

Glutathione Levels of the Human Crystalline Lens in Aging and Its Antioxidant Effect against the Oxidation of Lens Proteins

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This paper reports the role of glutathione (GSH) in the crystalline lens as an antioxidant against the oxidation of lens protein. GSH levels in normal lenses decreased gradually with increasing age, from approximately 5 μmol per g lens (wet weight) to 3 μmol per g lens (wet weight). On the other hand, levels of oxidized GSH in the lenses increased until the age of 40. After that, it remained almost constant at the level of approximately 0.9 μmol per g lens. Protein-bound GSH levels in both soluble and insoluble lens proteins dropped noticeably in the 50-year and older age groups, although there were significant differences in levels between both fractions. A decrease of tryptophan and tyrosine residues in lens proteins was proportional to a decrease in GSH levels in the lens as a result of aging. Those residue levels in the cataractous lenses were approximately half those in the normal lens proteins, and GSH levels in such lenses were almost one-tenth that in the normal lens.

This study revealed that GSH may play an important role in preventing the oxidation of lens proteins from various oxidants. Furthermore, it is conceivable that these normal changes in GSH levels in the lenses increase the vulnerability of the lens to senile cataractogenesis.

Keywords human lens; glutathione; aging; lens protein oxidation; oxidized glutathione

Recent studies have demonstrated that in the crystalline lens, glutathione (GSH) may be closely related to the maintenance of lens transparency through its action as a scavenger against oxidants invading the lens.¹ It has been demonstrated that GSH may prevent lens protein aggregation due to disulfide bond formation.² Many investigators reported that the levels of GSH in lenses decreased with increasing age and/or with the advance of cataracts, indicating that this phenomenon may result in a diminution of the protective activity of the lens against oxidative damage by various oxidants, thus allowing the easy oxidation of lens proteins. It has been reported also that this disorder of the redox system in the lens may be one of the causes of cataractogenesis.³ To clarify the exact relationship between GSH content, the oxidation of lens proteins and cataract formation, it is first necessary to have knowledge of the GSH levels in normal lenses. However, there is not much information available on lens GSH as a function of age in the human. Thus, I attempted a more accurate measurement of GSH levels in the normal human lenses in aging, applying the high performance liquid chromatography method of Toyooka *et al.*⁴

Recently, some investigators reported that tyrosine residues in protein decreased as a result of oxidation.⁵ Many researchers also reported that tryptophan residues in lens proteins were easily decomposed by oxidation.⁶ Thus, I subsequently attempted to determine whether a lowering of GSH in the lens related to the oxidation of lens proteins.

This paper reports the changes in levels of reduced and oxidized GSH, protein-bound GSH and free thiol groups in normal human lenses as a function of age, and also the relationship between the activity of GSH reductase and the level of GSH. In addition, the level of thiobarbituric acid (TBA)-reactive substances was also determined, because it has been reported that the levels of TBA-reactive substances in the lenses are an important indicator for determining the degree of a lens proteins' oxidation.⁷

Materials and Methods

Human Lenses Normal human lenses were obtained from the Jules Stein Eye Institute Eye bank, University of California, Los Angeles. Upon removal 4—8 h after death, the eyes were stored at -80°C until use. The normal human lenses were dissected from the frozen eyeballs just before use. Human cataractous lenses were obtained from routine cataract surgery at the Jules Stein Eye Institute, having been removed during anterior capsulotomy. The lens cortex was removed with a mechanical infusion/aspiration unit and the lens nucleus was expressed entirely and retrieved.

Chemicals GSH and its oxidized form (GSSG), *N*-acetyl-tyrosine-ethyl ester and *N*-acetyl-tryptophan-ethyl ester were purchased from Tokyo Kasei, and 7-fluoro-4-sulfomoyl-2,1,3-benzoxadiazole (ABD-F) was from Dojin, Japan. 1,3-Diphenyl-2-thiobarbituric acid and 1,1,3,3-tetraethoxy propane were from Sigma. All other chemicals were of analytical grade or of the highest purity available.

Preparation of Lens Fractions Lenses were quickly homogenized in 1.5 ml of 1.15% KCl in an ice bath, and the supernatant was used as a soluble protein fraction. The soluble protein was exhaustively washed out of the resulting pellet by resuspension in 1.15% KCl and centrifugation. After washing the pellet 7 times, no protein was detected in the supernatant, as analyzed either by colorimetry or by the absorbance at 280 nm. This final pellet was used as an insoluble fraction. Both fractions were immediately used for analysis.

Preparation of Sample for Analysis of Free GSH and GSSG Samples for free GSH assay were prepared as follows. After a known amount of the internal standard (cysteamine) had been added to an aliquot of the soluble protein fraction, the solution was treated with 10% trichloroacetic acid (TCA) containing 1 mM EDTA to reach a final concentration of 5% TCA. The solution was subsequently centrifuged to remove the precipitate, and free GSH in the resulting supernatant was determined by HPLC as described below.

Samples for free GSSG assay were prepared as follows. After a known amount of the internal standard had been added to an aliquot of the original soluble fraction, the solution was divided into two fractions. One fraction was used to determine free GSH by the method described above, and the other was used to assay the total thiol group of GSH plus GSSG by the following method. After the soluble protein had been removed by treatment with TCA and centrifugation, the pH of the resulting supernatant was adjusted to 8.0 with 3 N NaOH. The solution was treated with an appropriate volume of 2% NaBH_4 in 10 mM sodium borate for 2 h at ambient temperature. After that, the pH of the solution was adjusted to 4 with 1 N HCl to decompose NaBH_4 . The total thiol groups of free GSH and GSSG were determined by HPLC as described below. Free GSSG concentration was calculated as the difference between the concentrations of the total thiol groups (GSH plus GSSG) and free GSH.

Preparation of Protein-Bound GSH Sample The original soluble and insoluble fractions were dialyzed against pure water at 4°C for 24 h in a stream of nitrogen gas. During dialysis, the outer solution was changed at 4-h intervals. After dialysis, samples were treated with NaBH₄ under the same conditions described above. The solution or suspension was then treated with TCA following the addition of a known amount of the internal standard, and centrifuged to remove the precipitate. GSH in the resulting supernatant was assayed as protein-bound GSH by HPLC as described below.

Determination of GSH and Other Thiol Compounds by HPLC GSH and other thiol compounds were determined by HPLC according to the method of Toyooka *et al.*,⁴⁾ after the thiol compounds had been converted to ABD-F derivatives. To a 1.5 ml reaction vial were added 50 μl of 1 mM ABD-F in 0.1 M sodium borate, pH 8.0, 30 μl of the sample solution, 10–50 μl of 2 μM cysteamine as an internal standard, and 70 μl of 0.1 M sodium borate containing 2 mM EDTA-2Na, pH 8.0. The reaction mixture was thoroughly mixed, and after the tube was capped it was heated at 50°C for 10 min. The reaction tube was then cooled in ice-water, 60 μl of 1 N HCl was added to the tube, and a 10 μl aliquot of the resulting solution was injected onto the HPLC column. The HPLC conditions were as follows: the column was a Wakosil 5C₁₈-200T (4.6 × 300 mm), and the eluent used was acetonitrile: 50 mM potassium biphthalate = 8:92 (v/v), pH 4.0. The flow rate was 1.0 ml/min. The ABD-F derivatives were detected with a fluorescence detector at 510 nm (Ex = 380 nm). Concentrations of the ABD-F derivatives were calculated from the value of the integrated peak area as compared with that of the internal standard.

Determination of Free Thiol Groups in Soluble Protein Levels of free thiol groups, which are of cysteine residues in proteins, were determined as follows. The original soluble fraction was treated with ABD-F according to the method of Toyooka *et al.*,⁴⁾ and subsequently dialyzed against pure water, which was changed 4 times at 4-h intervals. After dialysis, the amount of protein-bound ABD-F was determined by fluorometry (Ex = 380 nm, Em = 510 nm) to be free thiol groups. GSH-ABD-F, synthesized as described above, was used as a standard.

Assay of Activity of GSH Reductase After the lens had been homogenized in 1.0 ml of 0.05 M phosphate buffer, pH 7.2, the resulting supernatant obtained by centrifugation at 23000 × g for 20 min at 4°C was used as an enzyme solution for the assay. The activity of GSH reductase was assayed by the method of Rathbun *et al.*⁸⁾ To 200 μl of sample solution were added 825 μl of 70 mM phosphate buffer (pH 7.2), 150 μl of 33 mM GSSG and 75 μl of 0.2 mM FAD. Then, 150 μl of 1.0 mM NADPH was added to the solution. After the solution was mixed well, changes in the absorbance at 340 nm were measured over a time-course for 5 min. The enzyme activity (nmol/min) was calculated from the formula, $\Delta A/5 \text{ min}/(6.22 \times 1000)$, where ΔA is the difference between the absorbances at 0 and 5 min.

Determination of Tyrosine and Tryptophan Residues in Lens Protein by the Method of Magnetic Circular Dichroism The content of tyrosine and tryptophan residues in lens proteins was determined by the method of magnetic circular dichroism (MCD) using a spectropolarimeter J-600, Japan Spectroscopic Co., Ltd., Japan. MCD conditions were as follows: flow rate of nitrogen gas = 6 kg/cm², magnetic field = 11.67 kG, light path = 10.00 mm, scans = 7 times, and polarity = negative. For details of the determination of the tyrosine: tryptophan ratio in proteins by MCD, the paper of Barth *et al.* should be referred to.⁹⁾ As standards for the calibration curve, *N*-acetyl-tyrosine-ethyl ester and *N*-acetyl-tryptophan-ethyl ester were used.

Determination of Thiobarbituric Acid (TBA)-Reactive Substances The content of TBA-reactive substances in the lenses was measured according to the method of Nakashima *et al.*¹⁰⁾ The soluble protein fraction, 1 ml, was placed in a glass test tube with a glass stopper, and 1 ml of HCl-sodium acetate buffer, pH 2.0, and 0.5 ml of 0.12 M 1,3-diphenyl-2-thiobarbituric acid was added. The solution was heated in boiling water for 30 min and then cooled in an ice bath. Methyl-isobutyl ketone, 4 ml, was added to the solution, which was then shaken vigorously. The mixture was centrifuged at 1000 × g for 10 min and the organic solvent layer was separated and its absorbance was measured at 538 nm. 1,1,3,3-Tetraethoxy propane was used as a standard.

Protein Assay Soluble and insoluble proteins were determined by the method of Lowry-Folin.¹¹⁾

Results

Change in Levels of Free GSH and GSSG in Human Lenses in Aging Figure 1 shows the HPLC patterns of

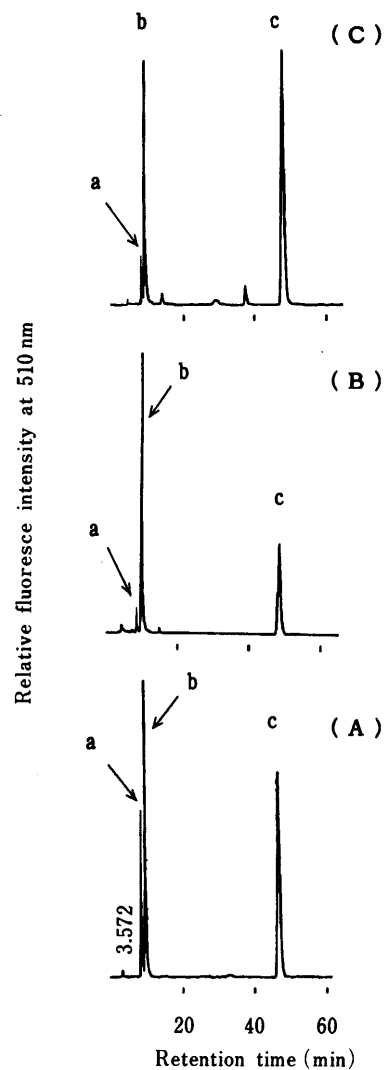


Fig. 1. HPLC Patterns of ABD-F Derivatives of Authentic Standards (A), Free GSH Sample (B, Lens of 42-year-old) and Protein-Bound GSH Sample (C, Lens of 42-year-old)

HPLC conditions and preparation method of ABD-F derivatives were described in text in detail. (a), cysteine ($t_R = 8.67$ min); (b), GSH ($t_R = 9.82$ min); (c), cysteamine ($t_R = 47.19$ min, internal standard) used as an internal standard. The amounts of cysteine, GSH and cysteamine in the authentic sample were 540, 480 and 535 pmol, respectively. The amount of the internal standard in the free GSH and protein-bound GSH samples was 535 and 48.2 pmol, respectively. Other peaks in the chromatograms were unidentified materials with fluorescence at 510 nm (Ex = 380 nm).

authentic standards (A), a sample of free GSH of the lens (B) and a sample of protein-bound GSH of the lens (C). As can be seen, GSH ($t_R = 9.81$ min) was completely separated from cysteine ($t_R = 8.67$ min). The level of GSH decreased gradually with increasing age, from approximately 5 μmol per g lens (wet weight) to approximately 3 μmol per g lens (wet weight) as shown in Fig. 2A.

For reference, GSH levels in cataractous human lenses from subjects of different ages and with various lens colors were also measured, as shown in Fig. 2B. As can be seen, free GSH levels in all three types of cataractous lenses were less than one-tenth the level in a normal lens. The GSH levels in white cataractous lens decreased increasing age. Regarding the other two types of cataractous lenses, their GSH levels remained almost constant in aging. There was also a significant difference in GSH level between the other two cataractous lenses.

As shown in Fig. 3, GSSG levels in the lenses rose,

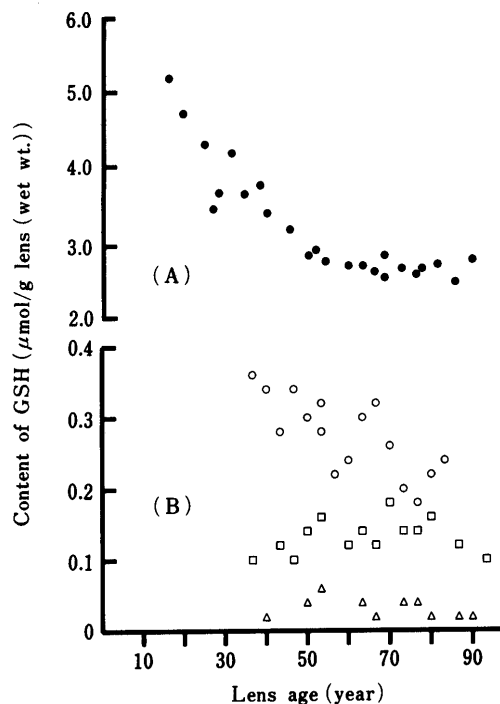


Fig. 2. Free GSH Level in Normal Human Lens

Samples were prepared as described in Materials and Methods. Each dotted datum is of one lens. ● (A), normal lenses; (B), cataractous lenses: ○, white; □, yellow; △, brown.

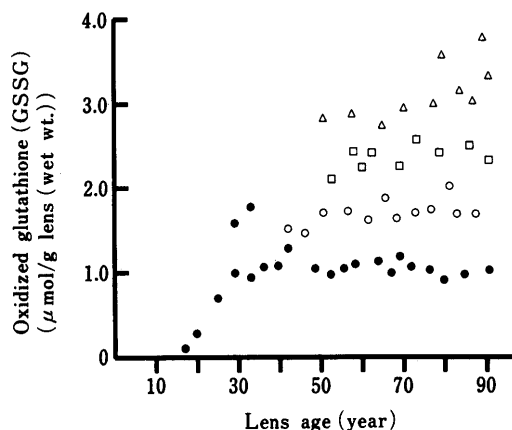


Fig. 3. Oxidized GSH (GSSG) Level in Normal Human Lens

Samples were prepared as described in Materials and Methods. Each datum is of one lens. ●, normal lens; ○, white cataractous lens; □, yellow cataractous lens; △, brown cataractous lens.

apparently with increasing age, until the age of 40. After that, the levels remained constant at approximately $0.2 \mu\text{mol}$ per lens. GSH/GSSG ratios as an indication of the degree of oxidation decreased exponential-functionally with increasing age, for example, 25 in the 10-year age group, 5 in the 30-year age group and 3 in the 50-year age group.

Protein-Bound GSH and Free Thiol Group Levels in Normal Human Lens The GSH levels of binding to soluble and insoluble proteins of normal human lenses were measured. The present study showed that in normal lenses the content of protein-bound GSH was larger at a younger age than that at an older one, and that the lenses at ages 10–30 showed approximately a two-fold content compared to that of the lenses of ages 70–90, as shown in Fig. 4.

The bound GSH level in the soluble fraction is approxi-

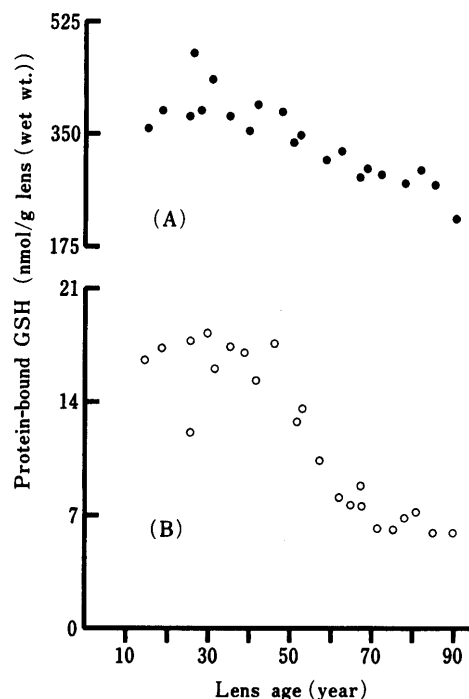


Fig. 4. Protein-Bound GSH Levels in Soluble and Insoluble Proteins of Normal Human Lens

Each datum is of one lens. The preparation method of protein-bound GSH is described in Materials and Methods in detail. (A) ●, soluble proteins; (B) ○, insoluble proteins.

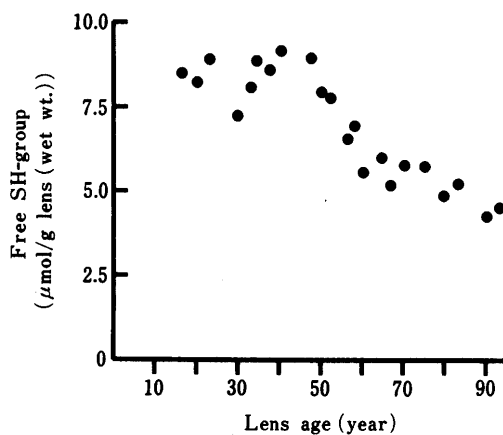


Fig. 5. Free SH-Group Levels in Soluble Proteins of Normal Human Lens

The SH-group levels were determined by fluorometry at 510 nm ($E_x=380 \text{ nm}$), using the ABD-F derivative of GSH synthesized as a standard (see Materials and Methods in detail). Each datum is of one lens.

mately 20-fold higher than in the insoluble fraction, although the data is scattered, and there is a significant difference in the concentration of protein-bound GSH between both fractions. The level of protein-bound GSH decreased with increasing age. Especially, the level dropped between the ages of 40 and 60. This fact indicates that the GSH content in the lens decreased apparently at an age of more than 40.

Next, the content of free SH-groups, or the SH-groups of cysteine residues in the soluble protein of the normal lens was determined. As shown in Fig. 5, in the normal lenses in this group remained almost constant at approximately $3.0 \mu\text{mol/g lens}$, between the ages of 10 to 40. The level of the free SH-group in lenses of subject more than

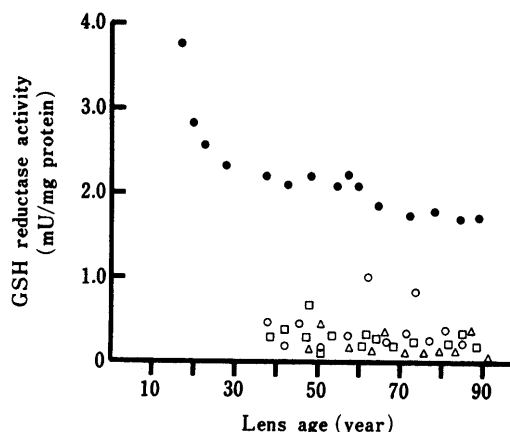


Fig. 6. Activity of GSH Reductase in Normal and Cataractous Lenses
 Assay of GSH reductase is described in Materials and Methods in detail. Each datum is of one lens. ●, normal lenses; ○, white cataractous lenses; □, yellow cataractous lenses; △, brown cataractous lenses.

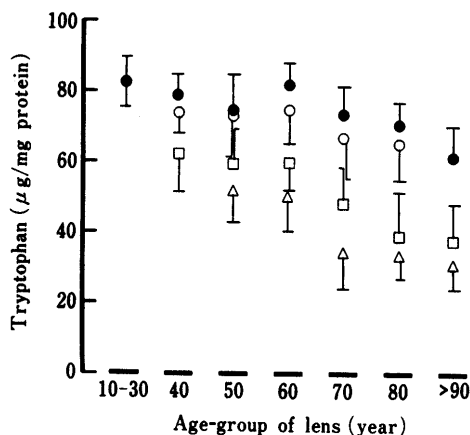


Fig. 7. Level of Tryptophan Residues in Proteins of Normal and Cataractous Lenses

Each dotted datum represents the mean \pm S.D. of three lenses of the same age group obtained from separate experiments, respectively. Ages of the lenses used in the 10–30 age group were 17, 24 and 32 years. There was no data on cataractous lenses of the young age group or from white lenses of the 90-year age group, because I was not able to obtain. ●, normal lens; ○, white cataractous lens; □ yellow cataractous lens; △ brown cataractous lens.

50 years old decreased gradually with increasing age. This phenomenon may be due to an enlargement of the number of disulfide bond formations between cysteine residues.

Activity of GSH Reductase in Normal and Cataractous Human Lenses In normal lenses of the young age-group, subjects between 10 and 30 years of age, the activity of GSH reductase decreased precipitously, from 3.75 munits/mg lens protein at the age of 17 to 2.4 munits/mg lens protein at the age of 30, as shown in Fig. 6. After that, GSH activity further decreased with increasing age. In cataractous lenses, the activity of GSH reductase was very low, approximately one-tenth compared to that of normal lenses. The trend of changes in GSH reductase activity in aging was similar to that of the GSH levels.

Levels of Tryptophan and Tyrosine Residues in the Protein of Normal and Cataractous Human Lenses For determining the content of tryptophan and tyrosine residues in the lens proteins, three lenses of similar age were used. In both normal and cataractous lenses, the level of tryptophan in lens protein decreased with increasing age, although there was a significant difference in its content among those

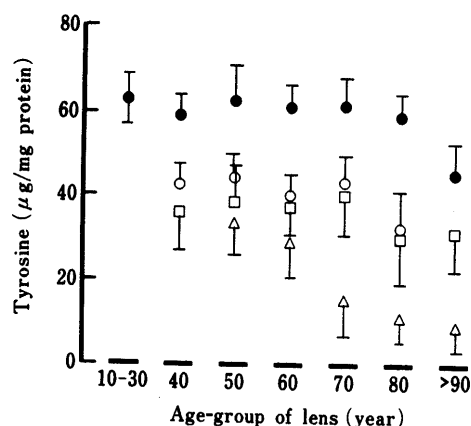


Fig. 8. Level of Tyrosine Residues in Proteins of Normal and Cataractous Lenses

Each dotted datum represents the mean \pm S.D. of three lenses of the same age group obtained from separate experiments, respectively. Ages of the lenses used in the 10–30 age group were 17, 24 and 32 years. There was no data on cataractous lenses of the young age group or from white lenses of the 90-year age group, because I was not able to obtain. ●, normal lens; ○, white cataractous lens; □ yellow cataractous lens; △ brown cataractous lens.

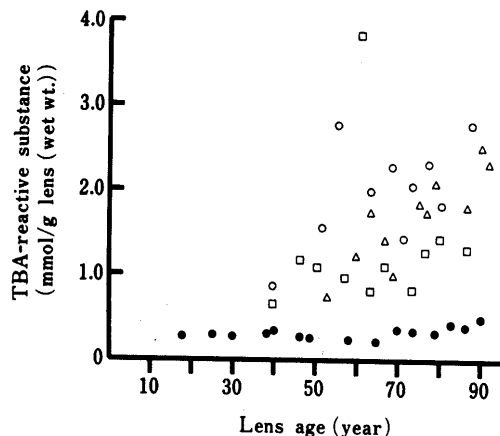


Fig. 9. Concentration of TBA-Reactive Substances in Normal and Cataractous Lenses

The sample used in this experiment was an aliquot of the same used for the assay of GSH. Each dotted datum is of one lens. ●, normal lens; ○, white cataractous lens; □, yellow cataractous lens; △, brown cataractous lens.

lenses, as shown in Fig. 7. Normal lenses always showed a higher level of tryptophan than did cataractous lenses. Especially, the yellow and brown lenses of more than 60 years ages showed a significant and statistical diminution of tryptophan level ($p < 0.05$) as compared with normal lenses. For example, in the lenses of the 70-year age group, the level in normal lenses was approximately $73 \mu\text{g/mg lens protein}$, but only about $32 \mu\text{g/mg lens protein}$ in cataractous brown lenses.

Regarding the levels of tyrosine residue in lens proteins of normal and cataractous lenses, these also showed a tendency similar to that of the tryptophan levels. In the brown lenses of more than 70 years of age, the tyrosine level ($18.2 \pm 7.6 \mu\text{g/mg lens protein}$) decreased remarkably, becoming almost one-third that of the normal lens ($61.2 \pm 7.3 \mu\text{g/mg lens protein}$).

Changes in TBA-Reactive Substance Level in Normal and Cataractous Human Lens Finally, the content of TBA-reactive substances was determined as an indication of the extent of oxidation occurring in the lens protein.

The level of TBA-reactive substances in the normal human lenses remained constant at approximately 0.3 mmol/g lens (wet weight) for long time, from ages 10 to 90, although a slight increase was observed after the age of 70, as shown in Fig. 9. In the cataractous lenses, the content level was higher than that of normal lenses, and increased significantly from approximately 1.0 mmol/g lens at a young age to approximately 2.0 mmol/g lens. This trend was remarkable in the white and brown lenses.

Discussion

It is well known that free GSH decreases in many types of cataractous lenses or in normal lenses as a factor of aging. Many investigators have speculated that the loss of GSH in the lens may induce oxidation and the subsequent aggregation of lens proteins.¹⁻³⁾ In a recent publication detailing a study of GSH levels in the human lens, Pao *et al.*¹²⁾ reported data on 3 fresh normal lenses of a very advanced age. Rathbun *et al.*¹³⁾ also reported the level of total reduced acid-soluble GSH and GSSG in the human lens as a function of age. However, reports on sufficient evidence on the relationship between the loss of GSH and oxidation of lens proteins have not been extensive. Therefore, I have attempted to examine the correlation between them both using several normal and cataractous human lenses, which I was fortunate to obtain.

In the previous paper, I reported that the amount of insoluble proteins with large molecular weights in the lens increases with aging and with the advance of cataracts.¹⁴⁾ Kamei *et al.* postulated that under normal conditions, GSH may bind to the cysteine residues of lens proteins and protect the formation of disulfide bonds between cysteine residues, whereas under abnormal conditions, the loss of GSH and its disulfide bonds between cysteine residues may increase, and proteins may thus aggregate or become insoluble. The current experiment revealed that the correlation between a decrease in the level of GSH and a decrease in the level of protein-bound GSH or a decrease in the level of a free thiol group is proportional. I confirmed that this fact supports my hypothesis. I also inferred that the loss of GSH in aging and the advance of cataract may be caused by a lowering of GSH reductase activity followed by the formation of GSSG.

Recent studies of the effects of ultraviolet radiation on lenses or on lens proteins showed a loss of tryptophan residues of the lens proteins followed by the yellowing and crosslinking of their proteins.^{6,15)} Other recent experiments confirmed that aerobic photo-oxidation destroys tryptophan residues and causes a loss of thiol groups followed by the crosslinking of lens proteins.^{6b,c)} Furthermore, it has been reported that under the same conditions, tyrosine residue is also oxidized and its level decreased.⁵⁾ The correlation between the loss of GSH and the oxidation of lens protein was also examined according to the decrease of these oxidizable amino acid residues. In the present experiment, I observed that the levels of tyrosine and tryptophan residues decreased as the level of GSH in the lens decreased. I confirmed that the loss of GSH in the lens was accompanied by the oxidation of lens proteins, indicating that GSH plays an important role in protecting lens protein from oxidation by many oxidants.

The antioxidant effect of GSH was then examined from

a different point of view, that is, according to changes in the level of TBA-reactive substances, since it is a well known fact that the level of this material reflects the level of lipid peroxide. The level of TBA-reactive substances in the cataractous lenses is very high, but not in normal lenses. This fact suggests that GSH may also prevent the oxidation of lipids in the lens.

The normal lenses used in the present experiment were eyebank specimens taken within 4 to 8 h of death. In 1991, Rathbun *et al.*¹²⁾ reported the level of total reduced acid-soluble GSH and GSSG in the human lens as a function of age. The values in Fig. 2A for GSH as almost same as those reported in that study. From this fact, I am convinced that the GSH level in lenses taken within 4 to 8 h of death and stored at -80°C remains unchanged.

In conclusion, the current work clarified that a lowering of GSH accelerates the oxidation of lens materials such as proteins and lipids. However, I am not yet sure when this decrease in GSH level occurs, before or after other materials are oxidized. I believe that GSH may obviate disulfide bond formation occurring as a result of protein-protein binding and protein aggregation through disulfide bonds between cysteine residues. It is conceivable that the loss of GSH in aging increases the vulnerability of the lens to cataractogenesis.

In addition, I have to mention that in the present experiment, the datum of each age is the value of only one lens, because it is hard to obtain several normal lenses of the same age. I know that biochemical changes in human lenses show great individual variation. However, I firmly believe that these data are useful to further research on crystalline lens and cataract formation.

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