NONENZYMATIC GLYCOSYLA'TION, SULFHYDROLY
OXIDATION, AND AGGREGATION OF LENS PROTEINS
IN EXPERIMENTAL SUGAR CATARACTS*

By V. M. MONNIER, V. J. STEVENS,‡ AND A. CERAMI

From the Laboratory of Medical Biochemistry, The Rockefeller University, New York 10021

In studies of human cataracts, Dische and Zil (1) first noted that the proteins of the human cataractous lens were associated with a marked increase in sulphydryl oxidation to form disulfide bonds. The correlation of disulfide bonds and cataract formation has been combined subsequently by a number of other workers studying animal and human cataracts (2–6). These disulfide bonds can act as intermolecular crosslinks and have been proposed to be partly responsible for the polymerization of the lens proteins in the formation of high molecular weight aggregates. Protein aggregates >150 × 10^6 daltons have been detected in aged normal lenses as well as in human cataracts (7, 8) and recently a disulfide-linked high molecular weight protein associated with the fiber cell membrane has been isolated from human cataracts and characterized (9). One of the physical characteristics of protein aggregates of this size is their ability to scatter light. Based on theoretical consideration, Benedek (10) has hypothesized that the formation of protein aggregates of mol wt > 50 × 10^6 daltons could scatter light sufficiently to account for cataract formation in the lens. This is substantiated by the work of Spector and colleagues (11) who found that light backscattering of the lens parallels the increase in high molecular weight protein in the aging lens.

In contrast to studies of human senile cataractogenesis, little is known of the physical state of the lens proteins of the sugar cataracts formed in diabetes and galactosemia. Preliminary evidence of electron dense aggregates and a decrease in low molecular weight proteins have been reported in cataracts of galactosemic rats and point to a potential role of protein aggregates in sugar cataract formation (12–14). The prevalent theory to account for the cataract formation in these experimental models is the accumulation of polyols (sorbitol and galactitol) in the cells which is believed to cause osmotic swelling, vacuolization, and rupture of lens fibers (15). However, the mechanism responsible for the formation of light scattering elements after the putative osmotic shock have not been demonstrated. We have recently proposed that in the diabetic cataract of the rat, the aggregation of lens proteins via disulfide bonds is accelerated after the increased nonenzymatic glycosylation of proteins under hyperglycemic conditions (16).

In the present work, we have extended our in vitro studies to an examination of the lens proteins of cataracts from diabetic and galactosemic rats for the presence of

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nonenzymatic glycosylation of e-amino groups of lysine residues and high molecular weight protein aggregates linked by disulfide bonds. In addition, the effect of reducing agents on the light scattering properties of protein solution from cataracts was also studied.

Methods

Chemicals. Dithioerythritol, Tris, and alloxan were obtained from Sigma Chemical Co., St. Louis, Mo. Dimercapto succinic acid, thioglycolic acid, penicillamine, and glutathione were supplied by Aldrich Chemical Co., Inc., Milwaukee, Wis. β-mercaptoethanol was obtained from Eastman Kodak Co., Rochester, N. Y. Samples of purified and isotopically labeled Adenovirus and SV 40 virus were kindly provided by Dr. J. M. Blanchard. β-galactose was obtained from Eastern Chemical, Div. of Guardian Chemical Corp., Hauppauge, N. Y.

Cataractous Lenses. Lenses were obtained from normal, diabetic, and galactosemic female Sprague-Dawley rats (Taconic Farms, Germantown, N. Y.). Diabetes was induced by intravenous injection of a freshly prepared solution of alloxan (40 mg/kg body weight, dissolved in 0.01 M sodium phosphate buffer, pH 3.5) into female rats (60 g). Galactosemic cataracts were induced by feeding female rats (100 g) with a diet (Purina Lab Chow) containing 33% β-galactose. Nuclear cataracts appeared 8-10 wk after the onset of diabetes or 3-5 wk after galactose feeding. The lenses were tested immediately after appearance of the first nuclear opacities.

Rat Lens Suspension. Fresh rat lenses were decapsulated and placed into a test tube containing 1.5 ml buffer A (Tris-HCl 0.1 M, pH 8.3) per lens. The buffer had been previously deaerated and equilibrated with nitrogen. The lenses were gently teased apart with Pasteur pipettes and stirred with a stream of nitrogen applied at the surface of the liquid until a homogenous suspension of the cataractous proteins was obtained (~45 min). Loss of liquid through evaporation was replaced. After disruption of the lens, insoluble material was allowed to settle by gravity for 60 min. The supernate was used for the experiments.

Absorbance of Light by Rat Lens Suspension. The effect of reducing agents on the absorbance of light by cataractous rat lens suspension was measured at 550 nm using a Zeiss PM6 spectrophotometer (Carl Zeiss, Inc., New York). A 0.75-ml aliquot of the lens suspension was placed into a 1-cm quartz cuvette and the reducing agent, dissolved in buffer A, was added to a final concentration of 50 mM. The solution was rapidly mixed and the absorbance was measured at regular intervals. The control and reduced samples of the experiments were performed simultaneously on the same batch of lens proteins.

Sucrose Density Gradient Centrifugation. Sucrose density gradients of 5-60% were prepared according to the procedure of Takemoto and Azari (6). 500 μl of the rat lens suspension (with or without 30 mM dithioerythritol (DTE))1 were layered on the top of the gradient which in addition to the sucrose contained buffer A (with or without 30 mM DTE). The samples were centrifuged at 25,000 rpm for 18 h at 20°C using a SW 27 rotor in a Spinco model L2-65N (Div. of Beckman Instruments, Palo Alto, Calif.) ultracentrifuge. The tubes were perforated at the bottom with a 20-g needle and 6-drop fractions were collected. 1 ml of buffer A was subsequently added to each tube and the optical density measured at 280 nm. When DTE was present, each fraction was dialyzed twice (Spectrapor No. 3 tubing) against 4,000 ml buffer to remove the DTE, allowing the protein concentration to be measured at 280 nm.

Gel Filtration Chromatography. After centrifugation at 400 g for 10 min at room temperature, an aliquot of 500 μl of the supernate of lens proteins from normal and cataractous lens was loaded onto a Sepharose 2B column (40 × 1 cm) (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) previously equilibrated with Tris-HCl buffer 0.1 M, pH 8.6. The column was eluted with this same buffer at a flow rate of 4 ml/h. The absorbance at 280 nm was monitored in the fractions (1.2 ml) to which 50 μl of NaOH, 10 M, were added to prevent spurious readings through light scattering. The void volume of the column was determined with blue dextran.

1 Abbreviation used in this paper: DTE, dithioerythritol.
Quantification of Glycosylated Lysine Residues. Each cataractous or normal lens was homogenized in 0.05 M sodium phosphate buffer, pH 7.0. The homogenate was separated into soluble and insoluble fractions by centrifugation at 6,500 g for 45 min., and the fractions were reduced with 2 mg tritiated NaBH₄ (8 μCi/mmol). This both labeled the glycosyl adduct and prevented its dissociation. Excess borohydride was removed by dialysis at 4°C for 3 d.

Modified lysine residues were detected by amino acid analysis with a Beckman model 119C analyzer (Beckman Instruments, Inc., Fullerton, Calif.) (16). Stream division was used for the recovery of radioactive products. The elution time of ε-amino galactosyl lysine (Nε-1-(1-deoxygalactitolyl)-lysine) was the same as the glucosyl adduct whether it was obtained from a standard synthesized from α-t-boc-lysine (17), or from an acid hydrolyzed sample of lens crystallins which contained 14C-galactose.

The relative amounts of modified lysines were determined by measuring the tritium incorporated into the glycosylated lysine residues after NaB₃H₄ reduction. The fraction of lysine residues which were modified was estimated from peak areas of the chromatograms. Protein concentration was measured by the method of Lowry et al. (18) using bovine serum albumin as a standard.

Results

The formation of sugar cataracts has been hypothesized to involve the nonenzymatic glycosylation of the ε-amino groups of lysine residues on crystallins, followed by the formation of high molecular weight protein aggregates.

The amount of glycosylated lysine residues was measured in normal and cataractous lenses from galactosemic and diabetic animals (Table I). Lysine residues in both the soluble and insoluble proteins of the cataractous lenses were modified to a much greater degree than the normal lenses. Four to six times more tritium was incorporated into the crystallins of the galactose cataracts. This corresponds to the modification of 7–8% of the lysine residues or 0.3 mol/mol protein (assuming a mol wt of 20,000 daltons/subunit). At the time of cataract formation in the diabetic animals, the amount of modified lysines was 8–14 times that found in the control lenses. This represents the modification of 10–15% of the total lysine residues or 0.6 mol/mol protein.

To investigate the mechanism responsible for light scattering in the cataractous lenses of diabetic and galactosemic animals it was necessary to prepare a lens homogenate containing the scattering elements as close to their original state as possible. The use of a tissue homogenizer on normal rat lens leads to the formation of tissue fragments which scatter light (Fig. 1 a). This artifactual opacity renders the preparation unsuitable for analysis of the light scattering elements. However, if a normal rat lens is teased smoothly apart in a deaerated buffer stirred by a stream of nitrogen, the insoluble material will settle and a clear solution of lens proteins (Fig. 1 b) is obtained. This method was used to prepare diabetic or galactosemic cataracts. A light scattering suspension of lens proteins was obtained from diabetic cataracts (Fig. 1 c). Upon addition of 30 mM DTE, a large part of the turbidity of the solution could be reversed (Fig. 1 d). A turbid solution which could similarly be reversed with DTE, was obtained from the cataractous lens of galactosemic rats (not shown). The effect of various reducing agents on the amount of light scattering in the suspension of cataractous lens proteins was studied by measuring the absorbance at 550 nm. DTE, followed by thioglycolic acid and penicillamine, were found to be the most effective in decreasing the scattering of light in solutions of lens proteins from diabetic cataracts (Fig. 2). No effect was obtained with dimercaptosuccinic acid and glutathi-
Radioactivity ($^3$H) per milligram protein associated with $N^6$-1-($\alpha$-dideoxyhexitolyl)lysine eluted from an amino acid chromatography column. Homogenized rat lenses were separated into soluble and insoluble fractions. Cataractous lenses from diabetic or galactosemic animals were compared with normal lenses fromagematched control animals. Values are mean cpm/mg protein ± SD, n = 5.

In galactosemic cataracts, both DTE and glutathione were effective in reducing the light scattering of the suspension (Fig. 3). This is in contrast with the proteins from diabetic animals where glutathione was not effective.

The effect of DTE was next tested on the separate soluble and insoluble fraction of the suspension obtained from galactose cataracts. After fractionation by centrifuging at 400 g, 10 min., the insoluble portion was suspended in 3 ml of deaerated buffer A containing Triton X-100 (Sigma, St. Louis, Mo.) and vortexed under nitrogen for 1 min. This procedure assured that protein aggregates were solubilized from any tissue fragments. The effects of 50 mM DTE on the absorbance (550 nm) of the soluble and insoluble fraction of the cataract were then measured. The cuvettes were constantly mixed between the measurements to prevent settling of insoluble material. As demonstrated in Fig. 4, the absorbance of the soluble and insoluble fractions from galactose cataracts could be decreased to 100% (base line) and 50%, respectively. The absorbance of the unreduced lens fractions remained constant. This experiment confirms the results obtained with the unfractionated preparation described first. Furthermore, it can be estimated that the disulfide-linked scattering of light contributed by the insoluble fraction (58% of total lens proteins) is 2.6 times greater than the soluble fraction (42% of total lens proteins).

The possibility that the light scattering of the cataractous suspension was a result of high molecular weight aggregates was next investigated. Protein aggregates varying in size from 36 to $176 \times 10^6$ daltons were detected with sucrose gradients in the suspension of proteins from galactose cataracts (Fig. 5). Similarly, high molecular weight species were detected in the suspension obtained from diabetic lenses. The addition of DTE (50 mM) to the solution of diabetic lens proteins resulted in a decrease in the amount of high molecular weight aggregates and a concomitant increase in the amount of low molecular weight materials (Fig. 6).

The presence of high molecular weight aggregates was also detected in the soluble
fraction of galactose cataracts with gel filtration chromatography on Sepharose 2B (Fig. 7). The protein eluting in the void volume of the column have a minimal size of ~40 million daltons. Again reduction with 50 mM DTE lead to the disappearance of the protein peak in the void volume indicating that these aggregates were linked by disulfide bonds. The high absorbance of low molecular weight species in Fig. 7 is due to the presence of DTE. Protein aggregates were not detected in the void volume of chromatograms of proteins from normal lenses.

Discussion

In a recent study on the potential role of nonenzymatic glycosylation for diabetic cataractogenesis we reported the formation of high molecular weight aggregates in solutions of bovine lens crystallins which had been incubated with glucose or glucose-6-phosphate. The absorbance at 550 nm of these solutions as well as the size of the aggregates could be reversed in part by treatment with mild reducing agents. We hypothesized that glycosylation enhanced the susceptibility of lens proteins to sulfhydryl oxidation. We have now extended our in vitro studies to cataractous lenses of diabetic and galactosemic rats.

The results presented here demonstrate the presence of protein aggregates of high molecular weight in experimental cataracts induced by both diabetes and galactosemia. These aggregates have a size of 40–176 million daltons and according to the theory of Benedek (10), are large enough to scatter light. They are in part linked by
FIG. 2. The effect of mild reducing agents (50 mM) on the absorbance at 550 nm of a suspension of lens proteins from cataracts of diabetic rat. GSH (glutathione), DMS (dimercaptosuccinic acid), PA (Penicillamine), TGA (thioglycolic acid), DTE (dithioerythritol). The control was incubated in absence of a reducing agent. Control and reduction experiments were performed simultaneously on the same batch.

FIG. 3. The effect of glutathione (○) and dithioerythritol (●) on the absorbance of a suspension of lens proteins from cataracts of galactosemic rats. The control (○) was incubated without reducing agent.

disulfide bonds as various reducing agents can decrease both the amount of light scattering and the amount of high molecular weight protein aggregates. The inability of reducing agents to achieve complete clearing of the solution and disaggregation of the aggregates can be explained by incomplete cleavage of disulfide bonds, noncovariant aggregation, or nondisulfide protein crosslinks. Covalent bonds other than disulfide bonds have been noted in human cataracts as well (6).

The mechanisms leading to sulfhydryl oxidation are of obvious interest for the formation of high molecular weight aggregates. One mechanism based on our observation in vitro (16) would be that the nonenzymatic glycosylation imparts to the lens proteins an increased susceptibility to sulfhydryl oxidation and the formation of disulfide bonds. It is possible that the modification of amino groups leads to a conformational change of the protein molecule and an unmasking of sulfhydryl groups. Other evidence supporting the importance of amino groups in maintaining the state of the crystallin molecules is the fact that chronic administration of cyanate, a carbamylating agent, leads to cataract formation in humans and experimental animals (19) that are morphologically similar to diabetic cataracts.

The nonenzymatic glycosylation is probably only one of several factors involved in cataract formation in diabetes and galactosemia. Indeed, the more rapid onset of the galactosemic cataract with less modification of lysine residues than in diabetes points
FIG. 4. The effect of 50 mM DTE (○) on the absorbance at 550 nm of the soluble and insoluble fraction from cataractous lenses of galactosemic rats. The control was incubated without DTE (●).

FIG. 5. The elution profile from sucrose gradient centrifugation of normal (○) and cataractous (●) lens from galactosemic rats. The lens supernate in 0.1 M Tris, pH 8.3, was layered on top of a 5-60% linear sucrose gradient. Centrifugation was performed for 18 h at 27,000 rpm at 20°C. Adenovirus and SV40 were used as molecular weight markers.
to the role of other factors in hastening cataractogenesis in galactosemia. The ability of the lens to maintain a reducing environment is probably critical for cataract formation and can significantly affect the time of onset of cataract formation. It would appear that in addition to leading to the glycosylation of lens protein, the galactosemic state causes a more rapid deterioration than the diabetic state in the ability of the cell to maintain glutathione levels which combat sulfhydryl oxidation and protein aggregation. The fact that aldose reductase inhibitors can retard but not prevent cataract formation suggests that these agents could retard the fall in gluta-
thione levels. However, the demand for reduced glutathione is eventually so overwhelming that protein aggregation proceeds. With this respect, the ability of aldose reductase inhibitors to scavenge radicals (20) could be of importance in maintaining glutathione levels. This is substantiated by the recent observation by Creighton and Trevithick (21) that glutathione and antioxidants could prevent cortical cataractogenesis under high glucose environment in vitro.

From the studies reported here it appears that once lens protein aggregates have formed, glutathione is not very effective in breaking protein-protein disulfide bonds in vitro. This points to the possible use of exogenous agents to prevent or decrease the opacity of cataracts in vivo.

Summary

The formation of sugar cataracts has been hypothesized to involve the nonenzymatic glycosylation, sulfhydryl oxidation, and aggregation of lens proteins. Cataractous lenses of diabetic and galactosemic rats were analyzed for glycosylated lysine residues in crystallins. A five- and a ten-fold increase in glycosylated lysine residues was measured in galactose and diabetic cataracts, respectively. The modification was predominant in the insoluble fraction of the lens homogenate.

The proteins were further examined for the presence of disulfide bonds and high molecular weight aggregates. After careful disruption of the lens in a nitrogen environment, a cloudy solution was obtained from cataractous lenses whereas a clear solution was obtained from normal lenses. The absorbance at 550 nm of the solution of both the galactosemic and the diabetic cataracts could be decreased by ~50% with the addition of dithioerythritol (50 mM). The presence of high molecular weight aggregates was ascertained by sucrose gradient centrifugation and gel filtration chromatography. The proteins were heterogenous in size and showed a mol wt range of 36 to >176 million daltons. Treatment with dithioerythritol induced a marked decrease in the amount of high molecular weight proteins.

These data suggest that sugar cataracts of experimental animals have, in common with human cataracts, the presence of high molecular weight aggregates which are in part linked by disulfide bonds.

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