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Swelling Studies on the Cornea and Sclera: The Effects of pH and Ionic Strength

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ABSTRACT The biophysical properties of the cornea and sclera depend on the precise maintenance of tissue hydration. We have studied the swelling of the tissues as a function of pH and ionic strength of the bathing medium, using an equilibration technique that prevents the loss of proteoglycans during swelling. Synchrotron x-ray diffraction was used to measure the average intermolecular and interfibrillar spacings, the fibril diameters, and the collagen D-periodicity. We found that both tissues swelled least near pH 4, that higher hydrations were achieved at lower ionic strengths, and that sclera swelled about one-third as much as cornea under most conditions. In the corneal stroma, the interfibrillar spacing increased most with hydration at pH values near 7. Fibril diameters and D-periodicity were independent of tissue hydration and pH at hydrations above 1. Intermolecular spacings in both tissues decreased as the ionic strength was increased, and there was a significant difference between cornea and sclera. Finally, we observed that corneas swollen near pH 7 transmitted significantly more light than those swollen at lower pH levels. The results indicate that the isoelectric points of both tissues are close to pH 4. The effects of ionic strength can be explained in terms of chloride binding within the tissues. The higher light transmission achieved in corneas swollen at neutral pH may be related to the fact that the interfibrillar fluid is more evenly distributed under these conditions.

INTRODUCTION

The cornea and sclera together form the outermost covering of the eye and withstand both the internal and external force of the eye to maintain the shape of the eyeball and to protect the contents from mechanical injury (Maurice, 1984; Komai and Ushiki, 1991). Unlike other connective tissues, including sclera, the cornea is transparent. Anything that alters the underlying structure of the cornea, such as swelling, will affect the mechanical and optical properties and affect the function of the eye. When the stroma swells it loses its transparency (Kinsey, 1948; Hodson et al., 1991; Hodson, 1996; Elliott and Hodson, 1998). This is because of increased light scattering, which is thought to be caused by a nonuniform distribution of water and disruption of the collagen packing (Benedek, 1971).

Corneal swelling has been studied extensively in our laboratory by using the technique of placing the corneal stroma in distilled water (Goodfellow et al., 1978; Elliott et al., 1980; Whitburn, 1981; Sayers et al., 1982) and bathing solutions with different ionic strengths and pH levels (Elliott et al., 1980). However, swelling by direct immersion in the bathing solutions was found to cause a significant loss of soluble protein and proteoglycans (PGs) from the stroma (Hughes, 1983). More recent studies have shown that equilibrating the tissue to a given hydration by the use of a bounding membrane essentially prevents the loss of PGs (Wall, 1990; Fullwood, 1992). This equilibration technique is thus a much improved method for assessing the effects of various factors on the cornea, and especially on the swelling behavior and structure of the tissue. Using this method, we are now able to show how water is distributed in the stroma under different conditions. We also present, for the first time, equivalent data on the swelling in the sclera.

X-ray diffraction (XRD) is a noninvasive technique that can give information about structure and arrangement within connective tissues. The main advantage is that this technique can be used to study tissues in a hydrated and unprocessed state, so it can avoid artefacts due to fixation, dehydration, embedding, staining, etc. (Meek et al., 1991). Another advantage is that measurements from x-ray patterns from a tissue represent an average value of the whole thickness of the sample through which the x-rays pass.

Corneal structures, including the interfibrillar spacings (IFS), fibril diameters, and intermolecular spacings (IMS) of collagen, have been intensively studied (Goodfellow et al., 1978; Sayers et al., 1982; Meek et al., 1991; Fratzl and Daxer, 1993). In this paper, we have examined bovine corneal stroma and sclera to understand the effects of pH, ionic strength, and tissue hydration on the molecular and fibrillar structure of the tissues.

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MATERIALS AND METHODS

Samples

Fresh bovine eyeballs were obtained from an abattoir, and the cornea and sclera (between the insertion of rectus muscles on the sclera and the equator) were excised from the eye within 3 h of death. By scraping with a scalpel, epithelium and endothelium were removed from the cornea, and choroid was removed from the sclera. Some of the tissues were wrapped in

TABLE 1 Hydration of equilibrated bovine cornea at various pH values (μ = 0.03)

PEG (%)	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
0	21.59 ± 5.77	3.86 ± 0.29	5.99 ± 0.42	9.36 ± 0.28	9.67 ± 0.42	10.69 ± 0.32
0.5	18.83 ± 7.23	3.30 ± 0.58	5.61 ± 0.32	7.06 ± 0.10	7.86 ± 0.55	8.25 ± 0.58
1.5	11.46 ± 6.73	3.11 ± 0.73	4.42 ± 0.52	5.27 ± 0.44	5.04 ± 0.24	5.82 ± 0.22
2.5	9.54 ± 5.94	2.46 ± 1.28	2.91 ± 1.15	3.80 ± 0.07	3.85 ± 0.15	4.32 ± 0.16
5.0	3.29 ± 1.49	1.68 ± 0.67	2.31 ± 0.40	2.25 ± 0.03	2.43 ± 0.11	2.56 ± 0.24
7.5	1.74 ± 0.57	1.53 ± 0.07	1.74 ± 0.05	1.70 ± 0.02	1.82 ± 0.02	1.96 ± 0.03
15.0	0.86 ± 0.03	0.89 ± 0.04	0.91 ± 0.03	1.00 ± 0.00	0.95 ± 0.01	0.99 ± 0.02

clingfilm and stored at −40°C until they were used. Some were placed in a desiccator and were dried over silica gel at room temperature (Goodfellow et al., 1978). Constant dry weight was obtained after at least 2 weeks. The tissues were kept in the desiccator until they were used.

The tissue hydration (H) can be calculated from

$$H = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}}$$

All specimens used in this work were weighed before being wrapped and stored. This allowed us to calculate the tissue hydration in the fresh state. For XRD experiments, samples were weighed again. This allowed us to calculate the tissue hydration at the time of the experiment.

Tissue equilibration

A variety of different equilibration solutions were used, depending on the experiments. For the experiment concerning the effect of pH on the cornea and sclera, the solutions were either a citric acid/Na_2HPO_4 buffer (pH 3, 4, 5) or an Na_2HPO_4/NaH_2PO_4 buffer (pH 6, 7, 8). The free ion concentration of each species was calculated from the accumulative association constants of Sillén and Martell (1964) and Portzehl et al. (1964), using Perrin's computer program. Sodium chloride was added if necessary, so that the final ionic strength (μ) was 0.03 for all solutions. Concentrations of 0, 0.5, 1.5, 2.5, 5, 7.5, 15, and 25% polyethylene glycol (PEG) (20 kDa; BDH Ltd., Warwicks., England) were used to adjust the hydrations of the tissues (Meek et al., 1991).

For the experiment concerning the effect of hydration on the IMS, the cornea and sclera were equilibrated at pH 7.4 and ionic strength 0.03.

For the experiment concerning the effect of ionic strength on the cornea and sclera, the buffer system used for equilibration was Na_2HPO_4/NaH_2PO_4 (pH 7.0). Sodium chloride was added to the buffer to set the final ionic strength at μ = 0.03, 0.15, 0.3, and 1.0. For each ionic strength, a series of solutions were made with PEG concentrations of 0, 2.5, and 5%.

Equilibration was carried out by placing the tissue in 14-kDa cutoff dialysis tubing and leaving it for 4 days at 4°C in the solutions (Meek et al., 1991).

X-ray diffraction

All of the XRD work was carried out using the synchrotron x-ray source of the Council for the Laboratory of the Research Councils at Daresbury, England. During x-ray exposure, the tissues were held in airtight cells. The x-ray beam was always directed along the optical axis of the cornea. For sclera, patterns were collected with the beam directed perpendicular to the scleral surface.

Low-angle diffraction patterns were obtained using a 3-m or a 6-m evacuated camera, radiation of wavelength 0.154 nm, and a focused beam with dimensions 0.25 × 4 mm or 0.5 × 4 mm. High-angle diffraction patterns were obtained using a 11–12-cm helium-filled camera, radiation of wavelength 0.1488 nm, with a collinated beam 0.5 mm in diameter. The low-angle patterns were recorded using a two-dimensional detector with 512 × 512 pixels and wire-to-wire resolution 1 mm, with exposure times of 0.5–20 min. The data were analyzed to obtain the IFS, fibril diameters, and D-periodicity (Huang et al., 1996; Huang and Meek, 1996). The high-angle patterns were recorded on Caeverken AB x-ray film (Caeverken, Strängäss, Sweden), using exposure times of 3–7 min; the IMS was calculated from each pattern (Meek et al., 1991). The diffraction system was calibrated from the 67-nm meridional spacing in rat-tail tendon (low-angle) or the 0.305-nm lattice reflection in powder diffraction patterns of calcite (high-angle).

Measurement of light scattering

An estimate of changes in the light scattered by the cornea was made using an Ultrascan XL laser microdensitometer (LKB Instruments, Gaithersburg, MD), which produced linear scans across the tissue. The wavelength of light produced by the laser was 633 nm. The shape of the beam was rectangular, 50 μm × 800 μm. To reduce the effects of light reflection and scattering from the irregular surface, the tissue was placed on a clean glass slide, and the posterior surface of the cornea made contact with the slide smoothly, without any bubbles. The laser was directed at 90° to the posterior surface of the cornea. The background due to the slide was subtracted from the light absorbance value.

Either a Scheffe F test or a Student t-test was performed. p values below 0.05 were regarded as statistically significant.

RESULTS

Hydration of the corneal stroma and sclera

Tables 1 and 2 show the hydrations of equilibrated corneal stroma and sclera at various pH values. The data are displayed in graphical form in Fig. 1, in which the trends are more readily visible. The lowest hydrations were seen at pH

TABLE 2 Hydration of equilibrated bovine sclera at various pH values (μ = 0.03)

PEG (%)	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
0	7.15 ± 0.15	2.35 ± 0.05	2.33 ± 0.04	2.43 ± 0.07	2.48 ± 0.15	3.02 ± 0.14
0.5	6.09 ± 0.09	2.02 ± 0.06	2.04 ± 0.02	2.02 ± 0.07	2.26 ± 0.05	2.46 ± 0.21
1.5	5.79 ± 0.02	1.85 ± 0.03	1.94 ± 0.10	2.18 ± 0.29	2.22 ± 0.40	2.32 ± 0.22
2.5	5.08 ± 0.10	1.58 ± 0.02	1.59 ± 0.02	1.59 ± 0.02	1.75 ± 0.01	1.83 ± 1.15
5.0	1.35 ± 0.06	1.31 ± 0.05	1.58 ± 0.03	1.31 ± 0.02	1.49 ± 0.04	1.53 ± 0.04
15.0	0.86 ± 0.01	0.80 ± 0.10	0.83 ± 0.07	0.85 ± 0.02	0.85 ± 0.03	0.91 ± 0.02

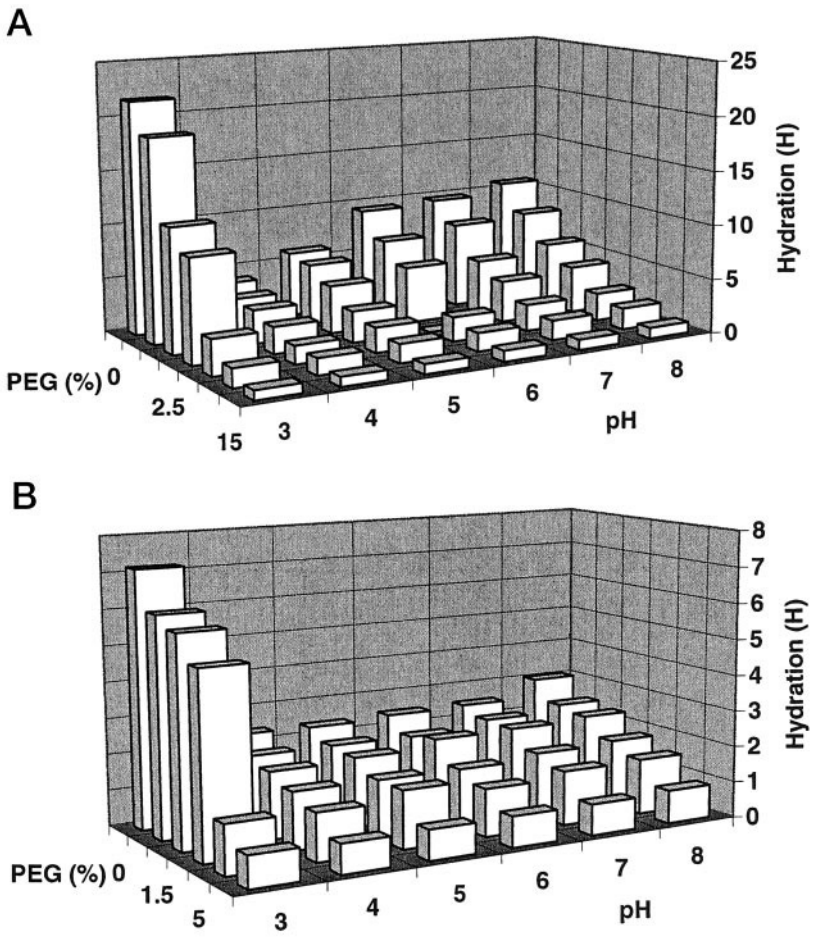

FIGURE 1 Hydration of equilibrated bovine cornea (*a*) and sclera (*b*) at varying pH ($\mu = 0.03$, $n = 4$ for each measurement).

4 at a given PEG concentration for both cornea and sclera. Away from this pH, the hydrations increased. Scleral hydrations were significantly lower than those achieved by the cornea under the same conditions.

The effects of ionic strength on the hydration of the cornea and sclera are shown in Table 3. The trend was that higher hydrations were seen at lower ionic strengths after equilibration at a given PEG concentration, although the difference in hydrations between $\mu = 0.155$ and $\mu = 0.3$ did not reach the significant level statistically. Scleral hydrations were significantly lower than those achieved by the cornea under the same conditions.

TABLE 3 Effects of ionic strength on hydrations of bovine cornea and sclera equilibrated at pH 7.0

PEG concentration	Hydration, mean ± SE ($N = 3$)			
	0.03μ	0.155μ	0.3μ	1.0μ
0%				
Cornea	9.76 ± 0.24	7.91 ± 0.03	8.02 ± 0.22	5.58 ± 0.42
Sclera	3.20 ± 0.21	2.93 ± 0.07	2.90 ± 0.34	2.81 ± 0.01
2.5%				
Cornea	4.52 ± 0.09	3.63 ± 0.27	3.21 ± 0.01	2.75 ± 0.02
Sclera	1.90 ± 0.10	1.78 ± 0.10	1.67 ± 0.07	1.54 ± 0.10
5%				
Cornea	2.80 ± 0.16	2.60 ± 0.17	2.35 ± 0.16	2.04 ± 0.01
Sclera	1.63 ± 0.01	1.30 ± 0.04	1.24 ± 0.01	1.30 ± 0.05

Low-angle XRD

Fig. 2 shows the IFS for the corneas equilibrated at various pH values from 3 to 8, as a function of hydration. The corneal stroma at pH 7 (near physiological pH) showed the largest increase of the IFS with hydration. IFS at pH 3 and 4 increased much more slowly with hydration. The fibril diameter is calculated from the first subsidiary maximum in the low-angle x-ray pattern (Meek et al., 1991). It was impossible to distinguish the subsidiary peak in some patterns from samples equilibrated to very low hydration at any pH or equilibrated to very high hydration at low pH. Accordingly, only some patterns were analyzed for fibril diameters. The results showed that both fibril diameters and the D-periodicity of corneal collagen were essentially independent of hydration and pH when H was above 1 (Table 4). Low-angle equatorial XRD patterns could not be obtained from the sclera because of the wide variation in fibril diameters.

High-angle XRD

High-angle XRD allowed us to calculate changes in the IMS in fresh bovine corneas and sclera equilibrated at pH 7.4. The change in IMS with hydration has two stages. When the hydration is above ~1, the IMS do not change much in

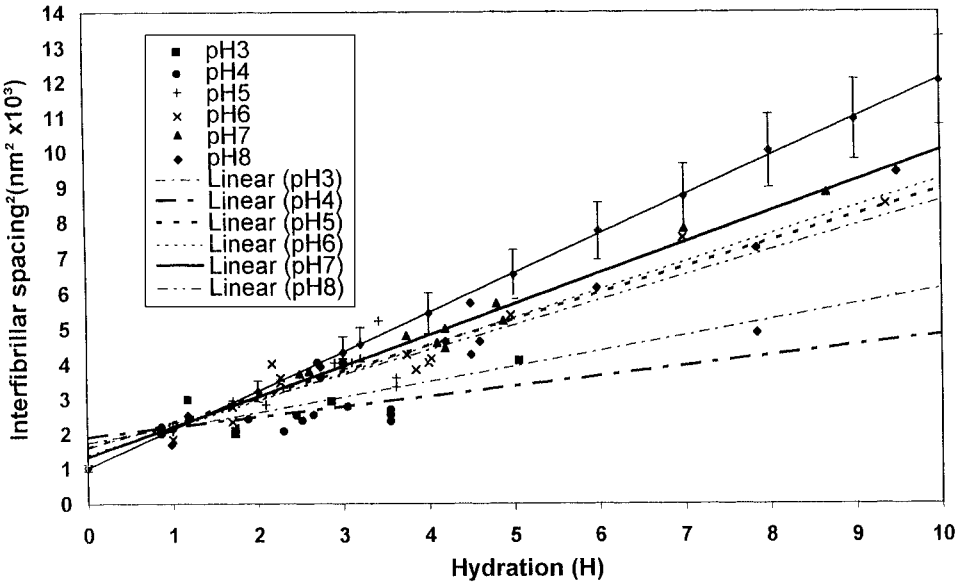

FIGURE 2 Interfibrillar spacing of bovine cornea equilibrated at different pH levels ($\mu = 0.03$). The line with error bars is the theoretical line. For an explanation, see the Discussion.

either tissue; however, when $H < 1$, IMS decrease significantly. Because there is virtually no change in IMS when $H > 1$, the IMS data were combined, giving a mean value of 1.75 ± 0.04 nm ($n = 12$) for the cornea and 1.65 ± 0.02 nm ($n = 9$) for the sclera. Statistically, there was a significant difference in the IMS between the two tissues. When $H = 0$, the mean spacing was 1.25 ± 0.02 nm ($n = 2$) for the cornea and 1.27 ± 0.01 nm for the sclera.

High-angle XRD shows the IMS results for both cornea and sclera equilibrated at various pH levels (from 3 to 8). The two-stage changes described above were also observed at all other pH values. The mean values of the IMS in both tissues at $H = 0$ and $H > 1$ are listed in Table 5. There was no statistical difference between any two pH values for the same tissue, but the IMS were generally lower in the sclera than the cornea when $H > 1$.

The IMS of collagen fibrils in the cornea and sclera decrease with the ionic strength (Table 6). Because the IMS at a given ionic strength do not change with the tissue hydration and all of the tissue hydrations were above 1, the data from each ionic strength were combined. There were significant differences in the IMS at various ionic strengths in both tissues, except between $\mu = 0.155$ and $\mu = 0.3$ for the cornea, probably because of the smaller ionic strength difference (twofold) and smaller sample number. Comparing the results from the cornea with those from the sclera, there were significant differences between the two tissues at any ionic strength (Fig. 3).

TABLE 4 Fibril diameters and D-periodicity in corneas equilibrated at various pH ($\mu = 0.03$)*

pH	Fibril diameter (Mean ± SE (nm))	D-periodicity (Mean ± SE (nm))
3	39.3 ± 0.4 $n = 4$	64.29 ± 0.12 $n = 8$
4	38.7 ± 0.4 $n = 3$	64.42 ± 0.10 $n = 8$
5	39.0 ± 0.4 $n = 4$	64.56 ± 0.32 $n = 8$
6	38.5 ± 0.9 $n = 9$	64.44 ± 0.35 $n = 9$
7	39.1 ± 1.1 $n = 6$	64.76 ± 0.31 $n = 9$
8	39.5 ± 0.6 $n = 7$	64.51 ± 0.10 $n = 10$

*Hydration (H) is above 1.0 in all of the samples.

Light scattering of corneal stroma at various pH levels

Light scattering of the corneas at various pH levels and PEG concentrations ($\mu = 0.03$) is shown in Fig. 4. The results show that the corneas at pH 6, 7, and 8 scattered much less light (i.e., were more transparent). The corneas at pH 4 scattered the most light and stopped swelling at hydration around pH 3. At pH 3 and 5 (near pH 4) the corneas also scattered light much more than the corneas at pH 6, 7, and 8.

DISCUSSION

The effects of the equilibration on the corneal stroma and sclera

Corneal stroma has an innate tendency to imbibe fluid and swell. When it swells it loses its transparency (Hodson et al., 1991). The ability to swell and the transparency of fresh corneal stroma are both unusual properties for a connective tissue (Maurice, 1969; Elliott et al., 1980). The swelling pressure has been measured in rabbit, bovine, and human corneal stroma and has been found to be 50 mmHg at normal thickness (Dohlman et al., 1962; Hedbys and Dohlman, 1963; Fatt and Goldstick, 1965) and 62 mmHg by a dialysis method similar to ours (Midelfart, 1987). However, it could be as high as 300 mmHg at $H = 2.0$, and it drops very sharply at higher stromal hydrations. At the molecular level, the swelling pressure is due primarily to glycosaminoglycans (GAGs) that make up the carbohydrate moiety of the PGs, although a significant proportion has been attrib-

TABLE 5 Intermolecular spacings of fibril collagen in equilibrated bovine cornea and sclera at pH 3–8

	Intermolecular spacing (mean ± SE nm)			
	Cornea		Sclera	
pH	$H = 0$	$H > 1$	$H = 0$	$H > 1$
3	1.27 ± 0.04 ($n = 2$)	1.77 ± 0.02 ($n = 4$)	1.30 ($n = 1$)	1.68 ± 0.03 ($n = 3$)
4	1.25 ± 0.02 ($n = 2$)	1.75 ± 0.05 ($n = 4$)	1.27 ($n = 1$)	1.66 ± 0.03 ($n = 4$)
5	1.28 ± 0.03 ($n = 2$)	1.68 ± 0.07 ($n = 3$)	1.25 ($n = 1$)	1.67 ± 0.02 ($n = 4$)
6	1.27 ± 0.03 ($n = 2$)	1.69 ± 0.04 ($n = 4$)	1.26 ($n = 1$)	1.65 ± 0.02 ($n = 4$)
7	1.28 ± 0.00 ($n = 2$)	1.71 ± 0.05 ($n = 3$)	1.28 ($n = 1$)	1.64 ± 0.03 ($n = 3$)
8	1.27 ± 0.02 ($n = 2$)	1.73 ± 0.07 ($n = 4$)	1.29 ($n = 1$)	1.64 ± 0.01 ($n = 3$)

uted to a chloride-binding ligand of unknown composition (Hodson, 1996). The chloride-binding ligand is not associated with the collagen molecules (Hodson et al., 1992). Hodson (1971) supposed that no effective cross-links exist in the cornea and suggested that the swelling pressure is due entirely to the Donnan-osmotic pressure between the stroma and the external solution. The pressure arises from the unequal distribution of small permeated ions, which in turn results from the presence of a fixed charge concentration in the stroma, due in part to the GAGs (Elliott et al., 1980).

Distribution of water within the swollen cornea

The volume of the cornea at a given hydration may be expressed in terms of the dry volume (Meek et al., 1991) as follows: hydration, H, is defined from

$$H = \frac{\text{mass of water}}{\text{mass of dry cornea}} = \frac{V_w}{V_c \times \rho_c} \quad (1)$$

where V_w and V_c are the volumes occupied by the water and the dry cornea, respectively. The density of water is taken as 1 g/ml, and ρ_c is the density of the dry cornea. Because the dried corneal stroma can be considered to consist of two parts, collagen and extrafibrillar material, ρ_c can be expressed as

$$\rho_c = \frac{M_{col} + M_{ext}}{V_{col} + V_{ext}} \quad (2)$$

$$= \frac{\rho_{col}V_{col} + \rho_{ext}V_{ext}}{V_{col} + V_{ext}} \quad (3)$$

where M_{col} and M_{ext} are the mass per unit volume of the collagen and the extrafibrillar material in the dried cornea, respectively. V_{col} and V_{ext} are the volume fractions of the collagen and the extrafibrillar material in the dried cornea, respectively.

We can thus calculate ρ_c if ρ_{col}, ρ_{ext}, V_{col}, and V_{ext} are known. Worthington (1984) reported values of $\rho_{col} = 1.41$ g ml^{-1} and $\rho_{ext} = 1.06$ g ml^{-1}. V_{col} and V_{ext} can be calculated by making the assumptions that the dry collagen fibrils pack with a "pseudohexagonal" arrangement, and that they are distributed evenly throughout the tissue. In this case, the volume of tissue associated with each fibril (corresponding to the volume of a "unit cell") is $i^2/1.12$ (to a close approximation), where i is the nearest-neighbor distance (Meek and Leonard, 1993). V_{col} can be expressed as

$$V_{col} = 1.12\pi r^2/i^2 \quad (4)$$

where r is the radius of the dry collagen fibril, and XRD indicates that $i = 34$ nm ± 2 nm in bovine cornea at $H = 0$ (Meek et al., 1991; Fullwood, 1992). (It is possible that the dry fibrils are hexagonally close packed, in which case the volume associated with each one will be $i^2/1.15$, and this will lead to a 2.7% increase in the calculated value of V_{col}.) The fibril radius, r (at $H = 0$), can be calculated to be ~13.5 nm according to the percentage reduction in the intermo-

TABLE 6 Intermolecular spacings (IMS) of fibril collagen in bovine cornea and sclera equilibrated at various ionic strengths (pH 7.0)

		IMS, mean ± SE, nm (number of samples)			
		PEG concentration			
Ionic strength		0%	2.5%	5%	Total
0.03 μ	Cornea	1.74 ± 0.01 (3)	1.76 ± 0.02 (2)	1.78 ± 0.0 (2)	1.76 ± 0.02 (7)
	Sclera	1.71 ± 0.0 (2)	1.70 ± 0.03 (3)	1.73 ± 0.02 (2)	1.70 ± 0.03 (7)
0.155 μ	Cornea	1.73 ± 0.01 (2)	1.72 ± 0.01 (3)	1.73 ± 0.0 (2)	1.73 ± 0.01 (7)
	Sclera	1.69 ± 0.0 (2)	1.68 ± 0.01 (2)	1.67 ± 0.01 (2)	1.68 ± 0.01 (6)
0.30 μ	Cornea	1.72 ± 0.01 (3)	1.73 ± 0.01 (3)	1.72 ± 0.0 (2)	1.72 ± 0.01 (8)
	Sclera	1.63 ± 0.0 (2)	1.65 ± 0.01 (2)	1.65 ± 0.02 (2)	1.65 ± 0.03 (6)
1.00 μ	Cornea	1.67 ± 0.0 (2)	1.64 ± 0.0 (2)	1.64 ± 0.0 (2)	1.65 ± 0.01 (6)
	Sclera	1.61 ± 0.02 (2)	1.60 ± 0.01 (2)	1.56 ± 0.01 (2)	1.60 ± 0.02 (6)

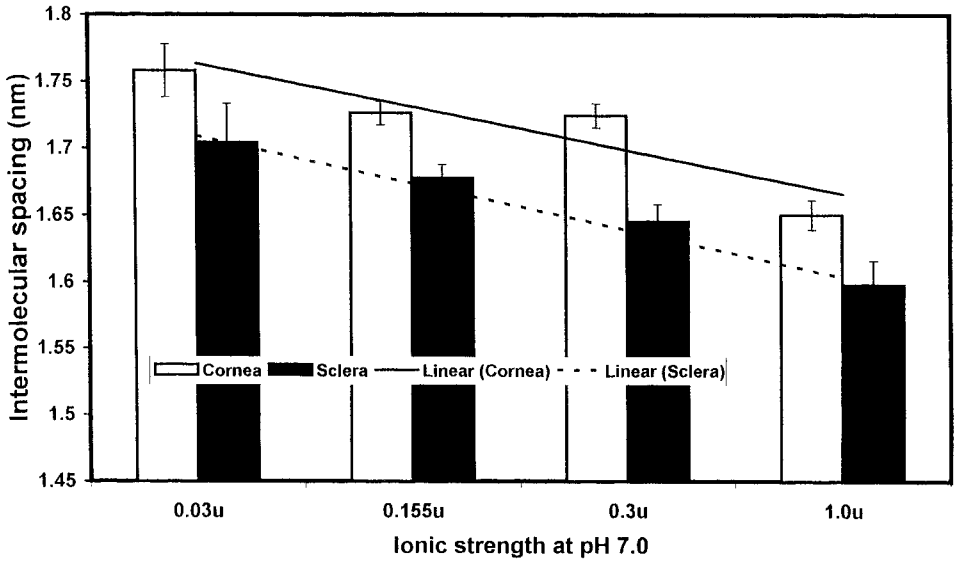

FIGURE 3 Comparison of the intermolecular spacings in collagen fibrils in bovine cornea and sclera equilibrated at different ionic strengths (pH 7.0, $H > 1$).

lecular spacings obtained between $H = 3.2$ (physiological hydration) and $H = 0$ (dried state). This leads to the value $V_{col} = 0.555$, and because, for dry cornea, $V_{col} + V_{ext} = 1$, $V_{ext} = 0.445$.

Using these values in Eq. 3, we get the result $\rho_c = 1.254$.

Equation 1 may thus be expressed as

$$V_w = V_c \times 1.254H \tag{5}$$

Let V_H represent the volume of the hydrated cornea; then

$$V_H = V_c + V_w = V_c(1 + 1.254H) \tag{6}$$

assuming that the volume occupied by the dry material in the cornea does not change when the tissue is hydrated. V_c is the volume occupied by the dry cornea, but Eq. 6 should apply equally to any volume of the tissue, so it will apply to the volume associated with each fibril, $i^2/1.12 = 1032$ nm^2. It is thus possible, using Eq. 6, to calculate the expected increase in volume with the increase in hydration (*line A* in Fig. 5). From Eq. 6 it follows that, at physiological hydration $H = 3.2$, the dry materials should occupy one-fifth of the volume associated with each fibril. The water will occupy four-fifths. The relative volume fractions of dry material and water, V_w, can be calculated from Eq. 6 for other hydrations; these are given in Table 7.

Cellular water, ~15% of total water in the tissue at physiological hydration (Maurice and Riley, 1970), does not contribute to the volume increase of the "unit cell," although it contributes to the total corneal volume. This fraction of

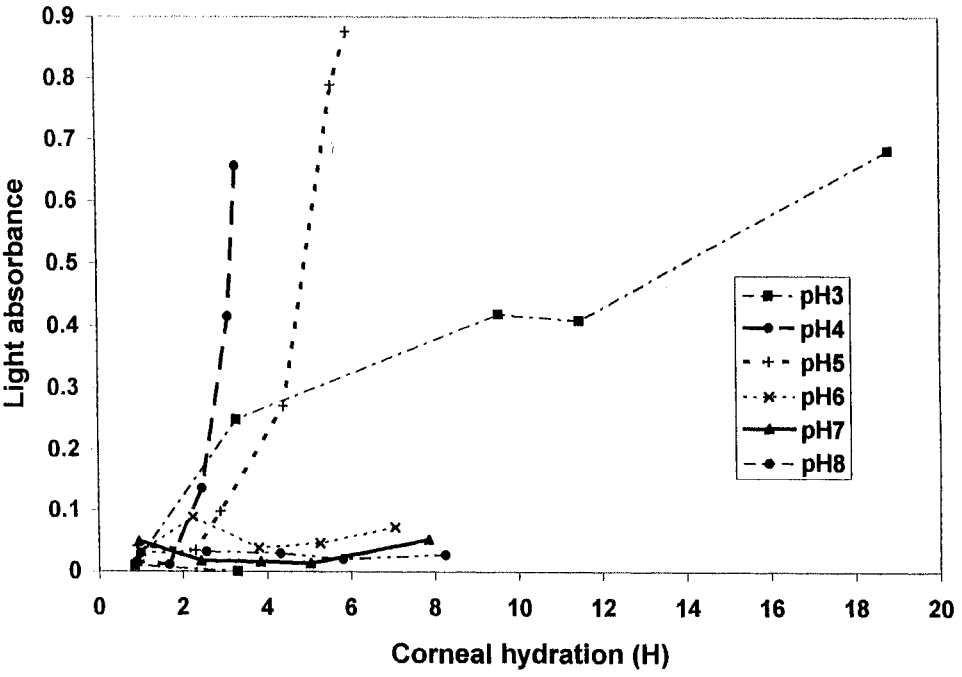

FIGURE 4 The light absorbance of bovine cornea equilibrated at different pH levels as a function of hydration.

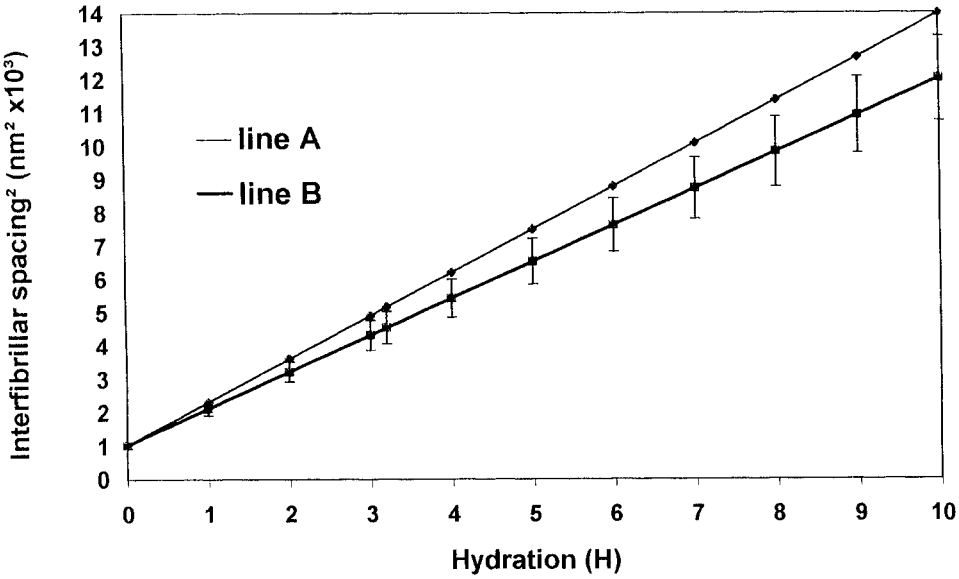

FIGURE 5 Two theoretical lines showing interfibrillar volume changes of bovine cornea with hydration. Line *A* is calculated with the assumption that all water is evenly distributed between the collagen fibrils; line *B* is calculated on the assumption that 15% of the water is not located between the fibrils. The calculation depends on the volume of the interfibrillar spacing at $H = 0$, which is taken as 34 ± 2 nm (Meek et al., 1991). The confidence limit of this value thus decides a limit to the accuracy with which line *B* can be plotted, which is indicated by error bars.

water must be deducted from the total water in the cornea to assess the effects of added water on the interfibrillar spacings. So, on the assumption that the volume of the cellular water changes proportionally with that associated with each fibril, Eq. 6 may be rewritten as

$$V_H = V_c + V_w = V_c(1 + 1.254H - 1.254H \times 15\%)$$
$$= V_c(1 + 1.066H) \qquad (6')$$

Relative volume fractions associated with each fibril can be calculated from Eq. 6′ for a variety of hydrations; these are listed in Table 7.

At $H = 3.2$, the total volume associated with each fibril (the volume of dry material and the volume of water) = 1032 nm^2 × (1 + 1.066 × 3.2) = 4552 nm^2. Similarly, points at other hydrations can be calculated; these are plotted in Fig. 5 (*line B*).

TABLE 7 Volume fractions of dry material and water in the cornea

Hydration of cornea (H)	Volume Fraction by Eq. 6*		Volume Fraction by Eq. 6′*	
	Dry Material	Water	Dry Material	Water
0	1.00	0.00	1.00	0.00
1	0.44	0.56	0.48	0.52
2	0.29	0.71	0.32	0.68
3	0.21	0.79	0.24	0.76
3.2	0.20	0.80	0.23	0.77
4	0.17	0.83	0.19	0.81
5	0.14	0.86	0.16	0.84
6	0.12	0.88	0.14	0.86
7	0.10	0.90	0.12	0.88
8	0.09	0.91	0.10	0.90
9	0.08	0.92	0.09	0.91
10	0.07	0.93	0.09	0.91

*Volume fraction of dry material = V_c/V_H; volume fraction of water = $1 - V_c/V_H$.

We can now compare the data from the XRD experiments with the theoretical volumes at any hydration. The data from the corneas equilibrated at pH 7 and at physiological ionic strength (Meek et al., 1991) are used and plotted in Fig. 6. The least-squares best-fit line is very close to the theoretical line. This indicates that most of the water (except the cellular portion) in the corneas that have been equilibrated near physiological ionic strength and pH distributes evenly between the fibrils over a large range of hydrations. The absence of significant lakes in corneas swollen by equilibration near physiological pH needs further investigation, perhaps by electron microscopy or confocal microscopy, but such a hypothesis might explain the relatively low levels of light scattering that we have observed in these corneas. Similarly, the data from the corneas equilibrated at pH 3–8 can be compared with the theoretical line (Fig. 2) to explain the higher levels of light scattering observed.

Effects of pH on the equilibrated bovine corneas and sclera

Fresh bovine corneal stroma and sclera equilibrated at pH 4 are found to give the lowest hydration, at a given PEG concentration and at the same ionic strength ($\mu = 0.03$), compared with other pH values. Away from this pH, the hydrations increase in both tissues (Fig. 1). However, the final hydration of the equilibrated corneal stroma is much higher than that of the sclera at a given pH and PEG concentration.

Elliott et al. (1980), who used predried corneal stroma and observed the swelling as function of time and ionic strength at various pH levels, also found a minimum swelling near pH 4. The explanation of this swelling behavior was that the isoelectric point is close to pH 4 and that the net fixed electric charge is zero at this point. The corneal stroma or sclera can be considered as a polyelectrolyte gel. When

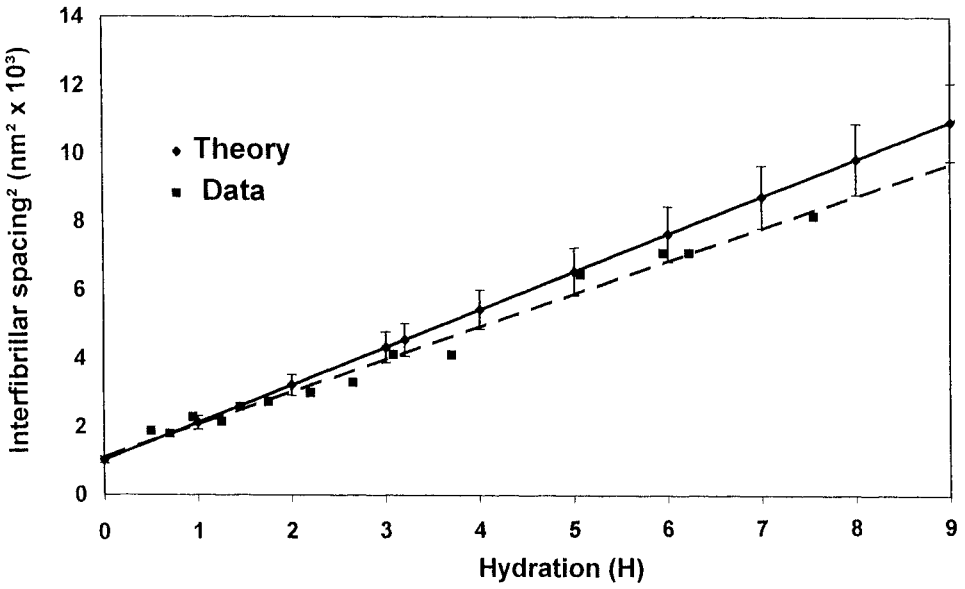

FIGURE 6 The theoretical volume (see line *B* in Fig. 5) and the data from low-angle XRD of equilibrated bovine cornea at pH 7 and $\mu = 0.15$ (Meek et al. (1991). The experimental interfibrillar volume data are shown as squares with the least-squares, best-fit line, which is very close to the theoretical line.

the tissue is equilibrated at the isoelectric point, electrovalent attractive forces are produced between the equal numbers of positive and negative charges (i.e., zwitterion pairs; Katchalsky, 1954), which results in tighter, less swollen tissue. This is consistent with our results.

Elliott et al. (1980) also found that the final hydration increases with the ionic strength of the bathing solution at pH 4 ($H = 2.75$–3.0 at $\mu = 0.06$; $H = 4.0$–4.5 at $\mu = 0.3$). According to their results, the final hydration of the corneal stroma equilibrated in the solution with ionic strength below $\mu = 0.06$ at this pH would be expected to be below $H = 2.75$–3.0. However, the hydration observed in our study (PEG = 0%, $\mu = 0.03$, pH 4) is higher than that ($H = 3.88$). There are two possible reasons for this. One is that fresh corneas were used in our study, so there are no effects due to predrying of the cornea (unpublished result). The other is that the leaking of PGs from the stroma into the bathing solution was prevented by our use of dialysis tubing.

Hydrations at pH away from the isoelectric point are higher because of the reduction in the number of zwitterion pairs and the increase in net fixed charges in the tissue. The net charge will be negative above the isoelectric point and positive below it and will affect the swelling in two ways. First, the fixed charge will require more counterions in the stroma to maintain electrical neutrality, resulting in the accumulation of small permeant ions. This will cause an excess internal osmotic pressure and will increase the swelling. Second, the reduction in the number of attractive zwitterion pairs will cause a reduction in the attractive forces and thus will loosen the stroma, again causing increased swelling (Elliott et al., 1980).

The hydration results from the sclera indicate that the isoelectrical point is also around pH 4 and that the swelling is following similar principles, but at a lower level. There are several reasons for the low final hydrations of the sclera. First, most of the swelling pressure in sclera is caused by PGs, which have charged groups on their GAGs. The sclera contains fewer PGs because the concentration is extremely low, ~0.1% (Piez, 1984). GAGs in the human sclera measured by the hexosamine concentration were five to six times lower than that in the cornea (Borcherding et al., 1975). The protein content of the PGs is between 40% and 60% of their dry weight (Cöster and Fransson, 1981; Cöster et al., 1981). Second, the sclera has a higher collagen content and larger fibrils with a smaller combined surface area than the cornea. If the PGs are bound to the fibril surface with a density similar to that of the cornea, the sclera would swell less. Structural differences in the sclera may also limit the swelling, because the fibrils in the sclera form bands, and these bands often branch and interweave with each other.

One interesting finding was that the light scattering increased more rapidly with hydration (even below physiological hydrations) at pH values around the isoelectric point. In contrast, the light scattering at higher pH values (pH 6, 7, 8) changed slowly with hydration, when light scattering was measured from the corneal stroma as a function of hydration at various pH levels. According to current models, corneal transparency at a given wavelength depends on certain structural parameters such as fibril diameters, the density of fibril packing, the position of each fibril relative to its neighbors, and the refractive indices of the collagen and the interfibrillar matrix, and changes in one or more of these factors may be sufficient to increase light scattering.

Larger fibril diameters are expected to scatter more light. Pitie (1947) has studied the behavior of collagen isolated from the cornea, which shows swelling maxima at pH 4 and pH 11 and a minimum at pH 7. However, high-angle XRD results presented here indicate that there are no significant changes in the intermolecular spacing of the collagen fibrils at $H > 1$, although slightly larger average values are seen at pH 3 and pH 4. So it is presumed that no significant changes

occur in the fibril diameter. This means that the fibrils preferentially absorb the initial water and then remain at a relatively constant diameter, which is supported by studies of the cornea at neutral pH (Meek et al., 1991; Fratzl and Daxer, 1993). The results from low-angle XRD also show that the D-periodicity remains unchanged with hydration, indicating no significant changes in the dimensions along the axis of the fibrils during swelling, although the average value of the D-periodicity at pH 3 is slightly lower than that at pH 7 and pH 8.

The packing of the fibrils is another important parameter. A number of XRD studies have been carried out on bovine corneas (Goodfellow et al., 1978; Sayers et al., 1982; Meek et al., 1991) and human corneas (Fullwood et al., 1992; Fratzl and Daxer, 1993) and have shown that IFS are a linear function of hydration. This is consistent with our XRD results from bovine corneas equilibrated at different pH values. However, at lower pH (3, 4, 5), the correlation between the interfibrillar spacing and the hydration is poor. One fact is that, at a given hydration, the interfibrillar volume of the tissue equilibrated at pH away from the physiological level is lower than that expected theoretically, particularly at pH 3 and 4, and the slopes of IFS versus hydration at lower pH are less steep than those at higher pH (Fig. 2). This suggests either that a larger portion of the water goes not into the interfibrilar space but into the another space, such as lakes (regions devoid of collagen fibrils), or that a larger change in the packing arrangement of the fibrils leads to some fibrils being so disorganized that they do not contribute to the XRD pattern. The lakes would act as light-scattering centers (if they reach sizes greater than half the wavelength of light), and these may cause scattering of sufficient magnitude to account for the cloudiness of the stroma. However, the light scattering in the corneas equilibrated near pH 4 is much greater than those equilibrated near pH 7, even when the tissue hydration is near or below physiological levels. This suggests that the fibrils are very disorganized. This would result in regions with higher fibril density and other regions with lakes. Both would be expected to increase light scattering. Clearly, much work still needs to be done to answer questions such as, What are the exact changes in the stroma? Are structural changes reversible? etc.

Effects of ionic strength on equilibrated bovine cornea and sclera

We have studied the effects of ionic strength on the hydration and IMS of fibril collagen in bovine corneal stroma and sclera. The final hydrations and IMS are significantly different at different ionic strengths (pH 7) at a given osmotic pressure in both the cornea and the sclera. The final hydration is higher when the ionic strength is lowered from $\mu = 1.0$ to $\mu = 0.03$. The hydration decreases with the PEG concentration at a given ionic strength. IMS decrease with ionic strength. However, unlike hydration, the IMS does not change much when the osmotic pressure in the equilibration solution is changed at a given ionic strength.

The simplest explanation of our hydration results is that a chloride-binding ligand exists in bovine corneal stroma and sclera that exhibits first-order kinetics, a dissociation constant, K_m of 300 mM, and a total capacity of 75 mEq/liter at physiological hydration, $H = 3.2$ (Hodson et al., 1992). When the ionic strength (NaCl concentration) in the equilibration solution is increased, more chloride ions bind to the ligands and become ligand-chloride ion complexes, and the cation exchange capacity of the stroma increases correspondingly. Because at any particular salt concentration the dry tissue exchange capacity (the product of the cation exchange capacity and the hydration, i.e., $Q \times H$) is constant (Hodson et al., 1992), the higher the ionic strength in the equilibration solution, the lower the final hydration that can be achieved.

The dependence of IMS on ionic strength has not been reported before. It is generally accepted that an increase in IMS will lead to a proportional increase in the fibril diameter. In this study, the fibril diameter was measured to be 39.0 ± 0.8 nm at $\mu = 0.03$, pH 7, which is 4.3% higher than the value of 37.4 ± 1.4 nm obtained from equilibrated cornea at $\mu = 0.15$ (Fullwood, 1992). Because the fibril diameters do not change much over a large range of hydrations, we can predict the fibril diameter in the cornea equilibrated at a specific ionic strength if the IMS is known. For example, if an intermolecular spacing of 1.76 nm corresponds to a fibril diameter of 39.0 nm in the cornea equilibrated at $\mu = 0.03$ at pH 7, the fibril diameters at $\mu = 0.155$, $\mu = 0.3$, and $\mu = 1.0$ will be 38.3 nm, 38.2 nm, and 36.6 nm, respectively, according to the data in Table 6.

The reason for the dependence of the IMS on the ionic strength of the bathing medium is not clear. The swelling pressure between the fibrils is higher at lower ionic strength, and, at a given ionic strength, the IMS remain unchanged. This suggests that there is a fixed balance inside and outside collagen fibrils. Higher ionic strength in the equilibration solution will reduce the interfibrillar swelling pressure and thus increase the IMS. However, the concentration of the ligand-chloride ion complex also increases when the ionic strength is increased. This may create a larger ionic gradient (such as Na^+) from the inside to the outside of the collagen fibril (with a lower ionic concentration within the fibril) because the chloride ligand is located in the matrix molecules. This, in turn, would explain the decrease in IMS at higher ionic strengths and vice versa.

Another possible explanation is a direct effect of salt through electrostatic double-layer repulsion. In the absence of thermal motion, sufficient counterions would become firmly attached to the surface of a colloidal particle to neutralize its charge, but thermal agitation prevents the formation of such a compact electrical double layer. In the locality of a charged colloidal particle there is a balance between the electrical forces, which are tending to attract counterions and repel coions, and thermal motion, which tends to produce a uniform distribution of these ions. Excess

counterions near the charged particle surface screen the electrostatic attraction for counterions farther away from the particle surface, with the result that the concentration of excess counterions, and hence the electric potential, drops off rapidly at first and then more slowly with increasing distance. The combined effect of electrical forces and thermal agitation is, therefore, to create a diffuse electrical double layer (Gouy-Chapman double layer). An increase in the electrolyte concentration magnifies the above screening effect and so leads to a compacting of the diffuse double layer, i.e., a more rapid decay of potential (Shaw, 1968). Leikin et al. (1994) reported direct measurements of forces between collagen triple helices in native and reconstituted fibers. At low osmotic pressures, interaxial spacings decreased with increased ionic strength, which is consistent with our results. However, it is claimed that a direct effect of salt through electrostatic double-layer repulsion is unlikely, given the insensitivity of both force-decay lengths and force magnitudes to ionic strength at high osmotic stress. Rather, salt either is mediating the attractive force or is preferentially excluded from the space between collagen helices, so as to apply an extra osmotic pressure in addition to the PEG stress.

If hypertonic drops are instilled in eyes with corneal edema and early epithelial edema, some clearing of vision can often be achieved. This is because enough water is temporarily extracted out of the epithelium to smooth the surface and reduce the diffraction of light by the microcysts (Dohlman, 1994). Clinically, 5% sodium chloride drops for daytime use and ointment of the same strength at night are commonly used. According to our data, it seems that sodium chloride should be better than other hypertonic measures for corneal edema, because it may also reduce the swelling pressure in the stroma and decrease the fibril diameter if used frequently. It may thus be beneficial for stromal edema and improve transparency. Because this agent is almost always harmless, it may be worth trying, even when there is doubt about its efficacy.

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