Changes in the Refractive Index of the Stroma and Its Extrafibrillar Matrix When the Cornea Swells

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ABSTRACT The transparency of the corneal stroma is critically dependent on the hydration of the tissue; if the cornea swells, light scattering increases. Although this scattering has been ascribed to the disruption caused to the arrangement of the collagen fibrils, theory predicts that light scattering could increase if there is an increased mismatch in the refractive indices of the collagen fibrils and the material between them. The purpose of this article is to use Gladstone and Dale's law of mixtures to calculate volume fractions for a number of different constituents in the stroma, and use these to show how the refractive indices of the stroma and its constituent extrafibrillar material would be expected to change as more solvent enters the tissue. Our calculations predict that solvent entering the extrafibrillar space causes a reduction in its refractive index, and hence a reduction in the overall refractive index of the bovine stroma according to the equation $n'_s = 1.335 + 0.04/(0.22 + 0.24\,H')$, where n'_s is the refractive index and H' is the hydration of the swollen stroma. This expression is in reasonable agreement with our experimental measurements of refractive index versus hydration in bovine corneas. When the hydration of the stroma increases from $H = 3.2$ to $H = 8.0$, we predict that the ratio of the refractive index of the collagen fibrils to that of the material between them increases from 1.041 to 1.052. This change would be expected to make only a small contribution to the large increase in light scattering observed when the cornea swells to $H = 8$.

INTRODUCTION

The cornea is the major refracting lens in the eye, responsible for some two-thirds of the eye's total dioptic power. Measurements of corneal refractive index have been made by a number of researchers in a variety of animal species, and most authors report values very close to 1.375 (Maurice, 1957; Farrell and McCally, 2000; Sivak, 1988).

The cornea not only refracts most of the incident light, but it also transmits >95% of this light. Corneal transparency has been the subject of much study over the years (Maurice, 1957; Hart and Farrell, 1969; Smith, 1969; Feuk, 1970; Benedek, 1971; Twersky, 1975; Worthington, 1984; Freund et al., 1986, 1995). It is now generally accepted that transparency depends on the destructive interference of light scattered away from the forward direction and that this, in turn, requires a certain amount of short-range ordering of collagen fibril positions (Hart and Farrell, 1969; Farrell and McCally, 2000). In Farrell's model, the scattering cross section per unit length for an isolated fibril, σ, may be expressed as (Farrell and McCally, 2000):

$$\sigma = \frac{k^3(n_f + n_e)^2}{8n_e^4}\left\{1 + \frac{2}{(m^2+1)^2}\right\} \times \frac{R_0^2\rho_s^2}{\rho^2}\left\{\frac{(1-f_f^s)M_c - f_f^s M_g}{(1-f_f^s)}\right\}^2, \quad (1)$$

where k is the modulus of the scattering vector, $m = n_f/n_e$, ρ_s is the mass density of the stroma, ρ is the number of fibril axes per unit area in a cross-section cut, R_o is the refractive increment (the change in refractive index with solute concentration), f_f^s is the volume fraction occupied by the hydrated fibrils in the stroma, and M_c and M_g are the mass fractions of dry collagen in the fibrils and biomolecules in the extrafibrillar matrix, respectively. From this equation it is clear that the scattering cross section depends on n_f, n_e, and hence m, i.e., the refractive indices of the hydrated collagen fibrils, of the extrafibrillar matrix, and their ratio.

Apart from the uniform refractive index model (Smith, 1969), all other explanations of corneal transparency assume that there is a significant difference in the values of n_f and n_e. Unfortunately, the two components (collagen fibrils and extrafibrillar matrix) cannot easily be isolated and examined in their physiological state, so it is not possible to obtain direct measurements of their refractive indices accurately (Maurice, 1957). Instead, their values must be estimated from known physical and chemical properties of the stroma and its constituents. Maurice (1957) found the refractive index of dry collagen, n_c, to be 1.55, and went on to calculate n_f as 1.47 and n_e as 1.345. In a later article, these values were refined to $n_f = 1.51$ and $n_e = 1.345$ (Maurice, 1969).

Worthington (1984) used Gladstone and Dale's law of mixtures together with known values of the relative weights and densities of the corneal components to calculate the refractive indices. This was later refined by Leonard and Meek (1997) to give values of $n_f = 1.416$ and $n_e = 1.356$ for bovine corneal stroma. These were close to the values for human corneas reported by Freund and co-workers (Freund et al., 1995; $n_f = 1.407$ and $n_e = 1.352$). However, all the methods mentioned above rely on assumptions, many of which are now known to be incorrect. With measurements of volume fractions computed directly from x-ray diffraction

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data, Leonard and Meek (1997) used Gladstone and Dale's law to calculate the refractive indices for four species. They also reported average values from 40 species of $n_f = 1.416$ and $n_e = 1.359$. Very little difference in refractive indices was found between the species studied.

There is much uncertainty as to the mechanism by which light scattering increases as the cornea swells. Several changes occur in the tissue, some of which probably affect scattering more than others. For example, the hydrations of the various components are altered to differing degrees and their refractive indices change accordingly. The fibrils move further apart so that the phase difference between the various scattered waves is altered, changing scattering cross sections (Farrell and McCally, 2000). The number density and volume fraction of the fibrils both decrease. Freund et al. (1986) have produced a robust method for calculating corneal transmission from normal and swollen corneas, and have tested the method on slightly swollen corneas (up to 25%; Freund et al., 1991). However, to precisely relate changes in structure to changes in transparency as the cornea swells, it is necessary to know how the extra water is distributed (inside/outside fibrils, within ''lakes''; Meek et al., 1991; Huang and Meek, 1999), how the collagen fibril arrangement changes (Freund et al., 1991; Meek and Quantock, 2001), and how the refractive indices change. The purpose of the present article is to address this last issue. Only when we know how the refractive indices change as fluid enters the stroma can we model how this affects light scattering (Benedek, 1989).

In this article we develop a simplified theoretical model of the corneal stroma consisting of hydrated, pseudo-hexagonally packed collagen fibrils embedded in a homogeneous, hydrated matrix. First, we apply Gladstone and Dale's law of refractive indices to calculate the volume fraction occupied by solvent in the physiological stroma. Along the way, we calculate values for a number of important structural parameters in the cornea. In the second part of the article, we apply the same law to determine a relationship between the refractive index of the whole stroma, and later the refractive index of the extrafibrillar matrix, as a function of tissue hydration. In the final part of the article, we compare the results of the theoretical variation of stromal refractive index versus hydration, with experimentally measured values.

Gladstone and Dale's law of mixtures applied to stroma at physiological hydration

According to Gladstone and Dale's law, the refractive index of a composite may be expressed as the partial sum of the refractive indices of its components $n_1, n_2, \ldots n_N$, each weighted by the volume fraction occupied by that component, $f_1, f_2, \ldots f_N$ (Maurice, 1957; Worthington, 1984):

$$n_{tot} = n_1 f_1 + n_2 f_2 + \cdots + n_N f_N. \quad (2)$$

Gladstone and Dale's law allows us to calculate a number of different volume fractions and refractive indices. Applying this law to the corneal stroma, and substituting known values for the refractive index of the bovine cornea, the refractive index of dry collagen and the refractive index of salt solution (solvent) (Table 2), the following expressions can be derived (the derivation of these equations is given in detail in Leonard and Meek, 1997, their Eqs. 9 and 10; however, the equations have been slightly modified from those in Leonard and Meek, 1997, by replacing the refractive index of water, 1.333, by the refractive index of salt solution, 1.335):

$$n_f = 1.335 + 0.212 f_c^f \quad (3)$$

$$n_e = 1.335 + \frac{0.042 - 0.212 f_c^s}{1 - f_f^s}, \quad (4)$$

where f_c^s and f_f^s are the volume fractions of dry fibrillar material in the stroma and hydrated fibrils in the stroma, and f_c^f is the volume fraction of collagen molecules in a fibril (see Table 1). The volume fractions f_c^s and f_f^s are related by:

$$f_c^f = f_c^s / f_f^s. \quad (5)$$

The value of f_c^f can be found by considering a unit cell along the length of a collagen fibril (Katz and Li, 1973),

$$f_c^f = \rho M_c / 5 D p_m^2 N_A \sin\gamma, \quad (6)$$

where ρ is the partial specific volume of collagen, M_c is the molecular weight of collagen, D is the collagen axial periodicity, p_m is the center-to-center lateral spacing of

TABLE 1 Definitions and values for various volume fractions when the stroma is at physiological hydration

Symbol	Meaning	Value
f_c^f	Volume fraction of collagen molecules in a hydrated fibril	0.37*
f_c^s	Volume fraction of dry fibrillar material in the stroma	0.12*
f_p^s	Volume fraction of dry extrafibrillar material in the stroma	0.10*
f_f^s	Volume fraction of hydrated fibrillar material in the stroma	0.32*
f_e^s	Volume fraction of hydrated extrafibrillar material in the stroma	0.68*
f_{iw}^s	Volume fraction of intrafibrillar solvent in the stroma	0.20
f_{ew}^s	Volume fraction of extrafibrillar solvent in the stroma	0.58
f_w^f	Volume fraction of solvent in a fibril	0.63
$f_p'^e$	Volume fraction of dry extrafibrillar material in the swollen extrafibrillar matrix	Function of tissue hydration
$f_p'^s$	Volume fraction of dry extrafibrillar material in the swollen stroma	Function of tissue hydration
$f_e'^s$	Volume fraction of hydrated extrafibrillar material in the swollen stroma	Function of tissue hydration

*Leonard and Meek (1997).

TABLE 2 Definitions and values for refractive indices when the stroma is at physiological hydration

Symbol	Meaning	Value
n_s	Refractive index of stroma at physiological hydration	1.375*
n_f	Refractive index of hydrated fibrils	1.413
n_e	Refractive index of hydrated extrafibrillar matrix	1.359
n_c	Refractive index of dry collagen	1.547†
n_p	Refractive index of dry extrafibrillar material	1.485
n_w	Refractive index of solvent (salt solution)	1.335‡

*Sivak (1988).
†Maurice (1957, 1969).
‡Farrell and McCally (2000).

collagen molecules within a fibril, γ is the packing angle of the collagen molecules, and N_A is Avogadro's number. Substituting known values of these parameters and the value of p_m obtained from wide-angle x-ray diffraction, Leonard and Meek (1997) obtained the value $f_c^f = 0.37$.

Leonard and Meek (1997) considered the stroma to consist of ''unit cells'' representing the average volume occupied by each fibril (thus neglecting the contribution of keratocytes to the stromal volume). With this model, they estimated the volume fraction per unit length of fibrils in the stroma, f_f^s, from the interfibrillar center-to-center Bragg spacing, p_i, and the fibril diameter, a, both of which can be measured from low-angle x-ray diffraction patterns from the cornea (Gyi et al., 1988; Meek and Leonard, 1993), using

$$f_f^s = \pi a^2/(4 \times 1.12\, p_i^2). \quad (7)$$

The factor 1.12 relates the Bragg spacing from a liquidlike arrangement of fibrils (Worthington and Inouye, 1985) to the equivalent mean center-to-center spacing of the fibrils in a pseudo-hexagonal lattice. For bovine cornea at physiological hydration they calculated a value of $f_f^s = 0.32 \pm 0.08$. Although the uncertainty was rather large, the mean value was close to the average value of the fibril volume fraction from 40 species ($f_f^s = 0.28 \pm 0.03$). Using the calculated values of f_c^f, $f_c{}^s$, and f_f^s specific for cow (Table 1), we can substitute into Eqs. 3 and 4 to obtain $n_f = 1.413$ and $n_e = 1.359$.

By compartmentalizing the hydrated stroma into hydrated collagen fibrils, dry extrafibrillar matrix, and extrafibrillar solvent, we can, for completeness, calculate the volume fractions of fibrillar and nonfibrillar fluid, although for the purposes of later arguments, separation into fibrillar and nonfibrillar compartments is not really necessary. Gladstone and Dale's law may be written as

$$n_s = n_f f_f^s + n_p f_p^s + n_w f_{ew}^s, \quad (8)$$

where the meaning of the symbols is defined in Tables 1 and 2.

We also know that

$$f_f^s + f_p^s + f_{ew}^s = 1, \quad (9)$$

from which we can use known values of f_f^s and f_p^s to calculate $f_{ew}^s = 0.58 \pm 0.08$. The estimated uncertainty is based on the precision of the value for f_f^s.

We therefore know values of all the terms in Eq. 8 except n_p, which can thus be determined, giving the value $n_p = 1.485$. The uncertainties in the values of f_f^s and f_{ew}^s imply that this figure has a precision of better than 4%.

The next step is to consider the compartmentalization of the solvent and the collagen within each fibril. Using the fact that $f_c^f + f_w^f = 1$, we see that $f_w^f = 0.63$. This value is written in Table 1.

Finally, we can calculate the volume fraction of intrafibrillar solvent in the stroma (f_{iw}^s) by using the fact that $f_{iw}^s = f_w^f \times f_f^s$. This value, $f_{iw}^s = 0.20$, is also presented in Table 1.

Gladstone and Dale's law applied to swollen corneas

Dependence of stromal refractive index on tissue hydration

Since the volume fractions of intrafibrillar and extrafibrillar solvent in the stroma are 0.20 and 0.58 respectively (Table 1), the total volume fraction of solvent in the stroma, f_w^s, is 0.78. If the cornea swells such that the volume of solvent increases by a factor P to $(1 + P) \times$ its initial value, then to a good approximation its hydration (weight of water/dry weight) also increases by a factor P (since the mass of the additional ions introduced is negligible compared with the mass of the stroma).

In this section, we will use the convention that primed notation refers to the values of parameters in the swollen cornea. Thus using V to represent the initial volume of the stroma, V_w for the initial volume of solvent in the stroma and V' for the new (swollen) volume of the stroma, we can write

$$V' = V + PV_w. \quad (10)$$

Hence,

$$V'/V = 1 + Pf_w^s, \quad (11)$$

where V_w/V is the volume fraction of the total solvent content of the stroma, f_w^s.

With the value for f_w^s given above, we get

$$V'/V = 1 + 0.78\,P. \quad (12)$$

It has been shown previously that, above physiological hydration, swelling of corneal collagen fibrils is negligible (Meek et al., 1991). If the additional solvent therefore does not go into the fibrils, the refractive index of the fibrils remains unchanged but the extra solvent in the extrafibrillar space will cause a reduction in the value of n_e. Furthermore, because the volume of the stroma is now greater, the various stromal volume fractions will change. The purpose of this section is to determine what changes occur in the volume fractions and the refractive indices, and hence to derive an

expression for the expected change in the refractive index of the stroma as the tissue swells.

Equation 12 states that when the stroma swells, its volume increases by the factor 0.78 P. The volume fractions can be defined in terms of the new volume of the fibrils, V'_f, and the new volume of the swollen stroma as follows:

$$f'^{s}_{f} = V'_f/V'. \tag{13}$$

Since we assume the fibrils do not swell (Meek et al., 1991), the new volume of the fibrils, V'_f, is the same as the original volume of the fibrils, V_f, and substituting from Eq. 12,

$$f'^{s}_{f} = V_f/\{(1 + 0.78\,P)V\} = f^{s}_{f}/(1 + 0.78\,P), \tag{14}$$

where we have used the definition $f^{s}_{f} = V_f/V$. Similarly, since the volume occupied by dry extrafibrillar material, V_p, does not change, the new volume fraction of the dry extrafibrillar material in the stroma, f'^{s}_{p}, can be defined as

$$f'^{s}_{p} = V_p/V' = V_p/\{(1 + 0.78\,P)V\} = f^{s}_{p}/(1 + 0.78\,P). \tag{15}$$

Finally, since $f'^{s}_{ew} = 1 - (f'^{s}_{f} + f'^{s}_{p})$, we get

$$f'^{s}_{ew} = 1 - \{(f^{s}_{f} + f^{s}_{p})/(1 + 0.78\,P)\}. \tag{16}$$

With these new volume fractions, we can apply Gladstone and Dale's law to the swollen stroma:

$$n'_s = f'^{s}_{f} n_f + f'^{s}_{p} n_p + f'^{s}_{ew} n_w \tag{17}$$

or

$$n'_s = n_w + \{f^{s}_{f} n_f + f^{s}_{p} n_p - (f^{s}_{f} + f^{s}_{p}) n_w\}/(1 + 0.78\,P)\}. \tag{18}$$

This can be simplified by substituting the values from Tables 1 and 2:

$$\begin{aligned} n'_s &= 1.335 + (0.32 \times 1.413 + 0.10 \times 1.485 \times \\ &\quad (0.32 + 0.10) \times 1.335)/(1 + 0.78\,P) \\ &= 1.335 + 0.04/(1 + 0.78\,P). \end{aligned} \tag{19}$$

This equation shows how the refractive index of the stroma should vary as a function of the fractional increase in solvent above physiological hydration (P). To reexpress this in terms of the physiological value of the tissue hydration (H_{phys}) and the hydration of the swollen cornea (H'), we use the fact that:

$$H' = (1 + P)H_{phys} \tag{20}$$

or

$$P = (H'/H_{phys}) - 1.$$

Using the known value $H_{phys} = 3.2$ and substituting into Eq. 19 gives:

$$n'_s = 1.335 + 0.04/(0.22 + 0.24\,H'). \tag{21}$$

This relationship is plotted in Fig. 1. It should be noted that the model predicts that the refractive index of the swollen stroma depends on knowledge of only two parameters in the physiological stroma, the refractive index (obtained by setting $P = 0$ in Eq. 19), and the total volume fraction of the solvent (the value 0.78 in Eq. 19). The value for n_s used in the present work was taken from Leonard and Meek (1997), where no uncertainty was quoted. However, other measurements, including those from the present work, suggest this figure is accurate to $>\pm 0.2\%$. Our value for the volume fraction of solvent in the stroma was derived using some values for which the uncertainty was also not quoted. The only other determination we have seen for this quantity comes from Worthington (1984) who quotes 0.819 (again without a precision estimate), which differs from our value by 5%. Using these uncertainties, we have estimated the precision with which our theoretical relationship (Eq. 21) is known, and these confidence limits are included in Fig. 1.

Dependence of n_e and m on tissue hydration

As can be seen from Eq. 1, the important refractive indices, as far as transparency of the cornea is concerned, are those of the collagen fibrils (n_f) and the extrafibrillar matrix (n_e) as well as their ratio (m). It is now possible to calculate how these vary as solvent enters the stroma. On the assumption that the fibrils themselves do not swell (Meek et al., 1991), n_f will remain unchanged (i.e., $n'_f = n_f$). To calculate the dependence of n'_e on P, we start by applying Gladstone and Dale's law to the extrafibrillar matrix,

$$n'_e = n_w + f'^{e}_{p}(n_p - n_w), \tag{22}$$

where f'^{e}_{p} is the volume fraction of dry proteoglycans, etc., in the swollen extrafibrillar matrix.

But $f'^{e}_{p} = f'^{s}_{p} / f'^{s}_{e}$ where volume fractions are defined in Table 1, and from Eq. 15 and the known value of f^{s}_{p},

$$f'^{s}_{p} = \frac{f^{s}_{p}}{1 + 0.78\,P} = \frac{0.10}{1 + 0.78\,P}. \tag{23}$$

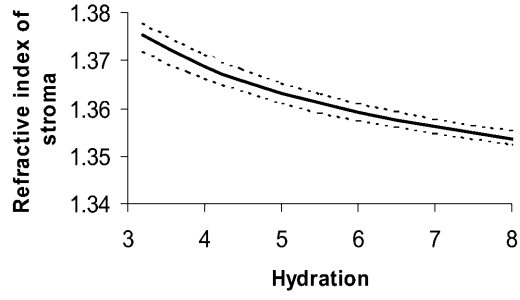

FIGURE 1 Refractive index of stroma as a function of tissue hydration calculated using Gladstone and Dale's law. Dotted lines indicate confidence limits of the solid curve.

Also, since $f'^{s}_{e} + f'^{s}_{f} = 1$, we substitute for f'^{s}_{f} using Eq. 14 and write

$$f'^{s}_{e} = 1 - \{f^{s}_{f}/(1 + 0.78\,P) = 1 - 0.32/(1 + 0.78\,P), \tag{24}$$

using the known value of f^{s}_{f}.

We can thus use Eqs. 23 and 24 to determine f'^{e}_{p}:

$$f'^{e}_{p} = \frac{0.10}{0.68 + 0.78\,P}. \tag{25}$$

We can now substitute Eq. 25 into Eq. 22, insert known values from Table 1, and get the following:

$$n'_{e} = 1.335 + 0.015/(0.68 + 0.78\,P). \tag{26}$$

As before, this can be expressed in terms of H':

$$n'_{e} = 1.335 + 0.015/(0.244\,H' - 0.1). \tag{27}$$

This relationship is plotted in Fig. 2. The confidence limits represent the effects of the uncertainties in the values of n_p, f^{s}_{f}, and $(f^{s}_{iw} + f^{s}_{ew})$.

Finally, the ratio m' can be expressed as:

$$m' = n'_{f}/n'_{e} = 1.413/\{1.335 + 0.015/(0.244\,H' - 0.1)\}. \tag{28}$$

This relationship is plotted in Fig. 3.

Measurement of corneal refractive index

To test the relationship derived in Eq. 21 (Fig. 1), the refractive indices of swollen bovine corneas were measured as a function of tissue hydration.

Samples

Fresh bovine eyeballs were obtained from the abattoir and the corneal discs were excised from the eyes within 3 h of death. The endothelium and epithelium were removed by scraping with a scalpel and the epithelium side tagged using cotton. The corneas were wrapped in clingfilm and left at 4°C until needed.

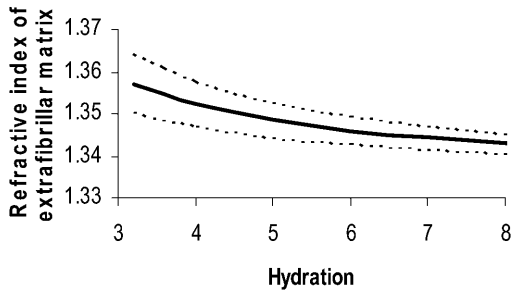

FIGURE 2 Refractive index of extrafibrillar matrix as a function of tissue hydration calculated using Gladstone and Dale's law. Dotted lines indicate confidence limits due to uncertainties in experimental data.

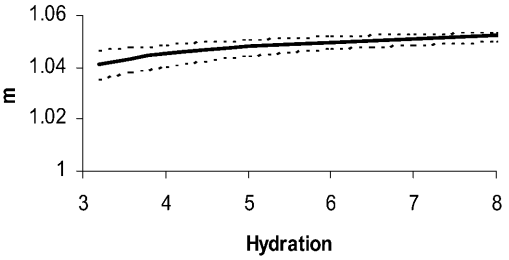

FIGURE 3 Refractive index ratio, m, as a function of tissue hydration, calculated using Gladstone and Dale's law. Dotted lines indicate confidence limits due to uncertainties in experimental data. The value $m = 1$ corresponds to the situation where the refractive indices of the collagen fibrils and the extrafibrillar matrix are equal. This was found not to be the case for either the physiological or the swollen stroma.

Tissue equilibrium

The individual corneas were placed in 14-kDa cutoff dialysis tubing, which was carefully smoothed to ensure no air bubbles were trapped inside. Each piece of dialysis tubing was clamped at both ends and then placed into an equilibration solution containing a fixed concentration of polyethylene glycol. Buffer equilibrium solutions of Na_2HPO_4/NaH_2PO_4 at pH 7.1 were used and NaCl was added as required to reach a final ionic strength (μm) of 0.03 as previously described by Huang and Meek (1999). Concentrations of 0, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 2.7, 3.0, and 3.5% polyethylene glycol (20 kDa, BDH Ltd., Warwickshire, England) were used to adjust the hydration of the tissues (Meek et al., 1991). The refractive index of the swollen tissue was then measured as described below. The corneas were reweighed to allow an average hydration during the course of the experiment to be calculated. They were then placed in an oven at 60°C until a constant dry weight was obtained.

Tissue hydration (H) was calculated using the following equation:

$$H = \frac{Average\,Wet\,Weight - Dry\,Weight}{Dry\,Weight}$$

Refractometry

A bench-top Abbe 60 Series Refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, England) was used for the experiment. This was calibrated using a silica test plate of known refractive index, supplied with the instrument, and the calibration was checked using a series of sugar solutions of known refractive index. The instrument was standardized before each experiment by adjusting the illumination to give a clear black/white boundary from distilled water. Transmitted illumination was from a bench lamp and reflected illumination from an in-built LED light source to observe the critical angle. This resulted in one side of the field of view from each cornea appearing black and the other white. All measurements were made at room temperature.

The cornea was placed on the refractometer stage tag-side-(anterior stroma)-up and the refractive index measured by adjusting the LED light source until a good borderline quality was observed. The cornea was then placed posterior-side-up and the refractive index was measured in the same way.

The average refractive index measurements from anterior and posterior stroma as a function of tissue hydration are shown in Fig. 4 *a*, and the predicted relationship (Eq. 21) is superimposed for comparison. A Pearson linear correlation analysis (Fig. 4 *b*) yielded a significant positive correlation of 0.78 ($p < 0.01$) between the experimental points and their corresponding theoretical values.

DISCUSSION

Leonard and Meek (1997) used their versions of Eqs. 3 and 4 to calculate the values $n_f = 1.413$ and $n_e = 1.357$. Corneal keratocytes are thought to occupy ~10% of the stromal volume (Kaye, 1969) and this was neglected in Leonard and Meek's calculations based on their x-ray diffraction data. However, if we assume the refractive index of the cells matches that of the extrafibrillar space, we can reapply Gladstone and Dale's law, taking into account the volume occupied by the keratocytes. If 10% of the stromal volume is not available to the fibrils, the fibrillar volume fraction is reduced from 0.32 to 0.29 and the volume fraction of the extrafibrillar material is increased from 0.68 to 0.71. From Eqs. 3 and 4, the effect of these changes is to leave the value of n_f unaltered, but to reduce the calculated value of n_e to 1.358. This represents <0.1% change in the value of the refractive index when the presence of cells is taken into account. For this reason it was decided to neglect the effects of keratocytes in the present work.

The refractive index of a polymer solution (such as the extrafibrillar matrix of the corneal stroma), n_e, can be expressed in terms of the specific refractive increment of the constituent proteins (R_o) and on their concentration (c):

$$n_e = n_w + R_o c. \tag{29}$$

For the cornea at physiological hydration, Eq. 25 gives the value $f_p^e = 0.147$ (setting $P = 0$). The density of the extrafibrillar material when dry is 1.06 gm/ml (Leonard and Meek, 1997), so its concentration is 1.06×0.147, which equals 0.156 gm/ml. The value of R_o for most proteins is ~0.18 ml/gm (Farrell and McCally, 2000). Substituting for these values, Eq. 29 gives $n_e = 1.362$ (taking the values of all parameters to three decimal places in the calculations). Within the precision of the volume fractions quoted in Table 1, we regard this to be in reasonable agreement with the value of $n_e = 1.359$ calculated in the present work.

We have shown both from a theoretical standpoint and experimentally how the average refractive index of the corneal stroma is reduced as the tissue swells. The agreement between experiment and theory, though showing a significant correlation, is clearly not exact (Fig. 4). Inspection of the distribution of data points in Fig. 4 *a* suggests that there is a shallower slope in the experimental data compared to the experimental curve. It is well-known that the anterior stroma swells very little in vitro (Müller et al., 2001), so its refractive index will not change much as the cornea as a whole swells. Conversely, most of the swelling takes place below these anterior layers, so changes in tissue hydration should primarily be reflected in changes in the refractive index of the posterior lamellae (Patel et al., 2000). This being the case, it is interesting to plot the swelling data for the anterior and the posterior stroma separately (Fig. 5). Despite the scatter in the experimental data points, it is evident that the theoretical expression fits the posterior swelling data (Fig. 5 *a*) better than the anterior data (Fig. 5 *b*), as expected.

Some caution is needed when applying the results to corneas swollen in vivo. For example, bulbous keratopathy, a condition where the stroma swells after surgical intervention, is known to be accompanied by changes in the composition of the extrafibrillar matrix (Quantock et al., 1991) which, in turn, may be expected to alter the refractive index in a way not predicted by the current analysis. However, the contribution of the nonaqueous fraction of the extrafibrillar matrix to the refractive index is relatively small (the matrix is very hydrated and becomes more so as the

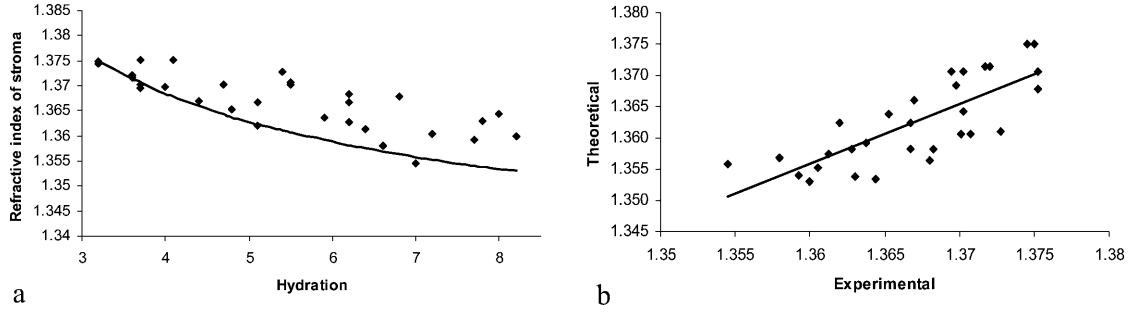

FIGURE 4 (*a*) Experimental values of the refractive index of bovine stroma (each point represents the average of anterior and posterior measurements from a given cornea) compared with the theoretical variation predicted by Eq. 21 (*continuous line*). (*b*) Linear regression of experimental and theoretical data in Fig. 4 *a* indicates a correlation coefficient of 0.78.

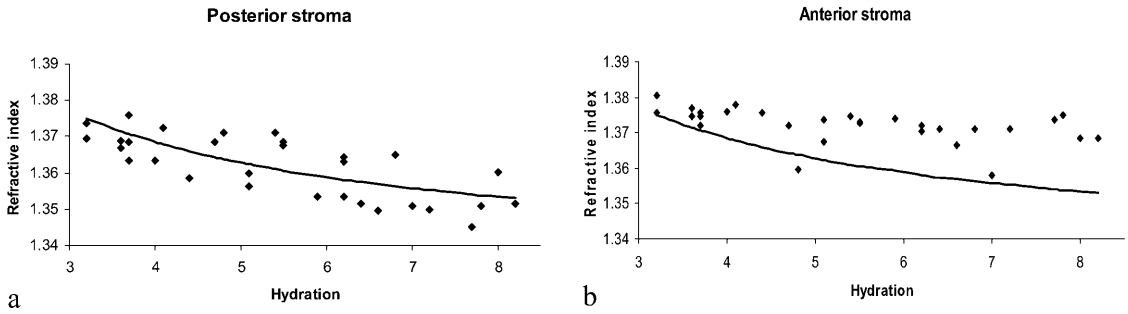

FIGURE 5 Refractive index measured at the posterior stroma (*a*) and anterior stroma (*b*). In each case, the continuous line is the theoretical prediction (Eq. 21).

tissue swells) and it will thus make only a small contribution to the refractive index.

In this article we have derived a simple expression that relates the refractive index of the corneal stroma to the increase in the volume fraction of solvent as the tissue swells (Eq. 19). From this it can be seen that only two parameters are required to specify the new refractive index, the refractive index of the stroma at physiological hydration and the volume fraction of solvent in the physiological stroma. We have calculated our value for the latter (78%) from the volume fractions of a number of other constituents, and it is in good agreement with the value of 77.2% estimated from the chemical composition of the stroma (Leonard and Meek, 1997). The change in refractive index with corneal swelling was previously studied by Fatt and Harris (1973), who produced a formula relating corneal refractive index to corneal thickness. However, their equation is asymptotic to $n_s = 1.342$, whereas, at very large hydrations, the refractive index of the stroma must approach that of the solvent, as does our Eq. 19.

In the bovine cornea, the refractive index is different on the epithelial and endothelial sides, as previously reported for human and porcine corneas (Patel et al., 1995; Watanabe and Uozato, 2001). This refractive gradient was shown by Patel and co-workers to have no practical importance in terms of the power of the normal cornea and may simply arise from the differential hydration between the anterior and posterior cornea (Castoro et al., 1988).

Changes in the refractive index of the extrafibrillar matrix as the cornea swells would be expected to affect light scattering. According to Eq. 1, an increase in m, the ratio of the refractive indices of the fibrils and the matrix, would cause scattering to increase, and it is possible that this contributes to the increased light scattering observed in swollen corneas. The actual effect of such a change is difficult to assess because the swelling will be accompanied by changes in other parameters on which transmission depends, such as order in the packing of the fibrils and their number density. However, if we consider the effects of changes in refractive index alone, assuming these other parameters remain constant, the transparency model of Freund et al. (1986) predicts that the change in refractive indices between $H = 3.2$ and $H = 8$ reported here would cause an ~5% increase in light scattering (Leonard, 1996; Meek et al., 2003), considerably less than the increase actually observed. It appears, therefore, that changes in refractive index of the extrafibrillar material make only a small contribution to the observed increase in light scattering when the cornea swells.

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