Lysosomal hydrolases in tears and the lacrimal gland: effect of acetylsalicylic acid on the release from the lacrimal gland.

N. J. VAN HAERINGEN AND E. GLASIUS.

Free and lysosomal activities of 10 acid hydrolases have been determined in human lacrimal gland tissue. The enzyme activities appeared mainly in the lysosomal fraction, and the proportions correspond very well with those found in the tear fluid. Acetylsalicylic acid at blood levels of about 1 mM produced a significant lowering of the \( \beta \)-hexosaminidase concentration in tears. It is suggested that acetylsalicylic acid may have a stabilizing effect on lacrimal lysosomes, resulting in a diminished release of lysosomal enzyme from the lacrimal gland.

Lysosomal hydrolases are present in human tears, and the main source of these enzymes is to be found in the lacrimal gland. \(^1\) \(^2\) This was demonstrated in assays of samples of tear fluid obtained by nonmechanical stimulation with tear gas. The conjunctival epithelium may act as a second source, but this is only noticeable if tear samples are obtained under slight epithelial damage with filter paper strips. This paper presents further evidence for the origin of lacrimal lysosomal hydrolases and the effect of acetylsalicylic acid as lysosomal stabilizer in vivo.

**Materials and methods.** Human lacrimal gland tissue was obtained occasionally from orbital operations in the University Eye Clinic, Amsterdam.

Tear fluid was obtained from normal volunteers after stimulation with vapor of 2-chloroacetophenone and collected in glass capillaries, discarding the first 10 \( \mu \)l. The next specimens—minimally 20 \( \mu \)l of tear fluid—could be regarded as lacrimal gland fluid in which admixture with conjunctival secretions was reduced to the minimum.

Tissue homogenates were prepared with 0.25M sucrose, free activities could be assayed without measurable breakdown of lysosomes. The total activities were measured under the same conditions in the presence of 0.1% Triton X-100 and centrifuged at 1000 \( \times \) g for 5 min. The pellet was then treated with 0.1% Triton X-100 and centrifuged at 1000 \( \times \) g for 10 min. The Triton X-100 supernatant was used for the estimation of the amount of enzymes discarded with the debris. In all experiments the enzyme activity in the debris was less than 5% of the total activity. The combined sucrose supernatants were centrifuged at 50,000 \( \times \) g for 30 min. The pellet was treated with 0.1% Triton X-100, and this extract was used for the determination of the lysosomal fraction.

Latency of the glycosidase activities was determined in a 50,000 g pellet under conditions in which lysosomes had appeared to be stable. In a 15 min incubation at 37\(^\circ\) C and at pH 5.2 in 0.25M sucrose, free activities could be assayed without measurable breakdown of lysosomes. The total activities were measured under the same conditions in the presence of 0.1% Triton X-100.

The 50,000 g supernatant was split into two parts, and these were dialyzed against, respectively, 0.05M acetic acid and citrate-phosphate bufl-
fer, pH 5.0. The dialyzed supernatant was used for determination of the cytoplasmic fraction. Addition of 0.1% Triton X-100 to dialyzed cytoplasmic fraction did not interfere with the enzyme assays. Any loss in activity due to dialysis could not be demonstrated. Therefore 0.25M sucrose was added again in the dialysate, and enzyme assays were compared with determinations in the non-dialyzed 50,000 g supernatant. The enzyme values did not differ more than 5%, although the activities were lower than in dialyzed supernatant because of the suboptimal assay conditions caused by the presence of sucrose.

Lysosomal hydrolases were determined fluorimetrically with the corresponding 4-methylumbelliferyl derivatives as substrates, following methods described previously.2 The assays were linear for the incubation times used and proportional to the amount of tear fluid or tissue extract. Quenching did not interfere, as appeared from the recovery of fluorescence in assays where a known amount of 4-methylumbelliferone was added afterward to the cuvet in the fluorimeter. All enzyme activities were calculated in international units per liter of tear fluid or kilogram of tissue. One enzyme unit is the amount which catalyzes the transformation of 1 μmol of the substrate per minute. The substrate concentrations and pH values were as follows:

1. In 0.1M citrate-phosphate buffer—1.6 mM at pH 4.5 for β-hexosaminidase2 (β-hex); 0.6 mM at pH 5.0 for α-fucosidase3 (α-fuc); 5.3 mM at pH 5.0 for α-galactosidase2 (α-gal); 0.7 mM at pH 4.5 for α-mannosidase2 (α-man); and 0.5 mM at pH 4.0 for β-galactosidase2 (β-gal).

2. In 0.1 M acetate buffer—4.2 mM at pH 5.0 for acid phosphatase2 (phos); 1.5 mM at pH 4.3 for

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Fig. 1. Proportions of acid hydrolases in tear fluid and lacrimal gland tissue. For each enzyme the mean ± S.E.M. (three experiments) are indicated by the sides of a rectangle. The correlation is significant both for the total (●) activity (r = 0.865, p < 0.01) and the lysosomal (□) fractions (r = 0.787, p < 0.01).

Table I. Lysosomal fraction and latency of acid hydrolases in lacrimal gland tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of total activity in lysosomes*</th>
<th>% apparent latency†</th>
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<tbody>
<tr>
<td>β-hex</td>
<td>69.3 ± 3.5</td>
<td>88</td>
</tr>
<tr>
<td>phos</td>
<td>90.4 ± 1.5</td>
<td>70</td>
</tr>
<tr>
<td>α-fuc</td>
<td>67.1 ± 5.3</td>
<td>83</td>
</tr>
<tr>
<td>α-gal</td>
<td>81 ± 15</td>
<td>86</td>
</tr>
<tr>
<td>β-gluc</td>
<td>90.0 ± 6.5</td>
<td>52</td>
</tr>
<tr>
<td>α-man</td>
<td>78.6 ± 1.9</td>
<td>83</td>
</tr>
<tr>
<td>β-gal</td>
<td>89 ± 13</td>
<td>81</td>
</tr>
<tr>
<td>α-gluc</td>
<td>73.5 ± 1.5</td>
<td>67</td>
</tr>
<tr>
<td>sulf</td>
<td>84.8 ± 2.2</td>
<td>80</td>
</tr>
<tr>
<td>β-gluc</td>
<td>92.1 ± 1.5</td>
<td>60</td>
</tr>
</tbody>
</table>

*Mean of three experiments ± S.E.M.
†Calculated from the difference between total and free activities in 50,000 g pellet (for conditions see text).
Results. The activities of the lysosomal hydrolases in lacrimal gland tissue and lacrimal gland fluid are presented in Fig. 1. The total enzyme activity as well as the lysosomal fractions of lacrimal gland tissue show a significant correlation with the enzyme activities in tear fluid. In Table I, the lysosomal fractions of the enzymes are given, together with the apparent latency, measured in a 50,000 g pellet.

Within 1 hr after an oral dose of 1 gm of acetylsalicylic acid (Ca-salt), a marked decrease in enzyme activity for β-hex in tears occurred (Fig. 2). Since acetylsalicylic acid is rapidly metabolized to salicylate, drug levels were determined with a salicylate assay. In one experiment salicylate levels in blood and tears were determined after 30 min; the concentrations were 1.0 mmol/L in plasma and 0.7 mmol/L in tears. For control of a possible effect of salicylate on the enzyme activity of tears in vitro, the effect of 1 mM salicylate was checked in enzyme assays of all 10 lysosomal enzymes investigated. No alteration of enzyme activity or protein content could be demonstrated when salicylate had been added to tear samples.

Discussion. The significant correlation between the concentrations of the 10 acid hydrolases investigated in this study in tear fluid and lacrimal gland tissue is further evidence for the lacrimal gland as source for these enzymes. Because both the total enzyme activity and the lysosomal fraction reveal a significant correlation of the same probability, it cannot be decided from these figures whether the lacrimal enzymes are derived from the lysosomes or from the cytoplasm. The near absence, however, of cytoplasmic enzymes in stimulated tear fluid and the mostly strong lysosomal localization of acid hydrolases in the lacrimal gland (Table I) are suggestive for the lysosomes as the source of these enzymes. The apparent latency in the lysosomal fraction confirms the isolation of structure-bound enzyme activities.

It has been demonstrated that anti-inflammatory agents have a stabilizing effect on lysosomes. In our experiments (Fig. 2) within 1 hr after administration of acetylsalicylate a marked lowering of the β-hex level in tears occurred, which must be the expression of a stabilizing effect on the lysosomes. Because the β-hex concentration was also diminished in relation to the total protein in tear fluid, it can be concluded that the effect is specific for the lysosomal production and not directly related to a general influence on the tear production rate, as has been described after long-term use of acetylsalicylic acid. The stabilizing concentrations of salicylate in blood and tears in our experiments were the same as that reported by Dewar and Barron to stabilize retinal lysosomes.

Salicylate had no effect on the activity of the lysosomal enzymes after their release in tears, thus indicating that its effect is to stabilize lacrimal lysosomes rather than inhibit the activity of enzymes after release.

We thank H. J. F. Peeters, M.D., University Eye Clinic, Amsterdam, for supply of lacrimal gland tissue.

From The Netherlands Ophthalmic Research Institute, Amsterdam, The Netherlands. Submitted for publication Dec. 21, 1979. Reprint requests: N. J. van Haeringen, Ph.D., The Netherlands Ophthalmic Research Institute, P. O. Box 6411, 1005 EK Amsterdam, The Netherlands.
The effects of increased hydrostatic pressure upon normal and regenerated rabbit corneal endothelium. WILLIAM H. SCHUTZEN AND DIANE L. VAN HORN.

The effect of increased hydrostatic pressure upon the ability of normal and regenerated endothelium to deturgescence was studied. Stromal deturgescence occurred as a biphasic response when hydrostatic pressure at the endothelial surface was increased above baseline values. Initially there was a rapid phase of stromal thinning which was dependent upon hydrostatic pressure and endothelial function. This was followed by a slower phase of corneal thinning which was independent of hydrostatic pressure at the endothelial surface for pressures between 15 and 50 mm Hg. The slow phase of thinning represents the steady-state ability of the endothelium to deturgescence the stroma. Regenerated rabbit endothelium functioned similarly to normal endothelium in deturgescing the stroma. In addition, short-term increases in hydrostatic pressure at the endothelial surface did not produce ultrastructural changes in normal or regenerated corneal endothelial cells.

The specular perfusion microscope provides an accurate method for the determination of alterations in corneal thickness due to incomplete perfusion media, drugs, and variations in hydrostatic pressure. Using the specular microscope, Bowman and Green reported that swollen, denuded endothelial corneas deturgesc multiple times as hydrostatic pressure at the endothelial surface is increased from 5 to 50 mm Hg. In contrast, Hodson reported that a pressure change from 6 to 37 mm Hg has no effect on the rate of corneal deturgescence.

Elevated intraocular pressure has been shown to produce ultrastructural damage during perfusion of the endothelium in vervet monkey. However, hydrostatic pressures as high as 60 to 80 mm Hg have been reported to produce no detectable endothelial damage in perfused rabbit corneas.

In light of these conflicting results, the purpose of the present paper is to describe the effects of increased hydrostatic pressure upon the function and ultrastructure of normal and regenerated rabbit corneal endothelium during in vitro perfusion.

Methods. New Zealand white rabbits (1.5 to 2.0 kg) without corneal defects were sedated with ketamine HCl (20 mg/kg). Following topical application of 0.4% benoxinate HCl, a 5 mm diameter probe cooled in liquid nitrogen (−196°C) was applied to the central region of the right cornea for 15 sec. This is sufficient to destroy all the endothelial cells in the area of the probe. The left cornea was not frozen. The endothelium of the frozen cornea was considered regenerated when corneal thickness returned to prefreezing levels (about 7 days).

Preparation of corneas for perfusion. The corneal epithelium was removed by gently scraping the corneas with a Gill knife. The corneas were then dissected from the globes and mounted in a specular microscope pressure-perfusion apparatus. The endothelial surfaces were perfused with glutathione-bicarbonate-Ringer solution without adenosine at a rate of 0.097 ml/min at 37°C.

Induction of stromal swelling. The de-epithelialized surface of the corneas was exposed to 0.9% NaCl for 60 min to induce stromal swelling. During this time, the hydrostatic pressure at the endo-