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Hydrogen Peroxide Removal by the Calf Aqueous Outflow Pathway

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Previous studies have shown that aqueous humor of calf and human eyes contains about 25 μM hydrogen peroxide. We have studied the removal of hydrogen peroxide by the outflow pathway of intact, freshly enucleated, calf eyes. Eyes were immersed under silicone oil that had a density greater than water and medium containing various agents was perfused into the anterior chamber. Medium passing through the trabecular meshwork and out the cut ends of the aqueous veins was trapped by the silicone oil and harvested. By measuring the concentration of hydrogen peroxide in the anterior chamber and in the emerging medium, we were able to study the rate of removal by the outflow structures and the effect of inhibitors on this rate. At 1 mM hydrogen peroxide, the amount emerging was undetectable by our methods. At 10 mM, the results were inconsistent, suggesting that tissue damage may have been occurring. At 5 mM, the concentration in the emerging medium was reduced 150–1000-fold, depending on time and conditions. This rate of removal could be reduced by 3-aminotriazole, reaching a maximum inhibition of about 50% at 80 mM. Addition of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) to further inhibit removal did not yield reliable results unless the concentration of H_2O_2 was lowered below 5 mM. Using loss of lactate dehydrogenase activity as a measure of cell damage, we found a 30% drop in activity after perfusing with BCNU, diamide, and 3-aminotriazole, followed by 3 hr with 10 mM hydrogen peroxide. The results suggest that physiological concentrations of hydrogen peroxide in aqueous humor are totally removed by the aqueous outflow tissue. The great capacity of this tissue to detoxify hydrogen peroxide has potential implications for both normal and abnormal function. Invest Ophthalmol Vis Sci 29:976–981, 1988

Hydrogen peroxide (H_2O_2) has been found to be a normal constituent of aqueous humor in many species.[1] In normal human and calf aqueous, a level of 25 μM H_2O_2 has been measured.[2] The presence of catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase in calf trabecular meshwork (TM) strongly suggests that the TM is capable of detoxifying H_2O_2.[3-6] However, demonstrating the presence of these enzymes in the TM does not prove that the TM removes H_2O_2 from the aqueous humor as it passes through the outflow structures. The suggestion that it in fact does so was supported by unpublished observations by us and by others (Frank Giblin, personal communication) that excised calf TM rapidly removes added H_2O_2.

In order to address this question directly we have perfused intact enucleated calf eyes with H_2O_2 using a system devised by Johnson et al.[7] that enabled us to measure the H_2O_2 concentration in the fluid that passed through the outflow pathway.

Materials and Methods

Chemicals

Hydrogen peroxide (30%) and horseradish peroxidase (Sigma Type VI) were obtained from Sigma Chemical Co. (St. Louis, MO).

The amount of H_2O_2 in stock solution was routinely measured by $KMnO_4$ titration.[8] All other chemicals were reagent grade.

Tissue

Freshly enucleated calf eyes were obtained from a local slaughterhouse, packed in mixed liquid/frozen saline. Eyes for perfusion were trimmed of extraocular muscle and other loose tissue and kept cool until

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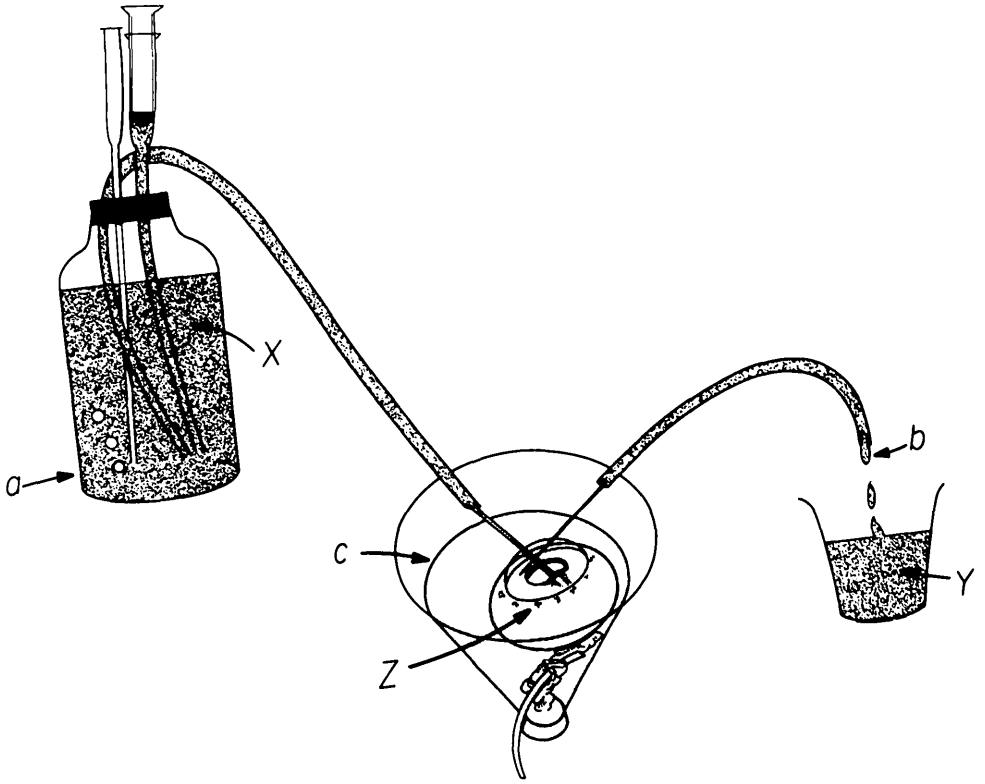

Fig. 1. Apparatus for perfusing eyes under silicone oil. X: medium entering anterior chamber; Y: medium leaving anterior chamber, Z: perfusate collecting under silicone oil. The vertical distances from a to b and from b to c were both equivalent to 10 mm Hg.

used. Calf TM was harvested as previously described.[9] Cornea, lens, and iris were also dissected; they were then blotted dry, weighed, and finally washed twice in cold Dulbecco's phosphate buffered saline with 5.5 mM glucose (Solution A).

Measurement of H_2O_2 Concentrations

The concentration of H_2O_2 was measured by the method described by Giblin et al.[1] In brief, the absorbance at 610 nm of 40 μM 2,6-dichlorophenolindophenol in 50 mM phosphate buffer, pH 6.6, was quenched by the addition of ascorbic acid (50 μl of a 20% solution in 4 mg% metaphosphoric acid). After addition of 50 μl of sample (diluted if necessary to <80 μM H_2O_2) the absorbance was again recorded. Five microliters of 5 mg/ml horseradish peroxidase solution were then added, and the increase in absorbance resulting from oxidation of the dye was measured. A calibration curve was constructed from known concentrations of H_2O_2.

Studies on Excised Tissues of the Anterior Chamber

Cornea, lens, iris, and TM were incubated at 25°C in beakers containing 3 ml of solution A, with 1 mM H_2O_2. Samples of 50 μl were withdrawn at appropriate intervals for the measurement of H_2O_2.

Enucleated Calf Eye Perfusions

Method 1: An inflow reservoir (X), was fitted with a Marriotte device to maintain a constant head of pressure (see Fig. 1). This was then connected by a length of polyethylene tubing to a 20 gauge needle, which was passed through the cornea and into the posterior chamber. A second 20 gauge needle, connected to a collection reservoir (Y) by a piece of polyethylene tubing of identical length and diameter, was inserted into the anterior chamber with its tip in front of the TM. The heights of the reservoirs were adjusted to give a difference of about 10 mm Hg between them, and a mean height of about 15 mm Hg above the eye. This produced a continuous flow of about 1 ml/min through the aqueous compartment. In this way, the removal of H_2O_2 by the non-outflow tissues of the anterior chamber could be overcome and the effective level of H_2O_2 in the anterior chamber "clamped" at the same level as in the inflowing medium (X).

An intact eye, prepared as described above, was then washed two times with 0.9% NaCl, dabbed dry and submerged under silicone oil. The eye was perfused with 1, 5, or 10 mM H_2O_2 in solution A and the emerging perfusate (Z) in the oil was collected. The effluent from the first 30 min was discarded and then hourly collections were made for 3 hr. Contaminating oil was sedimented by centrifugation at 1100 *g*,

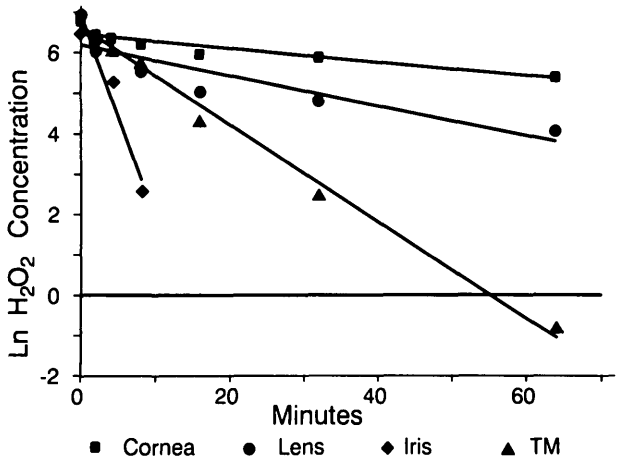

Fig. 2. Removal of H_2O_2 by excised tissues of the anterior chamber. Tissue was incubated at 25°C in Solution A containing 1 mM H_2O_2, using five TM and one each of the other tissues. Ordinates are $\log_e$ of H_2O_2 concentration in μM. Lines were fitted by least squares.

and H_2O_2 concentrations from Y and Z were measured.

Method 2: This earlier method was essentially the same as Method 1 except that the inflow reservoir was placed at 15 mm Hg above the eye and there was no flow through the second needle. The second needle was used simply to sample the contents of the anterior chamber.

Enzyme and Substrate Assays

After perfusion with various media, TM was dissected, weighed, and homogenized as described previously.[5,9]

Catalase[3]*:* Activity was determined at 25°C by following the absorbance of H_2O_2 at 240 nm. One unit was defined as the amount of enzyme that decomposes 1 μmol H_2O_2/min at an initial concentration of 30 mM at pH 7.0 and 25°C.

Glutathione reductase[5]*:* Activity was measured by following the decrease in NADPH absorbance at 340 nm. The final assay mixture contained 0.1 M phosphate buffer, pH 7.0, 2.5 mM oxidized glutathione (GSSG), 0.15 mM NADPH, 1 mM EDTA, and 0.1 ml of enzyme extract. One unit of glutathione reductase was defined as the amount of enzyme that oxidized 1 μmol of NADPH per minute at 25°C.

Lactate dehydrogenase (LDH)[10]*:* Activity was measured by following the decrease in absorbance of NADH at 340 nm. The final reaction mixture contained 50 mM phosphate buffer, pH 7.5, 2 mM pyruvate, 0.5 mM NADH, and 10 μl enzyme extract in a final volume of 0.5 ml. One unit of LDH was defined as the amount of enzyme that oxidized 1 μmol of NADH per minute at 25°C.

Measurement of TM glutathione (GSH): This was measured as non-protein SH. TM was homogenized in four volumes of 20 mM EDTA, with trichloroacetic acid added to a final concentration of 10%. The sample was centrifuged at 15,000 *g* for 10 min. The supernatant was analysed according to the method of Sedlak and Lindsay.[11]

Table 1. Rate of removal of H_2O_2 by excised tissues of the eye

	Iris	*TM*	*Lens*	*Cornea*
Rate (min^{-1}/tissue)	0.498	0.0240	0.0362	0.0169
Weight-specific rate (min^{-1}/g)	4.61	1.76	0.0412	0.0617

The various tissues were dissected and incubated in 1 mM H_2O_2 as described in *Methods* and in Figure 2. Values are −slope of $\log_e$ of concentration versus time except for TM value which is −slope/5 (see Fig. 2).

Results

Removal of H_2O_2 by Excised Tissues

H_2O_2 was rapidly removed when excised iris, cornea, lens, and TM were incubated in solution A with 1 mM H_2O_2 (Fig. 2). The rate of H_2O_2 removal per tissue was: iris > lens > TM > cornea but per gram wet weight was iris > TM > cornea > lens (Table 1).

Stability of H_2O_2 in Silicone Oil

In order to check for the stability of H_2O_2 collecting on the oil surface during the enucleated eye perfusions, a series of experiments were conducted where solutions of a known H_2O_2 concentration were mixed with oil, separated from the oil by centrifugation, and then allowed to stand at 25°C for at least 12 hr. At the end of the 12 hr, no decrease in H_2O_2 concentration could be measured.

Removal of H_2O_2 at Various Concentrations

To overcome the avid removal of H_2O_2 by the tissues bordering the TM, the anterior chamber was continuously exchanged (perfusion Method 1). Thus a steady state of either 1, 5, or 10 mM H_2O_2 was established in the anterior chamber. At 1 mM H_2O_2 flowing in (X), the concentration of H_2O_2 at site Z was below quantifiable levels (<1 μM). At 5 mM H_2O_2, the mean effluent values were 15 ± 4 μM at hour 1, 23 ± 6 μM at hour 2, and 38 ± 5 μM at hour 3 (results from three separate eyes ± SD). Perfusing with 10 mM H_2O_2, the effluent values were scattered and frequently much lower than at 5 mM inflow concentration. We speculate that perhaps the TM cells were being ruptured, therefore leaking detoxification enzymes (eg, catalase, glutathione peroxidase) into the outflow pathway.

Removal of H_2O_2 From Accumulated Perfusate by Sclera

Much of the collecting perfusate does not rise to the top of the oil but is trapped by surface tension on the surface of the eye. Introducing medium with various concentrations of H_2O_2 onto unperfused eyes under oil showed that the sclera was capable of appreciable rates of removal. However, calculating the contribution of this removal to the overall removal showed that the likely error from ignoring this effect would be small. This conclusion was supported by inhibiting the scleral removal by soaking a set of eyes in 100 mM 3-aminotriazole (AT) in Dulbecco's phosphate buffered saline. Results obtained when these eyes were perfused with 5 mM H_2O_2 were indistinguishable from those obtained with eyes soaked in Dulbecco's PBS without AT.

Defining a Rate of Removal

Before studying the effect of an inhibitor it is necessary to arrive at a satisfactory definition of rate of removal in order that comparisons can be made. If we simplify the outflow pathway and represent it as a simple tube (Fig. 3) whose walls remove H_2O_2 at a rate proportional to its concentration, then at a fixed rate of flow, the concentration (C) of H_2O_2 at some distance s along this tube is given by $C = C_Ye^{-ks}$ where C_Y is the concentration in the anterior chamber. For a tube of length s', the concentration C_Z in the emerging medium is given by $C_Z = C_Ye^{-ks'}$. In this fashion we arrive at a dimensionless measure of the rate $ks' = \ln C_Y - \ln C_Z$. The analysis can be further refined by considering the tube to have regions with different values of k (uveal meshwork, trabecular meshwork, juxtacanalicular, aqueous plexus, collector channels, etc.). For n regions, each of its own length s_i and removal constant k_i, we obtain a composite measure of removal:

$$\sum_{i=1}^{n} k_i s_i = \ln C_Y - \ln C_n \qquad (1)$$

where C_n is the concentration in the medium emerging from segment n. If segment n is the last segment, then $C_n = C_Z$. The factor(s) k_i are sensitive to the flow rate. Unfortunately, given the necessity of perfusing the anterior chamber at 1 ml/min, there was no ready way to measure flow through the TM. We were forced to assume that all eyes had the same facility and perfuse them all at the same pressure. Even with these limitations, it is clear that $\ln (C_Y/C_Z)$ is the most appropriate measure of the ability of the outflow pathway to remove H_2O_2.

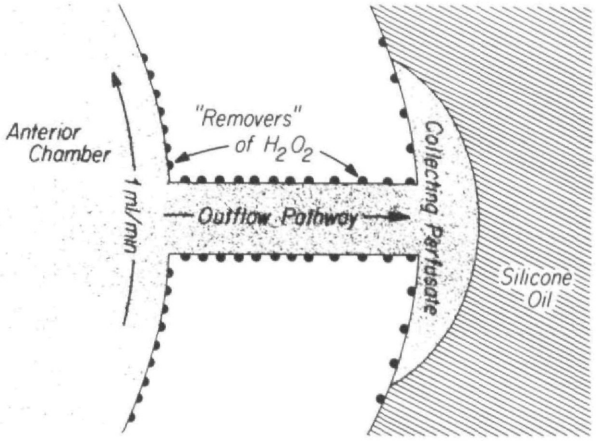

Fig. 3. Schematic representation of H_2O_2 removal by anterior structures of eye.

Effect on H_2O_2 Removal of Inhibiting Catalase

In the light of the previous results, a concentration of 5 mM H_2O_2 was chosen for these experiments. Eyes were perfused as before but with AT, a specific inhibitor of catalase, added at concentrations ranging from 20 to 80 mM (Fig. 4). Even at 80 mM, the inhibition of H_2O_2 removal was only 50%. The rise at 20 mM was unexplained. As the rise was the same magnitude as the spread between the first and third hours of perfusion it was probably due to undetected changes in perfusion parameters between the time of the control experiment and the inhibition sequence.

Effect on H_2O_2 Removal of BCNU and AT

In a separate series of experiments, eyes were divided into four groups and perfused according to

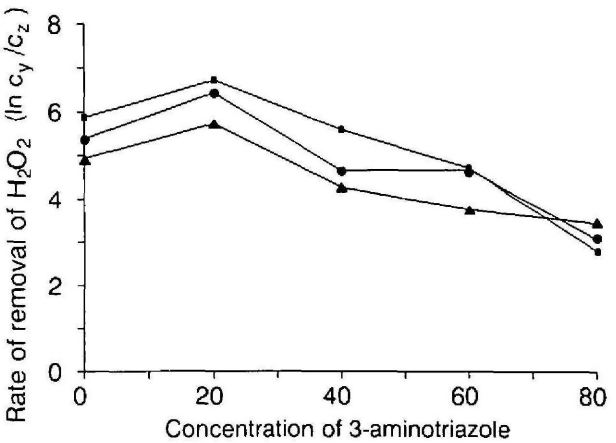

Fig. 4. Inhibition of H_2O_2 removal by AT. Eyes were perfused with 5 mM H_2O_2 as described in *Methods*. The concentration of H_2O_2 in the anterior chamber (Y) and in the perfusate (Z) was measured and the $\log_e$ of the ratio calculated. The rate of flow through the anterior from X to Y (Fig. 1) was adjusted so as to maintain the concentration at Y equal to that at X. 3AT was added as indicated and $\log_e$ ratio obtained at 1 hr (■), 2 hr (●), and 3 hr (▲) plotted versus concentration of 3AT.

Table 2. Inhibition of H_2O_2 removed by BCNU and aminotriazole

	Concentration of H_2O_2				
	At Y (mM)		*At Z (μM)*		*lnC_Y/C_Z*
Normal saline	3.50	0.7	9	1	6.0
BCNU, diamide	0.76	0.1	40	5	2.9
AT	0.51	0.07	70	10	2.0
AT, BCNU, diamide	0.45	0.07	70	17	1.9

Each value represents the mean ± SD from four separate eyes. Eyes were perfused according to Method 2 for 1 h with 0.9% saline containing 5 mM glucose and with additions as indicated. BCNU was 1 mM, diamide 10 mM, and AT 20 mM. H_2O_2 was introduced and the levels increased until measurable concentrations (>1 μM) appeared in the outflow (Z in Fig. 1). The anterior chamber was then sampled (Y in Fig. 1) and the concentration of H_2O_2 determined. These experiments were not carried out with continuous anterior chamber exchange.

Method 2 for 1 hr with BCNU, an inhibitor of glutathione reductase, diamide, an oxidant specific for GSH, and AT in various combinations as shown in Table 2. The drugs were dissolved in 0.9% NaCl because BCNU is inactivated in phosphate buffer. All four groups were then followed by H_2O_2 dissolved in solution A at steadily increasing concentrations. When measurable levels of H_2O_2 were found in the perfusate (Z) (>1 μM), a determination of the H_2O_2 level in the anterior chamber (Y) was then made (Table 2). Since these experiments were performed without using anterior chamber exchange to stabilize the H_2O_2 concentration in the anterior chamber, the exact concentrations reaching the TM are uncertain. However, the results are in general agreement with those obtained with the previous method.

The TMs were harvested after perfusion, and catalase, glutathione reductase and GSH were measured. The results are shown in Table 3. Perfusion with AT inhibited catalase by 95%. Although glutathione reductase was inhibited only 70% by BCNU, diamide did deplete the TM of GSH.

Even after catalase inhibition and GSH depletion, H_2O_2 entering the outflow pathway at site Y could not be fully recovered at site Z. A residual 5% activity

Table 3. Catalase, GSH and glutathione reductase in TMs perfused with AT, BCNU, diamide

	Control	*Experimental*
Catalase	884 (100%)	44.2 (5%)
Gluthathione reductase	0.12 (100%)	0.036 (30%)
GSH	0.55 (100%)	not detectable

Units are: Glutathione reductase: (μmol NADPH/min/gm tissue); Catalase: (μmol H_2O_2/min/g tissue); GSH: (μmol/g tissue).

Controls: Eyes (four) were perfused with 0.9% NaCl with 5.5 mM glucose for 1 hr. The TMs were then dissected, pooled and analysed for enzyme and GSH content as described in *Methods*.

Experimental: Eyes (four) were perfused as for controls but with the addition of 20 mM AT, 1 mM BCNU and 10 mM diamide to the medium.

Table 4. LDH content in eyes perfused with AT, BCNU, diamide, then H_2O_2

	No H_2O_2		*1 mM H_2O_2*		*10 mM H_2O_2*	
Control			8.3	1.3	8.0	0.9
Experimental	8.0	0.7	7.1	1.5	5.6	0.9*

* $P < 0.05$. Unit: μmol NADH oxidized/min/g tissue at 25°C.

Control: Eyes (four) were perfused with 0.9% NaCl with 5.5 mM glucose for 1 hr, then with H_2O_2 at the level indicated for 3 hr.

Experimental: Eyes (four) were perfused as for control eyes but with the addition of AT, BCNU and diamide during the first hour.

of catalase could remove a great deal of H_2O_2, possibly as high as 80%, depending on the guesses for various parameters of outflow. In addition, the H_2O_2 may be reacting with various tissue components in quantities large enough to remove measurable amounts from the aqueous. In order to assess tissue damage, the LDH activity was measured in TM excised after perfusion. Perfusion for 1 hr with BCNU, diamide, and AT, followed by 3 hr with 1 mM H_2O_2 did not cause a significant decrease in LDH content. However, repeating the perfusion with 10 mM H_2O_2 did cause a 30% decrease in LDH content ($P < 0.05$) (Table 4).

Discussion

H_2O_2 is rapidly removed by most tissues of the calf anterior chamber, in particular the iris (Fig. 2, Table 1). Therefore the steady state level of 25 μM typically observed in calf aqueous humor must result from a fairly energetic process of formation. The data presented here do not allow a calculation of in vivo rates because cut tissue may release enzymes destructive to H_2O_2, and geometry and stirring are too different from that in the intact anterior chamber.

The technique of Johnson et al,[7] in which perfusate emerging from perfused eyes is collected under silicone oil (Fig. 1), was readily adapted to this study. It may be a generally useful procedure for answering a number of metabolic questions concerning the outflow pathway. In this application, the very high rate of H_2O_2 removal made the rate difficult to measure. Only by raising the level to 5 mM could reproducible and measurable rates be obtained. However, the clear implication of this study is that as H_2O_2 at a physiological concentration passes through the TM, it is removed essentially in toto by the time aqueous humor reaches the other side of the collector channels.

The dose-response curve of H_2O_2 removal versus concentration of AT (Fig. 4) shows that even at 80 mM AT, H_2O_2 removal is inhibited only 50%. The remaining activity against H_2O_2 is presumably from glutathione peroxidase. Attempts to further inhibit

H_2O_2 removal by adding BCNU produced inconsistent results, presumably because at 5 mM H_2O_2 with the defensive enzymes inhibited, the tissue was being damaged. Part of the removal of H_2O_2 may also be due to nonenzymatic reactions with various targets of oxidative damage such as proteins and lipids. The slight decrease in rate of removal with time may reflect damage to the enzyme systems or saturation of sites of nonenzymatic removal, or both.

In a separate series of experiments, shown in Table 2, the concentration of H_2O_2 was varied until detectable amounts appeared in the perfusate. This effectively scaled the exposure to H_2O_2 to the capacity of the variously inhibited tissues to remove it. Despite the fact that these experiments were not performed with anterior chamber "flow-through" to clamp the H_2O_2 concentration, the results are reasonably consistent with those obtained in the more rigorous system. Despite the fact that each treatment separately inhibited H_2O_2 removal, we were surprised to find that adding BCNU to AT produces no detectable additional inhibition of H_2O_2 removal. This probably reflects limitations of this system rather than a real lack of additive effect.

Loss of LDH from TM was used as a marker for cell damage. Only in the presence of 10 mM H_2O_2 and only after catalase and glutathione reductase were both inhibited could any significant loss of LDH be seen. Using only BCNU and diamide, Kahn et al[12] had to perfuse calf eyes with 25 mM H_2O_2 for several hours before a reduction in outflow facility could be detected. Using cultured human cells, Polansky et al[13] showed damage to cells by overnight exposure to 0.1 mM H_2O_2 after treatment with AT.

These results indicate that calf TM has an impressive capacity to defend itself against damage from H_2O_2. The great capacity is presumably a consequence of great need. If this capacity is compromised in the elderly human TM, damage to TM from H_2O_2 could conceivably play a role in the pathogenesis of glaucoma.

Key words: hydrogen peroxide, calf, trabecular meshwork, eye perfusion, 3-aminotriazole, silicone oil

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