

# Synchrotron x-ray diffraction studies of the cornea, with implications for stromal hydration

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**ABSTRACT** The intermolecular and interfibrillar spacings of collagen in bovine corneal stroma have been measured as a function of tissue hydration. Data were recorded from low- and high-angle x-ray diffraction patterns obtained using a high intensity synchrotron source. The most frequently occurring interfibrillar spacing varied from 34 nm in dry corneas to 76 nm at  $H = 5$  (the hydration,  $H$ , is defined as the ratio of the weight of water to the dry weight). The most frequently occurring intermolecular Bragg spacing increased from 1.15 nm (dry) to  $\sim 1.60$  nm at normal hydration ( $H \approx 3.2$ ) and continued to increase only slowly above normal hydration. Most of the increase in the intermolecular spacing occurred between  $H = 0$  and  $H = 1$ . Over this hydration range the interfibrillar and intermolecular spacings moved in tandem, which suggests that the initial water goes equally within and between the fibrils. Above  $H = 1$  water goes preferentially between the fibrils. The results suggest that, even at normal hydration, water does not fill the interfibrillar space uniformly, and a proportion is located in another space or compartment. In dried-then-rehydrated corneas, a larger proportion of the water goes into this other compartment. In both cases, it is possible to postulate a second set or population of fibrils that are more widely and irregularly separated and therefore do not contribute significantly to the diffraction pattern.

## INTRODUCTION

The connective tissue layer of the cornea, the stroma, is made up of several hundred lamellae. Each lamella is  $\sim 2 \mu\text{m}$  thick and is composed of uniformly oriented collagen fibrils which appear to be  $\sim 25\text{--}26$  nm in diameter in transmission electron micrographs (e.g., Jakus, 1954). The basis of stromal transparency was first discussed in relation to the ultrastructure by Maurice (1957, 1984) who considered two theoretical models. In one model the fibrils are very hydrated so that their refractive index is comparable to that of the ground substance lying between them, the tissue would therefore have a uniform refractive index. This would imply that the (thin section) electron microscope images significantly underestimate the diameter of the constituent collagen fibrils in the normal physiological state of hydration. In the second model the fibrils are arranged in a strictly regular lattice, and the transparency results from the destructive interference of the scattered light. The latter model was preferred because (a) it explains how large molecules readily diffuse through the stroma, (b) it explains the observed high structural birefringence of the stroma, (c) it better explains the clouding of the tissue under stress. Other workers (Hart and Farrell, 1969; Benedek, 1971) have made theoretical calculations showing that transparency occurs even if the fibrils are distributed in a more or less orderly way rather than with strict regularity.

From time to time, investigators have revived the hypothesis that the fibrils are highly swollen (Smith, 1969; Lythgoe, 1976) and further investigation is desir-

able. X-Ray diffraction is a noninvasive technique which can give information about structure and arrangement to complement electron-microscopic observations. This information can conveniently be considered in two domains: the low-angle pattern and the high-angle pattern. In a fibrous structure such as the stroma, the low-angle pattern arises from the fibrils and from the arrangement of these fibrils within the tissue as a whole. A low-angle pattern from corneal stroma was published by Maurice (1957), who obtained reflections that were probably the third and fifth orders of the collagen axial periodicity, judging from subsequent work. More recent investigations have shown that the low-angle pattern has two components: diffraction from the axial structure of the collagen fibrils, and diffraction from the interfibrillar arrangement (Goodfellow et al., 1978). These components are considered in detail in Meek et al. (1981a, b) and Sayers et al. (1982). Similar results have also been obtained by Worthington and Inouye (1985), discussed by Worthington (1984), and by Marchini et al. (1986).

High-angle x-ray patterns give information of a different scale which concerns the arrangement of atoms and groups of atoms into molecules. This technique makes it possible, in principle, to establish the separation of the molecules that compose the collagen fibril and to determine how this separation changes between the dry and the normally hydrated condition of the tissue.

There is no modern high-angle x-ray study of corneal stroma. Hertel (1933) carried out a limited investigation

at various tissue hydrations and was able to identify rings which appear roughly to correspond to spacings in the now well-investigated x-ray pattern of the collagen molecule (e.g., Ramachandran, 1967). Hertel froze his specimens and cooled them to liquid air temperatures so that artifacts resulting from the crystallization of water are possible, and the structural information is probably not relevant to natural hydration. Adler et al. (1949) outlined results which apparently are in conformity with those of Hertel, but they published little detail of their findings. Agarwal et al. (1972) examined dry cornea, but were unable to study fresh tissue because of their instrumental setup. Accordingly, we decided to carry out a series of observations over a greater range of hydrations than employed by Hertel, taking advantage of the notable improvements in technique that have occurred since his study.

## MATERIALS AND METHODS

Bovine eyes were obtained from a local abattoir, and the corneas were excised at the limbus. 11 mm diam discs were cut from the centers of the corneas which were then scraped to remove the surface epithelial and endothelial layers. The discs were placed into 14KD cut-off dialysis tubing and immersed in solutions of polyethylene glycol (PEG, 20KD MW) (0%, 0.5%, 2%, 4%, 7%, 10%, 15%, 20%, 25%) containing 0.15 M NaCl. Equilibration was carried out at 4°C for at least 4 d. The corneas were then brought to room temperature, removed from the dialysis tubing, weighed and placed in air-tight cells. Matching preparations of pure steer skin collagen strips (Katz and Li, 1973) and rat-tail tendons were made for comparison. The hydration was specified by the parameter  $H$ , defined as the ratio of the weight of water to the dry weight. Specimens were examined at the SERC synchrotron (Daresbury, UK). Low-angle diffraction was carried out on Station 8.2 using a 3-m evacuated camera, radiation of wavelength 1.54 Å and a focussed beam with dimensions 4 mm × 0.5 mm. High-angle diffraction experiments were performed on Station 7.2b using a 12-cm helium-filled camera, radiation of wavelength 1.488 Å with a focussed beam collimated to 1 mm diam. Diffraction patterns were recorded on Caeverken AB photographic film (Caeverken, Strängnäs, Sweden), using exposure times of 4 or 5 min (high angle) or 30 s (low angle). The diffraction system was calibrated from the 3.05-Å lattice reflection in powder diffraction patterns of calcite (high-angle) or the 67-nm meridional spacing in rat-tail tendon (low-angle). Dimensions of the x-ray patterns were measured using an Ultrascan XL Laser Microdensitometer (LKB Instruments Inc., Gaithersburg, MD) which produced linear scans across the diffraction rings. From each of these scans the most frequently occurring Bragg spacing (corresponding to the maximum intensity in the x-ray reflection) was calculated.

## RESULTS

### High-angle pattern

The diffraction pattern of the cornea, as well as that of the tendon and the collagen strips, shows the intensity maxima expected from wide-angle reflection from colla-

gen (Ramachandran, 1967). The corneal pattern is radially isotropic, and in the dried tissue the reflections correspond to spacings of 0.29, 0.40, 0.45, 0.7, 1.0, and 1.15 nm.

The steer-skin collagen strips and dry rat-tail tendon produce similar spacings (0.29, 0.4, 0.45, 0.6, 1.0, and 1.08 nm), but these are now organized axially and equatorially. Fig. 1 gives a comparison between the two patterns, bovine corneal and tendon. From this comparison it is easy to see the rings in the corneal pattern that correspond to axial and equatorial collagen spacings.

The corneal spacings at 0.29, 0.4, and 1.0 nm correspond to the axial atomic and molecular arrangement and are not detectably altered by the changes in the hydration of the cornea. However, the intensity distribution that correlates with the equatorial intermolecular packing undergoes pronounced changes. The most prominent feature is the sharp reflection ranging from 1.15 to 1.18 nm in dry corneas that, by analogy with tendon, is a lattice reflection arising from the packing of molecules. The spacing of the reflection increases over the range of hydration from dry to "physiological" levels as determined in fresh corneas ( $H = 3.2$ – $3.5$ ), where the average dimension is  $\sim 1.60$  nm. Only limited increase in the intermolecular separation occurs above physiological hydration (Fig. 2). The matching preparations of tendon undergo a similar increase in the intermolecular Bragg spacing; the spacing reaches a maximum value of 1.5 nm at around  $H = 2.5$ , beyond which the intermolecular spacing in the tendon does not increase. Similar results were obtained with steer-skin collagen strips using a conventional x-ray source (Cooke, P., unpublished results).

The molecules within the fibrils in the corneal stroma do continue to move apart beyond  $H = 2.5$  (Fig. 2) reaching a maximum spacing of  $\sim 1.7$  nm. It may be presumed that the spacing increase ceases at a point when the covalent cross-links between adjacent collagen molecules reach their maximum extension. The increase in intermolecular spacing is possibly accompanied by an alteration in the mode(s) of intermolecular packing in hydrated corneas ( $H > 2.0$ ). This possibility is indicated by the sharp intermolecular reflection becoming more diffuse (Fig. 3). This suggests that intermolecular disorder increases as the tissue continues to imbibe water although it should be noted that an increase in intermolecular spacing, giving a lower molecular density, would always cause some increase in the breadth of the reflection (Woodhead-Galloway, 1982). At higher hydrations, and with longer exposures, a diffuse ring corresponding to an average isotropic spacing of  $\sim 0.33$  nm is observed in the pattern at wider angles.

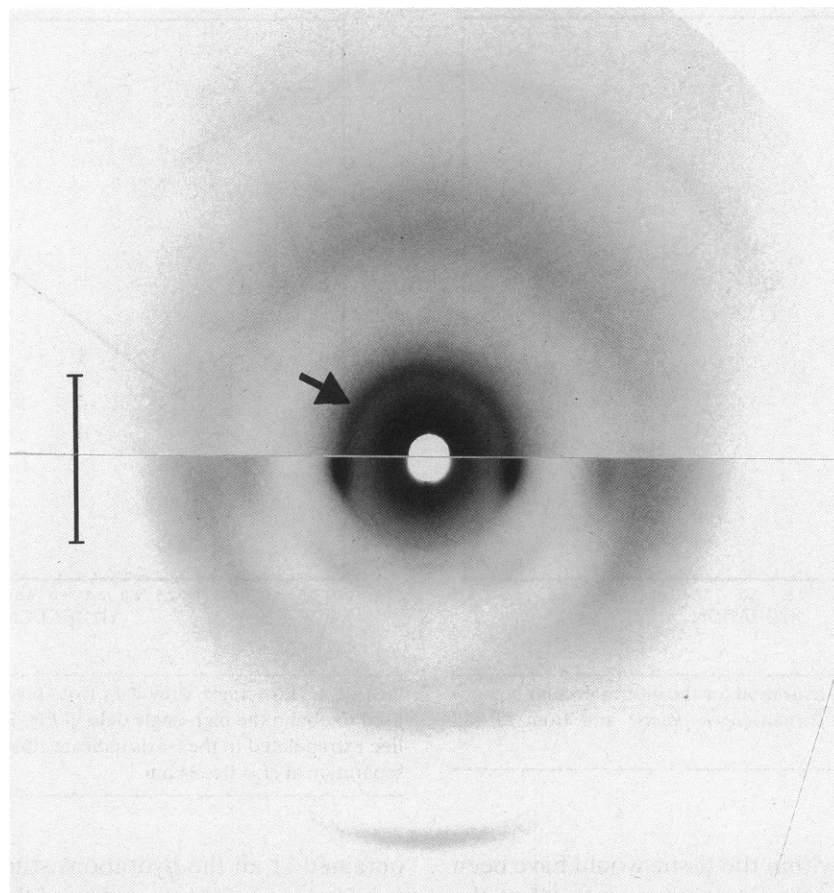


FIGURE 1 A comparison between the high-angle pattern of dry bovine cornea (*upper half*) and of rat tail tendon (*lower half*). The outer ring that appears in both halves (as a meridional reflection in the tendon pattern) is the collagen meridional reflection at 0.29 nm. The sharp inner equatorial in the tendon pattern (at 1.08 nm) coincides with a strong inner ring in the corneal pattern (*arrowed*). The fiducial mark corresponds to  $0.7 \text{ nm}^{-1}$ .

### Low-angle pattern

The mean interfibrillar spacing,  $i$ , of a liquid-like array of collagen fibrils is obtained from  $Ri = 1.12$ , where  $R$  is the reciprocal space coordinate of the first maximum in the low-angle diffraction pattern (Worthington and Inouye, 1985).<sup>1</sup> Fig. 4 shows  $i^2$  plotted as a function of tissue hydration. The specimens used to obtain these data were the same ones used to obtain the intermolecular data. From Fig. 4 the interfibrillar spacing in dry cornea is estimated as  $34 \text{ nm} \pm 2 \text{ nm}$ . This value is some 15% lower than that obtained by Sayers et al. (1982) who used a different experimental protocol to examine the

<sup>1</sup>For a hexagonal packing arrangement, the equivalent expression would be  $Ri = 1.15$ . Although it is not possible to decide from a single x-ray reflection what type of fibril packing occurs, Worthington and Inouye (1985), using up to five reflections from the low angle pattern, have concluded that fibrils are arranged in a two-dimensional liquid-like array.

effects of tissue hydration. They considered that the interfibrillar spacing at zero hydration corresponds to the diameter of the fibril on the assumption that when water is removed from the tissue the fibril surfaces are in contact.

## DISCUSSION

### High-angle pattern

Earlier studies of the high-angle x-ray pattern from corneal stroma were carried out using methods different from those employed here; the specimen hydration was altered by air drying, by immersion in water, or by equilibration with water vapor in an atmosphere of air or helium over saturated salt solutions. The x-ray patterns were obtained using a conventional source (Cooke, P., unpublished results; Fullwood et al., 1990). This had a number of disadvantages; as the hydration was changed,

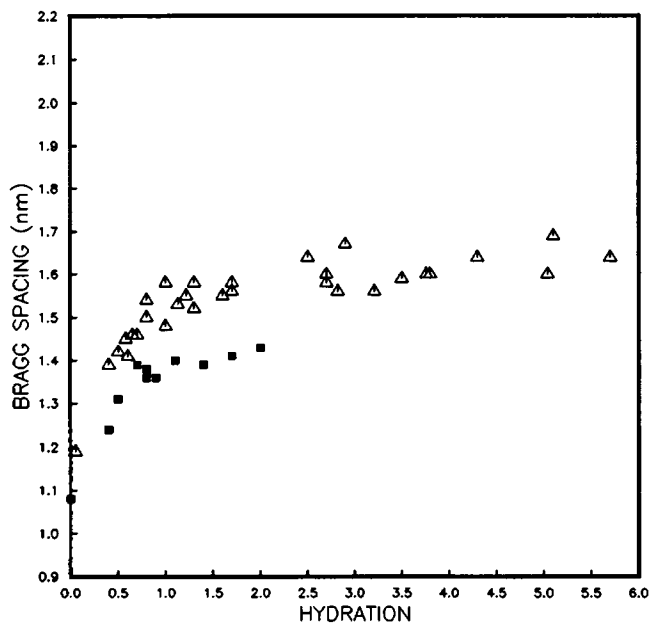


FIGURE 2 Bragg spacing vs. hydration for the intermolecular equatorial reflection from bovine cornea (*open points*) and from rat-tail tendon (*filled points*).

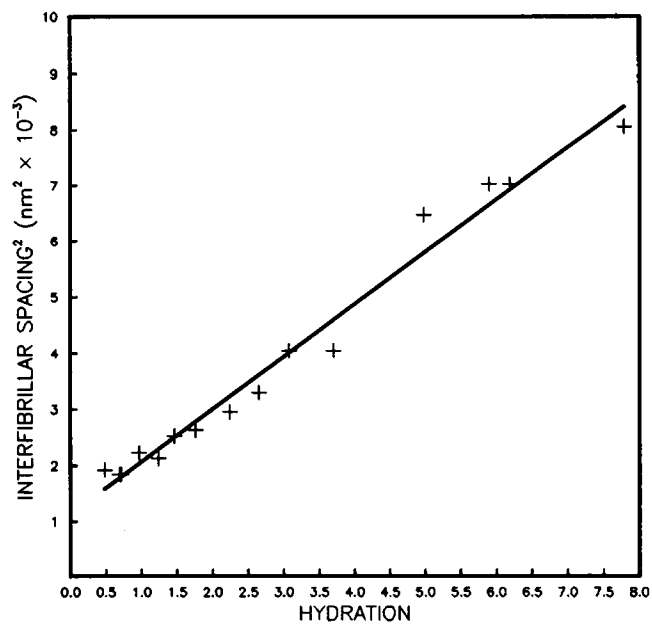


FIGURE 4 Low-angle x-ray data from several of the same specimens used to obtain the high-angle data in Fig. 2. The least-squares best-fit line extrapolated to the y-axis indicates that the center-to-center fibril separation at  $H = 0$  is 34 nm.

the salt concentration within the tissue would have been expected to change and this is now known to affect the intermolecular as well as the interfibrillar spacings (Meek, K. M., N. J. Fullwood, A. J. Quantock, and R. S. Wall, unpublished results). Also, the patterns obtained with a conventional source were diffuse and often difficult to measure. The synchrotron source has allowed each exposure time to be reduced from several hours to a few minutes and much sharper patterns have thus been

obtained at all the hydrations studied. The new results have led to a reinterpretation of the earlier x-ray data.

The present study shows that the collagen molecules move from a separation giving rise to a Bragg spacing at 1.15 nm in the totally dry state to 1.60 nm at physiological hydration ( $H \approx 3.2$ ). This implies that fibril diameters increase by  $\sim 40\%$  on hydration. Dry fibrils with radii of 13 nm (as seen in the electron microscope) should therefore have expanded to 18 nm in the normal

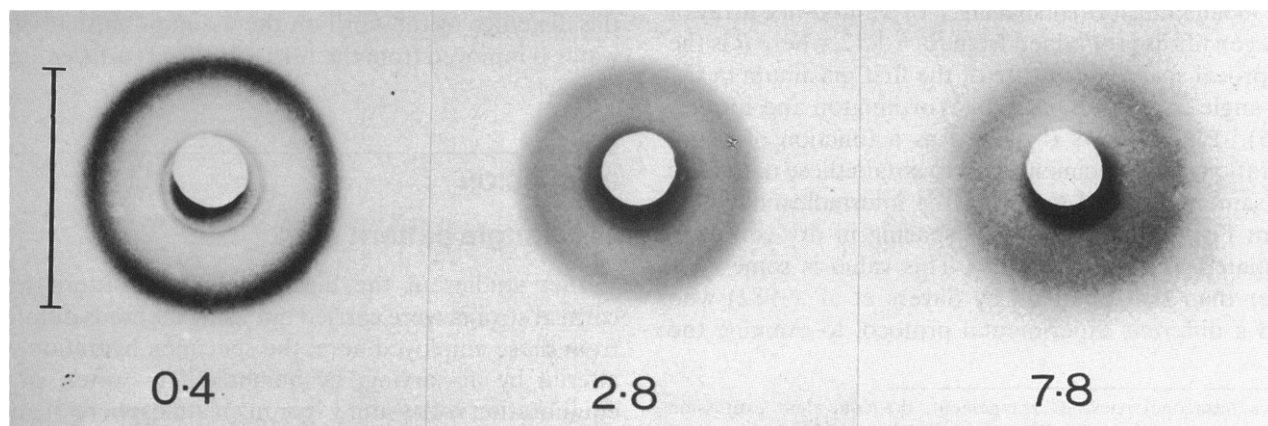


FIGURE 3 High-angle reflection resulting from the molecular packing at different hydrations in bovine cornea. As the hydration (given below each ring) increases, the ring becomes more diffuse. The fiducial mark corresponds to  $0.7 \text{ nm}^{-1}$ .

state. This is equivalent to an increase of 92% in the dry volume of the individual fibrils which presumably corresponds to the incorporation of water in the hydrated fibrils. This is in substantial disagreement with the 55% increase in volume from dryness to normal hydration calculated from the change in birefringence by Maurice (1957).

X-Ray studies at low angles of diffraction have suggested that the diameter of the dry collagen fibril in cornea is either 40 or 26 nm depending on the way the x-ray data are interpreted (Sayers et al., 1982). Sayers et al. favored an air-dried fibril diameter of 40 nm. This value represents the mean center-to-center spacing at closest approach measured from the low-angle x-ray diffraction pattern because in this dehydrated state the surfaces of the collagen fibrils were assumed to touch. Sayers et al. (1982) showed that this diameter, on the assumption that it increased with hydration, could also explain the equatorial x-ray intensities of the reflections seen in the fiber diagrams that they obtained from (bovine) cornea. In the present work we have used improved methods to equilibrate the tissue hydration and we now believe the distance of closest approach of the fibrils to be closer to 34 nm. Furthermore, it ought to be borne in mind that the distance of closest approach need not be the same as the dry fibril diameter if the fibrils do not touch when dry, i.e., if some interfibrillar material supports them. Worthington and Inouye (1985) quoted a value of 39 nm for the diameter of "untreated" (i.e., hydrated) bovine cornea (their Table 3).

Fibril diameters may also be calculated using a space-filling argument. The volume of the cornea at a given hydration may be expressed in terms of the dry volume as follows: Hydration,  $H$  is defined from

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

where  $V_w$  and  $V_c$  are the volumes occupied by the water and the dry cornea, respectively,  $\rho_c$  is the density of the dry cornea and the density of water is taken as  $1 \text{ g ml}^{-1}$ . Maurice (1957) has shown that  $\rho_c = 1.36 \text{ g ml}^{-1}$ . Eq. 1 may thus be expressed as

$$V_w = V_c \times 1.36 \times H. \quad (2)$$

Let  $V_H$  represent the volume of the hydrated cornea, then

$$V_H = V_c + V_w = V_c(1 + 1.36H) \quad (3)$$

assuming that the volume occupied by the dry material in the cornea does not change when the tissue is hydrated (e.g., by dissolution of tissue components).

From Eq. 3 it follows that, at physiological hydration ( $H \approx 3.2$ ), the dry material should occupy 1/5.4 of the

tissue volume. On the simplifying assumption that the fibrils are packed hexagonally, taking a unit cell of the stroma we may write this relationship as:

$$\pi r_0^2 = \frac{1}{5.4} \cdot \frac{\sqrt{3} i_H^2}{2}, \quad (4)$$

where  $r_0$  is the radius of the dry fibril and  $i_H$  is the center-to-center fibril spacing at normal hydration. This reduces to  $r_0/i_H = 0.23$ . If the dry fibril radius is 13 nm,  $i_H = 57 \text{ nm}$  assuming all the dry material is in the fibril. If some material is not in the fibril,  $i_H$  will be somewhat greater.

If, on the other hand, the dry fibrils have a radius of 17 nm as suggested from low-angle diffraction, this would rise to  $\sim 24 \text{ nm}$  on hydration to  $H = 3.2$ . Using the expression  $r_0/i_H = 0.23$ , the corresponding center-to-center fibril separation should then be 74 nm. This is somewhat greater than the interfibrillar spacing measured directly from low-angle x-ray diffraction patterns which is  $\sim 64 \text{ nm}$  at  $H = 3.2$  (Fig. 4). Furthermore, a dry fibril radius of 17 nm is difficult to reconcile with the 12–13 nm fibrils almost universally observed in thin sections in the electron microscope except as preparation artifacts of electron microscopy.

In the recent electron microscope study of corneal collagen by Craig et al. (1986) using low-temperature procedures, the radii of the fibrils were  $\sim 19 \text{ nm}$ , i.e.,  $\sim 50\%$  larger than those seen by conventional dehydration and embedding procedures. The authors pointed out that these larger values gave much better agreement with the low-angle x-ray results of Sayers et al. (1982) and of Worthington and Inouye (1985). They therefore suggested that the conventional electron micrographs showed dehydrated fibrils and that their new values were appropriate for the hydrated fibrils used for x-ray studies.

In the bovine cornea low-angle x-ray pattern (Sayers et al., 1982) and neutron diffraction pattern (Elliott et al., 1982) there is occasionally some evidence of orientation-effects or arcing of the normally ring-like reflections. On a few occasions we have observed traces of similar arcs in the high-angle pattern. These also probably arise from artifacts resulting from accidentally straining the cornea, and do not indicate a real preferred orientation of the fibrillar structure.

The diffuse ring at 0.33 nm that occurs in highly swollen corneas does not correlate with any spacing in the pattern of native collagen (Ramachandran, 1967), nor does its character suggest that it arises from any small molecule such as NaCl, etc. This ring occurs when the collagen pattern is indistinct and diffuse (for example, the rings at 0.45 and 0.29 nm disappear at  $H = > 4$ ) and when longer x-ray exposures have been used in an

effort to see more. This ring probably arises from the water itself, which is the most abundant component. The x-ray scattering curves of water (Morgan and Warren, 1938) show a pronounced maximum in this region.

### Correlation between the low-angle and high-angle patterns

Are the changes in the interfibrillar spacing that accompany hydration of the cornea consistent with the changes in the intermolecular packing that are observed in the wide angle x-ray pattern? We consider two possibilities. The interfibrillar and intermolecular spacings could shift simultaneously as the cornea becomes hydrated; this would suggest that the fibrils become hydrated and their surfaces remain either in contact or closer together than would be the case if the water went mostly between the fibrils. Alternatively, the intermolecular spacing might increase at the same rate as the interfibrillar separation while the fibrils preferentially absorb the initial water but might thereafter remain at a constant value while additional water is absorbed preferentially in the interfibrillar spaces. In an effort to distinguish between these two possibilities, both the intermolecular and the interfibrillar spacings are plotted as a function of hydration (Fig. 5). The two sets of data are normalized at  $H = 0$ . Fig. 5 indicates that the course of hydration has a similar

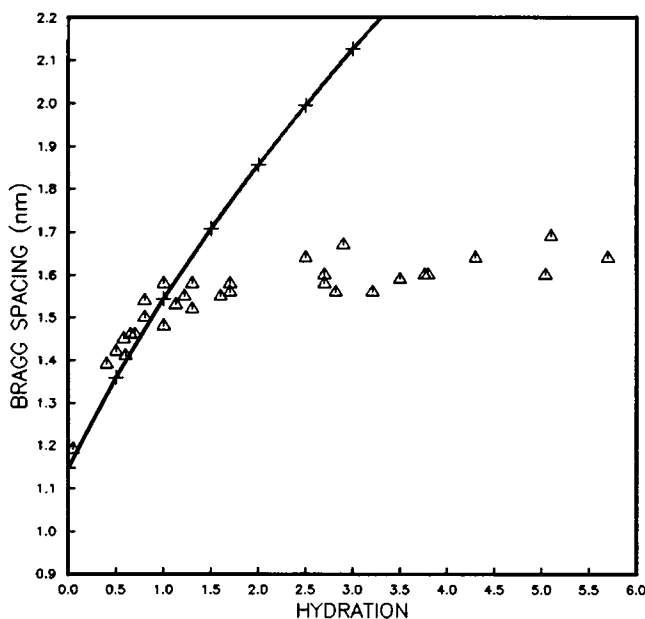


FIGURE 5 Corneal high-angle data, as in Fig. 2, with the addition of experimental low-angle data from Fig. 4; points calculated from the best-fit line are shown as plus signs. The two sets of data are normalized at  $H = 0$ .

effect on the intermolecular and interfibrillar spacings in the range  $H = 0-1$ . If collagen fibrils touch at  $H = 0$  (Sayers et al., 1982) this initial water would be absorbed totally within the fibrils, the surfaces of which would therefore remain in contact. On the other hand if the fibrils are still separated in the dry state, the results show that the initial water would have to be absorbed equally within and between the fibrils. As hydration increases above 1, however, proportionally less water is absorbed into the fibrils.

These data may have implications for the transparency of cornea, if considered with the information on the packing of fibrils obtained from low-angle x-ray diffraction patterns (Sayers et al., 1982), whose analysis of the low-angle pattern provided two major conclusions: First, that the diffracting fibrils in fresh or rehydrated corneal stroma are packed with short-range order extending over distances of three fibril diameters. Second, that much of the water is not localized between the fibrils, but rather in spaces ("lakes") between the domains of fibrils packed in groups of four (on the average) or between the stromal lamellae.<sup>2</sup> If a large portion of the water is localized between the lamellae, the cornea should become iridescent due to thin-layer light interference and this is not observed (Maurice, 1957). Thus, it would be necessary for the "extra" water to be situated elsewhere in the structure of the stroma. The low-angle diffraction patterns obtained during the current work indicate that the interfibrillar spacing is 34 nm in corneas at  $H = 0$  (extrapolated) and 64 nm at  $H = 3.2$ . These interfibrillar spacings suggest that the volume associated with each fibril (corresponding to a unit cell) increases by a factor of  $\sim 3.5$  while the volume of the tissue as a whole increases by a factor of 5.4 determined using Eq. 3. Fig. 6 extends this relationship to show how the volume associated with the fibrils (as determined by x-ray diffraction) increases as a function of hydration (line a). The data are taken from Fig. 4. For comparison, the increase expected if water goes uniformly between all the fibrils in the cornea is also shown (line b). The points on line b, however, have been calculated using Eq. 3, and thus depend on the value of the interfibrillar distance at  $H = 0$  which we have measured to be  $34 \pm 2$

<sup>2</sup>Worthington and Inouye (1985) give an alternative explanation of the experimental data from low angle diffraction. They assume that the nearest-neighbor number changes as the cornea hydrates. This paper is not the appropriate place to discuss this in detail however. The essential difference between Worthington and Inouye's approach and that of Sayers et al. (1982) is that Worthington and Inouye supposed that the higher order equatorial (interfibrillar) reflections arose from the fibril transform while Sayers et al. (1982), having considered that possibility, believed that the intensity data were better explained if these reflections arose from interference effects between several fibrils.

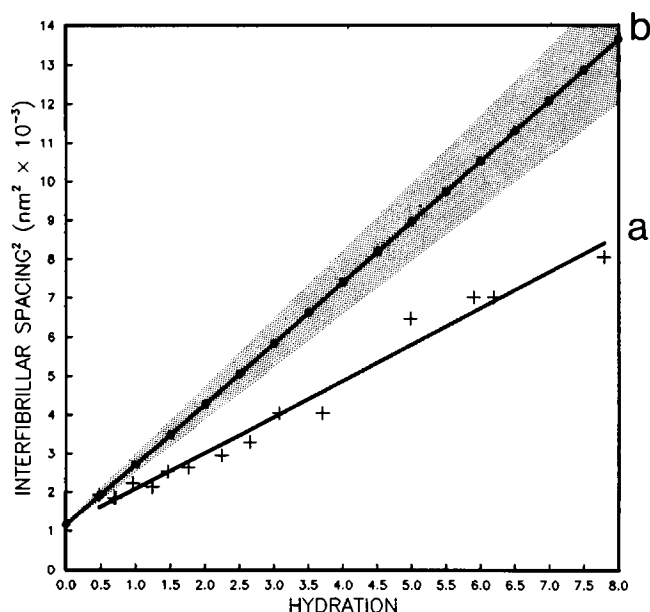


FIGURE 6 Corneal interfibrillar volume from low-angle data (crosses). Line *a* is the least squares, best fit through the data. Line *b* is the theoretical interfibrillar volume calculated by assuming the water to be absorbed uniformly throughout the tissue; calculated points are shown as filled circles. In this calculation we have supposed the corneal tissue to swell in two dimensions only, as is well supported by the low-angle data. The two sets of data are normalized at  $H = 0$ . The confidence limits of the calculated data are indicated by the shaded area.

nm. The confidence limit of this value, therefore, sets a limit to the accuracy with which line *b* may be plotted, which is indicated in Fig. 6 by the shaded area around the line. Even within the accuracy of the measurements it can be seen that, especially above  $H = 1$ , there is a significant divergence of the experimental data and the calculated line, indicating that some water must be located in another space or compartment. For dried-then-rehydrated corneas the interfibrillar spacing increases much less than for fresh swollen corneas (Sayers et al., 1982) so more of the water must be localized in this other space. In both cases it is possible to postulate a second set or population of fibrils that do not reach a uniform equilibrium spacing, and are therefore more widely and irregularly separated and do not contribute significantly to the diffraction pattern. This second population of fibrils would correspond to the "lakes" which have been invoked by Sayers et al. (1982), among others. There is no way of telling if these predominantly water spaces are completely free of fibrils or contain a few fibrils in no particular relative order. It seems possible that over the range of hydration from zero to about  $H = 1$ , the stroma accumulates the water uniformly as suggested by the correspondence between the

behavior of the molecular and interfibrillar spacings (Fig. 5). Only at levels of hydration  $> H = 1$  could the fibrils separate to allow the formation of lakes.

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