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

Gadiparthi N. Rao and Edward Cotlier

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 nonenyzmatic 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-Castiñei as and Miranda-Rivera[14] and Truscott[15] on the conte ts of free amino groups in cataractous lenses stand c n- tradictory to each other. Because of these discrepanc es in the conclusions, we have determined the glycosyla d product, 5-HMF, and free ϵ-amino groups in solu le and insoluble proteins of extracapsular diabetic a d nondiabetic human cataractous lenses. The results re reported in the present communication.

Materials and Methods

Trinitrobenzenesulfonic acid (TNBS), 2-thioba i- turic acid, sodium borohydride were purchased fr m Sigma Chemical Company; St. Louis, MO. All ot er reagents used were of analytical grade.

Human cataractous lenses which were removed y intracapsular cryosurgery were procured from the N w York Hospital, New York. The normal human len es were supplied by the Eye Bank for Sight Restorati n, Inc., New York. The cataractous lenses were classif d according to Cicchetti et al.[16] The capsule of the norr al and cataractous lenses was removed. The decapsula d lenses were homogenized individually in double s- tilled water using Potter-Elvehjem homogenizer a d centrifuged for 1 hr at 10000 × g in a Sorvall RC- B refrigerated centrifuge at 0°C. The pellet was wash d with double distilled water and centrifuged again as above. The supernatants were combined. The sup r- natant and the pellets were referred to as soluble a d

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

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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 Liener[17] 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 al[20] 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 Zil[21] 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 al[10] 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 al[12] 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 (HMWα), 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-Rivera[14] and Truscott[15] 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 al[27] have reported twofold increase in glycosylation in soluble and insoluble proteins of diabetic lenses. Kasai et al[28] 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 al[29] 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 al[27] with respect to the twofold increase. Recently, Liang and Chylack[30] 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 carbamylation[31] and isopeptide bond formation[32] 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

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