Effect of transcorneal pressure on small-angle light scattering from rabbit cornea*

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Small-angle light scattering (SALS) measurements from the central region of the rabbit cornea are presented. These measurements are unique since a transcorneal pressure is applied. The variation of the SALS patterns with pressure suggests that a new explanation of their origin should be considered. I_+ scattering changes markedly with the application of pressure and slight changes are also noted in $I_\parallel$ scattering. At less than 1 mmHg pressure ('zero' pressure), the I_+ pattern is a five-lobed cloverleaf, with one lobe at 0° scattering angle, and the others oriented along the polarization directions with intensity maxima at 1.8° scattering angle. The intensity of this pattern decreases dramatically as the pressure increases and a second cloverleaf pattern, aligned at 45° to the polarization direction becomes apparent. It is suggested that the waviness of the corneal stroma lamellae, noted in electron micrographs of tissue fixed in the absence of pressure, be considered as the morphological feature responsible for the 'zero' pressure pattern. The second cloverleaf pattern may be associated with stromal cells.

INTRODUCTION

The cornea is the clear, tough window in the wall of the eye. The outer corneal surface consists of a cellular layer called the epithelium, and the inner surface is lined by a second cellular layer called the endothelium. The epithelium and endothelium account, respectively, for approximately 9% and 1% of the total thickness (which is about 0.4 mm in the rabbit and about 0.5 mm in man). The central, or stromal, region comprises the remaining 90% of the corneal thickness.

The stroma is a lamellar structure. The average lamella is 2 μm thick. Within each lamella a matrix of collagen fibrils is embedded in a muco-poly(saccharide) ground substance. These fibrils, which are about 20 nm in diameter in the rabbit[1], lie essentially parallel to one another and to the corneal surface. The axes of fibrils in adjacent lamellae make large angles with one another. The refractive index of the fibrils differs slightly from the ground substance and so they scatter light. Although the individual fibrils are inefficient scatterers, they are so numerous that the scattering would be sufficient to render the cornea opaque if they were randomly arranged[2,3].

Modern theories recognize that corneal transparency is the result of destructive interference among the light waves scattered in all except the forward direction[1–8]. These interference effects arise because the spatial arrangement of the fibrils is partly ordered. We have recognized that light scattering provides a non-invasive means for probing structure and that light scattering can be used to test the predictions of the various structural models which have been advanced[6,8]. Our approach in this research has been to calculate the scattering expected from the structures shown in electron micrographs of stromal tissue, to make careful light scattering measurements under controlled physiological conditions and, finally, to compare the results. There is no *a priori* guarantee of agreement because the fixation and embedding procedures required for electron microscopy could possibly damage the tissues. Up to now, however, there has been agreement, and we have been able to show that the light scattering measurements are consistent with the short ranged ordering of fibrils shown in electron micrographs of normal corneas and with the occurrence of regions void of fibrils ('lakes') pictured in electron micrographs of less transparent swollen corneas[6,8]. Corneal light scattering argues against long range order models for corneal transparency[2,5] and is inconsistent with a general disorganization of fibrils as a mechanism for transparency loss in cold-swollen corneas[2,6,8].

Other researchers, notably Chang, Keedy and Chien[9] and Bettelheim and Kaplan[10] have been attempting to elucidate corneal structure by using the small-angle light scattering (SALS) methods which have been a powerful tool in understanding the morphology of polymers[11]. Bettelheim's group has primarily investigated bovine cornea[10] and Chien's[9] has examined rabbit cornea. The results for rabbit[9] and bovine[10] corneas suggest different morphologies for the two species. However, comparisons of the observed scattering patterns with model structures for which the scattering patterns are known and with structures known histologically have been less than satisfactory.

SALS probes structural features of larger dimensions (of the order of several microns) than the light scattering methods which we have previously employed. Nevertheless our success to date with the approach of comparing light scattering measurements with predictions based on structures revealed in electron micrographs has led us to investigate the possibility of applying similar methods to the SALS properties of cornea. Initially we have set out to discover the influence of physiological conditions, particularly intraocular pressure, on the SALS properties of the cornea. Our motivation stems from the observation that in conventionally

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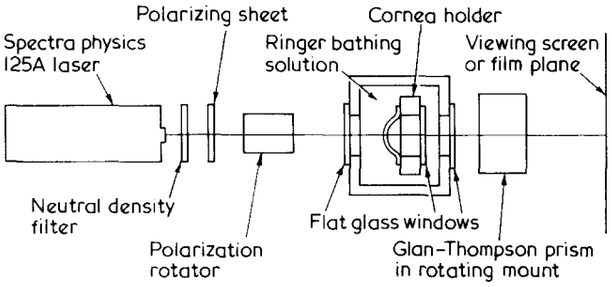

Figure 1 A schematic representation of the SALS apparatus

fixed corneal stroma, the lamellae are wavy and the waves have a fairly regular period of several microns[12,13]. Such structural features could possibly influence SALS, although no calculations have been made. The normal transcorneal pressure is not maintained during the conventional electron microscopy fixation procedure and there has been the suggestion, as yet unconfirmed, that the waviness is reduced when the cornea is fixed under pressure[14].

The primary purpose of this paper is to show that, indeed, intraocular pressure has a significant effect on SALS from the cornea and thus to point out the necessity of carefully controlling experimental conditions in future attempts to probe corneal structure by this method. Also, we note that previously proposed model structures are inconsistent with the observed scattering patterns, and suggest they be examined in terms of a new structural model.

EXPERIMENTAL

Specimen preparation

Adult albino rabbits weighing approximately 3 kg were used for the experiment. The animals were sacrificed with an overdose of sodium pentabarbitol (Nembutal) administered intravenously. The eyes were enucleated immediately after death. The corneas were excised and mounted in a specially designed Lucite holder which permits a hydrostatic pressure difference to be maintained across the cornea. The details of the excision procedure and the design of the Lucite holder are discussed by Farrell *et al.*[6]. The holder was inserted in the apparatus where it was held in a small tub having flat glass windows to permit the entrance of the incident laser beam and the exit of the scattered light. The cornea was bathed on both surfaces with Krebs bicarbonate Ringer solution containing glucose[6].

The SALS apparatus

The SALS apparatus is shown schematically in *Figure 1*. The vertically polarized output of the Spectra Physics 125A was attenuated to approximately 5 mW with a 1.0 neutral density filter. The attenuated beam (about 2 mm in diameter at its $(1/e)^2$ points) was passed through a sheet polarizer whose purpose was to attenuate further any depolarized light emanating from the laser. The polarization direction of the incident light could be varied by means of the Spectra-Physics polarization rotator. The scattered light passed through a Glan–Thompson prism (2.5 cm aperture, Karl Lambrecht Inc.) before being recorded on film. Two types of pattern, denoted by I_+ and $I_\parallel$, were measured. The polarization vectors of the incident and scattered radiation are parallel for the $I_\parallel$ patterns and are oriented at 90° with respect to one another for the I_+ patterns. I_+ patterns were recorded directly on 35 mm film held in a Nikon F camera body (with lens removed). The more intense $I_\parallel$ pattern was projected onto a ground glass screen and photographed with the Nikon and a 55 mm, f3.5 macro lens. Tri-X film developed in Microdol-X for 10 min at 70°F was used. Exposures, unless noted otherwise, were 1/1000 sec for I_+ patterns and 1/60 sec for the $I_\parallel$ patterns.

Method

The excised and mounted cornea was placed in the apparatus and a very slight hydrostatic pressure (≤1 mmHg) was applied to maintain normal corneal curvature. This is called the 'zero pressure' condition. The incident light beam was aligned normal to the corneal surface and passed through its centre.

SALS measurements (especially those of I_+ scattering) are known to be affected by birefringence; however, this effect is minimized when the polarization direction of the incident radiation is aligned parallel or perpendicular to the local optical axis[9,10,15]. The direction of the optic axis was located by determining the angle of the rotator for which the transmission was minimized in the I_+ configuration. At this setting, the optic axis is either parallel or perpendicular to the direction of polarization of the incident beam. All subsequent measurements, except those which purposely examined the effect of altering this condition, were made with this rotator setting. Increasing the pressure difference across the cornea did not significantly alter the direction of the optic axis. I_+ and $I_\parallel$ patterns were photographed at zero pressure and at 18 mmHg, which is the normal intraocular pressure of the rabbit. Additional I_+ patterns were photographed at 9 mmHg.

RESULTS

The I_+ patterns at zero, 9 and 18 mmHg are shown in *Figure 2*. At zero pressure the scattering pattern is a five intensity maxima cloverleaf, with one of the maxima at a scattering angle of 0°. The lobes of the cloverleaf are aligned with the polarization directions. This pattern persists as the transcorneal pressure is increased; but its intensity decreases dramatically and apparently monotonically. Moreover, as the pressure is increased a second cloverleaf pattern emerges. It appears likely that this pattern may also be a five-lobed cloverleaf. This is difficult to tell with certainty however, because the inner pattern is superimposed on the weak outer five-lobed pattern and the central region is overexposed to show these weak outer features. Consequently, subsequent discussions of this interior pattern are limited to the four lobes which are aligned at 45° with respect to the polarization direction. The intensity maxima occur at a smaller scattering angle than in the other pattern. The pattern alterations are essentially simultaneous with the pressure changes.

The positions of the intensity maxima were determined by scanning the negatives with a microdensitometer. The positions of the maxima in the outer pattern occur at scattering angles of 0° and 1.8° at all three pressures, and those in the inner pattern are at 1.4° at the elevated pressures. These values are for the true scattering angle and have been corrected for refractive effects in the bath and for the deviation introduced by the analysing prism.

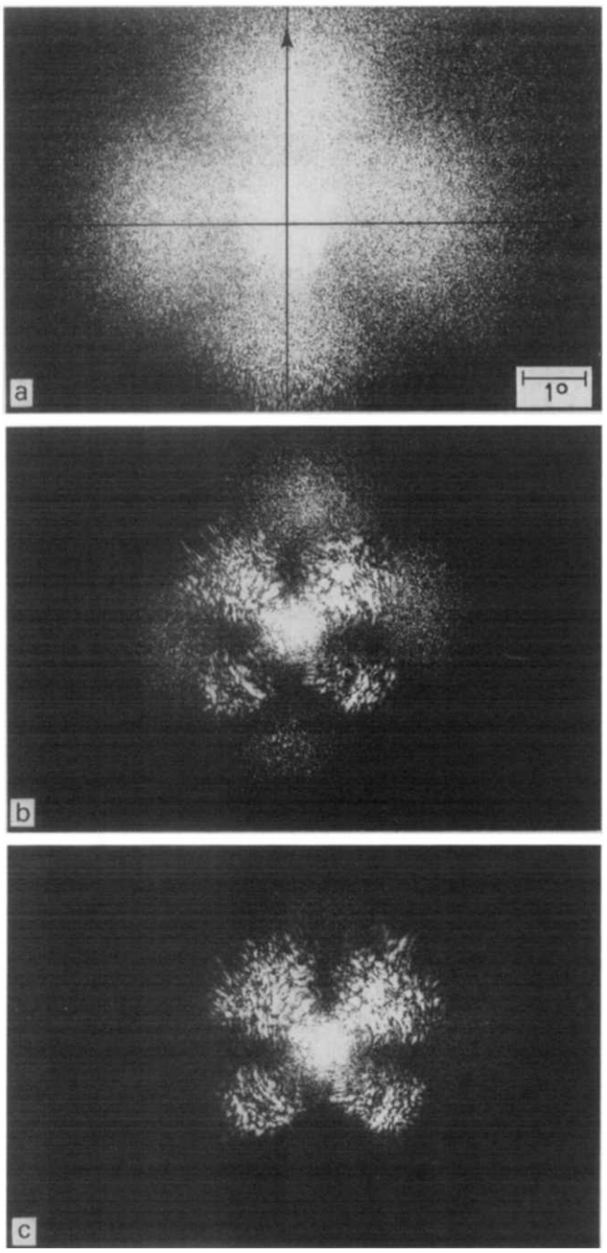

Figure 2 The effect of increasing transcorneal pressure on the I_+ scattering pattern. (a), (b) and (c) are at zero, 9 and 18 mmHg, respectively. The arrows show the directions of the polarizer axes. The scale for the true scattering angle is given in (a). Each picture received the same photographic and enlarging exposure and the same development

The $I_\parallel$ scattering patterns at zero and 18 mmHg are shown in *Figure 3*. The scattering is essentially uniform in these cases; however, a weak cross or cloverleaf pattern superposed on this background is discernible at zero pressure. This pattern, whose lobes are aligned at 45° with respect to the polarization direction, disappears as the pressure increases.

We also examined the effect of birefringence on the I_+ SALS patterns To accomplish this the polarizer and analyser were rotated in tandem, in 30° increments, away from the setting which gave minimum transmission. SALS patterns were obtained in this manner under the zero and 18 mmHg pressure conditions, and these patterns are reproduced in *Figure 4*.

Certain minor variations from the typical results shown in *Figures 2* and *3* were noted in a few of the corneas which we investigated. In some cases the outer pattern was more apparent at increased pressure, although its intensity was always markedly less than at zero pressure. Also, in one cornea, the outer pattern lobe intensities did not decrease uniformly when the pressure was increased. Rather, the intensity of two of the opposing lobes decreased more than the other two opposing lobes. Finally, the weak lobe structure in the zero pressure $I_\parallel$ pattern was more apparent in some corneas than in others. Nevertheless it was never discernible at a pressure of 18 mmHg.

DISCUSSION

The unique feature of our experiments is that a hydrostatic pressure difference is maintained across the cornea. Consequently, care must be exercised in making direct comparisons with other experiments. However, we note that the five intensity maxima cloverleaf pattern observed at zero pressure and reproduced in *Figure 2* is similar to the type A pattern which Chang, Keedy and Chien reported for the rabbit cornea[9], with respect both to the number of lobes and to their orientation relative to the polarization direction[16]. The

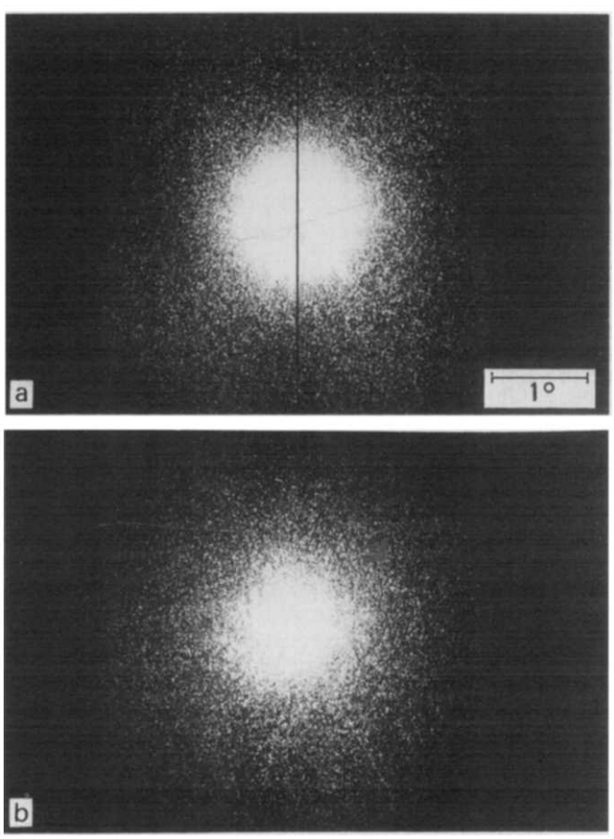

Figure 3 The effect of increasing transcorneal pressure on the $I_\parallel$ scattering pattern. The zero pressure pattern is shown in (a) and the pattern at 18 mmHg is shown in (b). The arrow shows the direction of the aligned polarizers and the scale for the true scattering angle is given in (a). Each picture received the same photographic and enlarging exposure and the same development

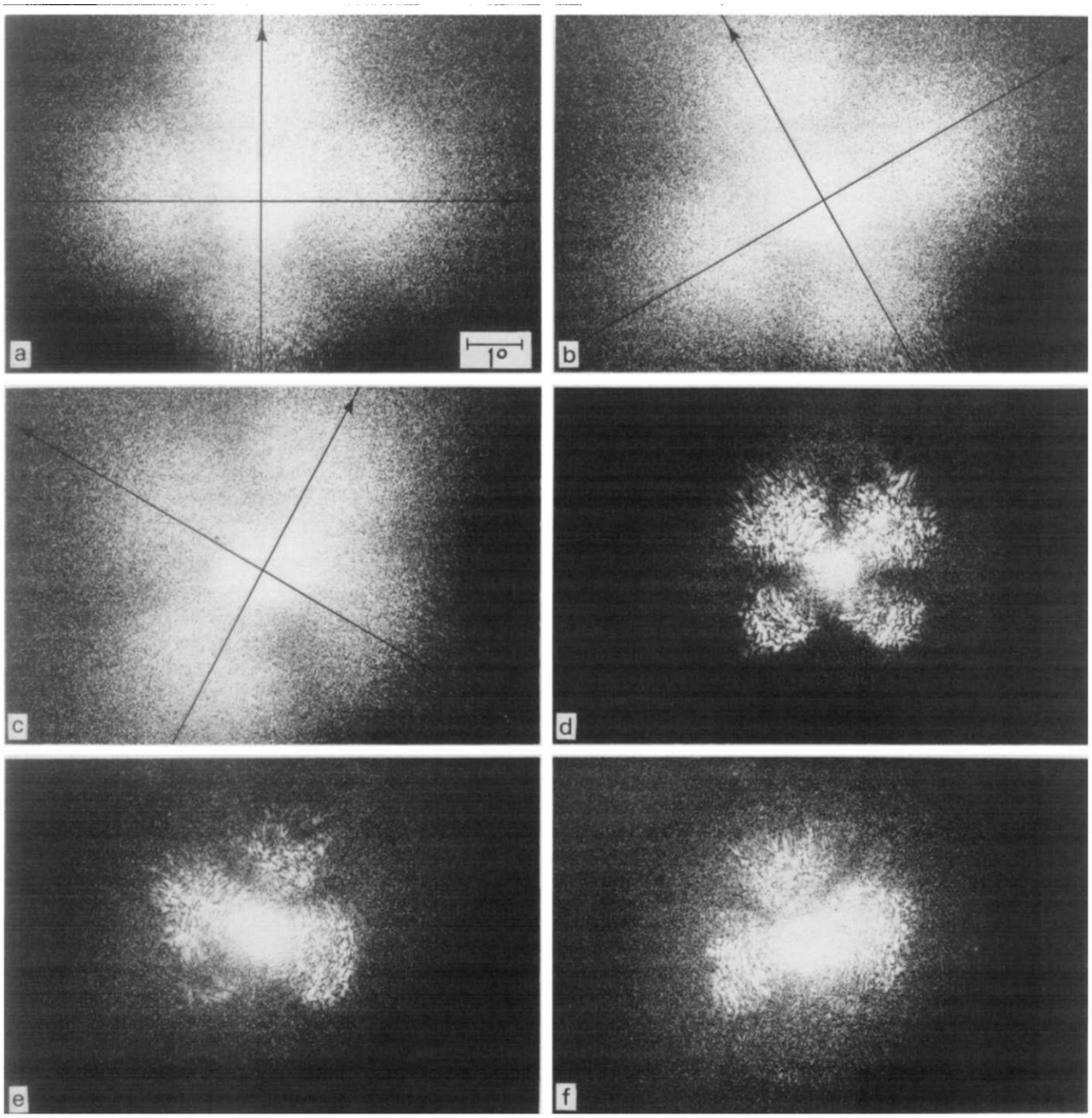

Figure 4 The I_+ scattering patterns at different orientations of the polarizer and analyser. The zero pressure results are shown in panels (a), (b) and (c). The results at 18 mmHg are shown in (d), (e) and (f). In (a) and (d) the polarizer is aligned either parallel or perpendicular to the optic axis and therefore the effects of birefringence are minimized[9,10,15]. These two pictures are the same as in *Figure 2a* and *Figure 2c*. In (b) and (e) and in (c) and (f), the polarizer and analyser have been rotated in tandem in 30° increments from the setting in (a) and (d). Each picture received the same photographic exposure (1/500 sec) and enlarging exposure and the same development

positions of the intensity maxima at 0° and 1.8° scattering angles also compare favourably with the 0° and 2° obtained by these investigators. This type of I_+ pattern has also been reported for the rat[17], but is different from that of bovine cornea[10]. In Chang *et al.*'s experiment this pattern was always observed when the polarizers were aligned with the slow optic axis, which is the optical configuration which we used. Chang *et al.* reported that, in the central cornea, the five-maxima cloverleaf changed to an intense circular pattern as the polarization direction was moved away from alignment with the optic axis; however, we did not observe this effect (cf. *Figure 4*).

Chang *et al.*[9] suggested that 'the morphology which gives rise to the type A pattern is most likely a random assembly of incomplete spherulites with sheaf-like textures'. They based this on the SALS theory advanced by Picot, Stein, Motegi and Kawai[18]. However, the observed alignment of the cloverleaf pattern relative to the polarization direction conflicts with the predictions of this model. The behaviour of our outer I_+ pattern with increasing pressure, when coupled with electron microscopic evidence and the above noted conflict, suggests that an alternative structural interpretation of the SALS should be considered.

Corneal lamellae appear wavy in electron micrographs

Figure 5 **A low magnification electron micrograph of corneal stroma of a rabbit. This cornea was fixed in the absence of a transcorneal pressure according to the procedure given in ref 6. The waviness of the lamellae is apparent and a few keratocytes are shown in cross-section**

prepared by standard techniques (cf. *Figure 5*); however, it has been suggested by J. Francois[14] that fixation of the cornea under its normal intraocular pressure appears to flatten out the lamellae. Structures of dimensions comparable to the periodicity of these waves would be expected to affect SALS measurements. In fact, Maurice[13] offered them as a possible explanation for the cross-striated appearance of the cornea under a phase-contract microscope. The decreased intensity in the outer SALS pattern with increasing pressure shown in *Figure 2* is consistent with scattering from these waves. However, firm conclusions must await confirmation of Francois' communication and detailed calculations of the scattering to be expected from such structures.

The interior I_+ cloverleaf pattern which appears in the present experiments at elevated transcorneal pressures, has not been observed previously. The interpretation of this pattern in terms of corneal structure is not evident; however, we note that a similar I_+ pattern, in which the lobes are oriented at 45° with respect to the polarization direction, is predicted for scattering from two-dimensional spherulites. A complete spherulite (disc) leads to a four-lobed cloverleaf I_+ pattern, whereas an incomplete spherulite (sheaf) leads to a five-lobed cloverleaf I_+ pattern[18]. As we noted in the Results section, it cannot be determined unequivocally whether this inner pattern has four or five lobes. Nevertheless, this is immaterial if one only wishes to determine the characteristic dimensions of the scattering elements, since the size parameter $w = (2\pi a/\lambda) \sin\theta$ obtains in both cases[18]. Here, a is the radius of the scattering element, λ is the wavelength in the scattering medium (the refractive index of cornea[1,2] is 1.375) and θ is the scattering angle[15,18]. In this model, the maximum intensity occurs is when $w = 3.9$. Thus, if this were the correct model, the scatterers would have diameters of ~20μm. This conjecture is not inconsistent with histological evidence. Stromal cells, the majority of which are keratocytes, are very flattened in the plane of the stroma and might act like two-dimensional spherulitic scatterers (either complete or incomplete). They occupy only a few percent of the stromal volume and have dimensions[13] of the order of 20μm. Although the I_+ inner pattern is consistent with scattering from such structures, comparison of the measured $I_\parallel$ pattern with the prediction of such a model is complicated by the background scattering in this configuration.

In conclusion, we have demonstrated that intraocular pressure strongly influences SALS from the cornea. Certain previously unobserved features become evident as the pressure is increased, and the intensity of the pattern present at zero pressure is drastically reduced at elevated pressures. This reduction in intensity suggests that wavy lamellae be considered as a possible scattering mechanism in attempts to determine the structural basis for SALS from the cornea. There is a clear need for further calculations and experimentation to understand fully these new observations. The present experiments underline the importance of conducting corneal (and other biological) experiments under well-defined conditions, and examining the effect of these conditions on the experimental outcome.

ACKNOWLEDGEMENTS

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