The effects of intracameral injection of hydrogen peroxide were examined using different techniques in young (4 to 6 weeks of age, \( \pm 500 \) g) and adult (3 to 6 months of age, 2.5 to 3.5 kg) rabbits. A response occurred that included changes in intraocular pressure, increased permeability of the iris vasculature, and swelling of the ciliary processes. The initial fall in intraocular pressure was the same in young and adult rabbits and the time to recovery of normal intraocular pressure was statistically the same in young rabbits as in adults. The loss of iris vascular integrity was more pronounced in adult animals, as judged by the appearance of fluorescein in the iris and anterior chamber. Microscopy revealed that the ciliary processes of young rabbits were unaffected by hydrogen peroxide, whereas in adult animals considerable swelling of the ciliary processes occurred. No polymorphonuclear leucocytes were observed in micrographs, ruling out their involvement in the response. The rate of loss of injected hydrogen peroxide from the anterior chamber was significantly longer in adult relative to young animals. Young animals had statistically higher catalase activity (U/mg wet weight) in iris and corneal endothelium than older animals. Differences exist in both the anterior segment response to, and the rate of clearance of, intracameral hydrogen peroxide injection in rabbits of at least two different ages that may reflect the differences found in tissue catalase concentrations. Invest Ophthalmol Vis Sci 29:335-339, 1988

Hydrogen peroxide is present in aqueous humor at concentrations between 0.03 and 0.07 mM, with higher concentrations found in certain patients with cataracts. In vitro studies have demonstrated the damaging effects of oxyradicals and hydrogen peroxide on the corneal endothelium. Hydrogen peroxide induced acute corneal swelling at concentrations of 0.3 to 0.5 mM in glucose-containing media, and at concentrations of 0.05 mM in non-glucose-containing media. In addition, intracameral injections of hypoxanthine/xanthine oxidase, generating hydrogen peroxide and superoxide anion, increased iris vascular permeability and possibly blood-aqueous barrier permeability, as well as inducing leucocyte infiltration.

The effects of hydrogen peroxide both in vivo and in vitro on various tissues of the anterior segment, and the possible relationship of the continued exposure of these tissues with time to pathological changes (eg, cataract), led us to examine the effects of intracameral hydrogen peroxide on tissues of the anterior segment.

**Materials and Methods.** Young albino rabbits, 4 to 6 weeks of age, ranged from 450 to 900 g; adults, 3 to 6 months old, ranged from 2500 to 3500 g. Hydrogen peroxide, either 6 \( \mu l \) or 10 \( \mu l \) of a 0.1 M solution, was injected intracameral to give a final aqueous humor concentration of 3.3 mM assuming an aqueous volume of 160 \( \mu l \) in young animals and 200 \( \mu l \) in adult animals. Animals were sedated with intramuscular ketamine (60 mg/kg), and topical tetracaine hydrochloride was used. All control eyes received an equal volume injection of sterile distilled water (the vehicle for hydrogen peroxide). Injections were made using a glass Gilmont (Great Neck, NY) syringe with a metal/plastic 30 gauge needle, thus very much reducing the possibility that OH radicals could contribute to the observed effects. Our investigations using animals conformed to the ARVO Resolution on the Use of Animals in Research.

Intraocular pressure (IOP) was measured in conscious rabbits using a calibrated Alcon (Fort Worth, TX) pneumotonograph; topical anesthesia 0.5% tetracaine hydrochloride was used, and washed off the eye after 5 to 10 seconds with 1 ml of 0.9% saline. Anterior segment fluorescein angiography was performed following the intravenous injection (via marginal ear vein) of 1 ml/kg of 10% sodium fluorescein solution with photographs taken every minute over 5 min.

For electron microscopy, tissues were fixed for 30 min at room temperature in a 2% glutaraldehyde, 1% paraformaldehyde solution containing 0.05% calcium chloride in 0.0625 M sodium cacodylate buffer at a pH of 7.3 and further fixed for 4 hr at 4°C after cutting tissue wedges. Tissues were rinsed with cold buffer and stored overnight at 4°C in fresh buffer before post-fixation for 2 hr in cold 2% osmium tetroxide in the same buffer. Tissues were bulk stained with 5% aqueous uranyl acetate, and embedded in Spurr medium.

Catalase was determined with a spectrophotometric method using a Beckman (Fullerton, CA) DU7. Animals were perfused with heparinized Ringer to remove erythrocytes. Tissues were kept ice-cold following dissection until analysis. They were extracted in 2 ml phosphate buffer (pH 7) with 0.1% Triton X-100 by homogenization and sonication. The preparations were centrifuged at 1500 g for 15 min at 4°C. Aliquots of supernatant were added to quartz cu-
Intraocular pressure following intracameral injection of hydrogen peroxide or distilled water

Table 1. Intraocular pressure following intracameral injection of hydrogen peroxide or distilled water

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>Experimental</th>
<th>Control</th>
<th>Experimental</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IOP % Change</td>
<td>IOP % Change</td>
<td>C-E</td>
<td>IOP % Change</td>
<td>IOP % Change</td>
<td>C-E</td>
</tr>
<tr>
<td>0</td>
<td>33.7 ± 1.7</td>
<td>37.0 ± 0.8</td>
<td>—</td>
<td>27.0 ± 2.4</td>
<td>28.2 ± 2.4</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>17.0 ± 2.5*</td>
<td>-49.6</td>
<td>24.5 ± 1.6*</td>
<td>-33.8</td>
<td>+15.8</td>
<td>14.3 ± 1.4*</td>
</tr>
<tr>
<td>2</td>
<td>20.3 ± 3.1*</td>
<td>-39.2</td>
<td>23.2 ± 2.5*</td>
<td>-37.3</td>
<td>+1.9</td>
<td>13.0 ± 1.3*</td>
</tr>
<tr>
<td>3</td>
<td>24.5 ± 3.4*</td>
<td>-27.3</td>
<td>27.2 ± 4.0*</td>
<td>-26.5</td>
<td>+0.8</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>29.3 ± 3.3</td>
<td>-13.1</td>
<td>28.3 ± 2.3*</td>
<td>-20.8</td>
<td>-7.7</td>
<td>25.7 ± 3.4</td>
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<tr>
<td>6</td>
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<td>+1.5</td>
<td>26.8 ± 1.9*</td>
<td>-27.6</td>
<td>-29.1</td>
<td>22.5 ± 2.1</td>
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<tr>
<td>12</td>
<td>34.2 ± 2.0</td>
<td>+1.5</td>
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<td>-42.7</td>
<td>-44.2</td>
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</tr>
<tr>
<td>24</td>
<td>34.7 ± 0.9</td>
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<td>-37.3</td>
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<tr>
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<td>-30.5</td>
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<tr>
<td>144</td>
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<td>37.0 ± 1.0</td>
<td>0</td>
<td>-3.9</td>
<td>28.5 ± 1.3</td>
</tr>
<tr>
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<td>+9.2</td>
<td>37.8 ± 1.1</td>
<td>+2.2</td>
<td>-7.0</td>
<td>31.0 ± 0.9</td>
</tr>
</tbody>
</table>

IOP values are the mean ± SEM of six rabbits per group. % Change is the percentage fall from the 0 time IOP and are represented as experimental IOP - control IOP/experimental X 100. Experimental eyes were injected with either 6 μl (young) or 10 μl (adult) of a 0.1 M hydrogen peroxide solution; control eyes were injected with equal volumes of distilled water depending on the age of the rabbit. C - E, control minus experimental percentage change. *Statistically different from 0 time IOP.

Results. Hydrogen peroxide concentrations were determined in the aqueous humor after injection. Samples were taken at 18 seconds and 1, 3, and 10 min. In adult animals extrapolation to zero time gave a concentration of 3.3 mM (n = 3 at each time period), while in young animals the concentration was 3.2 mM (n = 2 at each time period). The t 1/2 for anterior chamber loss of hydrogen peroxide was 32 seconds for the young and 86 seconds for adult rabbits. Baseline values for hydrogen peroxide were reached in the adult rabbit by about 10 min.

The IOP response to intracameral hydrogen peroxide is shown in Table 1. Statistical analysis with analysis of variance using repeated measures failed to indicate any difference between the IOP response of young and adult rabbits. There is a tendency, however, for the adult rabbits to show a slower recovery of IOP compared to young rabbits. The higher initial IOP in the young compared to adult rabbits supports earlier studies showing that IOP decreases by about 10 mm Hg over the initial 10 to 16 week growth period.

Young eyes displayed a higher basal fluorescence level in control eyes after dye injection (compare Fig. 1A, C). Experimental eyes of both young and mature rabbits, when assessed at equal time periods after injection of hydrogen peroxide, demonstrated an increased level of iris fluorescence as compared to their paired controls (Fig. 1B vs. 1A, and 1D vs. 1C). Hydrogen peroxide injections resulted in a more rapid influx of fluorescein into the iris tissue of adult as compared to young rabbits (compare Fig. 1B, D).

The ciliary processes of young animals showed less swelling whereas those of adult animals showed considerable edema (Fig. 2). There was no apparent disruption of either the pigmented or non-pigmented epithelium, although the pigmented epithelium of the adult rabbit was attenuated. The flatter non-pigmented epithelium in the edematous adult ciliary processes is probably a reflection of the vortex anatomy. The reaction was followed for 60 seconds at 240 nm.

The results of catalase determinations on tissues from untreated animals are shown in Table 2. On a U/mg wet weight basis, the iris and corneal endothelium show a significant decrease in catalase levels with age. This is not reflected in the ciliary processes, or in any tissue analyzed on a U/mg solubilized protein basis.

Discussion. The fluorescein angiography and the microscopy show that the response to hydrogen peroxide in the tissues of younger animals is less dramatic than that in adults. Iris fluorescein angiography demonstrates the increased leakiness of the iris vessels after intracameral hydrogen peroxide in both groups of rabbits. In the younger animals there is a greater baseline fluorescence after intravenous dye injection, although there is a lesser response to hydrogen peroxide (Fig. 1). The micrographs clearly show the

vettes containing 50 mM phosphate buffer, pH 7, and hydrogen peroxide (final concentration, 16 mM). The reaction was followed for 60 seconds at 240 nm. Protein concentrations were determined by the procedure of Lowry et al using bovine serum albumin as the standard.

The reaction was followed for 60 seconds at 240 nm. Protein concentrations were determined by the procedure of Lowry et al using bovine serum albumin as the standard.
Fig. 1. Iris fluorescein angiography of young and adult rabbit eyes after hydrogen peroxide injection into anterior chamber. (A) Young distilled water-injected control eye at 4 min after intravenous fluorescein injection. (B) Young treated eye at 4 min after intravenous fluorescein injection and 6 hr after hydrogen peroxide injection; paired eye to that in A. (C) Adult distilled water-injected control eye at 4 min after intravenous fluorescein injection. (D) Adult treated eye at 4 min after intravenous fluorescein injection and 6 hr after hydrogen peroxide injection; paired eye to that in C. The photographs show the typical response obtained in six young and six adult animals.

marked attenuation of the pigmented epithelium of the adult processes compared to younger tissue, and the edema is reflected in the flattening of the epithelial layers as well as the expansion of the stromal space, so that blood vessels are not detectable in close proximity to the base of the pigmented cells as they are in young tissues (Fig. 2).

The $t_{1/2}$ of hydrogen peroxide loss from the anterior chamber rules out the involvement of exit of hydrogen peroxide via conventional or unconventional outflow pathways since loss via these pathways occurs at only 2% of anterior chamber volume per minute. In the 10 min needed to reach background hydrogen peroxide levels in the anterior chamber (0.05 mM) only 20 $\mu$L of aqueous humor will have passed through the eye (assuming a flow rate of 2 $\mu$L/min), thus the "passive" loss (versus enzymic involvement after diffusion of hydrogen peroxide into tissues) would only account for a maximum of 10% of the total loss of hydrogen peroxide.

Although the IOP responses were statistically the same between the two groups, the tendency for the adult animals to recover more slowly to normal IOP (Table 1) may reflect the direct toxic effect of hydrogen peroxide on cells in the tissues bordering the anterior segment of the eye. This may relate to the known effects of hydrogen peroxide on ATPase$^9$ causing a prolongation of the response. Whether these results are a consequence of the ability to withstand the addition of hydrogen peroxide or due to a more rapid loss of the toxic agents in younger animals appears to be answered by the determination of catalase levels in untreated tissues.

Iris and corneal endothelium of younger animals contain more catalase than adults when expressed on a per mg wet weight basis (Table 2). Although enzyme levels expressed on a U/mg wet weight basis decreased with maturation in these two tissues, when expressed on a U/mg soluble protein basis the values remained relatively stable. Regardless of whether the catalytic capacity of the tissues decreases with age or the specific activity of the enzyme is maintained with growth but decreases on a wet weight basis due to either tissue water or protein content changes, young animals respond less to, and can better recover from, the same (if not greater) added quantity of hydrogen peroxide in the anterior chamber. The presence of more catalase may be the reason why the younger animals show a less vigorous reaction to hydrogen peroxide than do older animals, since the detoxification of the agent can occur more readily. The difference between young and adult rabbits in the $t_{1/2}$ for the loss of hydrogen peroxide from the anterior chamber is pronounced, and could be explained by
Fig. 2. Micrographs of ciliary processes after hydrogen peroxide injection into the anterior chamber. (A) Young animal, 6 hr after injection. Blood vessels are seen in normal close proximity to the basal surface of the pigmented cell layer. No changes are evident in the epithelium. (B) Adult animal, 6 hr after injection. The epithelial layers are flattened due to stromal edema, and the pigment epithelium is highly attenuated. The responses indicated in the micrographs are representative of six rabbits in each group, and were taken from the tips of the processes nearer the pupillary margin. Original magnification X4000.
the differences in tissue catalase activities. Detoxification of hydrogen peroxide is known to occur in the rabbit iris-ciliary body, although age-related differences were not examined. Collectively, the differences between the tissues bounding the anterior segment to hydrogen peroxide detoxification are strongly involved in modulating the responses of tissues bounding the anterior segment to hydrogen peroxide.

Detoxification of hydrogen peroxide can also occur by glutathione peroxidase, which is known to exist in many tissues of the eye, although age-related differences have not been examined. The relative roles of catalase and glutathione peroxidase in epithelial cells are not known. It has been reported that catalase and glutathione peroxidase have similar peroxidative activity at physiological hydrogen peroxide levels. Above physiological hydrogen peroxide levels catalase is assumed to perform the bulk of hydrogen peroxide detoxification.

Bhuyan and Bhuyan reported relative catalase and glutathione peroxidase activity levels of several ocular tissues on a units per mg protein basis. Their data indicate that at least 93% of the total peroxidative activity of catalase and glutathione peroxidase in iris, ciliary body and corneal endothelium was due to catalase. Certain patients with cataracts have a higher aqueous humor hydrogen peroxide concentration than do non-cataract subjects. The greater aqueous humor hydrogen peroxide levels may be the result of decreased catalase activity in anterior segment tissues with age.

**Key words:** hydrogen peroxide, rabbit eye, microscopy, catalase, age differences

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**References**