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Physical Changes in Bovine Lens Homogenate Following Ultraviolet Irradiation and Their Prevention by Some Compounds

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Exposure of the dialyzed supernatant of bovine lens homogenate to ultraviolet (UV) light led to increases in its turbidity, pigmentation, and viscosity. These photochemically induced alterations of lens proteins were prevented by glutathione, cysteine and *N*-acetylcysteine, but not by ascorbic acid, *S*-(1,2-dicarboxyethyl)-glutathione or dulcitol.

Keywords——UV irradiation; bovine lens homogenate; turbidity; protective effect; glutathione

Cataracts have many different causes,[1] for example, physical agents such as ultraviolet (UV) light, daylight, and X-rays, chemicals such as galactose, naphthalene, and glucocorticoids, and biochemical effects such as enzyme deficiencies, some of which are inherited. Whatever the cause, it appears that cataractous lens proteins are crosslinked by the formation of covalent bonds and the formation of crosslinked proteins causes lens opacification and light scattering.[2]

In this work, we have focused our attention only one photo-oxidation of lens proteins. Animal lenses are constantly exposed to sunlight by day. This exposure of the eyes to light causes many photochemical reactions that may result in irreversible damage to lenses if their defence and renewal systems are inadequate. Hollows and Moran found a positive correlation between the prevalence of senile cataract and levels of climatic ultraviolet radiation in Australia.[3] A positive correlation between cataract prevalence and sunlight was also observed in the Himalayas.[4] Since Pirie[5] reported that photochemical reaction may be involved in the process by which the human lens loses its transparency, numerous investigators have reported relationships between light and lens proteins. Zigman reviewed photochemical mechanisms in cataract formation,[6] and the problems of light and oxygen effects on the eye were discussed at an international symposium.[7] However, these reports have mainly dealt with pigmentation and fluorescence of lens proteins. Using a different approach, we have studied alterations in the physical properties of lens proteins produced *in vitro* by UV light. Dialyzed bovine lens homogenate was exposed to UV light under aerobic conditions, and various substances were tested in this system for ability to prevent appearance of turbidity of lens proteins. This is the first study based on such an approach. Our aim was to develop an *in vitro* system analogous to cataract formation and to test various compounds in this system for effectiveness in the prevention of cataracts.

Materials and Methods

Chemicals——*S*-(1,2-Dicarboxyethyl)-glutathione was kindly supplied by Senju Pharmaceutical Co. (Osaka). Dulcitol was purchased from Katayama Chemical Industries Co. (Osaka). *N*-Acetylcysteine was from Boehringer Mannheim, and other chemicals were from Wako Pure Chemical Industries (Osaka).

Bovine Lens Homogenate——Lenses were removed as soon as possible from bovine eyes freshly obtained from the slaughterhouse and were either used immediately or stored at $-20\,°C$. Lens (2 g) was homogenized in 5 ml of

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water at 0 °C with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 20000 × *g* for 30 min. The supernatant fluid, to which *N*-ethylmaleimide had been added to a concentration of 10 mM, was dialyzed overnight against 0.1 M potassium phosphate buffer (pH 7.4). This concentration of *N*-ethylmaleimide was the minimum required to mask SH-groups, as determined by the method of Ellman and Lysko.[8]

UV Light Irradiation——The dialyzed homogenate was placed in quartz test tubes (1.5 × 12 cm), and irradiated for 25 h at 25 °C from a distance of 10 cm with a bactericidal UV lamp (15 W, Mitsubishi Type 15F, which emits principally at 254 nm) under magnetic stirring. The intensity of 254-nm light at a distance of 50 cm was 128 μW/cm^2. One test tube was run in the dark as the control. Aliquots from the irradiated and control homogenates were tested at appropriate time intervals.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis——A 4% to 22.5% gradient slab of 2 mm thickness with 1% SDS was prepared according to the method described by Mostafapour and Reddy.[9] An aliquot (200 μl) of a ten-fold dilution of the irradiated lens homogenate was added at specified times to 25 μl of 20% 2-mercaptoethanol and 25 μl of 10% SDS, and the mixture was heated at 100 °C for 5 min. To each of 12 slots on the slab, 20 μl of the heated reaction mixture was applied. The glass plates were removed after the completion of migration, and the gel was placed in a staining solution consisting of 200 mg of Coomassie brilliant blue R 250 and 100 ml of 50% trichloroacetic acid for 40 min. Destaining was carried out in a solution of water : acetic acid : methanol (1950 : 150 : 200).

Results

Effect of UV Irradiation on the Turbidity and Absorption of Bovine Lens Homogenate

The turbidity of the irradiated lens homogenate was measured at 600 nm. Figure 1 shows the turbidity of the lens homogenate during UV irradiation; the unirradiated lens homogenate was clear and colorless even after 25 h, whereas the irradiated homogenate became increasingly opaque with time. In addition, the irradiated homogenate became yellow, then brown, with the passage of time. After 25 h, 1 ml of the irradiated or unirradiated sample was mixed with 0.5 ml of 0.5 N NaOH to obtain a clear solution. Both samples had absorption maxima at 302 nm attributable to aromatic amino acids. The maximum of the irradiated sample was a broad band that sloped gently down to 600 nm from 302 nm, whereas that of the control was a narrow peak. Figure 2 shows the changes in the absorption at 420 nm of the irradiated and control lens homogenates in alkali against time of irradiation. No color change was seen in the control lens homogenate. Human serum and egg white containing the same protein concentration as the lens homogenate were treated exactly as described above. However, only barely detectable turbidity was observed and no coloration took place in either case.

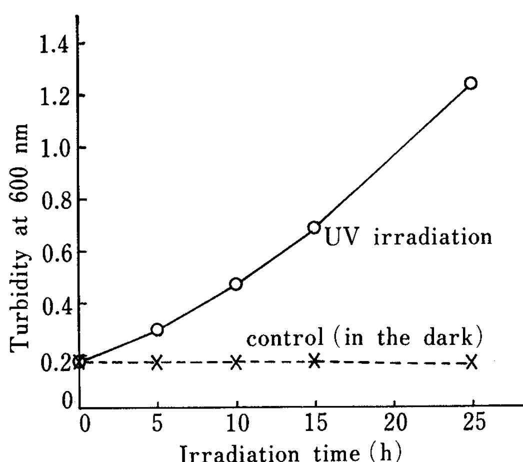

Fig. 1. Changes in Turbidity of Lens Homogenate on UV Irradiation

Bovine lens homogenate was prepared as described in Materials and Methods and exposed to UV light at 25 °C. The turbidity was measured at 600 nm (○—○). The control experiment was run in the dark (×---×).

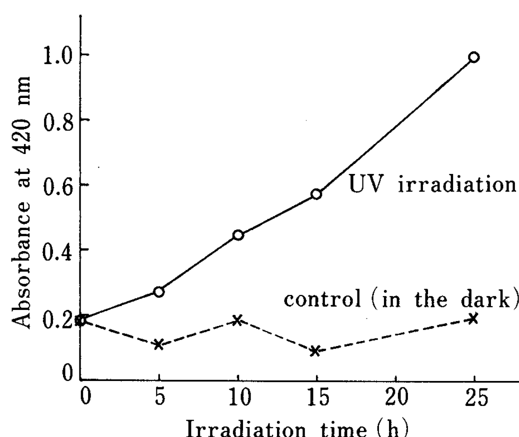

Fig. 2. Changes in Absorbance at 420 nm of Lens Homogenate on UV Irradiation

Lens homogenate was prepared and irradiated as described in Materials and Methods. Aliquots were taken at intervals and mixed with alkali, then the absorbance was measured at 420 nm (○—○). The control experiment was run in the dark (×---×).

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Protective Effect of Various Substances on the Turbidity and the Pigmentation of Lens Homogenate during Irradiation

Intrinsic protecting mechanisms against photochemical or oxidative damage must exist in normal lenses. We examined how substances of low molecular weight that are present principally in the eye protect against the development of turbidity and pigmentation of the lens homogenate preparation. The substance to be examined was added to the dialyzed bovine lens homogenate prepared as described in Materials and Methods. The concentration of each substance was 10 mM, and the irradiation procedure was the same as that described above. Figure 3 shows the change in turbidity at 600 nm with time, and Fig. 4 shows the pigmentation at 420 nm of lens homogenate in the presence of various substances.

We have determined the concentrations of *S*-(1,2-dicarboxyethyl)glutathione (DCEG), which was first isolated from calf lens by Calm and Waley in 1963,[10] in the lens of several animal species.[11] We found its concentration to be approximately 0.1 μmol per g of wet bovine lens, and we also found that the level of DCEG in rat lens during the formation of cataracts by galactose decreased to 50% of the control value within 48 h, to 30% on the 5th day, and to 25% on the 10th day.[11] As can be seen in Figs. 3 and 4, addition of DCEG to the lens homogenate appeared not to have prevented, but instead to have stimulated, the development of turbidity and pigmentation. This result is not explicable at present.

Ascorbate is present in the normal lens of various animals at concentrations of about 3—57 mg per 100 g of wet tissue.[7,12] It reduces oxygen to form H_2O_2, and this reaction is catalyzed by metal ions.[13] As shown in Fig. 3 the presence of ascorbate accelerated the increase in turbidity. Whether or not this effect was due to H_2O_2 was not determined. On the other hand, the exact measurement of pigmentation of the irradiated lens homogenate in the presence of ascorbate was impossible, because exposure of even ascorbic acid alone (10 mM) in 0.1 M potassium phosphate (pH 7.4) to light led to pigmentation.

Cataracts in diabetic and galactosemic animals are classified as sugar cataracts, and polyols are accumulated in lenses that have these cataracts. In addition, feeding of galactose

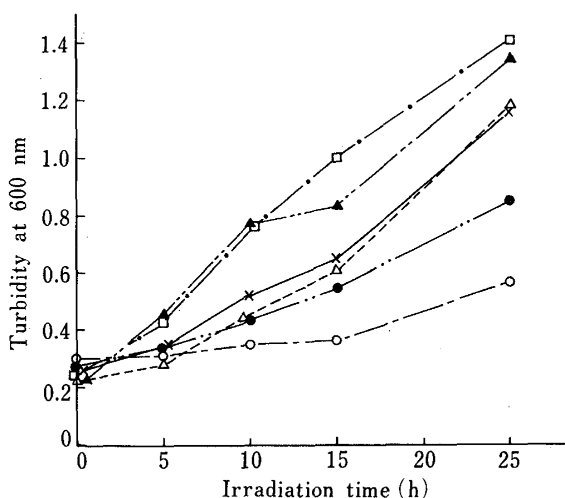

Fig. 3. Changes in Turbidity of Lens Homogenate by UV Irradiation in the Presence of Various Compounds

Bovine lens homogenate was prepared as described in Fig. 1 and a low-molecular-weight substance was added to the homogenate. The homogenate was exposed to UV light and turbidity was measured at 600 nm at intervals.

×——×, control; ○—-—○, GSH; ▲—-—▲, ascorbate; □—·—□, DCEG; ●—··—●, *N*-acetylcysteine; △---△, dulcitol.

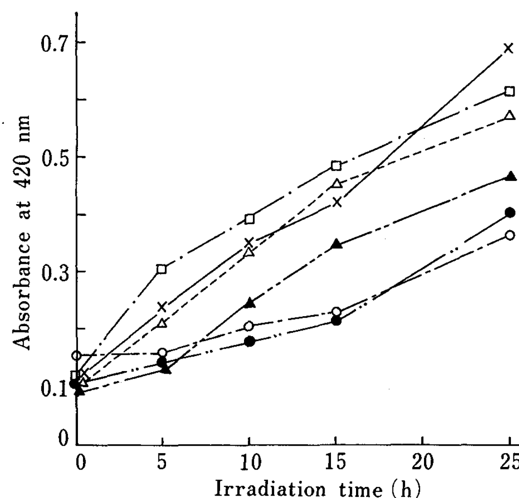

Fig. 4. Changes in Absorbance at 420 nm of Lens Homogenate on UV Irradiation in the Presence of Various Compounds

As described in Fig. 2 or in the text, bovine lens homogenate with test substance was prepared and irradiated with UV light.

×——×, control; ○—-—○, GSH; ▲—-—▲, cysteine; □—·—□, DCEG; ●—··—●, *N*-acetylcysteine; △---△, dulcitol.

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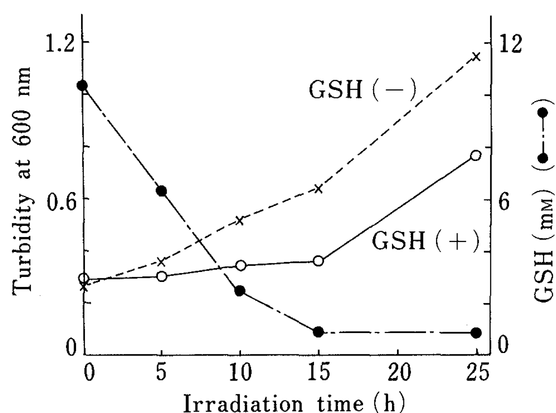

Fig. 5. Changes in Turbidity of Lens Homogenate on UV Irradiation with and without GSH, and Changes in GSH Level in Lens Homogenate

Turbidity was measured in the same way as described in the legend to Fig. 1. GSH level in lens homogenate was measured by the method of Saville.[22)]

leads to an accumulation of dulcitol.[14)] Polyol, sugar alcohol, sugars and ascorbic acid are known to be scavengers of free radicals. We therefore tested whether dulcitol affects photochemical changes in lens proteins and found, as shown in Figs. 3 and 4, that dulcitol has no effect on turbidity or pigmentation.

Reduced glutathione (GSH) is widely distributed in living cells and is present at concentrations of 0.5—10 μmol/g of wet tissues of various animals.[15)] GSH is present in lenses of various animals at concentrations 7—15 μmol/wet g,[16)] and appears to play a significant role in keeping the lens colorless and clear. The marked decrease in its concentration during cataract formation results from several factors.[16a,17)] In the present experiment, changes in turbidity and pigmentation were clearly suppressed in the presence of GSH (Figs. 3 and 4). GSH was thus found to be a potent inhibitor of the photo-oxidation reaction in lens. Addition of *N*-acetylcysteine, which may not be a genuine constituent of the lens, to the test system also prevented the photochemical alteration. Turbidimetric assay was not feasible with cysteine because of turbidity that resulted from the precipitation of cystine produced by the oxidation of cysteine in the test system. However, measurement of changes in absorbance at 420 nm was possible after the addition of alkali. As shown in Fig. 4, the pigmentation-preventing activity of cysteine was also appreciable, but was weaker than that of GSH.

Change in Glutathione Level in the Irradiated Lens Homogenate

As can be seen in Fig. 5, the concentration of GSH added to the lens homogenate decreased by 91% after 15 h of UV irradiation. As the concentration of GSH decreased, the turbidity of the homogenate gradually increased. However, the presence of ascorbic acid (3 mg/ml) maintained the GSH level at the added concentration (3 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.4) during exposure to UV light.

Changes in Specific Viscosity of the Lens Homogenate during UV Irradiation

In order to examine whether the crosslinking of lens proteins by UV light is prevented by GSH, the specific viscosity of lens homogenate was measured with an Ostwald viscosimeter at 25 °C. Samples of 2 ml of irradiated lens homogenate to which 1 ml of 0.5 N NaOH had been added were measured. The differences between the specific viscosities of the lens homogenate with and without GSH were 0.02, 0.03, 0.09 and 0.17 at 5, 10, 15 and 25 h, respectively. These results indicate that crosslinking of the lens proteins took place as the time of irradiation increased.

SDS–Polyacrylamide Gel Electrophoresis

Lens homogenate was prepared and irradiated as described in Materials and Methods. Samples at hourly intervals were subjected to SDS–polyacrylamide gel electrophoresis. As the time of irradiation increased, tailing occurred overall, and a broad band with a molecular

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weight of around 34000 daltons became thicker; these phenomena were prevented by the addition of GSH. Figure 6 illustrates the results of SDS–polyacrylamide gel electrophoresis of lens proteins irradiated for 25 h.

Discussion

During cataract formation, human lens proteins, in general, show gradually increasing levels of aggregation, pigmentation and fluorescence, and decreased SH group content. These phenomena have been shown to be caused or accelerated by photochemical reactions by a number of studies.

In order to elucidate these phenomena, many *in vitro* experiments have been performed. In 1971 Pirie first exposed human lens homogenate to sunlight and showed that the indole ring was split to yield N'-formylkynurenine.[5] Zigman *et al.* irradiated pure calf γ-crystallin, whole human and rabbit lenses, and calf aqueous humor *in vitro* with near-UV light. They reported increased pigmentation with increased exposure to light.[18] In 1980, the soluble protein fraction from the cortex of calf lens was exposed to near-UV light under both aerobic and anaerobic conditions. In this report, changes in fluorescence, UV spectra and mobility in SDS gel electrophoresis were documented.[19] Ziegler *et al.* also studied the effect of near-UV light on human lens crystallines and demonstrated an increase in blue fluorescence and covalent-crosslinking.[20] In recent years, Fujimori induced covalent cross-linking and blue fluorescent product formation of calf lens α-crystalline by exposure to near-UV light under aerobic conditions. He discovered that the formation of blue-fluorescent species and crosslinking of polypeptides were both inhibited by the presence of glutathione.[21] Many *in vitro* studies reported so far, some of which are cited above, have been concerned with fluorescence, coloration and crosslinking of lens proteins.

In the present study, we have investigated the changes in turbidity and viscosity of bovine lens proteins *in vitro* after UV irradiation; data on turbidity changes have not been reported previously. We think that turbidity experiments *in vitro* are desirable because cataract formation is essentially accompanied by an increase in the opaqueness of the lens proteins. As can be seen from Figs. 1 to 4, a positive correlation between the coloration and the turbidity change was observed. When lens proteins were irradiated with the UV lamp under aerobic conditions, many photochemical reaction occur. In particular, tryptophan, tyrosine, histidine and methionine are oxidized and degraded by radiation near the UV region.[6,20b] As shown in Figs. 3 and 4, various substances showed somewhat different effects on the development of turbidity and pigmentation in lens homogenate exposed to UV light. This is thought to be due to differences in their effects on different photochemical reactions. For example pigmentation may result for the most part from the degradation of tryptophan.[18a]

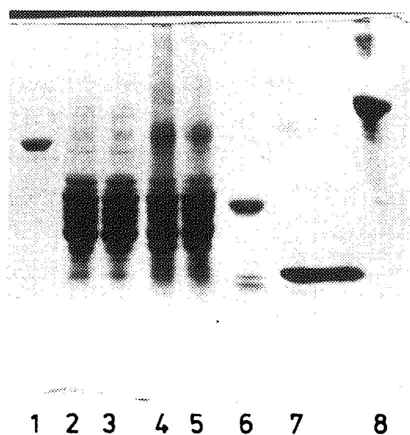

Fig. 6. SDS–Polyacrylamide Slab Gel Electrophoresis of Lens Homogenate Irradiated with UV Light

Bovine lens homogenate was prepared and irradiated with UV light for 25 h as described in the legend to Fig. 1. The irradiated homogenate was subjected to SDS–polyacrylamide gel electrophoresis.

1, alcohol dehydrogenase from yeast (m.w. 34000); 2, bovine lens homogenate without GSH at 0 time; 3, bovine lens homogenate with GSH at 0 time; 4, bovine lens homogenate without GSH after UV irradiation for 25 h; 5, bovine lens homogenate with GSH after UV irradiation for 25 h; 6, trypsin (m.w. 24300); 7, lysozyme (m.w. 11000); 8, bovine serum albumin (m.w. 68000).

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Except for experiments on the inhibition of alterations of ocular proteins exposed by near-UV light by ascorbic acid[18b)] and glutathione,[21)] no detailed study has been reported. We tested several substances other than ascorbate and glutathione in our system. It was found that the addition of glutathione prior to UV irradiation potently inhibited both turbidity development and coloration, as might be expected. Ascorbic acid accelerated the turbidity development of lens homogenate in this experiment, though it inhibited pigmentation of human lenses *in vitro*.[18b)] This discrepancy cannot be explained at present, though the experimental conditions differed from each other.

The photo-oxidation system in this report was prepared from lens homogenate treated with *N*-ethylmaleimide to mask SH groups before dialysis. In addition, we used a small commercially available UV lamp as a UV light source. This lamp did not heat the test tubes, and the temperature of the test tubes was maintained at 25 °C during irradiation. It is noteworthy that we succeeded in the photo-oxidation of lens proteins with this small lamp under simple and mild conditions, although near-UV light of high intensity has been used in many experiments.

The crosslinking of lens proteins molecules by UV irradiation was demonstrated by the observations of changes of specific viscosity and SDS–polyacrylamide gel electrophoresis behavior (Fig. 6). The changes in the turbidity of lens proteins can thus be attributed to crosslinking.

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