The effects of X-irradiation on lens reducing systems

Frank J. Giblin, B. Chakrapani, and V. N. Reddy

Studies have been made of the effects of X-ray on various lens reducing systems, including the levels of NADPH and glutathione (GSH), the activity of the hexose monophosphate shunt (HMS) and of certain enzymes, including GSH reductase, GSH peroxidase, and glucose-6-phosphate dehydrogenase (G-6-PD). It was found that during several weeks following X-irradiation but prior to cataract formation, there was very little change in the number of reduced -SH groups per unit weight of lens protein but that, with the appearance of cataract, there was a sudden loss of protein -SH groups. In contrast, the concentration of GSH in the X-rayed lens decreased throughout the experimental period. Similarly, the concentration of NADPH in the X-rayed lens was found to decrease significantly relative to controls 1 week prior to cataract formation, and the ratio of NADPH to NADP+ in the lens shifted at this time period from a value greater than 1.0 in the control lens to less than 1.0 in the X-rayed lens. A corresponding decrease occurred in the activity of the HMS in X-rayed lenses as measured by culture in the presence of 1-14C-labeled glucose. G-6-PD was partially inactivated in the X-rayed lens. Of the eight enzymes studied, G-6-PD appeared to be the most sensitive to X-irradiation. The data indicate that X-irradiation results in a steady decrease in the effectiveness of lens reducing systems and that when these systems reach a critically low point, sudden oxidation of protein -SH groups and formation of high-molecular-weight protein aggregates may be initiated.

Key words: rabbit lens, X-ray, pyridine nucleotides, sulfhydryl groups, hexose monophosphate shunt, glutathione reductase, glutathione peroxidase, glucose-6-phosphate dehydrogenase

The formation of high-molecular-weight (HMW) proteins is believed to be responsible for scattering of light and the resultant opacification of a cataractous lens in certain forms of cataracts.1-3 The mechanism by which these protein aggregates are formed is not known, but one possible contributing factor may be the oxidation of protein sulfhydryl (-SH) groups. An increased concentration of disulfide bonds has been found to be present in the HMW protein of X-ray-induced cataract, and more importantly, there is an indication that intermolecular bonding is involved, since treatment of the protein with disulfide bond-reducing agents results in the release of lower-molecular-weight crystallins.4 Additional support for the concept that oxidation of -SHs may play a role in protein aggregation comes from the observations of
Spector and Roy, 5 who found an increase in the disulfide content in HMW proteins of human cataractous lenses.

The present study was designed to provide possible clues to the mechanism of oxidation of protein -SH groups and the formation of HMW protein in X-ray-induced cataract in rabbits. A systematic investigation has been conducted on the effects of X-irradiation on various lens reducing systems which may normally act to maintain protein -SH groups in the reduced state. In this report, we present data on the levels of the reducing compounds nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and glutathione (GSH), the activity of the hexose monophosphate shunt (HMS), and the activities of several enzymes, including GSH reductase, GSH peroxidase, and glucose-6-phosphate dehydrogenase (G-6-PD) in normal and irradiated lenses. These parameters were examined at various times after X-ray until cataract formation, which occurred regularly 8 weeks later. The results suggest that a diminished supply of GSH and NADPH and decreased activities of G-6-PD and the HMS may promote the oxidation of protein -SH groups in this type of cataract.

**Methods**

The albino rabbits used in this study were 5 weeks of age at the time of X-irradiation. The X-irradiation factors employed have been described previously. 4 One eye was irradiated, and the area surrounding the eye was protected with a lead shield. The nonirradiated eye served as the control. A single 2000 rad dose was given to each rabbit.

Concentrations of protein -SH groups and GSH (nonprotein -SH groups) were determined with 5,5'-dithio-bis-(2-nitrobenzoic acid). 6 For this purpose, whole lenses were homogenized at 0 to 5°C in 0.02M sodium phosphate buffer, pH 7.4, containing 0.1M NaCl and 0.025M EDTA. Protein was assayed by the method of Lowry et al., 7 with bovine serum albumin used as standard.

The concentrations of pyridine nucleotides were determined with a "cycling assay" system employing thiazolyl blue (MTT) as a terminal electron acceptor. This method was originally described by Nisselbaum and Green 8 and was modified in the present study for use with lens tissue. A complete description of the modifications employed in the assay procedure is in preparation and will be shortly submitted for publication. Briefly, the cycling mixture consisted of MTT, phenazine ethosulfate (PES), and either ethanol and alcohol dehydrogenase for the determination of nicotinamide adenine dinucleotide (NAD+) and NADH or glucose-6-phosphate and G-6-PD for the determination of NADP+ and NADPH. The rate of the reduction of MTT to the corresponding formazan, which absorbs maximally at 570 nm, is proportional to the concentration of coenzyme.

The procedure for extraction of pyridine nucleotides was a modification of that used by Sippe 9. Either NAD+ and NADP+ or NADH and NADPH were assayed in individual lenses. For extraction of NAD+ and NADP+ the lens was homogenized in 5 vol of 0.23M KH2PO4 for 1 min in a boiling water bath, chilled, neutralized with an equal volume of 0.2N KOH, and centrifuged at 5°C for 30 min at 20,000 x g. The order of treatment of KH2PO4 and KOH was reversed for extraction of NADH and NADPH. The recoveries of each coenzyme in the procedure were determined by adding a known amount of coenzyme just prior to the homogenization of a normal lens in acid or base. The recoveries were nearly 100% for NAD+ and NADP+ and approximately 85% for NADH and NADPH.

The activity of the HMS was estimated in control and experimental lenses by determining the amount of 14CO2 liberated from lenses cultured in the presence of 1-14C-labeled glucose 10 (specific activity 14 μCi/mg; New England Nuclear Corp., Boston, Mass.). The lenses were cultured in capped tubes under sterile conditions for 8 hr at 37°C in 5 ml of KEI-4 medium 11 (6.94 mM glucose) containing approximately 45,000 cpm/ml radioactivity. The amino acid concentrations of the KEI-4 medium in the aforementioned paper are incorrectly given as four times the actual values; the values should read as milligrams per 4 L of medium. At the end of the culture period a vial containing 0.5 ml of hydroxide of hyamine was inserted into the tube; the tube was stoppered, and 1 ml of 2N H2SO4 was injected into the medium to release CO2. The released CO2 was allowed to be absorbed into the hydroxide of hyamine overnight. 12 The vials and contents were then added to scintillation fluid and counted in a liquid scintillation counter. The lenses were removed from the culture tubes, dried overnight at 85°C, and weighed. The amount of radioactivity
Table I. Weights of control and X-irradiated rabbit lenses

<table>
<thead>
<tr>
<th>Time after X-irradiation</th>
<th>Dry weight (mg)</th>
<th>Wet weight (mg)</th>
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<tr>
<td></td>
<td>Control</td>
<td>X-irradiated</td>
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<td></td>
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<td>X-irradiated</td>
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Results are expressed as the means ± S.D. The data for dry weights were obtained from lenses used in measurement of HMS activity (Fig. 2). The number of experiments is shown in parentheses.

released per lens was normalized to account for a slight day-to-day variation in the level of radioactivity present in the medium. A blank consisted of a vial of hydroxide of hyamine which was treated in the same manner as above except that a lens was not present in the culture medium.

The activities of glyceraldehyde phosphate dehydrogenase, lactate dehydrogenase (LDH), hexokinase, G-6-PD, phosphofructokinase, 6-phosphogluconate dehydrogenase, and GSH reductase were determined by measuring the change in absorbance at 340 nm of either NADH or NADPH as described by Bergmeyer. GSH peroxidase was assayed by the method of Lawrence et al.

Results

Under the conditions employed, 90% of the irradiated rabbits developed mature cataracts characterized by complete opacity and swelling 8 weeks (±3 days) after X-irradiation. One week prior to cataract development (7 weeks following X-irradiation), the X-rayed lenses were clear except for some haziness present in the posterior subcapsular region. These lenses, which weighed on the average 250 mg, were 20% lighter than the controls (312 mg) (Table I). However, 4 weeks after X-irradiation there was little difference in weight between control and experimental lenses, both weighing about 260 mg.

During the weeks following irradiation there was very little change in the number of reduced -SH groups per unit weight of protein (Fig. 1). In contrast, there was a dramatic decline in the concentration of GSH during the same period, so that 1 week prior to cataract formation the level of GSH in the experimental lens was only 25% of that in the control. It was only at the time of cataract formation (8 weeks after X-ray) that loss of protein -SH groups was observed; the decrease amounted to 35% relative to the control.

The sudden decrease in the number of reduced -SH groups per unit weight of protein and the previously reported finding of high disulfide content in the HMW protein of X-ray induced cataract prompted us to investigate changes in the reducing systems of the X-rayed lens which might be responsible for the observed phenomena. Table II summarizes the results of analyses for NADPH and other pyridine nucleotides in the lens 7 weeks after X-irradiation. The main feature of the data is that 1 week prior to cataract, there was a significant reduction in the concentration of NADPH in the lens (p < 0.001), amounting to a 42% decrease on a per gram wet weight basis. The decrease in NADPH on a per lens basis was higher (52%, data not shown) due to the fact that the X-rayed lens was 20% lighter than the control (7 weeks after irradiation). In addition to NADPH, the concentration of the other three coenzymes also decreased in X-irradiated lenses although to a lesser extent. On a per gram wet weight basis, the decreases in NADH, NADP+, and NAD+ ranged from 12% to 24%, somewhat less than that observed for NADPH. Of the four coenzymes analyzed, only the decrease in NADH was not significant (p < 0.05).

Table II also indicates that a shift occurred in the ratio of NADPH to NADP+ in the X-rayed lens 1 week prior to the appearance of cataract. In the control lens, at this time
Table II. Concentration of pyridine nucleotides in control and experimental rabbit lenses 1 week prior to the formation of X-ray-induced cataract

<table>
<thead>
<tr>
<th></th>
<th>Control (nmol/gm wet wt.)</th>
<th>X-irradiated (nmol/gm wet wt.)</th>
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<tbody>
<tr>
<td>NADP*</td>
<td>15.6 ± 1.1 (6)</td>
<td>12.5 ± 1.2 (6)</td>
</tr>
<tr>
<td>NADPH</td>
<td>17.7 ± 1.2 (8)</td>
<td>10.3 ± 1.2 (8)</td>
</tr>
<tr>
<td>NADPH/NADP*</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>NAD*</td>
<td>988 ± 79 (6)</td>
<td>748 ± 181 (6)</td>
</tr>
<tr>
<td>NADH</td>
<td>487 ± 71 (8)</td>
<td>428 ± 91 (8)</td>
</tr>
<tr>
<td>NAD*/NADH</td>
<td>2</td>
<td>1.8</td>
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Results are expressed as the means ± S.D. The number of experiments is indicated in parentheses. The pyridine nucleotide levels on a per lens basis can be calculated from the wet weights of lenses at 7 weeks, listed in Table I.

Table III. Activities of various enzymes in control and experimental lenses 1 week prior to X-ray-induced cataract

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control (units/gm wet wt.)</th>
<th>X-irradiated (units/gm wet wt.)</th>
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<tr>
<td>LDH (7)</td>
<td>51 ± 6.2</td>
<td>51 ± 6.0</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>40 ± 3.3</td>
<td>34** ± 3.6</td>
</tr>
<tr>
<td>Phosphatase dehydrogenase (7)</td>
<td></td>
<td></td>
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<tr>
<td>Phosphofructokinase (5)</td>
<td>19 ± 0.5</td>
<td>16** ± 0.9</td>
</tr>
<tr>
<td>GSH peroxidase (3)</td>
<td>1.2 ± 0.04</td>
<td>1.2 ± 0.08</td>
</tr>
<tr>
<td>6-phosphogluconate dehydrogenase (6)</td>
<td></td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>G-6-PD (8)</td>
<td>0.24 ± 0.01</td>
<td>0.17* ± 0.02</td>
</tr>
<tr>
<td>Hexokinase (6)</td>
<td>0.18 ± 0.01</td>
<td>0.15** ± 0.02</td>
</tr>
<tr>
<td>GSH reductase (5)</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
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</table>

Assays were performed on whole lenses; 1 enzyme unit corresponds to the conversion of 1 μmol substrate per minute at 30°C under optimal conditions. The number of experiments is shown in parentheses. Values marked with asterisks differ significantly from control: *p < 10^-3; **p < 10^-5.

period, the ratio of NADPH to NADP+ was 1.1, whereas in the X-rayed lens the value fell below 1 (0.8). Thus, in the precataractous lens, there was a significantly lower concentration of NADPH relative to NADP+. On the other hand, the ratio of the concentration of NAD+ to that of NADH was almost 2 in both control and X-irradiated lenses.

In view of the observed decrease in the level of NADPH, it was of interest to study the effect of X-ray on the activity of the HMS, which furnishes a continuous supply of NADPH in the lens. The 14CO2 liberated from the oxidation of 1-14C-labeled glucose was taken as a direct measure of HMS activity, since it has been well documented that the activity of the tricarboxylic acid cycle in the rabbit lens is minimal and that there is about 40 times more CO2 liberated from the oxidation of 1-14C-labeled glucose than from 6-14C-labeled glucose.10

Fig. 1 shows the activity of the HMS in control and experimental lenses at various times after X-irradiation. It may be seen that on a per lens basis, there was a slight increase in the HMS activity in control lenses over the 7 week period. This increased activity was due, in part, to the growth of the lens, the dry weight increasing from 55 mg at the be-
Fig. 2. Effect of X-irradiation on the activity of the HMS in the lens. Lenses were analyzed at various times following a single X-ray dose of 2000 rads to the rabbit eye. The activity of the HMS was compared by measuring $^14$CO$_2$ liberated from 1-$^14$C-labeled glucose. Controls consisted of nonirradiated contralateral lenses. Values of CO$_2$ liberated per lens are shown by circles, and those per milligram dry weight are shown by triangles; open symbols represent controls, and solid symbols represent experimental lenses. Each value is the mean of three to eight experiments, and the bars represent standard deviations.

In view of the differential growth of control and X-irradiated lenses, which was especially evident 7 weeks after X-irradiation (Table I), the results of HMS activity are also expressed on a per milligram dry weight basis. When the data are expressed in this manner (Fig. 2), a gradual decrease in HMS activity is noted in both control and X-irradiated lenses. However, the decrease in HMS activity was much greater in X-irradiated lenses; a 32% decrease relative to controls was observed at the end of 4 weeks, and this level of inhibition remained through 7 weeks following X-irradiation.

It is seen from the data in Fig. 3 that the activity of G-6-PD, the first enzyme present in the HMS, decreased gradually in the lens following X-irradiation. On a per lens basis, there was a 25% decrease in the enzyme activity at the end of 4 weeks, whereas a 35% decrease was noted 7 weeks following X-irradiation. On the basis of unit wet weight, the decrease in the activity was 20% and 27% 4 and 7 weeks after X-ray.

In addition to the activity of G-6-PD,
seven other enzymes were also assayed in lenses 7 weeks following irradiation or just prior to the formation of cataract. The activities of the various enzymes determined in whole lenses are shown in Table III. In contrast to a significant reduction in the activity of G-6-PD, the activity of 6-phosphogluconate dehydrogenase, the second dehydrogenase of the HMS, was not significantly affected. Similarly, the glycolytic enzyme LDH as well as two enzymes of GSH metabolism, GSH reductase and GSH peroxidase, were unaffected by X-irradiation. Some decrease in the activity of hexokinase, glyceraldehyde phosphate dehydrogenase, and phosphofructokinase was, however, noted. The decrease in the activity of these enzymes ranged from 14% to 18% relative to controls, compared with a 27% decrease in G-6-PD.

Discussion

A major objective of this work was to investigate the possibility that a decline in the activity of reducing systems in the X-irradiated lens may initiate the oxidation of protein -SH groups observed in cataractous lenses. The data clearly show the concentrations of GSH and NADPH, the ratio of NADPH to NADP+, and the activity of the HMS significantly decrease in the X-rayed lens prior to the appearance of mature cataract. In contrast, there is very little change in the level of protein -SH groups until the time of cataract formation, when a sudden loss of protein -SH groups occurs. This relative stability of protein -SH groups in the lens following X-irradiation has also been observed by other investigators. It is possible that the -SH groups of lens crystallins remain reduced at the expense of lens reducing systems and that when a critically low point is reached, the oxidation of protein -SH groups is promoted.

Merola and Kinoshita have also observed that the -SH groups of lens proteins are normally very stable; however, they found that the groups became very susceptible to oxidation after the proteins were denatured. A similar phenomenon may occur following X-irradiation of the lens; that is, at the point of cataract formation, an unfolding of lens proteins occurs, and protein -SH groups, previously buried, become exposed and oxidized. The decline in the lens reducing sys-
tems observed in the present study following X-ray may hasten the unfolding process and subsequent oxidation.

The cycling assay procedure used in this study for measurement of pyridine nucleotides was found to be highly suitable for lenses and more sensitive than previously employed fluorometric methods. The only published data for the concentrations of pyridine nucleotides in the rabbit lens are those of Bullard, who used a fluorescent assay technique with an extraction method identical to that employed in the present study. In general, the present procedure yielded higher values for NAD+ and NADH and lower values for NADP+ and NADPH. A major difference was that the ratio of NADPH to NADP+ (1.1) determined in the present study for normal rabbit lenses was considerably higher than the value of 0.4 reported in the earlier investigations.

The observed decreases in the concentration of NADPH and of the ratio of NADPH to NADP+ in the X-rayed lens can, for the most part, be attributed to the inactivation of the HMS. However, in attempting to explain the decrease in HMS activity, one must consider a number of factors. The observed inactivation of G-6-PD (27% on a unit weight basis) may have not been sufficient to account for the decrease in HMS activity. It has been reported that even though the activity of G-6-PD is relatively low in rabbit lens, the HMS can be significantly stimulated in vitro. Thus G-6-PD is probably present in excess in rabbit lens, and the inactivation of the HMS by X-irradiation may involve other factors such as the decreased activity of hexokinase, a lower level of NADP+, or possibly a reduction in glucose uptake.

Our data indicate that X-irradiation results in decreased activity of four of the eight enzymes assayed: G-6-PD, hexokinase, glyceraldehyde phosphate dehydrogenase, and phosphofructokinase. These results confirm earlier findings of X-ray–induced inhibition of G-6-PD, hexokinase, and glyceraldehyde phosphate dehydrogenase. In the present study, G-6-PD was inhibited to the greatest extent, suggesting that this enzyme is more sensitive to X-irradiation. The effect may vary within species, however, since in short-term studies of rat lenses, G-6-PD appeared to be unaffected by X-irradiation.

Van Heyningen et al. have reported that -SH–dependent enzymes in the lens are affected by X-irradiation earlier than those enzymes which do not require free -SH groups for activity. Consistent with this idea, we have observed that the activities of the enzymes G-6-PD, hexokinase, and glyceraldehyde phosphate dehydrogenase, which are known to be -SH–dependent, decreased following X-irradiation. LDH, although reported to be a -SH–dependent enzyme, was unaffected by X-ray in the present study as well as in that of Pirie et al. This is possibly due to the fact that the critical -SH groups of LDH are well protected within the enzyme. It is conceivable that lens crystallins may also be protected and less susceptible to oxidation by X-irradiation.

Our investigations show that the activity of the enzyme GSH reductase is unaffected by X-irradiation prior to cataract formation. Pirie et al. also studied the effect of X-ray on this enzyme and reported a decrease in activity. However, when their data are examined, it appears that inactivation of the enzyme was seen primarily in lenses which showed evidence of swelling. We also noted inactivation of GSH reductase at this cataractous stage (data not presented); however, we could not observe any change in the activity of the enzyme prior to the development of mature cataract. The latter finding is in contrast to the observations of Swanson, who noted a decrease in GSH reductase activity soon after X-irradiation of the lens. However, in the aforementioned study, the assays were performed on capsule-epithelium preparations. Since our assays were performed on whole lenses, the data are difficult to compare. The possibility that inactivation of the enzyme may be confined to lens capsule-epithelium is currently under investigation.

In any event, even though the activities of GSH reductase and GSH peroxidase in the lens may be unaffected by X-irradiation, it is
reasonable to conclude that the functions of these enzymes may be impaired, since the concentrations of their co-factors, NADPH (GSH reductase) and GSH (GSH peroxidase) are significantly lower than normal. Thus GSH reductase would be less effective in reducing oxidized GSH as well as the mixed disulfides of protein and GSH. Similarly, GSH peroxidase would be inhibited in its function of reducing hydrogen peroxide and organic hydroperoxides. The decreased effectiveness of these two enzymes could result in oxidative damage to lens epithelial proteins and lens crystallins.

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REFERENCES