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The Role of Soluble Proteins in Generating Aqueous Outflow Resistance in the Bovine and Human Eye

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Previous research has shown that wash-out in bovine and primate eyes can be greatly reduced by perfusing with buffer containing 5–15% serum. It was suggested that protein diffusion from the iris root might raise the in vivo protein concentration in the trabecular meshwork to a level much higher than in the anterior chamber. In this study, we investigated the protein concentration in effluent from the outflow pathways in bovine and human eyes, its possible relationship to wash-out, and whether the reduction of wash-out was caused by a bulk protein effect. Bovine and human eyes were placed under silicone oil and perfused with buffer. Outflow facility was continuously determined while effluent was periodically collected from the surface of the eye, and the soluble protein concentration in the effluent was determined. Separate studies were conducted perfusing either albumin or γ-globulin through bovine eyes. Theoretical models were developed to study the transport of protein into the perfusion fluid.

In the bovine eyes, the initial protein concentration in the collected effluent was approximately 1% that of serum, much lower than the 10–15% buffer in serum required to prevent wash-out. Furthermore, the rate of change of outflow facility showed a different dependence on perfused volume than did the protein concentration. Human eyes showed a much higher level of protein in the perfusate, that decayed over a much longer time period. A statistically significant correlation existed between outflow resistance and soluble protein concentration in both bovine and human eyes. However, modelling studies suggested that this correlation might be due to flow resistance setting the flowrate which then determines the protein concentration of the effluent. Separate experiments indicated that the decreased rate of wash-out caused by perfusion of 10–15% serum in buffer was not due to either albumin or γ-globulin alone. These results suggest that the reduction of wash-out observed in previous studies when serum proteins were perfused through bovine and monkey eyes was not due to the general level of serum proteins but may instead be due to interactions of a particular protein(s).

Key words: aqueous humor; facility; albumin; wash-out; hydrodynamics.

1. Introduction

When an in vivo or enucleated non-human eye is experimentally perfused, a phenomenon known as the 'wash-out' effect occurs whereby the outflow resistance of the eye progressively decreases (Erickson-Lamy et al., 1990), even if perfused with aqueous humor (Barany and Woodin, 1955). Johnson et al. (1993) were able to significantly reduce the rate of washout in the bovine eye by supplementing the perfusion fluid with 10–15% serum (protein concentration of approximately 6–10 mg; Sit, 1995). Kee et al. (1996) have made a similar observation in the living primate eye using perfusion fluid augmented with 5% autologous serum. These studies have shown that soluble proteins in the aqueous humor can influence the aqueous outflow resistance.

While these protein concentrations are significantly higher than those found in aqueous humor (about 1% of serum: Tripathi, Millard and Tripathi, 1989; Pavao et al., 1989), the actual protein concentration of the fluid passing through the trabecular meshwork under physiological conditions is unknown. The results of Freddo et al. (1990) and Barsotti et al. (1992) demonstrated that plasma-derived proteins from the ciliary body enter the aqueous humor at the iris root. This pathway has been recently confirmed using magnetic resonance imaging (Kolodny et al., 1996). This protein is added to the aqueous humor just before the fluid enters the trabecular meshwork. If these added proteins contribute to normal aqueous outflow resistance non-specifically and they are lost during experimental perfusions via progressive depletion (i.e. wash-out) of an anterior segment protein depot, we would expect that the initial protein concentration in the fluid passing through the trabecular meshwork of an in vivo or enucleated eye would be approximately 5–15% of serum. This concentration should then decrease progressively during experimental perfusions with protein-free perfusion fluid.

Based on this reasoning, our hypothesis was that in the bovine eye, the protein concentration in the fluid perfusing through the aqueous outflow pathway drops

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significantly over the course of an experimental perfusion, and as a consequence, outflow facility increases. In human eyes, the lack of washout suggests that either (i) the protein concentration of the fluid passing through the aqueous outflow pathway is unrelated to outflow resistance, or (ii) the protein depot in the human eye does not deplete appreciably over the course of a perfusion experiment.

While the dependence of outflow facility on volume perfused through the outflow pathways has been investigated (Johnson et al., 1991), the relationship between perfused volume and effluent protein concentration has not. In the current study, we perfused bovine and human enucleated eyes with protein-free media and collected the effluent as it emerged from the aqueous veins, while simultaneously measuring outflow facility. Once we had these two relationships (effluent protein concentration and outflow facility as functions of effluent volume), we compared them to evaluate the importance of protein concentration in generating flow resistance in the outflow pathway.

We also perfused bovine eyes with phosphate buffered saline augmented with either albumin or γ-globulin, the major protein constituents of both aqueous humor and plasma. Our objective was to determine whether the reduction of wash-out caused by the addition of plasma-derived proteins was due simply to a bulk protein effect (as represented by the albumin), or instead due to some particular constituent(s) in serum.

The result of our studies suggest that some particular component of serum is responsible for the reduction of wash-out found by Johnson et al. (1993) and Kee et al. (1996), rather than being a bulk protein effect. The absence of wash-out in the human may result from the very slow loss of this component from a depot.

2. Methods

Tissues and Materials

Enucleated bovine eyes from two-week old calves were obtained from a local abattoir (Arenas & Sons, Hopkinton, MA, U.S.A.). Eyes were transported in isotonic saline packed in ice, and were typically used within 6 hr of enucleation, except for one pair which was deliberately stored under moist chamber conditions in the refrigerator for 24 hr in order to determine the effect of post-mortem time.

Human eyes were obtained from the National Disease Research Interchange (Philadelphia, PA). Eyes were transported under moist chamber conditions, in an insulated container, and packed with ice. Eyes were excluded from the studies if they had any type of ophthalmic surgery, or were from diabetic donors. Human tissue was typically used within 24 hr post-mortem.

The perfusion fluid was either Dulbecco's phosphate buffered saline (Life Technologies, Grand Island, NY, U.S.A.) with 5·5 mM glucose added (DBG), or DBG to which bovine serum albumin (7·5 mg ml^{-1}; A2153, 96% electrophoretic purity, Sigma, St. Louis, MO, U.S.A.), or bovine γ-globulin (7·5 mg ml^{-1}; G5009, 99% electrophoretic purity, Sigma, St. Louis, MO, U.S.A.) had been added. Before use in an experiment, DBG solutions were filtered through a 0·2 μm filter (cellulose acetate, Costar Scientific Corp., Cambridge, MA, U.S.A.); albumin and γ-globulin solutions were ultracentrifuged at 100000 ***g*** for 30 min. In addition, all perfusion fluids were passed through a 0·2 μm Uniflo cellulose acetate syringe filter (Schleicher & Schuell, Inc., Keene, NH, U.S.A.) immediately prior to being infused into the eye.

Effluent Collection Experiments

Sixteen bovine eyes and 10 human eyes were used for these experiments. The method for collecting the effluent was based on that of Johnson et al. (1990). After eyes were received, they were cleaned of extraocular fat and muscle; the conjunctiva was carefully and completely removed. The eyes were then soaked in isotonic saline at 4°C for 1–2 hr to remove soluble proteins from the surface of the eye. Following this step, a suture was tied around the optic nerve and pulled through the spout of a funnel, and the end of the funnel was sealed with a rubber stopper. The eye-funnel assembly was then placed in a 50 ml beaker and placed in a water bath at 34°C. Cold cataracts in eyes were allowed to clear prior to cannulation.

Then, a perfusion and an exchange needle (23 gauge for bovine, 25 gauge for human; Becton Dickinson Vascular Access, Sandy, UT, U.S.A.) were placed into the eye, and the eye was covered with a heavier-than-water silicone oil (Dow Corning Fluid No. 710, Dow Corning Corp., U.S.A.). DBG was perfused from a syringe pump (model 22: Harvard Apparatus, Inc., South Natick, MA, U.S.A.) through a perfusion needle at a constant pressure of 10 mmHg for 6–10 hours, using a 10 ml gas-tight syringe for bovine eyes, and a 500 μl gas-tight syringe for human eyes (both from Hamilton Co., Reno, NV, U.S.A.). A perfusion pressure of 10 mmHg was picked as a compromise between the normal pressure drop across the aqueous outflow pathway (5 mmHg) and the normal inflation pressure of the eye (15 mmHg). Intraocular pressures were measured using pressure transducers (Microswitch pressure sensor, 142PC05G, Brownell Co., Woburn, MA, U.S.A.) connected via tubing to the cannulae.

The data acquisition and control system was similar to that described by Whale, Grodinsky and Johnson (1996). Instantaneous pressure and flowrate measurements were recorded, allowing continuous calculation and display of outflow facility. The data acquisition system is based on the Perfuser program (Whale,

1992), running on a Macintosh SE computer. This program controls the flowrate from the syringe pumps, maintaining either a constant pressure or a constant flowrate, as required. Pressure readings from the transducers are recorded, along with time and flowrate. Pressure signals were digitized using a MacADIOS-8ain A/D converter (GW Instruments, Inc., Somerville, MA, U.S.A.) and fed into the Macintosh SE via the SCSI port. The control algorithm is described in detail by Whale et al. (1996).

As the effluent exited the aqueous outflow system, it collected in pools at the top of the silicone oil layer. (The time lag for the appearance of effluent at the surface of the eye is estimated to be approximately 30 seconds and, for the present purposes, can be neglected.) The effluent could easily be distinguished from the silicone oil due to the different indices of refraction, even when small amounts of effluent were present. The effluent could then be preferentially pipetted off (5 3/4 inch disposable Pasteur pipet, VWR Scientific, Boston, MA, U.S.A.) due to the high viscosity of the silicone oil. Effluent from the first 30 minutes of perfusion was discarded; since it had to pass through the episcleral veins to reach the surface of the eye it was likely contaminated with blood. The first 30 minutes of perfusion acted to 'flush' out the system with fresh perfusion fluid. Samples of effluent were taken every 30 minutes for bovine, and every 60–90 minutes for human. Samples of effluent were frozen until protein assays could be performed. After each sample point, the syringe pump was paused, and the anterior chambers of the eyes were exchanged with fresh perfusion fluid to ensure that the fluid entering the outflow pathway had essentially zero protein content. The exchange was performed by setting an exchange reservoir to 2 cm H_2O above the set point pressure, and an exit reservoir to 2 cm H_2O below the set point pressure. Both lines were then opened, allowing the fluid of the anterior chamber to be rapidly exchanged, while keeping the average intraocular pressure approximately constant at the set point pressure. Approximately 3 ml was exchanged for bovine eyes, and 1 ml was exchanged for human eyes.

In these experiments, samples of fluid from the anterior chamber were required in order to determine how much protein was added to the fluid within the anterior chamber (this was expected to be minimal as a result of the anterior chamber exchanges). Two methods were used. If periodic samples (at the same time as the effluent samples) were desired, a third infusion needle was inserted into the anterior chamber prior to the start of the experiment, ensuring that the tip was placed in the anterior rather than posterior chamber. The tubing was cut short to minimize the fluid trapped in the tubing, and a clamp was used to close the line when not in use. Opening the line just before the exchange, allowed a small sample of fluid to flow out of the anterior chamber due to the positive IOP. Since protein concentrations in the anterior chamber samples were relatively low and constant, only a single anterior chamber sample was taken during later experiments. With this method, a syringe with needle was inserted into the anterior chamber at the end of the experiment, and a sample was removed.

Protein Assays

Samples were assayed using the Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, CA, U.S.A.), based on the method of Bradford (1976). Calibration standards were produced using lyophilized bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) for bovine eyes and lyophilized human albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) for human eyes. Readings were taken using a spectrophotometer (Model UV160U, Shimadzu Corp., Kyoto, Japan).

Perfusion Experiments with Albumin or γ-Globulin [Ritter, 1993]

Three pairs of bovine eyes were perfused with either DBG or DBG with albumin added (7·5 mg ml^{-1}), and three pairs of bovine eyes were perfused with either DBG or DBG with γ-globulin added (7·5 mg ml^{-1}). Perfusions were carried out following the same procedure for determining outflow facility as described above, however no exchange needle was used and the perfusion pressure was 15 mmHg. Perfusions were continued until approximately 10 ml of perfusion fluid had passed through each eye. The initial wash-out rate was determined following the procedure of Johnson et al. (1991), using data from the period following equilibration during which 4 ml of perfusion fluid passed through the eye.

Statistical Methods

Data from individual eyes were fitted to a regression model using a least squares analysis, and variance analysis was performed on the calculated parameters. The end time point at which an effluent sample was collected was used when correlating the protein concentration of the sample with other variables. Significance levels were determined by treating the model parameters as raw data, and calculating the significance of the mean for each parameter. This is commonly referred to as the NIH method, in reference to the standard practice at the National Institutes of Health (Feldman, 1988). This method is more suitable when individual specimen variability is considered to be important. Except where noted, all data analysis follows the NIH method.

Comparisons to determine statistical significance were done using a two-sided Student t test with a significance level of 0·05, comparing the quantity of interest with the null hypothesis (value = 0). All results are shown along with standard errors.

3. Results

Protein Concentration vs. Volume Perfused

Detailed experimental results can be found in Sit (1995). Figure 1 shows a typical example of the protein concentration in the effluent collected from a bovine eye and a human eye plotted as a function of perfused volume. Protein concentrations (C) were correlated with volume perfused (V) using a function of the form:

$$C(V) = C_0\, e^{(-V/V_0)} \tag{1}$$

where C_0 is the initial protein concentration in the effluent and V_0 is the 'wash-out volume constant' (analogous to a time constant). The data from each eye perfused with DBG were fitted to equation (1) using a least squares regression. For the data shown in Fig. 1, these parameters were found to be $C_0 = 756 \pm 34$ μg ml^{-1} and $V_0 = 10{\cdot}2 \pm 1{\cdot}1$ ml ($P < 0{\cdot}0001$ for V_0) for the bovine, while for the human, these parameters were found to be $C_0 = 1776 \pm 189$ μg ml^{-1} and $V_0 = 1{\cdot}84 \pm 0{\cdot}62$ ml ($P < 0{\cdot}05$ for V_0).

Then, the parameters determined for each eye (C_0 and V_0) were averaged. For the bovine eyes, the mean values were found to be $C_0 = 637 \pm 60$ μg ml^{-1} and $V_0 = 10{\cdot}0 \pm 1{\cdot}8$ ml ($n = 16$; $P < 0{\cdot}0001$ for V_0); Fig. 2 shows the bovine correlation along with the data from the individual eyes.

Only six of the nine human eyes showed statistically significant volume constants for the protein decay, likely due to the relatively small volume perfused through the human eyes (data from one eye were discarded as the protein concentrations were much higher than the other eyes, and protein concentration in the effluent was found to increase as a function of volume perfused). Thus, two methods were used to calculate the mean value of C_0 and V_0; for those six eyes in which wash-out of protein was definitely established, the mean parameter values were determined; we also pooled all of the data, and fitted them to equation (1). The results were similar: using the NIH method for the six eyes demonstrating protein washout, we found $C_0 = 2190 \pm 342$ μg ml^{-1} and $V_0 = 2{\cdot}1 \pm 0{\cdot}38$ ml ($n = 6$; $P < 0{\cdot}003$ for slope); using the pooled method, we found $C_0 = 2270 \pm 140$ μg ml^{-1} and $V_0 = 2{\cdot}0 \pm 0{\cdot}33$ ml ($n = 9$; $P < 0{\cdot}0001$ for slope). Figure 3 shows the NIH correlation along with the data from the nine individual eyes; a different symbol type is used for each eye.

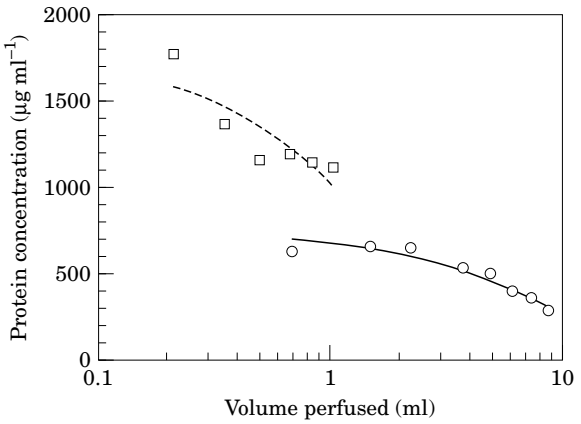

FIG. 1. Protein concentration in the effluent as a function of perfused volume. Squares are data from a human eye, circles from a bovine eye. The lines (– – –, human; ——, bovine) are the best fit to the data using equation 1. (Note the logarithmic scale on the abscissa.)

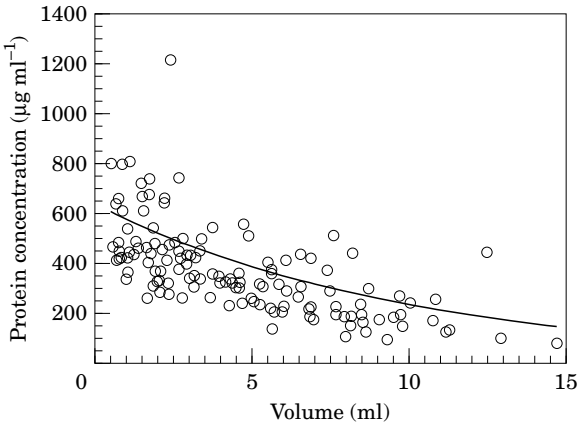

FIG. 2. Effluent protein concentration from all bovine eyes ($n = 16$) as a function of volume perfused; there are approximately eight measurements for each eye. Solid line is best fit to the data (equation 1).

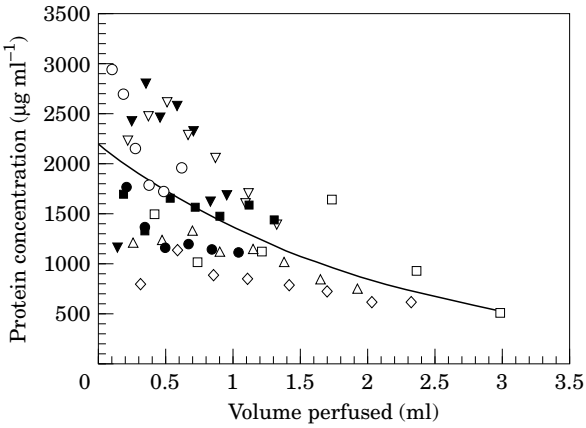

FIG. 3. Effluent protein concentration from all human eyes ($n = 9$) as a function of volume perfused; each eye is shown with a different symbol. Solid line is best fit to the data (equation 1) for the six eyes with statistically significant values of V_0.

Protein Concentration vs. Outflow Resistance

We also examined the relationship between protein concentration in the effluent (C) and outflow resistance (R = 1/outflow facility). If bulk concentration of protein (within physiological limits) generates outflow resistance, then we would expect to find a statistically significant correlation between these variables. Assuming a linear relationship,

$$R = a_1 + a_2 C \tag{2}$$

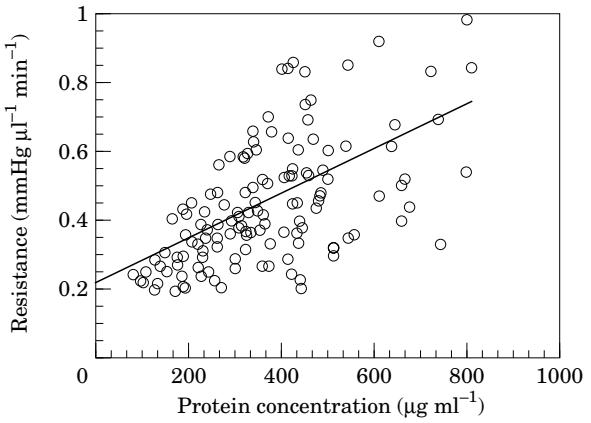

FIG. 4. Outflow resistance from all bovine eyes ($n = 16$) as a function of protein concentration in the effluent; there were approximately eight measurements on each eye. Solid line is best fit to the data (equation 2).

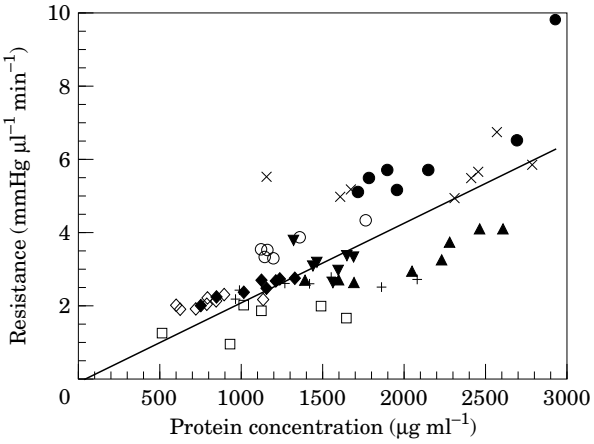

FIG. 5. Outflow resistance from all human eyes ($n = 9$) as a function of protein concentration in the effluent; a different symbol is used for each eye. No statistically significant relationship was found using the NIH method; the solid line is the best fit to the pooled data (see text in Theoretical Modelling).

the mean values of these parameters for the 16 bovine eyes were found to be $a_1 = 0{\cdot}220 \pm 0{\cdot}046$ mmHg μl^{-1} min^{-1} ($P < 0{\cdot}0001$), and $a_2 = 0{\cdot}652 \pm 0{\cdot}091$ mmHg μl^{-1} min^{-1} (mg ml^{-1}) ($P < 0{\cdot}0001$). This correlation is shown in Fig. 4. Thus these data are consistent with a relationship between protein concentration and outflow resistance in the bovine eye.

For the nine human eyes (Fig. 5), these parameters were found to be $a_1 = 1{\cdot}84 \pm 0{\cdot}58$ mmHg μl^{-1} min^{-1} ($P < 0{\cdot}01$), and $a_2 = 0{\cdot}912 \pm 3{\cdot}74$ mmHg μl^{-1} min^{-1} (mg ml^{-1}) ($P > 0{\cdot}4$), showing that no statistically significant linear relationship could be found between protein concentration and outflow resistance in the human eye. Note that while no relationship could be found examining each eye individually, as a group, there appears to be a strong relationship between outflow resistance and protein content of the effluent. We return to this point in the *Theoretical Modelling* section.

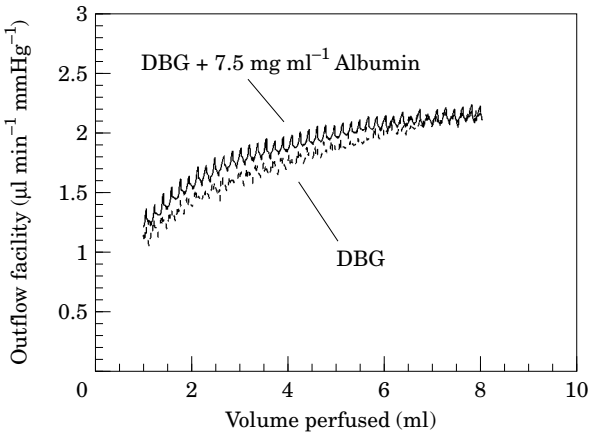

FIG. 6. Outflow facility in a bovine eye as a function of perfused volume comparing buffer (DBG) to buffer with albumin added.

Outflow Resistance vs. Volume Perfused

Our interest was to relate this wash-out of protein from the aqueous outflow pathway to the decrease in resistance that occurs during a prolonged perfusion in a bovine eye (the 'wash-out effect': for an example, see Fig. 6). Substituting equation (1) into equation (2) yields a relationship of the form:

$$R(V) = b_1 + b_2 e^{(-V/V_0)} \qquad (3)$$

where $b_1 = a_1$ and $b_2 = a_2 C_0$.

The mean values of these parameters for the 16 bovine eyes were found to be $b_1 = 0{\cdot}284 \pm 0{\cdot}055$ mmHg μl^{-1} min^{-1} ($P < 0{\cdot}0002$), $b_2 = 0{\cdot}585 \pm 0{\cdot}121$ mmHg μl^{-1} min^{-1} ($P < 0{\cdot}0003$), and $V_0 = 3{\cdot}5 \pm 0{\cdot}7$ ml ($P < 0{\cdot}0001$). The volume constant found for resistance wash-out (3·5 ml) is significantly smaller than that found for protein wash-out (10·0 ml): $P < 0{\cdot}005$. This indicates that, in the bovine eye, the protein concentration and the resistance decay at different rates with respect to the volume of fluid perfused.

Statistically significant results could not be found using equation (3) to fit the data from human eyes, consistent with the absence of wash-out in human eyes (Erickson-Lamy, 1990). For individual eyes, only three of the nine specimens exhibited a volume constant statistically significantly different from zero.

Effect of Extended Post-mortem Time

The human eye had a much greater concentration of protein in the effluent than did the bovine eye. This was unexpected as the aqueous humor of the human eye has a much lower protein concentration than does that of the bovine (human: 0·1 mg ml^{-1}, Tripathi et al., 1989; bovine: 0·5 mg ml^{-1}, Pavao et al., 1989). We thus investigated whether this result could be due to differences in post-mortem time. For the bovine eyes ($n = 1$ pair) stored under moist chamber conditions

for 24 hr, little difference was noticed between the protein concentration of the effluent compared with eyes with shorter post-mortem times. The data from this experiment were fitted to equation (1) to find that $C_0 = 385 \pm 72$ μg ml^{-1} and $V_0 = 17{\cdot}4 \pm 6{\cdot}6$ ml. These values are roughly comparable to the results from the bovine eyes with the shorter post-mortem times given above, and very different than the results from human eyes that had similar post-mortem times.

Effect of Albumin on 'Wash-out' in the Bovine Eye

Our previous studies (Johnson et al., 1993) demonstrated that serum proteins could decrease the rate of wash-out from the bovine eye. Here we examined whether serum albumin alone would have a similar effect. Figure 6 shows a typical result for a pair of bovine eyes with one eye receiving buffer and the other buffer with 7·5 mg ml^{-1} of albumin (approximately equivalent protein concentration to adding 10–15% serum to buffer). No significant difference in wash-out rate was found between control eyes (washout rates = 0·322, 0·282 and 0·417 μl min^{-1} mmHg^{-1} ml^{-1}) and the paired eyes perfused with albumin (washout rates = 0·362, 0·536, and 0·454 μl min^{-1} mmHg^{-1} ml^{-1}, respectively). These wash-out rates were consistent with those found by Johnson et al. (1993) for bovine eyes perfused with buffer (Johnson, 1993). We thus conclude that the decreased rate of wash-out caused by perfusion with 10–15% bovine serum is not due to albumin alone.

Effect of γ-Globulin on 'Wash-out' in the Bovine Eye

With 7·5 mg ml^{-1} γ-globulin, the wash-out rates were somewhat lower than the control eyes (control washout rates = 0·407, 0·241 and 0·356 μl min^{-1} mmHg^{-1} ml^{-1} and γ-globulin washout rates = 0·221, 0·157, and 0·270 μl min^{-1} mmHg^{-1} ml^{-1}, respectively). However, the differences were not statistically significantly different from one another and these wash-out rates were consistent with those found by Johnson et al. (1993) for bovine eyes perfused with buffer. Furthermore, the γ-globulin washout rates were much higher than those found by Johnson et al. (1993) for bovine eyes perfused with 10–15% serum in buffer. We thus conclude that the decreased rate of wash-out caused by perfusion with 10–15% bovine serum is not due to γ-globulin alone.

4. Theoretical Modelling

Our goal in these studies was to investigate the possibility that plasma-derived proteins in the aqueous humor contribute to the maintenance of aqueous outflow resistance. In fact, we did find a statistically significant relationship between the protein concentration in the effluent and aqueous outflow resistance (equation 2). However, this relationship might be due to outflow resistance controlling the flowrate and thereby the effluent protein concentration rather than vice versa. This seems especially likely since the protein levels found in the bovine effluent are not sufficiently high to generate a significant fraction of aqueous outflow resistance based on our previous studies (Johnson et al., 1993).

If proteins were being removed from a finite reservoir (e.g. the ciliary body stroma, Freddo et al., 1990), then over the course of a perfusion experiment, the protein depot would become depleted, and thus protein concentration of the effluent would be expected to decrease over the course of an experiment. Furthermore, the protein concentration of the effluent would depend on flowrate of fluid passing over the root of the iris carrying the protein away (high flowrates would have lower protein concentrations), and therefore protein concentration would depend on outflow resistance (when perfused at constant pressure). Both of these observations are consistent with our experimental results. In this section, we present scaling analyses that add support to these notions.

Protein Depot Length Scale Analysis

Our experimental results demonstrated that the protein concentration of the effluent was correlated with the volume of fluid perfused as indicated by equation (1). An estimate of the total mass of protein removed from the depot during an experiment can be obtained by integrating this equation with respect to volume of fluid perfused, V

$$M = \int_0^\infty C(V)\, dV = C_0 V_0 \tag{4}$$

This can be compared to an estimate of the total soluble protein contained in the ciliary body. Bill (1968) reported the osmotically effective albumin concentration in the ciliary body to be 74% of the plasma level. Assuming the fraction of the ciliary stroma available to albumin to be 40% (Freddo et al., 1990), we find the tissue concentration of albumin to be approximately 20 mg ml^{-1} tissue (C_{CB}) (assuming a plasma protein concentration of 70 mg ml^{-1}). The volume of the ciliary body is estimated as wH^2 where w is the circumference of the iris (5·5 cm in the bovine, Johnson et al., 1990, and 3·6 cm in the human, Moses, 1979) and H is the characteristic width of this depot (see Fig. 7) (we have assumed that the anterior/posterior dimension and the interior/exterior dimension of the protein depot are approximately equal size to one another and both can be characterized by H).

Then, an expression for H can be found as:

$$H = \sqrt{\frac{C_0 V_0}{w C_{CB}}} \tag{5}$$

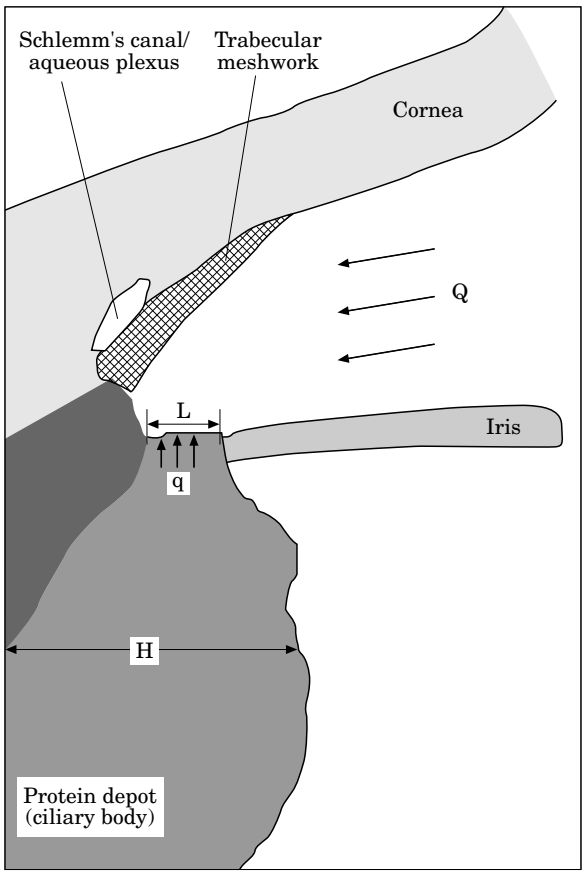

FIG. 7. Schematic of the bovine outflow pathway showing the model used to characterize the transfer for the protein flux (q) from the ciliary body stroma to the aqueous humor passing by at a flowrate Q.

Using the mean value of $C_0 V_0$ determined in the experiments (using all the bovine data, and using the 6 of 9 human eyes that had a statistically significant value of V_0), we find that H is approximately 2·2 mm in the bovine and 2·4 mm in the human. These values are higher than would be expected, although reasonable as order-of-magnitude estimates, and are consistent with the notion that the protein found in the effluent originated in the ciliary body region. In the bovine, the spaces of Fontana likely also contribute to the protein flux; in the human, the high value may be due to uncertainty in the value of $C_0 V_0$.

Relationship Between Flowrate and Concentration of Protein in Effluent

We here wish to determine the flux of protein (q) from the ciliary body to the aqueous humor. In general, we expect that a higher flowrate (Q) will lead to a lower protein concentration in the effluent. A detailed analysis of this mass transfer problem would include the resistance to transport through the ciliary body to the angle region and the resistance to transport from the angle tissue to the fluid passing by. After an initial equilibration period when resistance to transport in both regions will be important, the ciliary body region will begin to be depleted of protein, and diffusion in this region will become the rate limiting step. In this case, we expect the following scaling relationship to hold:

$$q \sim DA_{flux}(C_{\mathrm{CB}} - C)/\sqrt{Dt} \tag{6}$$

where $A_{flux} = wL$ is the area available in the angle for transport of protein between the ciliary body and the aqueous humor (L is the length of the area; see Fig. 7), C is the concentration of protein in the effluent, D is the diffusion coefficient of protein in the ciliary body (3×10^{-7} cm^2 sec^{-1} in the ciliary body; Freddo et al., 1990), t is time since the start of the experiment and $\sqrt{Dt}$ is proportional to the thickness of a protein depletion zone at the root of the iris.

The concentration of protein in the effluent, assuming that it all arises from the ciliary body, is related to flowrate as $C = q/Q$. And the flowrate is related to outflow resistance as $Q = \Delta P/R$. Thus, allowing that $C \ll C_{\mathrm{CB}}$ we find that:

$$\frac{C}{C_{\mathrm{CB}}} \sim \frac{A_{flux} R}{\Delta P}\sqrt{\frac{D}{t}} \tag{7}$$

Equation (7) shows that, as experimentally observed, the protein concentration in the effluent will decrease as a function of time. [For long times, we expect the decline in C to be exponential due to the finite size of the ciliary body that was not taken into account in equation 6]. Equation 7 also shows that as outflow resistance (R) increases (at fixed ΔP), the concentration of protein in the effluent will also increase. This relationship provides strong support for our suspicion that the correlation between protein concentration and outflow resistance, found using equation 2 for the bovine eyes, may not be due to effluent protein content affecting outflow resistance but the converse.

The question then arises, however, why no such relationship was seen in the human. Recall that we used the NIH statistical method to analyse that data. Since there was negligible wash-out of flow resistance in the human eyes examined, it would be difficult to determine the effect of changes in flow resistance on protein concentration for a particular eye, and thus it would be more appropriate here to use pooled data. When we repeated the statistical analysis of equation 2 using pooled human data (see solid line on Fig. 5), we found a highly significant relationship: $a_1 = -0{\cdot}12 \pm 0{\cdot}12$ mmHg μl^{-1} min^{-1} ($P > 0{\cdot}1$), and $a_2 = 2{\cdot}2 \times 10^{-3} \pm 0{\cdot}22 \times 10^{-3}$ mmHg μl^{-1} min^{-1} (mg ml^{-1}) ($P < 0{\cdot}0001$).

5. Discussion and Conclusions

Recent evidence has indicated that much of the protein in aqueous humor is added by diffusion from the ciliary body via the iris root (Freddo et al., 1990; Barsotti et al., 1992). This suggested that the protein concentration of aqueous humor entering the trabecular meshwork may be substantially higher than

the aqueous humor of the anterior chamber. Johnson et al. (1993) were able to prevent washout in bovine eyes by perfusing with a solution of 10–15% serum in buffer instead of buffer alone. Our goal in the current studies was to determine if there was a relationship between protein concentration in the fluid passing through the trabecular meshwork and washout in bovine eyes, and the lack of washout in human eyes. A series of perfusion experiments were conducted on human and bovine eyes in which the effluent was collected and assayed for protein content. We also examined the influence of two of the major proteins of aqueous humor, albumin and γ-globulin, on the rate of washout in the bovine eye.

Statistically significant correlations were found between effluent protein concentration and volume of fluid perfused for both bovine and human eyes. The apparent exponential decay of protein concentration as a function of effluent volume is highly suggestive of a finite protein depot being depleted (Figs 2 and 3). It is noteworthy that the volume constant characterizing effluent protein decay in the human ($V_0 = 2{\cdot}1$ ml) is significantly larger than the typical quantity of fluid perfused through a human eye during an experiment (less than 1 ml) while in the bovine eye these two values are comparable (each approximately 10 ml).

A statistically significant correlation was also found between outflow resistance and volume perfused for bovine eyes, based on an exponential model of resistance decay. However, that volume constant ($3{\cdot}5 \pm 0{\cdot}7$ ml) was significantly smaller than the volume constant from the bulk protein concentration vs. volume perfused correlation ($10{\cdot}2 \pm 1{\cdot}1$ ml), suggesting that outflow resistance and protein concentration may be governed by different mechanisms (since they decay at different rates). It could be, however, that a specific protein that decayed at a more rapid rate than did the bulk concentration of protein (perhaps due to a higher diffusivity) would show a better correlation with the decay in outflow resistance.

For human eyes, no statistically significant correlation was found between outflow resistance and volume perfused using the exponential model of resistance decay. This was consistent with the lack of washout found by previous researchers (Erickson-Lamy et al., 1990). This may have been due to the slow rate of decay of protein concentration (and consequently outflow resistance) in human eyes.

Another statistically significant correlation found was between outflow resistance and protein concentration in bovine eyes (Fig. 4). For human eyes, no statistically significant relationship was found using the NIH method of analysis, but when pooled data were used, a relationship was found (Fig. 5). Both of these findings were consistent with the notion that aqueous humor proteins control outflow resistance. However, the initial protein concentration of effluent in bovine eyes (1%) was found to be much less than the 10–15% serum solution required by Johnson et al. (1993) to prevent washout. This raised the possibility that, rather than aqueous humor proteins controlling outflow resistance, the protein concentration of the effluent was instead determined by the outflow rate and thereby depended on outflow resistance (when perfused at constant pressure).

Our modelling results added support to this hypothesis. We found that the protein concentrations in both bovine and human effluent were consistent with their having originated in a depot in the ciliary body region and with the concentration in the effluent decreasing as the protein depot becomes depleted. The higher initial protein concentration of the effluent from human eyes (four times higher than in bovine eyes) was likely due to the lower flowrate through that system.

Our experimental results indicate that the protein level found in the fluid passing through the bovine trabecular meshwork was much lower than the level necessary to prevent wash-out, and furthermore, that the decay of protein in the bovine effluent decreased at a different rate than did outflow resistance. These results suggest that in the bovine eye, the bulk protein level of fluid normally passing through the outflow pathway under physiological conditions does not affect outflow resistance. Further support for this finding comes from our studies perfusing bovine eyes with albumin and γ-globulin at protein concentrations similar to those previously used by Johnson et al. (1993). Neither protein affected the wash-out rate.

We conclude that, rather than outflow facility being determined by protein level in the fluid passing through the outflow pathway, instead outflow facility controls flowrate which in turn determines the protein level in the effluent. Over the course of a perfusion experiment, the protein depot in the ciliary body is depleted, leading to a progressive reduction in effluent protein concentration. In addition, if the outflow resistance falls (washout), the flowrate simultaneously increases, leading to a further decrease of the bulk concentration of protein in the effluent. The outflow facility would be controlled by a resistance causing mechanism other than the bulk level of aqueous humor proteins.

If aqueous humor outflow resistance is not related to bulk protein concentration in the perfusion fluid, why did Johnson et al. (1993) and Kee et al. (1996) find that 5–15% serum in buffer greatly reduced the wash-out phenomenon? One possibility is that a specific protein component is affecting outflow resistance rather than the bulk concentration of protein. When microporous membranes with pore sizes similar to those found in the inner wall endothelium are perfused with aqueous humor, they become progressively more resistive to flow (Johnson et al., 1986). This blockage occurred during perfusions with aqueous humor, but not with serum solutions at the same bulk protein concentration, and these investigators postulated that a particular aqueous humor protein

was responsible. It is intriguing to wonder whether that protein might be responsible for the effect of the high concentration of serum on wash-out in the bovine eye. The lack of wash-out in the human eye may result from the long time necessary to wash this protein out of the ciliary body region.

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