Factors Affecting the Pores of the Inner Wall Endothelium of Schlemm's Canal

Arthur J. Sit, Fides M. Coloma, C. Ross Ethier,* and Mark Johnson*

Purpose. A linear relationship between the density of pores in the inner wall of Schlemm's canal and aqueous outflow facility has been reported previously in a study in which investigators examined only eyes fixed at constant pressure, so that fixative flow rates differed from eye to eye. Because pores may form as a function of flow rate, the purpose in the current study was to verify the previous findings, using constant flow perfusions.

Methods. Outflow facility was measured in enucleated human eyes. Eyes were fixed under either constant flow or constant pressure conditions, microdissected to expose the inner wall of Schlemm's canal, and prepared for scanning electron microscopy. The density and diameter of pores in the inner wall were measured.

Results. Statistical analysis showed no correlation between outflow facility and either the density or the diameter of pores. Pore density decreased significantly during the hours after death. Examining only eyes for which experimentation was started within 20 hours of death, we found that pore density increased significantly with the volume of fixative that had been perfused through the outflow pathway.

Conclusions. The correlation found by Allingham et al between outflow facility and pore density in the inner wall endothelium was not confirmed. However, the relationship between pore density and volume of fixative perfused is consistent with and may be responsible for the finding in the previous study. Because fixation conditions can influence the apparent pore density in the inner wall endothelium significantly, the conclusion reached previously, that pores contribute only 10% of the aqueous outflow resistance, may require reevaluation. Invest Ophthalmol Vis Sci. 1997;38:1517–1525.

The precise route and mechanism by which aqueous humor crosses the inner wall endothelium of Schlemm's canal has been debated for more than 100 years.[1,2] When eyes are fixed at pressure, the inner wall contains giant vacuoles protruding from the endothelial layer into the lumen of Schlemm's canal.[3–5] Associated with these giant vacuoles, but also occurring in other areas, are pores passing through the inner wall. In a painstaking study of the inner wall endothelium of Schlemm's canal, using scanning electron microscopy, Bill and Svedbergh[6] found that these pores could account for only a small fraction (perhaps 10%) of the total aqueous outflow resistance. For this reason, the inner wall of Schlemm's canal was thought to be of only minor importance in determining outflow facility; this conclusion has been generally supported by results of other studies.[7–9] It is also consistent with results published by Grierson and Lee,[10] showing that pore density increases with pressure, whereas it is known that outflow facility actually decreases with intraocular pressure (IOP).[11]

However, it was reported recently by Allingham et al[12] that a linear relationship between pore density and aqueous outflow facility exists, and that pores in the inner wall are less frequent in eyes with primary open-angle glaucoma than in healthy eyes, suggesting that pores in the inner wall may be important in determining outflow facility. A limitation of the study of Allingham et al was the use of constant-pressure

*From the Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, and the *Departments of Mechanical Engineering and Ophthalmology, University of Toronto, Canada.*
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Reprint requests: Mark Johnson, Department of Mechanical Engineering, Room 3-264, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139.

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perfusions and constant-pressure fixation. Flow rate across the inner wall is linearly proportional to facility (at constant pressure); hence, the flow rate in that study varied from eye to eye. Because it is widely believed that the pores open in response to a transmural pressure difference generated by fluid flow across the inner wall endothelium,[10] the results reported by Allingham et al[12] could be explained by the the pores' opening passively in response to different flow rates, with outflow resistances controlled elsewhere in the eye. In these conditions, pore density would be a function of outflow facility, and not vice versa.

Our purpose was to extend the studies of Allingham et al[12] to understand better the factors influencing the formation of pores in the inner wall of Schlemm's canal by examining the inner wall endothelium of eyes perfused and fixed under a variety of conditions, including constant-flow fixation.

METHODS

Two series of experiments were conducted, series A at MIT and series B at the University of Toronto. Series A was performed explicitly to fulfill the stated goal. Series B was a set of control experiments developed in pursuit of a different question by one of the investigators (CRE).

Tissues and Materials

In series A, human eyes were obtained from the National Disease Research Interchange (Philadelphia, PA). Eyes were transported in a moist chamber packed in an ice-filled, insulated container. Eyes were excluded from the studies if donors had had ophthalmic surgery, were diabetic, or had a history of ocular disease. A total of 21 (10 pairs plus a single eye) normal human eyes, ages 5 to 97, were used. The time between death and enucleation ranged between 1.5 hours and 11 hours (median, 3.1 hours) and the time between death and the beginning of experimentation ranged between 8.3 and 34.3 hours (median, 20.2 hours).

The perfusion fluid used to obtain a baseline value for outflow facility was Dulbecco's phosphate-buffered saline (Life Technologies, Grand Island, NY) with 5.5 mM glucose added (DBG), prefiltered through a 0.2 μm filter (cellulose acetate, Costar Scientific, Cambridge, MA). The fixative was a modified Karnovsky's fluid of 2.5% glutaraldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, PA) and 2.0% paraformaldehyde (purified grade, Fisher Scientific, Pittsburgh, PA) in a Sörenson's phosphate buffer, with measured pH at 7.3. The final fixative solution was stored in a refrigerator and used within 24 hours of preparation.

In series B, human eyes were obtained from the National Disease Research Interchange (Philadelphia, PA) or the Ontario Eye Bank (Toronto, Canada). Transport conditions and exclusion criteria were identical to those for eyes used in series A. A total of 13 pairs of eyes were used. This report includes data from one eye per pair; the contralateral eye received a drug as part of another study. Age of donor eyes ranged from 74 to 90 years. The time between death and enucleation ranged from 1 to 10 hours (median, 5.8 hours) and the postmortem time ranged from 13 to 34 hours (median, 25 hours). Perfusion and fixative solutions were identical to those used in series A.

Experimental Procedure

The essential elements of the protocol for the experiments in series A follow. Outflow facility was measured while the eyes were perfused at a constant flow rate of 2 μl/minute, a rate selected to match the diurnally averaged normal flow rate of aqueous humor in humans.[13] The anterior chamber contents were then exchanged with fixative while the IOP was maintained carefully at the final pressure measured during the baseline perfusion. After the exchange, the eyes were perfused with fixative at a constant pressure equal to the final pressure achieved during the constant-flow perfusion while outflow facility was monitored. Because fixation decreased outflow facility, the use of constant-pressure perfusion to fix the eyes meant that a 2-μl/minute flow rate was maintained for only the initial stages of the fixation process; however, such a variation in flow rate was preferable to the high nonphysiologic pressures that would occur with true constant-flow fixation. Importantly, the effect of fixation on outflow facility was similar in all eyes, so that in this protocol, the postfixation flow rates for all eyes were comparable. We therefore term this technique *constant-flow* fixation. After fixation, the eyes were examined for pore density in the inner wall of Schlemm's canal, using scanning electron microscopy. The detailed data obtained from examination of all of the eyes in series A can be found in a previous report.[14]

The details for the experimental protocol are as follows. Methods used for data acquisition and the control system for perfusions were similar to those described by Whale et al,[15] allowing continuous calculation and display of outflow facility. New tubing and fittings were used for each experiment. Each eye was placed in a beaker, covered with saline, and the beaker placed in a water bath (34°C). For perfusion, two 25-gauge needles were inserted through the cornea, with the tip of the perfusion needle placed in the posterior chamber of the eye and the tip of the exchange needle placed in the anterior chamber. The line leading from the exchange needle was initially clamped. After perfusing from a reservoir for approximately 10 minutes at 10 mm Hg to reduce the filling time, the eyes were

perfused from syringe pumps at a constant flow rate of 2 μl/minute while outflow facility was measured, until a steady value was reached (usually in 60 to 90 minutes, but occasionally in less time). The outflow facility used in the statistical analysis was the average value recorded during the steady state period.

The eyes were then fixed at a constant pressure equal to the final pressure achieved during the constant-flow baseline (range, 3 to 43 mm Hg). The contents of the anterior chamber were first exchanged with fixative by placing the fixative-containing reservoir at a level 2 cm H_2O above the fixation pressure and the waste reservoir at a level 2 cm H_2O below the fixation pressure. This ensured that the average pressure in the eye during the exchange was never more than 2 cm H_2O away from the fixation pressure and thus reduced the possibility of pressure excursions during fixation. Both reservoir lines were then opened and approximately 2 ml of fluid was exchanged to thoroughly flush the anterior chamber and the fluid lines. A small amount of fluorescein sodium salt (approximately 10 mg/100 ml of fixative; Sigma Chemical St. Louis, MO) was added to help visualize the fixative and to ensure effective exchange.

After the exchange, the eyes were perfused from the syringe pumps at the fixation pressure. Because the flow lines were also filled with fixative, perfusion from the pumps resulted in fixative infusion, while allowing continuous measurements of outflow facility. To investigate the effect of duration and volume of fixative perfused, the duration of perfusion was varied from eye to eye, with a minimum duration of 60 minutes (to ensure complete fixation of the outflow pathway) to a maximum duration of 700 minutes. After completion of the perfusion, the eyes were cut open well behind the limbus and immersed in fixative for at least 12 hours.

In series B, eyes were prepared as described in Ethier et al.[16] Briefly, after removal of orbital connective tissue, eyes were placed in a beaker on a bed of saline-saturated gauze, and the beaker was placed in a water bath at 37°C. A 23-gauge butterfly infusion needle connected to a fluid-filled reservoir was inserted through the peripheral cornea so that the tip of the needle lay in the posterior chamber. A second 23-gauge needle was inserted through the peripheral cornea 180° from the first needle and was positioned so that its tip lay in the anterior chamber. The line leading from the second needle was initially clamped off. Eyes were perfused with DBG at a constant pressure of 8 mm Hg for 60 to 120 minutes, were retroperfused with DBG for 15 minutes, and were further forward-perfused with DBG at 8 mm Hg for 60 to 90 minutes. During forward perfusion at 8 mm Hg, outflow facility was measured using a previously described apparatus.[17] It is important to note that it has been demonstrated that retroperfusion with DBG does not alter outflow facility or outflow tissue ultrastructure.[16]

At the conclusion of the DBG perfusion, anterior chamber contents were exchanged with fixative, following a procedure virtually identical to that used in series A. In 7 of the 13 eyes, the exchange and subsequent constant pressure fixation were carried out at 8 mm Hg. In the remaining 6 of the 13 eyes, the exchange and fixation were carried out at an elevated pressure that was constant during the fixation procedure (range, 8 to 46 mm Hg). The duration of fixative infusion was 60 to 150 minutes in series B. During this time, outflow facility was not measured. After completion of fixation, the eye was hemisected at the equator and immersed in fixative for at least 12 hours.

Pore Counting

After fixation, eyes were microdissected and prepared for scanning electron microscopy, using standard techniques.[12,18] Briefly, the eyes were bisected along the equator and the lens dissected from the anterior segment. Two meridional wedges were taken from each quadrant. Schlemm's canal was opened by placing gentle tension on the ciliary body, which allowed access to the posterior margin of the canal without damaging the inner wall endothelium. After the canal was opened, samples containing iris, ciliary body, the outer trabecular meshwork, and the inner wall endothelium were microdissected free. Samples were then washed in buffer, postfixed in buffered 1% osmium tetroxide for 90 minutes, and washed again. Specimens were dehydrated with graded alcohols, critical-point dried, mounted on stubs, gold-sputter coated, and examined using a Hitachi S-570 scanning electron microscope (Nissei Sangyo America, Mountain View, CA).

For each eye, overview montages at a magnification $\times$1,500 were created for one sample per quadrant, yielding four samples per eye (Fig. 1). Dissection difficulties prohibited our obtaining one sample per quadrant of adequate quality in all eyes. Specifically, in 7 of 34 eyes, three samples were obtained, in 3 eyes, two samples were obtained, and in 2 eyes, one sample was obtained. Unlike Allingham et al,[12] pores were not counted directly from these montages. Instead, probable pores were identified from the montages and the original samples were then rescanned at $\times$10,000 to verify that each putative pore was nonartifactual. Openings in the endothelium were disregarded as artifactual tears if the edges were rough, torn, or notched. Because pores are typically elliptical, the major and minor axes were measured directly from the screen of the microscope at $\times$10,000. Length measurements were calibrated using two calibration grids for scanning electron microscopy (SIRA Institute, Chislehurst, UK) with densities of 19.7 and 2,160 lines per millimeter. Because

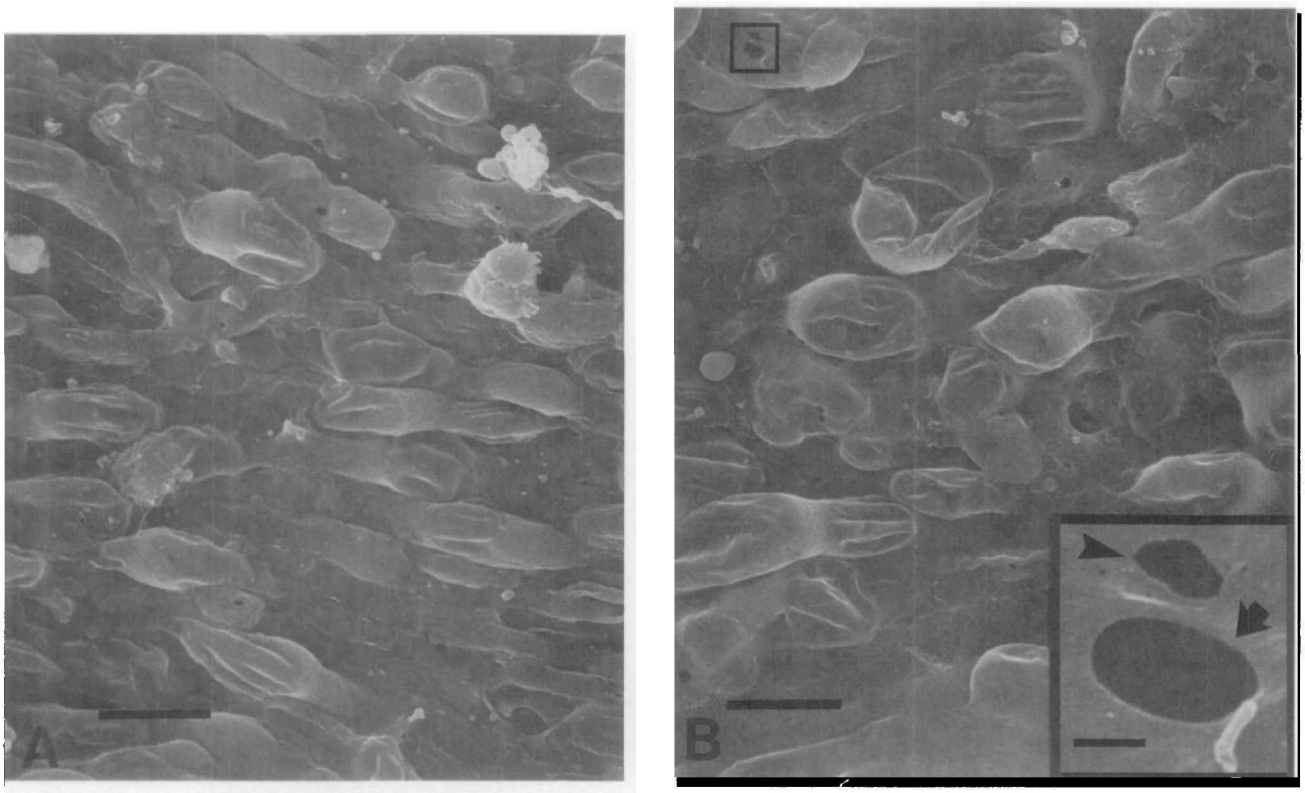

FIGURE 1. (A) Scanning electron micrograph of the inner wall endothelium of Schlemm's canal from an eye (Q5) that was perfused with a relatively low volume of fixative (0.115 ml) and was perfused beginning 14.3 hours after the donor's death. Scale bar = 10 μm. (B) Scanning electron micrograph of the inner wall endothelium of Schlemm's canal from an eye (Q13) that was perfused with a relatively high volume of fixative (0.258 ml) and was perfused beginning 13.4 hours after the donor's death. Scale bar = 10 μm. The inset shows an analyzed pore (*arrow*) and an artifactual pore (*arrowhead*). Scale bar = 1 μm.

circular pores will appear elliptical when view in tilted section, the pore diameter was taken as the major axis of the pore; however, results were also verified using the geometric mean of the major and minor axes as the pore diameter.

For each sample, the total area of inner wall, excluding regions where the inner wall endothelium had been torn off because of dissection artifacts, was measured from the overview montages. Typical total areas measured were 50,000 μm^2 per quadrant, or 200,000 μm^2 per eye. Pore densities (pores per unit of wall area) and mean pore diameters were then computed for each sample. Whole-eye pore density was determined by averaging the results from each sample; whole-eye mean pore diameter was computed as the density-weighted average of the mean diameter for each sample. Along with computation of the pore diameter, we computed the cube and the fourth power of the diameter of each pore for later statistical analysis.

Statistical Methods

The measured parameters for each eye included donor age (age, years), postmortem time (PM, hours), time between donor's death and enucleation (E, hours), pore density of the inner wall endothelium (N, pores/mm^2), average pore diameter (D, μm), outflow facility before fixation (C, μl/minuteute/mm Hg), outflow facility after fixation (C_F, μl/minuteute/mm Hg; series A only), fixation pressure (IOP, mm Hg), flow rate of buffer through outflow pathway before fixation (Q, μl/minuteute), volume of buffer perfused through the outflow system before fixation (V_B, ml), volume of fixative perfused through the outflow system (V_F, ml; series A only), and duration of fixative perfusion through the outflow system (T, minutes).

We also included one derived parameter in the data analyses: because series B did not measure V_F, we defined a new parameter V_F' (μl) = 0.5 · Q · T, which is an estimate of the volume of fixative perfused through the outflow system. (The rationale for the constant 0.5 is explained in Results.) In that V_F, V_F', and T were closely related and thus not statistically independent, we used only one of these parameters in any regression analysis. If one of these parameters yielded a statistically significant correlation, all were investigated to determine which gave the lowest *P* value.

We first performed linear regression analyses relating outflow facility to pore density and pore density to both fixation flow rate and fixation pressure. We then performed multivariate regression analyses (Systat for the Macintosh computer, version 5.2.1) examining the correlation of three of the variables (outflow facility, pore density, and pore diameter) with the remaining variables measured in both series. Variables that were not plausibly connected to the dependent variable were not included in the correlation (for example, fixation time could not affect prefixation outflow facility), nor were variables included that could be used directly to calculate the dependent variable (flow rate and pressure were not both included in correlations for outflow facility). We used an automated, forward, stepwise regression implemented on Systat using a tolerance (overall P value) of 0.01 and a P value for adding variables to the correlation of 0.01. In this scheme, at each step of the iteration, the most statistically significant variable not yet included in the correlation is added to the correlation while ensuring that the tolerance does not exceed 0.01. The most statistically significant variable is chosen by computing which of the remaining variables not yet added to the correlation would have the lowest individual P value (constrained to be <0.01) if it were added to the correlation. When no further variables could be added without having the tolerance exceed 0.01, the iteration was terminated, giving an overall P value for the correlation and an individual P value to each of the correlated variables. The residuals (the difference between the fitted value of the dependent parameter and its measured value) were examined after determining the best correlation; and, unless otherwise noted, the residuals appeared random when plotted against the independent variables.

Unless otherwise stated, all statistical analyses used the combined data from series A and series B. Both eyes from series A were used initially as independent data points, because in most pairs one eye had a lower fixation time at flow and was perfused with a lower volume of fixative than was the contralateral eye. However, to account for the dependence of the two eyes of a pair,[19] we reanalyzed the data using two methods. First, we analyzed those correlations not involving fixation time or fixation volume, using only one eye for each pair in series A (the eye perfused with the lower volume of fixative). Second, we followed the method suggested by Thompson[20] in which a corrected sample size (and corrected t value) is used to account for the correlation coefficient ρ between eyes of a pair: $n_{\text{eff}} = 2 \cdot M/(1+\rho) + n_{\text{single}}$, where M is the number of pairs and n_{single} is the number of unpaired eyes. All P values reported are from a two-tailed Student's t-test, and are from correlations in which data from each eye of a pair were assumed to be statistically independent. In the single case in which the P value calculated from either of the two methods that accounted for statistical dependence of eyes of a pair was greater than 0.05, it appeared to be attributable solely to low n. The entry for this case is marked with an asterisk in Table 1.

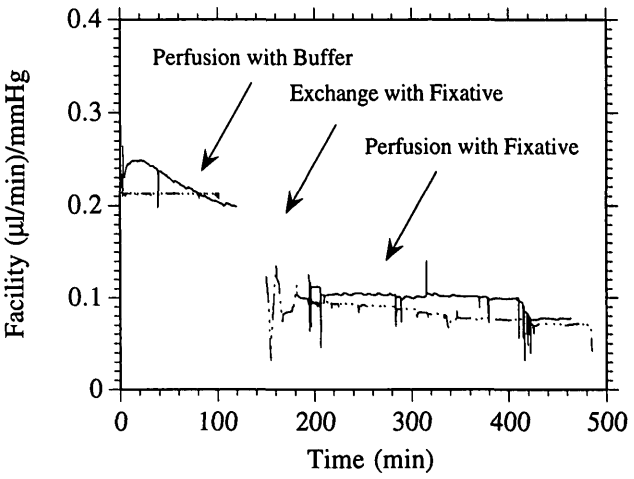

FIGURE 2. Outflow facility as a function of time for two eyes (not a pair) from series A. With fixation, there appears to be a relatively rapid drop in outflow facility, followed by either a relatively constant or slightly decreasing outflow facility as time passes.

RESULTS

Contrary to the findings of Allingham et al,[12] no correlation was found between outflow facility and pore density of the inner wall endothelium ($n = 34$; $P > 0.95$). This was not surprising in that we had postulated that the findings of Allingham et al might have been elicited by a relationship between fixation flow rate and pore density. However, we were surprised to find that we were also unable to find a relationship between pore density and either fixation flow rate or fixation pressure ($n = 34$; $P > 0.2$, $P > 0.35$, respectively).

We noticed in data analyses in our early studies that there appeared to be a relationship between pore density and the volume of fixative (V_F) perfused through the outflow pathway. This motivated us to examine in greater detail the relationship between pore density and the other parameters examined in the experiment. Because V_F was not measured in series B, we had to estimate this parameter for those experiments. This approach was not straightforward because outflow facility drops with fixation (Fig. 2). To account for this, we used data from series A to relate outflow facility before and after fixation. We found a mean ± SE ($n = 21$) of:

$$\mathbf{C}_F = (0.502 \pm 0.041)\mathbf{C} \qquad (1)$$

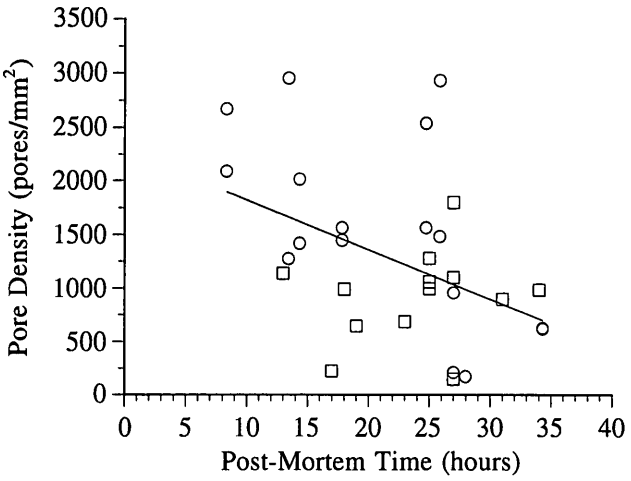

FIGURE 3. Pore density of the inner wall endothelium as a function of time elapsed after death. (○ = series A; □ = series B. The line indicates the best linear fit to the data. $P < 0.007$).

We used the ratio C_F:C to determine the coefficient 0.5 in the formula for V_F' as described in Statistical Methods.

A second difficulty in performing multivariate regression analyses was our finding that postmortem time had a significant effect on pore density ($P < 0.01$; Fig. 3). We were concerned that this correlation between postmortem time and pore density might affect or mask other correlations, and in an attempt to investigate this effect, we performed statistical analyses not only on the entire data set, but also on three subsets of the data: eyes with postmortem times of 15, 20, and 25 hours.

Using these subsets, we found outflow facility to be statistically correlated with IOP and Age:

$$\mathbf{C} = a_0 + a_1 \times \mathbf{IOP} + a_2 \times \mathbf{Age} \qquad (2)$$

with coefficients and significance levels shown in Table 1. A number of the eyes fixed at 10 mm Hg or less showed high residuals from the reported correlations.

Brubaker[11] reported an increase of outflow resistance with IOP; taking the inverse of this value as dC/dIOP, yields a value of approximately -0.005 μl/minuteute/mm Hg[2]. Gaasterland et al[21] reported a value for the age-related decline in "true" outflow facility of -0.0021 μl/minuteute/mm Hg per year. These values are generally consistent with those in our data.

No statistically significant correlation was seen when adding pore density to equation 2 ($P > 0.5$). A significant correlation was seen when pore diameter was added to equation 2 for the entire data set; however, this correlation was largely caused by outlying data collected from a single eye, as will be discussed later. For the data subsets (PM <25 hours, PM <20 hours, and PM <15 hours), no statistically significant correlation was seen when adding pore diameter to equation 2 ($P > 0.5$).

Examining next the relationship between pore density (N) and the other parameters, we found:

$$\mathbf{N} = b_0 + b_1 \times \mathbf{PM} + b_2 \times \mathbf{V_F}' \qquad (3)$$

with coefficients and significance levels as shown in Table 2.

Using V_F (volume of perfused fixative) for series A and V_F' (estimated volume of perfused fixative) for series B yielded relationships similar to equation 3 (see Fig. 4). Using T (fixation time) in the correlation instead of V_F' yielded:

$$\mathbf{N} = c_0 + c_1 \times \mathbf{PM} + c_2 \times \mathbf{T}' \qquad (4)$$

with coefficients and significance levels shown in Table 3. Multivariate regression including both V_F' and T could not distinguish which had the more statistically significant effect on N.

Using the major axis of the pore as the pore diameter (described in Methods), we found the pore diameter to be 1.16 ± 0.21 μm ($n = 33$; mean ± SD); using the geometric mean of the major and minor axes of

TABLE 1. Coefficients for Equation 2

Postmortem Time (hours)	*n*	a_0	a_1	a_2	P
All data	34	0.681 ± 0.058	−0.00459 ($P < 0.005$)	−0.00539 ($p < 10^{-6}$)	$<10^{-8}$
<25	19	0.688 ± 0.034	NS ($P = 0.038$)	−0.00659 ($P < 10^{-10}$)	$<10^{-10}$
<20	14	0.392 ± 0.058	−0.00324 ($P < 0.0005$)	−0.00242 ($P < 0.01$*)	10^{-6}
<15	9	0.424 ± 0.038	−0.00371 ($P < 0.0002$)	−0.00259 ($P < 0.002$)	10^{-4}

NS = not significant.
* $P > 0.05$ for one or both of the statistical methods, accounting for nonindepence of the eyes of a pair; a_0 = mean + SE; a_1, a_2 = mean value and statistical significance; P values in last column represent overall correlation.

TABLE 2. Coefficients for Equation 3

Postmortem Time (hours)	*n*	b_0	b_1	b_2	P
All data	34	2290 ± 360	−46.3 ($P < 0.01$)	NS ($P = 0.29$)	<0.01
<25	19	1500 ± 170	NS ($P = 0.11$)	NS ($P = 0.011$)	NS
<20	14	659 ± 256	NS ($P = 0.046$)	6710 ($P < 0.002$)	<0.002
<15	9	940 ± 250	NS ($P = 0.635$)	6160 ($P < 0.005$)	<0.005

NS = not significant.
b_0 = mean + SE; b_1, b_2 = mean value and statistical significance; *P* values in last column represent overall correlation.

the pore as the pore diameter, we found the mean diameter to be 0.85 ± 0.15 μm ($n = 33$). These averages exclude outlying data from one eye that had a significantly larger mean pore diameter than any of the other eyes ($D_{major} = 2.13$ μm, $D_{geometric} = 1.56$ μm; PM = 28 hours, E = 4.7 hours). Excluding data from this single eye from the regression analysis, D was not correlated with any of the other parameters in the entire data set or in any of the subsets.

Several theories have been proposed to relate the number and size of the pores of the inner wall endothelium to the flow resistance generated by this layer, including Poiseuille's law,[6] Sampson's law,[6] and the theory of funneling.[22] These relations give a scaling of outflow facility with pore number and size as $N \cdot D^4$, $N \cdot D^3$, and $N \cdot D$, respectively. We examined the correlation of outflow facility, before and after fixation, in the full set of eyes and in those with PM <20 hours, with IOP and Age, adding each of these three outflow facility-scaling relationships, and found no statistically significant relationships ($P > 0.5$ in all cases).

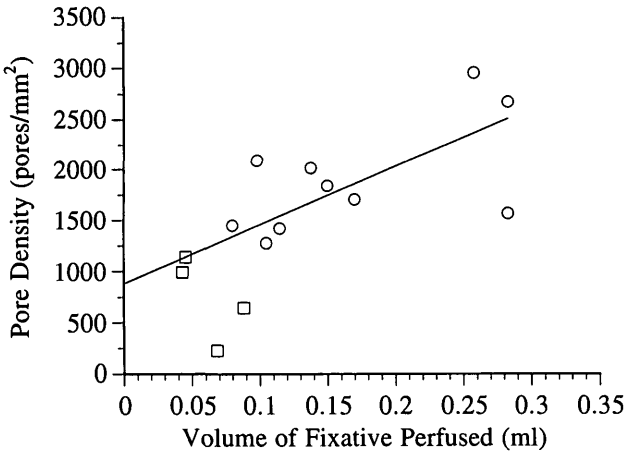

FIGURE 4. Pore density of the inner wall endothelium as a function of perfused volume of fixative for those eyes in which experiments were started within 20 hours of death. (○ = series A; □ = series B. The line is the best linear fit to the data. $P < 0.002$).

DISCUSSION

The current study was motivated by the work of Allingham et al,[12] in which a correlation between outflow facility and pore density in the inner wall endothelium was reported. This correlation was unexpected, inasmuch as it appeared to contradict previous conclusions[6,8,9] that, at most, 10% of total outflow resistance is localized in the inner wall endothelium. However, because Allingham et al perfused and fixed eyes at constant pressure, differences in flow rate from one eye to another could have been responsible for their observed correlation. We therefore repeated the study of Allingham et al, using a variety of fixation conditions with the goal of ensuring that their result was not related to their use of constant perfusion pressure.

Our major finding was that pore density in the inner wall endothelium in relatively fresh human eyes (postmortem time <20 hours) was correlated with the total volume of fixative that flowed through the inner wall endothelium during the fixation process (equation 3). For the fixation conditions that we used, we found no correlation between outflow facility and inner wall pore density, nor did we find a correlation between pore density and the flow rate of fluid crossing the inner wall endothelium.

The correlation between pore density and total fixative volume is used in the following explanation of the results of Allingham et al. In their study, all eyes were fixed at a constant pressure of 15 mm Hg for approximately the same period (60 minutes). Thus, allowing for the fact that fixation reduces outflow facility by 50% (equation 1), the volume of fixative that passed through those eyes can be estimated using the formula

$$V_F' = 0.5 \times C \times (15 \text{ mm Hg}) \times (60 \text{ minutes}) \quad (5)$$

In that N is linearly related to V_F' (equation 3), equation 5 implies that C is linearly related to N under the experimental conditions used by Allingham et al.

TABLE 3. Coefficients for Equation 4

Postmortem Time (hours)	*n*	c_0	c_1	c_2	P
All data	34	2290 ± 360	−46.3 ($P < 0.01$)	NS ($P = 0.36$)	<0.01
<25	19	1500 ± 170	NS ($P = 0.11$)	NS ($P = 0.018$)	NS
<20	14	620 ± 300	NS ($P = 0.024$)	6.72 ($P < 0.005$)	<0.005
<15	9	910 ± 260	NS ($P = 0.7$)	6.39 ($P < 0.005$)	<0.005

NS = not significant.
c_0 = mean + SE; c_1, c_2 = mean value and statistical significance; *P* values in last column represent overall correlation.

Using our data to calculate dN/dC for these conditions, we find a slope that is within approximately a factor of two of that seen in the relationship reported by Allingham et al. We conclude that results of Allingham et al are consistent with our finding that pore density increases with the volume of fixative that has passed through the outflow pathway. Our results are also consistent with those of other studies that have shown pore number to increase with perfusion pressure,[23] because in those studies, fixation time was held constant and thus, on average, those eyes fixed at higher IOP would have a greater volume of fixative passing through the outflow pathway.

It is not at all clear why fixation volume (or fixation time) increases the pore density of the inner wall. It may be that the pores of the inner wall continue to form during the fixation process; and thus, rather than a "snapshot" of the inner wall, we are viewing a "time-exposure" image. However, it is important here to recall our finding that fixation caused outflow facility to decrease approximately twofold, that this drop in facility occurred fairly rapidly, and that outflow facility then either remained constant or continued to decrease slowly as fixative flowed through the outflow pathway (Fig. 2). Grant[24] also found that fixative decreases outflow facility. If pores were involved in a process wherein they were continually forming and then were kept open by the fixative, and if pores play an important role in determining outflow facility, we would expect that outflow facility would have increased slowly during the fixation process; but this effect was not observed. It may well be that fixative-induced reductions in outflow facility are related to fixation effects in other parts of the outflow pathway. For example, it is known that fixation alters trabecular meshwork architecture, inasmuch as Inomata et al[25] have shown that microsphere transport through the juxtacanalicular region differs in fixed and unfixed eyes.

We also found that when eyes with postmortem times longer than 25 hours were included in the analysis, a correlation between inner wall pore density and postmortem time was seen. Further, unlike the situation with fresh eyes, inclusion of eyes with postmortem times longer than 20 hours eliminated (or masked) the correlation between pore density in the inner wall epithelium and fixative volume. This suggests that in eyes with postmortem times longer than 20 to 25 hours, artifacts occurring after death are influencing formation of pores in the inner wall. We note that length of postmortem time is negatively correlated with pore density, indicating that postmortem effects act to decrease the tendency of pores to form in the inner wall, thus suggesting that a certain level of viability of inner wall endothelial cells is a necessary condition for pore formation. Although the details of this process are unclear at present, it seems evident that minimizing postmortem time is important in the study of the pores of the inner wall endothelium.

Given the confounding effects of postmortem time and fixative volume (or fixation time), it is relevant to ask what the inner wall pore density is in vivo in human eyes. If we restrict attention to fresh eyes (postmortem time <20 hours), and extrapolate the equation relating pore number to fixation volume perfused (equation 3) back to zero volume (or equivalently, fixation time extrapolated to the beginning of the fixation process; equation 4), our study's results predict an in vivo pore density of 600 to 900 pores/mm^2. This value is appreciably less than commonly accepted densities of 1500 to 1800 pores/mm^2 for enucleated pressure-fixed human eyes,[6,12] although it is still higher than the pore density consistent with the generation of the bulk of aqueous outflow resistance (roughly 200 pores/mm^2: see calculation in Bill and Svedbergh[6]).

In summary, several factors affected the measured pore density of the inner wall epithelium. When data from all the eyes used in the study were considered, a correlation was found between pore density and postmortem time. When data from relatively fresh eyes only (postmortem time <20 hours) were considered, further possible artifactual influences on pore density included fixative volume per-

fused through the outflow system and fixation time. The implication of these findings is that the pore densities measured in typical perfusion experiments may not be representative of the pore densities in vivo, and that further exploration is required to determine whether the inner wall of Schlemm's canal is responsible for the generation of significant resistance to aqueous outflow. It is clear that further study of how fixation influences pore density is required; because if this effect were understood, we would be able to interpret the findings in existing studies better, using both monkey and human eyes.

Key Words

anterior segment, facility, glaucoma, morphometry, statistics

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