# AXIAL ELECTRON DENSITY OF HUMAN SCLERAL COLLAGEN

## Location of Proteoglycans by X-Ray Diffraction

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ABSTRACT The low angle meridional x-ray diffraction pattern from fresh human sclera was analyzed to ascertain if collagen-bound proteoglycans affect the axially-projected electron density distribution to the same extent as appears to occur in the cornea. The results showed that, unlike cornea, the electron density of the sclera is similar to that seen in rat tail tendon collagen. The proteoglycans were specifically stained using either Cuprolinic blue or Cupromeronic blue, both under critical electrolyte conditions. The tissue was then examined by electron microscopy and by low angle x-ray diffraction. The electron-optical observations suggested that proteoglycans associate with collagen near the d/e staining bands in the gap zone. A difference Fourier analysis from the x-ray results confirmed that these observations were not e.m. preparative artefacts and allowed a quantitative estimate to be made of the axial extent of the proteglycans in the wet tissue.

### INTRODUCTION

Although the sclera constitutes the major part of the outer protective coating of the eye, its lack of optical function has led to its morphology being largely overlooked except in comparisons with the adjacent cornea. The fibrous bands seen in the microscope are a continuation of the corneal lamellae which run uninterrupted across the limbus (Maurice, 1969). The scleral lamellae contain collagen fibrils with a diameter range from 30 to 300 nm (Schwartz, 1953) as opposed to the corneal collagen fibrils, whose diameter is uniformly 24 nm in all land vertebrates (Craig and Parry, 1981). It is thought that many corneal fibrils fuse together in the intervening limbus to form the broader fibrils found in the sclera.

Proteoglycans occur in the interfibrillar spaces in most connective tissues. Cartilages, for example, contain large proteoglycans consisting of keratan sulphate and chondroitin sulphate glycosaminoglycans attached to the same protein core, whose main roles seem to be space-filling and shock absorbing (Hardingham et al., 1986). Skin, tendon, and cornea, on the other hand, contain 'small' proteoglycans with only a few glycosaminoglycans (of a single type) attached to a small protein core. These proteoglycans may be involved in preventing the nucleation of calcification (Scott and Haigh, 1985*a*), limiting fibril diameters (Parry et al., 1982) and/or controlling interfibril spacings (Borcherding et al., 1975; Meek et al., 1986).

The scleral proteoglycans, although of the 'small' type, are different to those found in cornea and it is interesting to contrast the different optical and structural properties of the corneal stroma and sclera with the different proteoglycans in each tissue. This has become easier with the introduction of dyes which specifically stain proteoglycans (Scott, 1972; Scott, 1985). After enzyme digestion of the cornea with either chondroitinase or keratanase, Scott and Haigh (1985*b*) used the dye Cupromeronic blue to demonstrate binding of keratan sulphate proteoglycan to corneal collagen at the 'a' and 'c' electron-optical staining bands, and dermatan sulphate proteoglycan at the 'd' and 'e' bands. These results were confirmed in the wet tissue using x-ray diffraction techniques (Meek et al., 1986). Sclera, on the other hand, appeared to contain proteoglycans (dermatan sulphate) mainly at the 'd' band (Young, 1985; Scott and Haigh, 1985*b*).

In all electron-optical observations, the effects of dehydration, embedding, and counterstaining of the tissue are difficult to assess. X-ray diffraction, however, allows the specimens to be examined as near the native state as possible. It is thus a useful method of examining proteoglycan distributions to confirm and refine the electron microscopical results. The x-ray data are also an average over a very much larger region of the specimen and thus give information which is more representative of the whole sample. Furthermore, in systems consisting of both ordered and disordered proteoglycans, only the proteoglycans ordered in the direction of the collagen fibril axis can contribute to the meridonal x-ray diffraction pattern. In an earlier publication we showed how Cupromeronic blue (enhanced with sodium tungstate) is sufficiently electron-

dense to modify the low angle x-ray diffraction pattern from immature and mature rat tail tendons (Meek et al., 1985). We have now applied similar methods to locate the proteoglycans in human sclera.

## METHODS

Human sclera was obtained postmortem from donors between the ages of 50 and 70. The sclera was immediately frozen in liquid nitrogen–cooled isopentane and stored at −20°C until used.

### Electron Microscopy

Small (1–2 $mm^2$) pieces were dissected from a region ~0.5 cm from the limbus and stained overnight in 0.05% wt/vol Cuprolinic blue (BDH Ltd., Atherstone, Warwickshire, UK) containing 2.5% glutaraldehyde and 0.1 M $MgCl_2$ in 25 mM sodium acetate buffer, pH 5.7 (Scott et al., 1981). Controls were treated identically but with the omission of Cuprolinic blue from the mixture. The dyed tissues were rinsed three times, each for 5 min in the control solution. The pieces were then stained 'en bloc' in three changes of 0.5% wt/vol aqueous sodium tungstate for a total of 15 min. They were dehydrated in ethanol and embedded in Spurr resin. Thin sections (silver/gold) were cut on a Reichert-Jung Ultracut E ultramicrotome, positively stained with 1% aqueous uranyl acetate and examined with a Philips 301 electron microscope.

### X-Ray Diffraction

Small strips of sclera (~2 × 6 mm) were cut out and treated as described above except that Cupromeronic blue was used instead of Cuprolinic blue. The Cupromeronic blue was a gift from Professor J. E. Scott, Manchester University. It is believed to have a higher specificity than Cuprolinic blue and appears to produce finer proteoglycan filaments in the electron microscope. In all other respects it behaves similarly to Cuprolinic blue. Controls were processed in identical solutions but with the Cupromeronic blue omitted. Some samples were examined without any treatment (i.e., fresh).

X-ray patterns were obtained at the Science and Education Research Council synchrotron source (SRS) at Daresbury, UK. The advantage of using a synchrotron source has been discussed previously (Meek et al., 1985). The specimen-film distance was ~2.5 m, the wavelength of the radiation was 1.602 Å, and the beam dimensions were 1 × 4 mm. Exposures of 10 min were sufficient to record 10 orders of the low-angle pattern. The patterns were recorded on Ceaverken AB photographic film (Ceaverken, Strangnas, Sweden), which was then scanned using an Ultrascan XL laser microdensitometer (LKB Instruments, Inc., Gaithersburg, MD). Integrated intensities of the diffraction rings were made directly from the densitometer scans after subtraction of background scatter (Meek et al., 1985). No polarization or Lorentz corrections were made (Meek et al., 1981*a*).

The analytical procedures have been described in detail elsewhere (Meek et al., 1985; Meek and Chapman, 1985; Meek et al., 1986). Electron density (above water background) was calculated from

$$\rho(u) = F_0 + \Sigma F(h) \exp(i\Phi(h) - 2\pi hu), \qquad (1)$$

where $u$ is the axial coordinate specifying vector lengths in fractions of the D-period, (D = 67 nm) $F(h)$ are the structure factor amplitudes taken as the positive square roots of the observed intensities, $h$ is the order of the reflection and $\phi$ are the phases. Electron densities were smoothed by the factor $\exp(-h^2\beta)$ where $\beta = 0.001$, to reduce series termination errors (Sherwood, 1976). Zero order structure factors, $F_0$, were chosen so as to eliminate negative electron densities. The values of $F_0$ used are minimumn estimates but any additional contributions will not affect the variation of electron density along the D-period.

## RESULTS

The low angle meridional diffraction pattern from fresh human sclera is shown in Fig. 1. The x-ray beam was passed perpendicular to the plane of the sclera (the surface of the eyeball), and because collagen fibrils occur in all possible orientations within this plane, the diffraction pattern is a series of concentric circles. The corresponding integrated intensities are presented in Table I where they are compared with intensities from fresh rat tail tendon collagen. The first order scleral intensity is arbitrarily assigned the value 1,000 and the tendon data are scaled so as to equate the summed intensities with those from sclera. The close agreement between the intensities from sclera and from tendon suggests that at low resolution the corresponding electron densities are similar and this allows us to make the approximation that the phases are also similar. Phases for rat tail tendon have been published (Hulmes et al., 1980) and are quoted in Table I.

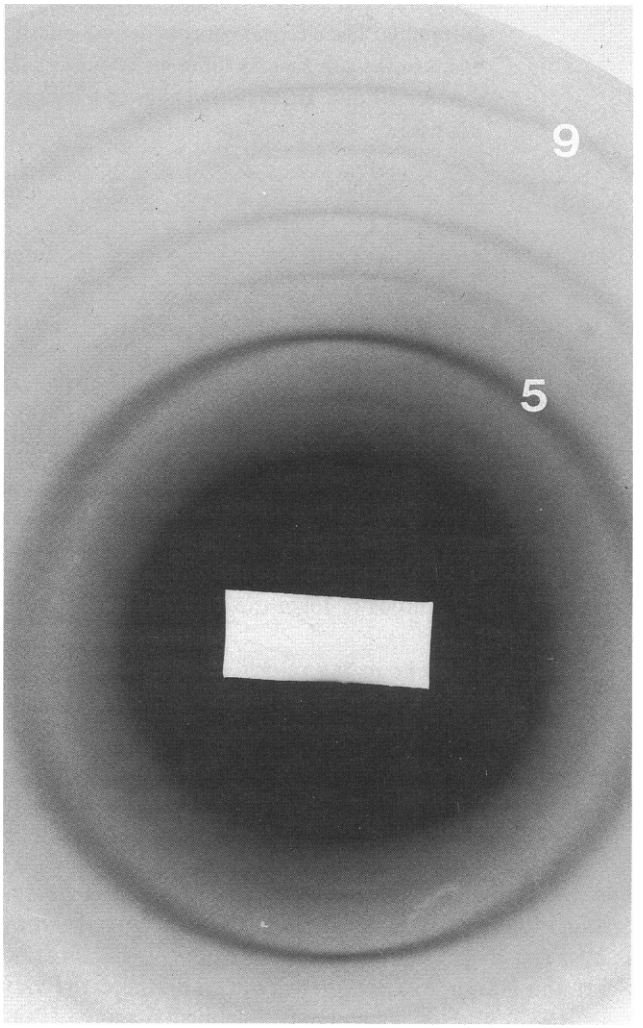


FIGURE 1 Low-angle meridional x-ray diffraction pattern from fresh human sclera. The fifth and ninth orders of the pattern are indicated. Due to the range of intensities encountered in the x-ray patterns it is not possible to reproduce all the orders on a single photographic print.

TABLE I
INTEGRATED X-RAY INTENSITIES FROM FRESH HUMAN SCLERA AND RAT TAIL TENDON

| Order ($n$) | Intensity (sclera) | Intensity* (rat tail tendon) | Phase‡ (rat tail tendon) |
|---|---|---|---|
| | | | *degrees* |
| 1 | 1000 | 1034 | 66.7 |
| 2 | 23 | 28 | 95.6 |
| 3 | 143 | 113 | 346.2 |
| 4 | 10 | 11 | 83.4 |
| 5 | 80 | 56 | 279.1 |
| 6 | 19 | 18 | 255.6 |
| 7 | 32 | 22 | 184.3 |
| 8 | 16 | 6 | 336.1 |
| 9 | 46 | 73 | 31.8 |
| 10 | 11 | 20 | 193.1 |

*Data taken from Hulmes et al. (1977). ‡Phases taken from Hulmes et al. (1980). The phases are relative to a zero origin.

Fig. 2 shows the electron density distributions for human sclera and for rat tail tendon, calculated from the data in Table I. The plots confirm that the axial electron density of scleral collagen is very similar to that in rat tail tendon collagen. This implies that the collagen axial structures, including the effects of natural cross-linking, etc., are homologous in the two tissues. Although scleral collagen is known to have nearly twice as many hydroxylysine-linked glycosides as tendon (Harding et al., 1980) these do not appear to contribute significantly to the diffraction.

Proteoglycans are also known to be bound to collagen. In fresh cornea, the presence of extra electron density compared with fresh tendon (Meek et al., 1981*b*) has been related to the sites of proteoglycan attachment (Meek et al., 1986). Any proteoglycan attachment in sclera, however, does not appear to contribute to x-ray scattering, possibly because it is associated with the surface of fibrils with a larger average circumference than found in cornea (Meek et al., 1983; Scott, 1984). Because of the similarity of the two electron densities in Fig. 2, it was not possible to produce a plot showing the difference between them. Such a plot would be highly dependent on the choice of scaling and hence would have little meaning. To locate any collagen-bound proteoglycans, human sclera was therefore stained with either Cuprolinic blue or Cupromeronic blue. Fig. 3 is an electron micrograph showing the relationship between the proteoglycan filaments and collagen fibrils. The result confirms those reported previously (Young, 1985) with filaments apparently associating with the collagen predominantly at the d or e bands. However, unlike Young (1985) we have observed very few filaments running parallel to the collagen linking consecutive d bands.

Human sclera was also stained for x-ray diffraction using Cupromeronic blue. The results are shown in Table II, where the first order intensity from the control is arbitrarily assigned the value 1,000. The scaling of the intensity data from sclera stained with Cupromeronic blue with respect to the control is discussed later. To produce

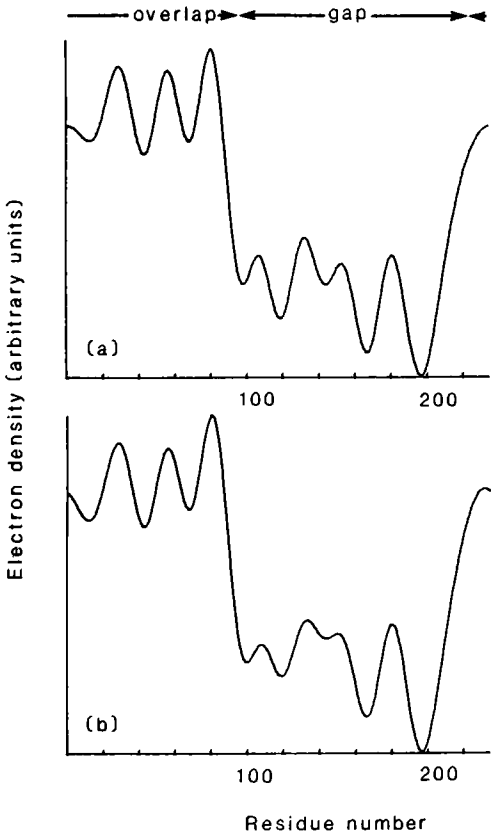


FIGURE 2 Axial distributions of electron density (above water background) along one D-period of the collagen fibrils in (*a*) rat tail tendon and (*b*) human sclera. *a* and *b* are plotted on the same (arbitrary) vertical scale. The gap and overlap regions are shown approximately.

the corresponding electron densities, suitable phases had to be found. Fortunately, scleral collagen is very similar to rat tail tendon collagen (hence the similarity of the electron density profiles in Fig. 2), and the changes in the electron density caused by treatment with the control solution (containing glutaraldehyde) were therefore predictable (Meek and Chapman, 1985; Meek et al., 1985). For the control sclera, the intensities were compared with those from identically treated mature rat tail tendon (Table II), after scaling by equating the summed intensities. The $R$ factor was used to assess the extent of agreement ($R = \Sigma \| F_S| - |F_T\| / \Sigma |F_S|$, where $F_S$ and $F_T$ are the structure factor amplitudes from sclera and tendon, respectively). Because $R$ was 0.13 it was considered acceptable to assume the phases for these tendons (Table I, Meek et al., 1985) were close to those for the control sclera. Using the same arguments, electron microscopy suggests that proteoglycan binding is similar in tendon and sclera so that staining with Cupromeronic blue might be expected to produce phase changes not unlike those observed in tendons. The comparison between stained sclera and stained tendon (Table II) gave $R = 0.21$ which for 10 orders of the diffraction pattern, is also considered to be sufficiently low to make the approximation that the phases are, within experimental resolution, the same (Meek et al., 1985; Chandross and

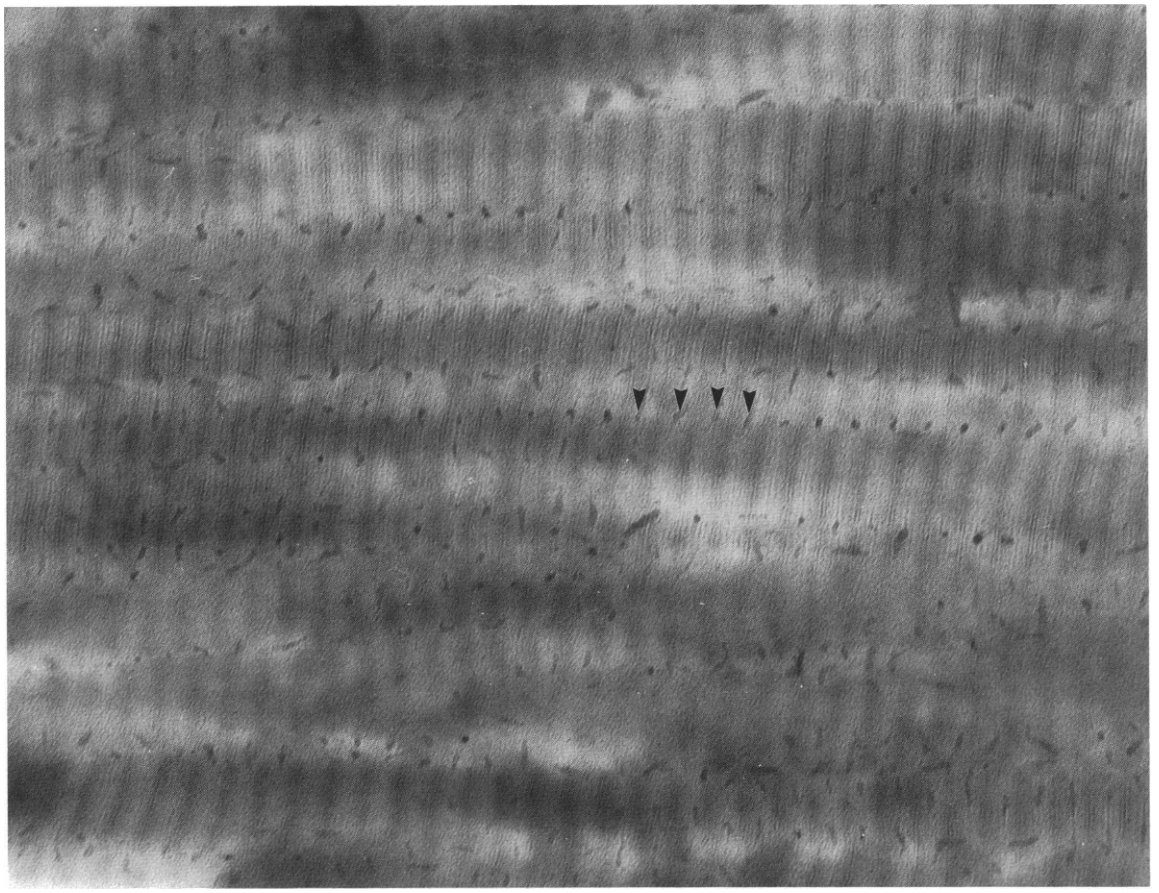

FIGURE 3 Human sclera stained with Cuprolinic blue and counterstained with uranyl acetate. The proteoglycan filaments (*arrowed*) appear to associate regularly with the collagen at the d/e staining bands (×108,000).

Bear, 1973). The relevant phases are therefore shown in Table II, and the corresponding electron density profiles in Fig. 4 *a*. The difference, which indicates the extra density caused by the dye, is shown in Fig. 4 *b*. The prominent feature is a peak centered near residue 180 (the d band), but the peak is broad and extends the complete width of the gap zone. Smaller superimposed peaks are probably artefacts of the scaling process.

TABLE II
INTEGRATED X-RAY INTENSITIES OF HUMAN SCLERA STAINED WITH CUPROMERONIC BLUE

| Order (*n*) | CUPROMERONIC BLUE Intensity | | Phase* | CONTROL Intensity | | Phase* |
|---|---|---|---|---|---|---|
| | Sclera | Tendon* | | Sclera | Tendon* | |
| | | | *degrees* | | | *degrees* |
| 1 | 1422 | 1574 | 301.6 | 1000 | 1151 | 66.2 |
| 2 | 136 | 88 | 161.8 | 43 | 39 | 90.6 |
| 3 | 116 | 187 | 5.0 | 201 | 144 | 353.5 |
| 4 | 103 | 31 | 6.3 | 59 | 6 | 56.0 |
| 5 | 132 | 162 | 297.6 | 209 | 166 | 290.1 |
| 6 | 154 | 36 | 228.1 | 85 | 59 | 236.8 |
| 7 | 121 | 54 | 199.6 | 113 | 120 | 194.0 |
| 8 | 33 | 48 | 343.8 | 91 | 98 | 341.4 |
| 9 | 112 | 169 | 39.7 | 173 | 169 | 39.5 |
| 10 | 54 | 34 | 195.6 | 34 | 56 | 195.0 |

*Taken from Meek et al. (1985). The phases are relative to a zero origin.

## DISCUSSION

Low angle meridional x-ray diffraction is a powerful tool to study the axially projected structure of collagen (Tomlin and Worthington, 1956; Ericson and Tomlin, 1959; Chandross and Bear, 1973), and the origins of the pattern are now known in great detail (Hulmes et al., 1977). The technique has been used to study collagenlike elastoidin (Ellis and McGavin, 1969; Chandross and Bear, 1979) and to assess the effects of dehydration (Tomlin and Worthington, 1956; Chandross, 1982), fixation (Stinson et al., 1979; Meek and Chapman, 1985) and staining (Ericson and Tomlin, 1959; Lam et al., 1978; Stinson et al., 1979; Meek and Holmes, 1983) on wet collagenous tissues. We have previously shown how Cupromeronic blue enhanced with sodium tungstate is sufficiently electron-dense to enable it to be located in rat tail tendons (Meek et al., 1985) and in bovine cornea (Meek et al., 1986), although in the latter case its contribution to the overall electron density was considerably lower. We have now shown that it also modifies the meridional intensities from human sclera.

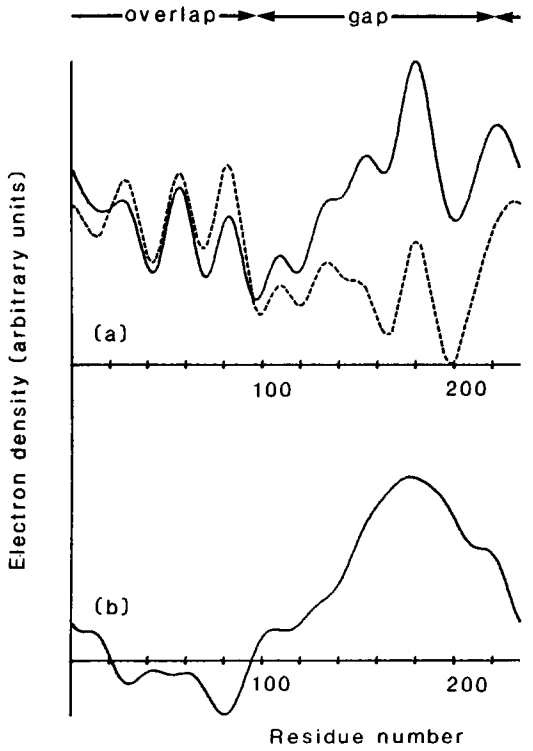


FIGURE 4 (*a*) Axial distributions of electron density (above water background) along one D-period of the collagen fibrils in human sclera treated with Cupromeronic blue (*solid line*) and its control (*dashed line*). (b) Difference electron density distribution between Cupromeronic blue–treated human scleral collagen and its control. *a* and *b* are plotted with the same (arbitrary) vertical scale. The gap and overlap regions are shown approximately.

To interpret the meridional intensity changes, it is necessary to scale the data sets relative to each other. We have discussed the merits of various methods of scaling in a previous publication (Meek et al., 1985). In the present work we have scaled tendon data with sclera data by equating summed scattered intensities (Lam et al., 1978; Chandross and Bear, 1979; Meek et al., 1981*a*). The underlying assumption in this method is that the number of electrons per unit cross-sectional area coherently scattered from a D-period is the same in both structures. The method was clearly not appropriate for comparing data from stained and unstained specimens, however, because of the presence of extra scattering from the stain electrons. To overcome the problem we adopted a scaling procedure which we used previously for our analysis of rat tail tendons (Meek et al., 1985), i.e., having noticed that the pattern of electron density of the overlap region was barely altered after Cupromeronic blue treatment, the data were scaled so that the overlap details matched out as far as possible. The assumption that most proteoglycan binding occurs in the gap was consistent with the location of the stained filaments seen in the electron microscope.

Dermatan sulphate is the major proteoglycan in the sclera (Coster and Fransson, 1981). X-ray diffraction has revealed that it is attached at the same axial location (the d/e bands) and to approximately the same extent as occurs in rat tail tendon. Using electron microscopy, it has also been located at this site in skin (Scott and Haigh, 1985*a*), heart valve (Nakao and Bashey, 1972), intervertebral disc (Scott and Haigh, 1986), and articular cartilage (Orford and Gardner, 1984). Proteoglycan binding in the cornea is more complex and although dermatan sulphate is bound at some d/e bands (Scott and Haigh, 1985*b;* Meek et al., 1986; Meek et al., 1987) a proportion of the gap zones remain unoccupied. It has been suggested that radial growth of collagen fibrils could be hindered by restricted access to the cross-linking sites, due to the proximity of the dermatan sulphate proteoglycan (Scott and Orford, 1981). This implies that tissues with less collagen-bound dermatan sulphate would contain fibrils with larger diameters, which is not the case in the cornea and sclera. A possible explanation is that corneal keratan sulphate proteoglycan plays a similar restrictive role to the dermatan sulphate proteoglycans.

The absence of dermatan sulphate proteoglycan at the d band has been noted in demineralized bone collagen (Scott and Haigh, 1985*a*). This has led to the suggestion that its presence in noncalcified tissues sterically inhibits the nucleation of calcification. This seems an even more likely possibility if the proteoglycans fill up not only the d band but a large part of the gap zone as suggested by the x-ray results. The deposition of calcium occurs in a number of connective tissue disorders including those of ocular tissues. In particular, its presence in sclera has been observed, where it can occur throughout the full thickness of the pathological tissue (Yanoff and Fine, 1982). This calcification may be due to a lack of some or all of the dermatan sulphate proteoglycans, or their abnormal arrangement with the collagen fibrils. The combination of electron microscopy and x-ray diffraction of the specifically stained tissues should provide a valuable approach for studying such disorders.

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