Glycation and Insolubility of Human Lens Protein

Akira KAMEI

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468, Japan.
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To learn whether glycation plays a role in insolubilization or in senile cataractogenesis, the reactivity of lens protein from normal and senile cataractous lenses and individual crystallin prepared from human lens with various sugars [glucose, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P) and fructose], and the insolubility of those proteins were determined. The reactivity of human lens protein to glucose was increased in a dose-dependent manner, and it was demonstrated that 17.9, 18.5 and 24 kDa proteins were susceptible to glycation with sugars. The study also showed that α-, β-crystallins and high molecular weight (HMW) aggregate obtained from cataractous lens have some weak reactivity against sugars. It was demonstrated that the proteins obtained from normal lens of older age and from cataractous lenses have higher insolubilities to glucose than do normal younger ones. Measurement of glycosylated protein by affinity column chromatography revealed that cataractous lenses contained a larger amount of glycosylated protein than normal ones.

These results suggest that there is an age-related increase of glycation in normal human lens protein, and that such glycation increases the amount of insolubilized protein with the effect of aging. The author also speculates that an abnormal acceleration of glycation in the human lens may induce senile cataract formation.

Keywords normal human lens; lens protein; crystallin; cataractogenesis; senile cataract; glycation; aging; protein insolubilization

Glycation of lens protein with various sugars in the lens is now suspected to be one of the main causes of lens protein aggregation or insolubilization, and its coloration in aging.[1] Glycation, formerly called non-enzymatic glycosylation or the Maillard reaction, is a post-synthetic modification of protein. Glycation is particular concern in an individual with elevated blood sugar level (diabetes mellitus, galactosemia) and may play a role in secondary complications of diabetes such as atherosclerosis and cataract.[2] It is further suggested that the senile cataract may be caused by the stimulation of such glycation.[3]

I previously reported following glycation of lens protein with various sugars in the human lens during aging.[3] These facts were estimated by the measurement of 1-deoxyfructosyl adduct and the fluorescent material that has been identified as these products in the middle stage of the Maillard reaction.[4] The lens contains a large amount of protein, approximately 35% to the total wet weight of the whole lens, and the protein was divided roughly into three types of crystallins: α-, β- and γ-crystallin. It is suggested that the modification of crystallins or changes of their characterization may be closely related to the lens protein insolubilization or to lens opacification, and that such proteins may be vulnerable to attack from glucose or other sugars, because they are exceptionally long-lived in the lens.[5] Thus, many investigators have studied the relationship between changes in characterization of crystallins and glycation.

The current paper reports the reactivity of each crystallin with various sugars and the involvement in the lens protein insolubilization by sugars. In addition, the possible role of glycation in the onset of cataract is discussed.

Materials and Methods

Lens Materials The normal and cataractous human lenses of different colors were kindly donated by Dr. J. Horwitz (Jules Stein Eye Institute, University of California, Los Angeles). Upon removal, these lenses were stored at −80 °C until use.

Materials D-[1-^{14}C]Glucose (2.4 GBq/mmol), D-[^{14}C(U)]glucose-1-phosphate (11.5 GBq/mmol), D-[1-^{14}C]glucose-6-phosphate (2.1 GBq/mmol) and D-[^{14}C(U)]fructose (12.7 GBq/mmol) were purchased from DuPont Company.

Preparation of Soluble Protein and Crystallin Fractions from Human Lens The soluble protein fraction of human lens was prepared according to the method described previously.[6] Briefly, after a decapsulated human lens was homogenized in 1.5 ml of 0.2 M phosphate buffer, pH 7.4, in an ice bath, the suspension was centrifuged at 17000 rpm for 30 min at 4 °C. The supernatant was used as a soluble protein fraction. All work was done on a clean bench using tools sterilized in an autoclave or by dry air sterilization.

Each human lens crystallin fraction was prepared by high performance liquid chromatography (HPLC) according to the modified method of Asselbergs *et al.*[7] Briefly, an aliquot of the soluble protein fraction was filtered through a membrane filter with a pore size of 0.45 μM. The filtrate was gel-chromatographed on a TSKG3000SW (0.45 × 60 cm) column and each fraction obtained was submitted to re-chromatography on the same column. The fraction at each peak position was pooled and used as purified crystallin. The final yiedls of purified samples, high molecular weight (HMW) protein, α-, β- and γ-crystallins were 6.3, 4.2, 7.4 and 5.4 mg per lens, respectively.

Reactivity of Lens Protein with Sugars This reaction was performed according to the modified methods of other investigators.[8] The reactivities of lens protein with sugars were determined from the uptake of [^{14}C]sugars into the protein in a glass tube with a sterilized stopper. In this experiment, 0.1 μmol of labeled sugar plus 9.9 μmol of unlabeled sugar were added to 2 mg of the lens proteins in 1 ml of 0.2 M phosphate buffer (pH 7.4), and the solution was slowly shaken at 37 °C. The reaction period was different according to the individual experiment. After the reaction, the reactant was treated with 1 mg of $NaBH_4$ for 30 min. The unreactive sugar was removed from the reactant by PD-10 column chromatography using 0.2 M phosphate buffer containing 0.1 M NaCl and 0.2% sodium dodecyl sulfate, pH 6.8, as an eluent. The reactivity of lens protein with sugar was determined from the radioactivity of the [^{14}C]labeled sugar incorporated into the protein.

Measurement of Insolubility of Human Lens Protein by Sugars Human lens protein (2 mg) and various concentrations of sugars were reacted in 2 ml of 0.2 M phosphate buffer, pH 7.4, at 37 °C under sterilized conditions. After an aliquot of the reactant had been taken out at regular intervals, the insolubility was determined from the turbidity of the reactant at 500 nm.

Assay of Glycosylated Protein in Human Lens The amount of glycosylated protein was determined with a Glyc-Affin-GHb kit, Seikagaku Kogyo, Ltd., Tokyo.[9]

Other Analyses The radioactivity of the incorporated [^{14}C]labeled sugar into the protein was measured with a Packard TRI-Carb liquid

scintillation counter using a conventional method. The protein concentration was measured by the method of Bradford.[10]

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli using 16% acrylamide gel of 1 mm thickness.[11]

HPLC was performed on a TSKG3000SW (0.45 × 60 cm) column. As an eluent, 0.2 M phosphate buffer containing 0.2 M NaCl, pH 7.2, was used with protein absorption monitored at 280 nm.

Results

Reactivity of Human Lens Protein with Glucose To determine suitable experimental conditions, 2 mg of human lens protein was reacted with the various concentrations of glucose containing 1% of [^{14}C]glucose at pH 7.4. Glucose concentrations are given in Fig. 1. The reactivity of 2 mg of lens protein with glucose was measured by the radioactivity of [^{14}C]glucose incorporated into the protein, and increased in a dose-dependent manner until 0.02 mmol of glucose. The reaction was almost maximal under the concentration conditions of more than 0.02 mmol glucose and 28-d reaction time as shown in the figure.

Then, the distribution changes in the molecular weight of the proteins reacted with glucose were analyzed by SDS-PAGE as illustrated in Fig. 2. According to the increase in glucose concentration, the proteins with molecular weight of 24, 18.5 and 17.9 kDa disappeared from the soluble fraction, while on the contrary, the protein with 25.4 kDa molecular weight appeared in the reactant to the contrary. This suggests that the three protein species that disappeared are susceptible to the glycation in the lens proteins.

Reactivity of Human Lens Protein with Various Sugars The experiments were carried out under the conditions of 0.02 mmol sugar containing 1% of [^{14}C]sugar and a 28-d reaction period. These conditions were based on the result of the experiment described above. As to the typical sugars observed in the glucose metabolism *in vivo*, the reactivities of their sugars with the protein obtained from normal and cataractous human lenses were determined. Reactivities of glucose, fructose and glucose-6-phosphate (G-6-P) with the protein of normal lens were almost similar and 2 to 3-fold higher than that of glucose-1-phosphate (G-1-P) as shown in Fig. 3. The incorporation of labeled sugars into

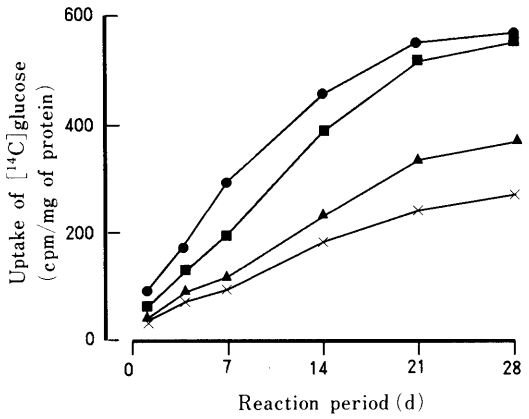

Fig. 1. Effect of Glucose Concentration on Reactivity of Human Lens Protein

Various amounts of glucose containing 1% of [^{14}C]glucose were each added to 2 ml of reaction solution: ×, 0.005 mmol glucose; ▲, 0.01 mmol glucose; ■, 0.02 mmol glucose; ●, 0.04 mmol glucose. The experiment data represent the mean of three separate experiments with different normal lenses of 50 year aged group.

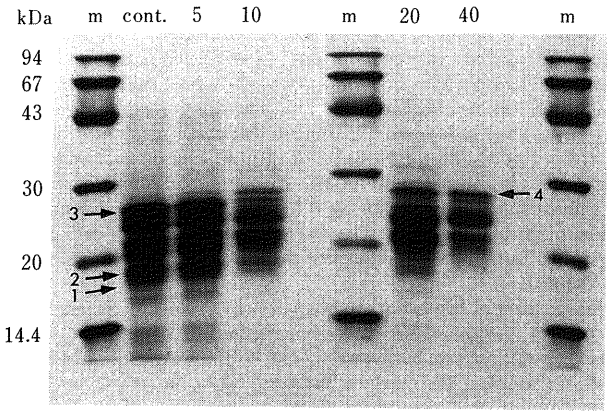

Fig. 2. SDS-PAGE of Soluble Fraction of Reactants at 28 d of Reaction Time

The numerical values at the top of each column of the chromatogram show the concentration (μmol) of glucose in 2 ml of reaction solution. m, indicates a marker. Four arrows, 1, 2, 3 and 4 indicate the position of proteins with 17.9, 18.5, 24 and 25.4 kDa, respectively.

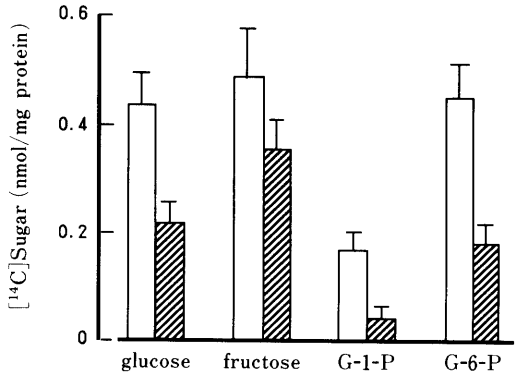

Fig. 3. Incorporation of [^{14}C]Labeled Sugars, Glucose, Fructose, G-1-P and G-6-P, into Soluble Protein of Normal and Cataractous Human Lenses

Each bar represents the mean ± S.D. of three separate experiments using different lenses of the 50 aged group. Detailed experimental conditions are described in the text. □, normal lens protein; ▨, yellow cataractous lens protein.

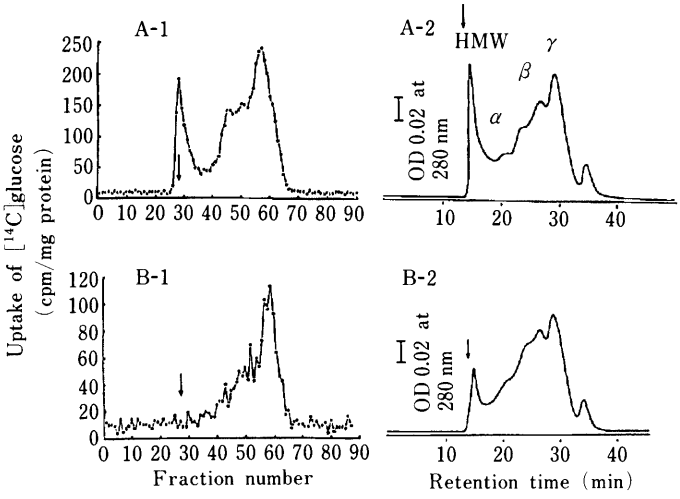

Fig. 4. HPLC Patterns of Protein of Normal and Cataractous Human Lens and Distribution of Glucose Incorporated in Lens Protein

These patterns are the reactant of 28 d. The arrows on the chromatograms indicate the position at the same retention time. HMW protein, α, β and γ indicate the position of HMW and each crystallin eluted by HPLC. Only yellow cataractous lenses were used.

TABLE I. Amino Acid Composition (%) of Soluble Protein of Human Lens

Amino acid	Normal		Cataractous[c]
	40[a]	70[b]	
Asp	9.74	9.83	9.83
Thr	4.13	4.02	3.95
Ser	7.66	7.85	7.97
Glu	14.46	14.70	14.74
Gly	9.21	9.23	9.26
Ala	5.36	5.40	5.44
Val	5.35	5.48	5.60
Cys	0.58	0.33	0.24
Met	2.44	2.77	2.85
Ile	4.88	4.87	5.00
Lue	7.45	7.58	7.63
Tyr	5.68	5.88	5.99
Phe	6.17	6.22	6.26
Lys	4.98	4.12	3.20
His	3.44	2.66	2.57
Arg	8.16	8.12	8.10
Pro	1.55	1.57	1.72
Total	101.24	100.63	100.35

The data represents the mean of two separate analyses. *a*) 40 aged-group, lenses of 42 and 45 year olds. *b*) 70 aged-group, lenses of 77 and 78 year olds. *c*) Yellow cataractous lenses of 46 and 50 year olds.

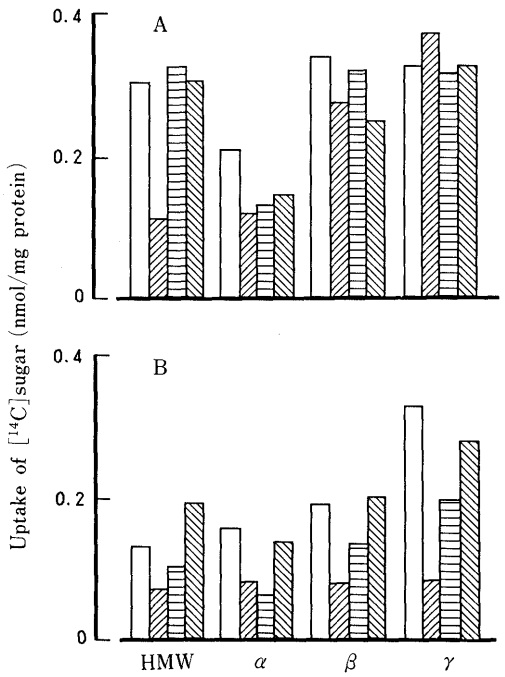

Fig. 5. Reactivity of Each Crystallin with Various Sugars, Glucose, G-1-P, G-6-P and Fructose

A and B show the uptake of sugars into crystallins of normal and yellow cataractous lenses, respectively. Detailed experimental conditions are described in the text. Each bar represents the mean of two separate experiments with the same crystallin fraction obtained from three different lenses of similar ages, 52 to 59 years old. □, glucose; ▨, G-1-P; ▤, G-6-P; ▧, fructose.

normal lens protein was always larger than that of cataractous lens protein, although there was considerable difference of incorporation rate.

Then, the distribution of incorporated sugars into the proteins were determined by HPLC. After 2 mg lens protein was reacted with 0.02 mmol sugar containing 1% of labeled sugar for 28 d, the reactant was chromatographed, fractionated and radioactivity of each fraction determined. Figure 4 shows patterns of the chromatogram and incorporation of glucose into the lens protein of normal (A-2 and A-1) and cataractous (B-2 and B-1) human lens. The uptake of glucose into the HMW aggregate of cataractous lens was almost negligible, while that into proteins with more than 24 kDa molecular weight ($t_R = 27.2$ min and fraction no. = 52), corresponding to α- and β-crystallins, was also lower than those of normal lens. A similar tendency was observed for other sugars, fructose, G-1-P and G-6-P (data not shown), although there was considerable difference of incorporation rate. As to this significant difference of reactivity with glucose between normal and cataractous lens proteins, the author's supposition is that in cataractous human lens glycation may progress further, and that the number of lysine and arginine residues reactive with sugar may decrease in the cataractous lens protein as compared to that of normal protein. To clarify whether this hypothesis is true or not, amino acid composition of the soluble proteins of normal and cataractous human lenses was analyzed. As shown in Table I, the content ratios of lysine and histidine residues apparently decreased in the protein of cataractous lenses and old aged normal lenses than in normal younger ones. This supports the hypothesis concerning a decrease in the number of amino acid residues.

Lens protein consists of various types of proteins, called crystallins. To investigate glycation of lens protein in detail, crystallins fractionated by HPLC were examined to determine their reactivity with sugars. Figure 5 shows the sugars incorporated into crystallins, and the radioactivity of the reactant was determined after the 28-d reaction at 37 °C. HMW protein, α- and γ-crystallins obtained from a normal lens incorporated preferentially sugars, although the incorporation of G-1-P into the HMW protein was approximatey 50% lower than those of others. The reactivities of crystallins obtained from cataractous lens were lower than those of normal lens, and glucose and fructose incorporated preferentially into the crystallins. This experiment also showed that the reactivities of glucose and fructose with any crystallin obtained from cataractous lens were almost 2-fold higher than those of G-1-P and G-6-P as shown in Fig. 5B.

Insolubilization of Human Lens Proteins by Various Sugars In this experiment, the relationship between glycation and insolubilization of lens protein was examined. First, insolubility of lens protein to various sugars was determined from the turbidity of reactant at 500 nm as shown in Fig. 6. G-6-P was revealed to be about twice as effective as glucose or fructose as a stimulator of lens protein insolubilization. G-1-P also insolubilized the lens protein, though its amount was significantly lower than those of other sugars. The insolubility of each crystallin to glucose, 0.01 mmol, was also determined under the condition of pH 7.4. Only γ-crystallin showed lower insolubility (Fig. 7). A significant difference of insolubility was observed between HMW proten and γ-crystallin; this phenomenon may be due to the difference in molcular weight. Insolubility of normal and cataractous human lens proteins by glucose as a function of age was then determined (Fig. 8). In all case of normal and cataract

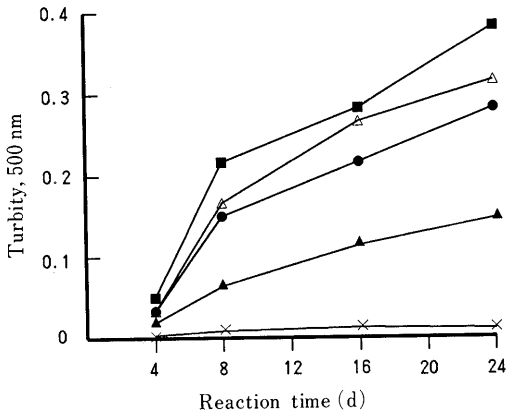

Fig. 6. Effect of Sugars on Insolubilization of Human Lens Protein

The reaction was done at 37 °C using 10 mg of normal lens protein and 0.02 mmol sugar in 2 ml of the reaction solution. The experimental data represent the mean of two separate experiments with two different normal lenses of similar age, a 54 and a 57 year old. ×, without sugar (control); ●, glucose; ▲, G-1-P; ■, G-6-P; △, fructose.

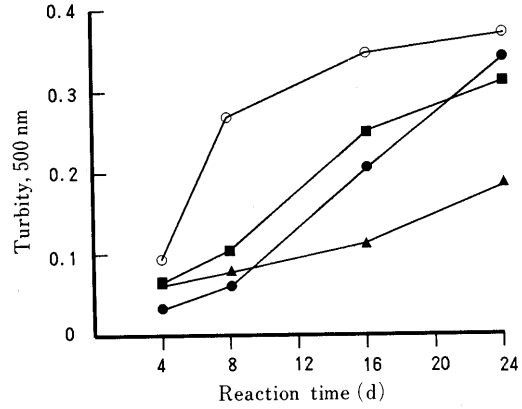

Fig. 7. Insolubility of Human Lens Crystallin Species by Glucose

The reaction was done using 5 mg of crystallin and 0.01 mmol glucose in 1 ml of the reaction solution. The experimental data represent the mean of two separate experiment with the same crystallin fraction obtained from three different lenses of similar age, 52 to 59 years old. ○, HMW; ●, α-crystallin; ■, β-crystallin; ▲, γ-crystallin.

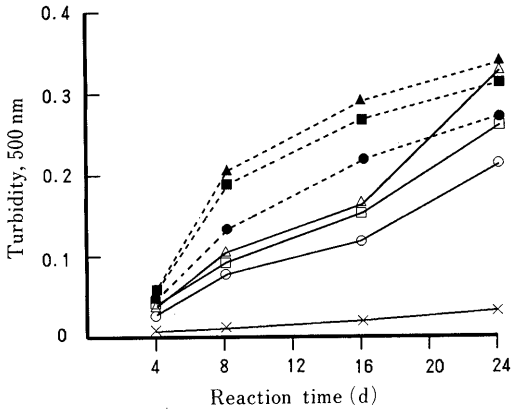

Fig. 8. Insolubility of Normal and Cataractous Human Lens Protein of Different Ages by Glucose

The reaction was carried out using 10 mg of human lens protein and 0.02 mmol glucose in 2 ml of reaction solution at pH 7.4. The experimental data represent the mean of two separate experiments with different lenses of the same aged group. ×, 40 aged-group, normal without glucose (as control); ○, 40 aged-group, normal; □, 60 aged-group, normal; △, 80 aged-group, normal; ●, 40 aged-group, cataract; ■, 60 aged-group, cataract; ▲, 80 aged-group, cataract. All cataractous lenses were yellow.

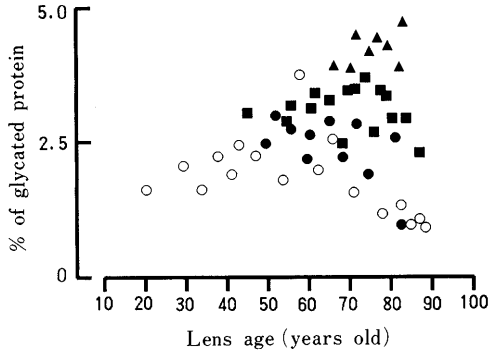

Fig. 9. Amount of Glycosylated Protein in Human Lens as a Function of Age or Advance of Coloration

The assay method is described in the text. Each dot is the percentage of glycated protein to total soluble protein of whole lens and the experimental value of individual normal and cataract lenses. ○, normal lens; ●, white or pale yellow cataractous lens; ■, yellow cataractous lens; ▲, brown cataractous lens.

lenses, the insolubility of the protein in older aged lens to glucose was always higher than that in a younger lens. It was also observed that the protein of the cataractous lens had a higher insolubility to glucose than that normal one.

Glycation of Human Lens Protein in Aging Finally, an attempt was made to determine the amount of glycosylated protein in human lens as a function of age. This experiment was done with a Glyc-Affin-GHb column that was developed to measure hemoglobin A_{1c} and has the affinity for glycosylated protein.[9] The percentage of glycosylated proteins to the total soluble protein in the normal lenses was less than 2.5%. The percentage of glycosylated protein in the cataractous lenses, on the other hand, was 2.5% to 5.0% of the total soluble protein of whole lens, and increased with the advance of coloration, yellow to brown, but not with aging. However, in the normal lenses the amount of glycosylated protein increased slightly until 50 years of age, and thereafter decreased during aging.

Discussion

The Maillard reaction or glycation has been detected in hemoglobin, erythrocyte membrane protein, collagen, albumin, serum protein and lens crystallin.[12] Glucosylation in particular, is a common posttranslational modification of body protein, in which glucose reacts directly with primary amino groups on protein to yield stable covalent adducts. Glycation in the lens protein is now suspected to be one cause of senile cataracts. Stevens *et al.* reported that a high glucose concentration *in vivo* or an increased glucose or G-6-P concentration *in vitro* leads the glycosylation of ε-amino groups of lysine residues in bovine and rat lens crystallins.[5d] *In vitro*, this glycosylation imparts an increased susceptibility of the crystallins to sulfhydryl oxidation. We reported that glycation of the lens protein may be one cause of human lens coloration in aging.[3a,b] McPherson *et al.*[13] and Awasthi and his colleagues[14] reported that glucose or fructose incubation with lens protein resulted in an increase in mean molecular weight of all crystallin fractions and the occurrence of a HMW aggregate partly formed by disulfide bonds. Furthermore, Chiou *et al.* reported that there is an age-related increase of non-enzymatic glycosylation in normal bovine

lens crystallins.[5c] Wolff and Dean also pointed out that glucosylation has been correlated with cataract formation as one complication in human diabetes.[15] The author attempted particularly to clarify the relationship between insolubilization and glycation of human lens protein in aging.

In the current experiment, it was observed that human lens protein could easily incorporate glucose, G-6-P and fructose. The protein of normal lens has the ability to incorporate a larger amount of sugars than that of a cataractous lens. HMW protein of cataractous lens scarcely reacts with sugars. The present experiment elucidated that this phenomenon might be the result of a decrease in the number of amino acid residues reactive with sugars, like lysine or arginine, since these residues have already reacted with some sugars. It was then found that glucose, G-6-P and fructose could insolubilize the lens proteins, and that the amount of the proteins insolubilized by glucose increased in a dose-dependent manner. G-1-P showed a similar effect. However, it is a well-known fact that the free hydroxy group at the C1 position of glucose must react with an amino group in the glycation. Thus, the insolubilization of lens protein by G-1-P is believed to be induced by another mechanism.

In this experiment, the author attempted to determine changes in the amount of glycosylated protein in normal and cataractous lenses during aging by affinity column chromatography. The column used in this method has an affinity for glycosylated materials, and has been widely used in the clinical field.[9] The amounts of glycosylated protein in normal lens increased slightly until approximately the 50 age-group, and thereafter gradually decreased. It is the authors assumption that this phenomenon is caused by insolubilization of such glycosylated protein.

In summary, there is an increase in the amount of glycosylated protein in senile cataractous human lens, and the progress of glycation appears to cause an increase in the amount of insolubilized protein. From these results, the author concludes that the abnormal acceleration of glycation in the lens may induce senile cataract formation, although further work is needed to assess the exact role of glycation in crosslinking or insolubilization of crystallins during cataract formation and aging.

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