Further Studies on the Flow of Aqueous Humor through Microporous Filters

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It has recently been shown that aqueous humor is able to obstruct flow through Nuclepore polycar-bonate filters having flow dimensions similar to those found in the juxtacanalicular tissue (JCT). We undertook studies designed to identify the component(s) of aqueous humor responsible for this ob-struction and to determine the mechanism of blockage. We conclude that aqueous humor contains two components (one of which is specific to aqueous) which, when simultaneously present, hydrophobi-cally bind to microporous filters and lead to filter blockage. Some implications for aqueous humor flow through the JCT are briefly discussed. Invest Ophthalmol Vis Sci 30:739–746, 1989

A central goal of glaucoma research is to localize and identify the cause (or causes) of the glaucoma-tous eye's subnormal aqueous outflow facility. This in turn requires the identification of the main resis-tive sites within the aqueous outflow pathway of the normal eye, as well as an understanding of how aqueous humor passes through these tissues. A tissue which has received a great deal of study in this regard is the trabecular meshwork, and in particular, the juxtacanalicular tissue (JCT). Morphometric analysis of the JCT in immersion-fixed primate eyes indicates that this tissue is permeated by an interconnected and complex network of apparently open "pores" having typical diameters in the range 0.1 to 1.0 μm.[1–3] Some time ago Barany and Woodin[4] suggested that the tra-becular meshwork contains glycosaminoglycans, which may play a role in determining outflow facility, a hypothesis consistent with recent calculations[3] showing that measured outflow resistance can be ac-counted for by assuming that the pores of the JCT are filled by an extracellular gel containing glycosamino-glycans and/or proteoglycans. The flow of aqueous humor through these pores has generally been consid-ered to occur in a relatively passive and noninteract-ing fashion, that is, the behavior of the aqueous humor was thought to be essentially like that of iso-tonic saline. However, the possibility of a more com-plex interaction was recently raised by Johnson et al[5] based on the finding that bovine and monkey aqueous humors progressively obstruct Nuclepore microporous membranes having pore diameters sim-ilar to the pores within the JCT (0.2 μm). It was noted that bovine plasma diluted to aqueous humor protein levels exhibited only a very small degree of filter blockage, and that addition of the nonspecific pro-tease papain to aqueous humor eliminated its filter blocking capability. It was therefore suggested that a protein or glycoprotein specific to the aqueous humor was responsible for this blockage.

Given that aqueous humor contains a specific filter-obstructing factor (or factors), it is natural to ask whether a similar blocking process could occur within the pores of the JCT in vivo. In order to ad-dress this question, we undertook a number of studies designed: (1) to more fully characterize, and if possi-ble, isolate, the factor(s) present in aqueous humor responsible for filter blockage; and (2) to identify the mechanism whereby this factor is able to obstruct microporous membranes. The goal was to improve our understanding of the filter-blocking process in order to enable a focused investigation of possible blockage in the JCT to be conducted. In this paper we report our findings regarding the filter-blocking pro-cess by aqueous humor and discuss the implications of these findings for the JCT.

Materials and Methods

In order to more fully elucidate the nature of the blocking process on microporous membranes we conducted a number of experiments in which either:

(1) the composition of the aqueous humor was altered in a known fashion; or (2) the properties of the test filter were altered in a controlled way. The effects of such alterations were then observed and inferences about the blocking process were made. In addition, an effort was made to synthesize a "mock aqueous" containing a number of the same components normally present in calf aqueous humor.

Collection and Processing of Aqueous Humor

Aqueous humor was collected from freshly enucleated calf eyes obtained from a local abattoir (J. T. Trelegan and Co., Cambridge, MA). Calves were 2–4 weeks of age at death and were killed by exsanguination after being stunned and inverted. Eyes were transported in a frozen/liquid isotonic saline mixture, and the aqueous humor was withdrawn within approximately 4 hr of death. Aqueous humor collection followed the protocol of Johnson et al.[5] Briefly, aqueous humor was directly aspirated from the anterior chamber and pooled, typically from 20–30 eyes. Eyes were rejected if they were noticeably bloody in the limbal region or had any other anterior segment abnormalities, as determined by simple visual inspection. The pooled aqueous humor (as well as other solutions used in this study) was then ultracentrifuged at 100,000 g for 1 hr at 4°C in an attempt to remove extraneous particulate material which could lead to artifactual filter blockage.

In one experiment, calf aqueous humor was separated into high and low molecular weight-containing fractions by using a Centricon-10 centrifugal microconcentrator with a nominal molecular weight cutoff of 10,000 daltons (Amicon Corp., Danvers, MA). Separation was effected by centrifugation at 5000 g for 1 hr at 10°C. The retentate (containing high molecular weight material) and filtrate (containing low molecular weight material) were diluted with prefiltered Dulbecco's phosphate buffered saline (DPBS) (Gibco Labs, Grand Island, NY) so that their volumes equaled the original aqueous humor volume, and were then ultracentrifuged and processed in the usual manner.

In another experiment, 9% chilled absolute ethanol was added to aqueous humor so as to precipitate Cohn fraction I,[6] which contains fibrinogen (and fibronectin).[7]

Protein Assays

Protein levels were determined by the method of Bradford[8] using a Bio-Rad kit (Bio-Rad, Richmond, CA) and a bovine plasma γ-globulin standard (Bio-Rad Standard I). Duplicate assay samples were prepared except on infrequent occasions when sample

volumes were insufficient; reported concentrations are the mean of the two samples. If the tested sample contained a substance which partially interferes with the assay (e.g., ethanol or dilute detergents) a standard curve in which the concentration of the interfering substances was equal to that in the test sample was used. Small (<20 μg) protein amounts were assayed using the Bio-Rad microassay technique. In this case sample volumes were usually too small to allow for duplicate assays.

Preparation of Plasma, Serum and Other Solutions

The following materials were obtained from Sigma Chemicals (St. Louis, MO) and used as received: bovine serum albumin (A0281, essentially fatty acid-free), bovine γ-globulin (G5009, bovine Cohn fraction II, 99% γ), bovine fibrinogen (F4789 Type IV, 99% clottable), bovine plasma fibronectin (F4789), Triton X-100 (T6878), lauryl sulfate (SDS; L4509), spermine (S4513), and human umbilical cord hyaluronic acid (H1751, Grade I). These materials were dissolved in DPBS or filtered DPBS. Bovine serum albumin, γ-globulin, fibrinogen and fibronectin were obtained as lyophilized powders; fibrinogen was dissolved by adding DPBS to lyophilized powder followed by gentle stirring. Typically not all fibrinogen went into solution; a small amount (estimated 5–10%) was present as flocculent precipitate removable by ultracentrifugation. Fibrinogen concentration was not directly measured after ultracentrifugation. Hyaluronic acid was allowed to dissolve overnight in filtered DPBS in the refrigerator.

Bovine lens γ-crystallins were obtained by ion-exchange chromatography of pooled lens homogenates (courtesy of Dr. John Thompson, MIT). They were stored in a 1.2 mg/ml buffered aqueous solution and preserved by addition of 0.02% sodium azide and 0.2 mM DTT.

Prepared solutions which were to be added to aqueous humor or perfused through filters were made up in filtered DPBS and then ultracentrifuged (100,000 g for 1 hr) prior to use. Exceptions were ethanol, Triton-X, and SDS, which were simply prefiltered through a 0.08 μm Nuclepore (Pleasanton, CA) polycarbonate membrane prior to use.

Calf serum was obtained by centrifuging (2000 g, 15 min) freshly collected whole calf blood to remove the red cells and allowing the fibrin to clot out of the plasma in a glass tube. Once no further fibrin formation was observed for one-half hour the serum was diluted with filtered DPBS and ultracentrifuged in the usual manner.

Calf plasma was obtained by collecting seven volumes of whole calf blood in one volume of 3.8%

sodium citrate, followed by immediate dilution 30:1 with filtered Ca- and Mg-free buffered saline. The red cells were removed by centrifugation (2000 g, 15 min) and the citrated plasma was ultracentrifuged in the usual manner.

In order to test for possible accidental introduction of unwanted particulate matter into test solutions a control solution was prepared for each experiment. The steps taken in the preparation of this solution mimicked as closely as possible the preparation of the test fluid. Hence, for calf aqueous humor experiments, control saline was prepared from filtered DPBS by repeatedly "mock handling" the saline in a cleaned syringe, pipetting it into an ultracentrifuge tube and ultracentrifuging it. In one experiment the cornea was punctured by the needle of a syringe containing filtered DPBS (no aspiration) in an effort to control for possible collection of corneal debris.

Testing of Aqueous and Other Solutions

In order to avoid artifactual blockage of the test membranes, great care was taken at every step to prevent introduction of extraneous particles. In addition to ultracentrifugation as described above, all apparatus were repeatedly prewashed with water filtered through a 0.08 μm Nuclepore polycarbonate membrane. Solutions were tested by perfusion at constant flowrate through various types of filters (see below). The exposed area of the filters was 0.71 cm^2 and the flowrate was 40 μl/min.

The pressure drop across the filter was measured continuously, from which the overall resistance, defined as the pressure drop divided by the flowrate, was calculated as a function of time. In this paper we present data in terms of a normalized resistance, obtained from the overall resistance by subtracting off the resistance of the filter holder, valves and tubing, and then dividing by the filter's resistance to isotonic saline. Hence, a normalized resistance value of one indicates that the test solution is behaving exactly as pure isotonic saline; normalized resistances greater than one are indicative of filter blockage.

We employed several different types of Nuclepore track-etched polycarbonate membranes in our tests. When originally produced these filters are nonwetting, and are rendered wettable by a process in which poly(vinylpyrollidone) (PVP) is adsorbed onto the pore walls. In the 0.2 μm pore size both regular wettable (hydrophilic) and nonwettable (hydrophobic) PVP-free filters were used. Nonwetting filters were prepared for perfusion by passing approximately 0.5 ml of prefiltered ethanol through them, followed by approximately 1.5 ml of prefiltered H$_2$O. All membranes were placed into an ultrasound bath of prefiltered H$_2$O for 4 min to ensure complete wetting. We
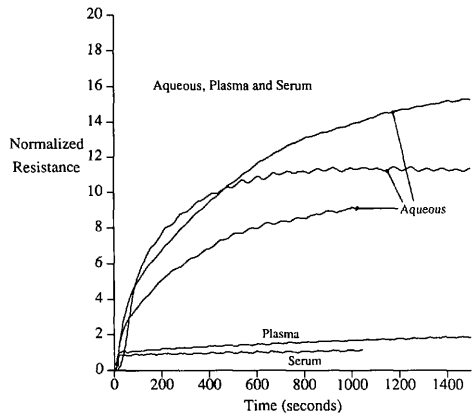

Fig. 1. The normalized resistance of calf aqueous humor, plasma and serum as a function of perfusion time on 0.2 μm Nuclepore filters. Each curve labeled "Aqueous" represents a different pooled batch of aqueous humor, tested on 0.2 μm wettable (hydrophilic) filters. Plasma: citrated calf plasma diluted 90:1 and tested on a 0.2 μm wettable filter. Serum: calf serum diluted 10:1 and tested on a 0.2 μm hydrophobic (PVP-free) filter. Original (predilution) protein concentrations: plasma 79 mg/ml, serum 97 mg/ml. See text for definition of normalized resistance.

also employed Millipore (Bedford, MA) Durapore 0.22 μm pore diameter filters of the hydrophobic (GVHP) and the hydrophilic (GVWP) varieties. Filter preparation protocols were as for the hydrophobic and hydrophilic Nuclepore membranes.

Results

Aqueous, Plasma and Serum

Figure 1 displays several representative traces of normalized filter resistance as a function of perfusion time for calf aqueous humor passing through 0.2 μm polycarbonate Nuclepore filters, each curve representing a different pooled batch of aqueous humor. Although there is evidently some interbatch variability, each aqueous humor sample produced readily discernible filter blockage, that is, behaved in a manner significantly different from that of saline. This blocking behavior has been confirmed in over 100 perfusion tests involving calf aqueous humor. Although it was not possible to identify the source of the interbatch variability, a weak positive correlation (r = 0.49, n = 63) between normalized resistance at 500 seconds and total aqueous humor protein content was noted. Interestingly, the intra-batch variability (ie, differences between two perfusions drawn from the same batch of aqueous humor) was small.

On two occasions the protein content of aqueous humor samples drawn from individual eyes was as-
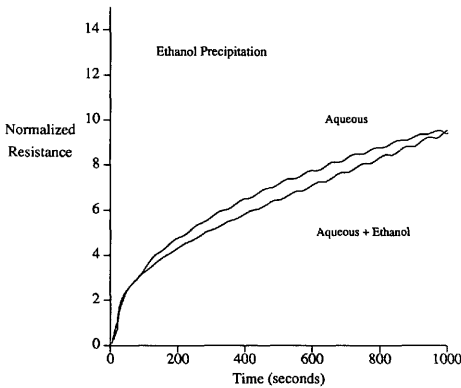
Fig. 2. Normalized resistance of untreated aqueous humor ("Aqueous") and aqueous humor from which Cohn fraction 1 (containing fibrinogen and fibronectin) has been selectively precipitated ("Aqueous + Ethanol"). Tests on 0.2 μm wettable filters.

sayed by the Bradford method prior to pooling. The mean aqueous humor protein concentrations and standard deviations on these two occasions were 537 ± 106 μg/ml (n = 28) and 992 ± 97 μg/ml (n = 27). In addition, the protein content of the pooled aqueous humor batches was routinely assayed, the mean and standard deviation for 50 such measurements being 820 ± 274 μg/ml.

In a previous publication[5] it was noted that calf serum diluted 100-fold (so that bulk protein levels were comparable to those in aqueous humor) exhibited little or no filter-blocking. This conclusion is extended by the results shown in Figure 1, where both
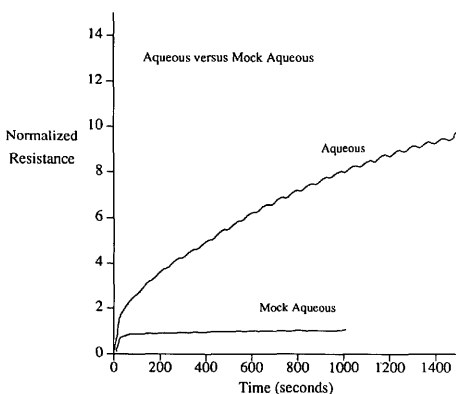

Fig. 3. Normalized resistance of aqueous humor ("Aqueous") and a mock aqueous solution ("Mock Aqueous") consisting of albumin, γ-globulin, fibrinogen and fibronectin (350, 100, 27 and 10 μg/ml, respectively). Tests on 0.2 μm wettable filters.

calf serum diluted 10-fold (ie, with bulk protein concentrations approximately ten times those of aqueous humor) and citrated calf plasma diluted 90:1 exhibit little or no blocking. In addition, citrated calf plasma diluted 90:1 which was recalcified immediately prior to perfusion showed only a very small amount of blockage (data not shown), suggesting that the clotting cascade does not play a role in filter blockage.

Modification of Aqueous Composition

Because fibrinogen (and fibrin) have a long rod-shaped geometry and are known to adsorb to polymeric materials,[9] they might be expected to play a role in filter blockage. In order to definitively rule them out as blocking candidates, aqueous humor which had its Cohn fraction I components (which include fibrinogen and fibronectin) precipitated by ethanol was tested.[6,7] The efficacy of the precipitation method was confirmed on a test solution of pure fibrinogen. Figure 2 shows that the ethanol-treated aqueous humor acted like the untreated fluid, confirming the fact that fibrinogen plays at most a minor role in filter blockage.

The results of the previous section are consistent with the hypothesis that the agent (or agents) responsible for filter blockage are absent (or present in very low quantities) from calf plasma. Accordingly, any attempts to synthesize a "mock aqueous" from blood-derived components should lead to a solution which does not obstruct filters. This was found to be the case; in Figure 3 the behavior of one such mock aqueous humor is compared to that of calf aqueous humor; very little blockage is observed. In fact, all of the following "mock aqueous" solutions (all made up in DPBS) have exhibited little or no filter blockage: (1) albumin (350 μg/ml), γ-globulin (100 μg/ml), fibrinogen (25 μg/ml) and thrombin (0.025 NIH units/ml), individually and in combination; (2) albumin (350 μg/ml), γ-globulin (100 μg/ml), fibrinogen (27 μg/ml) and fibronectin (10 μg/ml), individually and in combination; (3) lens γ-crystallins (300 μg/ml) alone, and in combination with albumin (700 μg/ml), γ-globulin (200 μg/ml) and hyaluronic acid (11 μg/ml).

The above experiments indicated that an albumin solution (made up in DPBS) does not obstruct filters, and one would therefore expect that the addition of albumin to aqueous humor would have no influence upon the blocking capacity of aqueous. Surprisingly, this was not the case. As shown in Figure 4, when aqueous humor was "spiked" with an albumin solution the blocking capacity of the aqueous humor was greatly enhanced (compared to that of aqueous humor to which the same volume of saline was added), even though the albumin solution on its own
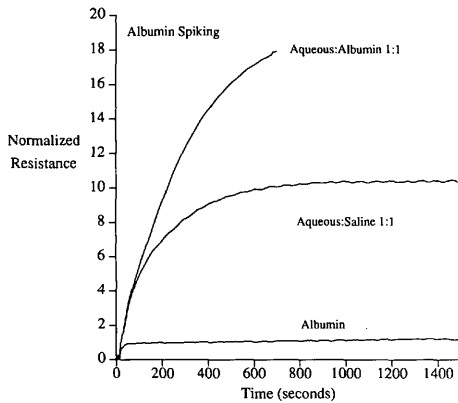
Fig. 4. Normalized resistance of calf aqueous humor "spiked" with albumin, of calf aqueous humor diluted with DPBS, and of a pure albumin solution. Prior to mixing protein concentrations were: aqueous 1166 μg/ml, albumin 3.5 mg/ml. Tests on 0.2 μm wettable filters.

Fig. 5. Normalized resistance of calf aqueous, and high and low molecular weight containing fractions (HMW and LMW, respectively). Tests on 0.2 μm wettable filters.

exhibited no filter blockage. A similar finding was noted when aqueous humor was "spiked" with lens γ-crystallins and an albumin/γ-globulin mixture.

As part of our efforts to isolate the material (or materials) in aqueous humor responsible for the blocking process we fractionated the aqueous humor into high and low molecular weight containing fractions using a Centricon-10 miniconcentrator. As shown in Figure 5, the low molecular weight (LMW) fraction (molecular weights < 10,000 daltons) showed little or no blocking, while the high molecular weight (HMW) fraction blocked quite extensively. In fact, the blockage of the HMW fluid was consistently greater than that of the unfractionated aqueous humor, yet when the HMW and LMW fluids were mixed they behaved like aqueous humor once again. We emphasize that the "excess" blocking capacity of the HMW fraction (over the aqueous humor) cannot be due to a concentration effect, since the HMW sample was diluted so as to have the same volume as the original aqueous humor sample from which it was extracted.

Alteration of Filter Properties

In Figure 6 we show the results of passing the same batch of aqueous humor through two 0.2 μm Nuclepore polycarbonate filters which differed only in their wettability. It can be seen that the blockage was significantly greater and occurred more rapidly on the hydrophobic (PVP-free) filter than on its hydrophilic counterpart. Similar results were obtained when calf aqueous humor was tested on hydrophobic and hy-
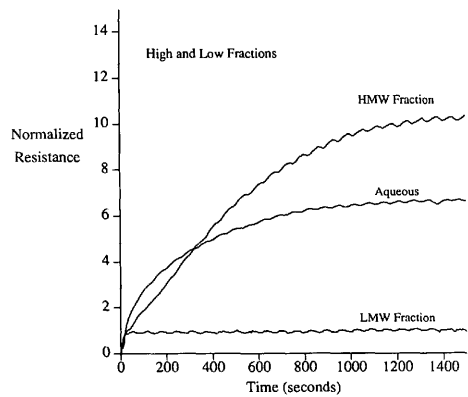
drophilic Millipore Durapore 0.22 μm filters; blockage was more rapid and of a significantly greater magnitude on the hydrophobic membranes. This finding indicates that filter surface chemistry plays a very important role in the blocking process, and suggests that it should be possible to alter the blocking process by exposing filters to agents which disrupt hydrophobic interactions, that is, surfactants. This was indeed found to be the case; in Figure 7 are shown the results of postperfusing a blocked 0.2 μm filter with a 1% solution of the nonionic detergent Triton-X. An immediate and complete elimination of the blockage was achieved, suggesting that blocking component(s) bound to the filter were eluted
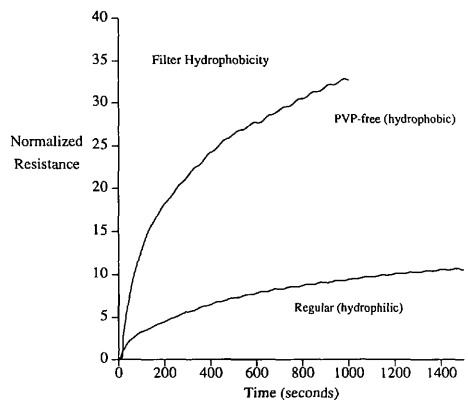

Fig. 6. Normalized resistance of calf aqueous humor perfused through 0.2 μm wettable (hydrophilic) filter and 0.2 μm PVP-free (hydrophobic) filter.
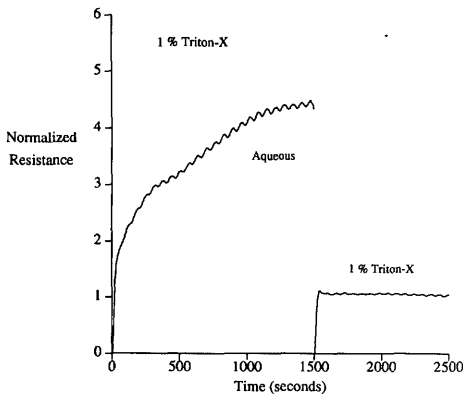
Fig. 7. Perfusion of 0.2 μm wettable filter with aqueous humor followed by postperfusion with 1% Triton-X solution.

from the filter by the Triton-X. A similar effect was noted with ionic detergents such as SDS, although Triton-X seemed to be most effective in disrupting blockage.

The above results suggest a protocol for isolating and collecting the blocking component(s) from aqueous humor, namely: allow a filter to become obstructed by perfusing it with aqueous humor, postperfuse it with several ml of DPBS, and then postperfuse it with Triton-X while collecting the filtered Triton-X. A similar technique has recently been employed to analyze the component(s) of aqueous humor and serum binding to filters by using two-dimensional gel electrophoresis.[10] In addition, we have attempted to measure the amount of protein bound onto the filter by assaying the eluate obtained during a Triton-X postperfusion. The amount of protein eluted from the filters (and hence presumably participating in filter blockage) was less than the sensitivity of the protein assay we used, which we estimated to be approximately 1 μg.

In addition to hydrophobic binding, it is possible that blocking component(s) could ionically bind to the filter. To test this possibility we: (1) postperfused blocked filters with 2 M NaCl and $CaCl_2$; and (2) pretreated the filters with the polycationic material spermine in an attempt to reduce or reverse the negative charge known to be present on the pore walls.[11] Postperfusion with two molar salts had no effect (compared with postperfusion with isotonic saline), while pretreatment of filters with spermine caused a moderate but statistically significant decrease in the blocking process (65% mean decrease in blockage at 500 sec relative to perfusion on non-pretreated filters, $P < 0.005$, two-sided t-test, n = 4). In general, attempts to disrupt ionic interactions (as described above) had a relatively minor effect on filter blockage when compared to experiments in which hydrophobic interactions were altered.

Discussion

We interpret the above results to mean that the blockage of microporous membranes by calf aqueous humor involves at least two components, one (or both) of which hydrophobically (and to a lesser extent ionically) bind to the filter surface. The reasoning behind this statement is as follows. First, the two-component nature of the blocking process is suggested by the observation that aqueous humor "spiked" with various protein solutions (albumin, lens γ-crystallins or γ-globulin) exhibits increased blocking, even though those protein solutions by themselves do not block filters. We feel that the most likely explanation of this effect is that the albumin, γ-globulin and lens γ-crystallins were interacting with some other component (or components) present in aqueous humor to cause filter blockage. Secondly, the hydrophobic nature of the blocking process is strongly suggested by the aggressive and rapid blockage seen when aqueous humor is perfused through hydrophobic membranes (compared to results on hydrophilic membranes), as well as the efficacy of surfactants in modifying filter blockage. This is consistent with previous observations of certain proteins adsorbing to a greater extent onto hydrophobic than onto hydrophilic substrates.[12] Finally, the ionic contribution to the blocking process is suggested by the moderate changes to blockage rate seen after pretreatment of the filters with spermine.

For the purposes of discussing the two-component nature of the blocking process, let us label these two components A and B. Since albumin is effective in "spiking" aqueous humor, and is also normally present in aqueous humor, it seems likely that albumin can play a role in the blocking process. For definiteness, let us arbitrarily call albumin component A. From the observation that both albumin and lens γ-crystallins are effective at "spiking" aqueous humor, yet do not by themselves or in combination block filters, we conclude that lens γ-crystallins can also function as component A, in place of albumin. In other words, it is more correct to speak of two *classes* of blocking components, class A and class B, each of which could (in general) contain several members. Blocking occurs when one member of class A is able to interact with one from class B.

Since there are class A members (eg, albumin) in plasma and serum, it seems likely that plasma and serum's inability to block is because they lack members of class B (or contain them in insufficient

quantity). In other words, we postulate that the distinguishing characteristic of aqueous humor is that it contains B, which in the presence of albumin (and other class A members), confers blocking ability. This implies that contamination of a nonblocking solution (one lacking B) with blood will not produce blocking ability. Thus, we conclude that the blocking capability of aqueous humor is not a consequence of blood leakage into the anterior chamber postmortem, although this could increase the magnitude of the blocking. In light of the above discussion, it is not surprising that "mock aqueous" was unable to block filters, since it lacked the unknown substances of class B.

The existence of two interacting classes of blocking components (A and B) raises a number of interesting possibilities. We have focused our attention on proteins as the blocking candidate(s), since proteases eliminate blocking. However, it is possible that members of class B are not proteins, and the action of protease is simply to degrade proteins in class A. For example, a two-component mechanism which is consistent with the data is blockage of filter pores by "bricks and mortar," in which certain proteins present in aqueous play the role of "bricks" and are bound to each other by the "mortar." Proteases could attack the "bricks," yet the component which is specific to the aqueous could be the "mortar." Identification of class B members is evidently of some interest and is the subject of current studies.[10]

Results of protein elution measurements from filters indicate that less than 1 μg of protein is needed to cause a substantial (10-fold or more) increase in normalized filter resistance. Using a different protein assay and a slightly different protein elution technique, Pavao et al[10] measured 1.9 to 3.5 μg of protein binding to each filter. We conclude that a small quantity (ie, microgram amounts) of protein can effect a large increase in filter resistance.

Experiments in which the Centricon-10 minicon-centrator was used to fractionate the aqueous humor show that removal of the low molecular weight components (<10,000 daltons) actually enhances the blockage of filters by calf aqueous. We interpret this result to mean that calf aqueous humor contains a low molecular weight inhibitor of blocking. The mode of action of this inhibitor is unknown, although it could be as simple as competing for hydrophobic binding sites with the blocking components. In short, for the purposes of interpreting the above data, calf aqueous humor must be regarded as a complex, multicomponent mixture. This viewpoint is consistent with modeling studies of the blocking process.[13]

Aqueous humor protein levels measured in this study (mean = 820 μg/ml) are somewhat higher than values previously reported for bovine aqueous humor: 582 μg/ml[14] and 650 μg/ml.[15] This discrepancy is probably due in part to the fact that aqueous humor protein levels are known to increase somewhat after death.[16] In this connection it is important to note that calf aqueous humor collected within 5 min of death also exhibits blocking behavior.[5] Furthermore, we have previously shown that monkey aqueous humor collected from live anesthetized animals causes filter blockage.[5] As we have argued above, the blocking capacity of aqueous humor cannot be due to contamination by blood or blood-derived components, and thus it appears unlikely that the results are due to a postmortem breakdown of the blood–aqueous barrier.

If such a blocking process is occurring within the trabecular meshwork of the eye, it should be possible to effect an increase in facility by perfusing the eye with materials which disrupt hydrophobic interactions. Based on experiments with filters, one obvious candidate is Triton-X. Unfortunately, Triton-X is not well tolerated by the cells of the outflow pathway,[13] and thus attempts to show an increase in facility due to Triton-X perfusion have proved impossible. Further experimentation in this area will require a surfactant which is better tolerated by trabecular meshwork cells.

One implication of the above work is that blocking within the trabecular meshwork would occur at hydrophobic sites (if such blocking does occur). In this regard it is of interest to note that collagen and elastin are relatively hydrophobic materials,[17,18] and thus present themselves as possible binding substrates within the JCT. If binding does occur, blocking components within the aqueous humor could interact with or even form part of an extracellular gel within the pores of the JCT. Investigations are currently underway to determine if such binding does occur.

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