The following article by Marano and Matschinsky represents the last paper for the issues of Diabetes/Metabolism Reviews *that dealt with Diabetic Complications. The Editor*

Biochemical Manifestations of Diabetes Mellitus in Microscopic Layers of the Cornea and Retina

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I. INTRODUCTION

The eye is a major site for the development of complications associated with diabetes mellitus. The morphology and function of nearly every ocular structure appears to be affected. The lens undergoes progressive opacification. The cornea's resiliency to stress is impaired.[2] The basement membrane of the ciliary epithelium is thickened.[3–5] The iris is afflicted by neovascularization and lacy vacuolization of its pigmented epithelial layer.[3,5] The inner and outer barriers of the retina are pathologically disturbed.[6–8] Endothelial cell hypertrophy,[6] pericyte loss,[9,10] basement membrane thickening,[4,7,8] and increased permeability[7,8] compromise the inner retinal barrier of the retinal microvessels. Basement membrane thickening[4] and disruption of the morphology of the retinal pigmented epithelium impinge on the function of the outer retinal barrier.[11,12] Visual psychophysical tests reveal diabetes-induced deficits in the neurosensory retinal components.[13] Considering this list, it is thus apparent that the structures of the eye are representative of the gamut of potential cellular responses to the diabetic condition. Represented by the mural cells of the retinal microvasculature are those cells which are destroyed by diabetes mellitus. Endothelial cells of the retinal microcirculation respond with proliferation. Cell dysfunction rather than cell destruction characterizes the corneal endothelium's response to diabetes. Pathological lesions and dysfunction occur in the RPE in response to glucose intoxication. The progression from reversible to irreversible lesions is apparent in the lens exposed to prolonged hyperglycemia.

The underlying biochemical events linking diabetes mellitus to the development of these global ocular complications has eluded researchers just as such pathogenetic mechanisms remain obscure in other tissues afflicted by diabetic complications, such as the nerve, kidney, and vasculature. Thus, the analysis of the pathological biochemical pathways remains an active area of diabetes research. A brief review of currently postulated mechanisms explaining the pathological consequences of diabetic complications is now in order before the specific aspects of certain diabetic ocular complications are discussed.

II. CURRENT HYPOTHESIS REGARDING DIABETIC COMPLICATIONS

Several working hypotheses have been proposed to account for diabetic complications. They arose from intensive morphological and biochemical investigations in both human diabetics and animal models of the disease.

Accumulation of polyols to osmotically active concentrations in the presence of hyperglycemia characterizes the *osmotic hypothesis*.[1,14,15] The early work of van Heynigen in the lens of rats fed a high hexose diet or rendered diabetic by alloxan injection associated "sugar" cataracts with enhanced aldose reductase activity and polyol accumulation.[16,17] The hyperosmotic effect of excess polyols in the lens cytosol was thought to cause the typical lens pathology. These concepts were gener-

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alized and applied to explain the development of impaired nerve conduction velocity[18–20] and depressed renal function in diabetics,[21] since it became apparent in humans and animals with diabetes that sorbitol and fructose accumulated in the nerve and renal structures exposed to a diabetic milieu.[21,22] Several other working hypotheses to explain the biochemical basis of diabetic complications have been developed to replace or complement the view that hyperactivity of the polyol pathway and hyperosmotic shock are the primary cause. The so-called *myoinositol depletion hypothesis*, according to which activation of the polyol pathway due to hyperglycemia is accompanied by a reduction in free cellular myoinositol levels, arose from the earlier work evaluating the osmotic hypothesis.[15] An absolute or relative deficiency of insulin,[23,24] hypersecretion of growth hormone or other growth factors such as IGF-1 and FGF,[25–27] male sex steroids,[28] and thyroxine[29] have been invoked to explain the development of at least some of the tissue pathology associated with diabetes, a view that may be described as the *hormonal hypothesis* of diabetic complications. The *nonezymatic glycosylation* of proteins has been implicated in the disruption of enzyme function, extracellular matrix biochemistry, transport protein properties, and the immunogenicity and permeability characteristics of tissues affected by diabetes.[30,31] Thus, there exist at least four distinct concepts which have been invoked as explanations for diabetic complications. The relative importance of these mechanisms for the development of the diverse manifestations of complications is currently being worked out.

Because diabetes-associated tissue pathology markedly improves when aldose reductase inhibitors are administered or when myoinositol supplementation is offered to diabetic animals, the depletion of tissue myoinositol and the activation of the polyol pathway have attracted the widest attention in the area of diabetic complications research for the past 25 years (e.g., Refs. 21 and 32–37). Stated briefly, the hypothesis suggests that glucose accumulates in tissues despite a lack of insulin; high intracellular glucose enhances flux through the polyol pathway and leads to a rise in the levels of intracellular polyols because of the increased reduction of glucose to sorbitol by the enzyme aldose reductase which has a low affinity for glucose.[15,38,39] Through yet-to-be-defined mechanisms, the elevation in sorbitol results in a critical depletion of myoinositol accompanied by a reduction in Na^+,K^+ATPase activity and increased intracellular Na^+.[40–42] As postulated by Greene et al., myoinositol depletion interferes with the turnover of the phosphoinositides and the production of the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3).[15,43] The decreased availability of DAG and impaired Ca^{2+} homeostasis results in a reduction of protein kinase C activity. The phosphorylation state of proteins either directly or indirectly (through a cofactor) is thought to modify the activity of Na^+, K^+ATPase.[43] The electrochemical gradient for Na^+ is disrupted, thus affecting Na^+-coupled membrane transport systems, including that for myoinositol.[33] Additionally, hyperglycemia competitively inhibits myoinositol uptake, thus contributing to myoinositol depletion in a self-reinforcing cycle. In a modification of this formulation, Winegrad proposed that hyperglycemia depletes a relatively small, discrete pool of myoinositol that is required for the synthesis of phosphatidylinositol,[44] which is believed to regulate the activity of Na^+, K^+ATPase.[44–46] Depletion of this pool of myoinositol may not be reflected in alterations of the total intracellular content of myoinositol.

In studies of the effects of elevated glucose on the blood flow and vascular permeability of granulation tissue in a skin chamber, Williamson and coworkers have made observations which reinforced the activation of the polyol pathway and myoinositol depletion as pathogenetic mechanisms in the expression of diabetic complications.[47] Incubation with 1 m*M* myoinositol in the presence of 30 m*M* glucose normalized increases in blood flow and reduced the increase in vascular permeability observed with high glucose alone.[47] Williamson also observed that 1 m*M* pyruvate was as effective as 1 m*M* myoinositol. The observations of improvement in diabetes-induced dysfunction with pyruvate is a remarkable new finding. Earlier studies with human erythrocytes appear to be relevant for these observations. Impairments in glucose metabolism in human red blood cells incubated with 50 m*M* glucose were observed, and it has been reported that pyruvate was able to reverse the effects of high glucose.[48,49] In these earlier studies using human erythrocytes incubated with 50 m*M* glucose, Winegrad and collaborators demonstrated increased conversion of glucose to sorbitol and its subsequent oxidation to fructose via the NAD^+-dependent sorbitol dehydrogenase. Activation of the polyol pathway led to a decrease of the NAD^+/NADH ratio and corresponding alterations of the pyruvate/lactate ratio because the lactate dehydrogenase step appears to

be influenced by the altered redox state.[49] Since lactate dehydrogenase as well as the glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase systems are in equilibrium, the decrease in NAD^+/NADH results in a decrease in the ratio of 3-PGA/G-3-P.[49] Glycolysis is inhibited and triose phosphates, as manifested by increased concentrations of G-3-P, DHAP, and fructose-1,6-diphosphate, accumulate under conditions of elevated extracellular glucose and hexitols.[49] The relevance of this observation is underscored by the provocative finding that 1 m*M* pyruvate restores the ratio of NAD^+/NADH to a more oxidized state in the red blood cell as can the inhibitor of sorbitol dehydrogenase, barbital.[49] Furthermore, 1 m*M* pyruvate, presumed to serve a metabolic function similar to that cited in the erythrocyte, can, like myoinositol supplementation, normalize or reduce the pathological change in tissue function associated with diabetic complications.[47]

These explanations for the aberrant biochemistry underlying the development and the maintenance of diabetic complications may not be applicable to all tissues. The elaboration of these mechanisms may be complicated by morphological heterogeneity of the individual tissue in question or the inherent ability of the tissue to respond adaptively to the variety of factors disturbed by diabetes mellitus.

Over the years our laboratory has applied the powerful methodology of quantitative histochemistry to associate discrete changes in cellular biochemistry to pancreatic B-cell insulin secretion.[50,51] The same approach has now been applied to help characterize the biochemical events associated with the development of diabetic complications in a variety of different tissues from experimentally induced and spontaneously diabetic animal models.[52] Since most tissues affected by diabetic complications consist of complex arrangements of heterogeneous cells, we have focused on the eye in which discrete and homogenous populations of cells known to be affected by the diabetic condition reside in the retina and the cornea. Biochemical effects of diabetes, that fall within the framework of myoinositol depletion hypothesis explaining diabetic complications, were studied by using the techniques of quantitative histochemistry on the individual layers of the retina (seven layers of the retina proper and the retinal pigmented epithelium (RPE) and choricapillaries) and the cornea including the epithelium, stroma, and endothelium. Those well-defined homogenous cell samples were dissected from alloxan-treated rabbits with diabetes of acute and chronic duration. In the retina, the results in terms of glucose and polyol accumulation, free cellular myoinositol levels, and parameters of Na^+ homeostasis, were consistent with current formulations of the myoinositol depletion hypothesis explaining diabetic complications. However the corneal layers, which accumulated polyols in diabetic animals in a typical manner, exhibited a paradoxical elevation of myoinositol levels when diabetes was severe and extended for 70 days.

III. MORPHOLOGY AND FUNCTION OF THE DIABETIC RETINA

Diabetic retinopathy has traditionally and exclusively been attributed to the microvascular changes of the inner retinal barrier, characterized histologically at the level of the endothelial cell with its associated hypertrophy and increased deposition of basement membrane material and at the level of supporting pericyte with its loss.[6–10] Fundoscopic examination of fluorescein angiography of the diabetic retina reveals gross evidence of capillary occlusion, microaneurysms, hard and soft exudates, intraretinal hemorrhage, and neovascularization.[3,4]

Little attention has been directed to the effects of diabetes on the nonvascular retina comprised of the neural and photosensitive cellular elements. However, psychophysical evaluation of visual function has revealed neurosensory losses of color vision,[53,54] contrast and spatial sensitivity,[55,56] dark adaptation,[57] and perimetry[58] in diabetic patients. It is conceivable that at least some of these impairments are a direct result of the diabetic state rather than caused indirectly by the impaired microcirculation.

Histological examination of the outer retinal barrier has uncovered extensive disruption of the arrangement of the RPE cells under conditions of both experimental and spontaneous diabetes. The basement membranes of the RPE and the choriocapillaries undergo progressive thickening with increasing duration of diabetes.[4,59] Basilar infoldings of the plasmalemma of the RPE cells become more pronounced, with areas of necrosis and attempted repair becoming more evident.[11,12] Sucrose continues to be excluded from the RPE in the diabetic eye.[60] Similar findings appear in the RPE of experimentally induced galactosemic animals.[61] Interestingly, the apical tight junctions of the RPE are not affected by either metabolic condition.[61] However, in studies using fluo-

rescein angiography or horseradish peroxidase as intravsacular markers to assess the barrier function of the RPE, it has been reported to be intact or disturbed by the presence of diabetes.[62–64] Furthermore, the transport capacity of the RPE, measured by vitreal fluorophotometry, appears to be compromised in the diabetic.[6] Under normal conditions, vitreally injected fluorescein is transported from the retina to choroid across the RPE. This apparent impairment of fluid transport across the RPE has been evoked as the cause of macular edema encountered in the diabetic.[65] Thus the outer retinal barrier composed of the RPE and choriocapillaries, responsible for the maintenance of the extracellular milieu of the photoreceptor and neural components of the retina, is clearly affected by diabetes.

In agreement with the disruption of the morphology and function, an abnormality in the electrophysiological response of the retina to light is precipitated in the presence of diabetes. The electroretinogram (ERG) reveals alterations in the b wave and the oscillatory potentials on the ascending limb of the b wave, which originate from the inner nuclear layer of the retina most likely from the Muller cells and bipolar cells.[66] Both the b wave and the oscillatory potential derive their nutrient-energy supply from the inner retinal blood supply, which has been documented to be compromised in diabetes.[66] Ischemia precipitated by disruption of the inner vascular supply to the retina or the inability of the inner retinal blood vessels to autoregulate would render the supplied tissue hypoxic.[67–69] Yet, the documentation of impaired visual function in diabetic subjects precedes or develops closely with the clinically detectable microangiopathy.[53–56] Therefore, impaired retinal vascular permeability may or may not be the primary factor precipitating the alterations in visual function, the b wave and oscillatory potential of the ERG of diabetics, and the measured neurosensory deficits. However, the probability of progressing to end-stage proliferative retinopathy and eventual blindness was significantly increased in those diabetics with low oscillatory potentials coupled with higher vascular permeability and capillary nonperfusion as measured by fluorescein angiography.[66] The c wave of the ERG emanating from the RPE is also affected by a diabetic environment.[35,70] Under normal circumstances, the apical membrane of the RPE undergoes a passive hyperpolarization in response to the decrease in extracellular potassium in the outer retinal extracellular space following light-induced hyperpolarization of the photoreceptor cells.[71] The c wave of the ERG is a manifestation of the light-induced ionic changes, and the c wave amplitude progressively decreases with duration of diabetes.[35] An electrophysiological impairment of the light peak has been variably documented.[72–74] Occurring several minutes after the c wave, the light peak is the cummulative result of the depolarization and conductance increase in the basal aspect of the cell membrane of the RPE.[75,76] The standing potential, which is the potential difference between the cornea and the optic nerve recorded in the dark, is decreased in diabetes and other disorders affecting the RPE.[77,78]

An interconnection between the pathological effects of diabetes on the RPE of the outer retinal barrier and the development of an abnormal extracellular milieu for the photosensitive and neural components of the retina as well as for the endothelial cells and pericytes of the inner retinal barrier has not been systematically evaluated. Alterations in the transport properties of the RPE, as a consequence of disturbances in ion homeostasis, could possibly result in the disruption of water homeostasis, of the supply of nutrients to and the removal of metabolites from the retinal microenvironment, and could constitute plausible contributing factors for the observed pathology of the neural and vascular components of the retina in diabetic retinopathy.[79–81] Based on the extensive clinical and experimental evidence of retinal changes induced in the presence of diabetes mellitus, we searched for potential biochemical correlates that might explain these disruptions of retinal morphology and function.

IV. BIOCHEMISTRY OF THE DIABETIC RETINA

The biochemical mechanisms underlying the morphological and functional disturbances of the vascular and nonvascular retina precipitated by the presence of diabetes mellitus were little understood until the last few years. Application of the microanalytical techniques of quantitative histochemistry helped to clarify certain aspects of the effects of diabetes on the biochemistry of the retinal layers. The levels of glucose, sorbitol, myo-inositol, sucrose (following an intravenous bolus), Na^+, and K^+, and the activity of ouabain-inhibitable Na^+, K^+ATPase and of Mg^{2+} ATPase were measured in freeze-dried, microdissected samples of the choroid, RPE, and 7 microscopically defined retinal layers of normal and alloxan-diabetic rab-

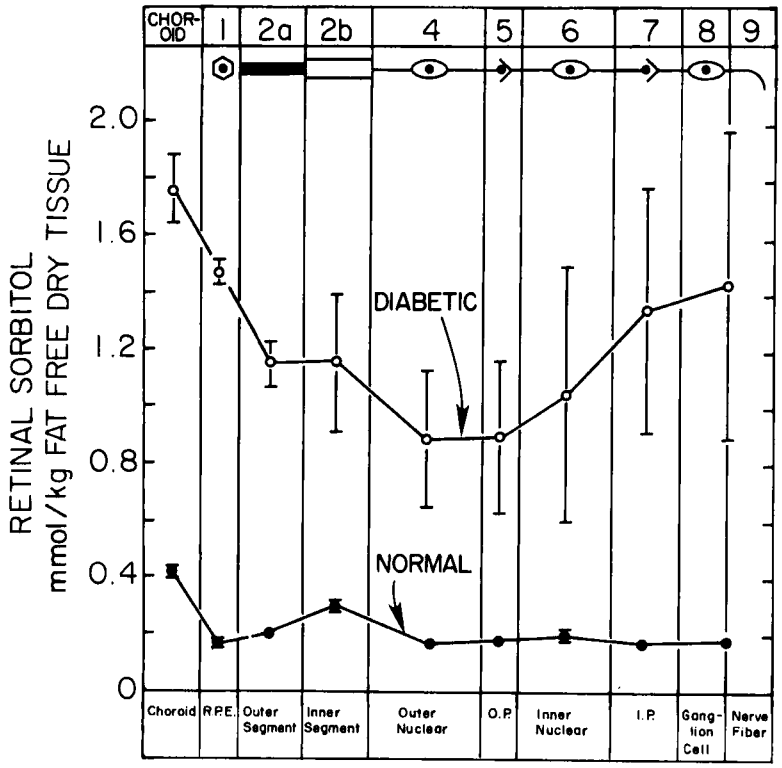

Figure 1. Profile of sorbitol in retinas of normal and alloxan-diabetic rabbits. Eight samples of each retinal layer from the right eye of three normal and three diabetic rabbits were analyzed (MacGregor et al., 1986).[60] Data represent the mean ± SEM of the three average values for each retinal layer. Fasting blood glucose was 7.4 ± 0.7 mmol/L for normal and 25.4 ± 1.5 mmol/L in the diabetic rabbits.

bits of 17 days duration and relevant biochemical parameters of the serum and vitreous were also studied.[42,60]

Alloxan-induced diabetes of 17 days duration in the rabbit was associated with the accumulation of glucose and sorbitol in all layers of the retina (Ref. 60; Figure 1). As for the normal rabbit retina, there was a smooth diffusional profile for glucose across the retina of the diabetic rabbit, proceeding from concentrations of glucose roughly equivalent to choroidal levels (28 mmol/kg, lipid-free dry weight) and falling to vitreal concentrations (17 mmol/kg, lipid-free dry weight) in the innermost retinal layers. The barrier function of the RPE appeared to be intact, since sucrose in the choroid approximated serum levels but was totally excluded from all other layers.[60] Sorbitol accumulation was not restricted to the intracellular compartment. Serum sorbitol concentrations were at least 4-fold greater in diabetic as compared with normal rabbits, whereas the vitreous accumulated sorbitol to levels approximately 40-fold greater in diabetics as compared with normals, amounting to as much as 0.3 m*M*.[60] Myoinositol levels were depressed by 22–40% in the retinal layers of the diabetic rabbits compared with nondiabetic controls despite serum and vitreal levels that were unaffected by diabetes. Although all layers of the retina exhibited reductions in myoinositol content, the greatest decline was observed in layers with levels > 20 mmol/kg dry weight, such as the outer plexiform layer, the site of synapse between the photosensitive rods and cones, the bipolar neurons, and the horizontal cells; the inner nuclear layer, composed of the cell bodies of the bipolar cells in addition to Muller and amacrine cells; the inner plexiform layer, the site of synapse between bipolar and ganglion neurons; the ganglion cell layer; and the nerve fiber layer comprising the optic nerve (Ref. 60; Figure 2). Total Na^+ increased to levels significantly above control only in the RPE layer of the diabetic retina, whereas total K^+ levels were unaffected in any retinal layer (Ref. 42; Figure 3). The total ATPase activity was reduced in the inner and outer segments, the outer nuclear layer, and the outer plexiform layer of diabetic retinas.[42] A decline in Mg^{2+} ATPase activity followed the pattern established for total ATPase except in the

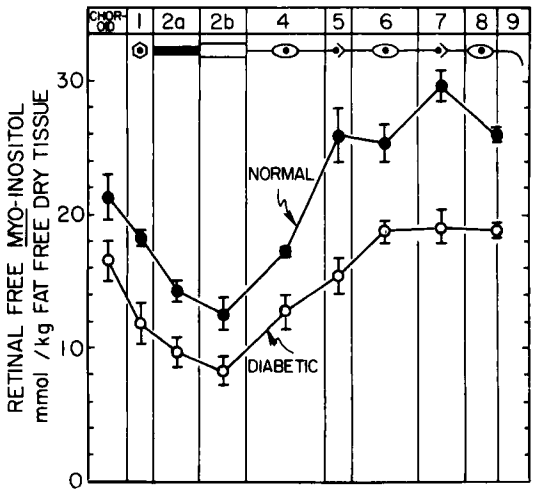

Figure 2. Profile of myoinositol in retinas of normal and diabetic rabbits. Twelve samples of each retinal layer from each of five normal and six diabetic rabbits were assayed for myoinositol content (MacGregor et al., 1986).[60] The structure of the figure is similar to that of Figure 1.

inner segment in which the enzyme activity was unaffected by diabetes. Significant decreases in Na^+, K^+ATPase activity were measured in the RPE and the outer nuclear and plexiform layers of the retina.[42] The response of the neural retinal layers to diabetes in the rabbit cannot be secondarily attributed to changes in the microcirculation of the inner retinal barrier, since the rabbit is characterized by an avascular retina, except for a central streak which was not analyzed.

In light of the limitations of acute experimental models of diabetes for the understanding of the long-term complications of diabetes, the myoinositol results from these acute studies were re-evaluated in chronically alloxan-diabetic rabbits of 70 days duration.[52] Diminished free myoinositol characterized the RPE of the severely diabetic rabbit, defined by a requirement of insulin to prevent ketosis and dehydration. However the mildly diabetic group not requiring insulin administration exhibited no myoinositol loss.[52] Whether these chronic changes of myoinositol were also present in the other retinal layers is not known. Furthermore, the impact of the altered myoinositol on Na^+ content and ATPase activity of the RPE was not evaluated. Similar findings of glucose toxicity (i.e., sorbitol accumulation and myoinositol depletion) were reproduced by incubating cultured feline RPE cells in 26 m*M* glucose, an effect which could be mimicked by incubation of the cells in 1 m*M* sorbitol but not by incubation with 26 m*M* of the nonmetabolizable analogue of glucose, 3-0-methylglucose, and was reversed in the presence of an inhibitor of aldose reductase (Ref. 82; Figure 4). Human RPE cells in culture also respond with myoinositol depletion when incubated with 45 m*M* glucose or in the presence of 1 m*M* sorbitol and low glucose.[83] Yet ouabain-sensitive Rb^{86+} uptake by human RPE cells was unaffected by either condition and may depend upon factors present in situ but not under these in vitro culture conditions.[83] The apparent effect of sorbitol is compatible with similar observations of myoinositol depletion made by Yorek et al. in neuroblastoma cells in culture and incubated with 1 m*M* sorbitol.[84] Metabolically the sorbitol effect could be reconciled to alterations in the redox state and glycolytic flux in the RPE as a consequence of its NAD^+-linked oxidation to fructose as proposed independently by Travis et al.[49] and Williamson et al.[47] What is all the more enticing about the effect of extracellular sorbitol is its pronounced elevation (280 μM compared with 7 μM) in the vitreous of diabetic rabbits versus their nondiabetic controls.[60] Thus, in situ exposure of retinal elements to marked elevations of extracellular sorbitol is a physiological possibility.

In addition to the changes in retinal metabolism characterized by glucose and polyol accumulation, myoinositol depletion, increased intracellular Na^+, and reduced Na^+,K^+ATPase activity, Bresnick suggests that ischemia-induced hypoxia

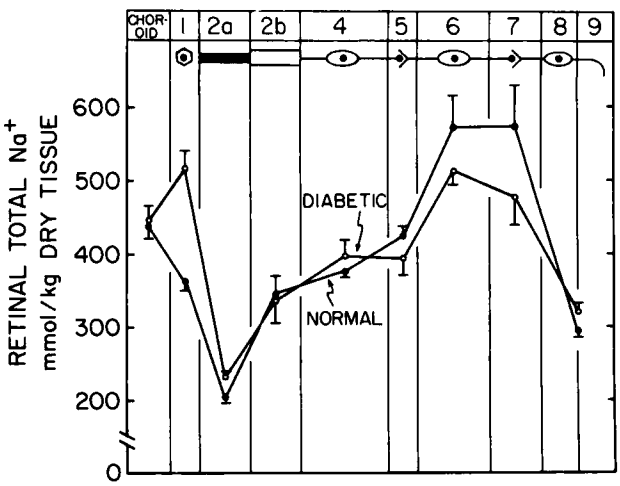

Figure 3. Profile of total sodium in retinas of normal and diabetic rabbits. Nine samples of each retinal layer from each of nine normal and eight diabetic rabbits were analyzed (MacGregor and Matschinsky, 1986).[42] Sodium was measured using atomic absorption with a carbon rod atomizer. The structure of the figure is as described for Figure 1.

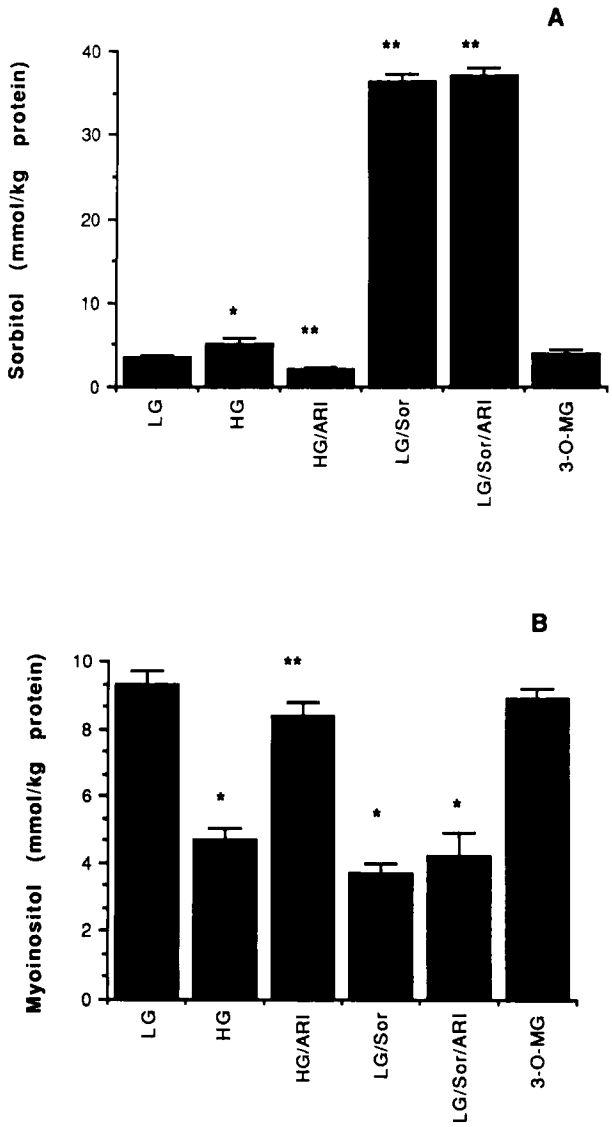

Figure 4. Sorbitol (**A**) and myoinositol (**B**) levels in cultured cat RPE cells incubated with glucose, sorbitol, and the aldose reductase inhibitor sorbinil. Confluent primary cultures were exposed to 6 m*M* glucose (LG), 26 m*M* glucose (HG), 26 m*M* glucose plus 200 μM sorbinil (HG/ARI), 6 m*M* glucose plus 1 m*M* sorbitol (LG/Sor), 6 m*M* glucose plus 1 m*M* sorbitol plus 200 μM sorbinil (LG/Sor/ARI), or 6 m*M* glucose plus 26 m*M* 3-0-methylglucose (3-0-MG) for 14 days. The results are the mean ± SEM for 6–18 cultures derived from 3–7 cats and exposed to the respective media conditions (MacGregor et al, 1988).[82] (*$p < 0.05$ compared with the LG group, **$p < 0.05$ compared with the HG group).

of the inner retinal layers may lead to the release of the excitatory neurotransmitters, glutamate and aspartate, leading to depolarization and eventual destruction of surrounding neuronal elements as a similar situation ensues in the hypoxic brain.[85,86] Alternatively, the diabetes-induced changes in cellular metabolism, reflected in enhanced polyol pathway activity, glucose accumulation, and altered ion homeostasis, as described above, could contribute to the accentuated release of glutamate and aspartate from the neural retina. The proposal is provocative and deserves further consideration, particularly since glutamate concentrations in the retina range from 20 to 60 mmol/kg dry weight, with the highest content documented for the ganglion cell layer whose efferent fibers comprise the optic nerve.[87] Glutamate dehydrogenase and glutamate-dependent transaminase are also very active in certain retinal layers.[87] Furthermore, application of either aspartate or glutamate results in the selective disappearance of the b wave component of the ERG,[88,89] which is derived from the inner retinal layers and is reduced in the presence of diabetes.[66,90]

The significance of the biochemical changes induced by the presence of diabetes became more apparent when the effects of treatment of streptozotocin-diabetic rats with aldose reductase inhibitor (ARI), sorbinil, or myoinositol supplementation on the electrophysiological response of the RPE over a period of 6 weeks were explored.[35] The trend toward a decrease in the magnitude of the c wave of the untreated diabetic rat was arrested by either treatment (Ref. 35; Figure 5). This effect can be explained by a depression of the activity of the polyol pathway by the ARI, sorbinil, and a fall in sorbitol levels coupled perhaps to a reversal of the reduced redox state as proposed earlier to occur with pyruvate supplementation or barbital administration to red blood cells.[49] Secondly, myoinositol supplementation could prevent the depletion of intracellular stores of the cyclitol.[23] In view of the myoinositol depletion hypothesis, the combined effect would be a correction of impaired Na^+, K^+ATPase activity presumably by the availability of sufficient (poly)phosphoinositides and/or the lipid-derived second messengers, e.g. IP_3 and DAG.[15] There was a partial correction of the depressed c wave, yet not a total reversal (Ref. 35; Figure 5). The findings of altered Na^+ and K^+ homeostasis in the RPE of the diabetic animal are significant in light of the dependence of nutrient transport to the neural and photosensitive retinal elements by the RPE on the appropriate transepithelial electrochemical gradients established by Na^+,K^+ATPase at the apical and basal membranes of the RPE.[89] Na^+ and Ca^{2+} movements are directed from choroid to retina, whereas potassium

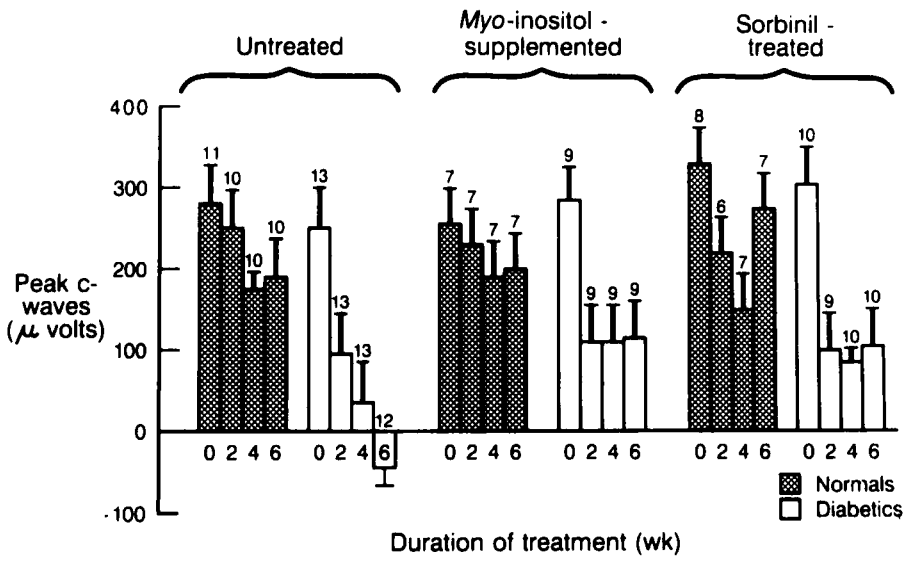

Figure 5. Peak c wave amplitudes of the ERGs of normal and diabetic rats during treatment with myoinositol supplementation or the aldose reductase inhibitor sorbinil. Data represent the mean ± SEM of 6–13 animals per treatment group (MacGregor and Matschinsky, 1985).[35]

and chloride fluxes and associated water movements are in the opposite direction.[91,92] Disturbances in these ionic gradients might affect the supply of nutrients to the microenvironment of the photoreceptor and neural retinal elements as well as to the inner retinal barrier in addition to disrupting fluid movements across the RPE from the retinal and subretinal spaces. The possible consequences of such alterations are the impaired function of the photoreceptor and neural retina as well as the development of macular edema and an adverse microenvironment for the endothelium and pericytes of the capillaries comprising the inner retinal barrier, although the possible connection between these pathologies of the diabetic retina has yet to be systematically studied.

V. MORPHOLOGY AND FUNCTION OF THE CORNEA

The suggestion that the cornea is a target of diabetic complications arose from morphological observations at both the clinical and experimental levels. The diabetic cornea is characterized by decreased sensitivity[93,94] and the increased incidence of sterile, neurotrophic ulcers.[95] Corneal complications following vitrectomy, such as, clouding of the epithelium, which necessitate its removal, delayed postoperative healing of the epithelium with recurrent or persistent defects, superficial ulceration, and vascularization, are frequently encountered in the diabetic population.[96,98] Dysfunction of the corneal endothelium (CEN) in diabetics manifests itself by preoperative increases in corneal thickness[99] and postoperative stromal edema.[97,98] Specular microscopic examination of the CEN revealed an increase in cell area (polymegathism) and an increase in the variation in cell shape (plemorphism) in patients with either IDDM or NIDDM.[100,101] The CEN is normally constructed of a monolayer of cells arranged in a regular hexagonal pattern, which appears to be necessary for the optimal function of the endothelium in maintaining the hydration and the transparency of the cornea.[102,103] Disruption of this regular pattern has been proposed to account for the increase in corneal thickness measured in diabetic patients.[102] The maintenance of constant corneal hydration is achieved by a pump-leak mechanism in which the fluid leaking into the stroma as a consequence of intraocular pressure is balanced by its efflux mediated through an active transport mechanism now localized to the endothelial layer.[104,105] Fluid transport across the endothelium is an energy-dependent process requiring intact cellular metabolism.[106]

Corneas of diabetic and galactosemic animals exhibit pathologies of the cornea reminiscent of those observed in human diabetics. The denuded corneas of both diabetic and galactosemic animals are plagued by a reduced repair ability of the epithelial layer.[107,108] The delayed healing of the

epithelium may in part be a reflection of changes in the composition and thickness of the basement membrane in diabetes.[109] Alloxan-diabetic dogs as well as streptozotocin-diabetic rats display the polymegathism and pleomorphism of the corneal endothelial layer documented for the human diabetic population.[110,111] These alterations in endothelial cells are reversed in part upon administration of an aldose reductase inhibitor to the STZ-diabetic rat,[112] suggesting a role for polyol accumulation in these morphological disturbances. Spontaneous stromal edema is not routinely encountered in diabetic animals and if it occurs may perhaps be a consequence of stress induced by various experimental interventions. The absence of edema in these animals suggests that despite altered cell morphology, the function of the corneal endothelium to maintain the appropriate degree of corneal hydration under normal, unstressed conditions is compensated.

VI. BIOCHEMISTRY OF THE CORNEAL LAYERS

The metabolic state of the corneal layers has been investigated using the two distinct approaches of noninvasive redox fluorometry on one hand and quantitative histochemistry on the other. The former, as introduced by Chance and collaborators, depends on the fluorescence intensity of oxidized flavoproteins (FP) and reduced pyridine nucleotides (PN).[113–115] The ratio of FP/PN is used as a measure of the redox state, and extrapolations are made to the P potential of the tissue under observation.[115] Quantitative histochemistry, coupling enzymatic metabolite and cofactor assays to oil well techniques and enzymatic cycling, provided the sensitivity for the direct analysis of ATP, ADP, AMP, and Pi in addition to NAD^+, NADH, $NADP^+$, and NADPH in the epi- and endothelial layers of the cornea.[116] The epithelium and endothelium of the cornea demonstrated distinct differences in both the concentration and ratio of pyridine nucleotides (NAD^+/NADH and $NADP^+$/NADPH). The aerobic epithelium possessed a 2.5-fold greater concentration of NAD^+ plus NADH than the endothelium, whereas the NAD^+/NADH ratio indicated a more oxidized state in the endothelial layer compared with the epithelium.[116] Despite these differences in pyridine nucleotides, the response of either layer to cyanide-induced anoxia was very similar. Anoxia resulted in significant increases in NADH accompanied by a decline in NAD^+ and a 12% loss of total pyridine nucleotides in both the epi- and endothelium of the cornea.[116] Remarkably, the P potential was little affected in either the epithelial and endothelial layers, implying a substantial glycolytic reserve.[116] A similar assessment of the metabolic state of the corneal epi- and endothelium was made from the qualitative changes in fluorescence measured by the technique of redox fluorometry.[116] The significance of these findings to the development of diabetic complications in both the epithelium and endothelium of the cornea is probably best reflected in the observations of both Winegrad and his group[49] and of Williamson and his colleagues[47] of the ability of 1 m*M* pyruvate to prevent the alterations in the redox state and glycolytic activity and changes of blood flow and vascular permeability that are caused by exposure to chronically high glucose levels. Application of quantitative histochemistry as well as noninvasive redox fluorometry could provide important insights into the biochemical basis of diabetic complications.

Even though knowledge of the biochemical basis of the diabetic lesion of the corneal epithelium and endothelium is scarce, several pertinent observations have been made. The enzymes of the polyol pathway have been localized to the epithelial and endothelial layers of the cornea.[117] As is true for other tissues afflicted by diabetic complications, glucose transport across the epithelium appears to be insulin insensitive.[118] The insulin sensitivity of endothelial cell transport is not yet known. Glucose and sorbitol have been demonstrated to accumulate in the corneal epithelium incubated with 35 m*M* glucose in vitro[118] or isolated from alloxan-diabetic rabbits.[119] Prompted by these observations, our laboratory applied the sensitive techniques of quantitative histochemistry to evaluate biochemical manifestations of diabetes in the corneal layers of the alloxan-diabetic rabbit.

Acute diabetes of 17 days duration resulted in the marked accumulation of sorbitol in the epithelium, stroma, and endothelium of the rabbit cornea (Ref. 52; Figure 6A). However, despite evidence of enhanced polyol pathway activity, which was most marked in the stroma and endothelium, myoinositol levels remained close to normal or were elevated relative to the myoinositol content of the corneal layers isolated from nondiabetic controls (Ref. 52; Figure 6B). After 70 days of alloxan diabetes, rabbits were characterized as severe, requiring insulin to prevent ketosis and dehydration, or as mild, not requiring insulin. The aqueous humor concentrations of sorbitol and glucose were markedly elevated in both groups of

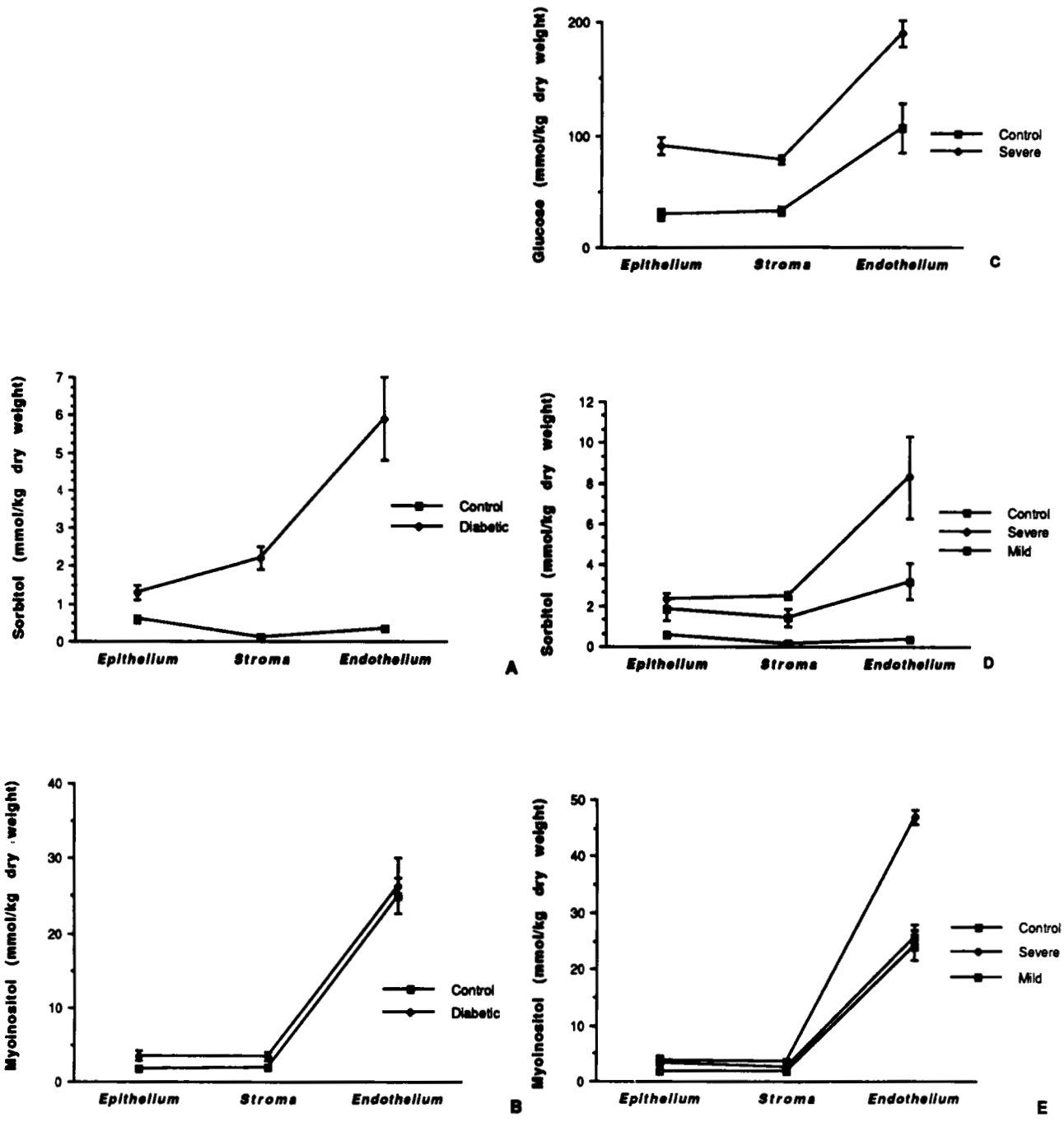

Figure 6. Glucose, sorbitol, and myoinositol levels in the corneal layers of rabbits with experimental diabetes of 17–70 days duration. The corneal epithelium, stroma, and endothelium were microdissected from lyophilized sections of the cornea from control and diabetic rabbits characterized in Table I. Glucose, sorbitol, and myoinositol were measured in corneas of acutely diabetic (**A, B**) and chronically diabetic (**C–E**) rabbits with mild or severe manifestations of the disease (Matschinsky et al., 1987).[52]

diabetic rabbits, thus as with the retina providing an in situ exposure to both elevated extracellular sorbitol and glucose, with the former now recognized to be cytotoxic in *tissue culture* preparations (Refs. 82–84; Table I). Both mildly and severely diabetic rabbits had elevated levels of glucose and sorbitol in the epithelial, stromal, and endothelial layers of the cornea (Ref. 52; Figures 6C and D).

Table I. Effect of Acute and Chronic Experimental Diabetes on Glucose, Sorbitol, and Myoinositol Levels of the Serum and Aqueous Humor of the Alloxan-diabetic Rabbit

	Experimental condition			
			70 Days	
Parameter measured	Control	17 Days	Severe	Mild
Serum				
Glucose (m*M*)	13.0 ± 1.3	30.5 ± 3.5	39.4 ± 3.0	30.0 ± 2.1
Myoinositol (m*M*)	0.077 ± 0.008	ND	0.053 ± 0.005	0.045 ± 0.004
Aqueous humor				
Glucose (m*M*)	7.9 ± 0.7	ND	29.2 ± 3.0	21.7 ± 9.0
Sorbitol (m*M*)	0.084 ± 0.007	ND	1.11 ± 0.06	0.68 ± 0.2
Myoinositol (m*M*)	0.22 ± 0.01	ND	0.20 ± 0.01	0.21 ± 0.01

Note: The data represent the mean ± SEM of samples obtained from 4–9 animals. ND represents not determined. Fasted, white New Zealand rabbits were administered alloxan (125 mg/kg, i.v. in water). They were considered diabetic 3 days after injection if fasting blood glucose levels exceeded 18 m*M*. With chronic diabetes, rabbits were divided into severe and mild groups based on their dependence or lack of dependence on insulin to prevent ketosis and dehydration. At 17 and 70 days, blood was collected from the anesthetized rabbits, and the eyes were removed for dissection and microanalysis (Matschinsky et al., 1987).[52]

The myoinositol content of the cornea was unaffected in the presence of intracellular accumulation of glucose and sorbitol in the corneal layers of the mildly diabetic rabbits (Figure 6E). Paradoxically, the myoinositol content of the corneal epithelium, stroma, and endothelium of the severely diabetic rabbits rose twofold despite pronounced elevations of cellular glucose and sorbitol (Ref 52; Figure 6E). The effects of diabetes on Na^+ handling and ATPase activity have not yet been assessed in the corneal layers in situ. The connection between these biochemical changes and the inability of the corneal layers to withstand trauma and yet maintain the appropriate degree of hydration for unimpeded vision in the absence of stress is far from understood.

VII. SUMMARY

Biochemical evidence of glucose toxicity was found in the retinal and corneal layers of diabetic rabbits. It can be reasonably assumed that the observed changes are causally related to the morphological and physiological diabetic pathologies of the retinal and corneal cells. Intracellular glucose is greatly increased, and the polyol pathway activity appears to be enhanced, resulting in an accumulation of intracellular sorbitol, which can be assumed to be oxidized to fructose. Accompanying the alterations of glucose metabolism are disturbances in myoinositol and Na^+ handling by the affected structures. The detailed relationship of the observed metabolic effects of hyperglycemia to changes in cellular ion handling and the observed morphological and functional disturbances has yet to be elucidated. The morphologically and functionally discrete populations of RPE and CEN cells, which are readily amenable to experimental manipulation *in situ* and *in cell culture* may serve as unique models for systematic examination of the causes and the consequences of diabetes leading to ocular complications in particular and to the complications of other more complex tissues such as nerve and kidney. The present data show that the findings in one population of cells may not be completely reproducible in another as can be seen in the diverse myoinositol responses of the retinal and corneal layers to diabetes mellitus. The diverse responses perhaps reflect unique adaptive capabilities of individual tissues to the diabetic condition. It is a challenge for complications research to fully appreciate diverse responses of various tissues to persistent glucose intoxication and to delineate meticulously the time courses of such heterogeneous responses, which might result in debilitating pathology in certain cases but in a compensated chronic disease state in others. The corneal endothelium and the RPE are relatively resilient structures compared with the mural and endothelial cells of the retinal microvessels which are destroyed by the diabetic condition. Factors and components that protect tissues against the persistent effects of hyperglycemia need to be uncovered. Success in such an endeavor could be of benefit in the management of diabetic complications.

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