Contribution of Glycation to Human Lens Coloration

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To study the contribution of glycation or the Maillard reaction to the spontaneous coloration of human crystalline lens in aging, we determined 1-deoxyfructosyl adduct and the fluorescent material, which are produced in the early stage of glycation, in the proteins of normal and colored human lenses of different ages. The amount of both glycation products in the lens increased significantly in proportion to aging or the advance of lens coloration. The insolubility of lens protein also increased with the advance of glycation. In addition, the present study showed that glucose and glucose-6-phosphate have higher reactivities with human lens protein than fructose and glucose-1-phosphate.

This paper demonstrates that the deeper colored or older aged lens contains larger amounts of glycation products, and that glycation between lens protein and various sugars in vivo may be a serious factor in human lens coloration or insolubilization of lens protein.

Keywords human lens; normal lens; cataractous lens; glycation; Maillard reaction; lens coloration; 1-deoxyfructosyl adduct; fluorescent material

It is well known that the crystalline lens coloration of white to brown is observed only in human. An understanding of the mechanism of lens coloration is desired because advanced coloration induces a loss of vision. Photooxidation of tryptophan residues in the lens protein has been suspected of being a serious factor affecting coloration.1) Recently, glycation has also been proposed as one cause of human lens coloration, because lens protein is exceptionally long-lived in the lens and may be vulnerable to attack from glucose, its derivatives or other sugars.2) Glycation is a nonenzymatic binding reaction between proteins and sugars; it is called the Maillard reaction or nonenzymatic browning reaction, and occurs widely throughout the natural world.3) In the initial stage of the Maillard reaction, the condensation product, an aldosylamine, undergoes an Amadori rearrangement to form a 1-deoxyfructosyl adduct (DFA) or fructosamine.4) In the later stage of the Maillard reaction, the Amadori product undergoes dehydration and rearrangement to form advanced glycosyl end products (AGE), and subsequently yellow-brown fluorescence and protein cross-links that decrease the protein solubility.5c,5) It was also reported that AGE may be produced by the reaction of protein with 3-deoxyglucosone that is an intermediate in the Maillard reaction.6) The proposed mechanism of the Maillard reaction is summarized in Fig. 1.3–7

![Reaction mechanism diagram]

Fig. 1. The Process of the Maillard Reaction or Glycation

The amnono analogue of compound IV derived from glucose is called DFA or fructosamine.

Similar phenomena have been observed in senile cataractous human lenses.8) These include protein aggregation with a decrease in its solubility, production of nondisulfide covalent cross-links between proteins, advanced coloration from white to brown, and a dramatic increase in protein-bound yellow-brown fluorescence not attributable to tryptophan. It is also well known that normal human lenses become yellow in aging. From studies on glycation in animal lens proteins or aged senile cataractous lenses,9,10) the possibility of the involvement of glycation in human lens coloration has been considered, but no conclusion can yet be drawn. To clarify the relationship between glycation and lens coloration, we believed it indispensable to follow the extent of glycation in the normal human lens in aging. There are few reports of such studies, because it is hard to obtain a large number of normal human lenses of different ages. We were fortunate in obtaining several of these, and with them we attempted to clarify whether glycation occurs in the human lens.

The current paper reports the extent of glycation in the human lens in aging, or the advance of coloration by measuring DFA and fluorescence material that have been identified as products in the initial stage of the Maillard reaction.3c,4c,5) The involvement of glycation in the lens protein insolubilization and reactivity of various sugars with human lens proteins were also examined in this study.

Materials and Methods

Lenses Used Human normal and colored lenses (or senile cataractous ones) were kindly donated by Dr. Horwitz, Jules Stein Eye Institute, University of California, Los Angeles. Briefly, normal human lenses were obtained from autopsy eyes which were removed 4 to 8h following death. Upon removal, the eye was stored at −80 °C until use. Cataractous lenses were also obtained during routine cataract surgery at the same institute. Bovine lenses were purchased from a local slaughterhouse and stored at −80 °C until use.

Materials D-[1,14C]Glucose (2.4 GBq/mmol), D-[14C(U)]glucose-1-phosphate (11.3 GBq/mmol), D-[14C]glucose-6-phosphate (2.1 GBq/mmol) and D-[14C(U)]fructose (12.7 GBq/mmol) were purchased from DuPont Company. 1-Deoxymethylfructose used as a standard material for DFA determination was from Sigma Chemical Co. Polysine (＞M.W. = 8000) was also from Peptide Institute, Inc., Japan.

Preparation of Soluble and Insoluble Protein Fractions from Human and Bovine Lenses The soluble and insoluble protein fractions of human and bovine lenses were prepared according to the method described previously.10) Briefly, after a human lens was homogenized in 3.0ml
of 0.2 m phosphate buffer, pH 7.4, the suspension was centrifuged at 17000 rpm for 20 min at 4°C. The supernatant was used as a soluble protein fraction. The soluble proteins were exhaustively washed out of the resulting pellet by 7 repetitions of suspensions in the same buffer and centrifugation. After washing the pellet, the water-soluble protein could no longer be detected in the pellet by measurement of protein content, by colorimetry with a Bio Rad assay kit or by the absorbance at 280 nm. The pellet was then dissolved in 3.0 ml of 6 M urea. A small amount of the insoluble material was removed by centrifugation at 27000 rpm for 20 min at 4°C. The resulting supernatant was used as an insoluble protein fraction. One bovine lens was also homogenized in 7.0 ml of 0.2 m phosphate buffer, pH 7.4, and its soluble and insoluble protein fractions were treated by the same procedure. Except for centrifugation, all work was carried out on a clean bench using tools sterilized in an autoclave or by dry air sterilization.

Glycation between Lens Protein and Sugars This reaction was done according to the methods of other investigators.8,11) Regarding glycation between human lens protein and glucose, 0.1 μmol of [¹⁴C]glucose plus 0.1 mmol unlabelled glucose was used to react with 10 mg of protein in 3 ml of 0.2 m phosphate buffer, pH 7.4. The reaction was undertaken at 37°C under a sterilized condition for 28 d in a dark place to avoid the effect of light. An aliquot of the reactant was taken from the sample solution at regular intervals. In the experiment of incorporation of [¹⁴C]-labeled sugars into human lens protein, 0.01 μmol of sugar plus 0.01 mmol of unlabeled sugar were added to 1 ml of the solution containing 2 mg of lens protein. The solution was then filtered into a sterilized glass tube through a 0.45 μm Millipore filter, and the filtrate was shaken at 37°C for 28 d in a dark place. After the reaction had finished, the reactant was treated with 1 mg of NaN₃ for 30 min.11) The unreactive sugars were removed from the reactant by PD-10 and TSKG3000SW column chromatography followed by using 0.2 m phosphate buffer containing 0.1 M NaCl and 0.2% sodium dodecyl sulfate (SDS), pH 6.8, as an eluent. The main peak was pooled and lyophilized. The reactant was dissolved in an appropriate volume of 0.1 m phosphate buffer, pH 6.8, containing 0.1 M NaCl and 0.2% SDS, and its radioactivity was measured.

Reaction of Glucose with Polysyrine The detailed procedure was described elsewhere.21) Briefly, 0.1 mmol glucose and 5 mg of polysyrine was dissolved in 1 ml of 0.2 m phosphate buffer, pH 7.4, and filtered through a 0.45 μm Millipore filter into a sterilized glass tube with a sterilized stopper. Then, the solution was slowly shaken at 37°C for 10 d in a dark place.

Other Analyses Amino acid composition was analyzed with a Hitachi 835 amino acid analyzer. Fluorescence intensity was determined with a Hitachi MPF-2 fluorescence spectrophotometer. The radioactivity of the incorporated [¹⁴C]-labeled sugar into the protein was measured with a Packard TRI-CARB liquid scintillation counter using a conventional method. DFA was measured by the method of Baker et al.4) Briefly, 0.1 ml of the sample solution was mixed with 1.0 ml of 0.25 mm nitrotriazolium blue in 0.1 m bicarbonate buffer, pH 10.35. After the solution had been shaken at 37°C for 15 min and then cooled, the amount of DFA was determined from the absorbance at 530 nm and 1-deoxymorpholino-fructose was used as a standard material. The protein concentration was measured by the method of Bradford.22)

Results Identification of Reliable Indications of Glycation Before investigating the extent of glycation in human lens protein, a preliminary experiment was done to confirm whether the DFA level and fluorescence intensity in the glycation product are directly proportional to the extent of glycation or the amount of sugar incorporated into protein, because there was no information on the determination of DFA in lens protein. This experiment was undertaken using [¹⁴C]glucose and bovine lens protein that consists of crystallin species similar to those of human lens protein. Figure 2 reveals the correlation among the amount of incorporated glucose, the DFA level, and fluorescence intensity at 440 nm (Ex = 340 nm) in the time-course of glycation.

This finding shows that the DFA level and fluorescence intensity in the glycation product increased proportionally with an increase in the amount of incorporated sugar, and that these two factors are reliable indications by which to determine the extent of the glycation.

Variation in Content of Maillard Reaction Products in Normal and Colored Human Lens Protein in Aging and with Advance of Coloration Human crystalline lens has various colors from white to brown as shown in Fig. 3. Although there are many types of lenses of various colors, three types with typical colors of white, yellow and brown were picked out and used in the experiment. The amount of glycation product in the lens protein was determined from the DFA level and fluorescence intensity at 440 nm (Ex = 340 nm), which the above experiment had confirmed to be indicative the extent of glycation.

Figures 4A and 4B show the DFA level in the soluble and insoluble protein fractions of normal and colored human lenses. The levels in both proteins increased with aging. In the soluble protein, however, there was no significant difference in levels between normal and colored lenses at a similar age, except for the yellow lens that was conspicuous for its higher level of DFA. In the insoluble proteins, on the other hand, the colored and aged yellow and brown lenses showed higher DFA levels than in normal and white ones.

The fluorescent material produced by the Maillard
reaction in human lens protein was also determined from fluorescence intensity at 440 nm (Ex = 340 nm) as another indication of the reaction. Figure 5A shows the fluorescent spectrum of the reactant of polylysine with glucose in the 10-d reaction, which was used as a typical fluorescent spectrum of the Maillard reaction product. Figure 5B also shows the almost similar spectra of the soluble proteins in normal and colored lenses. The spectra of the insoluble lens proteins also showed a similar pattern to that of the polylysine-glucose reactant (data not shown). This indicates that the fluorescence of colored insoluble protein excited at 340 nm might be because it is a product of the Maillard reaction.

Then, using a portion of the lens protein prepared for determining DFA level, the correlation among aging, coloration and the content of the fluorescent material in the lens protein was determined from the fluorescence intensity at 440 nm (Ex = 340 nm). In both soluble (Fig. 6A) and insoluble (Fig. 6B) proteins, the fluorescent material increased with aging and the advance of coloration. Its contents in the proteins of deeper colored and aged lenses were always higher than those of the normal and white ones. The DFA levels in the soluble protein of yellow and brown lenses were approximately 3-fold that of other lenses.

This tendency was similar to that of DFA levels in the insoluble proteins. However, a discrepancy between the DFA and the fluorescent material levels in the soluble proteins of the brown lens was observed, because the DFA level was very low in spite of the high level of fluorescent materials. These facts show that the Maillard reaction or glycation has taken place in the human lens protein with aging and the advance of coloration. Furthermore, from the disappearance of DFA in the brown lens it is also suggested that the Maillard reaction might progress to a further stage in the deeper colored lenses, and that in the later stage of the reaction DFA may be converted to the advanced glycosyl end product. These facts were also supported by an amino acid composition analysis of the soluble proteins as shown in Table I. Lysine and histidine residues in the lens protein decreased in proportion to the advance of lens coloration. Arginine residues in the brown lens protein in addition to those of both amino acid residues, reduced significantly as compared to those in other lenses. This fact also suggests the existence of the Maillard reaction in the lens and the progress of its reaction to a further stage with the advance of coloration. These assumptions were made after referring to many papers reporting the reactivity of various sugars with proteins and basic studies on the Maillard reaction or glycation.

It was also observed that the content ratios of cysteine residues reduced to approximately one-half the value of normal lens. Many investigators have reported that this phenomenon might result from the formation of disulfide.
TABLE I. Amino Acid Composition (%) of Soluble Protein of Human Lenses

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Normal(^{b})</th>
<th>White</th>
<th>Yellow</th>
<th>Brown</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>9.76</td>
<td>9.82</td>
<td>9.85</td>
<td>9.93</td>
</tr>
<tr>
<td>THR</td>
<td>4.17</td>
<td>3.87</td>
<td>4.03</td>
<td>4.06</td>
</tr>
<tr>
<td>SER</td>
<td>7.59</td>
<td>7.77</td>
<td>8.16</td>
<td>8.30</td>
</tr>
<tr>
<td>GLU</td>
<td>14.58</td>
<td>14.63</td>
<td>14.85</td>
<td>15.06</td>
</tr>
<tr>
<td>GLY</td>
<td>9.22</td>
<td>9.24</td>
<td>9.28</td>
<td>9.44</td>
</tr>
<tr>
<td>ALA</td>
<td>5.36</td>
<td>5.40</td>
<td>5.48</td>
<td>5.81</td>
</tr>
<tr>
<td>VAL</td>
<td>5.38</td>
<td>5.55</td>
<td>5.65</td>
<td>5.84</td>
</tr>
<tr>
<td>CY5</td>
<td>0.56</td>
<td>0.25</td>
<td>0.24</td>
<td>0.21</td>
</tr>
<tr>
<td>MET</td>
<td>2.59</td>
<td>2.84</td>
<td>2.89</td>
<td>2.32</td>
</tr>
<tr>
<td>ILE</td>
<td>4.86</td>
<td>4.98</td>
<td>5.02</td>
<td>5.33</td>
</tr>
<tr>
<td>LUE</td>
<td>7.50</td>
<td>7.58</td>
<td>7.67</td>
<td>7.58</td>
</tr>
<tr>
<td>TYR</td>
<td>5.71</td>
<td>5.92</td>
<td>6.05</td>
<td>6.96</td>
</tr>
<tr>
<td>PHE</td>
<td>6.16</td>
<td>6.04</td>
<td>6.26</td>
<td>6.32</td>
</tr>
<tr>
<td>LYS</td>
<td>4.95</td>
<td>4.62</td>
<td>3.22</td>
<td>2.25</td>
</tr>
<tr>
<td>HIS</td>
<td>3.45</td>
<td>3.07</td>
<td>2.56</td>
<td>2.35</td>
</tr>
<tr>
<td>ARG</td>
<td>8.23</td>
<td>8.12</td>
<td>8.05</td>
<td>6.58</td>
</tr>
<tr>
<td>PRO</td>
<td>1.56</td>
<td>1.66</td>
<td>1.71</td>
<td>1.69</td>
</tr>
<tr>
<td>Total</td>
<td>101.63</td>
<td>101.36</td>
<td>100.97</td>
<td>100.03</td>
</tr>
</tbody>
</table>

The data represents the mean of two separate analyses. \(^{a}\) Ages of normal lenses were 48 to 50. \(^{b}\) Ages of white, yellow, and brown cataractous lenses were 42 to 47, 52 to 56, and 72 to 80, respectively.

The incorporation of \(^{14}\)C-labeled sugars, glucose, fructose, glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P), into the Soluble Proteins of Normal and Cataractous Human Lenses Each bar represents the mean ± S.D. of three separate experiments. Detailed experimental conditions were described in the text. <sup>•</sup> normal (age 62 to 65); <sup>□</sup> white (age 60 to 67); <sup>△</sup> yellow (age 62 to 66); <sup>▲</sup> brown (age 66 and 68) lenses.

**Fig. 8.** Incorporation of \(^{14}\)C-labeled sugars, glucose, fructose, glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P), into the Soluble Proteins of Normal and Cataractous Human Lenses

**Discussion**

Nonenzymatic glycation has been shown to occur with hemoglobin to form hemoglobin A\(_1c\) and with a variety of other body proteins, *e.g.*, collagen and nerve protein.\(^{14}\) Furthermore, nonenzymatic browning has been hypothesized to occur in long-lived proteins such as crystallin and collagen in diabetes.\(^{15}\) Such coloration is also observed in human crystalline lens, which colors pale yellow in aging under normal conditions or white to brown in cataract. Glycation\(^{22}\) and photooxidation of tryptophan residues in the lens protein\(^{17}\) have been the main suspects as causes of lens coloration; however, not much corroborating evidence has been found for this.

Abraham *et al.* reported that nonenzymatic glycation does not progress in human lens in aging, nor contribute to the lens coloration.\(^{24}\) However, our study revealed that DFA and fluorescent substance in the human lens, which are products in the early stage of the Maillard reaction, apparently increase in aging or with the advance of coloration. This fact suggests that glycation may contribute to human lens coloration. Furthermore, the content of arginine residues, in addition to lysine and histidine ones, in the brown lens protein diminishes significantly as compared to those in white and yellow lens proteins. In the initial stage of the Maillard reaction, it was reported that lysine or histidine reacts predominantly with sugars.\(^{7}\) From this point of view, a decrease of arginine residues in the protein of brown lenses suggests that the glycation may progress further in deeper colored lenses. It is necessary to determine the advanced glycosyl end product to clarify the exact contribution of glycation to human lens coloration, because it has been observed widely that the pigmentation bonds between lens proteins.\(^{13}\)

**Insolubility of Lens Protein in Aging or with Advance of Its Coloration** In this experiment, the insolubility of lens protein in aging or with the advance of coloration was determined from the content ratios of insoluble protein in the lens. Figure 7 shows the percent ratios of the insoluble protein to the total protein of the whole lens. Findings indicate that an increase in insolubility of lens protein is directly proportional to aging and the advance of coloration.

**Reactivity of Sugar with Human Lens Protein** Using \(^{14}\)C-labeled sugars and the soluble protein fraction of human lens, this experiment was done to determine the reactivity of various sugars with lens proteins. Figure 8 shows the incorporated \(^{14}\)C-labeled sugar levels in the 28-d reaction product. In all cases, normal lens protein incorporated larger amounts of sugars than seen in the colored ones, although there was considerable variety of the incorporated levels depending on the sugars. In colored lenses, the incorporated sugar levels decreased with the advance of coloration, yellow to brown.

These findings show that free glucose and glucose-6-phosphate have considerably higher reactivity with lens protein than fructose and glucose-1-phosphate.
in the protein by glycation is produced in this products in the later stage of the Maillard reaction.\textsuperscript{3,5} At the present time, however, it is not possible to determine such material, because there is no method of analysis for the advanced glycosyl end product and no information on its detailed structure.

In conclusion, this investigation revealed that glycation between lens protein and glucose or its derivatives may be one of the causes of human lens coloration and of lens protein insolubilization in normal lens with aging. Further understanding of nonenzymatic browning \textit{in vivo} is needed so that strategies for interfering with the process on long-lived proteins can be devised.

\textbf{Acknowledgment} We thank Dr. Horwitz, Jules Stein Eye Institute, University of California, Los Angeles, who provided normal and colored cataractous human lenses.

\textbf{References}


