An Internal Report* on
The Effect of GAGases on Outflow Facility in the Bovine Eye

by Mark Johnson[1] and Haiyan Gong[2]

[1]Fluid Mechanics Laboratory, MIT
[2]Boston University School of Medicine

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Abstract

Barany was the first to point out that testicular hyaluronidase increased outflow facility in bovine eyes. It has long been assumed that this was an effect on the extracellular matrix within the aqueous outflow pathway. Based on experiments conducted in primate eyes in which we found no effect of either Streptomyces hyaluronidase or chondroitinase ABC in primates, we were motivated to retrospectively reexamine both our own previous data concerning the effects of GAGases on bovine outflow facility and also to reexamine reports in the literature.

After GAGases incubation for several hours, we could not find evidence that GAGases 0increases outflow facility in the bovine beyond the wash-out that naturally occurs during perfusions. More over, for shorter time periods of incubation, the evidence is not convincing that these enzymes have definite effect. It may be instead that GAGases do not have their primary effect on the glycosaminoglycans of the outflow pathway, or they may affect junctions in the inner wall where it would be difficult to detect its effect morphologically, and these junctions may naturally open during wash-out.

This is a review of data from experiments conducted at MIT between November of 1989 and November of 1992. While we are here concerned with the effects of GAGases on outflow facility in the bovine eye, it should be noted that the experiments herein described were not conducted for that purpose, but instead were aimed at looking at the effects of prolonged perfusion of bovine eyes with aqueous humor following GAGase treatment. The hypothesis then being explored was that GAGase treatment would remove the hydrophilic lining of the flow pathway in the aqueous outflow system, leaving them more hydrophobic, and thus more inclined to become obstructed by proteins in the aqueous humor (Johnson et al., 1986; Ethier et al., 1989). **The present manuscript is written as an internal report since the methods are not sufficiently consistent from one experiment to another to be reported in a peer-reviewed journal.**

The principal motivation for this data review is the recent findings by our group, in collaboration with Dr. Paul Kaufman's group at the University of Wisconsin Medical School in Madison, that Streptomyces hyaluronidase and chondroitinase ABC appear to have no effect on outflow facility, either acutely or chronically, in the primate eye (Hubbard et al., 1997). As glycosaminoglycans have been thought to be involved in aqueous outflow resistance for over 40 years (Barany, 1953; Barany and Scotchbrook, 1954), this finding suggested a retrospective review of data from our laboratory on the effects of GAGases on bovine outflow resistance, since that was the specie in which the GAGase effect was first demonstrated.

Methods

Enucleated bovine eyes from two-week old calves were obtained from a local abattoir (Arenas & Sons, Hopkinton, MA) and transported on ice. Perfusions were begun within eight hours post-mortem.

The eyes were placed into a gauze-filled beaker, wetted until the cornea was just covered with saline, and then placed into a constant temperature bath (34 °C). A 23 gauze needle was then inserted through the cornea, passing through the iris, and into the posterior chamber. This needle was connected to a reservoir of Dulbecco's phosphate buffered saline (Life Science Technology, Inc., Chagrin Falls, OH) with 5.5 mM glucose (DBG) which was used to fill the eye. After 10 minutes of equilibration, the needle tip was moved into the anterior chamber. A second 23 gauge needle was placed into the anterior chamber to be used for exchanging the contents of the anterior chamber. The tubing connected to this needle was clamped off except when an exchange was occurring.

Perfusions were conducted using a computer-controlled syringe pump (Model 944, Harvard Apparatus Co., South Natick, MA) that can be run either in a constant flow mode or constant pressure. It should be noted that in cases where data is from a pair of eyes being perfused at "constant pressure", only one of the eyes is held at constant pressure (red or orange line in the graphs), while the second eye (green line in the graphs) receives the exact same flowrate as does the first.

A baseline perfusion was conducted at a constant pressure of 15 mm Hg (except where noted) for approximately 30-60 minutes. Then the contents of the anterior chamber were exchange with the appropriate enzyme and allowed to incubate for 30-60 minutes. A second exchange was then performed with the fluid to be perfused with (either aqueous

humor or DBG) and the perfusion was then conducted at constant flowrate using the flowrate determined at the conclusions of the constant pressure baseline; this second perfusion was conducted for 1-7 hours. In those cases that aqueous humor was used to perfuse the eyes, the aqueous humor had been collect from bovine eyes, pooled, ultracentrifuged at 100,000xg for 1 hour and then frozen until use.

Enzymes. The enzymes used were chondroitinase ABC (C2905, Sigma, 1 unit/ml DBG), testicular hyaluronidase (Wydase), Streptomyces hyaluronidase (H1136, Sigma, 65 - 110 units/ml DBG), or heparinase (H8891, Sigma, 2 units/ml DBG). The enzymes solutions were either ultracentrifuged at 100,000xg for 30 minutes or filtered through a 0.2 μm filter before use.

The activity of one batch of the chondroitinase ABC was confirmed by Dr. Ed Crean of the Howe Laboratory at the Massachusetts Eye and Ear Infirmary. Dr. Crean also examined the activity of the Streptomyces hyaluronidase and testicular hyaluronidase, for 1 hour and 6.5 hours, at pH 5 and 7, on chondroitin sulfate and hyaluronic acid, and found that while both enzyme preparation were active, for perfusion conditions (1 hour perfusion at pH 7), the Streptomyces hyaluronidase would remove all of the hyaluronic acid but would have not an effect on the chondroitin sulfate, while the testicular hyaluronidase would partially remove the hyaluronic acid and again have no effect on the chondroitin sulfate.

We also evaluated the activity of the chondroitinase ABC and the Streptomyces hyaluronidase, filtering the enzymes before use, and then placing them on bovine trabecular meshwork samples and using a hyaluronic acid assay. The Streptomyces hyaluronidase was completely effective and the chondroitinase ABC was partially effective (hyaluronic acid not being its primary substrate), as compared with control solutions.

Morphology. In one case, the eyes were examined following enzyme treatment, using standard electron microscopic preparation techniques (Johnson et al., 1993)

Results

Chondroitinase ABC. Two experiments were conducted in which the effects of chondroitinase ABC on outflow facility could be directly compared with a control solution (exchanging anterior chamber with DBG instead of ABC). The control is important because of the "massage effect" in which outflow facility of a bovine eye can be increased by manipulation of the eye, even simply exchanging the contents of the anterior chamber (Barany and Woodin, 1955).

Figure 1 shows an apparent increase in outflow facility caused by the ABC although the control eye has reached the same outflow facility within an hour of the

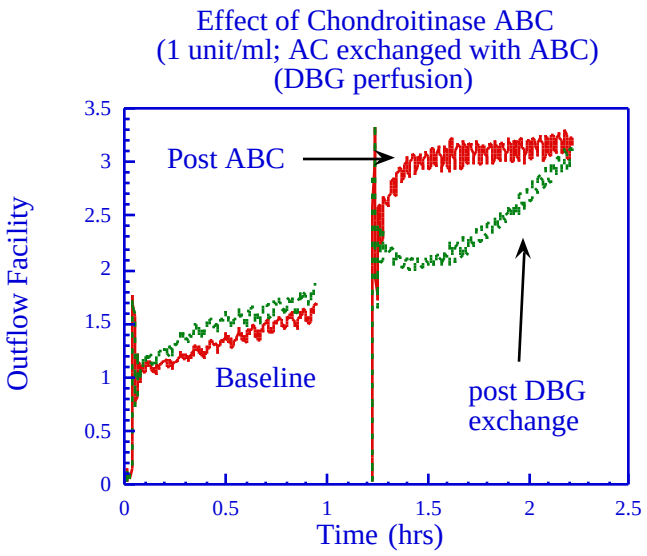

Figure 1 (exps. 136, 137)

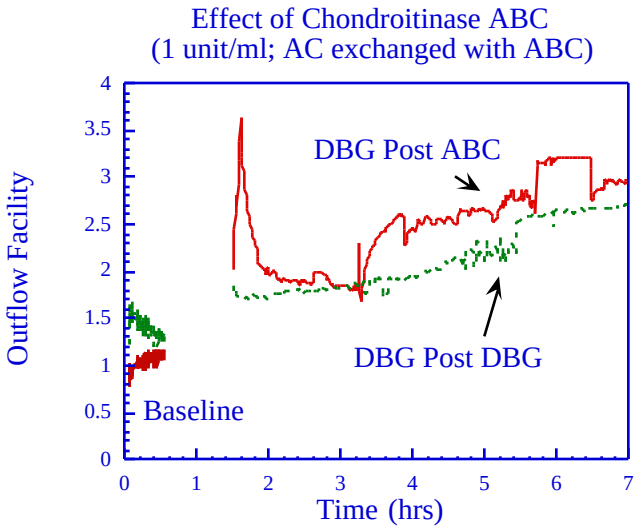

Figure 2 (exps. 156, 157)

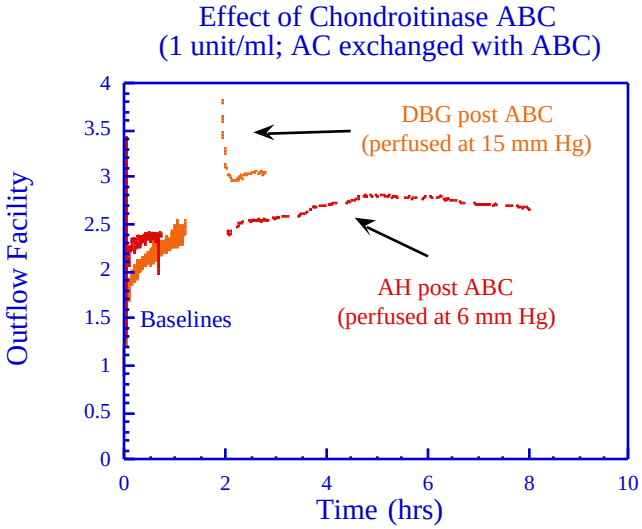

Figure 3 (exps. 141, 142, 160, 161)

initial exchange. The second experiment (Figure 2) appeared to show less of a difference between the ABC-treated eye and the control eye, but it should be noted that the baseline facility in the experimental eye was somewhat lower than that in the control eye (1.1 vs. 1.35 µl/min/mm Hg, respectively). In terms of a percentage increase in facility, the enzyme-treated eye increased in facility approximately 75% while the control eye increased only 30%. Note also that in this second experiment, there is a longer time between the initial exchange and the second perfusion (1 hour) than that in Figure 1 (15 minutes).

Both of these experiments are consistent with ABC transiently increasing outflow facility but within an hour or so, the wash-out effect in the control eye catches up. This would suggest that the GAGase effect acts on the same locus as does the "wash-out" effect, merely speeding up the process. However, there is a second interpretation, and that is that the ABC has no effect on outflow facility and the higher facility seen in Figures 1 and 2 in the eyes receiving ABC is due to statistical variability.

Two other "uncontrolled" experiments are consistent with this latter interpretation. Figure 3 shows two eyes (not perfused as a pair), one perfused at 6 mm Hg, the other at 15 mm Hg. In the first case, there seem to be little or no effect of the GAGase treatment above the very slow wash-out, while in the second, there appears to be more of an effect, but it is again consistent with the wash-out trend seen in the baseline.

Thus, the results with chondroitinase ABC, an enzyme that should remove chondroitin sulfate (4-sulfate and 6-sulfate), dermatan sulfate and hyaluronic acid, show either no effect or an effect little indistinguishable from wash-out within one hour. It is also interesting that the single experiment done at a low perfusion pressure showed no effect of the enzyme treatment at all.

Streptomyces hyaluronidase. Two experiments were conducted in which the effects of Streptomyces hyaluronidase on outflow facility could be directly compared with a control solution. As can be seen in Figure 4, both sets of eyes show negligible effects of the Streptomyces hyaluronidase when compared with the their respective controls. Most significant is the eye perfused with 65 units/ml of Streptomyces hyaluronidase as compared with its control (the pair of eyes shown in orange and light green): even though the period

for the exchange is quite brief (approximately 15 minutes), no difference can be seen between the enzyme-treated eye and the control. (For this last pair of eyes, each eye was *individually* perfused at constant pressure during the baseline period, and then at constant flow at the flowrate determined at the end of the baseline period).

Thus, the results with Streptomyces hyaluronidase, which should remove only hyaluronic acid, shows the enzyme to have no measurable effect.

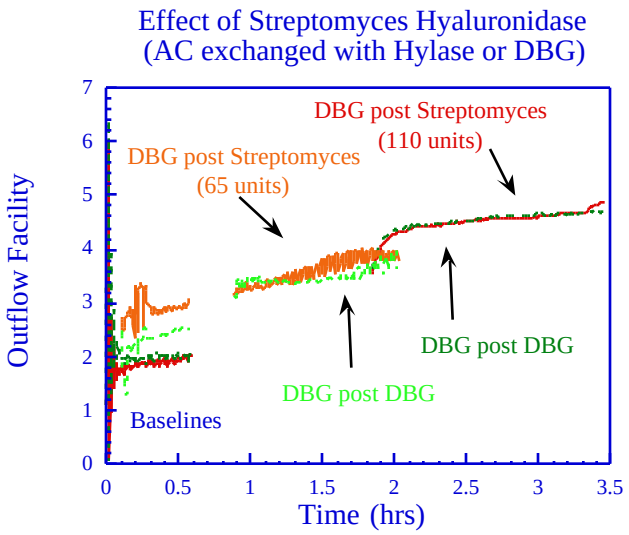

Figure 4 (exps 165, 166, 175, 176)

Testicular hyaluronidase. Several uncontrolled experiments (Exps. 143, 144, 146, 147) showed testicular hyaluronidase to increase bovine outflow facility by between 75 and 375%; however, without a control, the extent of the massage effect and wash-out in each of these eyes could not be determined.

Figure 5 shows a pair of eyes in which testicular hyaluronidase increased outflow facility significantly more than did a control solution, and the enzyme-treated eye maintains a higher outflow facility for many hours*. Note, however, that the two graphs cross approximately 5 hours after anterior chamber exchange.

A second pair of eyes examining the effect of testicular hyaluronidase showed a similar effect (Figure 6), namely, that the enzyme seems to have a larger effect on outflow facility than did an exchange with a control solution.

Thus, all experiments using testicular hyaluronidase showed a significant effect on outflow facility, and in those that had an appropriate control, the testicular hyaluronidase increased outflow facility to a greater extent than did the control.

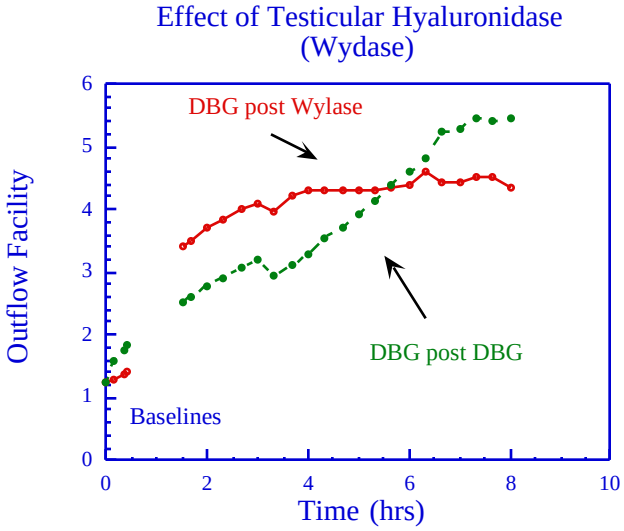

Figure 5 (exps. 150-153)

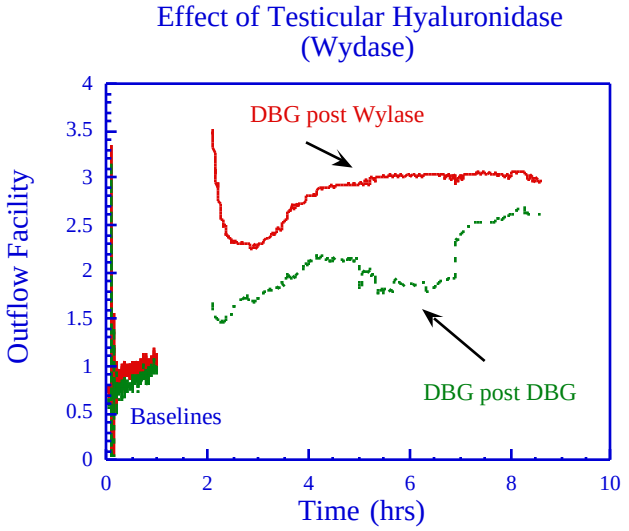

Figure 6 (Exps. 154, 155)

* The original data on computer for this experiment was lost, so the graph was recreated from data available in the experimental notebook.

Figure 7 (Exps 146, 147)

In one set of experiments, we examined the morphological effects of perfusion with testicular hyaluronidase on the bovine outflow system.. Figure 7 shows the inner wall of the aqueous plexus, the bovine equivalent of Schlemm's canal (seen in the upper left hand corner of the micrograph), of an eye perfused with hyaluronidase. Extensive vacuolization of the inner wall was seen as well as weakening and loss of attachments of the inner wall cells to the juxtacanalicular region (lower left hand corner of micrograph). Similar findings have been made by Grierson et al. (1979) as mentioned in the discussion section.

Comparison to Testicular Hyaluronidase to Streptomyces Hyaluronidase. The data thus far described has suggested that (a) chondroitinase ABC may have an acute effect on outflow facility but is not distinguishable from wash-out within one hour, (b) Streptomyces hyaluronidase appears to have no affect on outflow facility (as compared to control) and (c) testicular hyaluronidase appears to increase outflow facility, compared to control, for a relatively long period of time. As a test of this hypothesis, we examine two data sets that allow a direct comparison between the effects of testicular hyaluronidase as opposed to Streptomyces hyaluronidase on paired calf eyes.

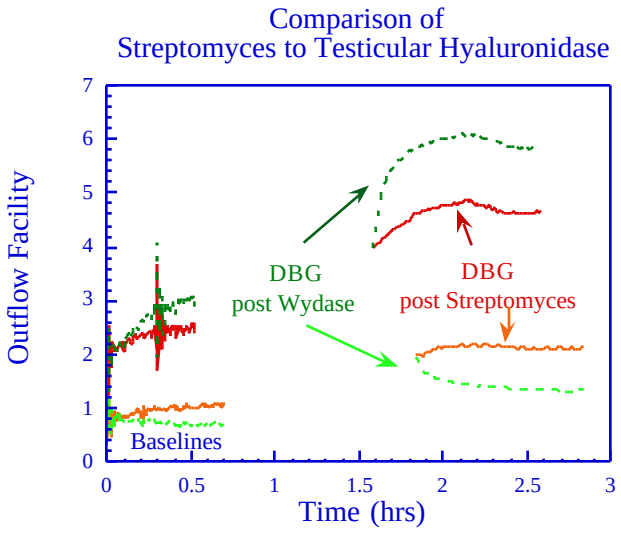

Figure 8 (Exps. 167, 168, 171, 172)

The results of this comparison are surprising (Figure 8). While one pair of calf eyes showed a somewhat greater effect of the testicular hyaluronidase than did the Streptomyces hyaluronidase (red and green lines), the second pair showed the opposite effect (orange and light green lines). Furthermore, the percentage increase in outflow facility is the same in all of these cases (roughly a doubling).

Thus, 1 hour following exchange, there is no apparent difference between Streptomyces hyaluronidase and testicular hyaluronidase, at least, in this limited data sample.

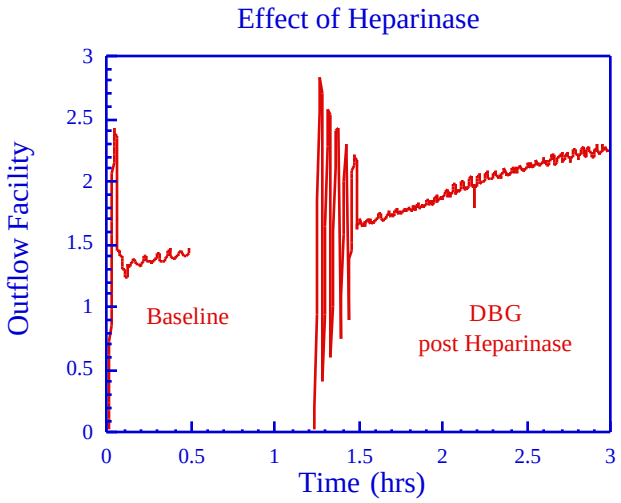

Figure 9 (exps 173, 174)

Heparinase. An experiment was conducted perfusing an eye with heparinase since heparan sulfate is a constituent of basement membrane, and Schlemm's canal is known to have a basement membrane. The results showed (Figure 9) no apparent effect of the heparinase (2 units/ml). It should be noted however, that this was the result of a single experiment and that enzyme activity was not verified biochemically or histochemically.

Conclusions

Barany (Barany, 1953; Barany and Scotchbrook, 1954) first called attention to the possible importance of glycosaminoglycans as playing a role in generating aqueous outflow resistance. Barany found that testicular hyaluronidase decreased the outflow resistance of bovine eyes by about a factor of two; Pedler (1956) reported a similar result. While testicular hyaluronidase has been reported to increase outflow facility in a number of species (guinea-pigs: Melton and DeVille, 1960; primates, Peterson and Jocson, 1974; dog: Van Burkirk and Brett, 1978; rabbits: Knepper, 1984), the evidence suggests little effect on human eyes (Pedler, 1956; Grant, 1963).

Barany's observation has led to numerous studies aimed at determining the precise role that glycosaminoglycans play in generating aqueous outflow resistance, and whether altered glycosaminoglycan metabolism plays a role in the pathogenesis of primary open angle glaucoma. Interestingly, there have been only two studies that have used highly specific GAGases to determine precisely which GAGs are involved in generating outflow resistance. Knepper (1980) found that chondroitinase AC, chondroitinase ABC and Streptomyces hyaluronidase increased outflow facility in the enucleated rabbit eye in a dose-dependent manner. In the primate eye, Sawaguchi et al. (1992) reported that chondroitinase ABC decreased IOP compared with control eyes receiving heat-inactivated enzymes.

Thus our group was surprised when we found no acute effect of either Streptomyces hyaluronidase or chondroitinase ABC on IOP or outflow facility in the primate eye (Hubbard et al., 1996). The data which we review here in the current report shows that we have also been unable to show a convincing effect of these enzymes on outflow facility in the bovine eye. However, it is important to recall that this manuscript summarizes data retrospectively, and thus the experiments summarized here were not designed to determine the effects of GAGases on aqueous outflow facility in the bovine eye. Nonetheless, it is quite interesting that in the specie in which the hyaluronidase sensitivity of the barrier to aqueous outflow was first demonstrated, it is difficult to conclusively show any effect of these four different GAGases on outflow facility (above the wash-out and massage effects that occurs in the control eyes).

In attempting to resolve this dilemma, it is useful to reexamine Barany's methods, as those studies were done many years ago, and techniques have changed significantly in the interim. Firstly, Barany (and most later investigators) used testicular hyaluronidase, an enzyme known to have significant protease activity (Keiser and Hatcher, 1979), even though Barany and Scotchbrook claim that their preparation was protease-free. In fact, a variety of proteases are known to affect aqueous outflow resistance (plasmin: Pandolfi, 1967; alpha-chymotrypsin: Hamanaka and Bill, 1988).

Second, Barany placed his perfusion needles in the anterior chamber since the chamber-deepening effect (Moses, 1977) was not known at that time. This would have led to variable results reflecting different degrees of chamber deepening. In fact, there may have been some effect of the testicular hyaluronidase on the hyaluronic acid in the vitreous and this might enhance the chamber deepening in the enzyme-treated eyes. However, the facility increasing effect of testicular hyaluronidase has been demonstrated in primate eyes in which anterior chambering deepening was prevented (Peterson and Jocson, 1974).

Finally, the perfusion fluid was calcium-free. It is known that calcium-free perfusion fluid acts as a chelating agent, separating tight junctions and leading to increased outflow facility (Bill et al., 1980). It may be that the junctions in hyaluronidase-treated eyes were more sensitive to this chelating effect. Evidence showing an effect of hyaluronidase on cell junction is apparent in figure 7, although it is not known whether this was due to an effect on GAGs in the cell junctions, or to a calcium chelation affect. However, Grierson et al. (1979) perfused hyaluronidase in a calcium-containing medium into baboon eyes, and reported effects very similar to that seen in figure 7. This suggests that, while testicular hyaluronidase may have an affect on cell junctions, it is likely not due to calcium chelation.

One final line of reasoning lends further doubt to the notion that testicular hyaluronidase increases outflow facility by degrading extracellular matrix GAGs in the outflow pathway. It has been reported (Pedler, 1956), and the data we summarized here confirm that the 'wash-out' effect and hyaluronidase treatment of an eye are not additive in their influence on outflow facility ; in fact, if one eye of a pair is perfused with buffer while the other is first treated with hyaluronidase and then perfused, they will both, after sufficiently long time, end at approximately the same outflow facility. Yet we have found (Johnson et al., 1993) that in bovine eyes perfused for 5 hours (and demonstrating significant wash-out), no change in the levels of sulfated glycosaminoglycans could be detected. Furthermore, Gong and Freddo found only negligible quantities of hyaluronic acid in the outflow pathway. Thus, the dilemma has been that wash-out appears to have the same locus of activity as does testicular hyaluronidase, yet no evidence of loss in glycosaminoglycans in the outflow pathway can be demonstrated following wash-out.

It may be instead, that testicular hyaluronidase does not have its primary effect on the glycosaminoglycans of the outflow pathway (e.g. acting as a protease), or it may affect junctions in the inner wall where it would be difficult to detect its effect morphologically. Further studies should be done to determine whether testicular hyaluronidase treatment in bovine eyes in which anterior chamber deepening is prevented and the perfusion fluid contains calcium has any greater effect than does chondroitinase ABC or Streptomyces hyaluronidase, and if so, what is the locus of its action.

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