

Free Epsilon Amino Groups and 5-Hydroxymethylfurfural Contents in Clear and Cataractous Human Lenses

Gadiparthi N. Rao and Edward Corlier

The free ϵ -amino groups and 5-hydroxymethylfurfural (5-HMF) contents were determined in soluble and insoluble proteins of clear human lenses and diabetic and nondiabetic senile cataractous lenses. The free ϵ -amino group content of soluble proteins in diabetic cataracts was decreased by 37% ($P < 0.01$), whereas in nondiabetic senile cataracts it did not differ from that of clear lenses. The free ϵ -amino group content of insoluble proteins both in diabetic and nondiabetic cataracts was decreased significantly ($P < 0.001$, $P < 0.015$, respectively). The 5-HMF content of soluble proteins in diabetic cataracts was increased by 52% ($P < 0.001$), whereas in nondiabetic cataracts it did not change from that of clear lenses. The 5-HMF content of insoluble proteins in diabetic as well as in nondiabetic cataracts was increased significantly as compared to that of clear lens ($P < 0.001$, $P < 0.001$, respectively). The soluble protein of diabetic and nondiabetic cataracts was decreased with an increase in the insoluble protein content. These results suggest that nonenzymatic glycosylation plays a role in the conformational change of lens proteins in both diabetic and nondiabetic cataracts. *Invest Ophthalmol Vis Sci* 27:98-102, 1986

Nonenzymatic glycosylation is a condensation reaction between reducing sugars and free amino groups. Following the demonstration that hemoglobin undergoes post-translational nonenzymatic glycosylation *in vivo*,¹ a variety of proteins such as rat albumin, human collagen, and basement membrane proteins were shown to be subjected to this reaction under physiological conditions.²⁻⁴ It was also shown that the rate of nonenzymatic glycosylation is influenced by a number of factors such as the availability of reducing sugar, life-span of individual proteins, pH, temperature, etc.^{5,6} Nonenzymatic glycosylation was also shown to be involved in pathogenic complications of diabetes mellitus, like browning of collagen and collagen-rich tissues with increasing stiffness.^{7,8} It was further suggested that nonenzymatic glycosylation plays a role in experimental sugar cataractogenesis.^{9,10} However, studies from different laboratories could not support the hypothesis that glycosylation is involved in cataract formation.¹¹⁻¹³

Furthermore, the recent reports by Garcia-Castiñeiras and Miranda-Rivera¹⁴ and Truscott¹⁵ on the contents of free amino groups in cataractous lenses stand contradictory to each other. Because of these discrepancies in the conclusions, we have determined the glycosylated product, 5-HMF, and free ϵ -amino groups in soluble and insoluble proteins of extracapsular diabetic and nondiabetic human cataractous lenses. The results are reported in the present communication.

Materials and Methods

Trinitrobenzenesulfonic acid (TNBS), 2-thiobarbituric acid, sodium borohydride were purchased from Sigma Chemical Company; St. Louis, MO. All other reagents used were of analytical grade.

Human cataractous lenses which were removed by intracapsular cryosurgery were procured from the New York Hospital, New York. The normal human lenses were supplied by the Eye Bank for Sight Restoration, Inc., New York. The cataractous lenses were classified according to Cicchetti et al.¹⁶ The capsule of the normal and cataractous lenses was removed. The decapsulated lenses were homogenized individually in double distilled water using Potter-Elvehjem homogenizer and centrifuged for 1 hr at $10000 \times g$ in a Sorvall RC-2B refrigerated centrifuge at 0°C . The pellet was washed with double distilled water and centrifuged again as above. The supernatants were combined. The supernatant and the pellets were referred to as soluble and

From the Eye Biochemistry Laboratories, Department of Ophthalmology, Cornell University Medical College, New York, New York.

Presented in part at the 1984 Annual Meeting of the Association for Research in Vision and Ophthalmology, April 30, 1984, Sarasota, Florida.

Submitted for publication: February 6, 1985.

Reprint requests: Gadiparthi N. Rao, PhD, Department of Medical Microbiology and Immunology, Albert B. Chandler Medical Center, University of Kentucky, Lexington, KY 40536-0084.

insoluble proteins, respectively. The supernatants and the pellets (suspended homogeneously in 2.0 ml of double distilled water) were dialysed (M.W. cut-off 3,500) against water for 48 hr and lyophilized. The lyophilized material was used for the determination of free ϵ -amino groups, 5-HMF and protein.

Free ϵ -amino Groups

The free ϵ -amino groups were measured according to the procedure of Kakade and Liener¹⁷ as described by Eklund.¹⁸ To 1.0 ml of the sample containing 1 mg of protein, 1.0 ml of 4% (W/V) NaHCO_3 (pH 8.5) and 1.0 ml of 0.1% aqueous TNBS solutions were added and incubated for 2 hr at 40°C. After incubation, 4.5 ml of conc HCl was added and hydrolyzed for 90 min at 110°C. The hydrolysates were cooled to room temperature and centrifuged at 3000 rpm for 10 min. The supernatants were extracted twice with 10 ml of ether in order to remove α -TNP amino complexes. The aqueous solution was kept in hot water till residual ether evaporated. The optical densities of these aqueous solutions were measured at 346 nm on a Gilford spectrophotometer against a blank prepared in the same way as that of the test except that the HCl was added prior to TNBS addition. In the determination of free ϵ -amino groups in insoluble proteins, 1.0 mg of the latter was suspended homogeneously in 1.0 ml of 4% NaHCO_3 and submitted to the rest of the procedure as mentioned above. The free ϵ -amino group content in the samples was calculated using the molar extinction coefficient value of 1.46×10^4 ¹⁷ and the values are expressed as μmol per mg protein.

5-hydroxymethylfurfural

The 5-HMF content in soluble and insoluble proteins was determined by the method of Dolhofer and Wieland.¹⁹ Four milligrams of protein dissolved in 4.0 ml of buffer (soluble protein was dissolved in 0.1 M sodium phosphate buffer, pH 7.0 and insoluble protein was suspended homogeneously in 0.05 M Tris-HCl buffer containing 0.1 M KCl and 7 M urea, pH 8.5) and divided into 2 equal halves (2.0 ml each). The first half was treated with 19 mg of sodium borohydride for 1 hr at room temperature. Trace amounts of octanol-1 were added to prevent frothing. The second half without sodium borohydride treatment served as a control. The test and the control samples were dialyzed for 18 hr at 4°C against 4 litres of 0.9% NaCl with 2 changes. After dialysis, the volumes of the test and control samples were adjusted to 3.0 ml with 0.9% NaCl (the protein content in the test and control is same) and incubated with 0.45 ml of glacial acetic acid for 24 hr at 100°C. Following hydrolysis, 0.75 ml of 3.0

M TCA was added and centrifuged at 3000 rpm for 30 min. Then, 1.0 ml of the supernatant was incubated with 0.5 ml of 50 mM 2-thiobarbituric acid for 30 min at 40°C. After completion of the incubation, the solutions were cooled to room temperature, and the optical densities were measured at 443 nm. The amount of 5-HMF in the samples was calculated using its molar extinction coefficient value of 4×10^4 , and the values are expressed as nmol per mg protein.

The protein content was determined by the method of Lowry et al²⁰ using bovine serum albumin as a standard.

Results

The soluble, insoluble, and total protein and free ϵ -amino groups and 5-HMF contents of clear human lenses and diabetic and nondiabetic senile cataracts are shown in Tables 1 and 2. The soluble protein content of types B, C, and D of nondiabetic senile cataracts was decreased by 31.66, 33.15 and 55.99% respectively ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively), and in diabetic cataracts it was decreased by 32.58% ($P < 0.05$) as compared to the values of the age-matched clear lenses. On the other hand, the insoluble protein content of B, C, and D types of nondiabetic senile cataracts was increased by 82.72, 128.07, and 224.56% respectively ($P < 0.05$, $P < 0.001$, $P < 0.001$, respectively), and in diabetic cataracts it was increased by 50.61% ($P < 0.05$, Table 1). However, the total protein content in both diabetic and nondiabetic senile cataracts did not differ significantly from that of clear lenses (Table 1). The ratio of insoluble to soluble protein was increased in all types of cataracts, being the highest in type D (Table 1).

Free ϵ -amino group content of soluble proteins of nondiabetic senile cataracts did not differ from that of clear lenses, whereas in diabetic cataracts it was decreased by 37% ($P < 0.01$, Table 2). The free ϵ -amino groups of insoluble proteins in B, C, and D types of senile nondiabetic cataracts were decreased by 31.79, 30.43, and 23.64% respectively ($P < 0.05$, $P < 0.05$, $P < 0.05$, respectively). The free ϵ -amino groups of insoluble proteins of diabetic cataracts were decreased by 48.91% ($P < 0.001$, Table 2) as compared to the values of clear lenses. The 5-HMF content of soluble proteins of all the 4 types of senile nondiabetic cataracts did not change from that of clear lenses, whereas in diabetic cataracts it was increased by 52% ($P < 0.001$, Table 2). The 5-HMF content of insoluble proteins of types B, C, and D senile nondiabetic cataracts was increased by 25.17, 27.24, and 33.10% respectively ($P < 0.05$ for type C and $P < 0.01$ for type D), and in diabetic cataracts it was increased by 81.72% ($P < 0.001$, Table 2).

Table 1. Soluble and insoluble protein contents in clear human lenses and senile and diabetic cataracts

	Protein (mg/lens)			Ratio of insoluble/soluble
	Soluble	Insoluble	Total	
Clear human lens (64-72 yr)*	35.6 ± 1.16 (5)	11.4 ± 1.08 (5)	47.0 ± 1.73 (5)	0.32 ± 0.03 (5)
Cataract†				
Type A (60-75 yr)	28.33 ± 1.85 (6)	12.83 ± 1.38 (6)	41.17 ± 2.64 (6)	0.457 ± 0.043 (6)
% change	-20.42	+12.54	-12.40	+43.75
P<	NS	NS	NS	NS
Type B (66-75 yrs)	24.33 ± 2.17 (6)	20.83 ± 0.80 (6)	45.17 ± 2.84 (6)	0.880 ± 0.058 (6)
% change	-31.66	+82.72	-3.89	+175.00
P<	0.05	0.05	NS	0.001
Type C (62-68 yrs)	23.80 ± 2.13 (5)	26.0 ± 2.39 (5)	49.8 ± 4.38 (5)	1.01 ± 0.054 (5)
% change	-33.15	+128.07	+5.96	+243.75
P<	0.05	0.001	NS	0.001
Type D (60-76 yrs)	14.6 ± 1.25 (5)	37.0 ± 1.38 (5)	51.6 ± 1.75 (5)	2.61 ± 0.259 (5)
% change	-58.99	+224.56	+9.79	+715.63
P<	0.01	0.001	NS	0.001
Diabetic cataract (52-69 yrs)	24.0 ± 2.63 (6)	17.17 ± 2.22 (6)	41.17 ± 3.46 (6)	0.77 ± 0.14 (6)
% change	-32.58	+50.61	-12.40	+140.63
P<	0.05	0.05	NS	0.001

* No significant variation was measured in the protein content within any one category of cataractous lenses with respect to the age group studied.

† Cataract Type A = yellow with moderate opacities; B = light brown with moderate opacities; C = brown with intense opacities; D = dark brown with

intense to total opacities; diabetic cataracts analyzed in this study are light brown in color with moderate to intense opacities.

Parentheses indicate number of lenses analyzed.

Table 2. Free epsilon amino groups and 5-hydroxymethylfurfural contents in clear human lenses and in senile and diabetic cataracts

	Free epsilon amino groups*		5-hydroxymethyl furfural†	
	Soluble	Insoluble	Soluble	Insoluble
Clear human lens (64-72 yr)‡	0.140 ± 0.010 (5)	0.368 ± 0.028 (5)	0.233 ± 0.014 (5)	0.290 ± 0.020 (5)
Cataract§				
Type A (60-75 yr)	0.138 ± 0.010 (6)	0.372 ± 0.026 (6)	0.254 ± 0.013 (6)	0.342 ± 0.013 (6)
% change	-1.43	+1.08	+9.01	+17.90
P<	NS	NS	NS	NS
Type B (66-75 yr)	0.139 ± 0.012 (6)	0.251 ± 0.027 (6)	0.254 ± 0.009 (6)	0.363 ± 0.020 (6)
% change	-0.71	-31.79	+9.01	+25.17
P<	NS	0.05	NS	NS
Type C (62-68 yr)	0.130 ± 0.034 (5)	0.256 ± 0.017 (5)	0.289 ± 0.017 (5)	0.369 ± 0.026 (5)
% change	-7.14	-30.43	+24.03	+27.34
P<	NS	0.05	NS	0.05
Type D (60-76 yr)	0.139 ± 0.012 (5)	0.281 ± 0.016 (5)	0.229 ± 0.006 (5)	0.386 ± 0.028 (5)
% change	-0.71	-23.64	-1.70	+33.10
P<	NS	0.05	NS	0.01
Diabetic cataract (52-69 yr)	0.088 ± 0.004 (6)	0.188 ± 0.006 (6)	0.353 ± 0.005 (6)	0.527 ± 0.011 (6)
% change	-37.14	-48.91	+51.50	+81.72
P<	0.01	0.001	0.001	0.001

* μmol/mg protein

† nanomol/mg protein

‡ No significant variation was measured in the free ε-amino group and 5-HMF contents within any one category of cataractous lenses with respect to the age groups studied.

§ Cataract Type A = yellow with moderate opacities; B = light brown with

moderate opacities; C = brown with intense opacities; D = dark brown with intense to total opacities; diabetic cataracts analyzed in this study are light brown in color with moderate to intense opacities.

NS = statistically not significant.

Parentheses indicate number of lenses analyzed.

Discussion

Dische and Zil²¹ first reported increased disulfides in human cataracts, and since then a number of investigators confirmed the same in human senile and experimental animal cataracts.²²⁻²⁴ These disulfide bonds are thought to be responsible for the formation of high molecular weight aggregates in cataracts.²² Monnier et al¹⁰ demonstrated that lens crystallines upon glycosylation become more susceptible to oxidation leading to the formation of disulfide linked high molecular weight aggregates, which could explain cataractogenesis in diabetic condition. Ansari et al¹² have reported increased glycosylation of lens proteins in human cataracts; however, in these studies no correlation was found between glycosylation and sulfhydryl oxidation of lens proteins. On the other hand, increased glycosylation of aged bovine lens α -crystallins, particularly high molecular weight α -crystallin (HMW α), was reported by Chiou et al.¹³ Thus, increasing attention is being paid to nonenzymatic glycosylation of proteins, as this process was shown to be involved in many pathogenic complications of diabetes mellitus such as browning of collagen and collagen-rich tissues with increasing stiffness.⁷ It was further shown that the long-lived proteins like collagen, lens crystallins, basement membrane proteins, and basic nerve myelin protein are more susceptible to nonenzymatic glycosylation.^{25,26} As nonenzymatic glycosylation is a condensation reaction between free amino groups and reducing sugars, attempts were made by Garcia-Castiñeiras and Miranda-Rivera¹⁴ and Truscott¹⁵ on the determination of free amino groups in cataracts. However, their reports on free amino groups in cataracts are contradictory to each other. Our studies on the determination of the nonenzymatic glycosylated product, 5-HMF, revealed a significant increase in its content in soluble and insoluble proteins of diabetic cataracts and in insoluble proteins of nondiabetic cataracts. The decrease in the free ϵ -amino groups in soluble and insoluble proteins of diabetic cataracts and in insoluble proteins of nondiabetic cataracts further supports the occurrence of nonenzymatic glycosylation in cataracts. Recently Garlick et al²⁷ have reported twofold increase in glycosylation in soluble and insoluble proteins of diabetic lenses. Kasai et al²⁸ have demonstrated the nonenzymatic glycosylation of lens soluble proteins by incubating the proteins from diabetic and nondiabetic cataracts with glucose *in vitro*, and the extent of glycosylation was found more with proteins of the former type of cataracts. Mandel et al²⁹ reported an increased glycosylation of lens capsule in diabetics as compared to the nondiabetics. From our results it is evident that the extent of nonenzymatic glycosylation is more

(twofold higher) in diabetic cataracts as compared with clear lens values, indicating that the glycosylation reaction is proportional to the glucose level. Our results on 5-HMF in soluble and insoluble proteins of diabetic cataracts agree with the results of Garlick et al²⁷ with respect to the twofold increase. Recently, Liang and Chylack³⁰ by circular dichroism studies have shown a change in the tertiary structure of α -crystallin upon glycosylation. The increased nonenzymatic glycosylation of human skin collagen caused more stiffness and resistance to enzymatic hydrolysis.⁷ Based on all these findings, it may be mentioned that increased glycosylation leads to the conformational changes in lens crystallins, which further results in the opacification of the lens. It is interesting to note that the decrease in the free ϵ -amino groups in soluble and insoluble proteins of diabetic cataracts and insoluble proteins of nondiabetic cataracts could not be explained by nonenzymatic glycosylation reaction alone, since the rise in 5-HMF content was several hundredfold less than the decrease in free ϵ -amino groups. Hence, it is also tempting to speculate the involvement of other mechanisms such as carbamylation³¹ and isopeptide bond formation³² besides nonenzymatic glycosylation in cataracts, which could probably explain the decrease in free ϵ -amino groups. Or otherwise, it could be possible that the glycosylation reaction reached beyond the reducible Maillard products in cataracts, in which case the increase in 5-HMF would not be proportional to the decrease in free ϵ -amino groups.

The decrease in soluble protein contents of both diabetic and nondiabetic cataracts could be explained by an increase in insoluble protein content, as the total protein content of cataracts did not change significantly from that of age-matched clear human lenses. Further studies on the susceptibility of lens proteins, particularly individual crystallins to various chemical modifications like carbamylation and glycosylation, are of importance in order to understand the role of post-translational modifications of crystallins in cataractogenesis.

Key words: human clear lens, human diabetic and nondiabetic senile cataracts, nonenzymatic glycosylation, epsilon amino groups, 5-hydroxymethylfurfural

References

1. Bunn HF, Haney DN, Kamin S, Gabbay KH, and Gallop PM: The biosynthesis of human hemoglobin A_{1c}. Slow glycosylation of hemoglobin *in vivo*. *J Clin Invest* 57:1652, 1976.
2. Day JF, Thornburg RW, Thorpe SR, and Baynes JW: Nonenzymatic glycosylation of rat albumin. Studies *in vitro* and *in vivo*. *J Biol Chem* 254:9394, 1979.
3. Rosenbert H, Modrak JB, Hassing JM, Al-Turk WA, and Stohs

- SJ: Glycosylated collagen. *Biochem Biophys Res Commun* 91: 498, 1979.
4. Cohen MP, Urdanivia E, Surma M, and Ciborowski CJ: Non-enzymatic glycosylation of basement membranes. *In vitro* studies. *Diabetes* 30:367, 1981.
 5. Fluckiger R and Winterhalter KH: *In vitro* synthesis of hemoglobin A_{1c}. *FEBS Lett* 71:356, 1976.
 6. Monnier VM and Cerami A: Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science* 211: 491, 1981.
 7. Schnider SL and Kohn RR: The effects of age and diabetes mellitus on the solubility and nonenzymatic glycosylation of human skin collagens. *J Clin Invest* 67:1630, 1981.
 8. Monnier VM, Kohn RR, and Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus. *Proc Natl Acad Sci USA* 81:538, 1984.
 9. Stevens VJ, Rouzer CA, Monnier VM, and Cerami A: Diabetic cataract formation: potential role of glycosylation of lens crystallins. *Proc Natl Acad Sci USA* 75:2918, 1978.
 10. Monnier VM, Stevens VJ, and Cerami A: Nonenzymatic glycosylation, sulfhydryl oxidation and aggregation of lens proteins in experimental sugar cataracts. *J Exp Med* 150:1098, 1979.
 11. Pande A, Garner WH, and Spector A: Glycosylation of human lens protein and cataractogenesis. *Biochem Biophys Res Commun* 89:1260, 1979.
 12. Ansari NH, Awasthi YC, and Srivastava SK: Role of glycosylation in protein disulfide formation and cataractogenesis. *Exp Eye Res* 31:9, 1980.
 13. Chiou SH, Chylack, Jr LT, Tung WH, and Bunn HF: Nonenzymatic glycosylation of bovine lens crystallins: effect of aging. *J Biol Chem* 256:5176, 1981.
 14. Garcia-Castiñeiras A and Miranda-Rivera MN: Loss of free amino groups in the water-insoluble fraction of nuclear senile cataracts. *Invest Ophthalmol Vis Sci* 24:1181, 1983.
 15. Truscott RJW: Availability of protein amino groups in senile nuclear cataract. *Ophthalmic Res* 15:38, 1983.
 16. Cicchetti DV, Sharma Y, and Cotlier E: Assessment of observer variability in the classification of human cataracts. *Yale J Biol Med* 55:81, 1982.
 17. Kakade ML and Liener IE: Determination of available lysine in proteins. *Anal Biochem* 27:273, 1969.
 18. Eklund A: On the determination of available lysine in casein and rapeseed protein concentrates using 2,4,6-trinitrobenzene sulfonic acid as a reagent for free epsilon amino groups of lysine. *Anal Biochem* 70:433, 1976.
 19. Dolhofer R and Wieland OH: Improvement of the thiobarbituric acid assay for serum glycosylprotein determination. *Clin Chim Acta* 112:197, 1981.
 20. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265, 1951.
 21. Dische Z and Zil H: Studies on the oxidation of cysteine to cystine in lens proteins during cataract formation. *Am J Ophthalmol* 34:104, 1951.
 22. Truscott RJW and Augusteyn RC: The state of sulfhydryl groups in normal and cataractous human lenses. *Exp Eye Res* 25:139, 1977.
 23. Spector A and Roy D: Disulfide linked high molecular weight protein associated with human cataract. *Proc Natl Acad Sci USA* 75:3244, 1978.
 24. Giblin FJ and Reddy VN: High molecular weight protein aggregates in x-ray induced cataract: evidence for the involvement of disulfide bonds. *Interdisciplinary Topics of Gerontology* 12: 94, 1978.
 25. Kohn RR, Cerami A, and Monnier VM: Collagen aging in vitro by nonenzymatic glycosylation and browning. *Diabetes* 33:57, 1984.
 26. Cohen MP and Wu VY: Age related changes in nonenzymatic glycosylation of human basement membranes. *Exp Gerontol* 18: 461, 1984.
 27. Garlick RL, Jonathan SM, Chylack, Jr LT, Tung WH, and Bunn HF: Nonenzymatic glycation of human lens crystallin: effect of aging and diabetes mellitus. *J Clin Invest* 74:1742, 1984.
 28. Kasai K, Nakamura T, Kase N, Hiraoka T, Suzuki R, Kogure F, and Shimoda SI: Increased glycosylation of proteins from cataractous lenses in diabetes. *Diabetologia* 25:36, 1983.
 29. Mandel SS, Shin DH, Newman BL, Lee JH, Lupovitch A, and Drakes GHN: Glycosylation in vivo of human lens capsule (basement membrane) and diabetes mellitus. *Biochem Biophys Res Commun* 117:51, 1983.
 30. Liang JN and Chylack, Jr LT: Change in the protein tertiary structure with nonenzymatic glycosylation of calf α -crystallin. *Biochem Biophys Res Commun* 123:899, 1984.
 31. Harding JJ and Rixon KC: Carbamylation of lens proteins: a possible factor in cataractogenesis in some tropical countries. *Exp Eye Res* 31:567, 1980.
 32. Lorand L, Hsu LK, Siefrins GE, and Rafferty NS: Lens transglutaminase and cataract formation. *Proc Natl Acad Sci USA* 78:1356, 1981.