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The Non-Uniform Distribution of Albumin in Human and Bovine Cornea

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In our previous studies, we noted a non-uniform distribution of protein tracer preferentially entering the anterior stromal lamellae of the cornea from the limbus. Given other differences reported previously between the anterior and posterior lamellae of the cornea, and the number of corneal disorders in which abnormalities are preferentially confined to either the anterior or posterior lamellae, we were prompted to examine the distribution of albumin in normal human and bovine cornea.

The distribution of albumin in bovine and human cornea was studied immunohistochemically. Total soluble protein and albumin in the anterior 1/3 and posterior 2/3 of the central, middle and peripheral cornea of bovine eyes was measured biochemically. To aid in interpreting the findings, a theoretical model was developed based upon the combined effects of diffusive and convective transport.

Using immunohistochemical methods, in both bovine and human eyes, intense staining of albumin was found in the anterior 1/3 of the corneal stroma. There was a gradual reduction in staining intensity from the limbus to the central cornea in the anterior corneal stroma. Less staining was found in the posterior 2/3 of corneal stroma. Additionally, a greater concentration of soluble protein and albumin was found in the anterior stroma than in the posterior stroma of the bovine eyes by biochemical analyses. The theoretical model demonstrated that this distribution of protein required a difference in excluded volume fraction between the anterior and posterior stroma and was consistent with a convective flux originating at the limbus and passing through the corneal stroma.

The soluble proteins of the bovine and human cornea are preferentially concentrated in the anterior cornea and near the limbus. This distribution is likely due to differences in excluded volume fraction between the anterior and posterior stroma and a small convective flux passing through the cornea.

Key words: corneal stroma; albumin; bovine; human; modeling; transport.

1. Introduction

In our previous studies (Freddo et al., 1990; Barsotti et al., 1992) documenting the pathway for plasma protein entry into the normal aqueous humor of rabbit and monkey eyes, we noted a non-uniform distribution of protein tracer preferentially localized in and near the anterior stromal lamellae at the limbus. In further studies (Johnson et al., 1993) examining the distribution of albumin in the anterior segment, we noticed a distinct difference in the staining pattern between the anterior (A) and posterior (P) corneal stroma. Several biochemical (Bettelheim and Goetz, 1976; Castoro, Bettelheim and Bettelheim, 1988), physiological (Kikkawa and Hirayama, 1970; Turss et al., 1971; Lee and Wilson, 1981; Cristol, Edelhauser and Lynn, 1992) and ultrastructural (McTigue, 1967; Komai and Ushiki, 1991; Freund et al., 1992) differences distinguish the anterior and posterior lamellae of the cornea, and a number of corneal disorders preferentially involve either the anterior or posterior lamellae (McTigue, 1967; Freddo, Polak and Leibowitz, 1989). These differences prompted us to examine the distribution of albumin in normal human and bovine cornea to determine if A/P differences in protein concentration could be confirmed using immunohistochemical and biochemical techniques.

2. Materials and Methods

Materials

Thirty-two enucleated bovine eyes from two-week old calves were obtained from a local abattoir (Arena & Sons, Hopkinton, MA, U.S.A.). Thirty of these eyes were kept in a plastic bag, transported on ice and arrived within 4 hr of death. Eight bovine eyes were fixed 5 hr postmortem. Two bovine eyes were fixed immediately after enucleation. Eight bovine eyes were deliberately stored under moist chamber conditions in the refrigerator for various times (two eyes each for 24, 48, 72, or 96 hr) before fixation and used as a control for post-mortem distributional and autolytic changes. Blood samples from the same animal were also obtained.

Eight, normal human eye-bank eyes were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, U.S.A.). Intact eyes were

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wrapped in gauze wetted with saline, maintained at 4°C, transported on ice and arrived within 24 hr postmortem.

Immunohistochemistry

Eighteen bovine and eight human eyes were used. Each eye was bisected by an equatorial incision. After the lens was removed, the anterior segment of each eye was fixed in Carnoy's fluid for 12–18 hr and processed for paraffin embedding. Five-μm sections of bovine and human anterior segments were deparaffinized and rehydrated in phosphate buffered saline (PBS, pH 7·3) for 10 min. The sections were then incubated with monoclonal antibodies (anti-human serum albumin, Medix Biotech Inc., Foster City, CA, U.S.A. and anti-bovine serum albumin, Sigma, St. Louis, MO, U.S.A.) for 2 hr at room temperature. The sections were next washed 3 times in PBS and incubated with gold-conjugated secondary antibody (rabbit anti-mouse IgG conjugated with 5 nm gold, Amersham, Arlington Heights, IL, U.S.A.) for 4 hr at room temperature. The sections were again washed 3 times in PBS, and a silver enhancement solution (Amersham, Arlington Heights, IL, U.S.A.) was applied to the sections for 8–15 min. After washing, sections were counter-stained with hematoxylin and cover-slipped with Permount (Fisher, Fair Lawn, New Jersey). Photomicrographs were taken using a Leitz Orthoplan photomicroscope (Leitz-Wetzlar, Germany).

Control studies included: (1) Preadsorption of anti-albumin antibodies with bovine or human albumin, and (2) Substitution of the primary antibody with PBS or mouse IgG.

Corneal Stroma Extracts

The corneas were removed from 14 bovine eyes by cutting just central to the corneoscleral limbus, excluding all sclera. The epithelium and endothelium were scraped away with the edge of a scalpel. Three 5 mm diameter buttons were trephined from central, middle and peripheral cornea. The corneal buttons were flattened between two glass slides, then placed on dry ice and frozen. The endothelial side of the corneal button was marked using tissue marking dye (Fisher, Pittsburgh, PA, U.S.A.). The anterior 1/3 of the cornea was separated from the posterior stroma as accurately as possible in a lamellar dissection with a razor blade. Six buttons (3 anterior and 3 posterior) were obtained from each bovine eye and the wet weight of each corneal button was determined. One ml of PBS with 5·5 mM glucose was added to each glass vial and the vials were placed on a rotary shaker for 4 hr at 4°C. Eluates were centrifuged at 12000 g for 30 min. The resulting supernatant was used for protein analysis.

The total soluble protein content of the different segments of corneal stroma was quantified using the Bio-Rad (Hercules, CA, U.S.A.) protein assay (Bradford, 1976) with bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) as the standard.

Electrophoresis and Western Blots

SDS-PAGE was performed using 12% polyacrylamide mini gels (Laemmli, 1970) and sample volumes were normalized to tissue wet weight (100 μg wet tissue/lane). Molecular weight standards (Promega, Inc., Madison, WI, U.S.A.), purified bovine serum albumin (Sigma, St. Louis, MO, U.S.A.), and fresh bovine serum (obtained from the same animal from which eyes were enucleated, 1:1000 dilution) were loaded in adjacent lanes. Total protein was visualized using silver staining (Bio-Rad, Hercules, CA, U.S.A., Switzer, Merril and Shifrin, 1979).

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes (Towbin, Staehlin and Gordon, 1979) and reacted with anti-bovine serum albumin antibody (Sigma, St. Louis, MO, U.S.A.). After washing, the membrane was incubated with biotinylated horse-anti-mouse IgG (Vector, Burlingame, CA, U.S.A.) and washed. The membrane was then incubated with avidin-biotin complex and the albumin band identified by reaction with diaminobenzidine (DAB) solution (Kirkegaard & Perry Lab, Gaithersburg, MD, U.S.A.)

Statistics

Statistical comparisons were done using Tukey's paired comparison procedure (Box, Hunter and Hunter, 1978) for multiple comparison as implemented on SYSTAT 5 for the Macintosh (Tukey-Kramer HSD Multiple Comparisons, Evanston, IL, U.S.A.). Probabilities of 0·05 or less were considered to be statistically significant.

Results

Distribution of Serum Albumin in Bovine and Human Cornea

Pre-adsorption of the primary antibodies with albumin, or substitution with mouse IgG or PBS, resulted in no staining [Fig. 1(A)].

In the bovine eyes, the most intense staining of serum albumin was found in the anterior 1/3 of the corneal stroma [Fig. 1(B)]. Less staining was found in the posterior 2/3 of the corneal stroma. No staining was found in the basement membrane of the corneal epithelium or Descemet's membrane. In the human eyes, similar to the bovine eyes, the most intense staining of serum albumin was found in the anterior 1/3 of the corneal stroma. Toward the limbus, however, albumin was present at progressively deeper levels. A small amount of staining was found in the posterior 2/3 of central corneal stroma [Fig. 1(C)]. No

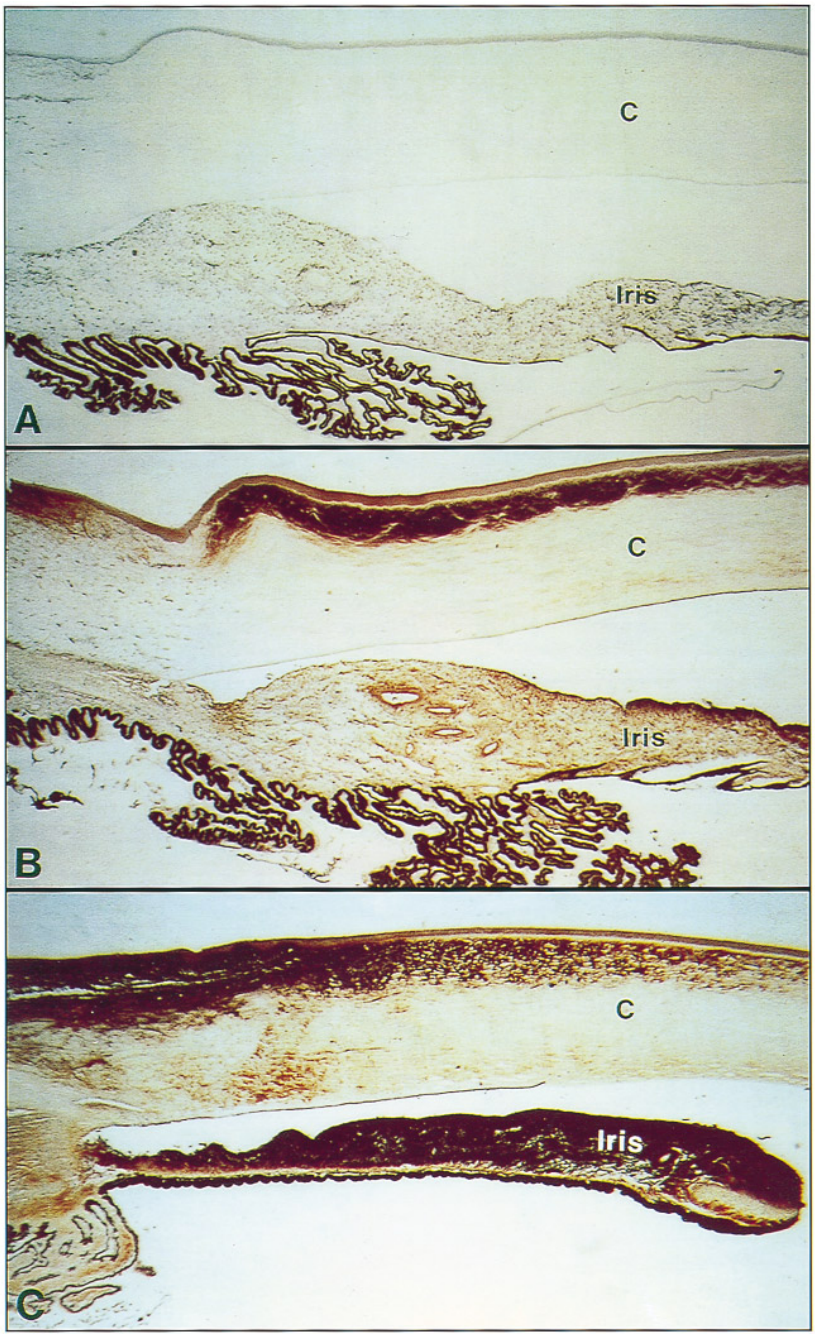

FIG. 1. Distribution of serum albumin in bovine and human cornea. (A) Controls; Substitution of mouse IgG for antibody to bovine serum albumin demonstrates no staining in bovine cornea. (× 28). (B) Serum albumin in bovine cornea; The antibody to bovine serum albumin, demonstrated by brown color, is distributed in the corneal stroma with the highest concentration being found in the anterior 1/3 of the cornea and limbal region. There was a gradual reduction in staining intensity from the limbus to the central cornea and diminished amount of staining in the posterior corneal stroma. No staining was found in the basement membrane of the corneal epithelium or Descemet's membrane. (× 28). (C) Serum albumin in human cornea; The antibody to human serum albumin, demonstrated by brown color, is distributed in the corneal stroma with the highest concentration being found in the anterior 1/3 of the cornea. Toward the limbus, albumin was present at progressively deeper levels. There was a gradual reduction in staining intensity from the limbus to the central cornea and diminished amount of staining in the central region of the posterior corneal stroma. No staining was found in Bowman's or Descemet's membrane. (× 29).

staining was found in Bowman's layer or in Descemet's membrane. In addition to the heterogeneous distribution seen anteroposteriorly, both bovine and human eyes exhibited a reduction in staining intensity progressing from the limbus to the central cornea [Fig. 1(B) and (C)].

No difference in the distribution of albumin was found between eyes fixed immediately and those fixed 5, 24, and 48 hr post-mortem. In the eyes fixed 72 hr post-mortem, albumin staining was found both anteriorly and posteriorly, although still with more staining in the anterior region. In the eyes fixed 96 hr

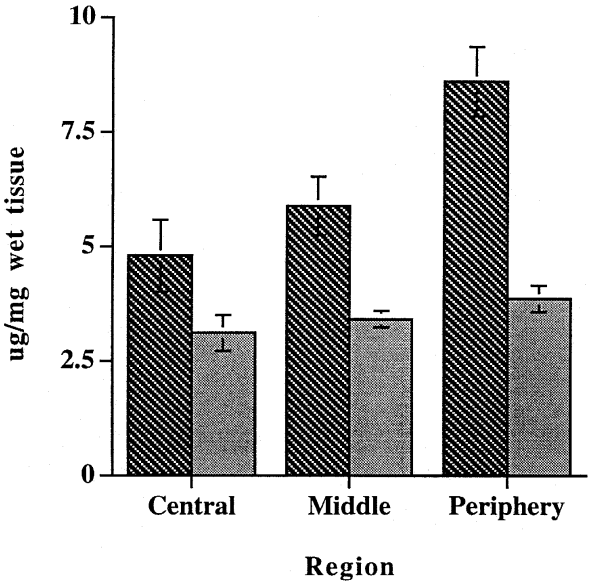

FIG. 2. Total soluble protein; Total soluble protein extracted from six segments of corneal stroma was determined using the Bio-Rad protein assay. ($n = 12$, Mean $\pm$ S.E.). In all three regions of corneal stroma (central, middle and peripheral), soluble protein content was greater in the anterior lamellae. A higher concentration of total soluble protein was found in the peripheral region of the anterior stroma compared with the central and middle regions. No significant differences were found in the three posterior regions of corneal stroma. ▨, Anterior, ■, posterior.

post-mortem, albumin staining was found throughout the corneal stroma. The corneas fixed after 24 hr post-mortem were found to have increased thickness.

Protein Assay

Data on total soluble protein content in the six segments of corneal stroma are shown in Fig. 2. Soluble protein content was higher in the anterior stroma than the posterior stroma in all three regions of cornea: central ($P = 0{\cdot}05$), middle ($P < 0{\cdot}01$) and peripheral ($P = 0{\cdot}0001$). A higher concentration of total soluble protein was found in the peripheral region of the anterior stroma compared with middle ($P < 0{\cdot}05$) and central regions ($P < 0{\cdot}005$). No significant differences in soluble protein were found between the three posterior regions of the corneal stroma.

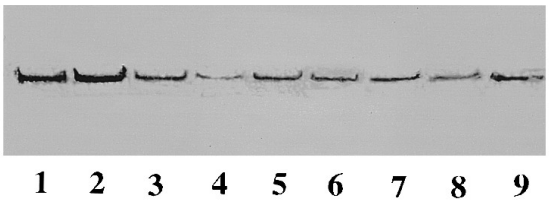

FIG. 4. Western blot. The monoclonal antibody, anti-bovine serum albumin reacted only with a single band in Western blot of corneal stroma extracts. This band had the same mobility as bovine serum albumin. Same results were obtained from 10 different corneal stroma extracts. (1) molecular weight mark, (2) purified bovine serum albumin, (3) central anterior stroma, (4) central posterior stroma, (5) middle anterior stroma, (6) middle posterior stroma, (7) peripheral anterior stroma, (8) peripheral posterior stroma, (9) bovine serum.

SDS-PAGE and Western Blot Analyses

Gel electrophoresis of extracts from the six different segments of bovine corneal stroma each displayed a prominent protein band with similar electrophoretic mobility corresponding to that of purified commercial bovine serum albumin and serum albumin (from the same animal from which eyes were enucleated) with a molecular mass of 66 kD (Fig. 3). The profiles are similar to those found by Wiig (1989).

The identification of this band as bovine serum albumin was confirmed by Western blot analysis. The monoclonal antibody, anti-bovine serum albumin, reacted only with a single band in Western blots of corneal stromal extracts. This band had the same mobility as bovine serum albumin (Fig. 4).

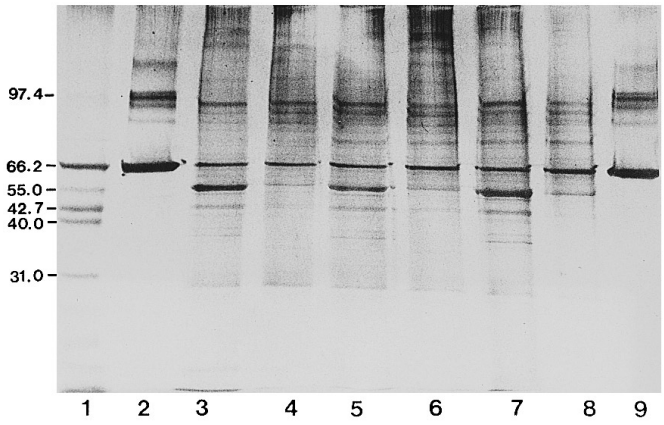

FIG. 3. Gel Electrophoresis; Silver-stained SDS/polyacrylamide gel electrophoresis from corneal stroma. (1) molecular weight mark, (2) purified bovine serum albumin, (3) central anterior stroma, (4) central posterior stroma, (5) middle anterior stroma, (6) middle posterior stroma, (7) peripheral anterior stroma, (8) peripheral posterior stroma, (9) bovine serum (1:1000 dilution). Same results were obtained from 11 different corneal stroma extracts.

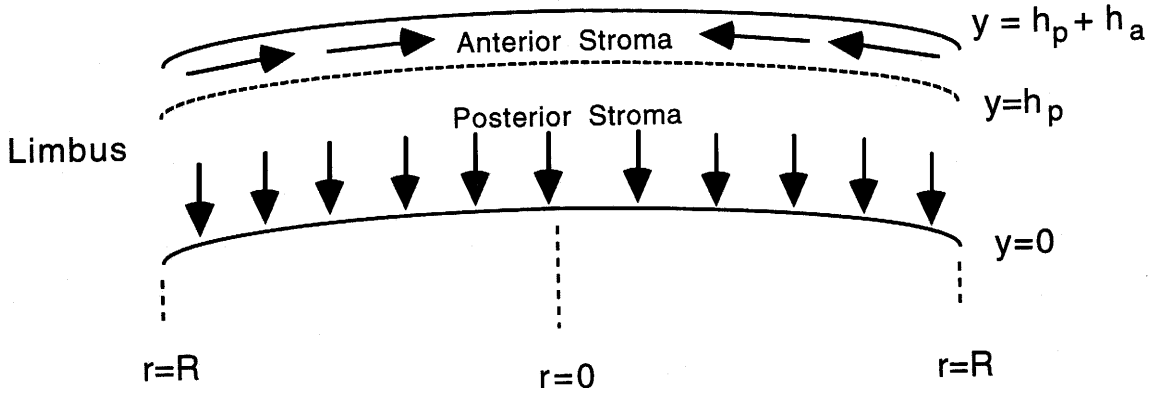

FIG. 5. Domain of analytical model. The analytical domain used to model the corneal stroma. The centripetal coordinate (r) extends parallel to the surface of the cornea from the central cornea ($r = 0$) to the limbus ($r = R$); the anteroposterior coordinate (y) extends from the corneal endothelium ($y = 0$) to the corneal epithelium ($y = h_p + h_a$). Arrows show presumed directions of protein transport.

A Model to Describe the Distribution of Soluble Proteins in the Corneal Stroma

Based on these findings, we developed an analytical model to aid in the interpretation of these data. Our goal was to develop a simple model that, nonetheless, captured the essential processes responsible for the distribution of soluble proteins we observed in the anterior and posterior corneal stroma. Maurice and Watson (1965) previously modeled the transport of albumin starting at the limbus and passing through the corneal stroma from $r = R$ to $r = 0$ assuming that the concentration was uniform in the A/P direction (see Fig. 5). They modeled this process as purely diffusional ($D_{albumin} = 1 \times 10^{-7}$ cm^2 sec^{-1}), with a transfer coefficient k_c, describing the loss of albumin from the cornea. Using this model, they were able to determine an empirical value for k_c consistent with their measured protein distribution showing a central corneal concentration of albumin approximately 1/3 that at the periphery.

We examined two extensions of the model of Maurice and Watson (1965). In the first, we defined two distinct regions for diffusion of albumin: a region comprising the anterior 1/3 of the corneal stroma with a diffusion coefficient characteristic of albumin in free solution ($D_a = 8 \times 10^{-7}$ cm^2 sec^{-1}) and a region comprising the remaining posterior lamellae of the corneal stroma with a diffusion coefficient for albumin of $D_p = 1 \times 10^{-7}$ cm^2 sec^{-1} (Maurice and Watson, 1965). Based on the postulated large difference in diffusivities, we assumed transport to be primarily in the centripetal direction (toward the central cornea) in the anterior stroma, and anteroposteriorly (or normal to the corneal surface) in the posterior stroma. Clearly, this two compartment model is a simplification of the corneal stroma which likely has smooth gradients in properties in the anteroposterior direction. To allow for this more physiological situation would require detailed knowledge of the spatial variations in corneal properties and adopting a more detailed model such as that of Klyce and Russell (1979).

The diffusional flux (transport per unit area: q) of albumin crossing the posterior stroma into the anterior chamber is limited by the diffusional resistance of the posterior stroma (h_p/D_p) and that of the corneal endothelium and associated boundary layer ($1/k_e$), where h_p is the thickness of the posterior cornea. This antero-posterior flux can be expressed as

$$q(r) = \left[\frac{c_a(r) - c_{ah}}{1/k_e + h_p/D_p}\right] \tag{1}$$

Here $c_a(r)$ is the concentration of albumin in the anterior cornea as a function of distance from the center of the cornea and c_{ah} is the concentration of albumin in the aqueous humor (assumed negligibly small: see Table I). Then, following the same approach as Maurice and Watson (1965), we find the distribution of albumin in the anterior corneal stroma to vary as

$$\frac{c_a(r)}{c_a(R)} = \frac{I_0\left[r\sqrt{k_e/D_a h_a\left(1 + \frac{k_e h_p}{D_p}\right)}\right]}{I_0\left[R\sqrt{k_e/D_a h_a\left(1 + \frac{k_e h_p}{D_p}\right)}\right]} \tag{2}$$

where R is the distance from the center of the cornea to the limbus, and I_0 is the modified Bessel function of the first kind of order zero. If k_e is very small (high diffusional resistance across corneal endothelium and associated boundary layer in anterior chamber), then this result reduces to the same formula given by Maurice and Watson with $(k_e/h_a)(D_p/D_a)$ equal to the coefficient k_c they introduced (there was an error in the original expression given by Maurice and Watson, later corrected, such that the radial signs were missing in the numerator and denominator).

To reproduce the observation of Maurice and Watson (1965), [$c_a(r = 0)$ is one-third of $c_a(r = R)$] in our two-layer model, we find that the diffusional resistance of the corneal endothelium ($1/k_e$) must be roughly 25-fold greater than the diffusional resistance of the posterior corneal stroma (h_p/D_p). However, in

TABLE I

Typical values of model parameters and variables

Parameters and variables	Description	Typical values[a]
Geometric		
R	Radius of Cornea	1 cm
h_a	Thickness of Anterior Stroma	0·015 cm[b]
h_p	Thickness of Posterior Stroma	0·06 cm[b]
r	Distance from center of cornea	0–1 cm
y	r/R	0–1
Transport		
μ	Viscosity	0·007 g/cm/sec
K_p	Hydraulic Conductivity	10^{-14} cm²
ΔP	Swelling pressure difference between cornea and sclera	50 mm Hg
k_e	Permeability of Corneal Endothelium	$1–7 \times 10^{-7}$ cm sec^{-1}
k_c	Corneal Transfer Coefficient $= k_e D_p / h_a D_a$	0·09–0·5 days^{-1}
D_p	Diffusion Coefficient of Posterior Stroma	1×10^{-7} cm² sec^{-1}
D_a	Diffusion Coefficient of Anterior Stroma	8×10^{-7} cm² sec^{-1}
v_p	Permeation Velocity in Posterior Stroma	$0·4–3 \times 10^{-7}$ cm/sec[c]
v_a	Permeation Velocity in Anterior Stroma	$0·1–1 \times 10^{-5}$ cm/sec[c]
Pep	Posterior Peclet No. $= (v_p h_p / D_p)(1 + D_p / k_e h_p)$	0·3–0·7
Pe_a	Anterior Peclet No. $= (v_p R^2)/(D_a h_a)$	3–27[c]
Q	Flowrate through Cornea	0·007–0·06 μl min^{-1}
Concentration		
c_{ah}	Concentration of albumin in aqueous humor	0·5 mg/ml[d]
$c_a(r)$	Concentration of albumin in anterior cornea	3–10 mg/ml[e]
$Z(y)$	$c_a(y)/c_a(1)$	0–1

[a] Values given are from the text unless otherwise noted; [b] based on Fig. 1; [c] based on Pe_p necessary to give $Z(0) = 0·33$ for given value of k_c; [d] (Pavao et al., 1989); [e] (Maurice and Watson, 1965).

this case, there would be only a negligible albumin concentration difference between the anterior and posterior cornea, in contrast to our experimental results. Furthermore, we find that k_c must be approximately 0·05 days^{-1}, somewhat lower than the range of values available in the literature (0·09–0·5 days^{-1}) (Maurice and Watson, 1965; Wiig, 1990). Thus, we conclude that the albumin distribution we observed in the corneal stroma is not due solely to different diffusion properties in the anterior and posterior stroma.

The results from our experiments in which we allowed the eyes to sit for 24–96 hr before examining the distribution of albumin in the corneal stroma suggested that the difference in protein concentration between the anterior and posterior stroma could be maintained far longer than the time scale necessary for diffusion equilibration between these two regions. The likely explanation of this phenomenon is the excluded volume effect which arises due to the high concentration of proteoglycans in the cornea, reducing the actual space available to the albumin in the corneal stroma. Thus, one possibility is that the albumin concentrations in the albumin-accessible spaces in the anterior and posterior corneal stroma are equal, although the actual albumin concentration may be significantly lower in the posterior stroma due to the higher excluded volume fraction in this region caused by an increased proteoglycan fraction (Castoro et al., 1988).

To evaluate this possibility, we used the single-layer model of Maurice and Watson (1965) with an average diffusion coefficient reflecting that in both the anterior and posterior stroma ($D_{ave} = (h_a^* D_a + h_p^* D_p)/(h_a + h_p)$) (the single layer model is now appropriate because the anterior and posterior layers are assumed to have the same concentration of albumin in the albumin-accessible spaces). We find, for the parameters given in Table I, that for $c_a(r = 0) = (1/3)^* c_a(r = R)$, that k_c must be approximately 0·13 days^{-1}. While this is in the range of measured values for k_c (Maurice and Watson, 1965; Wiig, 1990), it is nonetheless somewhat low. A value of k_c of 0·22 (Maurice and Watson, 1965) or 0·5 (Wiig, 1990) would give a concentration of albumin in the central cornea much less than the measured value of 1/3 the peripheral concentration.

While the excluded volume effect is likely responsible for the observed albumin difference between anterior and posterior corneal stroma, the radial concentration profile might be, in part, due to the convective flow through the anterior corneal stroma. The possibility of a convective flow through the cornea arises from the observation that the swelling pressure of the cornea is much higher than that of the neighboring sclera

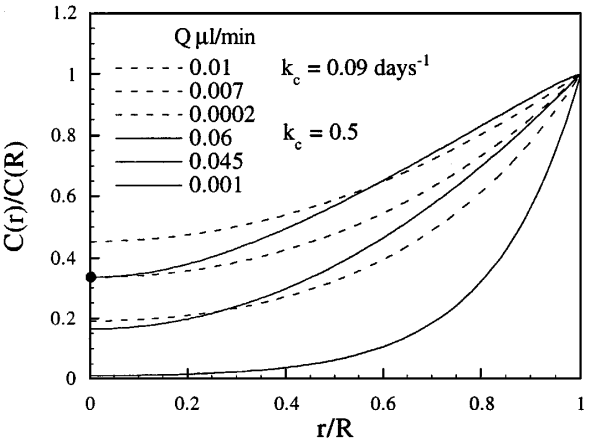

FIG. 6. Anterior cornea albumin distribution (theoretical prediction). Plot of the distribution of albumin in the anterior cornea, $c_a(r)$, as a function of flowrate through the corneal stroma for two values of K_c. The black spot marks the point where $c(r)/c(R) = 0{\cdot}33$.

(Maurice, 1984). As there is no anatomical barrier between these tissues, this suggests that there may be a small steady, osmotically-driven flow from the sclera to the cornea with the corneal endothelium pumping this fluid out of the cornea. Based on the anatomical considerations discussed above, the fluid would pass primarily through the more highly conductive anterior corneal stroma toward the central cornea while filtering through the more resistive posterior stroma; this flux of fluid would enhance the transport of albumin toward the central cornea above that due to diffusion alone.

To determine whether this process is consistent with our data for the corneal albumin distribution, a second two-layer model was developed. The details of this model are found in Appendix A. Briefly, transport in the anterior stroma is assumed to be primarily in the centripetal direction (both convective and diffusive), with transport across the posterior stroma occurring solely by diffusion in the antero-posterior direction (see Fig. 5).

Figure 6 shows a plot of the concentration of albumin in the anterior cornea as a function of the flowrate Q passing through the corneal stroma for values of the parameters shown in Table I. The two values of corneal albumin loss rate that are shown ($k_c = k_e D_p / h_a D_a = 0{\cdot}09$ and $0{\cdot}5$ days^{-1}) represent the range of values available in the literature (Maurice and Watson, 1965; Wiig, 1990); they correspond to $k_e = 1 \times 10^{-7}$ and 7×10^{-7} cm sec^{-1}, respectively. Kim et al. (1971) reported the permeability of the rabbit corneal endothelium to be between $0{\cdot}8 \times 10^{-7}$ and 3×10^{-7} cm sec^{-1} for macromolecules in the size range of albumin.

The results in this graph show that as k_c is increased, to keep $c(r)/c(R) = 0{\cdot}33$, Q must increase from $0{\cdot}007$ μl min^{-1} for $k_c = 0{\cdot}09$ days^{-1} to $0{\cdot}06$ μl min^{-1} for $k_c = 0{\cdot}5$ days^{-1} (the corresponding Pe_p increases from $0{\cdot}32$ to $0{\cdot}67$, respectively). We can check this prediction for consistency by estimating the flux of fluid through the posterior cornea using Darcy's law (Levick, 1987)

$$Q = \frac{\Delta P K_p A}{\mu L} \tag{3}$$

where K_p is the hydraulic conductivity of the cornea (approximately 10^{-14} cm^2) (Levick, 1987); ΔP, the swelling pressure difference between the sclera and cornea varies depending on corneal location, but is approximately 50 mm Hg (Wiig, 1989; Maurice, 1984), μ, is the viscosity of the extracellular fluid ($0{\cdot}007$ poise), A is the cross sectional area facing the flow and L is the length scale over which the pressure drop ΔP occurs.

The length scale L is unknown although we can bound its values. At one limit, we can assume the cornea to be homogeneous in the A/P direction, in which case, the pressure gradient is entirely in the centripetal direction. Then L becomes the radius of the cornea and $A = 2\pi R(h_a + h_p)$. This is consistent with the approach taken by Wiig (1989), and for the values in Table I, we find a flux of $0{\cdot}002$ μl min^{-1}.

However, our two-layered model has as its basis a significant compositional difference between anterior and posterior corneal stroma. If we assume that the anterior corneal stroma is highly conductive relative to the posterior stroma, then the majority of the drop in pressure will occur as the fluid crosses the posterior corneal stroma (note that several studies have demonstrated difference in swelling pressure between the anterior and posterior corneal stroma: Kikkawa and Hirayama, 1970; Lee and Wilson, 1981). In this case, length scale L becomes just the thickness of the posterior stroma (h_p) and A becomes πR^2. Then we find a flux of $0{\cdot}15$ μl min^{-1} for a bovine cornea with surface area of $2{\cdot}5$ cm^2. Thus, Darcy's law generates a range of flowrates through the corneal stroma ($0{\cdot}002$–$0{\cdot}15$ μl min^{-1}) which are consistent with the range of flowrates that our two-layered model predicts are necessary to produce the observed concentration distribution of albumin ($0{\cdot}007$–$0{\cdot}06$ μl min^{-1}).

Thus we conclude that the non-uniform A/P distribution of albumin in the cornea is caused by a differing excluded volume fraction between the anterior and posterior stroma, and that the radial distribution of albumin originates in a limbal source of proteins that are transported through the cornea by the effects of diffusion with a small convective contribution.

Discussion

Morphologic differences in lamellar structure between the anterior and posterior regions of corneal stroma have been reported in rabbit and human (McTigue, 1967; Komai and Ushiki, 1991; Freund et al., 1992). McTigue (1967) pointed out that the conjunctival substantia propia, which is representative of the dermal substantia propria, continues as part of

the corneal stroma. This dermal substantia propria contribution is represented by the superficial layers of the cornea which possess lamellae running in a more oblique angle with respect to each other than those in the posterior stroma. Clinically, certain corneal dystrophy's preferentially affect either the anterior or posterior stroma. (e.g. central crystalline dystrophy, McTigue, 1967; Freddo et al., 1989).

Previous studies have shown that in the rabbit, cat, and bovine corneas, the anterior lamellae have a lower swelling pressure than the posterior lamellae (Kikkawa and Hirayama, 1970). A difference in the amount of a relatively highly sulfated GAG fraction between the anterior and posterior parts of bovine corneal stroma (Anseth, 1961) has also been reported. Analyses of bovine corneas have indicated an increasing ratio of keratan sulfate proteoglycan to dermatan sulfate proteoglycan from the anterior to the posterior stroma (Bettelheim and Goetz, 1976; Castoro et al., 1988). The uneven distribution of GAGs in the stroma is likely responsible for the non-uniform swelling properties of the corneal stroma (Kikkawa and Hirayama, 1970; Lee and Wilson, 1981).

Several differences exist between the rabbit cornea and the corneas of humans and bovines examined in the present studies. For example, the rabbit cornea does not exhibit a classic Bowman's layer (Kaye and Pappas, 1962), while this layer is well-developed in the human cornea (Beuerman and Pedroza, 1996). Despite this difference, the rabbit, bovine and human corneas are similar in several ways, such as swelling pressure distribution (Wiig, 1989), hydroxyproline and total sulphated polyanion contents (Scott and Bosworth, 1990). In particular, the protein concentration gradient from limbal to central cornea in both human (Allansmith and McClellan, 1975) and rabbit (Maurice and Watson, 1965) cornea, lead us to suspect that protein kinetics are likely similar in all three species. The distribution of albumin we found in the bovine and human corneal stromas suggests that macromolecular transport kinetics in the corneal stroma are non-uniform. This finding, combined with our modeling studies, suggests that transport of albumin through the anterior stroma must occur much more readily than through the posterior stroma. Previously published data on protein kinetics (Fig. 6 in Raviola, 1983; Fig. 5 in Freddo et al., 1990) support this view. Indeed, the distribution of glycosaminoglycans in the cornea shows a region within the anterior stroma with significantly less GAGs than the surrounding cornea (Bettelheim and Goetz, 1976; Castoro et al., 1988).

Maurice and Watson (1965) proposed that albumin diffused into the cornea from the limbus and then was lost across the corneal surfaces. The conjunctival blood vessels in the limbal region are known to leak macromolecules (Raviola, 1983), and these vessels represent a likely source for most of the albumin found in the corneal stroma. Examining the rabbit cornea, Maurice and Watson (1965) modeled the transport of albumin starting at this limbal reservoir and passing through the corneal stroma (in the centripetal direction). They modeled a solely diffusional process, with a transfer coefficient describing transport across the corneal surfaces, and they assumed that the albumin distribution was uniform between the anterior and posterior surfaces of the corneal stroma.

Our modeling studies showed that the effects of differing diffusion coefficients between the anterior and posterior corneal stroma were not sufficient to explain the non-uniform distribution of soluble proteins in the corneal stroma. Instead, this distribution was likely due to a difference in excluded volume fraction between the anterior and posterior stroma. The distribution of albumin from limbus to corneal apex may be influenced by a small convective flux of fluid passing from the limbal region, through the corneal stroma, and exiting through the corneal endothelium. The difference in swelling pressure between the sclera and the corneal stroma (and the difference between anterior and posterior stroma) provides a driving force for this flow. Moreover, Wiig (1990), who examined the transport of radiolabelled albumin in the cornea of live rabbits, found strong evidence in support of a bulk, centripetal flow and of a centripetal pressure gradient driving this flow (Wiig, 1989).

This small flux of fluid passing through the cornea may have some physiological significance in so far as it aids in the delivery of nutrients and the removal of waste products from this region. It also represents an additional source of aqueous humor protein that originates from the leaky limbal vessels, passes through the cornea, and enters the anterior chamber across the corneal endothelium. It is interesting in this regard to compare the estimated fluid flux rate to that pumped by the corneal endothelium. Several studies have estimated the pumping rate of the corneal endothelium by inhibiting the pump with ouabain or by cooling the eye; the studies indicate a resulting swelling rate of approximately $0{\cdot}045\ \mathrm{mm\ hr^{-1}}$ (Mishima et al., 1969) or a flux through a bovine corneal endothelium with a surface area of $2{\cdot}5\ \mathrm{cm^2}$ of approximately $0{\cdot}2\ \mu\mathrm{l\ min^{-1}}$. Our predicted flux of $0{\cdot}007$–$0{\cdot}06\ \mu\mathrm{l\ min^{-1}}$ would represent between approximately 5 and 30% of this flow.

Several caveats could be raised regarding the model we have developed. First, we have assumed that the distribution of albumin in the cornea seen in the photomicrographs reflects its in vivo distribution. The possibility exists that the distribution seen is partially due to differing accessibility of the antibody to different regions of the cornea or the possibility of a non-linear relationship between albumin concentration and stain density. Furthermore, the substantial reduction in staining for albumin in the posterior stroma may simply reflect that the concentration there is below the threshold that this method can detect. However, these

effects should only alter the magnitude of the differences in albumin concentration between the anterior and posterior stroma rather than cause these differences.

A second caveat that we have considered, but not explicitly included in our model, is the 'excluded volume effect' (Maurice, 1969). The differences in the excluded volume fraction between the anterior and posterior cornea will make a significant contribution to the observed distribution of albumin, but to a degree not easily assessed without detailed information on the proteoglycan distribution. However, it is important to note that a differing excluded volume fraction between the anterior and posterior corneal stroma necessarily leads to a small convective flow through the cornea due to the differing swelling pressures in these regions.

Additional studies are planned to test the kinetics of our proposed model and alterations of corneal homeostasis that might ensue following perturbation of epithelial or endothelial barrier function.

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References

Allansmith, M. R. and McClellan, B. H. (1975). Immunoglobulins in the human cornea. *Am. J. Ophthalmol.* **80**, 123–32.

Anseth, A. (1961). Studies on corneal polysaccharides. III. Topographic & comparative biochemistry. *Exp. Eye Res.* **1**, 106–15.

Barsotti, M., Bartels, S. P., Freddo, T. F. and Kamm, R. D. (1992). The source of proteins in the aqueous humor of the normal monkey eye. *Invest. Ophthalmol. Vis. Sci.* **33**, 581–95.

Bettelheim, F. A. and Goetz, D. (1976). Distribution of hexosamines in bovine cornea. *Invest. Ophthalmol.* **15**, 301–4.

Beuerman, R. W. and Pedroza, L. (1996). Ultrastructure of the human cornea. *Microsc. Res. Tech.* **33**, 320–35.

Box, G. E. P., Hunter, W. G. and Hunter, J. S. (1978). *Statistics for experimenters*, John Wiley & Sons, Inc.: New York, NY, U.S.A.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–54.

Castero, J. A., Bettelheim, A. A. and Bettelheim, F. A. (1988). Water gradients across bovine cornea. *Invest. Ophthalmol. Vis. Sci.* **29**, 963–8.

Cristol, S. M., Edelhauser, H. F. and Lynn, M. J. (1992). A comparison of corneal stromal edema induced from the anterior or the posterior surface. *Refractive Corneal Surgery* **8**, 224–9.

Freddo, T. F., Polack, F. M., Leibowitz, H. M. (1989). Ultrastructural changes in the posterior layers of the cornea in Schnyder's crystalline dystrophy. *Cornea* **8**, 170–7.

Freddo, T. F., Bartels, S. P., Barsotti, M. F. and Kamm, R. D. (1990). The source of proteins in the aqueous humor of the normal rabbit. *Invest. Ophthalmol. Vis. Sci.* **31**, 125–37.

Freund, D. E., McCally, R. L., Farrell, R. A., Cristol, S. M., Edelhauser, H. F. and L'Hernault, N. L. (1992). Comparison of the ultrastructure in anterior and posterior stroma of normal human and rabbit corneas and its relation to transparency. *Invest. Ophthalmol. Vis. Sci.* **33** (suppl.), 1411.

Hedbys, B. O. and Mishima, S. (1969). The flow of water across the corneal layers. In (Maurice Langham. Ed.), *The cornea, macromolecular organization of a connective tissue, Pp.* 69–77. Johns Hopkins Press: Baltimore, U.S.A.

Johnson, M., Gong, H., Freddo, T. F., Ritter, N. and Kamm, R. (1993). Serum protein and aqueous outflow resistance in bovine eyes. *Invest. Ophthalmol. Vis. Sci.* **34**, 3549–57.

Kaye, G. I. and Pappas, G. D. (1962) Studies on the cornea I: The fine structure of the rabbit cornea and the uptake and transport of colloidal particles by the cornea in vivo. *J. Cell Biol.* **12**, 457–79.

Kikkawa, Y. and Hirayama, K. (1970). Uneven swelling of the corneal stroma. *Invest. Ophthalmol.* **9**, 735–41.

Kim, J. H., Green, K., Martinez, M. and Paton, D. (1971). Solute permeability of the corneal endothelium and Descemet's membrane. *Exp. Eye Res.* **12**, 231–8.

Klyce, S. D. and Russell, S. R. (1979). Numerical solution of coupled transport equations applied to corneal hydration dynamics. *J. Physiol.* **292**, 107–34.

Komai, Y. and Ushiki, T. (1991). The three-dimensional organization of collagen fibrils in the human cornea and sclera. *Invest. Ophthalmol. Vis. Sci.* **32**, 2244–58.

Laemmli, U. K. (1970). Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) **227**, 680–5.

Lee, D. and Wilson, G. (1981). Non-uniform swelling properties of the cornea stroma. *Curr. Eye Res.* **1**, 457–61.

Levick, J. R. (1987). Flow through interstitium and other fibrous matrices. *Quar. J. Exp. Physiol.* **72**, 409–38.

Maurice, D. M. (1969). The physical state of water in the corneal stroma. In *The cornea, macromolecular organization of a connective tissue* (Langham, M. ed.), pp. 193–204.

Maurice, D. M. (1984). The cornea and sclera. In *The Eye*. (Davson, H. ed.). pp. 1–158. Academic Press: New York, U.S.A.

Maurice, D. M. and Watson, P. G. (1965). The distribution and movement of serum albumin in the cornea. *Exp. Eye Res.* **4**, 355–63.

McTigue, J. W. (1967). The human cornea: A light and electron microscopic study of the normal cornea and its alterations in various dystrophies. *Trans Am. Ophthal. Soc.* **65**, 591–660.

Mishima, S., Kaye, G. I., Takahashi, G. H., Kudo, T. and Trenberth, S. M. (1969). The function of the corneal endothelium in the regulation of corneal hydration. In *The cornea, macromolecular organization of a connective tissue*. (Langham, M. ed.), pp. 207–35.

Pavao, A. F., Lee, D. A., Ethier, C. R., Johnson, M., Anderson, P. J. and Epstein, D. L. (1989). Two-dimensional gel electrophoresis of calf aqueous humor, serum and filter-bound proteins. *Invest. Ophthalmol. Vis. Sci.* **30**, 731–38.

Raviola, G. (1983). Conjunctival and episcleral blood vessels are permeable to blood-borne horseradish peroxidase. *Invest. Ophthalmol. Vis. Sci.* **24**, 725–36.

Scott, J. E. and Bosworth, T. R. (1990). A comparative biochemical and ultrastructural study of proteoglycan-collagen interactions in corneal stroma. *Biochem. J.* **270**, 491–7.

Switzer, R. C., Merril, C. R., Shifrin, S. (1979). A highly sensitive silver stain for detecting protein and peptides in polyacrylamide gels. *Anal. Biochem.* **98**, 231–7.

Towbin, H., Staehelin, T., Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4.

Turss, R., Friend, J., Reim, M., Dohlman, C. (1971). Glucose concentration of hydration of the corneal stroma. *Ophthalmic. Res.* **2**, 253–60.

Wiig, H. (1989). Cornea fluid Dynamics I: Measurement of hydrostatic and colloid osmotic pressure in rabbits. *Exp. Eye Res.* **49**, 1015–30.

Wiig, H. (1990). Cornea fluid dynamics II: Evidence for transport of radiolabelled albumin in rabbits by bulk flow. *Exp. Eye Res.* **50**, 261–7.

Appendix

We here solve for the concentration of albumin in the albumin-accessible spaces of the anterior cornea including the effects of diffusion in the centripetal direction. Applying conservation of mass to a differential element of the anterior corneal stroma yields:

$$\frac{d\left[rh_a v_a(r)\, c_a(r) - D_a\, h_a\, r\frac{dc_a(r)}{dr}\right]}{dr} = -r\left[\left(1-\rho\right) v_p(r)\, c_a(r) + \frac{(c_a(r)-c_{ah})}{\frac{1}{k_e}+\frac{h_p}{D_p}}\right] \quad \text{(A1)}$$

where h_a is the thickness of the anterior cornea, v_a is the velocity of the fluid in the anterior cornea passing centripetally toward $r = 0$, ρ is the reflection coefficient of the posterior stroma/corneal endothelium characterizing convective albumin transport through this region and v_p the velocity of the fluid passing in the A/P direction in the posterior cornea*.

This equation balances the changes in convective flux of albumin passing through the anterior stroma in the centripetal direction (first term on left hand side of A1) and changes in diffusive flux of albumin in this same direction (second term on left hand side) with the albumin convecting (first term on the right hand side) and diffusing (second term on right hand side) anteroposteriorly, passing from the anterior stroma through the posterior stroma and into the anterior chamber.

* We here consider only A/P transport through the posterior cornea and treat any centripetal transport through this region as negligible based on an order-of-magnitude analysis of the full convective-diffusion equation.

Mass conservation of the fluid flow allows us to relate v_a to v_p:

$$v_a(r) = \frac{rv_p}{2h_a} \quad \text{(A2)}$$

Equation (A1) is combined with equation (A2). Then, we assume that $\rho = 1$ (complete rejection of convectively transport albumin (Hedbys and Mishima, 1969) with all transport through the posterior stroma and corneal endothelium occurring by diffusion), and we further assume that c_{ah} is negligible.

We introduce $y = r/R$, a scaled distance in the centripetal distance, and $Z(y) = c_a(y)/c_a(1)$, the scaled anterior albumin concentration, which is unity at the periphery ($y = 1$). Using the definitions of the anterior Peclet number

$$Pe_a = v_a(R)\, R/D_a = v_p R^2/D_a h_a$$

and posterior Peclet number that includes the effects of the diffusional resistance of the corneal endothelium, $(k_e/h_a)\,(D_p/D_a)$

$$Pe_p = (v_p\, h_p/D_p)^*(1 + D_p/h_p\, k_e),$$

we find that equations (A1) and (A2) yield

$$\frac{1}{Pe_a}\frac{d}{dy}\left[y\frac{dZ}{dy}\right] + \frac{1}{2}\frac{d[y^2 Z(y)]}{dy} - \frac{1}{Pe_p}yZ(y) = 0 \quad \text{(A3)}$$

with boundary conditions that $Z'(y = 0) = 0$ and $Z(y = 1) = 1$.

We look for a solution using a Taylor series approach:

$$Z(y) = \sum_{i=0}^{\infty} a_i\, y^i \quad \text{(A4)}$$

By then substituting into equation (A3) and equating equal powers of y, we find that

$$Z(y) = \frac{1 + \sum_{n=1}^{\infty} \frac{(-Pe_a/4)^n (y)^{2n}}{n!^2} \prod_{j=1}^{n} (j - 1/Pe_p)}{1 + \sum_{n=1}^{\infty} \frac{(-Pe_a/4)^n}{n!^2} \prod_{j=1}^{n} (j - 1/Pe_p)} \quad \text{(A5)}$$

This series converges only slowly, and for the highest Peclet numbers considered in Fig. 6, fourteen terms of the Taylor series had to be used to reduce the remaining error to less than 3%.

With equation (A5), we can solve for the distribution of albumin in the anterior stroma as a function of the anterior and posterior Peclet number (Pe_a and Pe_p) or equivalently, as a function of the flowrate through the cornea, $Q = \pi r^2 v_p$, and the endothelial transport coefficient, k_e (increasing Q is equivalent to increasing v_p and thus increases both the value of Pe_p and Pe_a, keeping the other parameters shown in Table I fixed).