DAVID E. FREUND, RUSSELL L. McCALLY, and RICHARD A. FARRELL

LIGHT–SCATTERING TESTS OF STRUCTURE IN NORMAL AND SWOLLEN RABBIT CORNEAS

Theoretical and experimental results are compared to determine whether the features seen in electron micrographs of swollen corneas are accurate depictions of the cornea's ultrastructure or whether they have been distorted by the procedures used to prepare the micrograph. The recently developed method of direct summation of fields is used to compute the light scattering expected from the structures depicted in electron micrographs of normal, 15% swollen, and 25% swollen rabbit corneas. Comparisons of these theoretical predictions with experimental light-scattering measurements performed on freshly excised rabbit corneas show good agreement, supporting the idea that electron micrographs are a quantitatively accurate representation of the cornea's ultrastructure.

INTRODUCTION

For many years, our research has been concerned with determining what structural features of the cornea of the eye are responsible for its transparency and what structural alterations cause transparency loss when it swells or is otherwise changed by damage or disease. Since the cornea does not absorb light in the visible portion of the spectrum, answering these questions requires an understanding of the cornea's light-scattering properties. The ultrastructures from which light is scattered are traditionally viewed by means of electron microscopy. In principle, methods could be devised to calculate the scattering expected from these ultrastructures and thereby to gain insight into how they cause increased or decreased scattering. But the methods used to preserve the tissue and render it suitable for observation in the high-vacuum environment of the electron microscope could alter the structures so that they would not be accurate enough representations to allow quantitative scattering calculations. For this reason, we have been investigating whether light scattering itself can be used to probe the relevant structures within the cornea.

Because the wavelengths of visible light are larger than the structural details of the cornea, light scattering does not provide the spatial resolution afforded by electron microscopy, but does offer the distinct advantage that measurements could be made on fresh tissue. The measurements therefore are not subject to the artifacts that could affect electron microscopy. In fact, we have not abandoned electron microscopy. Rather, the approach that we have adopted is to make quantitative predictions of the scattering to be expected from the structures depicted in electron micrographs, together with predictions of how the scattering is expected to vary with parameters such as light wavelength and scattering angle, and then to compare the results with appropriate scattering measurements made on fresh tissue under carefully controlled conditions. In this manner the scattering measurements serve as a test of the depicted structures. Moreover, the method permits testing of the relative importance of various structural features as they affect transparency, as well as the investigation of other possible explanations of corneal transparency. This approach has been used previously for normal corneas, and good agreement was found between experimental light-scattering measurements and theoretical predictions.[1–3] That work has been summarized in previous *Technical Digest* articles.[3,4]

The structures in electron micrographs of swollen cornea are fundamentally different from those in normal cornea. For that reason, the theoretical techniques that were used to make quantitative light-scattering predictions for normal cornea[1,2] are not applicable to swollen cornea. We were able, however, to predict how the scattering would vary with light wavelength, on the basis of a simple model of the structures depicted in electron micrographs, and to show that other possible swelling mechanisms would produce different dependencies.[4,5] Scattering measurements on swollen corneas were consistent with the model based on the structures seen in electron micrographs,[4,5] but we had no way to make quantitative predictions of scattering to compare with the measurements. Recently, we developed a technique—called the direct summation of fields—that has alleviated this problem.[6] The new technique is general and is applicable to normal as well as swollen corneas. A detailed description of the new method, along with a preliminary application, was described in our most recent *Technical Digest* article.[7]

In this article we report the application of direct summation of fields to both normal and swollen rabbit corneas, with special emphasis on the results for swollen corneas. First, we discuss the structure of normal and cold swollen corneas, emphasizing the features that are important for calculating light scattering. We then pre-

sent the direct summation calculations for normal corneas and for corneas swollen to a thickness 15% and 25% greater than normal and compare them with the experimental results. We conclude by discussing the implications of these results *vis-à-vis* the prominent theories of corneal transparency and its loss upon swelling.

BACKGROUND

As represented in Figure 1, the cornea is the transparent portion of the eye's wall, having a thickness of about 0.4 mm in rabbits and about 0.5 mm in humans. Viewed from the front, it is roughly circular, with a diameter of approximately 11 mm in both rabbits and humans, and its spherical radius of curvature is about 7.5 mm in both species. Its curved interface with air provides about three-fourths of the eye's total light-focusing power. Thus, it is imperative that the cornea maintain its proper curvature.

The region of the cornea known as the stroma possesses the structure that enables the cornea to support the intraocular pressure and to maintain its curvature. The stroma constitutes approximately 90% of the cornea's thickness and, as shown in Figure 2, is a layered structure composed of many stacked sheets called lamellae. A few large flat cells, called keratocytes, are dispersed between the lamellae, and occupy from 3% to 5% of the stroma's volume. Each lamella is made up of long, thin, cylindrical collagen fibrils embedded in an optically homogeneous ground substance composed of water, mucoproteins, and various salts. The fibrils have nearly uniform diameters of about 25 nm. Within a given lamella, they run parallel to each other and to the surface of the cornea and extend over its entire width. Fibrils within adjacent lamellae make large angles with respect to each other. This fibrillar structure gives the cornea its mechanical strength and the ability to maintain its curvature.

Another property of the cornea, which is also necessary for normal vision, is its near-perfect transparency. It is not immediately obvious how the fibrils, needed for the cornea to maintain its structural integrity, are compatible with transparency. The relative refractive index between the fibrils and the homogeneous ground substance surrounding them differs only slightly from unity; typical estimates range from 1.04 to 1.1. Although it follows that the individual fibrils are very weak scatterers of light, Maurice[8] showed that, because their sheer numbers are so great, the cornea would be essentially opaque if the fibrils scattered light independently of one another. Maurice also recalled a property of perfect crystalline lattices: if the lattice spacing is smaller than half the light wavelength, only the zero-order Bragg condition can be satisfied, and light will pass through the lattice unscattered. Noting that the mean fibril spacing is much less than half the light wavelength, he postulated that the fibril centers within each lamella are actually arranged in a perfect two-dimensional hexagonal lattice. He attributed the apparent lack of crystalline order in electron micrographs of corneal lamellae (cf. Fig. 3A) to disruptions of the *in vivo* positions of fibril centers, introduced by the required tissue preparation.

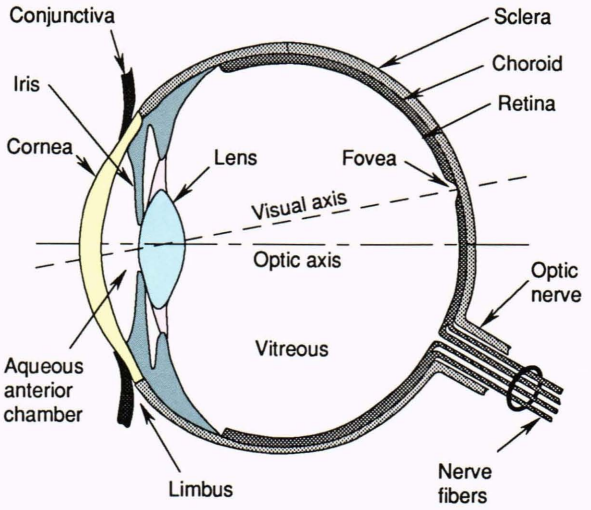

Figure 1. Diagram of the eye showing location of the cornea.

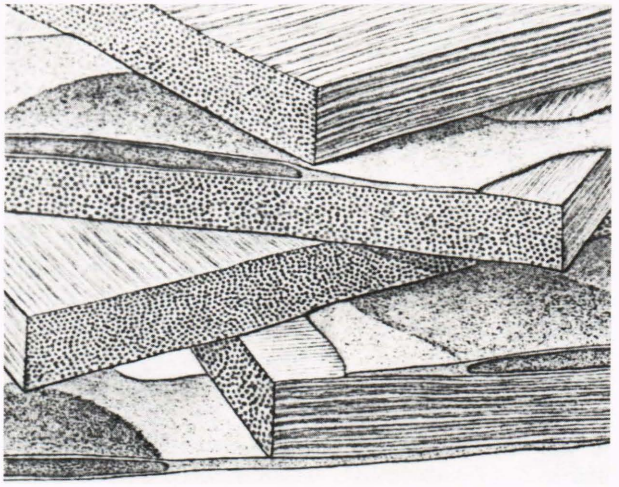

Figure 2. A schematic illustration of four lamellae from a normal cornea. The collagen fibrils are essentially of uniform diameter and, within a given lamella, are all parallel to each other and run the entire length of the cornea. Adjacent lamellae are oriented at large angles with respect to each other. Three keratocytes are also shown between the lamellae. (Reprinted, with permission, from Hogan, M. J., Alvarado, J. A., and Weddell, J. E., *Histology of the Human Eye,* W. B. Saunders, Philadelphia, p. 93; © 1971 by W. B. Saunders.)

Although they lack perfect crystalline order, the positions of the fibrils in electron micrographs do have local or short-range order that can be described by a radial distribution function $g(r)$, as shown in Figure 4. Hart and Farrell asked whether this short-range spatial ordering could, by itself, create enough destructive interference among the scattered waves to explain transparency. To answer the question, they derived a theory for normal corneas that was based on methods similar to those developed for calculating X-ray scattering from liquids.[1,9,10] Using the radial distribution function ob-

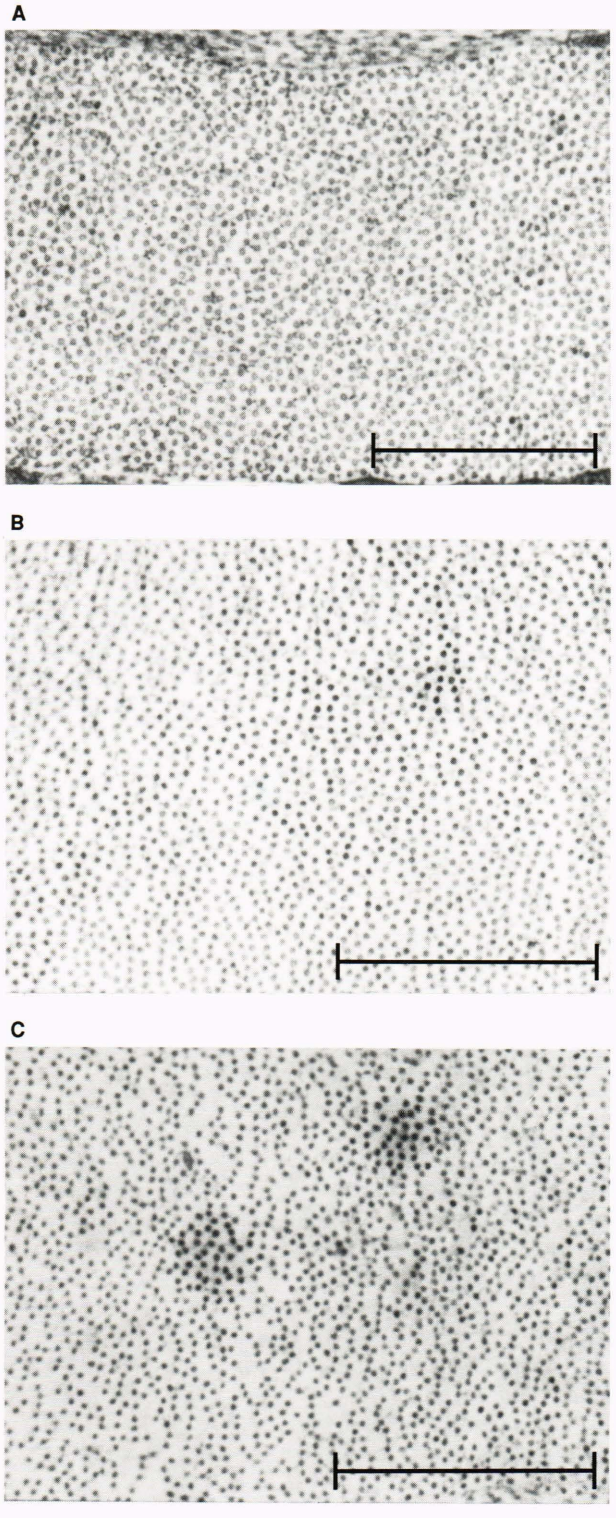

Figure 3. Electron micrographs of rabbit corneas at three different stages of swelling. Scale bar is equal to 1 μm. **A**. Normal unswollen cornea. **B**. Cornea swollen 15%. **C**. Cornea swollen 25%.

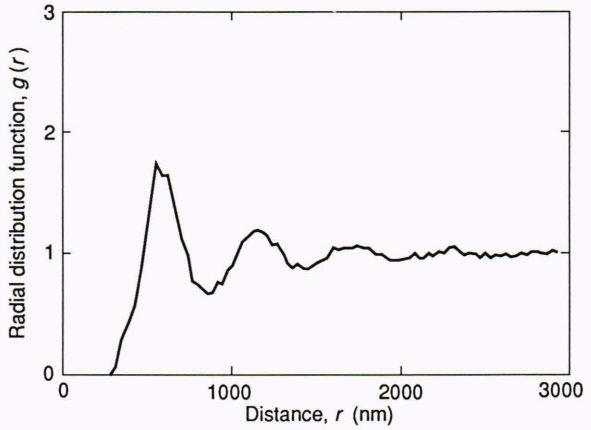

Figure 4. A typical radial distribution function, *g(r),* illustrating the short-range order observed in electron micrographs. It represents the relative likelihood of finding a fibril at a distance *r* from any given fibril and goes to unity at large distances.

tained from direct measurements of fibril positions in electron micrographs of normal corneas, they made quantitative predictions for light transmission that were in good agreement with measurements.[1,2] That agreement showed that the structures seen in electron micrographs of normal corneas are consistent with transparency and cannot be discarded as artifacts using arguments based on transparency theories.

When the cornea becomes more hydrated than in its normal state as the result of damage or disease, it swells and its transparency is diminished. Electron micrographs of swollen corneas show that the short-range spatial ordering that existed for normal corneas has been disrupted (see Figs. 3B and 3C). In particular, some regions contain no fibrils; these regions are called lakes. Such a distribution cannot be described by a radial distribution function, and the theory of Hart and Farrell[1] therefore cannot be applied to swollen cornea. The requirement for a more general theory led to our development of the new direct summation-of-fields method.[6,7]

LIGHT–SCATTERING MEASUREMENTS

The quantity determined for comparison with theoretical scattering calculations is the total scattering cross section per fibril (per unit length), $\sigma_T(\lambda)$, in which λ is the light wavelength. The quantity actually measured is the fraction of light transmitted by the cornea, F_T, which is the ratio of the light passing directly through the cornea to that which passes in the absence of the cornea.[1,2,4] The two quantities are related by

$$F_T = \exp(-\sigma_T \rho \Delta) , \qquad (1)$$

where Δ is the thickness of the cornea and ρ is the number density of fibril centers.[2,4,5] The apparatus and techniques for making the transmission and thickness measurements have been described earlier in the *Technical Digest* and elsewhere.[4,5,7,11] Two important assumptions are made to analyze the data properly.[4,5,11] For normal cornea we assume that rabbit-to-rabbit variations in F_T are entirely due to changes in corneal thickness (i.e., ρ

and $\sigma_T(\lambda)$ are the same for all normal corneas). These variations are accounted for by averaging the quantity $(1/\Delta)\ln F_T$ over the measured corneas. The average value of F_T is then computed using a value of 0.38 mm for thickness. For swollen corneas we use the fact that the number of fibrils is conserved, so that $\rho_0\Delta_0 = \rho\Delta$, in which the subscript zero refers to the cornea's state before swelling. The quantity averaged is

$$\left(\frac{1}{\Delta_0}\right) \ln F_T = -\frac{(\sigma_T \rho \Delta)}{\Delta_0} = -\sigma_T \rho_0 \, . \tag{2}$$

By analogy with normal cornea, the average value of the transmittance is computed from

$$\overline{F}_T = \exp\left[0.38 \left(\overline{\frac{1}{\Delta_0} \ln F_T}\right)\right], \tag{3}$$

where Δ_0 is in millimeters.

THEORETICAL CALCULATIONS

The newly developed method of direct summation of fields is used to compute the expected light scattering from the structures shown in electron micrographs of both normal and swollen corneas. Succinctly stated, this method allows one to approximate the ensemble average for the total scattering cross section, using only a single electron micrograph. The approximation is done, in essence, by partitioning the micrograph with an imaginary grid containing K grid elements, each of the same size and shape. The grid is then used to construct a sample with which to approximate the desired ensemble average. Since the details have been described previously, we will not reproduce them here; instead, we simply give the final expression for the scattering cross section.[6,7] Specifically, if one makes the approximation that all the fibrils have the same diameter, then the differential (or angular) scattering cross section per fibril (per unit length) at scattering angle θ_s is given by

$$\sigma(\lambda, \theta_s) = \frac{K\sigma_0(\lambda, \theta_s)}{(K-1)\overline{N}}\left[\overline{|\, S_b(\lambda, \theta_s)\,|^2} - |\,\overline{S_b(\lambda, \theta_s)}\,|^2\right], \tag{4}$$

where overbars denote sample averages and $\overline{N}$ is the average number of fibrils within a grid element. The quantity $S_b(\lambda, \theta_s)$ is a phase sum for the bth grid element in the partition, and is defined by

$$S_b(\lambda, \theta_s) \equiv \sum_j^{N(b)} \exp(i\mathbf{q}\cdot\mathbf{r}_j) \, , \tag{5}$$

where the summation runs over all the $N(b)$ fibrils within the bth grid element and $\mathbf{r}_j$ is the location (coordinate vector) of the center of the jth fibril. The scattering vector is denoted by $\mathbf{q}$ and is given by

$$\mathbf{q} \equiv \mathbf{k}_i - \mathbf{k}_s \, , \tag{6}$$

where $\mathbf{k}_i$ and $\mathbf{k}_s$ are the incident and scattered wave vectors, respectively. Finally, $\sigma_0(\lambda, \theta_s)$ in Equation 4 denotes the differential scattering cross section (per unit length) of an isolated fibril. In the dielectric needle approximation, with unpolarized light incident normal to the fibril axes,

$$\sigma_0(\lambda, \theta_s) = \frac{n^3(\pi a)^4(m^2-1)^2}{2\lambda^3}\left\{1 + \left[\frac{2\cos\theta_s}{m^2+1}\right]^2\right\}, \tag{7}$$

where n is the refractive index of the ground substance, a is the fibril radius, and m is the fibril's relative refractive index (i.e., the ratio of the fibril's refractive index to that of the surrounding ground substance).

The electron micrographs to be analyzed are scanned at the appropriate pixel resolution on an Optronics International rotating-drum scanner. The scanned images then are processed on a Macintosh II, using the image analysis program IMAGE (available without restriction from Wayne Rasband, Research Services Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland), together with Fortran algorithms that we have developed, to locate the fibril centers ($\mathbf{r}_j$ values) and the average fibril radius, a.[6,12] The values of n and m are then estimated using the Gladstone–Dale law of mixtures, as described previously.[2,5]

The total scattering cross section $\sigma_T(\lambda)$, which is needed to compare the theoretical predictions with experimental measurements, is related to the differential scattering cross section by

$$\sigma_T(\lambda) = \int_0^{2\pi} \sigma(\lambda, \theta_s)\, d\theta_s \, . \tag{8}$$

Thus, the total scattering cross section is found by using Equation 4 to evaluate $\sigma(\lambda, \theta_s)$ at a series of scattering

angles between 0 and 2π and then using numerical integration to obtain $\sigma_T(\lambda)$.

RESULTS AND DISCUSSION

Figure 5A compares the calculated and measured values of the transmissivity, F_T, as a function of the wavelength of incident light. The open circles are the averages of measurements taken on six different normal corneas,[4,5] and the solid circles are the average computed values from four different electron micrographs of normal corneas. Similarly, the open triangles are the averages of measurements taken on four different 15% swollen corneas,[4,5] and the solid triangles are the average computed values from three different micrographs of 15% swollen corneas. The open squares are the average measurements taken on three different 25% swollen corneas,[4,5] and the solid squares show the average computed values from three different micrographs of 25% swollen corneas. We note that Equation 1 is used for computing F_T, where the fibril number density for each calculation was determined directly from the electron micrograph by dividing the total number of fibrils within the grid by the area of the grid. The thickness was taken to be 0.38 mm for normal rabbit corneas. Effective thicknesses of 1.15 and 1.25 times this value were used for the 15% and 25% swollen corneas, respectively. Agreement between the calculated and measured results is excellent, especially since we have not adjusted (or normalized) parameters to make the calculated values fit the measurements, for example, at a specified wavelength. It could be argued that such an adjustment would be justified because of animal-to-animal variation in, among other things, number density of fibrils, or because the parameters used to determine the refractive indices of the ground substance and fibrils are not well known.

We previously reviewed alternative explanations for corneal transparency and its loss upon swelling.[4,5,11] We showed that transparency theories can be tested on the basis of their predictions for the wavelength dependence of the total scattering cross section. In particular, short-range order like that seen in electron micrographs of normal corneas leads to an inverse cubic dependence. The so-called equal–refractive-index explanation[13] and the modified hard-core theory of Twersky[14] also would have this same dependence since they postulate a short-range order. The equal–refractive-index theory has been rejected on the basis of scattering measurements for polarized light,[4,11] and the modified hard-core theory fails on the basis of its predictions for swollen cornea, as discussed below. Feuk[15] postulates that the fibrils actually have small random displacements from perfect lattice sites. This long-range ordering would lead to an inverse fifth-power dependence on wavelength. For swollen corneas, our analysis of Benedek's lake theory predicts that the presence of lakes would add a term to the scattering cross section that would vary as the inverse square of wavelength.[4,5,16] On the other hand, an extension of the modified hard-core theory[14] postulates that the lakes are artifacts and that the increased scattering arises from a randomization in the fibril positions. Such randomization would arise because the fibrils have more volume available to them, and it would lead to a decrease in destructive interference. This theory predicts the same wave-

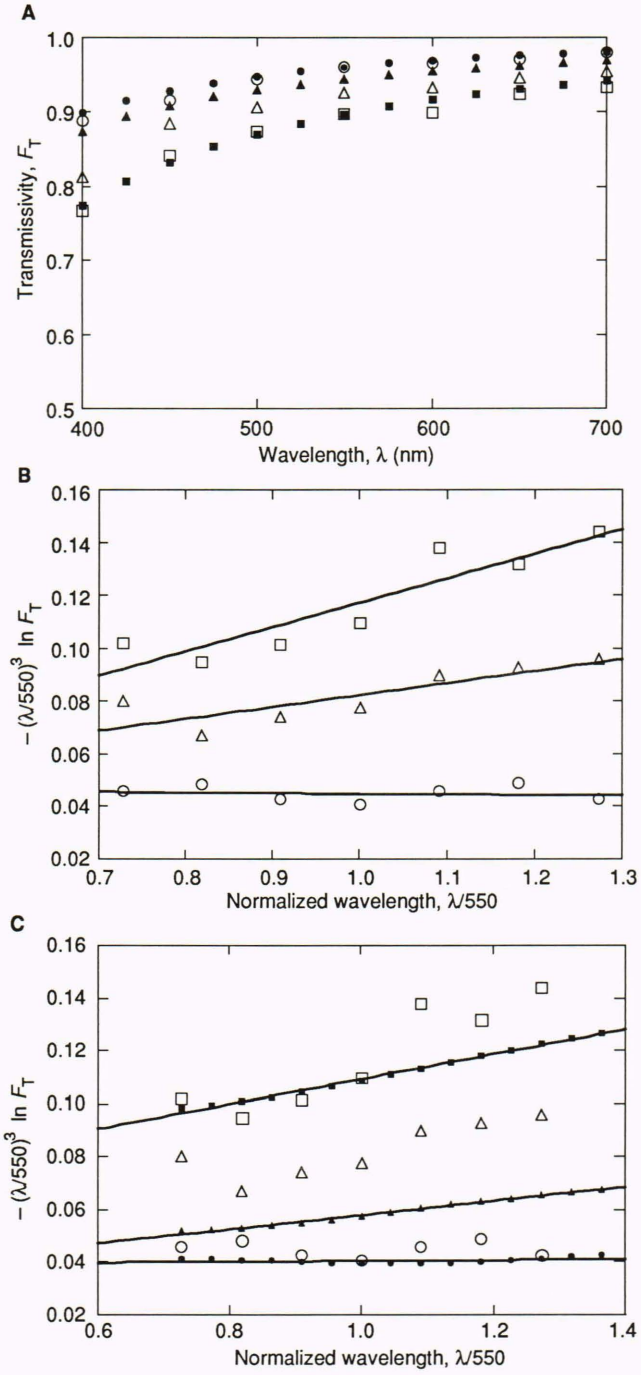

Figure 5. Light-scattering measurements (open symbols) and direct summation-of-fields calculations (solid symbols) for normal (circles), 15% swollen (triangles), and 25% swollen (squares) rabbit corneas. **A**. A comparison of the wavelength dependence for measured and calculated transmissivity for normal, 15% swollen, and 25% swollen corneas. **B**. Least-squares fit showing the wavelength dependence of the quantity $-(\lambda/550)^3 \ln F_T$ for the measured light-scattering data. The results support short-range order theories for normal corneas and the theory of lakes for swollen corneas. **C**. Comparison of the measured wavelength dependence for the quantity $-(\lambda/550)^3 \ln F_T$ with that predicted from light-scattering calculations based on structures seen in electron micrographs.

length dependence as for normal cornea (i.e., inverse cubic). Obviously, all of these predictions can be tested. Previous transmissivity tests, using experimental results for normal and swollen cornea and calculations for normal cornea, were in accord with the predictions based on the structures as revealed by electron microscopy. We show here that the new calculations for swollen corneas are also in accord with the structures depicted in electron micrographs.

Figure 5B shows a plot of $-(\lambda/550)^3 \ln F_T$ (recall that Equation 2 shows $-\ln F_T$ directly proportional to σ) as a function of $\lambda/550$, where $\lambda/550$ is a normalized wavelength. Multiplication by $(\lambda/550)^3$ would remove the inverse cubic dependence on wavelength expected if short-range ordering produces transparency, and it would turn an inverse-square dependence, such as that expected with lakes, into a linear dependence on wavelength. The straight lines are the least-squares fit through the corresponding data. The data for normal corneas are essentially constant, suggesting that the cross section indeed varies as the inverse cube of wavelength. The data for swollen corneas fall on straight lines with positive slope that increases with increased swelling, suggesting that the lead term indeed varies as the inverse square of wavelength. These results are in accord with short-range order for normal corneas and lakes for swollen corneas.

Figure 5C is the same as Figure 5B, except that the lines representing the least-squares fit through the data have been replaced with the corresponding plots of the theoretical calculation and their least-squares line fit. These results also are in good agreement with the experimental measurements and show that theoretical calculations based on structures seen in electron micrographs are not only capable of accurately predicting F_T (as in Figure 5A), but also of predicting the correct shape (wavelength dependence) of the scatterings. Again, it should be emphasized that the theoretical calculations have not been adjusted or normalized in any way. The results lend support to the idea that the structures seen in electron micrographs are accurate depictions of the cornea's true ultrastructure for both normal and swollen corneas. Indeed, given the results in Figures 5B and 5C, if any criticism is to be made about electron micrographs, it is that they may understate the significance of lakes in swollen corneas. The remarkable agreement for normal corneas seems very convincing for short-range order, as seen in electron micrographs.

Our future research in this area will be directed toward applying these and similar methods to the study of corneal diseases such as keratoconus and to the structures in corneal scar tissue.

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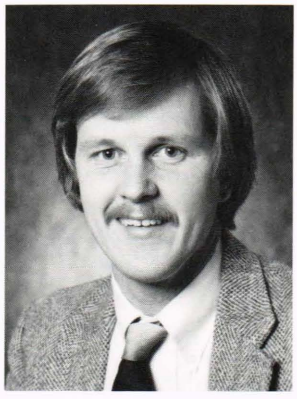

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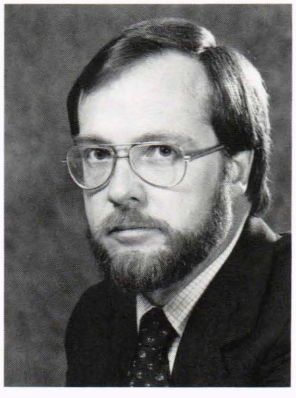

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