?)	galactosemia	12 months	deep
Chakrabarti and Sima, 1989	BB Wistar rats (M)	spontaneous	6 months	superficial and deep
Sima, et al., 1985	BB Wistar rats (M)	spontaneous	4-11 months	superficial and deep
Vinores, et al., 1988	BB Wistar rats (?)	spontaneous	1-12½ months	deep
Fischer and Gartner, 1983	Wistar rats (?)	strep-induced	12 months	superficial and deep
Sima, et al., 1988	Lewis rats (M)	strep-induced	14 months	superficial and deep
Tilton, et al., 1986	Sprague-Dawley rats (M)	strep-induced	9 months	deep

7. FIGURES

Figure 1: Diagram of the retina showing the location of the structures of interest. a=superficial capillary bed, b=deep capillary bed, c=photoreceptors, d=retinal pigment epithelium, e=Bruch's membrane, f=choriocapillaris. (adapted from Leeson and Leeson, 1981)

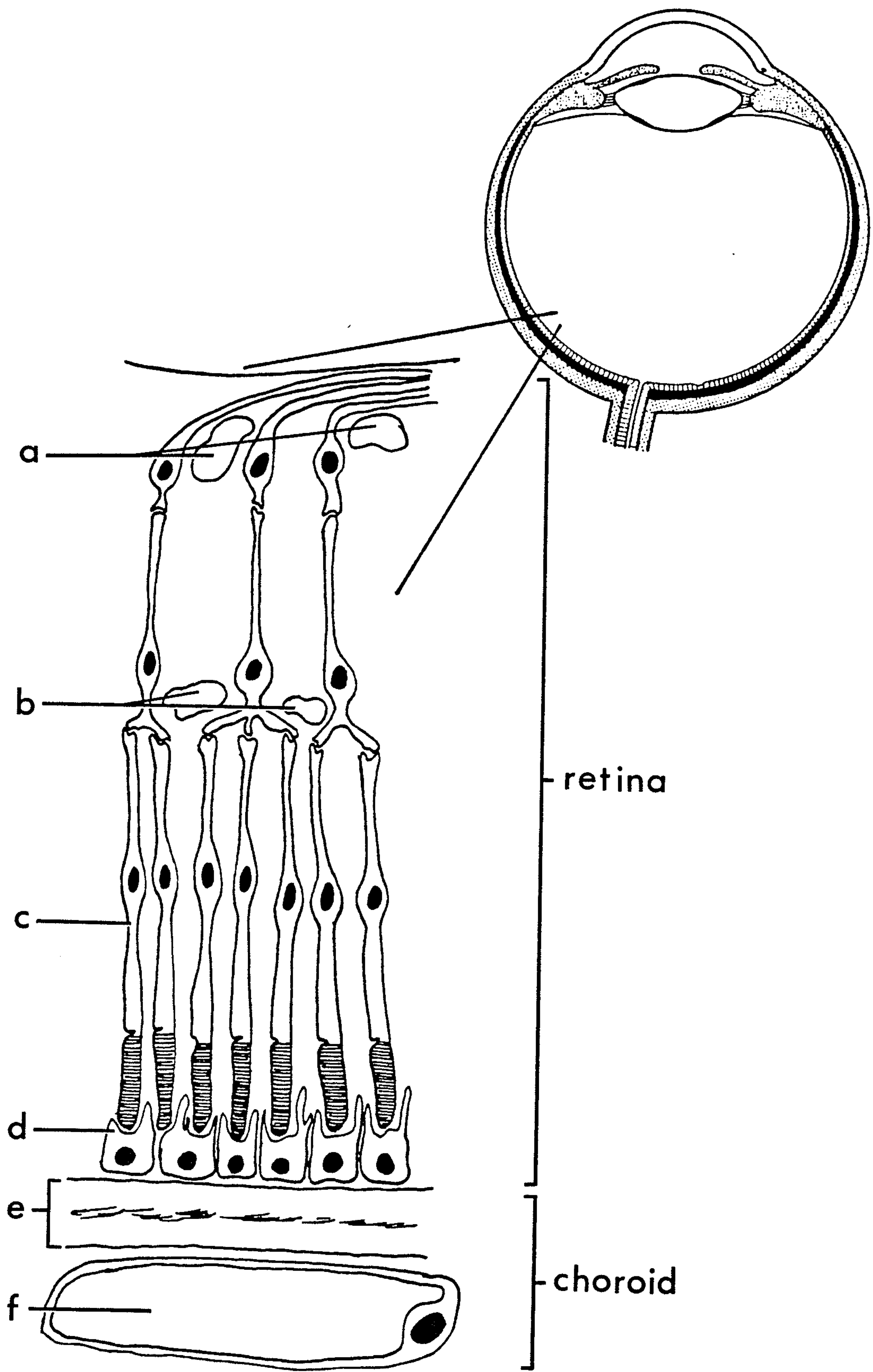

a
b
c
d
e
f
retina
choroid

Figure 2: Experimental protocol. Six animals were sacrificed at the beginning of the experiment to serve as zero time controls (CO). Experimental animals were allocated into two diet groups. One group received an 8% protein diet and the other, a diet containing 24% protein. Within each diet group, 4 treatment groups were established; uninjected control (C), streptozotocin-injected non-diabetic (STZ), untreated diabetic (DM), and insulin treated diabetic (DM+I).

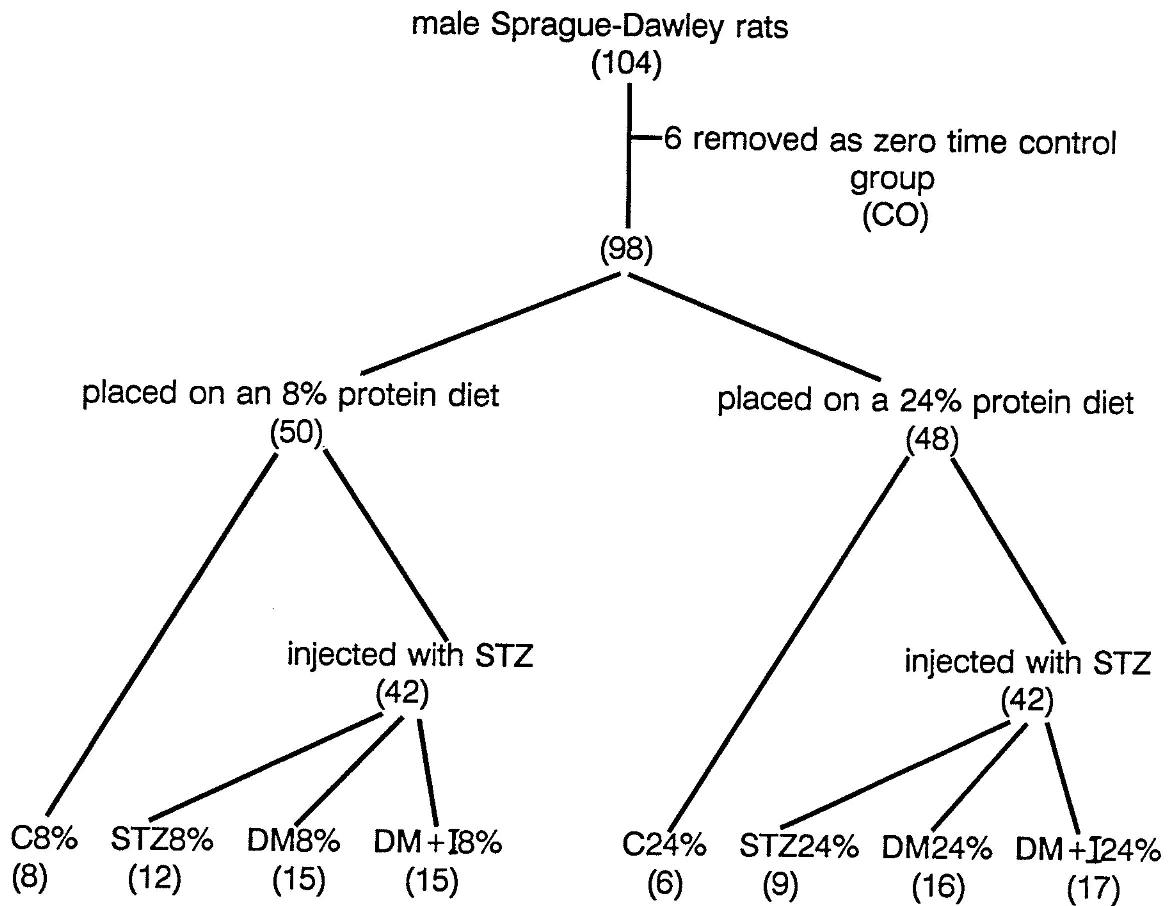
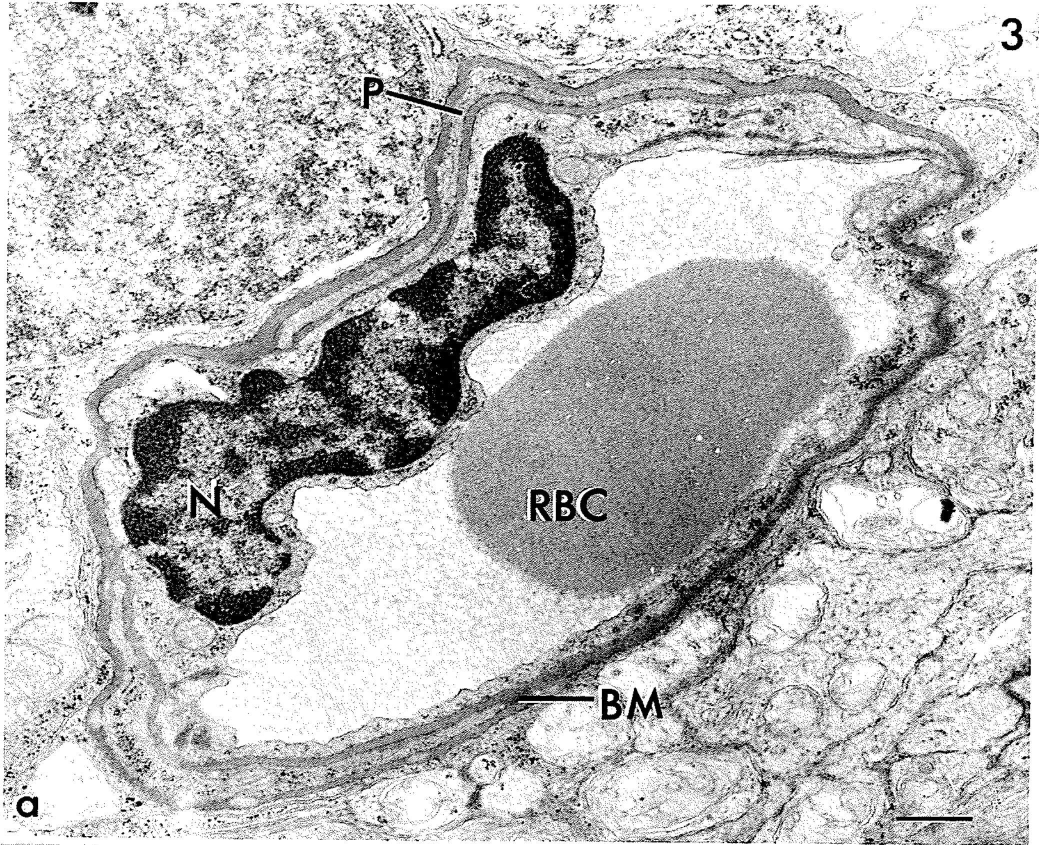

male Sprague-Dawley rats
(104)
6 removed as zero time control group
(CO)
(98)
placed on an 8% protein diet
(50)
placed on a 24% protein diet
(48)
injected with STZ
(42)
injected with STZ
(42)
C8%
(8)
STZ8%
(12)
DM8%
(15)
DM+I8%
(15)
C24%
(6)
STZ24%
(9)
DM24%
(16)
DM+I24%
(17)

Figure 3a: Electron micrograph of a capillary from the deep capillary bed of the retina. BM=basement membrane, N=endothelial cell nucleus, P=pericyte process, RBC=red blood cell. x17000. Scale bar represents 0.5μm.

3b: The same micrograph as above but with the basement membrane outlined in pen for easier tracing on the graphics tablet.


3
P
N
RBC
BM
a

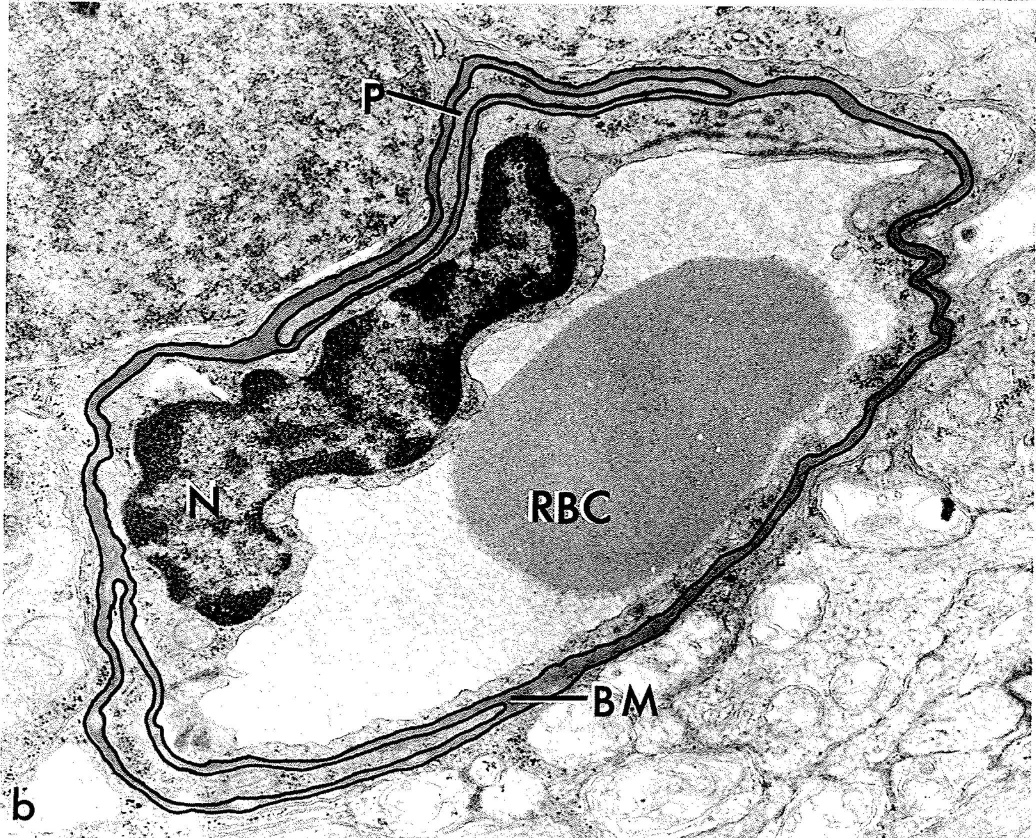

P
N
RBC
BM
b

Figure 4: Method for measuring the basal cell membrane area of the retinal pigment epithelium. A transparency with a grid printed on it was placed on the micrograph. The number of intersections between the cell membrane and the grid lines was counted and used to determine basal cell membrane area. BI=basal infoldings, Br=Bruch's membrane, CC=choriocapillaris, M=mitochondrion, Ph=phagosome, RPE=retinal pigment epithelial cell. x21500. Scale bar represents 1.0μm.

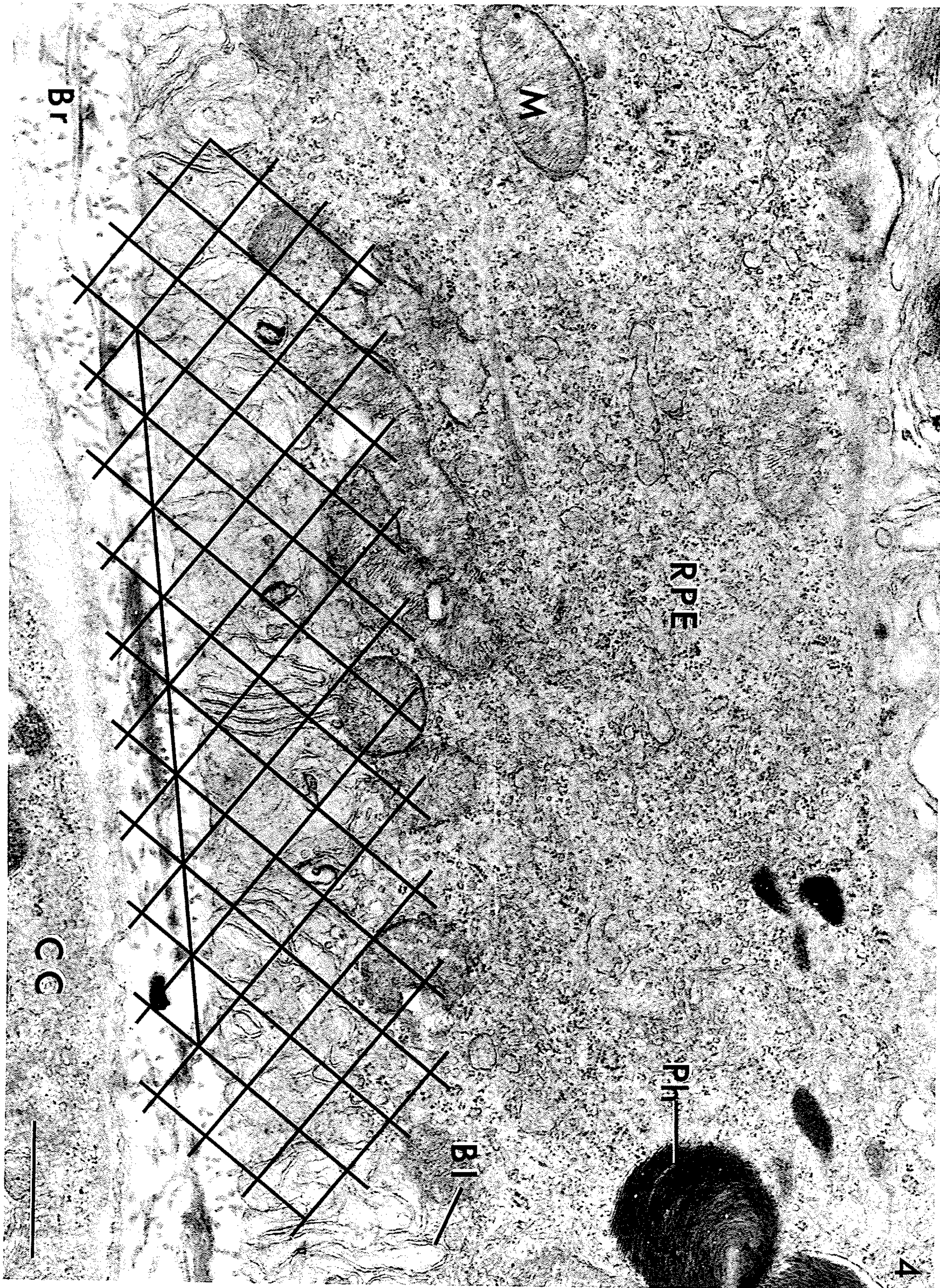

M
Br
RPE
CC
Pi
Bl
4

Figure 5: Method for measuring Bruch's membrane thickness. A transparency with parallel lines marked on it was placed on the micrograph. The thickness of Bruch's membrane was measured at the six places indicated and the mean of these six values was used in subsequent calculations. BI=basal infoldings, Br=Bruch's membrane, CC=choriocapillaris, M=mitochondria, RPE=retinal pigment epithelium, V=microvillar processes, <-->indicates the width of Bruch's membrane. x24000. Scale bar represents 1.0μm.

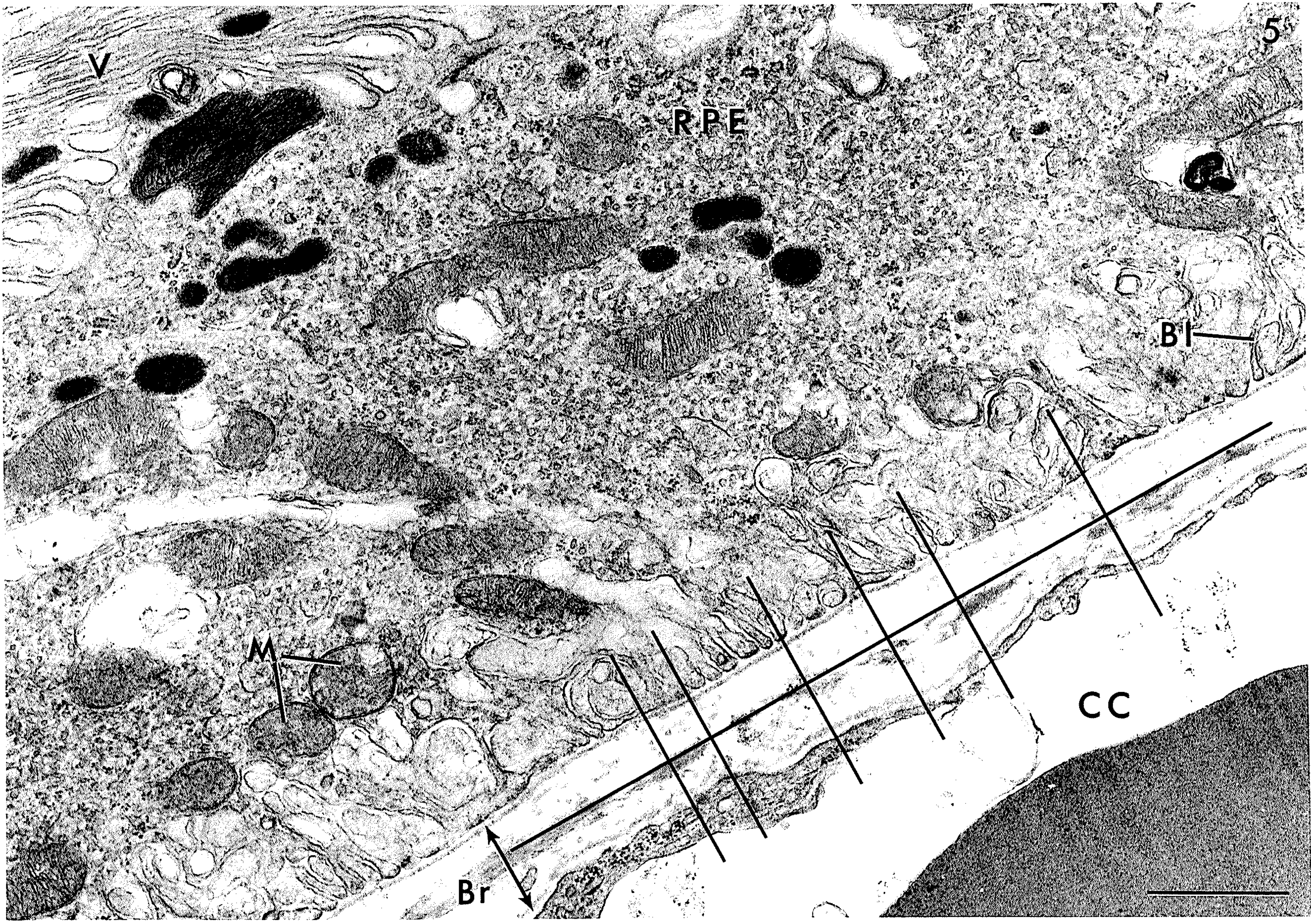
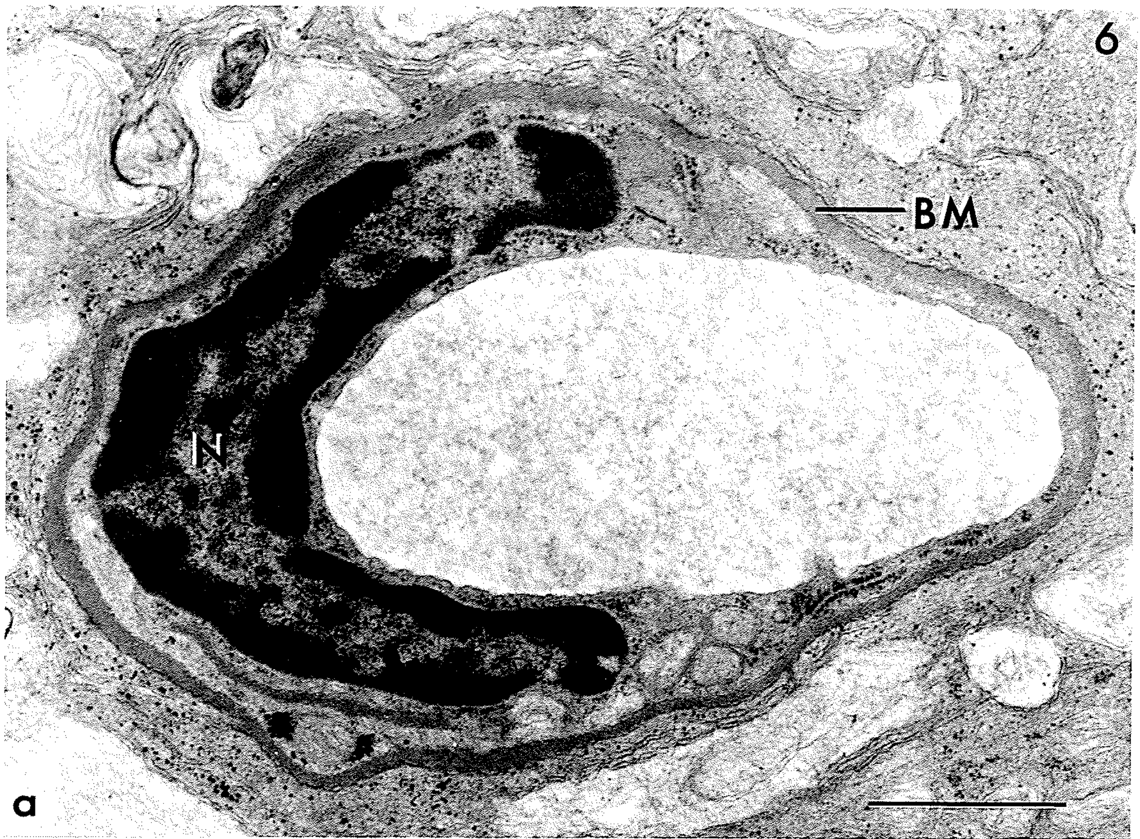

5
V
RPE
BI
M
CC
Br

Figure 6: Examples of capillaries from the deep capillary bed of the retina. BM=basement membrane, J=junction between endothelial cells, N=endothelial cell nucleus, P=pericyte process, RBC=red blood cell. x26000. Scale bar represents 1.0μm.


6
BM
N
a

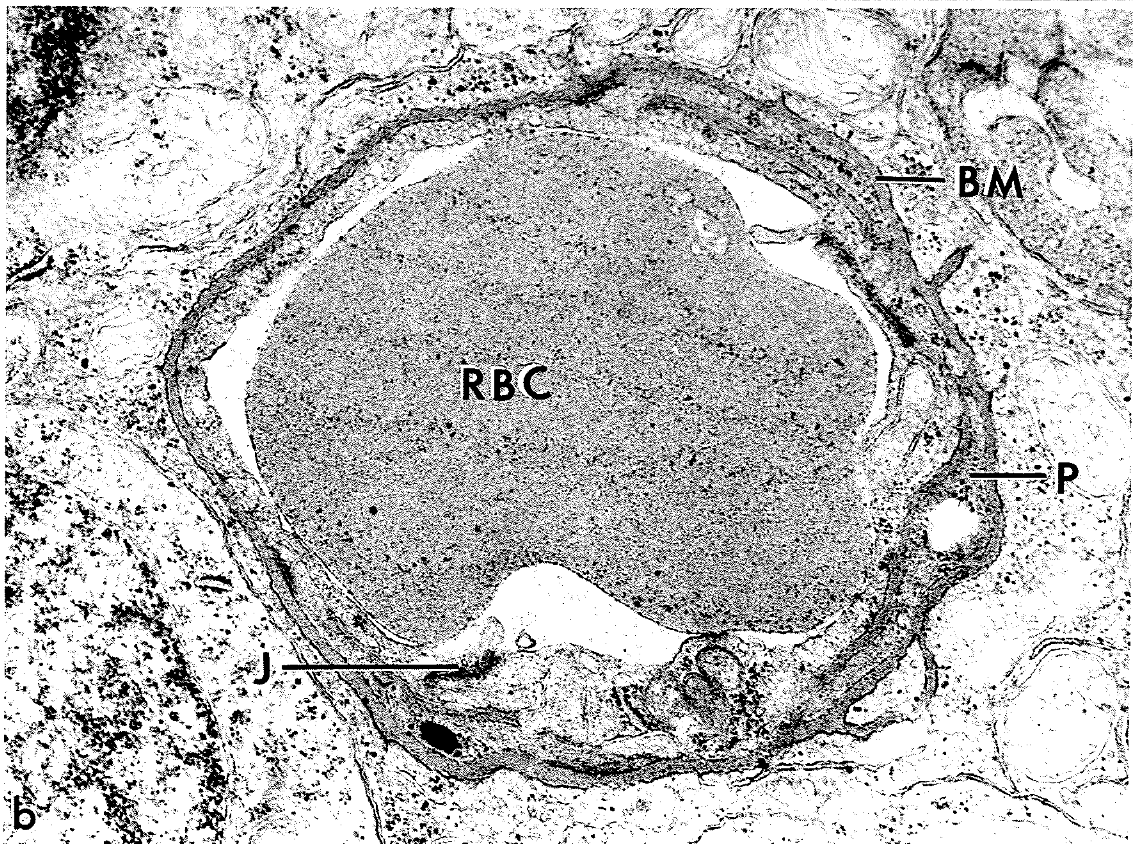
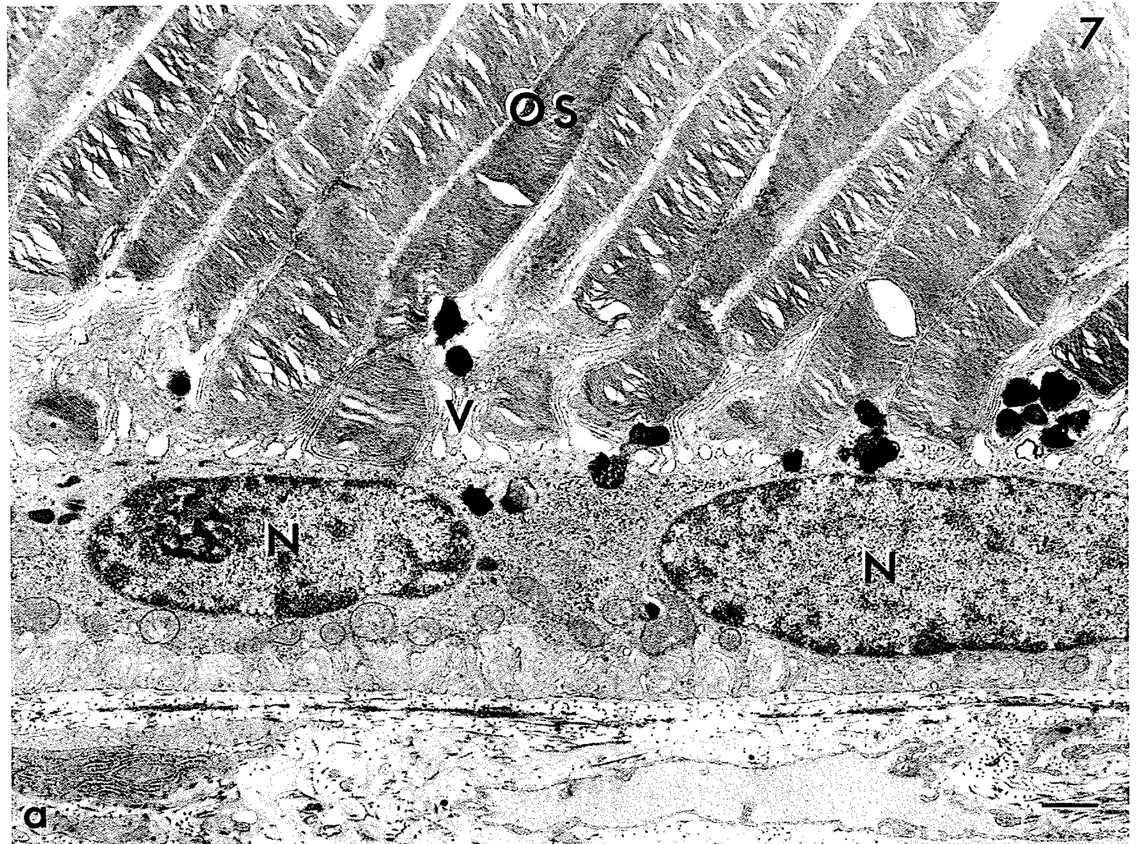

BM
RBC
P
J
b

Figure 7: Examples of retinal pigment epithelial cells. BI=basal infoldings, Br=Bruch's membrane, CC=choriocapillaris, J=junction between endothelial cells, M=mitochondria, N=nucleus, OS=photoreceptor outer segments, V=microvillar processes. a- x8000, b- x17000. Scale bars represent a- 1.0μm, b- 0.5μm.


7
OS
V
N
N
a

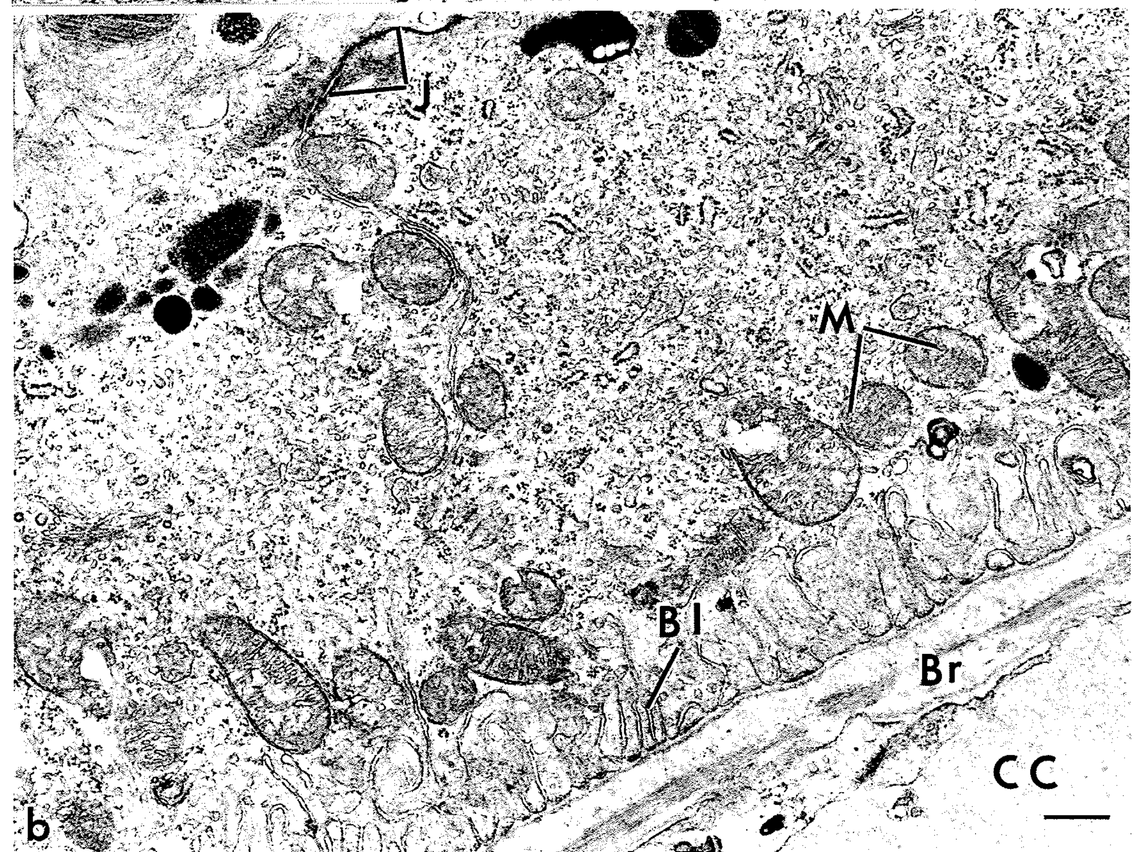

J
M
Bl
Br
CC
b

Figure 8: The components of Bruch's membrane. a=basement membrane of the retinal pigment epithelium, b=inner collagenous layer, c=elastic layer, d=outer collagenous layer, e=basement membrane of the choriocapillaris. The retinal pigment epithelium (RPE) and choriocapillaris (CC) are indicated for orientation. x30000. Scale bar represents 0.5μm.

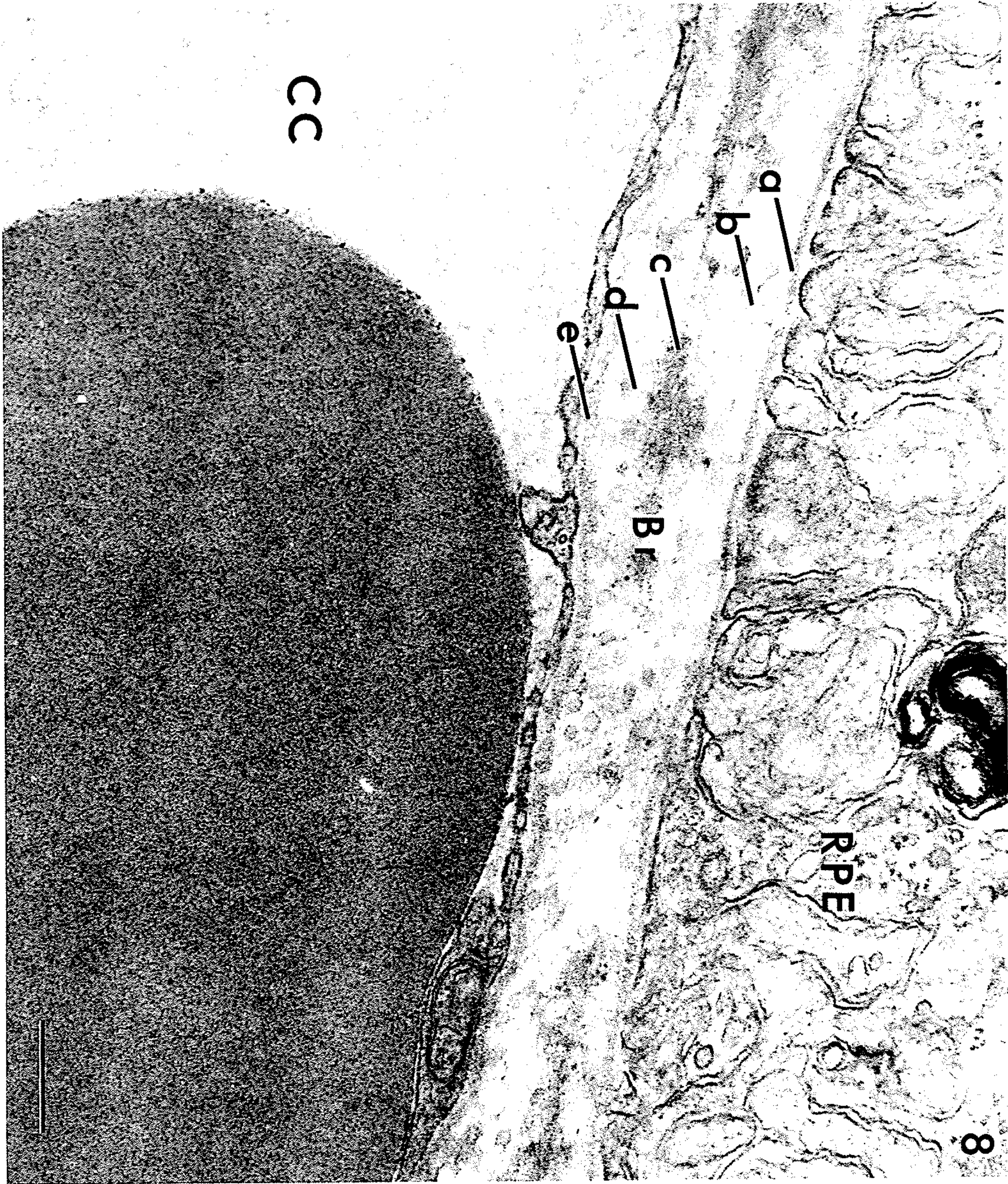
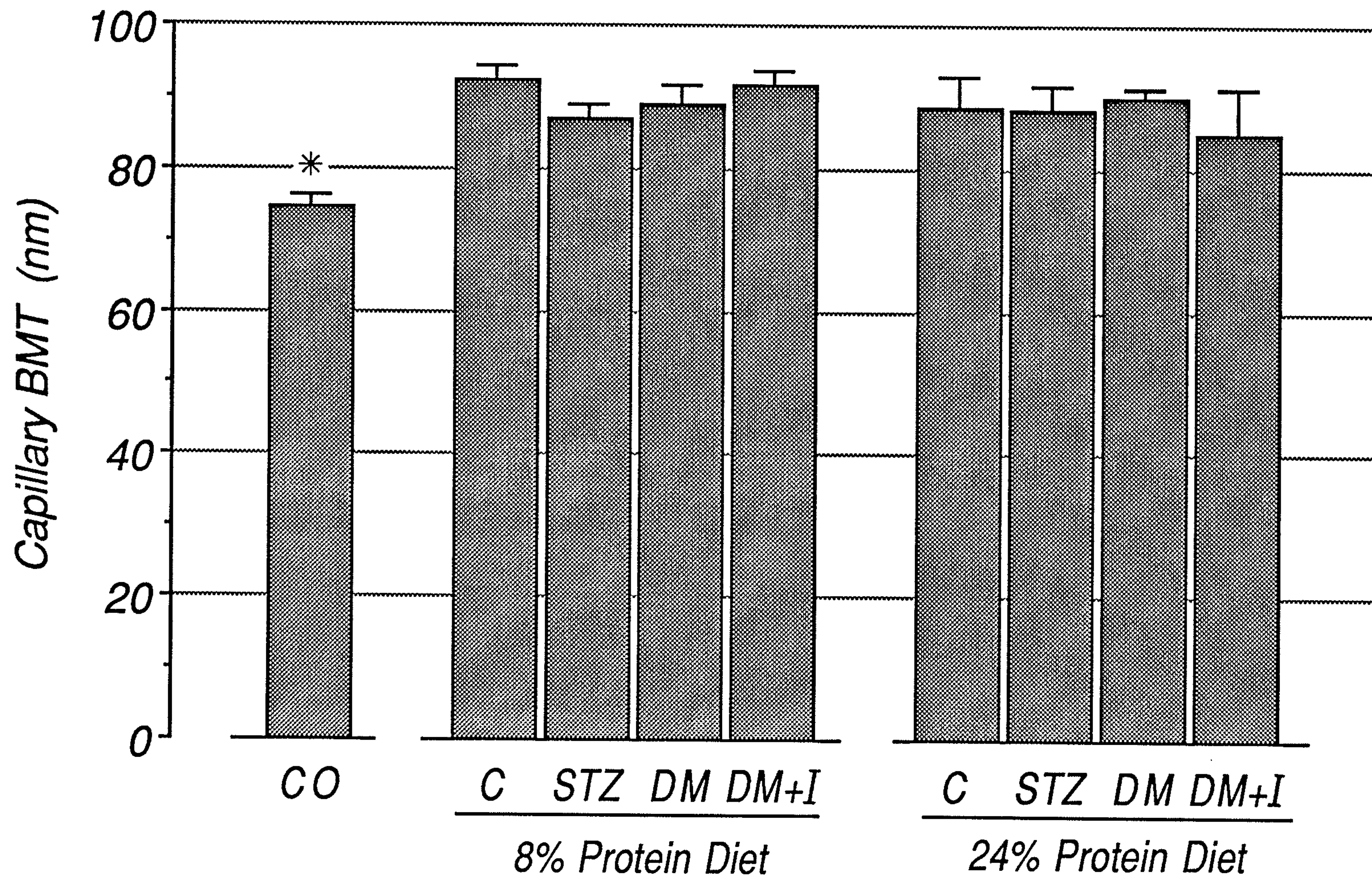

CC
a
b
c
d
e
Br
RPE
8

Figure 9: Mean retinal capillary basement membrane thickness (BMT) (nm) in zero time control (CO), control (C), STZ-injected non-diabetic (STZ), untreated diabetic (DM), and insulin treated diabetic (DM+I) groups on 8% and 24% protein diets. Error bars indicate $\pm$ standard error of the mean. *CO is significantly < all other groups ($p<0.05$).



100
80
60
40
20
0
Capillary BMT (nm)
*
CO
C
STZ
DM
DM+I
8% Protein Diet
C
STZ
DM
DM+I
24% Protein Diet

Figure 10: Mean retinal pigment epithelium (RPE) basal cell membrane area (BCMA) (L_c/L_b) in zero time control (CO), control (C), STZ-injected non-diabetic (STZ), untreated diabetic (DM), and insulin treated diabetic (DM+I) groups on 8% and 24% protein diets. Error bars indicate $\pm$ standard error of the mean. *DM24% is significantly $<$ C24%, DM8% and DM+I8% ($p<0.05$).

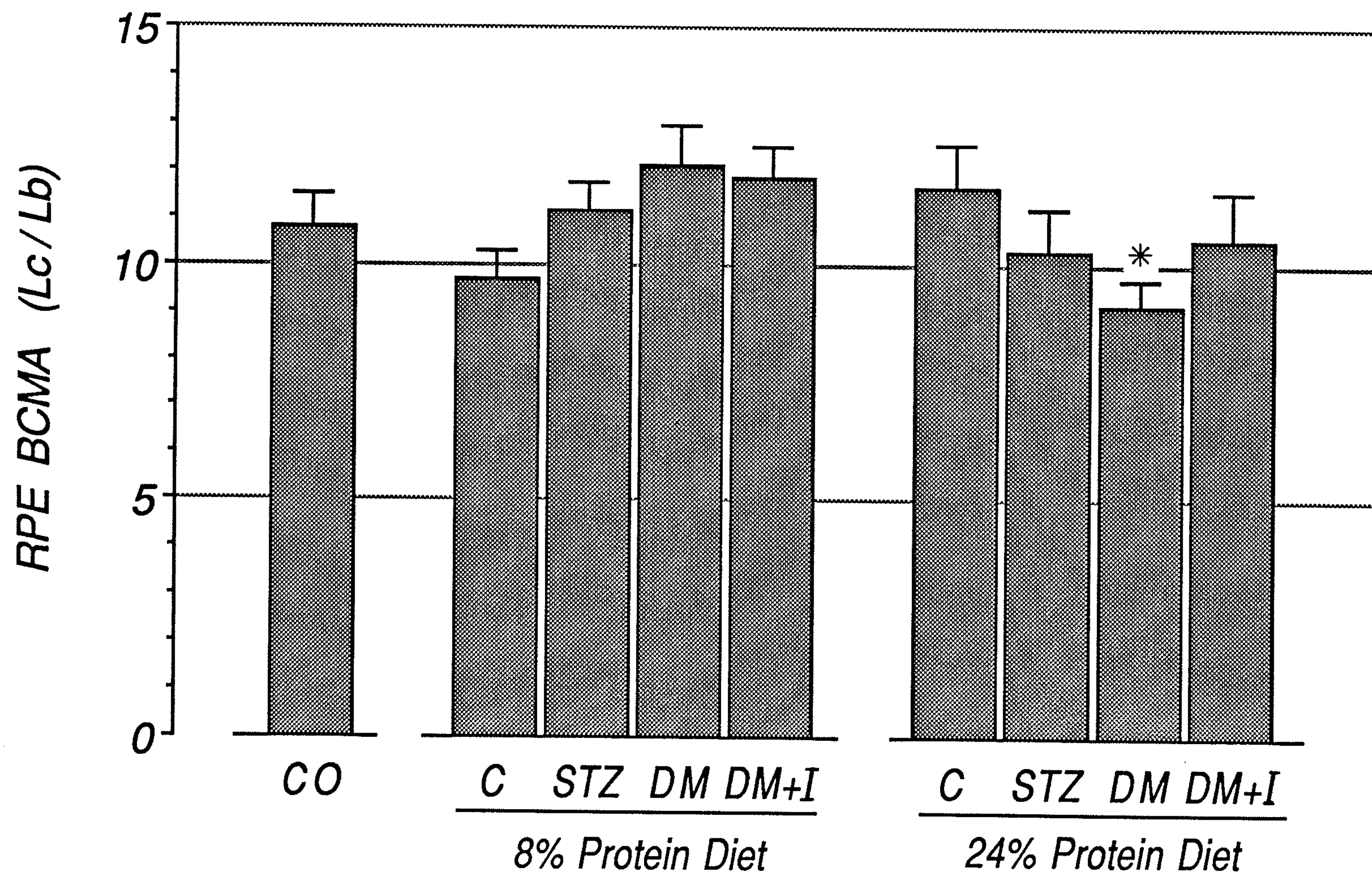
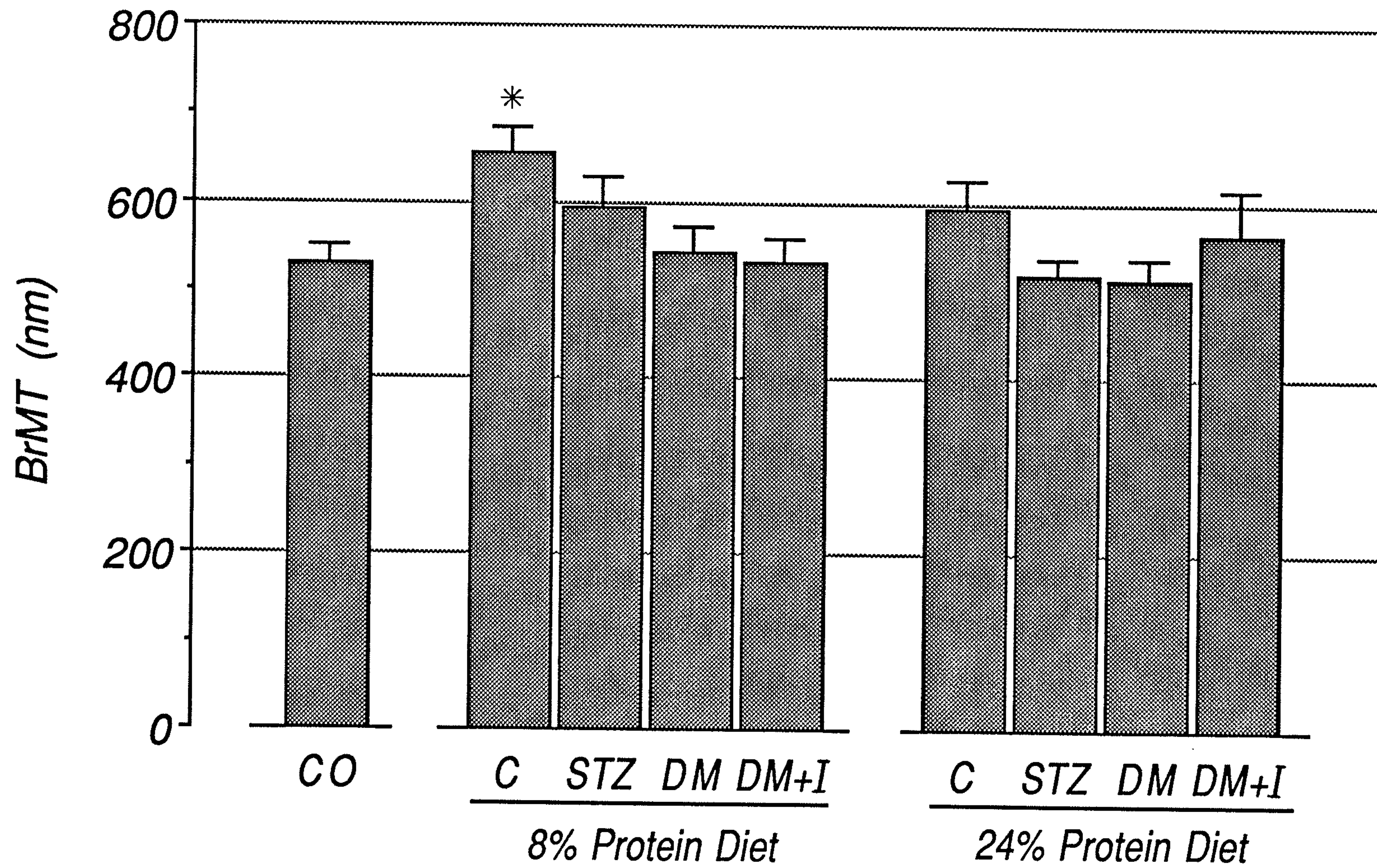

15
10
5
0
RPE BCMA (Lc/Lb)
*
CO
C
STZ
DM
DM+I
8% Protein Diet
C
STZ
DM
DM+I
24% Protein Diet

Figure 11: Mean thickness of Bruch's membrane (BrMT) (nm) in zero time control (CO), control (C), STZ-injected non-diabetic (STZ), untreated diabetic (DM) and insulin treated diabetic (DM+I) groups on 8% and 24% protein diets. Error bars indicate ± standard error of the mean. *C8% is significantly > CO, DM8%, DM+I8%, STZ24% ($p<0.05$), and DM24% ($p<0.01$).



800
600
400
200
0
BrMT (nm)
*
CO
C
STZ
DM
DM+I
8% Protein Diet
C
STZ
DM
DM+I
24% Protein Diet

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