Sorbitol pathway in the ciliary body in relation to accumulation of amino acids in the aqueous humor of alloxan-diabetic rabbits

V. N. Reddy, B. Chakrapani, and D. Steen

The activity of the enzymes, aldose reductase and polyol dehydrogenase, associated with sorbitol pathway were determined in the rabbit ciliary body and compared with the activity of these enzymes in the lens and the retina, a tissue which has the same embryologic origin as the ciliary body. Despite the presence of the two enzymes, no sorbitol could be detected in the ciliary body of diabetic rabbits. These results suggest that the accumulation of sorbitol in the ciliary body and presumably in the ciliary epithelial cells, which are responsible for transport, is not a contributory factor in the reduction of amino acid levels observed previously in the aqueous humor of these animals. The reasons why sorbitol does not accumulate in the ciliary body of diabetic rabbits despite the presence of the necessary enzyme systems are discussed.

Key words: sorbitol pathway, aldose reductase, polyol dehydrogenase, sorbitol, ciliary body, lens, retina, aqueous humor, alloxan diabetes, amino acid transport

Previous studies in this laboratory have shown that the rates of accumulation and the steady-state concentration of amino acids in the lens and aqueous humor are reduced in diabetic animals. The reduced capacity of diabetic lenses to accumulate amino acids has been ascribed to both a decrease in active transport and an increase in the permeability of the surface membranes of the lens as a secondary result of high concentration of sorbitol. van Heyningen has shown that sorbitol accumulates in lenses when the animals are made diabetic by alloxan administration. Since the lens membranes are relatively impermeable to sugar alcohols, the accumulation of these compounds leads to osmotic swelling of the lens, thus affecting the permeability of the membranes. If such an accumulation of sorbitol were to occur in the ciliary epithelial cells, it could account for the depressed level of amino acids in the aqueous humor of diabetic animals.

This communication deals with a study that indicates the presence of sorbitol pathway in the ciliary body, since both of the enzymes involved in this pathway, namely...
Sorbitol pathway and amino acid accumulation

aldose reductase and polyol dehydrogenase, are present in the tissue. However, sorbitol does not appear to accumulate in the ciliary body of diabetic animals, suggesting that the accumulation of this polyol in the ciliary epithelial cells is not a contributory factor in the reduction of amino acid levels observed previously in the aqueous humor of these animals.1, 2

Methods

All tissues used were obtained from albino rabbits weighing 1.8 to 2.3 kilograms. Diabetes was induced by injection of alloxan as described in a previous paper. Plasma glucose levels of injected animals were followed at frequent intervals, and only the animals which showed established hyperglycemia were used in the study. They were killed approximately eight to nine days after the initial injection of alloxan with the blood sugar values ranging from 350 to 700 mg. per cent.

For the enzymatic assays, ciliary body—iris, retina, and lenses from several normal rabbits were removed immediately after the death of the animals, weighed, and pooled separately. The tissues were homogenized in three volumes (w/v) of 0.01M sodium bicarbonate solution at 0° C. The homogenates were dialyzed for 16 to 20 hours against 4 L. of deionized water containing 10⁻⁴M β-mercaptoethanol. After dialysis the contents of the tubes were adjusted to a pH of 7.4 with 0.01M ammonium hydroxide and centrifuged for 20 minutes at 12,800 x g. The supernatants were removed and assayed spectrophotometrically for aldose reductase and polyol dehydrogenase. Except for the enzymatic assay, all operations subsequent to the removal of the tissues were carried out in a cold room at a temperature of 0 to 4° C.

Aldose reductase was assayed by a combination of the methods described by van Heyningen and by Hayman and Kinoshita. The experimental and the blank cuvette each contained 10 μmoles of tris hydrochloride, pH 7.4, 0.7 μmoles of nicotinamide adenine dinucleotide phosphate, reduced (NADPH), and the tissue extract. The reaction was started by adding 5 μmoles of α-glyceraldehyde as the substrate to the experimental cuvette. The final volume of the reaction mixture was 3.0 ml.

Polyol dehydrogenase assay, based on the reduction of nicotinamide adenine dinucleotide (NAD), was carried out according to Chakravorty and Horecker, except that phosphate buffer was substituted for glycine buffer and the final volume of the reaction mixture was 3.0 ml. The reaction was started by adding xylitol as substrate.

In the two assays, the change in absorbance at 340 mλ was followed at one-minute intervals. Protein content of the enzyme preparations was determined by the method of Lowry and associates with bovine serum albumin as standard. A unit of activity was defined as a change in optical density of 0.001 per minute.

α-Glyceraldehyde and xylitol were chosen as substrates for aldose reductase and polyol dehydrogenase, respectively, because of the low Km values of these compounds to the corresponding enzymes.

The level of hexoses and hexitols in the tissues of normal and diabetic animals was determined by gas-liquid chromatography (GLC) following their conversion to trimethylsilyl derivatives in the following manner: The tissues were homogenized in Nelson-Somogy reagent and homogenates centrifuged at 12,800 x g. Aliquots of the clear supernatant were transferred to test tubes and lyophilized. The contents of the tubes were further dried in a vacuum desicator and stored until ready to be used. The free sugars and sugar alcohols present in the dried extracts were converted to their trimethylsilyl derivatives by the method of Sweeley and associates. The silylated compounds were never used beyond 48 hours after preparation. Sample volumes ranging from 0.5 to 3.0 μl were chromatographed using a Becker-Delft GLC instrument. A stainless steel column, 4 feet 2 inches x ¼ inch outside diameter, containing Chrom W 50-100 mesh as a solid support and five per cent SE-30 (obtained from The Anspec Co.) as a stationary phase was employed. Purified nitrogen at a flow rate of 100 ml. per minute was used as a carrier gas. The chromatographic column was “conditioned” at 180° C. for 24 hours initially and two to four hours for subsequent uses. The samples were chromatographed at an injection port temperature of 120° C. and a programmed temperature of 140° to 170° C. per 0.4° C. per minute. Quantitation was done by comparing the peak heights of the compounds in the unknown sample with those of standards of freshly prepared silylated compounds of glucose, fructose, galactose, inositol, and sorbitol which were chromatographed simultaneously.

Results

Both of the enzymes involved in the sorbitol pathway, namely aldose reductase and polyol dehydrogenase, appear to be present in the ciliary body (line I, Tables I and II). For the purpose of comparison, the activities of these two enzymes were also assayed in the lens and the retina, a tissue of the same embryologic origin as the ciliary body (lines 2 and 3, Tables I and II).
Table I. Activity of aldose reductase in ocular tissues of normal rabbit

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity (units*/Gm. wet weight)</th>
<th>Specific activity (units/mg. protein)</th>
<th>Specific activity (units/unit of absorbance at 280 με)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliary body</td>
<td>133 (118-152)†</td>
<td>4.4 (3.4-5.2)</td>
<td>8.6 (5.5-11.3)</td>
</tr>
<tr>
<td>Retina</td>
<td>195 (122-236)</td>
<td>2.4 (1.7-2.9)</td>
<td>2.8 (1.6-3.7)</td>
</tr>
<tr>
<td>Lens</td>
<td>317 (235-392)</td>
<td>1.0 (0.7-1.4)</td>
<td>1.6 (1.0-2.2)</td>
</tr>
</tbody>
</table>

*A unit of enzyme activity is defined as a change in absorbance of 0.001 per minute under the conditions of the assay. All values represent average of three separate experiments with the range indicated in parentheses.

Table II. Activity of polyol dehydrogenase in ocular tissues of normal rabbit

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity (units*/Gm. wet weight)</th>
<th>Specific activity (units/mg. protein)</th>
<th>Specific activity (units/unit of absorbance at 280 με)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliary body</td>
<td>9.8 (6.6-13.8)†</td>
<td>0.31 (0.19-0.39)</td>
<td>0.61 (0.39-0.85)</td>
</tr>
<tr>
<td>Retina</td>
<td>9.1 (5 -11.3)</td>
<td>0.12 (0.06-0.15)</td>
<td>0.13 (0.07-0.18)</td>
</tr>
<tr>
<td>Lens</td>
<td>7.1 (4.4-12 )</td>
<td>0.02 (0.01-0.03)</td>
<td>0.03 (0.03-0.04)</td>
</tr>
</tbody>
</table>

*A unit of enzyme activity is defined as a change in absorbance of 0.001 per minute under the conditions of the assay. All values represent average of three separate experiments with the range indicated in parentheses.

Table III. Carbohydrate content of ocular tissues from normal and alloxan-diabetic rabbits

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ciliary body</td>
<td>Retina (6)</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.13 ± 0.06</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.03 ± 0.01</td>
<td>0.00 ± 0.07</td>
</tr>
<tr>
<td>Inositol</td>
<td>2.39 ± 0.50</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>1.32 ± 0.40</td>
<td>Trace</td>
</tr>
</tbody>
</table>

The values given are as μmoles per tissue normalized for the average weight of each tissue; ciliary body 45 mg., retina 73 mg., lens 300 mg.

On the basis of wet weight, the activity of aldose reductase in the ciliary body is lower than that in the retina and in the lens. However, the specific activity of the enzyme expressed on the basis of milligrams of protein or absorbance at 280 με is considerably higher in the ciliary body than in the other two tissues. The lower enzymatic activity expressed on a wet weight basis might be due to incomplete extraction of the enzyme from the ciliary body since there is a considerable amount of stroma in this tissue which is not completely homogenized in the assay procedure used.

The activity of polyol dehydrogenase in the ciliary body is approximately the same as in the other two tissues examined when the results are expressed on a wet weight basis. The specific activity in the ciliary body, however, is some 15 to 20 times higher than that in the lens. It may also be noted that the activity of the polyol dehydrogenase in the ciliary body as in the other two tissues is lower than that of aldose reductase.

In order to determine whether the level of sorbitol or any other carbohydrate increases in the ciliary body of diabetic animals, the carbohydrate levels in this tissue from both normal and diabetic rabbits were measured. The results of such estimations are shown in comparison with the corresponding values in the retina and in the lenses from the same animal (Table III). In the normal ciliary body as well as the retina, only glucose and inositol could be detected. Even in the diabetic animal, no
sorbitol was found in the ciliary body (or the retina), although there was a significant increase in the level of glucose. In diabetic lenses, in agreement with previous observations, there was a considerable increase in the level of sorbitol, glucose, and fructose and a decrease in the level of inositol.

Discussion

The sequence of reactions leading to the conversion of glucose to fructose via sorbitol involves the enzymes aldose reductase and polyol dehydrogenase and is generally referred to as sorbitol pathway (Scheme 1).

Since the first demonstration by Hers that placenta and seminal vesicles contain an enzyme that catalyzes the reduction of a variety of aldoses to their corresponding alcohols, the enzyme aldose reductase has been shown to be widely distributed in mammalian tissues. The second enzyme in the sorbitol pathway, polyol dehydrogenase, is also found in a number of mammalian tissues. The cells containing aldose reductase are potentially capable of converting glucose to sorbitol if the plasma level of aldose is abnormally elevated. The role of the aforementioned enzymes in the accumulation of sorbitol and fructose in diabetic lenses has been well documented.

The results of the present study show that both aldose reductase and polyol dehydrogenase are also active in the ciliary body. What is surprising, however, is that despite the presence of these enzymes, sorbitol could not be detected in this tissue from either normal or diabetic rabbits.

While the principal purpose of this investigation was to seek a correlation between polyol content of the ciliary body in diabetic rabbits and the previously observed decrease in amino acid accumulation in the aqueous humor, it is nevertheless interesting to consider why sorbitol does not accumulate in the ciliary body of diabetic rabbits despite the presence of the necessary enzymatic mechanism. The possibility that the sorbitol formed may simply leak out of the tissue seems unlikely since the cell membranes appear to be relatively impermeable to this compound. The reason for the absence of sorbitol in the ciliary body could also be due to the possibility that even in diabetic animals there may not be sufficient concentration of glucose present in the ciliary epithelial cells to produce significant quantities of sorbitol since it is well known that glucose is a very poor substrate for the enzyme aldose reductase and has a high Km value.

Still another possibility for the lack of accumulation of sorbitol in the ciliary body of diabetic animals may be that the sugar alcohol is readily converted by the polyol dehydrogenase to fructose, which could then be further metabolized. A comparison of the relative activities of aldose reductase to those of polyol dehydrogenase in the ciliary body, the retina, and the lens indicates that the ratio of aldose reductase to polyol dehydrogenase is highest for the lens and lowest for the ciliary body, with the retina having an intermediate value. Thus the relatively higher activity of polyol dehydrogenase in the ciliary body would favor the conversion of sorbitol to the corresponding keto-sugar.

Another important consideration in the formation of sorbitol from glucose is the characteristic metabolism of the tissue involved. The factors that favor sorbitol formation in a particular tissue are considered to be low hexokinase activity, high aldose reductase, and a high pentose shunt activity. It appears that the NADPH produced in the pentose shunt reaction is reoxidized in the conversion of glucose to sorbitol by aldose reductase as shown in Scheme 2.

\[ \text{Glucose} \xrightarrow{\text{Aldose reductase}} \text{Sorbitol} \xrightarrow{\text{Polyol dehydrogenase}} \text{Fructose} \]
Unlike the lens which has an active pentose shunt mechanism with limiting hexokinase reaction, the ciliary body has low pentose shunt activity. This difference in the metabolism of the ciliary body and the lens combined with the relatively higher activity of polyol dehydrogenase could conceivably account for the lack of accumulation of sorbitol in the former tissue. Whatever the mechanism that is responsible for the absence of sorbitol in the ciliary body and presumably in the ciliary epithelial cells of diabetic animals, it is clear that the mechanism by which the accumulation of amino acids is reduced in the aqueous humor of diabetic animals is not analogous to that operative in the lens. More specifically, the decrease in accumulation of amino acids in the aqueous humor of diabetic animals is not brought about by the hydration of ciliary epithelial cells as a result of osmotic effect of polyol accumulation, but probably due to an impairment of active transport mechanisms. Whether impairment of active transport in the ciliary body of diabetic animals is due to changes in energy metabolism or some other factors remains a subject for further investigation.

REFERENCES

13. Varma, S. D., and Reddy, V. N.: Phospholipid composition of aqueous humor, plasma and


