The effect of diamide on lens glutathione and lens membrane function

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The physiological role of glutathione (GSH), present in exceptionally high concentration in the lens, has never been clearly delineated. Using 86Rb we have studied the cation transport and permeability of rat lenses incubated in vitro with diamide, a relatively specific intracellular GSH oxidant. Lenses incubated without glucose with 0.4 mM diamide manifest a progressive decrease in GSH with time. In the presence of glucose this effect is minimized, presumably because the GSH level is maintained by the NADPH generated by the pentose shunt mechanism. When lenses are preincubated with diamide without glucose for 2 hours, a 55 to 57 per cent decrease in 86Rb uptake is observed. In addition, diamide also affects a 53 per cent increase in the 86Rb run-out of lenses incubated without glucose. In the presence of glucose both of these effects are minimized. Incubation with dithiothreitol leads to a small but definite increase in the 86Rb uptake of diamide-treated lenses. The observed membrane changes suggest the presence of certain sulfhydryl groups on the lens membrane which are unusually susceptible to oxidation when the GSH level is precipitously reduced. These membrane —SH groups are involved in cation pumping (86Rb uptake) as well as in determining the degree of membrane permeability (86Rb run-out). GSH would be necessary to maintain these —SH groups in the reduced form for proper membrane function.

Key words: glutathione, diamide, lens, cell membrane, membrane permeability, sulfhydryl, cation transport, rubidium, cataract, dithiothreitol.

T he physiological role of glutathione (GSH), present in exceptionally high concentration in the lens, has never been clearly delineated.1 Studies attempting to characterize the role of GSH in cell function2–9 have been hampered by the lack of specificity of the sulfhydryl reagents employed. Kosower and associates10 recently have introduced diamide as a relatively specific, stoichiometric, intracellular GSH oxidant (Fig. 1). With this compound a study was undertaken to determine what effect lowering the GSH level has on certain parameters of lens membrane function.

Methods

Charles River strain rats (Charles River Laboratories, Wilmington, Mass.) weighing 125 grams were killed by decapitation, and the globes were excised. The lenses were removed by a posterior approach and then transferred on a wire loop to an incubation test tube containing...
(CH₃)₂NCON=NCON(CH₃)₂ + 2 GSH \rightarrow (CH₃)₂NCONHNHCON(CH₃)₂ + GSSG

Fig. 1. Reaction of diamide with GSH.

3 ml. of control medium. In studies requiring several changes of medium and in 8²rubidium (⁸²Rb) uptake experiments, lenses were placed on a nylon mesh platform inside the incubation test tube (Fig. 2). This platform was adapted from the rabbit lens incubation apparatus of Chylack and Kinoshita.¹¹ Medium without glucose was composed of 50 mM trishydroxymethylaminomethane, 1.5 mM CaCl₂, 1.0 mM K₂HPO₄, 4.0 mM KCl, and 105.1 mM NaCl; the pH was adjusted to 7.4 with HCl. When the medium contained glucose, the concentration used was 5.5 mM. Medium for ⁸²Rb run-out experiments contained 1 × 10⁻⁴M ouabain and 10 mM nonradioactive Rb in place of potassium. Whenever necessary the osmolality was adjusted with appropriate amounts of NaCl so that the tonicity of all media was 287 ± 1 mOsm, as determined by an osmometer obtained from the Advanced Instrument Co. (Newton Highlands, Mass.).

Lenses were incubated at 37° C. in a final
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Fig. 3. Effect of \(4 \times 10^{-4}\)M diamide on the GSH of rat lenses incubated without glucose. The per cent of decrease in GSH is calculated by comparing the GSH content of paired control and diamide-treated lenses. Brackets indicate ±2 S.E.

volume of 5 ml. of medium. The final concentration of diamide (supplied by E. M. Kosower, Stony Brook University, Stony Brook, N. Y., and Alcon Laboratories, Ft. Worth, Texas), stored in the dark in a desiccator and freshly prepared for each experiment, was \(4 \times 10^{-4}\)M.

In Rb studies, tracer amounts of \(^{86}\)Rb (obtained from Iso-Serve Corp., Cambridge, Mass.) were added to the medium. \(^{86}\)Rb run-out experiments were based on the studies of Becker and Cotlier.\(^{12}\) The lenses were preincubated with tracer amounts of \(^{86}\)Rb for two hours, then rinsed once, and incubated with the above-described run-out medium. The medium was sampled at time zero to correct for radioactivity not removed in the changing process.

In all experiments paired lenses were used. At the end of the incubation period, the lenses were removed in paired sequence from the culture tubes, rolled on filter paper to remove adherent water and zonules, and weighed in a glass homogenizer on a Mettler balance. For GSH and radioactive assays the lenses were deproteinized in trichloroacetic acid. In ATP analyses the lenses were boiled in 3.0 ml. of water after homogenization, and the supernatant fluid assayed by the firefly luminescence method.\(^{13}\) GSH was determined by a microadaption of the nitroprusside method of Grunert and Phillips,\(^{14}\) employing \(\frac{3}{8}\) proportionate volumes. Lactate was determined enzymatically using lactate dehydrogenase and NAD (available from Boehringer-Mannheim Corp., New York, N. Y.).\(^{15}\)

Lense water was calculated as 61.1 per cent of the wet weight of the lens.\(^{10}\) The actual lens water in a small series of incubated control lenses for which both wet and dry weights were determined was within 2 per cent of this figure.

Results

Clarity and weight of lenses. Both control and diamide-treated lenses incubated with or without glucose without ouabain remain clear. There is no significant weight difference between control and experimental lenses, although there is a suggestion of a very slight loss of weight (less than 0.25 mg.) for the diamide-treated lenses incubated without glucose for 3 hours. In the \(^{86}\)Rb run-out experiments, the use of \(1 \times 10^{-4}\)M ouabain for 5 hours did lead to a faint anterior and posterior haze in both control and experimental lenses.

GSH data. Diamide causes a rapid oxidation of lens GSH. Within 7 minutes of exposure to diamide without glucose there is a 16 per cent decrease in total lens GSH...
Fig. 4. Effect of $4 \times 10^{-4}$M diamide on the GSH of rat lenses incubated with and without glucose. The per cent of decrease in GSH is calculated as in Fig. 3. Brackets indicate $\pm 2$ S. E.

Fig. 5. Effect of $4 \times 10^{-2}$ M diamide on $^{85}$Rb accumulation of rat lenses. Both the diamide and $^{85}$Rb are added at time zero. The $^{85}$Rb accumulation of lenses incubated without glucose is measured at 35, 60, and 180 minutes, and with glucose at 190 minutes. Brackets indicate $\pm 2$ S. E.
(Fig. 3). From 7 to 30 minutes there is an apparent plateau in the level of GSH, whereas from 1 to 3 hours there is an almost linear decrease in total lens GSH, after which another plateau is evident. In the presence of glucose the effect of diamide on total lens GSH is minimized, although still apparent (Fig. 4).

$^{86}\text{Rb}$ uptake data. Diamide-treated lenses incubated without glucose manifest a progressive impairment in $^{86}\text{Rb}$ accumulation with time (Fig. 5). At 35 and 60 minutes the levels to which $^{86}\text{Rb}$ accumulates in the control and diamide-treated lenses are not statistically different, whereas after 150 minutes of exposure to diamide $^{86}\text{Rb}$ accumulation is decreased by 46 per cent ($p < 0.01$). In contrast to the striking effect diamide has in the absence of glucose, the $^{86}\text{Rb}$ accumulation of diamide-treated lenses is only minimally decreased in the presence of glucose (Fig. 5).

It has been shown that Rb and K are handled identically by the Na-K pump mechanism in the lens. Theoretically, such a decrease in $^{86}\text{Rb}$ accumulation could be the result of decreased pump activity and/or increased permeability of the membrane to intracellular $^{86}\text{Rb}$. To help differentiate between these possibilities, short-term $^{86}\text{Rb}$ uptake experiments were conducted in which the lens/medium ratio of $^{86}\text{Rb}$ would not be greater than 1, so that loss of $^{86}\text{Rb}$ by passive diffusion would be minimized. These studies demonstrate that diamide causes a marked decrease in the $^{86}\text{Rb}$ uptake of the lens in the absence of glucose (Fig. 6). Lenses pretreated with diamide without glucose for 2 hours manifest a 55 per cent ($p < 0.01$) decrease in $^{86}\text{Rb}$ uptake compared to controls. After pretreatment lenses are transferred to fresh medium containing $^{86}\text{Rb}$ without diamide. Brackets indicate ±2 S.E.

Fig. 6. Short-term $^{86}\text{Rb}$ uptake of rat lenses pretreated with $4 \times 10^{-6}\text{M}$ diamide for 2 hours. After pretreatment lenses are transferred to fresh medium containing $^{86}\text{Rb}$ without diamide. Brackets indicate ±2 S.E.
0.01) and 57 per cent (p < 0.01) decrease in $^{86}$Rb uptake at 15 and 30 minutes, respectively. Again, the presence of glucose in the medium has an ameliorating effect, as the $^{86}$Rb uptake of similarly treated lenses is maintained at near normal values (Fig. 6).

$^{86}$Rb run-out data. In addition to a decrease in the $^{86}$Rb uptake process, diamide also effects an increase in the efflux of $^{86}$Rb from the lens. Diamide-treated lenses incubated without glucose manifest a 53 per cent increase in $^{86}$Rb run-out (p < 0.01) (Fig. 7). This effect is not demonstrated until at least 20 minutes after the diamide is added to the medium. In the presence of glucose the run-out of similarly treated lenses is increased only 20 per cent (Fig. 8). The difference in $^{86}$Rb run-out between diamide-treated lenses incubated with and without glucose is statistically significant (p < 0.01).

ATP and lactate data. Diamide has only a minimal effect on lens ATP levels. The ATP content of diamide-treated lenses incubated 3 hours without glucose is decreased only slightly from 1.31 to 1.02 μmoles per gram of lens (Table I). Simi-
larly treated lenses incubated with glucose have ATP levels equal to control lenses. The ATP content of control lenses incubated without glucose is 45 per cent less than that of control lenses incubated with glucose (Table I).

Diamide does not seem to affect the over-all glycolytic mechanism. Lactate production of diamide-treated lenses incubated with glucose is the same at 2 and 5 hours as that of control lenses (Table I).

Table I. Effect of 4 × 10⁻⁴M diamide on ATP content and lactate production of rat lenses

<table>
<thead>
<tr>
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<th>ATP (μmoles/Gm. lens)</th>
<th>Lactate (μmoles/5 ml. medium)</th>
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<tbody>
<tr>
<td></td>
<td>2 hours</td>
<td>5 hours</td>
</tr>
<tr>
<td>Controls without glucose</td>
<td>1.31 ± 0.08</td>
<td></td>
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<tr>
<td>Diamide without glucose</td>
<td>1.02 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Controls with glucose</td>
<td>2.37 ± 0.08 0.74 ± 0.02 1.16 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Diamide with glucose</td>
<td>2.48 ± 0.04 0.74 ± 0.02 1.06 ± 0.04</td>
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*The ATP content is measured after 3 hours of lens incubation. The 5 hour lactate measurements are from incubations with 1 × 10⁻⁴M ouabain (²³⁹Rb run-out experiments). Values are expressed as ± S.E.

Table I. Effect of 4 × 10⁻⁴M diamide on ATP content and lactate production of rat lenses

Effect of dithiothreitol. Incubation with dithiothreitol, a source of reduced —SH groups, leads to an increase in the ⁸⁶Rb uptake of diamide-treated lenses (Fig. 9). Both control and experimental lenses are pretreated with diamide without glucose for 2 hours. Control lenses then are placed in control medium and experimental lenses in control medium + 4 × 10⁻³M dithiothreitol for 105 minutes, after which short-term ⁸⁶Rb uptake studies are conducted. Dithiothreitol-treated lenses manifest a 14 per cent (p < 0.01) and 27 per cent (p < 0.02) increase in ⁸⁶Rb uptake at 15 and 30 minutes, respectively.

Discussion

Evidence has been presented that lowering of the GSH level does have deleterious effects in the lens. The oxidation of GSH by diamide results both in a decrease in Na-K pump activity and an increase in the permeability to cations, as indicated by the ⁸⁶Rb uptake and run-out studies (Figs. 5, 6, and 7). Although a significant de-

![Diagram](image-url)
crease in total lens GSH occurs within 7 minutes of exposure to diamide (Fig. 3), there is a delay of at least 20 to 30 minutes before the effect of diamide on both pump activity and membrane permeability becomes apparent (Figs. 5 and 7). This apparent lag period may suggest that the GSH level has to be reduced to a certain critical level before any change can occur. Alternatively, it may indicate that the oxidation of GSH does not effect, directly, the changes in the cation pumping activity and in permeability. Rather, these membrane phenomena may be secondary to another process, that of the normal oxidation of membrane sulfhydryl (—SH) groups, which in turn require reduction through interaction with GSH for proper membrane function (Fig. 9). Normally, the level of GSH is maintained by the NADPH generated by the direct oxidation of glucose through the pentose shunt (Fig. 10). This is probably the reason that despite the exposure of the lens to diamide, only minimal changes occur in the level of GSH as long as glucose is present in the medium (Fig. 4). Preliminary studies indicate that diamide, indeed, does stimulate pentose shunt activity in the rabbit lens.18

Diamide is regarded by Kosower and associates10 as a relatively specific, stoichiometric oxidant of intracellular GSH. In studies with the red blood cell Kosower and Kosower19 observed that 0.6 mole of diamide, compared to a theoretical 0.5 mole, is required to oxidize 1 mole of GSH. The reaction of diamide with GSH is instantaneous.

The possibility that interference in the generation of biological energy may be responsible for the changes observed has to be considered. However, diamide does not seem to interfere with lactate production from glucose (Table I). In addition, the ATP content of diamide-treated lenses incubated without glucose is decreased only slightly from 1.31 to 1.02 μmoles per gram lens (Table I). The magnitude of this difference may not be sufficient to account for the 55 to 57 per cent decrease in Na-K pump activity as measured in ⁸⁶Rb uptake studies (Fig. 6). This conclusion receives support from the observation that approximately a twofold difference in ATP levels of control lenses incubated with and without glucose (Table I) leads only to a 13 to 18 per cent difference in ⁸⁶Rb uptake (Figs. 5 and 6).

The explanation for the observed membrane changes which appears most attractive is that there is present on the lens membrane certain sulfhydryl groups unusually susceptible to oxidation when the GSH level is precipitously reduced. These membrane —SH groups are involved in cation pumping (⁸⁶Rb uptake) as well as in determining the degree of membrane permeability (⁸⁶Rb run-out) (Fig. 9). GSH would be necessary to maintain these membrane —SH groups in the reduced form for proper membrane function. This is in accord with the original suggestion of Barron and Singer20 that the general role of GSH is to maintain enzyme —SH groups in the reduced state. It is known that Na-K activated ATPase,21 located in

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Fig. 10. Relationship between pentose shunt activity and the reduction of oxidized glutathione (GSSG).
the cell membrane, contains a reactive —SH group. GSH, thus, may be required to maintain this —SH group in the reduced form. Alternatively, one may speculate that the proposed active transport membrane carrier system involves the regular oxidation and reduction of —SH groups for which GSH is required. Similarly, normal membrane permeability, as measured in the 86Rb run-out experiments (Figs. 7 and 8), may require that membrane —SH groups be maintained in the reduced state. The above described 20 to 30 minute lag period for these membrane effects (Figs. 5 and 7) might indicate that diamide does not directly alter the membrane —SH groups, but rather, affects these groups secondarily, as they undergo oxidation when the protective action of GSH is no longer available.

The increase in 86Rb uptake of diamide-treated lenses incubated with dithiothreitol (Fig. 9) tends to support this hypothesis, although it is not known whether the dithiothreitol has reacted with membrane sulphhydryl groups or with intracellular oxidized glutathione (GSSG). This increase in uptake is definite, although of small magnitude.

Kinoshita has demonstrated that, in the absence of glucose, the calf lens achieved energy production from the aerobic oxidation of endogenous substrates. Presumably, then, the observed small decrease in ATP of diamide-treated lenses incubated without glucose reflects some interference with aerobic metabolism (or, alternatively, increased utilization of ATP through an unknown pathway). The former possibly could result from the decreased activity of a sulphhydryl enzyme dependent on GSH for reduction of its —SH group.

From the results reported here, a new role of GSH in maintaining normal cation pumping and membrane permeability is brought to light. Loss of GSH, which occurs in practically all forms of cataract, results in defective lens membrane function. Cell membranes become leaky and are unable to pump cations normally. Lens fibers thus become unable to maintain their internal environment, and it is in this setting that opacities develop in experimental cataracts.

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REFERENCES

12. Becker, B., and Cotlier, E.: The efflux of


