The Flow of Aqueous Humor Through Micro-Porous Filters

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Flow resistance was measured as bovine and primate aqueous humor was passed through Nuclepore polycarbonate filters having flow dimensions similar to those found within the juxtacanalicular meshwork of the aqueous outflow network. The results indicate that aqueous humor has a greater flow resistance than isotonic saline; this greater resistance is attributable to proteins or glycoproteins in aqueous humor that obstruct the filters. If the same phenomenon is operative in the aqueous outflow network, it would help to explain discrepancies between calculated and measured aqueous outflow resistance. Invest Ophthalmol Vis Sci 27:92–97, 1986

Aqueous humor is composed of the soluble constituents of the blood (although in different ratios) and contains only a small amount of protein (100–600 μg/ml)[1,2] and glycosaminoglycans (1–4 μg/ml).[3] The aqueous humor leaves the eye through an outflow system or network consisting, from inside to outside, of (1) uveal and corneoscleral trabecular meshwork, (2) juxtacanalicular meshwork, (3) endothelial wall of Schlemm's canal, (4) Schlemm's canal, and (5) aqueous veins that extend from the canal through the sclera to join blood-containing veins on the surface of the eye. The normal drop in pressure through this outflow network is approximately 6 mm Hg; in chronic glaucoma the pressure drop can be as much as 60 mm Hg with nearly the same rate of flow. The basis for both normal resistance and the elevated resistance in chronic open-angle glaucoma has eluded researchers for more than 100 years.

Previous work has indicated that neither the trabecular meshwork, the endothelial wall, Schlemm's canal, nor the aqueous veins are likely to be major sites of flow resistance.[4–8] It is generally believed that the tortuous flow passages within the juxtacanalicular meshwork can account for much of the flow resistance; however, more recent calculations based on dimensions determined from electron micrographs indicate that on average the passages seen microscopically are too large to account for the measured resistance to flow.[9] This discrepancy may be due to the presence of an extracellular matrix gel filling much of the apparent open space in the juxtacanalicular meshwork.[10,11] In each of the studies wherein outflow resistance was calculated, the flow dimensions were determined from microscopy, and aqueous humor was assumed to behave like saline. Normal aqueous humor has a viscosity approximately equal to that of saline when measured in a viscometer.[12,13] Thus, it has been expected that aqueous humor would behave in a manner similar to saline as it flows through the small openings of the aqueous outflow network. We report here that when aqueous humor is passed through micro-porous filters having flow dimensions similar to those found within the juxtacanalicular meshwork, there is an obstruction of these filters and an increased flow resistance relative to that of isotonic saline. If the same phenomenon is operative in the aqueous outflow system of the eye, it would help to explain some troublesome discrepancies between calculated and measured resistance to outflow which have been associated with the assumption that aqueous humor behaves like saline in the small outflow channels of the eye. As we will show, the greater resistance to flow through micro-porous filters appears to be attributable to macromolecules in the aqueous humor that are affected by papain but not by hyaluronidase.

Materials and Methods

A series of experiments was conducted wherein various solutions were passed through Nuclepore poly-

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carbonate filters (Nuclepore Corp.; Pleasanton, CA). The pores in these filters are essentially uniform circular cylinders.[14] Pore diameters of 0.2 and 1.0 μm were chosen, as they span the smallest flow dimensions in the juxtacanalicular meshwork. The solutions passed through these filters included bovine and primate aqueous humor, hyaluronic acid, bovine aqueous humor and hyaluronic acid each pretreated with testicular hyaluronidase, bovine aqueous humor pretreated with papain, diluted bovine serum, and a control solution of Dulbecco's phosphate buffered saline (DPBS) prefiltered through a 0.08 μm Nuclepore filter.

All glassware, syringes, centrifuge tubes, pipettes, etc were carefully washed and rewashed using deionized water that had been pre-filtered through a 0.08 μm Nuclepore filter. We found this to be an extremely important step due to difficulties caused by even slight impurities. Only by following these procedures could a stable and repeatable baseline flow resistance be achieved with DPBS.

Solution Preparation

Pooled samples of bovine aqueous humor were obtained by withdrawing 300–500 μl of fluid from the anterior chamber of approximately 25 enucleated calf eyes. The eyes were obtained from Joseph T. Trelegan & Co.; Cambridge, MA. The animals were stunned by a blow to the head and killed by exsanguination. The eyes were transported on ice and used within 4 hr after death. In two experiments, the aqueous humor was collected immediately after death of the animals.

Primate aqueous humor was collected from 33 Rhesus monkeys (Macaca mulatta) anesthetized with sodium phenobarbital. All animals were utilized in accordance with the ARVO Resolution on the Use of Animals in Research. Approximately 150–200 μl of aqueous humor were collected from the anterior chamber of each eye using a 23-gauge needle and syringe. Tests on primate aqueous humor were done in a masked fashion such that the investigator testing the solutions did not know which fluid was primate aqueous humor and which fluid was the control solution.

Solutions of hyaluronic acid were prepared (400 μg/ml) by dissolving human umbilical cord hyaluronic acid (Sigma Chemicals; St. Louis, MO) in DPBS. The solution was allowed to sit overnight (refrigerated) to completely dissolve the hyaluronic acid. The hyaluronidase used was Wydase (150 USP/ml, Wyeth Lab.; Philadelphia, PA). Papain (P4762, Sigma Chemicals; St. Louis, MO) was prepared by dissolving the enzyme in DPBS (1 mg/ml).

When hyaluronidase or papain was added to other solutions, 0.5 ml of the enzyme was added to 1.5 ml of the test solution. The resulting solutions were then incubated for 5 min (at room temperature for hyaluronidase; 40°C for papain). For these enzymatic tests, a control solution was prepared by adding 0.5 ml of DPBS to 1.5 ml of the test solution.

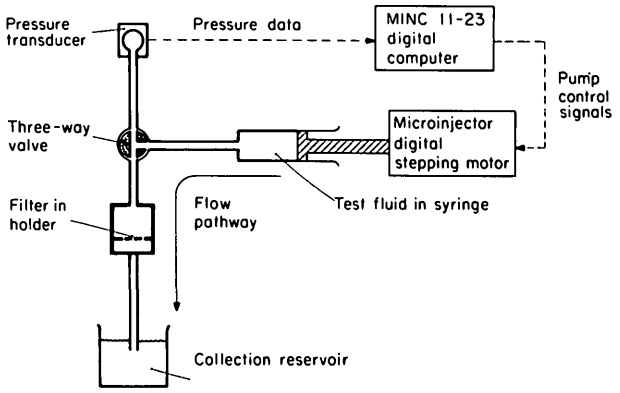

Fig. 1. Schematic of perfusion system.

Bovine serum was diluted one hundredfold to yield a solution whose protein content was similar to, or greater than, that of bovine aqueous humor. The protein level in the bovine aqueous humor was measured using the Bio-rad protein assay (Bio-rad; Richmond, CA) with a gamma globulin standard.

After preparation of the various solutions, they were centrifuged at 100,000 × g for 1 hr, following which the supernatant was collected. Control solutions (DPBS) went through identical procedures as test solutions.

Test Apparatus

All tubing in the test system was cleaned before each test with deionized water prefiltered through a 0.08 μm filter (Fig. 1). To ensure complete wetting of the Nuclepore filters, they were placed, in solution, into an ultrasound bath for 4 min.

The test solution was then placed in the syringe (Fig. 1), and the solution was used to fill the upstream half of the filter holder. The filter was then placed into the holder and the downstream half of the holder was attached. DPBS was used to backfill the system and eliminate bubbles. All perfusions were done at room temperature.

The pressure drop across the filter was measured (as a function of time) while passing the test solution through the filter at a constant flowrate (40 μl/min for the 0.2-μm filters; 100 μl/min for the 1.0 μm filters) using a stepping-motor driven syringe pump. The flowrate chosen was such that the flowrate per unit filter area was similar to that through the aqueous outflow network. Baseline resistances were determined with DPBS and found to be 0.072 mm Hg min/μl for the 0.2 μm filters and 0.0027 mm Hg min/μl for the 1.0 μm filters. (Note that these resistances do not in-

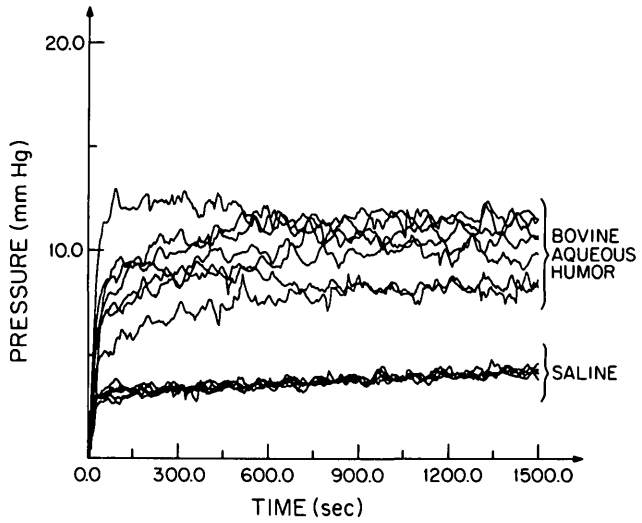

Fig. 2. Pressure drop of bovine aqueous humor or Dulbecco's Phosphate Buffered Saline (DPBS) as these solutions pass through Nuclepore polycarbonate 0.2 μm filters at 40 μl/min. In this and subsequent graphs, the rapid initial rise in pressure is a pressurization transient.

clude the small pressure losses in the tubing and valves.) No significant variation of baseline resistance was seen between different filters of the same pore size.

Results

Figure 2 shows the results of passing bovine aqueous humor (collected 4 hr post-mortem) or DPBS through 0.2-μm filters. The flow resistance of the aqueous humor increases to a level two to three times that of DPBS and seems to level off there. On the other hand, bovine aqueous humor passed through 1.0-μm filters showed no significant difference from DPBS. The slight increase in flow resistance of saline shown in the figure is presumably due to impurities in the system that were not eliminated when the glassware was cleaned. Figure 3 shows results for bovine aqueous humor collected immediately after death (again passing the aqueous humor through 0.2-μm filters). This "fresher" aqueous humor shows a slightly different character than the older fluid, but both behave in a distinctly different manner than did DPBS, and they each obstruct the filters to approximately the same degree. The protein level in the bovine aqueous humor was found to be approximately 500 μg/ml in agreement with values obtained by other investigators.[2]

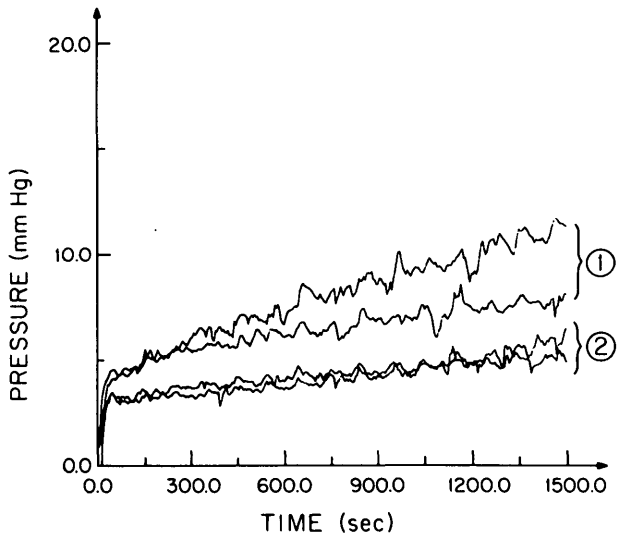

Fig. 3. Pressure drop of bovine aqueous humor (1) collected immediately post-mortem compared to that of bovine serum diluted 100 to 1 with DPBS (2) as these solutions pass through the 0.2 μm Nuclepore filters at 40 μl/min.

As mentioned in the section on methods, these results are for bovine aqueous humor that has been ultra-centrifuged. A series of tests was also run on bovine aqueous humor that was not ultra-centrifuged, but instead pre-filtered through 1.0 μm Nuclepore filters. The results (not shown here) indicate that this pre-filtered bovine aqueous humor obstructs both 0.2- and 1.0-μm filters to a much greater degree than does the ultra-centrifuged bovine aqueous humor. Whether the behavior of aqueous humor in vivo is more analogous to that of ultra-centrifuged or pre-filtered aqueous humor (if either) is at present unknown.

Figure 4 shows the results of passing primate aqueous humor (or DPBS) through 0.2-μm filters. Figure 4a shows the results of primate aqueous ultra-centrifuged in the usual fashion; Figure 4b shows the result for a sample of primate aqueous humor that was not ultra-centrifuged. These tests were done in a masked fashion, and in all cases, the investigator was able to distinguish between the control solution and the aqueous humor. This was an important step since, as Figure 4 indicates, the filter obstruction caused by primate aqueous humor is less (over the time scales tested) than that of bovine aqueous humor. However, in all cases, primate aqueous humor exhibited a higher flow resistance in 0.2-μm filters than did DPBS. Protein levels in the primate aqueous humor were not measured, but one of the investigators (DG) has measured primate aqueous humor protein concentration in a study conducted under nearly identical conditions[15] and found the protein level to be 333 μg/ml.

To test the hypothesis that hyaluronic acid might be responsible for this obstruction, solutions of hyaluronic acid were passed through the 0.2 μm filters in an attempt to duplicate the effect observed for aqueous humor. We found, however (Fig. 5), that in order to duplicate the rate at which aqueous humor obstructs these filters, we required solutions whose hyaluronic acid concentration was at least 50 times that of bovine aqueous humor. Hyaluronidase was added to samples of bovine aqueous humor and to prepared solutions

of hyaluronic acid. As seen in Figure 5, the enzyme eliminated most of the filter-obstructing effect of the hyaluronic acid solution, but it had only a modest effect on aqueous humor.

The protease papain was then added to samples of aqueous humor. Papain was chosen because of its general nonspecificity as a protease. As can be seen in Figure 6, the enzyme greatly reduces the filter-obstructing potential of the bovine aqueous humor. To determine if this effect was due to the general protein level of aqueous humor, the filter-obstructing potential of bovine serum was investigated. Bovine serum has a protein concentration approximately 100 times larger than does bovine aqueous humor; therefore, the serum was diluted one hundredfold to reduce it to aqueous humor protein levels. As Figure 3 shows, the diluted serum behaves much more like DPBS than like bovine aqueous humor.

Discussion

These experiments demonstrate that under certain experimental conditions, aqueous humor may exhibit flow behavior significantly different from that of isotonic saline. We interpret our results as indicating that macromolecules in aqueous humor are capable of obstructing flow through these filters. One candidate for the obstructing macromolecule is hyaluronic acid. To test this notion, hyaluronic acid solutions were passed through the filters, and it was found that hyaluronic acid concentrations of approximately 50 times that of aqueous humor were required to achieve the same filter-obstructing effect as that of bovine aqueous humor; in addition, the filter-obstructing potential of aqueous humor was only mildly affected by hyaluronidase. We

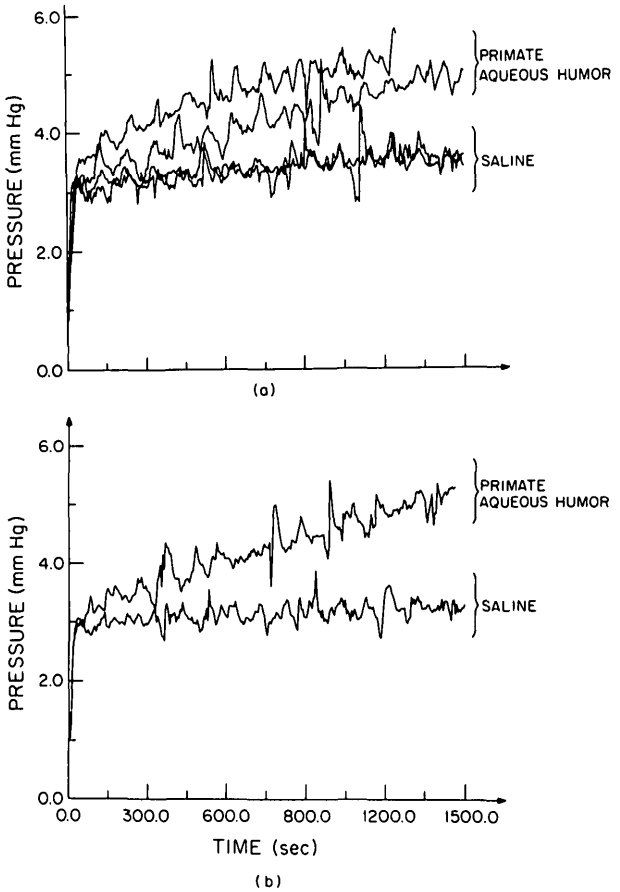

Fig. 4. Results from passing primate aqueous humor through the 0.2 μm Nuclepore filters at 40 μl/min. (a) Aqueous humor and DPBS prepared as described in the methods section; (b) Aqueous humor and DPBS not ultra-centrifuged.

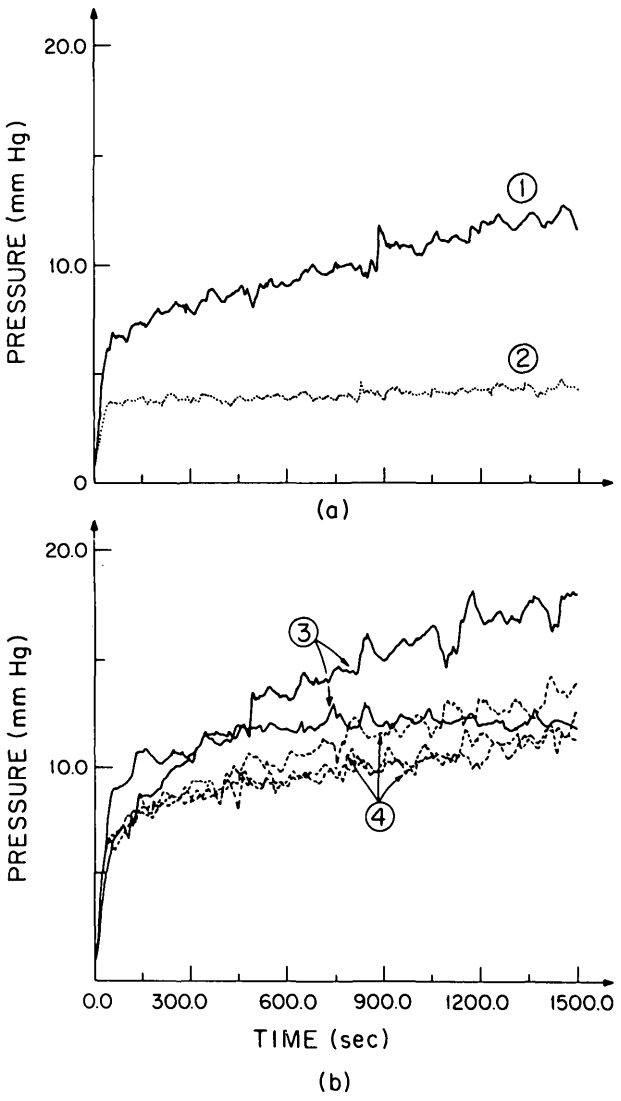

Fig. 5. Effect of hyaluronidase on the filter-obstructing effect of hyaluronic acid and bovine aqueous humor (0.2 μm Nuclepore filters; 40 μl/min). (a) Solid line (1): hyaluronic acid (HA) (400 μg/ml) diluted with DPBS (3 parts HA to 1 part DPBS); dotted line (2): hyaluronic acid to which hyaluronidase has been added (3 parts HA to 1 part enzyme); (b) solid lines (3): aqueous humor (AH) diluted with DPBS (3 parts AH to 1 part DPBS); Dashed lines (4): aqueous humor to which hyaluronidase has been added (3 parts AH to 1 part enzyme).

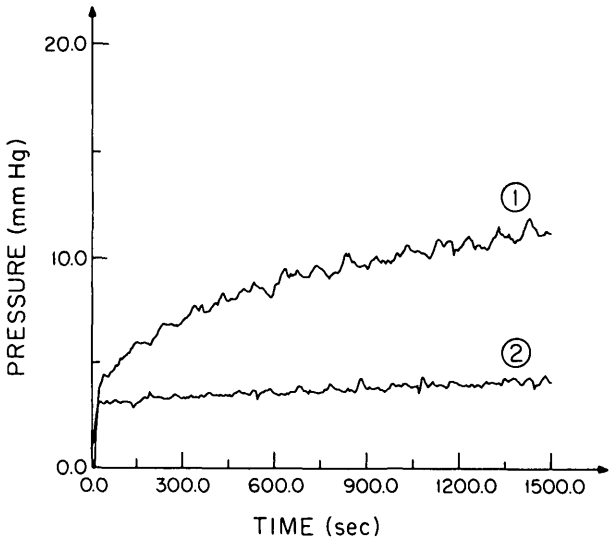

Fig. 6. Effect of papain upon the filter-obstructing effect of aqueous humor (0.2 μm Nuclepore filter; 40 μl/min). Upper curve (1): aqueous humor diluted with DPBS (3:1); Lower curve (2): aqueous humor to which papain has been added (3:1).

therefore conclude that hyaluronic acid plays at most a minor role in the observed filter obstruction.

Although aqueous humor appears to be relatively insensitive to hyaluronidase, its filter-obstructing potential is rapidly degraded by papain. We thus conclude that a protein or glycoprotein plays an important role in obstructing these filters. Other tests demonstrated that bovine serum with protein concentration similar to that of bovine aqueous humor showed very little filter-obstruction potential. This suggests that the proteins in aqueous humor responsible for obstructing the filters are either absent from bovine serum or present in very small quantities (as a function of total protein content) relative to bovine aqueous humor.

The concentration of macromolecules in aqueous humor is too low to affect the viscosity of aqueous humor significantly; however, when aqueous humor flows through filters with microscopic pore dimensions, the macromolecules may accumulate on the surface or within the pores of the filter and thus cause significant flow resistance. Aqueous humor may in fact contribute to the maintenance of the extracellular matrix gel of the juxtacanalicular meshwork.

The extent to which the results with bovine aqueous humor represent a postmortem change in the character of the aqueous humor has not yet been determined. However, experiments on primate aqueous humor—collected from live Rhesus monkeys—suggest that aqueous humor may indeed have flow properties different than isotonic saline when passing through submicron pores. In addition, the similarity between samples with and without ultra-centrifugation suggests that this higher resistance was not due to macroscopic contaminants. The quantitative differences between the character of the bovine and primate aqueous humor are curious and may be due to any of a number of causes, including species variability, postmortem changes in the bovine aqueous humor, or developmental differences due to the young age of the calves (several weeks old). However, it should be emphasized that both bovine and primate aqueous humor behaved differently from saline solution.

The experiments described here have been performed using Nuclepore filters that may interact with aqueous humor in an entirely different manner than does the aqueous outflow network. Nonetheless, the results suggest the possibility that aqueous humor may dramatically alter the outflow resistance of the aqueous outflow network (perhaps within the juxtacanalicular meshwork where the pores sizes are similar to those used in this study) by obstructing the narrow passages with proteins or glycoproteins. Although it is far from certain that a similar obstructive effect occurs in the living eye, these results suggest a novel approach to the problem of locating and perhaps therapeutically altering aqueous humor outflow resistance.

Troncoso, at the beginning of this century, suggested that glaucoma is caused by an excess of protein in aqueous humor.[16] There is evidence that certain secondary glaucomas may involve protein obstruction of the outflow channels.[17-19] Further studies on normal and glaucomatous eyes may demonstrate whether abnormalities in aqueous humor protein levels or their interaction with the aqueous outflow network could be involved in the pathogenesis of primary open-angle glaucoma. Regardless, our study suggests that aqueous humor, itself, may normally contribute to aqueous outflow resistance.

Key words: aqueous humor, aqueous humor proteins, filtration, aqueous outflow resistance, juxtacanalicular meshwork

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