Refractive Indices of the Collagen Fibrils and Extrafibrillar Material of the Corneal Stroma

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ABSTRACT Ultrastructural data from x-ray diffraction studies of the cornea were used to estimate the refractive indices of the collagen fibrils and extrafibrillar material of human, ox, trout, and rabbit corneas. X-ray diffraction measurements of the size and spacing of the collagen fibrils and the separation between the constituent molecules of the fibrils were taken from a previous species study. The tissue volume fractions occupied by the stromal components were estimated and their refractive indices were calculated using the Gladstone-Dale law of mixtures. For the fibrils and extrafibrillar material, the refractive indices in the human cornea were 1.411 and 1.365; for the ox 1.413 and 1.357; for the rabbit 1.416 and 1.357; and for the trout 1.418 and 1.364, respectively. An alternative estimate based on the physical properties and chemical composition of bovine cornea, accounting for interfibrillar type VI collagen and cellular water, produced similar estimates of 1.416 and 1.356 for the fibrils and extrafibrillar material, respectively.

INTRODUCTION

To fulfill its function as the "window of the eye," the cornea must be both tough and transparent. These dual properties are achieved through the ultrastructure of the corneal stroma, which constitutes ~90% of the thickness of the cornea. The stroma is made up of stacked lamellae, each containing parallel fibrils of collagen embedded in a viscous extrafibrillar material, or "ground substance," rich in proteoglycans and glycoproteins (Maurice, 1957).

Several mathematical treatments have been put forward to explain corneal transparency in terms of light scattering from the collagen fibrils (Maurice, 1957; Hart and Farrell, 1969; Smith, 1969; Cox et al., 1970; Feuk, 1970; Benedek, 1971; Twersky, 1974; Worthington, 1984; Freund et al., 1986; Freund et al., 1995). Any such calculation must consider the difference between the refractive index of the fibrils and the refractive index of the surrounding extrafibrillar material because this difference influences the amount of light scattered by each fibril. If the two refractive indices were identical, no scattering would take place. This explanation has been proposed as the reason for corneal transparency (Davson, 1949; Smith, 1969), but is not generally accepted because it fails to account for other optical properties of the cornea, namely its birefringence and its tendency to become opaque when mechanically distorted (Maurice, 1957).

Instead, the experimental evidence points to a small difference between the two refractive indices, and in this case each fibril will scatter light. The scattering cross section per fibril depends approximately on the ratio of the refractive index of the fibrils, n_f, to that of the extrafibrillar material, n_e, through a factor $(m^2 - 1)^2$, where $m = n_f/n_e$, (Hart and Farrell, 1969). Because m is close to unity, this term is rather sensitive to small changes in the values of the refractive indices.

The fibrils cannot be separated from the extrafibrillar material while maintaining physiological conditions, so it is not possible to make direct measurements of either refractive index. However, their values can be estimated using Gladstone and Dale's law of mixtures, provided that the refractive indices of the individual components are known along with their volume fractions in the tissue.

By using x-ray diffraction, it is possible to measure average values for the size of the collagen fibrils, the spacing between fibrils, and the spacing between their constituent molecules (Meek and Leonard, 1993). This information is sufficient to calculate the necessary volume fractions, and hence the refractive indices, of the fibrils and extrafibrillar material.

Alternatively, it is possible to estimate the volume fractions from the known physical and chemical properties of the cornea (Worthington, 1984). This method requires details of corneal composition such as the relative weights and densities of the various components, the proportions of fibrillar and nonfibrillar collagen in the tissue, and the amount of water contained in the stromal cells.

In this study we calculate the refractive indices of the collagen fibrils and extrafibrillar material from human, rabbit, ox, and trout corneas using x-ray diffraction data. The results for the ox are compared with estimates made using recent data on the physical and chemical composition of the bovine cornea.

Gladstone and Dale's Law of Mixtures

The refractive index, n_{tot}, of a mixture may be expressed as the partial sum of the refractive indices of its components,

Received for publication 5 August 1996 and in final form 12 November 1996.

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0006-3495/97/03/1382/06 $2.00

$n_1, n_2, \ldots, n_N$, weighted by the volume fraction of each component, $f_1, f_2, \ldots, f_N$, (Maurice 1957; Worthington 1984):

$$n_{tot} = n_1 f_1 + n_2 f_2 + \cdots + n_N f_N \quad (1)$$

Inasmuch as $f_1 + f_2 + \ldots + f_N = 1$, Eq. 1 may be rewritten:

$$n_{tot} = n_1 + f_2(n_2 - n_1) + f_3(n_3 - n_1) + \cdots f_N(n_N - n_1)$$

The stroma is considered to consist simply of fibrils and extrafibrillar material. Taking the fibrils to be a mixture of dry fibrillar material (collagen molecules) and water, and using the nomenclature given in Table 1, the refractive index of the fibrils is given by:

$$n_f = n_w + f_m(n_m - n_w) \quad (2)$$

The fluid between the molecules is approximated to water on the assumption that the large macromolecular constituents of the extrafibrillar material cannot penetrate between the closely packed collagen molecules in the fibrils. Small ions will probably penetrate, but their concentration would not be expected to have a significant effect on the refractive index of the intermolecular fluid. Such ions are present in the aqueous humour, which has a refractive index of 1.335 (Payrau et al., 1967), almost the same as water, which is 1.333.

The extrafibrillar material is considered as a mixture of dry nonfibrillar material and water:

$$n_e = n_w + f_n(n_n - n_w) \quad (3)$$

and the whole stroma as a mixture of dry fibrillar material, dry nonfibrillar material, and water:

$$n_s = n_w + f_c(n_m - n_w) + f_p(n_n - n_w) \quad (4)$$

There are several volume fractions because the tissue can be compartmentalized in different ways and each component has a different amount of water associated with it. In each case the volume fraction refers to the proportion of the given component in a hydrated compartment.

TABLE 1 Nomenclature for volume fractions and refractive indices within the corneal stroma

Symbol	Volume Fraction	Symbol	Refractive Index
f_c	Dry fibrillar material in the stroma	n_m	Dry collagen = 1.547*
f_p	Dry, nonfibrillar material in the stroma	n_n	Nonfibrillar material
f_n	Dry, nonfibrillar material in the extrafibrillar space	n_f	Hydrated fibrils
f_f	Hydrated fibrils in the stroma	n_e	Hydrated extrafibrillar material
f_m	Collagen molecules in a fibril	n_s	Hydrated stroma = 1.375#
		n_w	Water = 1.333

Values are given for those refractive indices, which have been independently measured and are known with some confidence.
*Maurice 1957; Maurice 1969.
#Sivak 1988; Maurice 1957.

In Eq. 2 the volume fraction f_m of collagen in the fibril is simply the volume fraction of dry collagen in the stroma divided by the volume fraction of hydrated fibrils in the stroma, $f_m = f_c/f_f$. (If the stroma has volume V_s and contains a total of N identical fibrils, then each fibril has a hydrated volume of $f_f V_s/N$, and the volume of collagen per fibril is $f_c V_s/N$. Therefore, the volume fraction of collagen in a fibril is $f_m = f_c/f_f$.) Similarly, in Eq. 3, f_n is the volume fraction of nonfibrillar material in the extrafibrillar volume, $f_n = f_p/(1 - f_f)$. Eqs. 2 and 3 become:

$$n_f = n_w + \frac{f_c}{f_f}(n_m - n_w) \quad (5)$$

$$n_e = n_w + \frac{f_p(n_n - n_w)}{1 - f_f} \quad (6)$$

Rearranging Eq. 4, we have

$$f_p(n_n - n_w) = (n_s - n_w) - f_c(n_m - n_w) \quad (7)$$

And substituting the left-hand side into Eq. 6:

$$n_e = n_w + \frac{(n_s - n_w) - f_c(n_m - n_w)}{1 - f_f} \quad (8)$$

The refractive index of the stroma, n_s, has been measured formany species (Maurice, 1957, 1969; Sivak, 1988), and although there is a variation of ± 0.005 between species, the mean value can be taken with some confidence to be $n_s = 1.375$. The refractive index of dry collagen is taken from Maurice, 1957; $n_m = 1.547$, and the refractive index of water is well-known: $n_w = 1.333$. These values can be substituted into Eqs. 5 and 8 to obtain expressions for n_f and n_e that are readily calculated:

$$n_f = 1.333 + \frac{0.214 f_c}{f_f} = 1.333 + 0.214 f_m \quad (9)$$

$$n_e = 1.333 + \frac{0.042 - 0.214 f_c}{1 - f_f} \quad (10)$$

Equations 9 and 10 come directly from Gladstone and Dale's law of mixtures. The numerical values depend only upon the refractive indices of the stroma and of dry collagen, which are both known with some confidence, and upon the assumption that the intermolecular fluid has the refractive index of water. The refractive indices of the fibrils and the extrafibrillar material can now be calculated given only the volume fractions and f_c and f_f. These can be obtained either from x-ray diffraction measurements or from corneal composition, if it is known.

Method 1: X-Ray Diffraction

The low-angle x-ray diffraction pattern from the corneal stroma consists of an intense innermost equatorial reflection

from which the average center-to-center spacing of the collagen fibrils can be calculated (Goodfellow et al., 1978; Gyi et al., 1988), and fainter subsidiary equatorial maxima that give information about fibril diameters (Worthington and Inouye, 1985). Superimposed upon these are a series of sharp meridional reflections arising from the axial periodicity (D-period) along the fibrils (Meek et al., 1982). The high-angle pattern consists of a single equatorial reflection from which the average separation of the collagen molecules within a fibril can be calculated (Meek et al., 1991).

X-ray diffraction data from human, rabbit, ox, and trout corneas were taken from a previous study (Meek and Leonard, 1993) and are presented in Table 2. The meridional reflections in the low-angle x-ray diffraction pattern indexed on $D = 65$ nm for each of the four species studied at physiological hydration. The fibril diameters given in Table 2 compare favorably with values measured by low-temperature electron microscopy (Craig et al., 1986).

The volume fraction of fibrils in the stroma, f_f, was estimated from the interfibrillar spacing, p_f, and fibril diameter, a, on the basis of a "unit cell" representing the average volume occupied by each fibril, as described previously (Meek and Leonard, 1993):

$$f_f = \pi a^2/(4 \times 1.12 p_f^2) \tag{11}$$

the area per unit length of a fibril being $\pi a^2/4$, and the area per unit length of the "unit cell," $1.12p_f^2$. The factor 1.12 enters through the assumption that the fibrils are packed with liquid-like order (Worthington and Inouye, 1985).

The volume fraction of molecules within a fibril was estimated from the intermolecular spacings shown in Table 2 and the Hodge-Petruska model of molecular packing (Hodge and Petruska, 1963). In this model collagen molecules 4.4 D in length are staggered with respect to one another by D, or some multiple of D, with "gaps" 0.6-D long between the head of one molecule and the tail of the next. This structure gives rise to alternating regions of low- and high-electron density of periodicity D, ("gap" and "overlap" regions) along the fibril axis, and accounts for the banded appearance of negatively stained collagen in the electron microscope.

To calculate the volume fraction of a fibril occupied by collagen molecules, a suitable unit cell is 5 D in length, as this contains a complete length of a collagen molecule and its associated gap region, (Katz and Li, 1973). The center-to-center intermolecular spacing, p_i, is 1.11 $\times$ the Bragg spacing on the assumption of "pseudohexagonal" packing (Klug and Alexander, 1974). If the packing angle of the molecules is denoted γ, the unit cell volume is:

$$U = 5Dp_i^2 \sin \gamma \tag{12}$$

The volume occupied by a single collagen molecule is:

$$V = \rho M_C/N_A, \tag{13}$$

where N_A is Avagadro's number, M_C is the molecular weight of the collagen, and ρ is the partial specific volume of collagen. The volume fraction of molecules in a hydrated fibril may therefore be written:

$$f_m = \rho M_C/5Dp_i^2 \sin \gamma N_A \tag{14}$$

Taking values for type I collagen from Katz and Li, 1973: M_C = 283 kDa; ρ = 0.71 ml/g; γ = 60°, the volume fraction can be calculated from x-ray diffraction data using Eq. 14. A variety of techniques have been used to measure the molecular weight of type I collagen yielding values of 283 kDa ± 3%, and the partial specific volume of collagen 0.71 ml/g has been confirmed using N_2 displacement, amino acid content, and pycnometric measurements on gelatin (Katz and Li, 1973).

The volume fractions derived from x-ray diffraction data on human, rabbit, ox, and trout corneas are presented in Table 3, along with the refractive indices calculated using Eqs. 9 and 10. The rightmost column of Table 3 shows average results from >40 species examined in a previous study (Meek and Leonard, 1993). The fibril volume fractions, f_f, were calculated from the interfibrillar spacing and fibril diameter as measured by x-ray diffraction, and carry an uncertainty of as much as ±30%. However, a value for all 40 species was inferred from a graphical average by plotting interfibrillar volume against "unit cell" volume (Meek and Leonard, 1993). For this larger sample the estimated uncertainty was reduced, giving an average value of f_f = 0.28 ± 0.03. Similarly, although the intermolecular spacing was approximately constant for all species, any individual spacing could only be measured to within ±0.1 nm. Averaging over all species reduced this uncertainty to ±0.03 nm. By using these average values, it was therefore possible to obtain a more accurate average value for the mean refractive indices (shown in the rightmost column of Table 3), and these do not differ greatly from the species-specific values.

Method 2: Corneal Composition

The value of f_c in Eqs. 9 and 10 can also be obtained from data on corneal composition. Table 4 shows the parameters involved.

TABLE 2 X-ray diffraction data from human, rabbit, ox, and trout corneas

	Human	Rabbit	Ox	Trout
Intermolecular Bragg spacing (nm)	1.63 ± 0.10	1.58 ± 0.10	1.60 ± 0.10	1.56 ± 0.03
Interfibrillar Bragg spacing (nm)	55.3 ± 4.0	58.8 ± 4.5	56.8 ± 4.5	45.4 ± 3.0
Fibril diameter (nm)	30.8 ± 0.8	38.8 ± 1.3	38.2 ± 1.0	24.7 ± 1.0

TABLE 3 The refractive indices of the fibrils and the extrafibrillar material for human, ox, rabbit, and trout corneas, calculated from X-ray diffraction data

		Human	Ox	Rabbit	Trout	40 species*
Fibril volume fraction	f_f	0.22 (±0.08)	0.32 (±0.08)	0.31 (±0.08)	0.21 (±0.08)	0.28 (−0.03)
Collagen volume fraction	f_c	0.08 (±0.03)	0.12 (±0.03)	0.12 (±0.03)	0.08 (±0.03)	0.108 ±0.008
Refractive index of fibrils	n_f	1.411 (±0.004)	1.413 (±0.004)	1.416 (±0.004)	1.418 (±0.004)	1.416 (±0.001)
Refractive index of extrafibrillar material	n_e	1.365 (±0.009)	1.357 (±0.010)	1.357 (±0.010)	1.364 (±0.009)	1.359 (±0.003)
Refractive index ratio	m	1.033 (±0.007)	1.041 (±0.008)	1.043 (±0.008)	1.040 (±0.007)	1.041 (±0.002)

Each set of values satisfies the Gladstone and Dale's law of mixtures for the stroma, (n_S = 1.375), while being compatible with the ultrastructural data of Table 2.

*The rightmost column shows the average result for >40 species examined in a previous study. (Meek and Leonard, 1993).

From a knowledge of the relative weights and densities of water, fibrillar material and nonfibrillar material in the stroma, the relative volumes occupied by each component can be estimated and used to calculate the volume fractions of dry fibrillar and nonfibrillar material in the stroma. This was carried out by Worthington (1984), using data from Maurice, 1957. Here, we use the same data, modified to take account of cellular water and the distribution of different collagen types, (Leonard, 1996).

From thickness versus hydration measurements, up to 15% of the water in the stroma is contained in the stromal cells (Huang, 1996), a figure also calculated from measurements of corneal salt concentrations (Davson, 1949). Although this makes up part of the total mass of water in the tissue, it is not associated with the fibril/extrafibrillar material matrix and does not contribute to the volume fractions, f_c and f_p. The cornea is assumed to contain 76.2% water (wet weight: dry weight ratio of 3.2) which, when reduced by 15%, gives the effective matrix water content shown in Table 4.

From measurements of hydroxyproline content, the dry weight of the stroma is split between collagen and noncollagenous material in the ratio 15:7 by weight (Maurice, 1957). However, we wish to divide the tissue into fibrillar and nonfibrillar material, and not all of the collagen is located within the fibrils. Although the predominant collagens in the stroma are fibrillar, (mainly type I with smaller amounts of type V and possibly type III, (Birk et al., 1990; Marshall et al., 1993)), it has been found that type VI is also present and may constitute up to a quarter of the total collagen content (Zimmerman et al., 1986; Cintron and Hong, 1988). Only about one-third of the type VI collagen molecule consists of the characteristic triple helix, the remainder being made up of globular domains. Approximately 10% of the collagen, as measured by hydroxyproline content, should therefore be attributed to type VI collagen. Since type VI collagen is located between the fibrils rather than within them, the weight ratio of dry fibrillar to dry nonfibrillar material has been adjusted from 15:7 to 13.5:8.5 to give the relative weights shown in Table 4.

The third column of Table 4 refers to relative areas per unit length of the tissue calculated by dividing the relative weight of each component by its corresponding density (Worthington, 1984). The total area to be considered to calculate the volume fractions is that of the matrix water plus the dry components, 64.8 + 10.4 + 8.7 = 83.9. On the basis of this corneal composition, the volume fraction of dry, fibrillar material in the stroma, f_c, is 0.124 and that of dry, nonfibrillar material, f_p, is 0.104.

Having estimated f_c from the corneal composition, and using the bovine-specific value f_f = 0.32 from Table 3, Eqs. 9 and 10 give the refractive indices n_f = 1.416 and n_e = 1.356. Table 5 summarizes the results of the x-ray diffrac-

TABLE 4 Physical properties and chemical composition of bovine cornea

Components	Relative Weights (Arbitrary units)	Densities (g/ml)	Areas per unit length (Arbitrary units)	Volume fractions
Cornea	100	1.05	95.2	1.000
Cellular Water	11.4	1.00	11.4	
Matrix Water	64.8	1.00	64.8	0.772
Collagen	14.6	1.41	10.4	f_c = 0.124
Extra Material	9.2	1.06	8.7	f_p = 0.104

TABLE 5 Refractive indices of the fibrils and extrafibrillar material from different authors.

Method	Volume Fractions		Refractive Indices		
	f_f	f_c	n_f	n_e	m
Method 1: X-ray Diffraction	0.32	0.120	1.413	1.357	1.041
Method 2: Corneal Composition	0.32	0.124	1.416	1.356	1.044
Maurice, 1957*	0.23	0.15	1.47	1.345	1.093
Maurice, 1969*	0.13	0.10	1.51	1.354	1.115
Smith, 1969*	0.33	0.08	1.384	1.369	1.011
Freund et al., 1995, rabbit#	0.45	0.12	1.391	1.356	1.026
Freund et al., 1995, human#	0.34	0.12	1.407	1.352	1.041

*Refers to values calculated by Worthington (1984) from published data.
#Refers to average values of the published data.

tion method and the corneal composition method for bovine cornea, together with some values given by other authors based on electron microscopy and/or birefringence measurements.

DISCUSSION

X-ray diffraction data provide a means of calculating the refractive indices of the collagen fibrils and extrafibrillar material of the corneal stroma, taking advantage of the fact that the intermolecular spacing of collagen can be measured directly from the hydrated tissue.

Nevertheless, the unit cell used to describe the molecular packing is somewhat simplified. Electron microscopy has suggested that the corneal collagen molecules are tilted with respect to the fibril axis (Marchini et al., 1986). Collagen molecules may also be crimped or kinked, particularly in the gap region (Fraser et al., 1987). However, the volume as calculated from the unit cell, or from Eq. 12, is conformationally independent. It is only necessary to know the mass of protein in the unit cell or elemental volume, and not the details of how the mass is actually distributed. The validity of the simple unit cell can be shown for the type I collagen of rat-tail tendon. When treated with phosphotungstic acid, rat-tail collagen gives a well-defined x-ray diffraction pattern that contains sufficient information to allow the definition of a triclinic unit cell volume of $7.01 \times 10^2 \text{nm}^3$ (Fraser et al., 1987). By comparison, entering typical data for tendon, ($D = 67$ nm, $p_i = 1.4$ nm, Katz and Li, 1973), into Eq. 12, we obtain the same unit cell volume of $7.01 \times 10^2 \text{nm}^3$. The agreement suggests that Eq. 12 provides a reasonable approximation for the unit cell volume for tissues, such as corneal collagen, that give a less detailed diffraction pattern.

Data from both x-ray diffraction and corneal composition lead to similar values for the refractive indices in bovine corneal stroma. In both cases the refractive index ratio is somewhat lower than those deduced by Maurice in his pioneering investigations into corneal transparency (see Table 5), owing to the different estimates of the volume fractions of collagen and hydrated fibrils in the stroma. The difference in the volume fraction of dry fibrillar material in the stroma, f_c, can be attributed partly to the changes made to the known corneal composition since the discovery of type VI collagen (Zimmerman et al., 1986; Cintron and Hong, 1988), and the development of more effective processes for extracting noncollagenous material from the stroma (Wall, 1990) both of which reduce the estimate of f_c.

The volume fraction of fibrils in the stroma, f_f, from x-ray diffraction, is similar to that obtained by Smith (1969), but higher than either of those obtained by Maurice, Table 5. The latter values derive from a comparison of experimental birefringence data with the theoretical curves expected to occur at various fibril hydrations (Maurice, 1957). However, the theoretical birefringence was calculated on the assumption that the hydration (and intrinsic birefringence) of the fibrils was independent of the corneal hydration. This assumption is not correct for dehydrated corneas. By monitoring the intermolecular spacing as corneas dry, it has been shown that fibril hydration remains relatively constant as the wet weight/dry weight ratio of the cornea drops from its physiological value (3.2–3.5) to values of ~ 1, but any further dehydration of the stroma results in a marked reduction in the intermolecular spacing (Meek et al., 1991).

From Eq. 9, the refractive index of a fibril depends on the proportion of the fibril occupied by collagen molecules, and hence the fibril hydration. Despite the fact that some animal species possess larger fibrils than others, the intermolecular spacing is uniform for all species measured (Meek and Leonard, 1991). This implies a uniform fibril hydration (larger fibrils contain a greater number of equally spaced molecules, rather than an equal number of molecules spaced further apart), and would explain the similarity between the refractive indices of the fibrils from the four species studied.

Few other refractive index estimates are available in the literature for comparison. The value for humans, obtained from x-ray diffraction data, compares reasonably with that used by Freund et al. (1995), (Table 5), estimated from Gladstone and Dale's law and measurements from electron micrographs. Similarly, the value for rabbit is in fair agreement with Freund et al. (1986), where $m = 1.051$.

Most models developed to investigate light scattering in the cornea have taken the necessary ultrastructural data from electron micrographs (Hart and Farrell, 1969; Freund et al., 1986; Freund et al., 1995). Preliminary investigations (Leonard, 1996), show that the refractive indices presented here, together with ultrastructural data obtained from x-ray diffraction, produce theoretical transmission spectra comparable to those in the literature.

We thank Liz Towns-Andrews, Sue Slawson and staff at CLRC, Daresbury, for their help in x-ray data collection.

The corneal project at the Open University is supported by the Medical Research Council and the Wellcome Trust.

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