Na^+-K^+-ATPase Pumping Activity Is Not Directly Linked to *myo*-Inositol Levels After Sorbinil Treatment in Lenses of Diabetic Rats

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SUMMARY

Changes in tissue levels of sorbitol, *myo*-inositol, and Na^+-K^+-ATPase enzyme activity have been implicated in the development of diabetic complications in animal models of the disease and in humans. The ability of the aldose reductase inhibitor sorbinil to reverse the hyperglycemia-induced changes in these lenticular metabolite and enzyme-activity levels in the streptozocin-induced diabetic rat was examined to determine what, if any, relationship exists between these changes. Two weeks of untreated diabetes did not change ouabain-inhibitable ATPase enzyme activity assayed in lens homogenates but did result in a decrease in the Na^+-K^+-ATPase transport activity as measured by ^{86}Rb uptake in the intact lens. This was accompanied by a 100-fold increase in the levels of sorbitol and significant decreases in the levels of *myo*-inositol, ATP, and glutathione in the lens. Whereas all of these changes could be reversed by sorbinil treatment, the dose required for restoration of the depleted *myo*-inositol level ($ED_{50} > 20$ mg · kg^{-1} · day^{-1}) was much higher than the dose required to reverse the other changes (ED_{50} range 2–5 mg · kg^{-1} · day^{-1}). These results suggest that the restoration of lenticular Na^+-K^+-ATPase activity is not secondary to a normalization of *myo*-inositol levels and may provide evidence that the two parameters are not strictly associated in diabetic tissues. *Diabetes* 36:1414–19, 1987

In diabetic tissues that are not dependent on insulin for glucose transport, elevated intracellular glucose levels lead to various metabolic alterations that have been proposed to play a role in the pathogenesis of diabetic complications. These include increased levels of sorbitol and fructose and decreased levels of *myo*-inositol, reduced glutathione, ATP, and Na^+-K^+-ATPase activity (1–11).

Several models have been proposed to explain how these metabolic alterations lead to the observed functional deficits in diabetic tissues that are important to the development of diabetic complications. Studies on the development of diabetic cataract led to the "osmotic hypothesis," which proposes that the accumulation of sorbitol, resulting from the action of the enzyme aldose reductase on the excess glucose, leads to osmotic swelling of the tissue and eventual membrane disruption. Other metabolic alterations were proposed to be secondary to these events (1). Support for this model comes from the use of aldose reductase inhibitors such as sorbinil in animal models of diabetic complications. Thus, with sorbinil it has not only been possible to prevent the occurrence of diabetic cataract but also to prevent and/or reverse many of the biochemical and functional deficits that occur in the nerve, kidney, and retina of the diabetic rat (2,4,6,8,9,11–13).

A second model, based on the effects of diabetes on peripheral nerve, suggested that diabetic neuropathy resulted from an intracellular deficit of *myo*-inositol (7,14). This deficiency was proposed to lead to an alteration in the metabolism of the phosphoinositides and thereby interfere with the normal structural and functional properties of the neural membrane. Studies utilizing dietary *myo*-inositol supplementation in which normal nerve function was maintained in diabetic animals lend support to this proposal. In addition, *myo*-inositol supplementation has been reported to prevent functional and metabolic changes in other diabetic tissues, e.g., the lens, kidney, and retina (15–17).

More recently, the observation that treatment of streptozocin-induced diabetic (STZ-D) rats with aldose reductase inhibitors can prevent the fall in nerve *myo*-inositol has led to a new proposal that integrates these two models in that the polyol-pathway flux during periods of hyperglycemia is at least partly responsible for the fall in intracellular levels of *myo*-inositol (7,18). This *myo*-inositol deficiency in turn leads to other metabolic abnormalities, including a fall in

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the Na^+-K^+-ATPase levels, which are thought to give rise to the observed functional deficits in the nerve (10). This construct accounts for the observed effects of both *myo*-inositol supplementation and aldose reductase inhibitor treatment and provides a mechanistic basis for the involvement of polyol-pathway activity in the pathophysiology of diabetic complications in tissues where sorbitol accumulation is not sufficient to exert a significant osmotic effect. However, the evidence for this model, particularly in tissues other than the nerve, is largely circumstantial.

The purpose of these studies was to test the validity of the latter model in the ocular lens of the STZ-D rat. Previous studies have demonstrated that hyperglycemia causes a rapid increase in the lenticular levels of sorbitol and fructose accompanied by a decline in the levels of *myo*-inositol, ATP, reduced glutathione, and Na^+-K^+-ATPase (1,8). The lens not only demonstrates the metabolic changes seen in other tissues, it is a particularly useful model in assessing the interrelationships because the biochemical changes are large and develop quickly compared with other tissues such as nerve. In these studies, the ability of the aldose reductase inhibitor sorbinil to reverse these changes was used to probe the interrelationships between the loss of *myo*-inositol and the fall in the levels of glutathione, ATP, and Na^+-K^+-ATPase.

MATERIALS AND METHODS

Animal studies. Male Sprague-Dawley rats weighing ~200 g (Charles River, Wilmington, MA) were maintained with free access to food (Prolab R-M-H 3000, Agway, Syracuse, NY) and water. Diabetes was induced by injection of STZ (85 mg/kg body wt, Upjohn, Kalamazoo, MI) in 0.01 M citrate buffer, pH 4.5, into the tail vein of fed rats. This dose of STZ produced severe hyperglycemia, with blood glucose levels ranging between 400 and 550 mg/dl. Sorbinil (CP-45,634, Pfizer, Groton, CT) was administered as an aqueous suspension by oral gavage at the indicated times and doses. The eyes were removed immediately after the animals were killed by cervical dislocation, and the lenses were dissected free from the eyeglobe with a posterior approach. Lenses used in the ^{86}Rb uptake and efflux experiments were immediately placed in TC199 medium (Gibco, Grand Island, NY). For studies on the Na^+-K^+-ATPase activity, the epithelium was peeled from the lens and homogenized in 0.1 ml of buffer containing 50 mM imidazole (pH 7.5), 0.1 mM EDTA, and 1 mM dithiothreitol. In the case of assaying whole-lens N^+-K^+-ATPase activity, the whole lens was homogenized in 1 ml imidazole buffer as described above. Sorbitol, *myo*-inositol, ATP, and glutathione were determined on lens extracts prepared by homogenizing each lens in 1.0 ml of 6% perchloric acid (PCA) followed by centrifugation and neutralization of the resultant supernatant with potassium carbonate. ED_{50} is defined as the dose of compound that can reverse 50% of the difference between normal and untreated diabetic rats.

Lens culture. Ouabain-sensitive Na^+-K^+-ATPase pump (or transport) activity was calculated from the initial rate of ouabain-inhibitable ^{86}Rb uptake. Lenses were preincubated for 10 min at 37°C in a TC199 medium in the presence or absence of 1 mM ouabain to allow equilibrium to be established. The medium was then replaced with a TC199 medium containing 34,000 counts per minute (cpm)/ml of ^{86}Rb (6.6 mCi/mg, New England Nuclear, Boston, MA), with and without 1 mM ouabain, and the lenses were incubated for another 30 min. Uptake was terminated by removal of the radioactive medium. The lenses were washed twice with ice-cold saline, dissolved overnight in 500 μl of 2.5% sodium dodecyl sulfate, and analyzed by liquid scintillation counting.

To determine the rate of ^{86}Rb efflux, lenses were preloaded by incubating at 37°C for 4 h in TC199 medium containing 1.1×10^6 cpm/ml ^{86}Rb. The lenses were rinsed once with 1.0 ml of TC199 medium containing 1 mM ouabain and then incubated with the same medium. Aliquots of the medium were removed at 0, 30, and 50 min, and the ^{86}Rb content was determined by liquid scintillation counting.

Analytical techniques. Protein was determined by the method of Lowry et al. (19). Plasma glucose was determined on an ABA 200 analyzer with the Glucose-UV clinical chem-

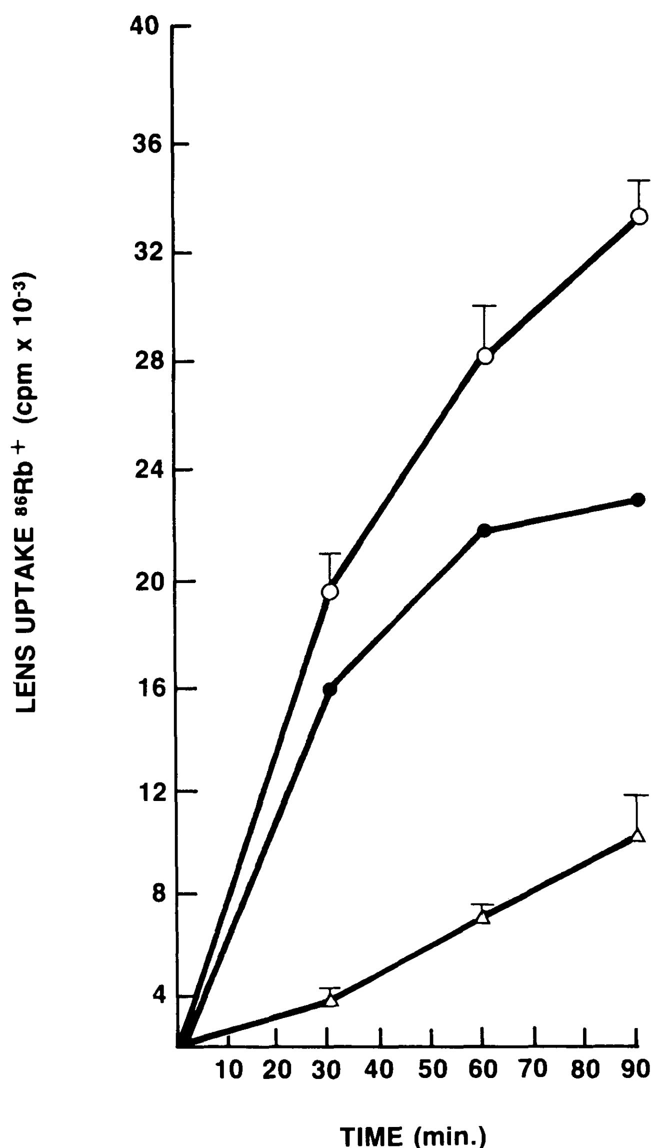

FIG. 1. Time course of lens ^{86}Rb uptake. Lenses were incubated in TC199 medium as described, and ^{86}Rb uptake was assessed over indicated periods in presence (△) and absence (○) of 1 mM ouabain. Ouabain-inhibitable uptake (●) was calculated as difference between measurements.

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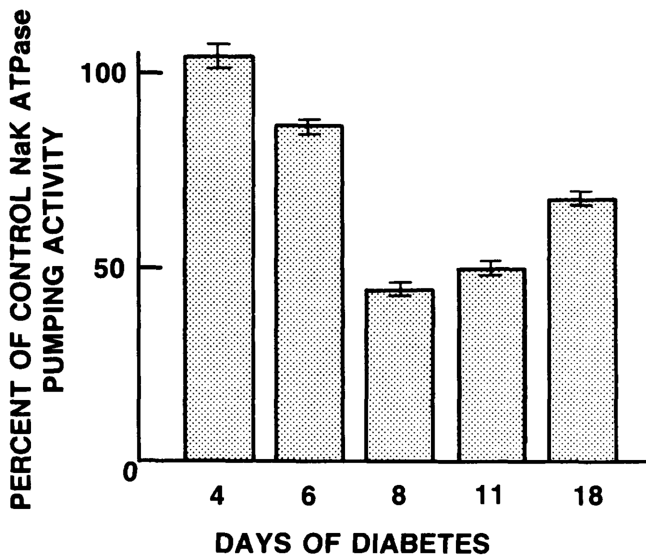

FIG. 2. Effects of diabetes on ouabain-inhibitable Na^+-K^+-ATPase–dependent ^{86}Rb uptake. Rats were made diabetic by single intravenous injection of streptozocin, and ouabain-inhibitable ^{86}Rb uptake was determined as described in MATERIALS AND METHODS at indicated time after induction of diabetes. Data are presented as percent of age-matched controls killed on same day.

istry reagent kit (Abbott, North Chicago, IL). Sorbitol in the PCA extracts was quantitated with sorbitol dehydrogenase as previously described (20). Na^+-K^+-ATPase enzymatic activity was measured by the pyruvate kinase–lactate dehydrogenase coupled assay as described by Cantley et al. (21) in the presence of 100 mM NaCl, 2.5 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES-Tris (pH 7.4), 1.4 mM phosphoenol pyruvate, 0.26 mM NADH, 10 μg/ml pyruvate kinase, and 10 μg/ml lactic dehydrogenase, at 37°C. Na^+-K^+-ATPase is defined as the difference between activities measured in the presence and absence of 1 mM ouabain. Omission of Na^+ from the assay gave a similar inhibition of enzyme activity as that caused by addition of ouabain.

Glutathione was determined with a modification of the method of Hissin and Hilf (22). An aliquot of 25 μl of the 6% PCA lens extract was added to 1.975 ml of sodium phosphate buffer (0.1 M, pH 8) containing 5 mM EDTA. The fluorescence (350 nm excitation, 420 nm emission) of the sample was determined 15 min after the addition of 100 μl of *o*-phthalaldehyde. ATP was measured by the high-performance liquid chromatography method of Anderson and Murphy (23). A sample of the 6% PCA lens extract was diluted with an equal volume of the mobile phase buffer (0.05 M ammonium phosphate, pH 6.0), and 10 μl were injected onto the column. *myo*-Inositol in the PCA extracts was determined by fluorimetrically monitoring the reduction of NAD by *myo*-inositol dehydrogenase (20). A 0.3-ml aliquot of the PCA lens extract was added to 1.0 ml of 0.1 M glycine buffer (pH 9.5) containing 0.5 mg/ml of bovine serum albumin and 1.2 mM NAD. The reaction was initiated by addition of 60 mU of *myo*-inositol dehydrogenase (Sigma, St. Louis, MO). After 30 min at room temperature, the fluorescence of the sample was determined (366 nm excitation, 425 nm emission) and compared with a standard curve covering the range 0–20 nmol of *myo*-inositol. Data are reported as means + SE. Student's *t* test was used to determine statistical significance.

RESULTS

Effects of diabetes on lenticular Na^+-K^+-ATPase and Na^+-K^+ pump activity. Previous studies have demonstrated that Na^+-K^+-ATPase is localized almost exclusively in the lens epithelium (24–26). The Na^+-K^+-ATPase enzymatic activity in the lenses of normal control rats and 2-wk diabetic rats were compared in both epithelium and whole

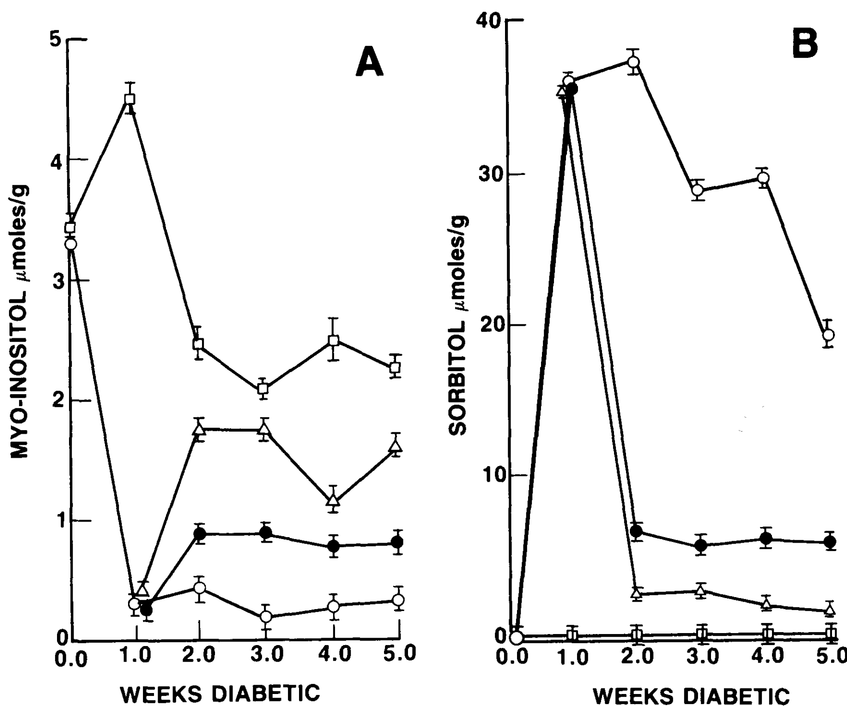

FIG. 3. Time course of effects of sorbinil on lens *myo*-inositol and sorbitol levels in diabetic rats. Rats were made diabetic as described in Fig. 2. One week later rats received sorbinil by oral gavage at a dose of either 5 (●) or 25 (△) mg · kg^{-1} · day^{-1}. Untreated diabetic rats were run in parallel experiments (○). Rats were killed at indicated times. Lens levels of *myo*-inositol (*A*) and sorbitol (*B*) were determined. Levels of lens *myo*-inositol in age-matched normal rats are also included (□).

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lens. There was no significant difference in either epithelium (normal 0.0675 ± 0.014 vs. 2-wk diabetic 0.068 ± 0.007 μmol/h) or whole lens (normal 0.079 ± 0.005 vs. 2-wk diabetic 0.096 ± 0.008 μmol/h) in terms of ouabain-inhibitable ATPase activity. Although there was considerable variability in the level of Na^+-K^+-ATPase activity level depending on the age of the rats, there was no significant effect of diabetes on this activity.

The transport activity of the Na^+-K^+-ATPase in the intact lens was determined as the ouabain-inhibitable uptake of ^{86}Rb. As shown in Fig. 1, this transport in a normal lens was linear for up to 60 min at 37°C and accounted for 70% of the total ^{86}Rb uptake determined in the absence of ouabain. The effects of STZ-D on this activity are shown in Fig. 2. The ouabain-sensitive uptake in diabetic lenses is expressed as a percent of the activity in age-matched nondiabetic controls. After 8 days, this activity was decreased by 50%. Although this effect was less marked at 18 days, the transport activity in the diabetic lenses remained significantly below that of the controls.

Previous studies have demonstrated that chronic diabetes leads to a disruption of the lens epithelial membrane and increased permeability to cations (1). To determine if the measured rates of ^{86}Rb uptake by the cultured lens were influenced by leakage of the ion, the efflux of ^{86}Rb from preloaded lenses was examined. There was no significant difference in the rates of efflux (means ± SE of 4 determinations) from diabetic (21.5 ± 6.8 vs. 23.1 ± 13.9) and normal (27.0 ± 9.8 vs. 29.8 ± 33.0) lenses during the first 2 wk after STZ administration. These ensure that the rates of ^{86}Rb uptake were a measure of the Na^+-K^+-ATPase transport activity not complicated by changes in ^{86}Rb efflux. All subsequent uptake determinations were performed on 2-wk diabetic animals.

Time course of metabolic changes in diabetic lens. Several investigators have demonstrated that treatment of diabetic animals with sorbinil can prevent or reverse the hyperglycemia-induced changes in sorbitol and *myo*-inositol levels in the rat lens, but the time course of these effects has not been well documented (8,27). To determine an appropriate time frame for the dose-response studies, the sorbitol and *myo*-inositol contents of diabetic lenses were examined at 1-wk intervals (Fig. 3). Under these conditions, maximal changes in both sorbitol and *myo*-inositol levels caused by diabetes are observed by 1 wk. After initiation of sorbinil treatment, new levels of both metabolites are achieved by 1 wk and do not change significantly during the next month. Based on these results, an experimental protocol involving 1 wk of untreated diabetes followed by 1 wk of sorbinil treatment was employed in our studies.

Dose response for sorbinil in reversing diabetes-induced changes in lens metabolites. In earlier studies on the involvement of the polyol pathway in the pathophysiology of diabetic cataracts, it has been demonstrated that sorbinil can prevent the increase in sorbitol and the decrease in *myo*-inositol, ATP, and reduced glutathione observed in the diabetic lens. These studies have employed relatively high levels of sorbinil that were thought to bring about maximal changes in these parameters. The purpose of these studies was to measure the dose response to sorbinil for each of the metabolites and the Na^+-K^+-ATPase transport activity over a wide range of sorbinil concentrations to determine if there is a causal relationship between their changes in diabetes (Table 1). After 2 wk of diabetes, there was a 100-fold increase in the level of sorbitol in the lens. This accumulation of sorbitol was reversed by sorbinil with an ED_{50} of ~2 $mg \cdot kg^{-1} \cdot day^{-1}$. The diabetic lenses also exhibited a 20% decrease in Na^+-K^+-ATPase pump activity (decreases up to 50% have been observed in other experiments). These changes were accompanied by a 70% decrease in reduced glutathione, an 80% decrease in oxidized glutathione, and a 30% decrease in ATP. Each of these changes displayed a similar sensitivity to reversal by sorbinil. The ED_{50} for each was <2.5 $mg \cdot kg^{-1} \cdot day^{-1}$, and in each case, near-normal

TABLE 1
Effects of sorbinil on sorbitol, *myo*-inositol, Na^+-K^+-ATPase pumping activity, glutathione, and ATP levels in streptozocin-induced diabetic rats

Sorbinil dose (mg/kg)	*myo*-Inositol (μmol/g)	Sorbitol (μmol/g)	^{86}Rb transport (counts/min)	Reduced glutathione (μmol/g)	Oxidized glutathione (μmol/g)	ATP (μmol/g)
Normal control	2.312 ± 0.07	0.391 ± 0.04	4115 ± 211	2.48 ± 0.11	0.46 ± 0.02	2.91 ± 0.13
Untreated diabetic	0.407 ± 0.13*	38.264 ± 1.78*	3285 ± 275†	0.78 ± 0.04*	0.09 ± 0.01*	2.11 ± 0.15‡
2.5	0.619 ± 0.06§	15.591 ± 2.65*§	4662 ± 263‡	1.99 ± 0.10*§	0.32 ± 0.02*§	2.99 ± 0.15‡
5.0	0.650 ± 0.08§	10.018 ± 1.12*§	3910 ± 288	2.31 ± 0.08*	0.29 ± 0.02*§	3.24 ± 0.25‡
10.0	0.974 ± 0.12*§	3.555 ± 0.83*‖	4326 ± 178‡	2.50 ± 0.36‡	0.36 ± 0.05*¶	3.12 ± 0.11‡
20.0	1.121 ± 0.15‡§	2.804 ± 0.55*‖	4644 ± 361‡	2.61 ± 0.10*	0.37 ± 0.03*¶	3.01 ± 0.15‡

Data are means ± SE of 4–8 separate values. All values for diabetic animals are significantly different from normal control values at confidence levels indicated.
*$P < .001$, †$P < .05$, ‡$P < .01$; except where noted, all values for sorbinil-treated animals are significantly different from untreated diabetic values at confidence levels indicated.
§$P < .001$, ‖$P < .01$, ¶$P < .05$; except where noted, all values for sorbinil-treated animals are significantly different from normal control values at confidence levels indicated.

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levels were achieved with 5 mg · kg^{-1} · day^{-1}. In contrast, the 80% decrease in *myo*-inositol observed in the diabetic lenses was much less sensitive to reversal by sorbinil, with an ED_{50} of >20 mg · kg^{-1} · day^{-1}. Even at a dose of 50 mg · kg^{-1} · day^{-1}, the *myo*-inositol levels were significantly less than those of nondiabetic control subjects (control, 2.312 ± 0.07 μmol/g tissue; 2-wk diabetic lens, 2.018 ± 0.06 μmol/g tissue).

DISCUSSION

The results presented herein suggest a dissociation between the effects of diabetes on the ATPase enzymatic and transport activities of the Na^+-K^+-ATPase in the rat lens. Several previous studies have reported a decrease in the enzymatic activity during sugar cataractogenesis (24,25,28), but that effect was not observed in this study. The reasons for this discrepancy are not clear but may be due to differences in experimental protocol. The earlier studies utilized long-term diabetic animals or galactosemic animals, both of which display significant tissue degeneration during the course of the experiment. In contrast, our studies were designed to examine the early biochemical events that may be more likely related to the primary pathologic lesion.

The decrease in pumping ability in the absence of detectable changes in the ATPase activity may reflect changes in the levels of the intracellular substrates for the enzyme rather than a decrease in the number or activity of the ATPase molecules. The accumulation of sorbitol leads to an increase in the water content of the lens, which could decrease the intracellular Na^+ concentration. This has been previously reported (1). In addition, as shown in Table 3, diabetes leads to a decrease in the level of ATP in the lens. These factors could act synergistically to bring about the observed decrease in the ^{86}Rb pumping activity of the lens. However, the inability to detect a change in the ATPase activity does not rule out the possibility that diabetes causes an irreversible inactivation of the enzyme. Based on the ATPase activity of the lens and a turnover number of 10,000/min (from purified Na^+-K^+-ATPase isolated from several tissues; 29), it is possible to calculate the concentration of ATPase molecules as 0.77 pmol/lens. In contrast, assuming that two K^+ are transported for each molecule of ATP that is hydrolyzed (30), it appears that the concentration of active pumping sites is only 0.1 pmol/lens or 13% of the total ATPase molecules. This seemingly small percentage of total Na^+-K^+-ATPase that is functional at any one time in the cell has also been reported for adipocytes (31). Thus, a 50% decrease in pumping ability in the diabetic lens could conceivably result from a 7% decrease in the total Na^+-K^+-ATPase activity. Such a decrease would probably not be detected in our studies. On the other hand, a large loss of total cellular Na^+-K^+-ATPase activity could occur without losing functional ATPase pumping activity. Thus, changes on total Na^+-K^+-ATPase should be interpreted with caution because they may not necessarily translate to changes in the amount of functional enzyme in the tissue.

The extent to which these biochemical changes are related to the formation of diabetic cataract is unknown. In the galactosemic animal, osmotic stress due to the accumulation of galactitol is probably the primary pathologic factor in sugar cataractogenesis. However, in the diabetic animal, where the onset of the cataract is much more protracted (4–6 wk vs. 4 days in galactosemic animals), the other biochemical changes that occur in the lens are probably involved in the process of cataractogenesis. Indeed, Ross et al. (32) have reported that administration of reduced glutathione to diabetic rats can prevent or delay the early morphologic changes observed in the lens, suggesting that the depleted levels of glutathione may play a key role in the formation of the diabetic cataract. The decrease in both the reduced and oxidized forms of glutathione suggest that the loss of reduced glutathione in the diabetic lens is at least partly due to a decrease in the rate of synthesis and/or increased rate of breakdown rather than a decreased ability to reduce the oxidized glutathione. Because glutathione biosynthesis is ATP dependent, the loss of glutathione may be secondary to the observed decrease in ATP levels. Cataractogenesis is characterized by abnormal distribution of Na^+ and K^+ across the fiber cell membranes (1). The changes in the Na^+-K^+-ATPase transport observed in this study could contribute to this condition in the diabetic lens. Again, these changes could be secondary to the decrease in lenticular ATP.

It is also significant that each of the above factors has been implicated in the pathophysiology of senile cataract in humans (33–36) and that genetic or chemically induced deficits in the Na^+-K^+-ATPase, reduced glutathione, or ATP levels will lead to cataract development in experimental animals (37–40). Thus, although these changes may only play a contributing role to the formation of diabetic cataract, they probably represent the primary pathogenic factors in the greatly increased incidence of senile cataract in the diabetic population (41).

It is evident that restoration of the glutathione and ATP levels and the Na^+-K^+-ATPase transport activity by sorbinil are not mediated by restoration of lens *myo*-inositol levels (Table 1). At 5 mg · kg^{-1} · day^{-1} of sorbinil, these parameters were nearly normalized in the diabetic lens, whereas the depleted levels of *myo*-inositol were unaffected. Thus, it seems unlikely that membrane alterations due to abnormal metabolism of phosphoinositides lead to the early biochemical changes that occur in the diabetic lens, although restoration of a small *myo*-inositol pool cannot be totally ruled out. However, Bucci et al. (15) have demonstrated that dietary *myo*-inositol supplementation can dramatically decrease the development of cataract in STZ-D rats (15). These results suggest that reversal of the biochemical changes brought about by aldose reductase inhibitor treatment and the prevention of functional changes observed with *myo*-inositol supplementation are mediated by independent mechanisms.

The relationship among flux through the polyol pathway, depletion of cellular *myo*-inositol, and the biochemical and functional changes observed in other diabetic tissues remains unknown. Obviously, because *myo*-inositol supplementation or aldose reductase inhibitor treatment can reverse many of these changes in the nerve, retina, and kidney and treatment with aldose reductase inhibitors can prevent loss of *myo*-inositol in these tissues, restoration of *myo*-inositol may be a key factor in the action of these inhibitors. However, in light of the results obtained in the lens, the protective effects of the two treatments may be brought

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about by distinct mechanisms, and the ability of sorbinil treatment to restore *myo*-inositol levels may be a secondary unrelated phenomenon. Examination of the time course of these changes in each of the tissues involved in diabetic complications and of the relative sensitivity of each of these changes to reversal by aldose reductase inhibitors may shed some light on this question.

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