The Changes in Ultrastructure and Transparency in Chemically or Physically Altered Rabbit Cornea

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The Changes in Ultrastructure and Transparency in Chemically or Physically Altered Rabbit Cornea.

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Abstract

This thesis describes an x-ray diffraction (XRD) and transmission electron microscopy (TEM) study of rabbit corneal ultrastructure. The healing of partial and full thickness wounds in both old and young subjects, with or without the application of mannose-6-phosphate (M6P) was followed. The implications for light scattering following the refractive surgery photorefractive keratectomy (PRK) or corneaplasty (hyaluronidase treatment) was also studied.

XRD was used to accurately measure the spacing and alignment of collagen and its constituent molecules whereas TEM was used to study the degree of stromal disorder and the measurement of either proteoglycans (PGs), or the positions and diameter of collagen fibrils following corneal alteration. Resultant haze was measured by slit-lamp or normal photography. From these measurements, corneal transparency was estimated using a theoretical light scattering model, the 'direct summation of fields' model, (Freund et al., 1986; 1995).

Both full and partial thickness wounds (caused by trephine or PRK respectively) showed a similar response to wound healing. In both types of wound fibrillar order moved towards a normal value with time. The mean PG size increased in response to both types of wound and more so in full thickness wounds. Neither type of wound returned to its normal haze value but stromal order did increase, indicating that healing took place. This was to a slower extent in the full thickness wounds probably due to increased wound volume. Full thickness wounds also demonstrated fibrils spreading across the wound from a tightly packed position at the wound edge, and an inability to swell. PRK on different age groups suggested an age dependent response to healing with younger animals displaying less haze, less PG content and having a wider variation in interfibrillar spacings. Tissue in this type of wound was shown to be highly hydrated, this was reduced by M6P with a concurrent decrease in PG content but no reduction in associated haze.

Corneaplasty resulted in a compression of the fibril packing leading to a small loss of transparency but deemed unlikely to cause visual impairment. PRK of both old and young corneas also resulted in a small loss of transparency leading to the conclusion that haze following PRK is not necessarily caused by fibril disorder.
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1. Introduction

This thesis describes the study of physically or chemically altered corneal tissue using principally x-ray diffraction and transmission electron microscopy. It concentrates mainly on the corneal stroma with particular emphasis on the size and arrangement of its constituent collagen fibrils and glycosaminoglycans.

The first study concerns the stromal architecture of a full thickness wound as revealed by x-ray diffraction, electron microscopy and slit-lamp photography. Relationships between the results gained by x-ray diffraction, electron microscopy and wound thickness under differing hydration are discussed with a view to elucidating the changes in molecular orientation during wound healing.

In the next study, the healing of a partial thickness anterior corneal wound caused by the ophthalmic procedure photorefractive keratectomy is assessed in both old and young subjects with a view to finding time-resolved ultrastructural differences between them. Using both x-ray diffraction and electron microscopy some ultrastructural parameters are incorporated into an existing theoretical model that explains light transmission through the cornea. This model has traditionally used data obtained from normal corneas rather than wounded corneas. Also, within this section, the effect of an anti-inflammatory drug on wound healing following photorefractive keratectomy is assessed.

The study is then extended to an experimental ophthalmic procedure in which the ultrastructure of chemically altered corneas is compared to that of normal corneas. Using the model of light transmission, its effectiveness as an ophthalmic procedure is assessed.
1.1 Anatomy

1.1.1 Anatomy of the Eye

The mammalian eye (Figure 1.1) is approximately spherical and for the rabbit measures about 13mm in diameter in the young (6 months) to about 20mm in the old (3 years). The cornea and sclera together form the tough outer coating of the eye, maintaining its shape and protecting the more delicate internal parts. The anterior surface of the cornea is in contact with the atmosphere, via the tear film, whereas the posterior surface is bathed in aqueous humour. After light passes through the cornea it is focused onto the back of the eye, the retina, by the lens. The vitreous humour, a gel like substance, occupies about 90% of the volume of the eye providing structural support and protection against mechanical shock. For clear vision, the cornea, aqueous humour, lens and vitreous humour must all be highly transparent.

![Figure 1.1: Anatomy of the mammalian eye. (Reproduced from Berman, 1991)](image-url)
1.1.2 Anatomy of the cornea

The cornea forms the primary refractive element in the eye and is composed of three cellular layers: epithelium, stroma and endothelium and two acellular layers: basal lamina and Descemet's membrane, each shown in Figure 1.2. The rabbit cornea is approximately 300µm thick through the centre.

![Diagram of cornea anatomy]

**Figure 1.2: Anatomy of the cornea.** (Reproduced from Berman, 1991)

The corneal epithelium is a non-keratinised, stratified squamous epithelium that forms a functional barrier between the tear film and the intraocular environment. This barrier prevents changes in tear film composition, and tear-born pathogens from affecting the intraocular environment. In addition, the epithelium acts as a structural barrier to the continual influx of fluid from the tear film (Mishima and Hedbys, 1967). Tight junctional complexes are found between the superficial epithelial cells, which create a highly
effective semi-permeable membrane on the cornea surface (McLaughlin et al., 1985). In addition, the active movement of ions by the epithelium augments the activity of the endothelium in maintaining corneal dehydration and transparency (Klyce and Bonnano, 1988). The epithelium in humans is 50 to 70μm thick and constitutes about 10% of the corneal thickness (Hogan et al., 1971).

The basal lamina (or basement membrane) is an acellular layer 0.08-0.12μm thick. Certain layers of this membrane bind osmium tetroxide and uranyl acetate very well such that, at lower magnifications it is clearly seen as a dark layer beneath the epithelium in electron micrographs. The basal lamina, as well as containing collagen (types IV and VII), also has high amounts fibronectin, laminin and perlecan (a heparan sulphate proteoglycan). These basement membrane components are thought to be important for cellular adhesion, migration and proliferation (Timpl and Brown, 1994).

Directly below the basal lamina is an acellular membrane, 8-12μm thick, called Bowman’s layer, but it is only found in primates and is thought to confer protection against injury and contribute to adhesion of the epithelium to the stroma (Olsen and McCarthy, 1994).

The stroma is the largest layer of the cornea occupying 85% to 90% of the total thickness. The primary structural components of the stroma are predominately collagen fibrils, type I (Newsome et al., 1982), collagen filaments type VI (Zimmerman et al., 1986) and associated proteoglycans which are dissolved in a ‘ground substance’, also containing, glycoproteins, soluble proteins, inorganic salts and water. The collagen fibrils have regular diameters and spacing, and are arranged into highly organised layers, called lamellae, which run from limbus to limbus at various angles parallel to the corneal
surface (Figure 1.3). The number and size of lamellae varies considerably between species. Human cornea consists of approximately 200 lamellae, each about 20μm thick (Maurice, 1957). It is this organisation of the collagen that provides not only for the transparency of the stroma but also the structural strength of the cornea.

![Figure 1.3: Electron micrograph of normal stroma, from a young rabbit. Each lamella contains fibrils with the same alignment. Staining: uranyl acetate and lead citrate. Scale bar = 200nm.](image)

The development, maintenance, and repair of the structural components of the stroma i.e. synthesis of collagen and constituents of the ground substance, are dependent upon the presence of functional keratocytes (Schofield et al., 1971; Church et al., 1980; Birk et al., 1984). These adapted fibroblasts are flattened in shape and lie between lamellae, connected to each other via long cell processes (Hogan et al., 1971). These cell processes allow cell-cell communication through the formation of gap junctions between adjacent cells (Watsky 1995; Petridou and Masur, 1996). Confocal microscopy suggests that
keratocyte density increases towards the peripheral and anterior regions of the stroma (Møller-Pedersen et al., 1994; Møller-Pedersen and Ehlers, 1995). In the normal mammalian cornea, keratocytes represent a stable population of cells with little or no mitotic activity, however, when the cornea is injured they are activated and begin to divide (Crosson, 1989).

Descemet’s membrane is the basement membrane of the endothelium. In rabbits it is approximately 4μm thick (less than 2% of the total corneal thickness). Descemet’s membrane is a trauma-resistant meshwork of collagen types IV, VI and VIII, partially produced by the adjoining endothelium.

The endothelium is a single layer of hexagonal-shaped cells that form a mosaic pattern across the posterior surface of the cornea in direct contact with the aqueous humour (Joyce, 1994). Endothelial cells are held together by two types of junctions, gap and occluding. Both junctions are found on the lateral side towards the stroma. The gap junctions provide strength between coupled cells whereas the occluded junctions create a ‘leaky’ barrier between the aqueous humour and the cornea which provides some resistance to the free flow of water from the aqueous humour (Watsky et al., 1990). The endothelial layer represents an important site for the regulation of stromal hydration by means of a fluid pump. The control of stromal hydration is an important process in maintaining corneal transparency (Maurice, 1989). The glycosaminoglycans within the corneal stroma confer an inherent tendency for the stroma to imbibe water, swell and eventually opacify. This is prevented by the barrier function of the endothelium and by actively pumping ions into the aqueous humour, which are followed by the passive efflux of water from the stroma. The mammalian endothelium is not self-renewing (except in
rabbits and cats (Van Horne et al. 1977)) and the endothelial cell density decreases with increasing age, partly due to a decrease in numbers and partly due to an increase in the size of ageing corneas (Murphy et al., 1984; Doughty, 1994).

1.2 Collagen

Collagens are a family of highly characteristic fibrous proteins, constituting a quarter of the total protein in mammals, and are produced by many cell types. For a molecule to be classed as a collagen, a major part of it must consist of the characteristic triple helical structure described below.

Three left-handed polypeptide chains (known as α-chains) are mutually staggered by one residue and each contains glycine at every third residue. This arrangement allows the chains to wrap around each other to form a right-handed triple helix, which is called tropocollagen (Figure 1.4). The structure of an α-chain may be written \((\text{Gly-X-Y})_n\), where X and Y may be any amino acid but are commonly proline and hydroxyproline; the Gly-Pro-Hyp triplet forms about 10% of the type I collagen triple helix. Glycine is the only amino acid with no side chains and its presence at every third residue in the sequence is essential for the formation of the secondary structure, the triple helix.

Twenty distinct types of collagen, the product of 33 genes have so far been found in vertebrates (Myers et al., 1997; Gordon et al., 1998). Types I, II, III, V and XI constitute the fibrillar collagens and are able to form into well defined striated fibrils. Types IV, VI to X and XII to XX represent the structurally diverse, nonfibrillar members which include the important sub-group the ‘fibril-associated collagen with interrupted triple helix’ (FACIT). For FACIT collagens it is thought that the helical part of the molecule binds to the surface of the striated fibrils whilst the non-helical domains project into the
interfibrillar matrix where they can react with other fibrils or proteoglycans (Zhan et al., 1995). FACIT’s therefore influence collagen fibrils and for the cornea specifically, contribute to its transparency.

Figure 1.4: Diagram of collagen type I synthesis. Collagen α-chains are initially synthesised in the form of pro-α-chains (top) that contain extension peptides that will later be removed. Three pro-α-chains combine to form a triple stranded helix (tropocollagen) within the cell before secretion into the extracellular matrix. Note that the carboxyl extension peptides of the triple stranded helix are covalently linked together. Extracellular tropocollagen molecules are arranged in a quarter staggered array in the striated fibril. Striated fibril shows detail of five bands (a-e) that comprise the 65nm repeat D period. (Reproduced from Marshall et al., 1993)
1.2 COLLAGEN

1.2.1 Collagen in the Cornea

At the present time six collagen types have been found in the stroma: types I, III, V, VI, XII (Marshall et al., 1993) and XIV (Myers et al., 1997). Types I, III and V are striated collagens with similar structures, the basic structural unit being the tropocollagen molecule as described in Figure 1.4.

Collagen accounts for about 70% of the total dry mass of the cornea (Maurice, 1969) the majority of which is most probably type I (Marshall et al., 1993). Attempts to quantify the other collagen types have not yielded consistent results. Conflicting results may have arisen from the application of different techniques or because studies have been carried out on different species in situ and in cell culture.

Small amounts of type III have been reported in foetal and neo-natal cornea and in the healing adult cornea (Newsome et al., 1981, 1982; Lee and Davison 1981, 1984), but its presence in the normal adult stroma remains controversial. It should be noted that the occurrence of type III collagen in the corneal stroma appears to be unique to the human eye.

The presence of type V collagen within the stroma is one of the many factors influencing fibril diameter during fibrillogenesis (Marshall et al., 1993). Type V collagen has a greater concentration in the cornea than the sclera, tendon, bone and dermis (Hong et al., 1979). The principle collagens in chick cornea are types I and V, co-distributed within the same fibril (Birk et al., 1986). Birk et al. (1990) have synthesised collagen fibrils in vitro using a mixture of types I and V showing that the average diameter of the heterotypic fibrils formed decreases as the ratio of type V collagen in the mixture is increased.
Type VI collagen has a helical region and globular domain and has been localised to fine filamentous structures in the human (Marshall et al., 1993), cow (Alper, 1988) and rabbit (Cintron and Hong, 1988) corneal stroma. These filamentous structures are located between and bound to striated collagen fibrils by mediation of the proteoglycan dermatan sulphate (Nakamura et al., 1997). Type VI collagen may constitute as much as 25% of the dry weight of the tissue (Zimmerman et al., 1986).

Collagens type XII and XIV are FACIT's. Type XII has been identified in undetermined amounts in mouse, chick and more recently in the healing stroma of rabbits (Zhan et al., 1995). Type XX has so far been limited to embryonic chick epithelium (Gordon et al., 1998).

1.3 Ground Substance
The ground substance surrounding the insoluble proteins in the stroma such as the collagen fibrils contains, water, proteoglycans, soluble proteins and inorganic salts.

1.3.1 Proteoglycans
Proteoglycans (PGs) are thought to play an important role in maintaining the collagen architecture of various extracellular matrix-rich tissues such as skin, tendon, muscle connective tissue and cornea. The transparency of the cornea depends on the small and uniform cross-sectional diameter as well as the small and uniform spacing of collagen fibrils (Maurice, 1957); PGs appear to be heavily involved in the development and regulation of this diameter and spacing (Hassell et al., 1983; Doane et al., 1992; Rada et al., 1993; Scott 1995; Chakravarti et al., 1998).

PGs are highly acidic macromolecules that possess at least one sulphated glycosaminoglycan (GAG) chain. The two major types of GAGs in normal adult rabbit
1.3 GROUND SUBSTANCE

corneal stroma are keratan sulphate (KS) and dermatan sulphate (DS). Each GAG is a polymer of a disaccharide which consists of N-acetylglucosamine and galactose in KS, and N-acetylgalactosamine and glucuronic acid or iduronic acid in DS (Cintron, 1989). The sugars in GAGs are sulphated to varying degrees, and the GAG chain is covalently linked to a core protein, which in recent years has been discovered to consist of a number of genetically distinct variations.

These core proteins determine the PG molecule’s conformation, the location of macromolecular and cellular binding sites, and the location of covalently linked sugar moieties (GAGs), which bestow specific properties on a PG (Hardingham and Fosang, 1992). Most core proteins associated with the cornea belong to the leucine-rich proteoglycan family; members so far include lumican, keratocan and osteoglycin, which are keratan sulphate proteoglycans (KSPGs) and decorin, a dermatan/chondroitin sulphate proteoglycan (D/CSPG). Typical weights are about 55000 daltons for KSPG and 45000 daltons for D/CSPG (Cintron, 1989). There are several examples that indicate that KSPGs play an important role in providing corneal transparency. KS content increases substantially during the acquisition of transparency in development (Coulombre and Coulombie, 1958), it has been found absent in opaque corneal wounds (Hassel et al., 1983) and it is absent in corneas of patients with macular corneal dystrophy type I (Klintworth et al., 1983; Nakazawa et al., 1984). Furthermore, the absence of lumican, the major corneal KSPG, from the corneal stroma of mice with a lumican null mutation results in the development of corneal clouding (Chakravarti et al., 1998). Changes in DSPGs are also involved with scar formation, such as its presence when stained with the cationic dye Cuprolinic blue (Scott, 1985), in full thickness corneal wounds in the form
of unusually large precipitates (Cintron et al., 1990). However, in the absence of DSPGs core protein, decorin, and with or without its associated GAG, fibril diameter is effected but not to a degree that adversely affects corneal transparency (Danielson et al., 1997).

Interestingly, the above observations from healing corneas recapitulates, partially, the chemical and cytochemical properties of PGs in normal developing cornea (Cintron and Covington, 1990; Noriyuki et al., 1994). DSPG, the major PG in foetal rabbit cornea, has a high charge density in comparison with the smaller quantities and low-sulphation of KSPG in this tissue (Gregory et al., 1988). The charge density and relative quantity of these PGs are the reverse of those in the adult. During development, the quantity and charge density of KSPG gradually rises, reaching the values of adulthood in the second to eighth week after birth. Wound tissue in the rabbit has supposedly smaller PGs than normal corneas with the exception of a population of DSPGs which are markedly larger than normal (Cintron et al., 1989). In addition, only chondroitin-4-sulfate, a minor type of GAG, is found in normal adult cornea, whereas both chondoritin-6-sulfate and chondroitin-4-sulfate are present in foetal and wounded tissue (Hassel et al., 1983). As in the developing cornea, KSPGs have a low-sulphation and their proportion to other PGs in wounded tissue is markedly lower than that found in the normal adult cornea (Cintron et al., 1990). These similarities in GAG synthesis (high D/CS, low KS) between foetal and wounded tissue suggests that they share at least some of the same basic mechanisms for the synthesis of the corneal stroma, its specific fibrillar organisation and transparency.

1.4 Corneal Transparency

Transparency is the quality or state of transmitting light without appreciable scattering so that bodies lying beyond are visible. Clearly, transparency is of paramount importance for
the cornea to achieve its basic function as the window in the eye wall through which the outside world is viewed.

1.4.1 Principles of Reflection and Refraction

Light rays pass through the transparent media of the eye, are absorbed by the rod and cone shaped cells of the retina, and initiate the perception of light, which is called sight. Light rays travel in straight lines within a homogenous non-absorbing medium (characterised by its refractive index, \( n \) and absorption coefficient, \( \alpha_{abs} \)) and their behaviour at an interface between two such media is governed by the laws of reflection and refraction. The refractive index and absorption coefficient of a medium can depend on the wavelength of the light.

![Diagram of reflection and refraction](image)

Figure 1.5: Light incident on a boundary between two homogeneous media is partially reflected and partially transmitted. The angles of incidence, \( \theta_i \), reflection, \( \theta_r \), and refraction, \( \theta_n \), are measured relative to the perpendicular to the surface. The direction of the perpendicular varies with location on a curved surface as illustrated in B. The reflected ray obeys the specular condition \( \theta_i = \theta_r \), and the transmitted ray is bent toward (away from) the normal passing into a medium of higher (lower) refractive index. (Reproduced form Farrell, 1994).
1.4 CORNEAL TRANSPARENCY

Figure 1.5 A illustrates that a ray incident on a planar boundary between two homogeneous media having refractive indices of \( n_1 \) and \( n_2 \) is partially reflected and partially transmitted. The law of reflection states that the angle of incidence, \( \theta_i \), is equal to the angle of reflection, \( \theta_r \), in respect to the normal, a line perpendicular to the interface; Snell’s law of refraction states that the angle of refraction, \( \theta_r \), is equal to the angle of incidence through the equation:

\[
\sin(\theta_r) = \frac{n_1}{n_2} \sin(\theta_i)
\]  \( (1.1) \)

These laws govern the directions of rays. For non-absorbing media, the amplitude of the reflected ray is a complicated function of the angle of incidence and the refractive indices, but for a perpendicular incidence it is directly proportional to the difference in the refractive indices, \( (n_2 - n_1) \). Figure 1.5 B, illustrates the behaviour of rays at a smooth curved interface between two homogeneous media. The same laws apply; however the direction of the normal depends on the location on the surface, as shown.

1.4.2 Light Scatter in the Cornea

Light rays passing through a non-absorbing homogeneous media have a constant amplitude. In other media light energy can be transformed into other forms of energy, such as heat, and these are called absorbers. The reciprocal of the absorption coefficient, \( 1/\alpha_{abs} \), is called the absorption depth and is an indicator of the distance to which radiation effectively penetrates the absorbing media. The absorption depth for visible light through the cornea is very high, effectively infinite.

The laws of geometric optics apply to light propagation through homogeneous media; however, most media are inhomogeneous because of local fluctuations in their density or
the intrinsic nature of composite materials. These local fluctuations change the propagation direction of some of the light incident on them; i.e. they scatter light out of the incident beam. Scattering differs from absorption in that light energy is redirected, not converted into another form of energy such as heat or light of a different colour. Therefore scattering has to remain low within the corneal stroma if transmittance is to remain high.

Three factors determine the normal corneas transparency to visible light. These are: a) Fibrils. The fibrils are the main nonspecular scattering elements in the cornea their radii (which are much smaller than the wavelength of light, for rabbit approximately 20 nm) and their refractive index (which is close to that of the surrounding ground substance) combine to make them weak scatterers. b) Corneal thickness. This is significant because as the incident beam passes through the cornea the amount of scattering produced is directly proportional to the number of scatterers it encounters therefore a thin cornea creates less scatter. c) Interference effects. Destructive interference among waves scattered by different fibrils reduces the amount of scattering from, that which would occur if the fibrils within the stroma scattered independently of one another. The interference factor defines the degree to which the scattering is reduced and depends on both the spatial distribution of fibril axes and on the spatial distribution of scatterer strength (Freund et al., 1995).

The assumption that fibrils scatter independently corresponds to the assumption that fibril positions are uncorrelated and distributed randomly with an ideal gas-like disorder. If this were the case, the interference factor would be 1, and for typical fibril sizes, the cornea would not transmit enough light for normal vision to occur (Maurice, 1957; Hart
and Farrell, 1969). Consequently, most contemporary theories of transparency for normal corneas are based upon the assumption that the fibril positions have some degree of spatial ordering (Maurice, 1957; Hart and Farrell, 1969; Feuk, 1970; Benedek, 1971; Twersky, 1975). Spatial ordering, if much smaller than the incident wavelength of light, produces destructive interference that lowers the interference factor and reduces the total amount of scattered light in all directions except the forward one, leading to increased transparency. Although the interference factor is an important element in explaining transparency, the other two factors previously mentioned are also important, since they will dominate specific areas of the cornea. An example of this is the Bowman’s layer; its fibrils have no axial alignment, therefore scatter light independently of each other creating an overall interference factor of 1. Despite this, the Bowman’s layer remains transparent. This is because of its thinness, (approximately 12μm) which allows any negative interference effects (a high interference factor) to be dismissable and its smaller fibril diameters (roughly two-thirds the diameter of those in the stroma) which effectively reduce scatter.

1.4.3 Theories in Corneal Transparency

The first of the theories which attempt to explain the near total absence of light scattering in the stroma, was suggested by Maurice (1957). In this seminal paper he proposed that in vivo, the corneal fibril axes were located at the positions of a perfect crystal lattice. Within this lattice the distance between nearest neighbour fibrils (about 50 to 60nm) is much smaller than the wavelength of light (about 400 to 700nm), such an arrangement would produce perfect transparency. The fact that the cornea is not perfectly transparent (otherwise ophthalmologists could not view the cornea with the slit-lamp) was explained
by Maurice to be scatter caused by keratocytes. However, normal keratocytes are now thought to be highly transparent due to the presence of two water-soluble proteins, transketolase and aldehyde dehydrogenase type 1 (Jester et al., 1999). These proteins are thought to contribute to corneal transparency at a cellular level, reminiscent of the enzyme-crystallins found in the lens. The short-range interactions of crystallins in the lens fibre cells minimise light scattering and promote undistorted transmission (Tardieu and Delaye, 1988)

Maurice dismissed the fact that electron micrographs such as Figure 1.3 do not show a crystal lattice order in the fibril arrangement, as an artefact of the preparative procedures needed to examine the tissue by electron microscopy. However, in the late 1960s, Hart and Farrell (1969) questioned whether the short-range order so obviously present in electron micrographs might be capable of explaining transparency. Using statistical techniques Hart and Farrell showed that the regularity in the spatial arrangements of fibril axes extended over distances of about 200nm and that these arrangements could be characterised by the radial distribution function \( g(r) \). The quantity \( g(r) \) measures the likelihood of finding a fibril axis within a specific distance \( r \) from a reference fibril axis. Using this function, the degree of destructive interference produced by the short-range order was found to be sufficient to render the cornea transparent i.e. it reduced the scattering to a level consistent with that, which is actually measured. Their calculation, however, does not prove that the short-range order shown in micrographs is correct; but it does mean that this structure cannot be rejected as artificial on the basis of a transparency argument.
Since Maurice’s early lattice theory and Hart and Farrell’s scattering calculations, several other models have been proposed to explain the cornea’s transparency. They can be grouped as to whether they are based on long-range or short-range order. In addition to the lattice theory, another long-range order theory includes a slight modification suggested by Feuk (1970) in which the fibrils are said to be randomly distributed around perfect lattice positions. The other short-range order theories include Benedek’s correlation area model (Benedek, 1971), Twersky’s modified hard-core model (Twersky, 1975) and the equal refractive index model (Smith, 1969) which strictly speaking is neither dependent on long-range or short-range order. In Benedek’s model, order in the relative positions of the fibrils is assumed to persist out to some radial distance \( R_c \), where \( R_c \) is assumed to be much smaller than the light wavelength. Benedek then introduced the concept of an effective correlation area \( A_e \), which he related to the actual radial distribution function. His model then predicts that the interference reduces the scattering by a multiplicative factor \((1-A_e/A_0)\) in which \( A_0 \) is the average area available per fibril.

Like Hart and Farrell’s calculation, this model also predicts that the distribution of fibrils shown in Figure 1.1 is consistent with transparency. In Twersky’s model it is postulated that the fibrils are covered with an impenetrable coating that is optically homogenous and has the same refractive index as the ground substance. This coating serves to keep the fibrils further apart than they would be if they were uncoated and there were no interactions between them. This forced increase in the hard-core diameter serves to increase the correlation’s in the fibril positions.

The theories outlined above are applicable only to fibrils that are of equal size and have a uniform or radially symmetrical spatial distribution. The direct summation of
fields model (Freund et al., 1986a) is a statistical technique that generalises these theories to assemblies of fibrils with an arbitrary distribution of diameters (that is, fibril scattering strengths) and with an arbitrary spatial distribution. Therefore this technique is especially beneficial for wounded tissue that may show large variability in fibril diameters and have voids in the spatial distribution of fibril axes, and for this reason it was the model of choice throughout this thesis for transparency calculations. The method is described in detail in Chapter 2.7, page 54.

1.5 Corneal Modification and Effect

1.5.1 Clinical Methods for Changing Corneal Refraction

The cornea must not only remain transparent but also refract incoming light, focusing it via the lens, onto the retina. The degree of refraction is ultimately determined by the anterior curvature of the cornea, an incorrect curvature causing the light to be focused either in front or behind the retina. Myopia is one such condition, resulting from a greater degree of refraction from the cornea than required, resulting in the light being focused in front of the retina. Placing a lens in front of the eye can correct refractive errors of this type. More serious refractive problems (or for cosmetic or practical reasons e.g. fashion model or fighter pilot) require a physical or clinical modification of the cornea.

Modification of the cornea can be achieved in a number of ways, all of which involve a reshaping of its anterior surface; a flatter anterior surface for myopia and a steeper surface for hyperopia or aphakia. Common surgical procedures are varied and problematic. Epikeratophakia, keratomileusis and keratophakia involve the suturing on of a suitably lathed lenticule, freezing and unfreezing of this additional tissue often causes swelling of the central corneal stroma and keratocyte death (Swinger and Barraquer,
Radial or transverse keratotomy is achieved by peripheral incisions to the cornea, but its shortcomings are lack of reproducible incision depths, corneal dehydration and endothelial damage (MacRae et al., 1985). Photorefractive keratotomy (PRK) and laser in situ keratomileusis (LASIK) are two surgical methods which make use of the fact that, although the cornea does not appreciably absorb visible light, it does however, absorb ultraviolet and infrared radiation. Using laser energy, a specific amount of the stroma is removed (ablated), resulting in a flattening of the cornea. In PRK the epithelium is also ablated. However these methods also have their problems, the major one being the unpredictable deposition of regenerated stromal tissue, which contributes to a regression of the refractive correction and the formation of subepithelial haze formed during healing of the wound (Malley et al., 1990; Rawe et al., 1992; Moller-Pederson et al., 1998).

1.5.2 Wound Healing Processes

Conceptually, corneal wound healing can be viewed as a sequence of overlapping biological processes that follow as a consequence of positive and negative signals derived from endogenous corneal cells and their surrounding environment. These signals include growth factors, cell-cell contact, cell-extracellular matrix contact and proteolysis.

Growth factors or cytokines, are proteins produced by cells in response to a variety of inducing stimuli. Secreted by producer cells, influencing the behaviour of target cells and usually acting within a few millimetres from their origin, cytokines play a major role in maintaining tissue homeostasis; their functions include regulation of cell proliferation, cell migration, cell differentiation, immune responses, cellular metabolism and wound healing. The most common types of cytokines found within healing corneas are: i) Fibroblast growth factor (FGF) which stimulates proliferation and migration of many
types of cells. ii) Epidermal growth factor/Transforming growth factor-α (EGF/TGF-α) which stimulate proliferation of many cell types. iii) Platelet-derived growth factor (PDGF) which stimulates proliferation and migration of connective tissue cells. iv) Insulin-like growth factor (IGF) which primes cells to respond to other growth factors and promotes cell survival. v) Transforming growth factor-β (TGF-β) which promotes fibrosis (Clemens 1991; Heath 1993).

Cell-cell contacts at specialised regions in cells, specifically the epithelium and endothelium, are called cell junctions. Cell junctions are classified into three functionally distinct groups (Alberts et al., 1994; Goodman, 1994). i) Occluding or tight junctions form a physical barrier that prevents the leakage of even small molecules between cells or tissue layers. ii) Adhering junctions, which include demosomes and adherens junctions. These are cytoskeleton mediated cell-cell interactions, providing strength to the corneal epithelium. iii) Communicating junctions, or gap junctions, allow chemical or electrical signals to pass between neighbouring cells.

Cell-extracellular matrix contact within the cornea is principally made from the adhesion molecules fibronectin and laminin, and also integrins (Alberts et al., 1994). Fibronectin promotes the attachment of fibroblasts and other cell types to the extracellular matrix (ECM), while laminin promotes the attachment of epithelial cells to the basal lamina. Integrins are the principle receptors used by animal cells to bind to the ECM. These are heterodimers, which function as transmembrane linker proteins that mediate bidirectional interactions between the adhesive proteins of the extracellular matrix and the actin cytoskeleton (Kupper, 1995; Giancotti, 1997).
Proteolysis, the degradation of a protein, is an essential component of tissue maintenance and wound repair. Two major groups of stromal proteinases are the matrix metalloproteinases (MMPs) and the serine proteinases. MMPs are dependent on Ca or Zn as cofactors, and serine proteinases have a highly reactive serine residue at their active site (Mignatti et al., 1996). MMPs degrade collagen types I, II, III, IV, V and VII, fibronectin, laminin and proteoglycans. Serine proteinases, in particular tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), cleave plasminogen to plasmin, itself a proteolytic enzyme (MacDougall and Martrisian, 1995).

The fundamentals of the wound healing process are constant in virtually all tissues. After a fibrin clot forms a provisional matrix, cells from the wound perimeter migrate into the clot, forming granulation tissue. Within this tissue are fibroblasts (activated keratocytes within the cornea) that acquire an activated phenotype and display cytoskeletal features consistent with the application of force upon their surrounding extracellular matrix (ECM). These forces mediate the contraction phase of wound healing, a process designed in part to minimise the surface area that needs to be re-epithelialized, for example fibroblasts can apply force to collagen-containing tissues through the integrin α2β1, a cell surface heterodimer that binds to collagen outside the cell and to the actin cytoskeleton inside the cell (Kupper, 1995). However, although the fundamentals may be similar there are still significant differences in the healing response to differing wounds, for example in GAG content and shape (Cintron et al., 1990), healing periods (Cintron and Kublin, 1977) and interfibrillar spacing (Rawe et al., 1994).

Immediately following a penetrating corneal insult (affecting both epithelium and stroma) the epithelium reorganises its basal cells (Dua et al., 1994), there is a loss of
necrotic or damaged cells, cell-cell attachments appear to ‘loosen’ and the epithelium becomes less packed (Crosson et al., 1986). At the same time fibronectin is apparent on the wound surface, probably derived from the tear fluid, and acts as a provisional matrix which enhances the migration and adhesion of epithelial cells over the wound bed (Fujikawa et al., 1981; Gipson et al., 1993). The epithelial cell-cell attachments, mainly desmosomes, generally stay intact and so the movement is sheet-like rather than as individual cells (Dua et al., 1994). Re-epithelialisation is complete in between 2 and 12 weeks depending on the wound type (Hanna et al., 1989; Tuft et al., 1989; Obata et al., 1994, Jain et al., 1996). Injury to the stroma results in the destruction of the connective tissue and the immediate loss of the keratocytes within and adjacent to the wounded area (Wilson and Kim, 1998). Damaged collagen fibrils are removed by infiltrating polymorphonuclear neutrophils, which secrete proteinases (Lee et al., 1981). Once re-epithelialisation is complete the keratocytes adjacent to the wound undergo ‘activation’ to form myofibroblastic cells which have the capacity to rapidly divide in order to rejuvenate cell numbers, to migrate into the wound area and to synthesise new stromal tissue (Jester et al., 1994). Keratocyte proliferation and production of new connective tissue usually peaks between 3 and 6 days post wounding (Tuft et al., 1989; 1993). It should be emphasised that all these components are part of a continuous process and the contribution of each is dependent on the exact nature of the wound.

As previously stated, the endothelium is required to maintain corneal deturgescence and optical transparency (Landshman et al., 1988; Huang et al., 1989). Following minor damage to the endothelium, the stroma swells and the cornea thickens as a result of decreased endothelial cell density adversely reducing total pump function (Laing et al.,
1.6 Introduction to Experiments

Three differing types of wounded or altered corneas were examined in four separate studies, firstly full thickness wounds, secondly partial thickness anterior wounds in old and young rabbits, thirdly partial thickness wounds following the application of mannose-6-phosphate and fourthly chemical alteration of the corneal curvature.

1.6.1 Full Thickness Wounds

In this experiment, the healing responses of full-thickness corneal wounds were studied. In this type of wound a 2mm circular full-thickness plug is excised from the centre of a
rabbit's eye. This differs from a non-penetrating wound since the full thickness plug contains all the separate layers of the cornea including the endothelium.

Full thickness penetrating wounds in rabbit cornea heal after a prolonged period to form transparent tissue (Cintron and Kublin, 1977) and therefore form an ideal experimental model in which to examine the ultrastructural changes during wound healing and how they may possibly relate to transparency. Different rabbits with similar wounds were studied over a period of 16 months; ultrastructural parameters were measured using x-ray diffraction (XRD) and transmission electron microscopy (TEM). The parameters measured within each wound were interfibrillar Bragg spacing, intermolecular Bragg spacing, fibril orientation across the wound centre, and in one case the area of an entire wound, GAG content and size at different wound depths, and wound opacity (haze).

The transparency of the corneal stroma is thought to be due to the regular spacing of the collagen fibrils and to their narrow, uniform diameters (Maurice, 1959; Farrell, 1994). Corneal wounds can be opaque because of their decreased ultrastructural order, and after an extended period of healing, transparency improves as ultrastructural order improves (Cintron et al., 1977). Previous x-ray diffraction studies of rabbit full thickness corneal wounds demonstrated a measurable increase in fibril order between the second and third month of wound healing (Rawe et al., 1994).

1.6.2 Partial Thickness Anterior Wounds

In these experiments, the healing responses of partial thickness anterior wounds were studied and the causes of post-operative haze following photorefractive keratectomy (PRK) and/or associated loss of transparency evaluated either in both old and young
rabbits, or in young rabbits with and without the application of the anti-inflammatory agent, mannose-6-phosphate.

For these types of wounds, an ultraviolet laser was used to accurately remove the anterior third of each cornea \textit{in vivo}, leaving the remaining two thirds of the cornea including the endothelium intact.

The argon fluoride excimer laser (193 nm) has been used as a tool for altering the refractive properties of the eye by reshaping its anterior curvature (Trokel et al.; 1983, Tuft et al., 1989). The radiation used in the excimer laser breaks up molecular bonds by a photochemical reaction, which causes an ablation of the target tissue. This process has been called photorefractive keratectomy (PRK). The characteristics of the argon fluoride excimer laser treatment are; (1) smooth ablated surface, (2) minimal damage to the adjacent tissue (Obata et al., 1994), and (3) precise submicron ablation (Waring, 1989). However, the major problem faced is that of subcellular corneal wound healing (Rawe et al., 1992; Malley et al., 1990; Moller-Pedersen et al., 1998). This causes a regression of the refractive correction, and the formation of a subepithelial haze that may be partly due to the regeneration of the corneal stromal tissue (Quantock et al., 1994, Tuft et al., 1993). The extent of deposition of regenerated tissue is unpredictable, but deeper ablations are reported to produce greater corneal opacification (Taylor et al., 1989; Tuft et al., 1989). This has implications for the visual acuity of the treated eye. For PRK to be successful the refractive correction must be predictable and the long-term implications must be evaluated.
1.6.2.1 PRK Wound Response in Old and Young Rabbits

Rabbit models have generally been used to study wound healing, but it is not known to what extent extrapolation can be made to wound healing in humans. Although wound closure rates at the epithelial level appear similar between rabbits and humans, it does seem to be the case that rabbits synthesise more collagen and subepithelial deposits post-laser ablation than humans do*. PRK is carried out on humans above the age of twenty-three, hopefully on a static globe, whilst animal studies have generally been carried out on young rabbits or young monkeys. Previous studies using the cationic dye Cuprolinic blue, immunocytochemistry and x-ray studies have tended to concentrate on young specimens, usually rabbits and for only relatively short periods of time after ablation (Rawe et al., 1992, Quantock et al., 1994 and Melles et al., 1995). It is possible however, that tissue plasticity affects the wound healing process, and that the level of development of the animal accounts for some differences in the response between rabbits and humans.

The purpose of this study was to examine haze and the changes in the packing of collagen fibrils as a possible cause of haze, after photorefractive keratectomy (PRK) treatment in both young and old rabbits for a period of up to 20 months.

It was proposed to test these concepts by measuring parameters such as interfibrillar spacing and fibril diameters of partial thickness wounds by x-ray diffraction (XRD) and transmission electron microscopy (TEM). Since the space between collagen fibrils is occupied, in part, by proteoglycans (PGs) and their function within the corneal stroma is related to their hydrophilic properties, location between collagen fibrils, and interaction

* J Marshall, personal observation. Professor J Marshall, St Thomas' Hospital, London.
with other macromolecules, the size and amount of the GAG part of PGs was also measured using TEM and image analysis as described in Chapter 2.1, page 32.

1.6.2.2 Application of Mannose-6-Phosphate (M6P) to PRK Wounds

Wound healing is a fundamental response to tissue injury that results in restoration of tissue integrity. This is achieved mainly by the activation of keratocytes and the synthesis of the connective tissue matrix, primarily collagen. Haze is a function of wound healing and an unwanted side effect of PRK, therefore by controlling keratocyte activation and collagen deposition haze may be prevented or reduced.

Drugs that inhibit the activation of keratocytes and collagen deposition can be classified as follows: steroids, non-steroidal anti-inflammatory drugs, collagen cross-link inhibitors, inhibitors of cellular migration, antimetabolites and immunologic mechanisms (Chang et al., 1998). Reports on the effects of steroids after PRK are conflicting, topical corticosteroids reduced the haze following PRK in rabbits (Tuft et al., 1989) but a more recent double-blind controlled trial demonstrated no effect of high-dose topical dexamethasone on haze or refraction after PRK (Gantry et al., 1992). The non-steroidal anti-inflammatory drugs, diclofenac and flurometholone were recently shown to influence corneal wound healing and reduce haze in rabbits after PRK (Nassaralla et al., 1995). According to Schipper et al. (1997), mitomycin C reduced the number of keratocytes in the treated corneas, leading to less collagen deposition but not to a reduction in haze. Bergman and Spigelman (1994) reported that the effect of 5-fluorouracil, another antimetabolite, on corneal haze was transient. Interferon-α 2β has been reported to reduce corneal haze after PRK whereas cyclosporin A had no effect on the inhibition of keratocyte activation or collagen deposition (Chang et al., 1998).
A new approach to modulate corneal wound healing using mannose-6-phosphate (M6P) was proposed by Sutton et al. (1996). In this pilot study five rabbits received topical M6P after PRK, and haze was shown to be significantly lower (p=0.05) at 4 weeks when compared to the controls.

M6P is a naturally occurring carbohydrate and has been identified as the major component of Aloe vera, an agent long known to aid healing and minimize scarring of the skin. Its mechanism of action has not yet been fully elucidated but it is thought to act by competing with latent Transforming Growth Factor Beta (TGFβ) at the Insulin-Like Growth factor II receptor (Vignon and Rochefort, 1992). Despite there being a number of growth factors capable of altering the healing process in a complex and as yet unknown manner, TGFβ is thought to play a key role, with its inhibition reported to prevent/reduce corneal haze in animal models (Jester et al., 1994; Sutton et al., 1996; Thom et al. 1997).

The purpose of this study was to repeat the Sutton experiment, replacing the non-viscous M6P solution with a more viscous gel-like solution of M6P. The hypothesis was that the longer the solution of M6P and the wounded cornea remained in contact, the greater M6Ps effect on the wound would be. A gel would therefore increase this contact time, since a less viscous solution would be quickly diluted and washed away by the reflex tearing. Hydration values of the wounded corneas with and without the applications of M6P were also studied, as Aloe vera is a known anti-inflammatory.

1.6.3 Chemical Alteration of Corneal Curvature

The organisation of collagen fibrils in the corneal stroma is responsible for the shape and transparency of the tissue. The spacing between the fibrils is thought to be governed by the interfibrillar proteoglycans (PGs) which, because of their swelling potential and their
association with collagen fibrils contribute to corneal rigidity (Borcherding et al., 1975; Scott, 1995). PGs are hybrid molecules consisting of a protein, to which are attached long chains of highly sulphated repeating disaccharides called glycosaminoglycans (GAGs). The most common types of GAGs found in the adult cornea are keratan sulphates and dermatan sulphates. ACS-005 hyaluronidase enzyme (Advanced Corneal Systems Inc., Irvine, California) is thought to break the chemical bonds between the sugars in the dermatan sulphate GAGs, softening the cornea, making it easier to reshape.

Corneaplasty (Karageozian et al., 1996) is a technique that is being developed to modify refractive errors in human patients. Corneaplasty is similar to orthokeratology both being procedures that involve changing the refractive properties of the cornea without cutting or ablating the tissue. Orthokeratology works by the fitting of a rigid contact (forming) lens over the cornea, which is much flatter than a conventional ‘alignment’ fitted lens, so to flatten the corneal apex. This lens must be worn continually, gradually replacing the lens with flatter ones at predetermined intervals, and this procedure is repeated until no further flattening is achieved. At this point a ‘Retainer’ lens is worn for a few hours each day (Grosvenor and Goss, 1999). Corneaplasty differs because it requires a single injection of enzyme, which softens the cornea allowing for a permanent re-shaping of the cornea without having to wear the forming lens again. The disadvantages of corneaplasty compared to orthokeratology are that prescription changes are not easily made and visual acuity may not remain optimal.

The first stage of corneaplasty is accomplished by softening the cornea with an injection of ACS-005 hyaluronidase. The second stage is to place a custom-designed forming lens over the softened cornea. The overall effect changes the corneal shape from
a baseline configuration to an optimised corneal shape for best visual performance in a matter of 1-2 days. The last stage is to administer a topically applied stabilising solution that stabilises the cornea to its optimised shape. This solution stabilises and maintains the shape of the remodelled cornea in a few days at which point the forming lenses and topical stabilising solution are no longer required and the vision of the patient should remain stable.

In contrast to corneaplasty, radial keratotomy involves making multiple radial incisions into the cornea with a scalpel, while photorefractive keratectomy relies on excimer lasers to remove surface tissue from the cornea. Both of these alternative methods can infrequently cause post-operative optical problems, such as an uneven anterior surface, fluctuation of vision, glare or haze (Ljubimov et al., 1998; Tuft et al., 1989).

Corneaplasty has been successfully tested in a rabbit model, and Phase IIa Human Efficacy Studies are underway (Karageozian et al., 1996).

In this study, synchrotron x-ray diffraction (XRD) and transmission electron microscopy (TEM) were used to measure the packing of the collagen fibrils of rabbit corneas that have been treated with ACS-005 enzyme. The corneas that have undergone this treatment are clinically clear, so it appears that removal of some of the corneal proteoglycans does not change corneal ultrastructure sufficiently to cause a significant increase in light scattering. To investigate this hypothesis, the direct summation of fields light scattering model (Freund et al., 1986a; 1986b; 1995) was used to predict how changes in collagen packing would be expected to affect the transmission of visible light through the corneas.
2. Methods

2.1 Transmission Electron Microscopy and Image Analysis

2.1.1 Dissection of Tissue

Dissection of the cornea is an important first step in the preparation of tissue samples for transmission electron microscopy. Firstly, the sample must be reduced to a very small size and the plastic nature of biological tissue means care must be taken not to cause mechanical damage by deforming the tissue by excess force or blunt scalpels when dissecting. Secondly, the tissue must be dissected in such a way that the original position or orientation of the sample within its original tissue/organ is known, allowing an accurate description of the tissue at a microscopic level.

A central disc was removed from each cornea using an 8mm trephine, this disc would then be dissected in to a further 8 segments (Figure 2.1). This results in extremely small tissue blocks with the minimum of mechanical damage allowing for a uniform fixation by immersion. At least two of these tissue blocks would be used for each fixation protocol.

Figure 2.1: Diagram of the removal of the central disc from dissected corneas and its further cutting into 8 pie pieces for fixation for transmission electron microscopy (TEM).
2.1.2 Fixation of Tissue

2.1.2.1 Standard Fixation

The corneal tissue blocks were fixed overnight (12 hours) in 2.5% glutaraldehyde, 0.1M phosphate buffer (pH 7.2), at 4°C, followed by 1.5 hours in 0.1M osmium tetroxide at 20°C.

2.1.2.2 Fixation and Staining of Glycosaminoglycans (GAGs)

Collagen fibrils are easily visible after fixation with osmium tetroxide (Agar Scientific, Stanstead, Essex) and counter staining with uranyl acetate and lead citrate, but proteoglycans (PGs), being at least partly in solution, are not. PGs (or at least the GAG part of PGs) were therefore stained with the specially designed reagent, Cuprolinic blue (BDH Chemicals, Poole, England). It is intensely blue, and contains a copper atom, which enhances its electron density, it is therefore useful in both light and TEM, although it is the subsequent attachment of tungstate ions that gives the stain sufficient electron density to be visible in the electron microscope.

Cuprolinic blue combines via its positive charges with all polyanions, including the PGs. Used in critical electrolyte concentrations by incorporating a certain amount of competitive cations in the staining solution, it specifically stains the polyanionic glycosaminglycan side chains of PGs and hyaluronan, an unsulphated GAG (Scott, 1985). The added cations compete with Cuprolinic blue for the negative charges on the polyanions, the three main polymer-bound anions found in most living tissue are carboxylate (\(-\text{COO}^-\)), phosphate ester (\(\text{PO}_4^2^-\)) or sulphate ester (\(-\text{SO}_4^2^-\)). These anions differ in their relative affinities to the stain Cuprolinic blue in the presence of a competitive cation enabling them to be sufficiently separated from each other. Mg\(^{2+}\) is particularly
useful as a competitive cation because sulphated polyanions are the last to give up their dye to this electrolyte, therefore sulphated PGs remained stained when polyanions containing carboxylate and phosphate ester have lost their stain (Scott, 1985). Using a concentration of 0.1M MgCl₂ ensures that all the GAGs present in the stroma including the polycarboxylate hyaluronic acid (if present) are stained. Cuprolinic blue not only stains GAGs but is also thought to precipitate them out of solution and, with the aid of glutaraldehyde, maintain their shape and position within the tissue.

The corneal tissue blocks were fixed and stained by Cuprolinic blue at critical electrolyte concentrations using, 0.05% Cuprolinic blue in 25mM sodium acetate buffer (pH 5.8) with 0.1M MgCl₂ and 2.5% glutaraldehyde for 24 hours at 16°C. The following day the tissue was rinsed 3x15 minutes and then fixed for 1 hour in 25mM sodium acetate buffer (pH 5.8) with 0.1M MgCl₂ and 2.5% glutaraldehyde. The tissue was then washed in 3x15 minutes of aqueous sodium tungstate (0.5%), followed by 15 minutes in 50% ethanolic sodium tungstate (0.5%).

2.1.3 Dehydration and Polymerisation of Tissue

Following either fixation protocol, the tissue blocks were dehydrated in ethanol from 50% to 100% in 10% steps for 15 minutes each and twice for 30 minutes at 100%. Infiltration with Spurr's resin (Taab Laboratories Equipment, Aldermaston, Berks.) was accomplished using a 3:1, 1:1 and 1:3 ethanol:Spurr's resin mixture for 8 hours each followed by two changes in 100% Spurr's resin mixture for 8 hours each. The tissue blocks were typically embedded on a flat-bed mould allowing precise positioning of the samples, and polymerised at 70°C for 8 hours (Spurr, 1969).
2.1.4 Sectioning and Counter-Staining of Tissue

The polymerised tissue blocks were sectioned (cut) in cross-section (anterior – posterior direction) on a Reichert-Jung Ultracut E ultamicrotome with a 2mm diamond knife to give slices of an approximate thickness of 50 nm (grey/silver interference colours). These slices were collected on 300 size mesh, uncoated 3mm grids (Athena Grids, Taab Laboratories Equipment, Aldermaston, Berks.).

Counter-staining of the tissue slices was achieved by placing the grids sample side down onto a drop of 2% uranyl acetate in a 70% ethanolic solution for 30 minutes at 37°C within a petri dish, followed by 1 minute on a drop of 70% ethanol, 50% ethanol, 25% ethanol and deionised water, all at room temperature. The grids were then allowed to dry before placing them sample side down on freshly prepared 2% lead citrate for 10 minutes followed by 3 x 1 minute on deionised water. All drop sizes were 0.2ml and were placed onto Laboratory film. The sample slices required no counter-staining if only GAGs were to be observed since the dye Cuprolinic blue plus tungstate has adequate electron density without the need for further staining.

2.1.5 Microscopic Observation of Tissue

The tissue slices were observed on a Jeol 1010 transmission electron microscope (TEM) at 80kv fitted with a lanthanum hexaboride cathode. Electron-optical images of the corneal stroma were generally taken at magnifications between 15000x and 30000x. Images could be stored either on film or digitally. The TEM was equipped with a charged-coupled device (CCD) camera (Kodak MegaPlus, Model 1.4i) which is able to digitise images in a 512 x 512 pixel raster in 256 grey levels. During acquisition of digital
images the contrast (gain) and brightness (offset) were set to a similar value (mean = 50%) for each image by adjusting the condenser on the TEM to minimise sample variation during analyses.

The software was calibrated by defining certain distances across a digital image of a calibration grid of known dimensions at magnifications either side of two image inversions i.e. at x10000 and x12000, followed by x25000 and x30000. For other magnifications, the software interpolated between these measurements.

2.1.6 Image Analysis

Histomorphometric analysis of the corneal stroma was implemented via an image processor running the software analySIS 3.0 (Soft Imaging System GmbH, Germany). This system could store up to 32 binary images or 8, 16 and 32 bit grey or colour images (8 bit grey images were used as standard). Numerous mathematical filters and morphological functions were available through an image-orientated language (Imaging C).

This system was used to analyse two separate components within the cornea, one, the size and relative positions of the collagen fibrils in cross section, the other, the mean size and percentage area covered by GAG precipitates within a two dimensional image of the stroma. Before either of the two ultrastructural components could be measured they were first separated from their surrounding background. Employing a set of image filters and morphological functions to the digital images in predetermined steps accomplished this. Comparing the resulting average diameter and area of collagen fibrils in cross-section assessed the order, type and degree of effect each of the filters and morphological functions had on a standard image of fibrils in cross-section. The final order of filters was
chosen due to its ability to both separate fibrils and smooth their edges whilst retaining a similar fibril diameter and area to the standard image before processing. This method of assessment was not ideal because comparisons were made between more than a thousand fibrils in cross-section from the filtered standard digital image in which noise was digitally suppressed (using various filters), and a few hundred judged by eye from the noisy unfiltered standard digital image using tracing paper and graph paper. The final order of the filters and their effect on both fibrils and GAGs are described in Table 2.1.

Limitations of the image analysis system are two-fold, one is the accuracy of background removal, and the other is operator experience in deciding what is background and what are measurable objects.

The limitations associated with the removal of background are due to the sensitivity of the CCD camera. An 8-bit camera can only distinguish between 256 shades of grey whereas the differing densities within an electron image are, theoretically, limited by the relative differences in the electron density (atomic number) of the atoms within the sample. Therefore increasing the camera to 16-bit would greatly increase the levels of detectable grey and the accuracy to which components within an image can be separated.

The decision as to what is background and what is an object is a subjective one and depends upon the operator of the image analysis system, because it is the operator who ultimately decides what is a fibril or a GAG precipitate and how big this object is. These decisions are subjective because they are based upon reason and experience e.g. fibrils in cross-section are round and GAGs are usually smaller than fibrils. Once these decisions have been made and conveyed to the computer, in terms of greyscale and area, the computer can then quickly and accurately find more of these objects.
### Procedure of Image Analysis

<table>
<thead>
<tr>
<th>Procedure of Image Analysis</th>
<th>Resultant image of collagen fibrils in cross-section</th>
<th>Resultant image of GAGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. An image is acquired by the CCD-camera and displayed onto the monitor.</td>
<td><img src="image1.png" alt="Resultant image of collagen fibrils in cross-section" /></td>
<td><img src="image2.png" alt="Resultant image of GAGs" /></td>
</tr>
<tr>
<td>2. The contrast of the image is then “Equalized” to modify gray values to create a well-distributed gray scale dynamic. Equalisation enhances the contrast of the original image where most gray values are concentrated, i.e., where most of the information is located. Contrast in other areas is suppressed.</td>
<td><img src="image3.png" alt="Resultant image" /></td>
<td><img src="image4.png" alt="Resultant image" /></td>
</tr>
<tr>
<td>3. A “Mean” filter is then applied to the image, this filter calculates the arithmetic mean of a pixel and its eight neighbours. This results in a suppression of noise. Because small image details are also suppressed, the resulting image appears to be smoother. Employed 3x3 matrix:</td>
<td><img src="image5.png" alt="Resultant image" /></td>
<td><img src="image6.png" alt="Resultant image" /></td>
</tr>
</tbody>
</table>

**Employed 3x3 matrix:**

```
1 1 1
1 1 1
1 1 1
```
4. The resulting image is then binarised. A binary image has only 2 grey levels; i.e. each pixel of an image may be set or not set. In order to binarise the image the program has to know which pixels of an image should be set to transfer a grey value image into a binary image. This information is given by a simple grey value threshold and was set between 0 and 80 for fibrils, and 0 and 50 for GAGs. All pixels lying within these thresholds are considered to be set and to correspond to a fibril or GAG precipitate. All other pixels correspond to the background.

5. Once binarised the image could then be put through a morphological filter, used to separate fibrils that are touching each other. The central techniques for particle separation are erosion (shrinking of an object), and dilation (expansion of an object), throughout this work a 3x3 lattice with 2 iterations was used. Firstly an erosion filter was used which “shaved off” perimeter pixels and removed connecting lines between objects.
6. Following erosion a dilation filter was implemented, causing small holes in the objects to close and small irregularities at the peripheries of the objects to be equalised.

7. Since the software is calibrated, measurements could then be made of either the fibrils or GAGs. This was achieved by setting the software to analyse all binarised objects within specific threshold values. These values were usually in units of area (300-4000nm² for fibrils, 50-1200nm² for GAGs). Any objects that fell between the specific threshold values were highlighted different colours could be set to represent different values in between the thresholds, to aid visual recognition. Parameters such as area and diameter could then be measured and incorporated into further analyses.

Table 2.1: Describes the methods employed to acquire information on both the fibril sizes and positions, and the PG sizes and amount from digital images of the corneal stroma.

<table>
<thead>
<tr>
<th>2.1.6.1 Assessment of Resultant Artefacts from Image Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artefacts from image analysis, or more specifically non-systematic errors, were kept to a minimum. The possible causes of these errors fall into three categories, 1) electron microscopy, 2) tissue inhomogeneity and 3) image analysis.</td>
</tr>
</tbody>
</table>
Non-systematic errors formed by electron microscopy come from tissue preparation and mechanical errors. For example, during tissue preparation section contamination can be caused by uranyl acetate crystals and lead precipitates. These artefacts can be kept to a minimum by making sure that all containers are kept clean from dust and lint, not allowing grids to dry between various staining steps and by using freshly prepared staining solutions. Stain density or penetration of the stain may also cause non-systematic errors. Stain density problems were alleviated by careful stain preparation, constant-staining times and at similar temperatures. Penetration of stains en bloc i.e. Cuprolinic blue, was assumed constant because of the thinness of the cornea and the excessive time in the staining solution (24 hrs). Mechanical errors cause variation in section thickness, in and between samples, which in turn affects resolution of the tissue, since resolution is inversely proportional to section thickness (assuming an even density). Differences in the resolution of collagen fibrils or GAG filaments at the same magnification may result in a change in threshold values which will affect the size of different resolved objects, assuming all images are properly focused. Other mechanical errors within the transmission electron microscope such as fluctuating and time-dependent beam intensities, astigmatism and hysteresis can cause resolving and magnification errors. Differing beam intensities were compensated by adjustment of the contrast and brightness for each digital image to a similar level. Astigmatism of the objective lens was assessed regularly using a holey film and adjusted using the stigmators. Hysteresis can cause magnification errors of ±10% but can be eliminated by taking a lens to saturation and then returning to the required magnification (Meek, 1970).
Tissue inhomogeneities such as differences in fibril diameters or GAG filament size and amount within a distinct area of the cornea (e.g. mid-stroma) or between several corneas from same species and of similar age are presently unknown. Therefore great care was taken to ensure that any measurements taken for use as a comparison were taken from similar areas within each cornea.

For errors relating to image analysis, the framing of the image was important. Images should be taken randomly throughout the area of interest (e.g. anterior stroma, mid stroma and posterior stroma). But taking images completely at random was impractical since certain areas of a section may be torn, obstructed from view by a grid bar or more importantly the image contained fibrils of an incorrect orientation. Images for GAG analysis were taken from areas displaying collagen bundles in cross section parallel to their fibril axis (the collagen fibrils were visualised in an unstained, translucent state), because a smaller and less constant number of GAGs would be seen in transverse sections. This is because (i) transverse PGs occur at specific locations with respect to the collagen fibril axis and (ii) a significant number of PGs are found longitudinally with respect to the collagen fibrils, and these filaments would not be accurately measured in transverse sections. The analysis of fibril diameters and their positions required a transverse image.

Once a correctly oriented image had been captured by the digital camera, and as long as the same parameters were used in binarising each image, the greatest source of error during the image analysis stage was estimated to be the manual separation of particles, especially the collagen fibrils. This occurs after implementing the dilation filter (see Table 2.1 page 38) and some particles are still not separated or large holes are not filled,
therefore requiring manual separation or filling with a digital paint brush. Each image required a similar amount of manual separation/filling, therefore it could be argued that this procedure forms a systematic rather than non-systematic error i.e. an equal amount of error was introduced into each image and for comparative studies these errors need not be taken into account. On average about 10 to 15 sets of two, occasionally three, fibrils required manual separation within each image.

2.1.6.2 Calculation of Image Measurement Errors

The sum of the errors mentioned above was estimated by comparing the results of image analysis from the mid-stroma of young rabbits. Diameters, GAG mean particle size and GAG percentage area covered were compared. Diameters of collagen fibrils vary little from the centre to the periphery of the cornea (Borcherding, 1975). For each image analysed the mean diameter or GAG particle size and percentage area covered was calculated. The results from the individual images were pooled together and compared to give a 95% confidence interval for each of the three parameters, which related to the inter-image variability. For fibril diameters this equalled ± 2.1nm, for GAG particle size it equalled ± 11.9nm$^2$ and for GAG percentage area covered it equalled ± 0.6%. These errors, representing inter-image variation within the methods outlined above, can therefore be added to any measured sample variation such as differences in fibril diameter or PG particle size. This is because any change in the mean value for these parameters (due to PRK wounding or chemical alteration) appear to result in the same spread or degree of variation as displayed by the normal values.
2.2 Statistics

2.2.1 95% Confidence Intervals
Confidence intervals were calculated to compare the normal distributions of fibril diameters and GAG size from control and modified stroma. The confidence interval is a range on either side of a sample mean. Using the mean, standard deviation and sample number of fibril diameters or GAG filaments from each population a 95% confidence interval was calculated for each population mean (Campbell, 1974).

2.2.2 Kruskal-Wallis and Student t-tests
The Kruskal-Wallis test was used to calculate the confidence limits in samples from more than two populations and the student t-test was used to evaluate the differences in means between two groups. Both are analytical procedures that concentrate on assessing the reasonableness of regarding two (or more) populations as the same in some respect, and are therefore significance tests. The particular hypothesis of equality, which is studied, is called the null hypothesis, which is that the population medians, or means, are equal. Therefore to decide whether the hypothesis of zero population difference is compatible with the sample values, the probability of such values occurring is obtained when the populations really are the same; if this probability is not too small the agreement between the null hypothesis and the samples can be regarded as reasonable and the null hypothesis accepted. If on the other hand this probability is small, the hypothesis is rejected. By convention as in the calculation of confidence intervals, a probability greater than 5% is regarded as reasonable, that is the null hypothesis is accepted if a probability greater than 0.05 is found. The probability of a random deviation of a particular size (from the population mean), decreases with the increase in the sample size therefore, small relations
can be proven significant only in large samples. Theoretically, the t-test can be used even if the sample sizes are very small (e.g., as small as 10 or less) as long as the variables are normally distributed within each group and the variation of scores in the two groups is not reliably different. The normality assumption was evaluated in each case by looking at the distribution of the data via histograms.

2.3 Calculation of the Radial Distribution Function

The radial distribution, denoted by $g(r)$, is the ratio of the average number density of fibril centres at a given distance, $r$, from any other fibril centre, to the bulk fibril number density, $\rho$. Therefore it represents the relative likelihood of finding two fibril centres separated by a distance, $r$. Because two fibrils cannot approach each other closer than touching, the function is zero at least twice the fibril radius.

Given the complete set of fibril co-ordinates $(x,y)$ from a digitised electron microscope image, $g(r)$ was calculated as follows. An arbitrary fibril centre was taken as the origin of a set of annuli, radius $r$ and thickness $\Delta r$, extending from $r = 0$ to about 500nm. The
number of fibril centres in each annulus was counted and divided by the area of the annulus, $2\pi r \Delta r$. The result was a histogram of the number density of fibril centres at each distance $r$ from the origin. The process was repeated using each fibril in turn as the origin, and the average histogram for all fibrils gave the radial distribution function (see Figure 2.2).

At a certain distance, $r$, for each fibril, the edge of the computerised electron microscope image was reached. For distances greater than $r$ the number of fibrils counted was artificially low. This effect would worsen when the reference fibril was near the edge of the image, since that would cause the distribution function to tail off at large $r$ when, in theory, it should stabilise to a constant value. This problem was overcome by converting the uncorrected function, $g'(r)$, to the corrected function, $g(r)$, using the formula of Fraser et al. (1964):

$$g(r) \approx \pi g'(r) / (\alpha - \sin \alpha)$$  \hspace{1cm} (2.1)

where $\cos(\alpha/2) = r/2S$ and $S$ is the radius of the circle containing all the fibrils.

The radial distribution function shows the value of $g(r)$ rise from zero (for $r = 0$) upwards until the nearest neighbour distance is reached, at which point there is a distinct peak. As $r$ increases further, the function undulates gently before stabilising to a constant value, the 'correlation distance' $r_c$. The value of $g(r)$ for $r > r_c$ represents the bulk number density of fibrils, $\rho$, for the whole area covered and is an important parameter in calculating the percentage transmission through the cornea (see direct summation of fields method Chapter 2.7 page 54.). The distinct peak followed by smaller undulations and eventual stability in the radial distribution histogram reflects the lattice structure of the cornea, with neighbouring fibrils being uniformly spaced, but without any long-range
order. The position of the primary peak in $g(r)$ was used to determine the average interfibrillar spacing from TEM images. A plot from co-ordinates gained from a typical TEM image of normal rabbit cornea and its corresponding radial distribution function are shown in Figure 2.3. Images analysed in this way contained about 1000 fibrils.

Figure 2.3: A plot of fibril co-ordinates and their radii from a normal rabbit mid-stroma (top) and its corresponding radial distribution function, showing its primary peak and correlation distance (above).
Tissue will swell and shrink during processing for TEM, and generally the tissue after polymerisation will be shrunken (Fullwood and Meek, 1994). Assuming this shrinkage occurred equally in two directions it can be compensated for by comparing the interfibrillar spacing determined from $g(r)$ from the radial distribution function, with the corresponding value determined from XRD. To correct for this discrepancy the $(x,y)$ fibril co-ordinates from digitised electron microscopic images were expanded equally in both the $x$ and the $y$ directions until the peak of the radial distribution function corresponded with the interfibrillar spacing form XRD (once the Bragg spacings were converted to interfibrillar spacings) (Leonard and Meek, 1997).

Having rescaled the fibril co-ordinates so that the radial distribution function agreed with the x-ray measurements, the bulk fibril number density was given by $g(r)$ in the stable region beyond the correlation distance, $r_c$, before normalisation. Following rescaling, $g(r)$ peaks at a larger interfibrillar spacing and stabilises at a lower fibril number density. This method of ‘expanding’ the electron images to account for shrinkage was based upon the assumption that the specimen contracted equally in all directions. The assumption seems reasonable since electron micrographs typically show an even distribution of fibrils with no preferred direction of contraction (Leonard and Meek, 1997). This assumption was assessed for each rescaled electron image by measuring the radial symmetry of the fibril positions.
2.5 Radial Symmetry Calculations

Radial symmetry was estimated by plotting the relative displacements of each fibril, from a micrograph, from every other fibril about a common origin. If radially symmetrical, the resulting graphs would be plotted by points surrounding a radially symmetrical circle, if not, they would be plotted by points surrounding an ellipse (see Figure 2.4). The degree to which the circles were radially symmetrical (circularity) was quantified by calculating the distance at the widest part of the circle divided by the distance perpendicular to that. Therefore any value greater than unity is an ellipse.

![Figure 2.4: Plots of the relative displacements of each fibril from every other fibril about a common origin from a digitised electron micrograph of normal stroma. In plot A y=y, x=x therefore circularity = 1. In plot B, y=y, x=x/2 therefore circularity = 2](image-url)
2.6 Synchrotron X-ray Diffraction

2.6.1 Synchrotron Radiation Source

The Synchrotron Radiation Source (SRS) at Daresbury was the world's first machine dedicated to the production and use of synchrotron radiation. Synchrotron light is produced at the SRS when an electron beam travelling close to the speed of light is accelerated in a magnetic field. A series of magnets is used to bend the path of the electrons into a circular shape. As they pass these "bending" magnets, the path of the electrons is deflected and they emit intense beams of light, known as synchrotron radiation. The beam is in the form of a cone in front of the electron, rather like a searchlight. The light covers a broad area of the electromagnetic spectrum, from infrared through to hard x-rays. Synchrotron x-rays are much more intense than those from a conventional laboratory source enabling experiments to be carried out in a very short time. This means many diffraction patterns from either one cornea or multiple corneas can be collected in a practicable length of time and without significant damage to tissue ultrastructure due to proteolysis or chemical preservation.

The limitations of the SRS are that (i) restrictions apply on its use, due to demand, therefore it is available only a few days every 2 or 3 months, (ii) the beam size has a minimum area of 2mm$^2$ for station 2.1 and 0.03mm$^2$ for station 7.2 therefore restricting the accuracy of measurements in small areas within the cornea especially of interfibrillar spacings and (iii) samples must either be frozen or kept on ice usually for days following their removal from the animal before being transported to the SRS.
2.6.2 X-ray Diffraction of the Cornea

X-ray diffraction patterns through the cornea consist of contributions from many different scatterers. Therefore, analysis of a corneal diffraction pattern yields the mean values of structural parameters averaged over a large number of fibrils or molecules. Also each cornea will have a certain amount of variation in fibril spacings and diameters, which causes the diffraction rings to spread out. This spread is caused by thermal vibrations of the molecules, the non-crystalline structure of the cornea and by differences in fibril diameter (Gyi et al., 1988). X-ray diffraction measurements of interfibrillar and intermolecular spaces therefore yield the mean value and its experimental error is expressed as the width of each diffraction ring.

2.6.3 Measurements of X-ray Diffraction Patterns

Up to seven different parameters were obtained and measured, from the corneal x-ray patterns. These were (i) intermolecular Bragg spacing, (ii) interfibrillar Bragg spacing, (iii) spread of interfibrillar spacing (iv) fibril diameter, (v) spread of fibril diameters, (vi) fibril orientation and (vii) fibril scattering intensity (fibril density).

2.6.3.1 Intermolecular Bragg Spacing

The intermolecular Bragg spacing, is a measure of the lateral separation of the molecules that make up the collagen fibrils. It was calculated from the first order equatorial reflection in the high-angle pattern (Meek et al., 1991). The values are left as Bragg spacings throughout, although, with the assumption that the molecules are packed in a 'pseudo-hexagonal' lattice, the Bragg spacing could be increased by a factor of 1.11 to give the mean centre-to-centre separation of the molecules (Maroudas et al., 1991).
2.6 SYNCHROTRON X-RAY DIFFRACTION

2.6.3.2 Interfibrillar Bragg Spacing
The interfibrillar Bragg spacing, is a measure of the lateral separation of neighbouring collagen fibrils and was calculated from the position of the innermost equatorial reflection (the lattice interference function) in the low-angle diffraction pattern. The interfibrillar spacing is known to vary with hydration (Goodfellow et al., 1978; Sayers et al., 1982), so corneas, where possible, were compared over only a narrow range of hydration (H= 2.3 to 3.8). This range covers the natural variation found within most species, including rabbit (Maurice, 1957). To calculate the most frequently occurring interfibrillar spacing, the fibrils were assumed to be arranged in a two-dimensional liquid-like array such that their centre-to-centre spacing is equal to the interfibrillar Bragg spacing multiplied by a factor 1.12. This type of packing is supported by both electron microscopy (Cox et al., 1970) and x-ray diffraction (Worthington and Inouye, 1985).

2.6.3.3 Spread of Interfibrillar Spacings
The spread of interfibrillar spacings was calculated by dividing the full height by the full width at half the full height of the lattice interference function, a low value indicating a wider variation of nearest neighbour interfibrillar spacing (Fratzl et al., 1993). This parameter allows a change in the variation of interfibrillar spacing to be examined between corneas.

2.6.3.4 Fibril Diameter
The fibril diameter was calculated from the fibril transform, that is the amplitude of x-rays diffracted by a single fibril. The fibril transform appeared in the low-angle equatorial diffraction pattern as one or more diffuse 'subsidiary' maxima (see Figure 2.9, page 67). The reciprocal space co-ordinate $R$ of the first maxima is given by
\[ R = \frac{5.14}{2\pi a} \quad (2.2) \]

where \( a \) is the fibril radius (Worthington and Inouye, 1985).

### 2.6.3.5 Spread of Fibril Diameters

Any change in the spread of the fibril diameters will lead to a broadening or narrowing of the first subsidiary maximum. Measurements were made of the full height divided by the full width at half height of this maximum. Increases in this parameter were then related directly to increase in the spread of fibril diameters (and vice versa).

### 2.6.3.6 Fibril Orientation

The fibril orientation at specific areas through the cornea can be approximated. Each stromal lamella in the cornea gives rise to a high-angle equatorial x-ray diffraction pattern with maxima perpendicular to the direction of the molecules of which the fibrils are composed. The maxima are perpendicular because the x-rays will scatter at right angles to the direction of their long axes (Newton and Meek 1998). Therefore if lamellae, or molecules, are aligned approximately parallel to each other in one specific direction through the cornea more than in any other direction, a pair of scattering maxima will be seen on the detector placed behind the sample. The molecular orientation can then be deduced from the angle of the line joining two maxima on an x-ray diffraction pattern. If there is more than one orientation of the molecules, for example between different lamellae, there will be more than one pair of diffraction maxima.

### 2.6.3.7 Fibril Scattering Intensity

The fibril scattering intensity (fibril density) is proportional to the number of fibrils at the point at which the diffraction pattern was taken. It also depends on the size and ordering
of the molecules within the fibrils and the square of the radius of the fibrils. The cross-sectional area of a fibril is proportional to the mass of collagen in a fibril if the molecular spacing is the same throughout the tissue. The molecular spacing would depend on intermolecular hydration; a decrease in hydration would increase the radial position of the collagen diffraction ring. Therefore, assuming there is no change in intermolecular hydration, the cross-sectional area is proportional to the mass of collagen in the fibril. Hence, the fibril scattering intensity is also proportional to the total mass of collagen in the x-ray beam at any one-measurement point (Newton and Meek 1998).

2.7 Transparency calculations: Direct Summation of Fields Method

Stromal light scattering depends sensitively on the fibrillar ultrastructure, especially on the degree of order in the spacing of the fibril centres and, to a lesser degree, on fibril size (Hart and Farrell, 1969). Light scattering calculations made from the positions of fibrils in transmission electron micrographs produce results that closely agree with the transparency measurements made on fresh (unfixed) tissue (Farrell et al., 1973; Freund et al., 1986).

Undeviated light is said to be light that exits an object in the same direction it entered. The total scattering cross-section per fibril per unit length, $\sigma_r$, is related to the fraction of light transmitted undeviated through the cornea by the equation:

$$F_T = e^{-\Delta \rho \sigma_r}$$  \hspace{1cm} (2.3)

where $\rho$ is the bulk number density of fibrils in the stroma, and $\Delta$ is the thickness of the stroma.
The problem facing theoreticians is to find the exact value for the scattering cross-section ($\sigma_t$). The method used in this thesis was developed by Freund et al. (1986) and later generalised to account for systems where there is a variation of the individual fibril diameters (Freund et al., 1995).

The details of the calculations are described in the original papers so only an outline of the method is given here. Starting with a TEM image from a small region of the stroma, typically an area of $1.6\mu m^2$ containing 900 to 1500 fibrils in cross section, the size and position of each fibril was measured using image analysis as described in Chapter 2.1.6 page 36. The area covered by the image is then divided up into a grid each grid element being the same size and shape. The field scattered by each fibril depends on its radius and thus varies from one fibril to the next. A superimposition, generated from the fields scattered by each fibril in their respective grid-elements (taking into account the phase differences introduced by the different fibril locations) can then be used to calculate the differential cross section per fibril, $\sigma(\theta_s)$. $\sigma(\theta_s)$ describes the scattering at one given angle, $\theta_s$, for all the fibrils in one grid-element. This procedure is then repeated for each grid element and the average over the whole grid equals the mean differential scattering cross section per fibril for the entire stroma. Integration of the mean differential scattering cross section per fibril from $\theta_s = 0$ to $2\pi$ radians gives the total scattering cross section per fibril, $\sigma_t$, from which the transmittance, $F_T$ can then be calculated using Equation 2.3.

For each grid-element, $b$, the sum of the scattered fields is:

$$S_b(q) = \sum_{j=1}^{N} E_j(\lambda, \theta_s)e^{iqa_j}/<\sigma_0(\lambda, \theta_s)>^{1/2}$$  (2.4)
where $q$ is the scattering vector; $|q| = 4\pi n_s \sin(\theta_s/2)/\lambda$, and is the $\theta_s$ scattering angle. $r_j$ is the position vector on the $j^{th}$ fibril, with respect to the bottom left hand corner of the grid-element, and $E_j(\lambda, \theta_s)$ is the field scattered by the $j^{th}$ fibril:

$$E_j(\lambda, \theta_s)^2 = \frac{n_j^3 \pi^2 (n_j)_{j^2}^2 (m^2 - 1)^2}{2\lambda^3} [1 + \frac{4\cos^2 \theta_s}{(m^2 + 1)^2}]$$

(2.5)

and summation is over all $N_b$ fibrils in the $b^{th}$ grid-element.

The differential scattering cross-section is found from the ensemble average for all boxes:

$$\sigma(\lambda, \theta_s) = \frac{K}{(K-1)N'} [\overline{|S_{(q)}|^2} - \overline{|S_b(q)|^2}]$$

(2.6)

where $K$ is the number of grid boxes and $N'$ is the average number of fibrils per box.

Integrating the differential cross-section from 0 to $2\pi$ gives the total cross-section:

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

(2.7)

From equations 2.3 and 2.5 it can be seen that $F_T$ depends on the bulk fibril number density, $\rho$, on the fibril radius, $a$, on the wavelength of light, $\lambda$, the thickness of the cornea, $\Delta$, and on the ratio of the refractive indices of the collagen ($n_j$) and ground substance ($n_b$), $m$. Values for $\rho$ and $a$ are obtained from electron micrographs, scaled to account for changes during processing as described in Chapter 2.4 page 48. A nominal thickness of 0.3mm was used for the thickness of all corneas, unless otherwise stated. Typically in transparency calculations, individual lamellae are not included, since fields
of differing angular orientation cannot interfere. Instead, the stroma is treated as a single lamella of the same thickness as the cornea comprising of parallel fibrils. The fibrils are treated as thin, infinitely long, dielectric cylinders, each having the same perpendicular orientation with respect to the propagation direction of the incident light.

Estimates are required for the refractive indices of the fibrils and the ground substance. Presently the most accurate values have been gained by x-ray diffraction measurements and for the normal rabbit cornea, \( n_f = 1.416 \) and \( n_g = 1.357 \) (Leonard and Meek, 1997). \( n_f \) is a function of the fibril hydration, if hydration increases, \( n_f \) will fall. The refractive index \( n_g \) may differ in pathological corneas due to differing concentrations of cytokines and digestive enzymes. The degree to which this occurs is presently unknown therefore the normal value for \( n_g \) was used for all the transparency calculations.

All parameters used in the above calculations are assumed to be uniform throughout the tissue.

It is pertinent to point out that the above transparency calculations (based upon the work of Freund et al. (1986;1995)) and those of Maurice (1957), Hart and Farrell (1969), Benedek (1971), Twersky (1975) and Farrell et al. (1973) are all limited, in some way, by the restrictive information contained in electron micrographs. Each electron micrograph represents a minute proportion of all the fibril diameters and their relative positions within a whole cornea and therefore at best contributes towards an approximation of corneal transparency. However, the use of electron microscopy remains essential to these calculations until a more representative method can be found.
2.8 Pachymetry

A KOI Model 3000x ultrasonic pachymeter was used to measure *in-* and *ex-vivo* corneal thickness with a resolution of 1 micron over a nominal range of 0.35 to 0.80 mm. The pachymeter was on loan, courtesy of Dr Russ McCalley (Applied Physics Laboratory, The John Hopkins University, Maryland, USA).

Theory of operation: The ultrasonic pachymeter operates on the “pulse-echo” principle. A short duration electric pulse excites a piezoelectric transducer. The transducer converts the electrical energy into mechanical vibrations (sound waves). These sound waves are coupled from a short delay medium in front of the transducer into corneal tissue by a coupling liquid (water). Sound waves travel through the cornea and are reflected back from the rear corneal surface. The same transducer receives the reflected sound waves and converts them to electrical pulses. The lapsed time $t$, between two successive echoes from the rear corneal surface is related to the thickness $x$, and the velocity $V$, at which sound waves travel through the cornea. The result is expressed by the relationship:

$$x = \frac{Vt}{2}$$

(2.8)

where, $x =$ the thickness of the cornea, $V =$ the velocity of ultrasonic sound waves in the cornea (0.1640 m/s) and $t =$ the measured round trip transit time.

The pachymeter was calibrated using a test block of a known thickness.

*In-vivo measurements*

The transducer was touched to the cornea using moderate pressure by hand, and held there for a few seconds until a stable reading was obtained. This was repeated three times and the average thickness calculated.
2.10 Healing of Full Thickness Wounds in Young Rabbits

Ex-vivo measurements

The excised cornea was held against the rounded end of a plastic pipette from which a square window (5mm x 5mm) had been cut out, thus allowing the transducer to be placed with moderate pressure against a small unsupported area of the cornea. This allowed just the thickness of the cornea to be measured and not its support. Again this was repeated three times and the average thickness calculated.

2.9 Calculation of Corneal Hydration

Wet corneas (whole or in part) were carefully weighed and then placed over silica gel in a sealed chamber for 4 to 6 days or until three consecutive weighing measurements separated by 12 hours gave the same reading.

Hydration values (H) were calculated thus

\[ H = \frac{W_M - D_M}{D_M}, \quad (2.9) \]

where \( W_M \) equals the weight of the cornea when wet and \( D_M \) equals the weight when dry.

2.10 Healing of Full Thickness Wounds in Young Rabbits

2.10.1 Details of Animal Treatments

2mm diameter wounds through the entire corneal thickness were produced in both eyes of eight adult albino rabbits by Dr Charles Cintron and colleague at the Schepens Eye Research Institute, Boston,
USA. The full method is described in Cintron et al., (1973). In brief, the rabbits were anaesthetised with intravenous injections of pentobarbital, and a 2mm-diameter full thickness plug was excised from the central part of each cornea with an Elliot trephine. A photograph of a 5 month wounded eye is shown above. The corneas healed forming a uniform plug of opaque avascular tissue, about the same diameter as the excised button.

Each animal was sacrificed after a period of 5, 10, 12 or 16 months with an overdose of pentobarbital and the wounded corneas with a scleral ring attached were excised. The orientation of the corneas in relation to their position in the eyes was marked on excision by a suture in the most superior part of the scleral rim. The corneas were then stored on ice and brought by Dr Charles Cintron from the Schepens Eye Research Institute, Boston, USA to the Central Laboratory of the Research Councils (CLRC) synchrotron source at Daresbury, UK.

2.10.2 X-ray Diffraction

2.10.2.1 Collection of Low-angle Data

The low-angle data were collected at station 2.1 at the CLRC synchrotron source, Daresbury, UK. Using a 2mm x 1mm collimated beam, scatter measurements from corneal collagen fibrils were taken. Exposure time was typically 3 minutes for each diffraction pattern.

The corneas were placed in an airtight polymethacrylate cell held between Mylar windows; a holder that could be moved horizontally using a remotely controlled stepper motor held the cell in front of the x-ray beam. Vertical translation of the tissue was accomplished by dextrous use of a calibrated screw. Exposing green indicator paper taped to the inside of the cell assessed the beam position relative to the wound; this was
repeated before exposing each cornea. This allowed the beam to be positioned to pass directly through the centre of the wound. The cell holder was then moved down in 0.6mm steps for up to 3mm, exposing separate areas of the wounded cornea to the x-ray beam. This was repeated for all 16 corneas. The accuracy of beam positioning was estimated to be ± 0.4mm. This was achieved by placing the cell over a sheet of graph paper. A fixed reference point was attained by drawing a template of the cell onto the graph paper clearly marking the top and bottom of the cell. The information from the green indicator paper displaying the shape and position of the beam relative to the cell was transferred to the template. This allowed an accurate positioning of each wound relative to the beam position. The beam position was checked with the green indicator paper at regular intervals to allow for drift of the beam.

The low-angle diffraction patterns collected from station 2.1 were analysed to measure the interfibrillar Bragg spacing, the average fibril diameter and to look for changes in the spread of fibril diameters.

2.10.2.2 Collection of High-angle Data

The high-angle camera at station 7.2 allows measurements from the scatter of collagen molecules within the fibrils, using a 0.2mm collimated beam and x-rays of wavelength 0.1488nm. Exposure time was typically 3 minutes for each pattern.

The corneas were contained in the same cells for both the low and high-angle cameras. To investigate different parts of the wound and surrounding tissue, the cornea was moved in the x direction by using the remotely operated motorised cell holder, as described previously. This time horizontal translation of the corneas was in 0.2mm steps. The position of the beam relative to the wound was determined at the beginning of each
experiment by exposing a piece of green indicator paper taped inside the cell and aligning it to a specific area outside the wound. This point would form the origin of a single or multiple line/transect (the origins were centrally inferior to the wound for the single transects and inferior-temporal for the multiple transects as shown in Figure 2.5). Using the same method as outlined for the low-angle camera, the accuracy of beam positioning was estimated to be ± 0.2mm.

X-ray scatter patterns were collected at points that were 0.2 to 0.6mm apart along straight transects across the wound. All measurements were taken from full thickness tissue and altogether 16 single transects across 16 individual corneas and 1 set of multiple (9) transects across 1 cornea were examined; their positions are shown on top of a typical wound in Figure 2.5.

Each stromal lamella in the cornea gives rise to a high-angle equatorial x-ray diffraction pattern because of interference between the x-rays scattered from the aligned molecules of which the fibrils are composed. Molecules aligned approximately parallel to each other, scatter x-rays at right angles to the direction of their long axes, which gives rise to the scattering maxima seen on a detector placed behind the sample. Therefore, molecular orientation can be deduced from the angle of the line joining two maxima on a

Figure 2.5: A magnified image of a full thickness wound showing the relative positions of each transect.
x-ray diffraction pattern. If there is more than one orientation of the molecules, for example between different lamellae, there will be more than one pair of diffraction maxima. Therefore, it follows that if there were an equal number of lamellae, with orientations equally spread over 360°, the resulting x-ray scatter pattern would be circular with an even circumferential intensity. In the human eye, two preferential directions for collagen fibrils were found by Meek et al (1987). A typical pattern from the human eye would show four intensity maxima superimposed on a diffraction ring arising from the interference of x-rays scattered from neighbouring collagen molecules, demonstrating an orthogonal, biaxial, angular distribution of collagen fibrils. Horizontally orientated collagen in the tissue within the cell holder, produces maxima at the top and bottom of the diffraction ring, vertically orientated collagen produces maxima to the left and right.

After the x-ray diffraction patterns were collected, the corneas were either immediately frozen at −20°C or processed for electron microscopy in both glutaraldehyde plus osmium tetroxide, and glutaraldehyde plus Cuprocinic blue.

2.10.2.3 Analysis of Low-angle Data
The low-angle x-ray diffraction patterns were collected on a multi-wire gas proportional detector and analysed using programs developed on-site at CLRC, Daresbury. Firstly, if more than one frame was captured for a specific exposure these frames were summed together. Then each experimental diffraction pattern was normalised to correct for the gradual decline in beam intensity during the course of the day. Normalisation was achieved by dividing every image’s intensity by its respective ion chamber reading. Following normalisation, each diffraction pattern was then divided by the detector response. The detector response was acquired by evenly illuminating the detector with a
Division of each diffraction pattern by the detector response pattern compensates for areas of differing sensitivity within the detector. The distance in reciprocal space from the centre of each diffraction pattern to the first equatorial reflection corresponds to the interfibrillar Bragg spacing. This distance was measured by a horizontal integration through a vertical transect. The vertical transect was attained by placing a rectangle through the middle of the pattern, stretching from one side of the reflection to the other (see Figure 2.6).

The differing intensities across the pattern were integrated horizontally and plotted as intensity versus distance across pattern (see Figure 2.7). The size of the rectangle was adjusted to gain the best intensity profile by balancing noise against broadening of the peak caused by the curvature of the diffraction pattern. Once the most representative of the intensity profiles from each of the patterns was achieved, the semi-transparent

Figure 2.6: A Low-angle x-ray diffraction image, collected by a multi-wire gas proportional detector. The inner ring is the first equatorial reflection from the interfibrillar spacings, the grey rectangle with a central black ellipse is the semi-transparent back stop and the red outlined rectangle is the vertical transect.
backstop peak was artificially set to $x=0$ and $y=100$ and the profile to the left of the backstop removed.

![Intensity profile](image)

**Figure 2.7: Intensity from a vertical transect across the centre of a low-angle diffraction pattern.**

To compensate for any scatter by the cell, an intensity profile for an empty cell was subtracted from the intensity profile of each diffraction pattern. To correct for differences in radial intensity across the pattern, since the measured intensities were integrated from a rectangle rather than a sector, each $y$ value was multiplied by its corresponding $x$ value ($y=y^*x$).

Within each intensity profile there are the reflections from the fibril transform (Bessel function) and the lattice interference function (equatorial reflection from aligned fibrils) but there is also a certain amount of background intensity which is at maximum in the centre of the pattern and decreases at wider angles. Presumably, this background scatter is incoherent scattering, as from a gas, and is produced by the less ordered material in the stroma such as the proteoglycans and glycoproteins. Therefore, reflections from the fibril
arrangement are superimposed upon a changing base line, which must be subtracted to find the true positions of the maxima. Fortunately, the background drops off quite smoothly, enabling a curve to be fitted beneath the intensity peaks using a power law, (see Figure 2.8).

![Intensity profile and background curve](image)

**Figure 2.8:** Graph showing the fitting of a background curve to an intensity profile from a low-angle diffraction pattern.

For calculating the interfibrillar Bragg spacing the fibril transform, \( F(R) \), must first be divided from the background-corrected intensity profile \( I(R) \)

\[
I(R) = |F(R)|^2 L(R), \tag{2.10}
\]

where \( L(R) \) is the interference function of the lattice. To do this a Bessel function was fitted to the first maxima of the fibril transform as shown in Figure 2.9. According to
2.10 HEALING OF FULL THICKNESS WOUNDS IN YOUNG RABBITS

Diffraction theory (Oster and Riley, 1952) the amplitude, $F(R)$, of x-rays diffracted by an infinitely long cylinder of uniform electron density is defined as:

$$F(R) = \frac{2J_1(Ra)}{Ra},$$  \hspace{1cm} (2.11)

where $2\pi Ra$ equals 5.14 (Worthington and Inouye, 1985) and $J_1$ is the first order Bessel function. Once the fitted Bessel function was divided from the intensity profile the position of the interfibrillar Bragg spacing could be measured from the first peak of the interference function of the lattice $L(R)$ with a typical accuracy of 3%. This value was then inverted into real space and calibrated using rat tail tendon whose first meridional reflection appears at 67 nm.

![Graph showing the fitting of a Bessel function to the first maxima of a fibril transform within an intensity profile.](image)

**Figure 2.9**: Graph showing the fitting of a Bessel function to the first maxima of a fibril transform within an intensity profile.
2.10.2.4 Analysis of High-angle Data

The high-angle diffraction patterns were collected on a Mar image plate and for full thickness wounds the fibril scattering intensity and orientation of the molecules within each pattern was measured.

To measure the fibril scattering intensity and orientation of the molecules within each pattern the high-angle x-ray diffraction patterns were analysed using a commercially available image-processing package (Optimas, Seattle, WA). The exact centre of each diffraction pattern, that is, the position corresponding to the axis of the straight-through beam, was found using the diffraction patterns gained from the Mylar windows of the cell holder. The centres of these patterns are easily found geometrically.

Each experimental diffraction pattern was normalised to correct for the gradual decline of the beam intensity during the course of each transect. Normalisation was carried out using the measured intensity of the Mylar ring. The intensity of the Mylar ring was measured in the same way as the intensity of the collagen ring, by using an Optimus program*. This program measured the intensity around the circumference of a sequence of 40 concentric and contiguous circles covering an area above and below the collagen ring and 15 circles covering an area above and below the Mylar ring. These circles were centred on the straight-through beam position and were of increasing radius, each circle having a width of 0.31 nm\(^{-1}\). The circumference of each circle was divided into 256 sectors and the average image intensity was recorded for each sector. This gave a 256x40 matrix of measurements for the collagen ring and a 256x15 matrix of measurements for

*All Optimus programs were written and kindly supplied by Dr RH Newton, Dept. Optometry and Vision Science, Cardiff, UK.
the Mylar ring for each diffraction pattern. Any single row of these matrices corresponded to the image intensity around a circle of a given radius. Any single column of these matrices corresponded to the image intensity spreading radially out from the smallest circle radius at a given angle relative to the vertical.

To calculate the intensity of the Mylar ring a straight line was fitted to the curve of intensity against the 15 concentric circles for each one of the 256 sectors (i.e. each row of the matrix). The straight line was subtracted from the curve and the integral under the resulting graph was calculated by summation of the 15 intensity values resulting in a 256x1 matrix of intensity values for each diffraction pattern. From the symmetry of the pattern, measurements at an angle $\theta$ and an angle $\theta + 180^\circ$ should be identical. Therefore, the matrix (256x1) was simplified by adding the first 128 and the second 128 entries and dividing by 2, to give a 128x1 matrix. Noise in the data was then reduced with a 10-point moving average smoothing. To avoid any effects to either end of the data due to smoothing it was padded with a copy of the last 20 values preceding the first value and a copy of the first 20 values proceeding the last value. After the 10-point moving average, these additional values were removed (Newton and Meek, 1998). Finally, the data was integrated by summation of the 128 values leaving just one value for the intensity of Mylar in each diffraction pattern. This value would then be divided from the final intensity value of the collagen ring, for normalisation.

To calculate the intensity of the collagen ring in each pattern, firstly the background scatter was removed. The background varies with radial distance from the centre of the pattern and is caused either by the scattering from non-fibrillar material within the cornea which is evenly distributed around $360^\circ$ and/or by the flare from the backstop which may
not be evenly distributed around 360° since it is impossible to ensure that the backstop remains entirely central on this particular camera. Removing the background separates out the scatter contributed by the fibrillar collagen (fibril scattering intensity). This was done by fitting a simple curve, using a power law, to points either side of the collagen peak in a scattering intensity versus radial distance plot. The resulting curve was then subtracted from the original data (see low-angle data analysis Chapter 2.10.2.3). Due to the uneven flare from the backstop an individual background was fitted and subtracted for each of the 256 radial directions recorded.

After background correction, the integral under the resulting graph was calculated by summation of the 40 intensity values using Simpson's Rule to give a better approximation to the area (Stephenson, 1973) resulting in a 256x1 matrix of intensity values for each diffraction pattern. As with the Mylar data only half the pattern was required, so the first 128 values were added to the last and divided by 2, giving a 128x1 matrix and the noise in the resulting data reduced with a 10-point moving average smoothing. Finally the resulting data was integrated by summation of the 128 intensity values and normalised by division of the corresponding intensity values for the Mylar ring. This background subtracted and normalised value was titled “fibril scattering intensity” for each diffraction pattern.

Fibril scattering can be broken down into scatter from two differing sources, one is “aligned fibril scatter”, and the other is “non-aligned fibril scatter”. Non-aligned fibril scatter is defined as the greatest scattering intensity common to all angles of molecular orientation at any one-measurement point in the tissue, i.e. the minimum in any intensity versus radial distance plot. Non-aligned fibril scatter is related to fibril density via the
background fibrils, which are uniformly distributed through 180°. Aligned fibril scattering intensity represents the remaining fibrils that adopt preferred orientations (Daxer and Fratzl, 1997). In Figure 2.10 the non-aligned and the aligned components of the fibril scattering intensity are shown separately shaded. The two differing sources can be subtracted from one another and displayed separately as in the example shown in Figure 2.11, page 72.

![Graph showing the variation of scattering intensity with the angle of orientation of the fibrils, labelled to illustrate the definition of aligned and non-aligned fibril scattering.](image)

**Figure 2.10:** Graph showing the variation of scattering intensity with the angle of orientation of the fibrils, labelled to illustrate the definition of aligned and non-aligned fibril scattering.
(Figure 2.11)
When studying the orientation of collagen fibrils it is pertinent to point out that the results gained are an approximation since they are derived from the scattering of collagen molecules, not fibrils. Since the exact orientation of the collagen molecules within a single corneal fibril is complex and not accurately known, it is not possible to consider the spread of angles of collagen molecules about the fibril axis. However, if the molecular angular distribution is small then the difference between this and the angular distributions of the fibrils will also be small. In type I collagen the angular spread is known to be small (Fraser et al., 1987).
2.10.3 Swelling of Wounded Corneas

Two excised corneas with their scleral rings attached (12 Aug OD and 22 OS) were thawed from -20°C, to room temperature then the thickness of each cornea was measured on and off the wounded area. Each cornea was placed in a weighing tray and weighed before adding a small volume of distilled water to the tray. When each addition of water was fully absorbed, any remaining water was removed by carefully dabbing the corneas with tissue paper. The corneas were then re-weighed in a dry weighing tray to calculate the amount of water added. The thickness of each cornea was measured on and off the wounded area again. This process was repeated each time adding more water to the corneas. The corneal thickness on and off the wounds was measured using a 3000x ultrasonic pachymeter.

2.10.4 Measurement of Haze

Photographic 35mm slides were taken of each of the rabbits eyes three or four days preceding their enucleation by Dr Charles Cintron. When developed each slide was converted into a 778 x 528 pixel, 24 colour digital image using a flatbed scanner. Using the imaging software running analySiS 3.1 GmbH, a horizontal intensity profile of 1 pixel width was taken across the centre of each eye, bisecting the pupil and the 2mm full thickness wound at the centre of the cornea. The intensity profile was measured in units of greyscale (0-255); a high intensity reading indicating a high degree of reflected and back-scattered light. Firstly, a baseline for each eye was established by measuring the amount of reflected and back-scattered light from a region, which corresponds, to the unwounded cornea plus the pupil. Next, the intensity of reflected and back-scattered light
was measured from a region of the wounded cornea plus the pupil for each eye. The difference in intensity between the two regions for each eye is related directly to back-scattered light from each wound, assuming all other parameters are the same. These relative differences in intensities were termed 'haze'. The degree of haze was calculated by dividing the highest intensity (on wound) by the lowest (off wound) as measured from the intensity profile of each eye.

It is not known if each exposure was within the linear region of the slide film response, therefore constant proportionality between each of the relative differences in intensity, could not be assumed. However, the images appear to display a full range of colours and slide film is very tolerant of over exposure.

2.11 Healing of Partial Thickness Anterior Wounds by Photorefractive Keratectomy In Old and Young Rabbits

2.11.1 Details of Animal Treatments

Photorefractive keratectomy (PRK) took place at St. Thomas' Hospital London and was performed by Anne Patmore; an Omnimed excimer laser (Summit Technology, Boston, Mass.) with a wavelength of 193 nm was used. The pulse energy resulted in a radiant exposure of 180 mJ/cm² at a pulse frequency of 10 Hz. The beam shape was circular with a fixed diameter of 6.0 mm. At least 2 hours before PRK was carried out on the right corneas of seven rabbits fentanyl I/M (0.3ml/kg), diazepan I/V (1mg/kg - 2mg/kg max) and 4 drops topical 1% proparacaine hydrochloride (right eye) was administered to each animal. Four young rabbits and three old rabbits were included in the study.

The animals were placed on a table and a wire lid speculum was used to hold the eye open. The corneal surface was wiped clean of debris using a swab and dried carefully.
The beam was aimed and focused on to the centre of the cornea and the laser activated for 400 pulses (corneal ablation rate approximately 0.25 \( \mu \text{m} \) per pulse). The laser ablated both the epithelium and the stroma. Chloramphenicol antibiotic drops were applied to the eye immediately after PRK surgery and were continued for a further 3 days.

Synchrotron x-ray diffraction (XRD) was used to study molecular and interfibrillar Bragg spacing within the rabbit stroma. Transmission electron microscopy (TEM) was used to study proteoglycan (PG) content and to provide data from which to predict the percentage transmission of visible light through the newly formed tissue of PRK-treated corneas. Corneal haze was quantified using slit-lamp measurements, carried out at St. Thomas' Hospital London.

Details of the four young rabbits, titled Y3, Y8, Y12 and Y20, and the three old rabbits, titled O3, O8 and O20 (where the figure represents the number of months between PRK and death) are given in Table 2.2

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Approx. age at PRK (months)</th>
<th>Approx. weight (Kg)</th>
<th>Date of PRK</th>
<th>Date of death</th>
<th>Treatment of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y3</td>
<td>3</td>
<td>2.5</td>
<td>12/3/96</td>
<td>29/5/96</td>
<td>h, ha</td>
</tr>
<tr>
<td>Y8</td>
<td>3</td>
<td>2.5</td>
<td>12/3/96</td>
<td>12/11/96</td>
<td>h, la, ha, em, cb, rdf, t</td>
</tr>
<tr>
<td>Y12</td>
<td>3</td>
<td>2.5</td>
<td>12/3/96</td>
<td>5/3/97</td>
<td>h, la, ha</td>
</tr>
<tr>
<td>Y20</td>
<td>3</td>
<td>2.5</td>
<td>12/3/96</td>
<td>10/11/97</td>
<td>h, ha, cb</td>
</tr>
<tr>
<td>O3</td>
<td>24</td>
<td>5.0</td>
<td>12/3/96</td>
<td>3/6/96</td>
<td>h, ha</td>
</tr>
<tr>
<td>O8</td>
<td>24</td>
<td>5.0</td>
<td>12/3/96</td>
<td>12/11/96</td>
<td>h, la, ha, em, cb, rdf, t</td>
</tr>
<tr>
<td>O20</td>
<td>24</td>
<td>5.0</td>
<td>12/3/96</td>
<td>10/11/97</td>
<td>h, ha, cb</td>
</tr>
</tbody>
</table>

h = haze, la = low-angle x-rays, ha = high-angle x-rays, em = transmission electron microscopy, cb = Cuprolinic blue staining, rdf = radial distribution function calculation, t = transparency calculation.

Table 2.2: Details of experimental procedures executed on each cornea

Each rabbit was sacrificed with an intravenous injection of Euthatal at 3 months, 8 months, 12 months or 20 months. Once dead the eyes were removed, kept on ice and immediately taken to the CLRC synchrotron facility at Daresbury U.K. for x-ray study or
alternatively frozen in liquid nitrogen-cooled isopentane and kept at -40°C until required. The frozen tissue was allowed to thaw before being placed into an x-ray beam or before fixation for TEM; it has previously been shown that freezing and thawing does not alter the collagen spacings in the cornea (Fullwood and Meek, 1994).

2.11.2 X-ray Diffraction
The defrosted and fresh corneas from young and old PRK treated rabbits were studied first at full thickness and then surgically dissected into 2 to 4 lenticules (semi-thick sections), and the anterior sections were studied. On one occasion, Mr Arun Brahma (a surgeon at the Manchester eye hospital) dissected layers straight from the eyeball of rabbit Y3 and no full thickness study was done. The full thickness and anterior semi-thick sections were mounted between Mylar windows in clear plastic cells, which were airtight to avoid tissue dehydration. The cells containing the corneal tissue were placed in the x-ray beam so that the x-rays passed through the centre of the tissue, along the optical axis.

2.11.2.1 Collection of Low-angle Data
Low-angle patterns were collected at Station 2.1 using a fixed camera length of 6m, an exposure time of 3 minutes and a 3mm x 1mm beam size. Diffraction patterns were taken from the centre of the wounds and collected on a multi-wire gas proportional detector.

2.11.2.2 Collection of High-angle Data
High-angle patterns were collected at Station 7.2, from full thickness corneas, with a camera length of 11cm an exposure time of 3 min and a 200μm diameter collimated beam. Diffraction patterns were collected on a MAR image plate detector.
2.11.2.3 Analysis of Low-angle Data

The interfibrillar Bragg spacing was calculated from the position of the innermost equatorial reflection (Worthington and Inouye, 1985) and rat tail tendon was used to calibrate the data. For the full method see Chapter 2.10.2.3.

2.11.2.4 Analysis of High-angle Data

The intermolecular Bragg spacing was calculated using programs developed on-site at CLRC, Daresbury. The distance in reciprocal space, from the centre of each high-angle diffraction pattern to the first order equatorial reflection corresponds to the intermolecular Bragg spacing (Meek et al., 1991). The method used to measure this distance is similar to that used in low-angle diffraction patterns i.e. a rectangle was placed vertically either side of the backstop, stretching from one side of the pattern to the other. The differing intensities across the pattern were integrated horizontally and plotted as an intensity profile (Figure 2.12). As in the method for low-angle data analysis, the size of the rectangle was adjusted to gain the best intensity profile by balancing noise against broadening of the peak. The centre of the opaque backstop, in the resulting intensity profile, was set to x=0, y=100. Background scatter was removed by subtracting a power law curve fitted to the lowest point closest to the backstop and to the points either side of the peak due to the Mylar windows of the specimen holder (Figure 2.13). To account for the gradual drop in beam intensity between each pattern, normalisation of each profile was achieved by dividing the intensity profile by the height of the Mylar peak assuming the Mylar was a constant thickness across the cell and that this thickness was stable in the x-ray beam. The resulting intensity profile was then converted to real space and calibrated with the 0.305nm spacing in calcite, the average intermolecular Bragg spacing
could then be read from the collagen peak position with a typical accuracy of 10% (Figure 2.14).

Figure 2.12: Graph showing the intensity profile across the centre of a high-angle x-ray diffraction pattern.

Figure 2.13: Graph showing the fitting of a background curve to an intensity profile for a high-angle x-ray diffraction pattern.
2.11.3 Theoretical Transparency of PRK Wounded Corneas

The direct summation of fields method was combined with the specially developed image processing technique to analyse TEM images of the anterior stromal regions of PRK-treated and normal rabbit corneas. The individual fibril positions and radii, fibril number density, and area fraction occupied by fibrils were obtained from the different regions. These data were used in the direct summation of fields method to calculate how the degree of spatial ordering in the fibril positions, scatter strengths (as measured by the interference factor) and light scattering differ across the stroma in the differing samples. It should be stressed that no assumptions were made regarding the homogeneity of the fibril distribution apart from radial symmetry so the presence of any inhomogeneities should be reflected in the calculated interference factor.

Figure 2.14: Graph showing the resulting collagen peak from a processed x-ray diffraction pattern from the intermolecular spacing within collagen molecules.
2.11.4 Slit-Lamp and Haze Measurements (back-scattered light from cornea)

The haze of each PRK-treated cornea was measured before PRK to establish a baseline and was then measured 16, 24, 36 and 570 days after PRK on at least one of both the old and young rabbits. The method of measuring haze is accurately described in Lohmann et al. (1993), a brief description is given below.

A charge coupled device camera (Photonic, EEV, London, Great Britain) was mounted at 40 degrees to the light path of a Haag-Streit slit-lamp and connected via a frame grabber (Wild Vision, Hawk V 10, London) to an Acorn computer (Archimedes 440, London). The slit lamp was positioned with a 20° angle of illumination from the temporal aspect of the cornea; care was taken to keep corneal light reflection out of the central field. To discriminate between reflected and scattered light, linear polarising filters were used. A fixed filter was located inside the CCD between the lens and the detector and a second switchable polarising filter was mounted in the light path distal to the mirror. This filter was divided into two portions with the plane of polarisation being at right angles between the upper and lower parts. By moving the switchable filter up and down the polarisers could be crossed or uncrossed. The images were recorded at 256 x 256 pixel resolution and eight-bit grey scale and stored on 3½ inch floppy discs.

By means of a cursor, areas for analysis could be delineated on the image displayed on a monitor. The images were digitised and then analysed using in-house software and expressed in relative units (grey levels).

The haze measurements were either conducted by myself, under supervision, or in my absence by Anne Patmore, at St Thomas’ Hospital, London.
2.12 Healing of Partial Thickness Anterior Wounds by Photorefractive Keratectomy with Mannose-6-Phosphate

2.12.1 Details of Animal Treatments

In this study 8 young New Zealand white rabbits (typical weight 2.0 kg), titled A1 to A8, underwent excimer laser treatment (PRK) to their right eyes using an Omnimed laser in an identical manner to those described in Chapter 2.11.1, page 75. Post-operatively 4 rabbits received, topically, mannose-6-phosphate (M6P) (Sigma Pharm. UK) to their right eye. A 3 ppm solution of M6P was made in a 2% hypromellose gel to prolong corneal surface exposure, containing 0.01% benzalkonium chloride (a preservative). The four remaining rabbits received an identical viscous solution without M6P to their right eyes. Drops were given hourly for the first 8 hours following PRK and then four times a day or until the epithelium healed over. Slit-lamp examinations and objective measurements of haze were made at 0, 7, 10, 14, 17, 21, 24, 28, 43 and 56 days in the same manner as described in Chapter 2.11.4. The rabbits were killed at 2, 4, 6 and 8 weeks by a lethal injection of Euthatal. All drugs were administered by Anne Patmore, St. Thomas’ Hospital, London.

The wounded area was excised from the cornea using an 8mm trephine. Half of the excised wound was processed for TEM for the study of proteoglycan content, a further quarter was sealed in cling film and placed on ice for 4-12 hours, before its hydration (H) was calculated (weighing was made to an accuracy of 5 x 10^-5 g). The last quarter was frozen in liquid nitrogen cooled isopentane slush and then stored at -40°C. All assessments were made in a masked fashion.
2.13 Chemical Alteration of Corneal Curvature Using the Enzyme Hyaluronidase

2.13.1 Details of Animal Treatments

All experimental procedures were carried out in accordance with the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research. Five New Zealand White rabbits (males), weighing between 1.5 and 2.0 kg, were included in the study. Two of the rabbits were injected with a 1000 IU of ACS-005 enzyme at four sites into the corneal rim of both eyes, a further rabbit was injected with a balanced salt solution (BSS) into the corneal rim of its left eye, leaving the right eye untouched. The two remaining rabbits had no injection in either eye.

The corneas and anterior segments were examined with a slit lamp in a masked fashion by a board-certified ophthalmologist (Dr. C. Kenney, Cedars-Sinai Medical Centre, Los Angeles, USA) at the days 0, 2, 4 and 7, the presence of any resulting haze was assessed by eye. After one week, the animals were sacrificed with intravenous injections of sodium pentobarbital (100mg/kg). All eyes were immediately enucleated and the corneal tissue carefully excised.

The corneas were preserved by either freezing and kept in dry ice, or by incubation in 0.1M phosphate buffered (pH 7.2) 4% paraformaldehyde (Table 2.3). They were then shipped from Cedars-Sinai Medical Centre, Los Angeles, to the Open University Oxford Research Unit, Oxford. The frozen tissue was allowed to thaw before being placed into an x-ray beam or before fixation for TEM.
Table 2.3: Corneal weights and treatments of chemically altered corneas

<table>
<thead>
<tr>
<th>Rabbit/Eye</th>
<th>Treatment</th>
<th>Fixative</th>
<th>Weight after excision (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/R</td>
<td>1000 IU</td>
<td>ACS-005 enzyme</td>
<td>4% Paraformaldehyde</td>
</tr>
<tr>
<td>1/L</td>
<td>1000 IU</td>
<td>ACS-005 enzyme</td>
<td>4% Paraformaldehyde</td>
</tr>
<tr>
<td>2/R</td>
<td>No Injection</td>
<td>4% Paraformaldehyde</td>
<td>Not recorded</td>
</tr>
<tr>
<td>2/L</td>
<td>No Injection</td>
<td>4% Paraformaldehyde</td>
<td>Not recorded</td>
</tr>
<tr>
<td>3/R</td>
<td>1000 IU</td>
<td>ACS-005 enzyme</td>
<td>Frozen</td>
</tr>
<tr>
<td>3/L</td>
<td>1000 IU</td>
<td>ACS-005 enzyme</td>
<td>Frozen</td>
</tr>
<tr>
<td>4/R</td>
<td>Balanced Salt Solution</td>
<td>Frozen</td>
<td>62.2</td>
</tr>
<tr>
<td>4/L</td>
<td>No Injection</td>
<td>Frozen</td>
<td>60.0</td>
</tr>
<tr>
<td>5/L</td>
<td>No Injection</td>
<td>Frozen</td>
<td>Not recorded</td>
</tr>
</tbody>
</table>

2.13.2 X-ray Diffraction

The defrosted corneas and the corneas kept in paraformaldehyde were mounted between Mylar windows in clear plastic cells, which were airtight to avoid tissue dehydration. The cells containing the corneas were placed in the x-ray beam so that the x-rays passed through the centre of the tissue, along the optical axis.

2.13.2.1 Collection of Low-angle Data

Low-angle patterns were collected at Station 2.1 using a fixed camera length of 6m, an exposure time of 3 minutes and a 3mm x 1mm beam size. Scattering patterns were collected on a multi-wire gas proportional detector.
2.13.2.2 Collection of High-angle Data

High-angle patterns were collected at Station 7.2, from full thickness cornea, with a camera length of 11cm an exposure time of 3 min and a 200μm diameter collimated beam. Diffraction patterns were collected on a MAR image plate detector.

2.13.2.3 Analysis of Low-angle Data

The interfibrillar Bragg spacing was calculated from the position of the innermost equatorial reflection (Worthington and Inouye, 1985) and rat tail tendon was used to calibrate the data. For the full method see Chapter 2.10.2.3 page 63.

2.13.2.4 Analysis of High-angle Data

The intermolecular Bragg spacing was calculated from the innermost equatorial reflection (Meek et al., 1991) and calcite was used to calibrate the data. For the full method see Chapter 2.11.2.4 page 78.

2.13.3 Swelling of Chemically Altered Corneas

To investigate the effects of any possible drying artefacts during transportation which might have affected the interfibrillar spacing of the collagen fibrils, and to examine changes in corneal swelling potential caused by the enzyme-treatment, the corneal tissue remaining after a portion had been removed for TEM was equilibrated against a solution of known osmolarity.

Equilibration was carried out by placing the corneas in 14 kDa cut-off dialysis tubing sealing both ends and leaving them for 5 days at 4°C in a solution of 2% polyethylene glycol (20 kDa) containing 0.15M NaCl. For normal, untreated rabbit corneas, this results
in tissue being equilibrated to a level near to physiological hydration with no detectable loss of interfibrillar constituents (Meek and Leonard, 1993; Fullwood, 1994).

2.13.4 Theoretical Transparency of Chemically Altered Cornea

A first approximation as to what occurs after the removal of hyaluronidase-sensitive proteoglycans from the cornea was made by applying the direct summation of fields method to predict the effect of this treatment on light scattering. For the calculation a nominal corneal thickness of 0.3 mm and the normal rabbit refractive indices for fibrils ($n_f=1.416$) and ground substance ($n_g=1.357$) (Leonard and Meek, 1997) were assumed.

For the full methods see Transparency Calculations Chapter 2.7, page 54.
3. Results and Discussions

3.1 Full Thickness Wounds

3.1.1 Haze

A significant decrease in the relative intensity of haze was found as the wound healed (Figure 3.1). All eyes were used to measure haze except 20 OS because the intensity profile across the wound peaked out above the maximum greyscale value of 255 due to a reflection from the camera flash. The photographs of the wounded eyes *in vivo* in the first column of Figure 3.2 show a gradual increase in the transparency of the wounded area as it heals over time, although at 16 months the wounds were still not completely clear. The slit-lamp images in the third column of Figure 3.2 indicate that the thickness of the wound and surrounding corneal tissue remains similar throughout the healing process. The centre column shows horizontal intensity plots across the image of each eye, allowing quantitative measurements of haze to be calculated.

Figure 3.1: Box plot showing the decrease in mean haze values following full thickness wounds (*p*=0.02 Kruskal-Wallis test).
### 3.1 FULL THICKNESS WOUNDS

<table>
<thead>
<tr>
<th>Rabbit number and eye (od = left os = right)</th>
<th>Horizontal intensity across eye and wound centre</th>
<th>Slit-lamp images of cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 month wounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 od</td>
<td><img src="image" alt="Horizontal profile (pixels)" /></td>
<td><img src="image" alt="Slit-lamp image" /></td>
</tr>
<tr>
<td>20 os</td>
<td><img src="image" alt="Horizontal profile (pixels)" /></td>
<td><img src="image" alt="Slit-lamp image" /></td>
</tr>
<tr>
<td>22 od</td>
<td><img src="image" alt="Horizontal profile (pixels)" /></td>
<td><img src="image" alt="Slit-lamp image" /></td>
</tr>
<tr>
<td>22 os</td>
<td><img src="image" alt="Horizontal profile (pixels)" /></td>
<td><img src="image" alt="Slit-lamp image" /></td>
</tr>
<tr>
<td>23 od</td>
<td><img src="image" alt="Horizontal profile (pixels)" /></td>
<td><img src="image" alt="Slit-lamp image" /></td>
</tr>
</tbody>
</table>
### 3.1 FULL THICKNESS WOUNDS

<table>
<thead>
<tr>
<th>Date</th>
<th>Image</th>
<th>Graph</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 os</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="graph1.png" alt="Graph" /></td>
<td>Horizontal profile (pixels)</td>
</tr>
<tr>
<td><strong>10 month wounds</strong></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="graph2.png" alt="Graph" /></td>
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</tr>
<tr>
<td>12 oct od</td>
<td><img src="image3.png" alt="Image" /></td>
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</tr>
<tr>
<td>12 oct os</td>
<td><img src="image4.png" alt="Image" /></td>
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<td>Horizontal profile (pixels)</td>
</tr>
<tr>
<td>21 od</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="graph5.png" alt="Graph" /></td>
<td>Horizontal profile (pixels)</td>
</tr>
<tr>
<td>21 os</td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="graph6.png" alt="Graph" /></td>
<td>Horizontal profile (pixels)</td>
</tr>
<tr>
<td>12 month wounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>--</td>
<td>---</td>
<td></td>
</tr>
<tr>
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<td><img src="graph1" alt="Graph" /></td>
<td><img src="image2" alt="Image" /></td>
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</tr>
<tr>
<td>12 aug od</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image3" alt="Image" /></td>
<td><img src="graph2" alt="Graph" /></td>
<td><img src="image4" alt="Image" /></td>
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<tr>
<td>12 aug os</td>
<td></td>
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</tr>
<tr>
<td><img src="image5" alt="Image" /></td>
<td><img src="graph3" alt="Graph" /></td>
<td><img src="image6" alt="Image" /></td>
<td></td>
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<tr>
<td>16 month wounds</td>
<td></td>
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<td></td>
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<td><img src="image8" alt="Image" /></td>
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<tr>
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<tr>
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<td><img src="graph5" alt="Graph" /></td>
<td><img src="image10" alt="Image" /></td>
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<td><img src="graph6" alt="Graph" /></td>
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</tr>
<tr>
<td>6 od</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td><img src="graph7" alt="Graph" /></td>
<td><img src="image14" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>
3.1 FULL THICKNESS WOUNDS

3.1.2 X-ray Diffraction

3.1.2.1 Low-angle

Low-angle diffraction patterns from the wound gave information on the full thickness average interfibrillar Bragg spacing and the spread of these spacings, the average fibril diameter and the full thickness fibril diameters spread. The corneas swelled during transit and since interfibrillar spacing depends on corneal hydration (Goodfellow et al., 1978) the hydration of the enucleated corneas at the time of their exposure to x-rays was estimated. This estimation was accomplished by comparing pachymetry readings between the original/in vivo corneal thickness (off-wound) with the final/ex vivo corneal thickness (off-wound) shortly before exposure to the x-ray beam. When expressing hydration as a ratio of in vivo to ex vivo off-wound thickness, any value over 0.7 was deemed acceptable i.e. not too swollen and included in the study (Table 3.1). The hydration (H) of the normal tissue was calculated to be 2.8 for Norm OD and 3.6 for Norm OS. Measurements of interfibrillar Bragg spacing from the acceptable corneas indicated that the spacing at the wound centre was much smaller than in normal tissue at all ages of wound healing. However, the spacing at the wound centre was shown to steadily increase as the wound healed (Figure 3.3). This increase was shown to be
significant between 5 and 16 months (p=0.02, student t-test), whereas there was no significant increase in the interfibrillar Bragg spacing in the normal tissue surrounding the wound (1mm from wound centre) (p=0.46, student t-test) see Figure 3.4. The spread of interfibrillar spacings can be compared by measuring the ratio of the low-angle lattice interference function peak height to peak width at half height. Figure 3.5 indicates that there was a trend for the spread of interfibrillar spacings to decrease as the wounds healed (indicated by a slight increase in the ratio of peak height to peak width) but not to any significance. The normal remained almost twice the value of the wounded corneas throughout the healing process.

### Table 3.1: Details of all corneas with full thickness wounds.

<table>
<thead>
<tr>
<th>Age of Wound (months)</th>
<th>Code</th>
<th>In vivo on-wound thickness (mm)</th>
<th>In vivo off-wound thickness (mm)</th>
<th>Ex vivo average off-wound thickness (mm)</th>
<th>Ratio of In vivo thickness /Ex vivo thickness off-wound</th>
<th>Suitable for interfibrillar spacing comparisons if ratio is &gt; 0.7 (Y or N)</th>
<th>The final destination of each eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20OD</td>
<td>0.205</td>
<td>0.334</td>
<td>0.496</td>
<td>0.673</td>
<td>N</td>
<td>TEM</td>
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<tr>
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<td>20OS</td>
<td>0.276</td>
<td>0.322</td>
<td>0.585</td>
<td>0.550</td>
<td>N</td>
<td>Frozen</td>
</tr>
<tr>
<td>5</td>
<td>23OD</td>
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<td>0.335</td>
<td>0.327</td>
<td>1.024</td>
<td>Y</td>
<td>TEM</td>
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<tr>
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<td>23OS</td>
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<td>TEM</td>
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<tr>
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<td>TEM</td>
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<tr>
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<td>0.334</td>
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<td>N</td>
<td>TEM</td>
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<tr>
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<td>0.353</td>
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<td>0.578</td>
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<td>TEM</td>
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<td>21OD</td>
<td>0.237</td>
<td>0.343</td>
<td>0.359</td>
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<td>TEM</td>
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<td>0.354</td>
<td>0.635</td>
<td>0.557</td>
<td>N</td>
<td>Frozen</td>
</tr>
<tr>
<td>12</td>
<td>12Au gOD</td>
<td>0.313</td>
<td>0.309</td>
<td>0.598</td>
<td>0.516</td>
<td>N</td>
<td>TEM</td>
</tr>
<tr>
<td>12</td>
<td>12Au gOS</td>
<td>0.300</td>
<td>0.325</td>
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<td>Swollen</td>
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<tr>
<td>16</td>
<td>20D</td>
<td>0.282</td>
<td>0.369</td>
<td>0.484</td>
<td>0.762</td>
<td>Y</td>
<td>TEM</td>
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<tr>
<td>16</td>
<td>20S</td>
<td>0.270</td>
<td>0.342</td>
<td>0.536</td>
<td>0.638</td>
<td>N</td>
<td>Frozen</td>
</tr>
<tr>
<td>16</td>
<td>60D</td>
<td>0.322</td>
<td>0.379</td>
<td>0.516</td>
<td>0.734</td>
<td>Y</td>
<td>TEM</td>
</tr>
<tr>
<td>16</td>
<td>60S</td>
<td>0.351</td>
<td>0.361</td>
<td>0.621</td>
<td>0.581</td>
<td>N</td>
<td>TEM</td>
</tr>
<tr>
<td>NA</td>
<td>Norm OD</td>
<td>NA</td>
<td>0.312</td>
<td>0.640</td>
<td>0.480</td>
<td>H=2.8*</td>
<td>TEM/Dried</td>
</tr>
<tr>
<td>NA</td>
<td>Norm OS</td>
<td>NA</td>
<td>0.309</td>
<td>0.550</td>
<td>0.561</td>
<td>H=3.6*</td>
<td>TEM/Dried</td>
</tr>
</tbody>
</table>

*H=Hydration
3.1 FULL THICKNESS WOUNDS

Corneal collagen fibril diameters do not depend on corneal hydration unless the corneas are desiccated (Meek et al., 1991; Fratzl and Daxer, 1993) which none of them were. Therefore, all of the corneas were included in the measurements of fibril diameter and in the fibril diameter spread calculations. The average fibril diameter did not significantly change within the scars as shown in Figure 3.6 (p=0.15 Kruskal-Wallis test). The spread of fibril diameters in different specimens can be compared by measuring the ratio of peak height to peak width at half height, of the low-angle subsidiary equatorial reflections. Figure 3.7 shows that there is a trend for the fibril diameter spread to decrease in the centre of the wound (as indicated by a slight increase in the ratio of peak height to peak width), as it heals, but not to any significance. The graph also shows a larger change in fibril diameter spread between the wound centre and the tissue (from 1mm onwards) surrounding the scar in all ages of wound healing.

Figure 3.3: Measurements of interfibrillar Bragg spacing from 'acceptable corneas' across full thickness wounds.
3.1 FULL THICKNESS WOUNDS

Figure 3.4: Box plots showing differences between interfibrillar Bragg spacing at 5 and 16 months in the wound centre (left) and outside the wound (right).

Figure 3.5: A box plot showing no significant change in the spread of interfibrillar Bragg spacings over 5, 10, 12 and 16 months from 'acceptable corneas'. The mean spread of interfibrillar spacings between 5 and 16 months is significantly lower than that of the normal (0.039 student t-test).
Figure 3.6: A box plot showing no significant change in the average fibril diameter at 5, 10, 12 and 16 months.

Figure 3.7: Fibril diameter spread. Ratio of peak height to peak width at half height increases during wound healing within the full thickness wound centres.
3.1.2.2 High-angle

**Single line transects**

The x-ray diffraction patterns form the single line transects across the centre of four 5 month (23os, 22od, 20od and 20os), two 10 month (12octod and 21od) and four 16 month (6od, 6os, 2od and 2os) wounds express two different results.

Firstly, the diffraction patterns gained from points at the wound edge are of a higher intensity than at the wound centre. The difference in intensity between the edge and the wound centre can be seen in plots of fibril scatter intensity versus distance across wound (Figure 3.8). This indicates the presence of less fibrillar scattering material at the wound centre than at the wound edge, since, as stated in the methods section, molecular spacing would depend on intermolecular hydration; a decrease in hydration would increase the radial position of the collagen diffraction ring. No such increase was noted. Sample 21os was scanned across several times (see multiple line transects, page 102) and the collagen peak from the averaged intensity profiles for each transect was found at the same point in reciprocal space (Figure 3.9). This result is supported by Rawe et al. (1994) who previously showed that molecules within a fibril from a 2mm full thickness wounded cornea had the same mean spacing as normal tissue.

Secondly, in the plots of aligned fibril scattering intensity versus distance across wound, there are generally two distinct peaks found approximately at 90° to the transect, on either side of the wound. These peaks indicate a high degree of preferred fibril orientation at the edges of wounds from all ages. The orientation of the collagen fibrils was in most cases tangential (10 out of 16) to the direction of the transect across the wound, as shown in the second column of Figure 3.8.
### Total fibril scattering intensities

<table>
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<tr>
<th>Angle of orientation (degrees)</th>
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<th>45</th>
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### Aligned fibril scattering intensities

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<td></td>
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</tbody>
</table>

---

3.1 FULL THICKNESS WOUNDS

---
3.1 FULL THICKNESS WOUNDS

23°

10 month wounds

21°

16 month wounds

2öd
3.1 FULL THICKNESS WOUNDS

Figure 3.8: The fibril scattering intensity (left column) and its corresponding proportion of aligned scattering intensity (right column) across full thickness wounded corneas.
3.1 FULL THICKNESS WOUNDS

To calculate the difference in the relative amounts of total aligned and total non-aligned fibrillar material across the whole of each wound, as a function of wound age, the total aligned and non-aligned fibril scatter were both divided by the total fibril scatter. The results are displayed in Table 3.2.

<table>
<thead>
<tr>
<th>Rabbit/wound age (months)</th>
<th>Total fibril scatter</th>
<th>Total aligned fibril scatter</th>
<th>Total non-aligned fibril scatter</th>
<th>Aligned scatter / Total fibril scatter ($R_1$)</th>
<th>Non-aligned scatter / Total fibril scatter ($R_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20od/5</td>
<td>23016.74</td>
<td>7977.36</td>
<td>15039.37</td>
<td>0.346</td>
<td>0.653</td>
</tr>
<tr>
<td>20os/5</td>
<td>42045.11</td>
<td>11015.82</td>
<td>30986.58</td>
<td>0.262</td>
<td>0.736</td>
</tr>
<tr>
<td>22od/5</td>
<td>56582.00</td>
<td>14407.01</td>
<td>42175.00</td>
<td>0.254</td>
<td>0.745</td>
</tr>
<tr>
<td>23os/5</td>
<td>167960.70</td>
<td>35032.05</td>
<td>132928.70</td>
<td>0.343</td>
<td>0.791</td>
</tr>
<tr>
<td>21os/10</td>
<td>341714.59</td>
<td>79619.50</td>
<td>256627.65</td>
<td>0.233</td>
<td>0.751</td>
</tr>
<tr>
<td>12octod/10</td>
<td>69615.29</td>
<td>11054.88</td>
<td>58560.41</td>
<td>0.158</td>
<td>0.841</td>
</tr>
<tr>
<td>2od/16</td>
<td>94113.94</td>
<td>15976.25</td>
<td>78114.57</td>
<td>0.169</td>
<td>0.830</td>
</tr>
<tr>
<td>2os/16</td>
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<td>32141.34</td>
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<td>0.764</td>
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<tr>
<td>6od/16</td>
<td>111658.00</td>
<td>24476.96</td>
<td>87093.24</td>
<td>0.219</td>
<td>0.780</td>
</tr>
<tr>
<td>6os/16</td>
<td>82906.44</td>
<td>11749.09</td>
<td>71157.35</td>
<td>0.142</td>
<td>0.858</td>
</tr>
</tbody>
</table>

Table 3.2: Relative amounts of aligned and non-aligned scatter compared to total fibril scatter, from single-line transects across each wound.

Figure 3.9: Averaged intensity profiles for each transect across the wound of 21os. Collagen peaks found between 1.58-1.60 nm.
Figure 3.10 below depicts the values of R1 and R2 when plotted against age of wound and a line fitted to each set of points. The results indicate a significant decrease in the relative amount of aligned fibrillar material (p=0.02 student t-test) and an increase in the relative amount of non-aligned fibrillar material (p=0.08 student t-test) between 5 and 16 months, as the wound heals.

Wound thickness as measured either by pachymetry in vivo (Table 3.1), or by the amount of total fibrillar collagen across the centre measured by total fibril scattering intensities (Table 3.2) are compared, both indicate no significant change as a function of wound healing. The standard deviations and errors for these measurements are shown in Figure 3.11.

Figure 3.10: To demonstrate an increase for R1 and a decrease for R2 as a function of wound healing, straight lines were fitted to the values of total aligned scatter / total fibril scatter (R1) and total non-aligned scatter / total fibril scatter (R2). Although there is no reason to suppose the changes are linear, the lines of 'best fit' are plotted to indicate the trends in the data points.
3.1 FULL THICKNESS WOUNDS

Figure 3.11: The mean total fibril scattering intensity across each wound at differing ages (graph on left) and the pachymeter readings of the wound centre *in vivo* (graph on right) both indicate no significant change as the wound heals.

Multiple line transects

The results of the normalised and background-subtracted intensity measurements around the circular diffraction patterns from collagen are shown in Figure 3.12. Because all of the measurements were made on full-thickness material, they represent an integration of orientations through the thickness of the tissue. Each graph in Figure 3.12 represents a single horizontal transect through the 10 month wound of rabbit 21os. Rabbit 21os was chosen because of its round shaped wound and its clear demonstration of fibril orientation from the single line transect measurement. Starting at the inferior/temporal wound edge, each transect is separated by 0.4 mm and nine transects were required to cover the entire wound. The y-axis represents the distance along the transect relative to the vertical origin (0) (see Figure 2.5 page 62) at the temporal edge of the wound. The x-axis plots the angle of orientation of scattering material relative to a vertical datum.
3.1 FULL THICKNESS WOUNDS
3.1 FULL THICKNESS WOUNDS

Figure 3.12: The contour values are the same in each graph and show the scattering intensity at any given angle for points along each of the nine transects. Transect 1 and 2 show an intensity peak moving through 180 degrees from one side of each transect to the other, indicating a gradual change in fibril orientation at the base of the wound. The intensity peak starts at 0/180° then 135°, 90°, 45° and back to 0/180° at the end of the transect. As the position of the transect moves towards the centre of the wound, transect 3 shows a similar intensity peak at 0/180° at the wound edges but less tangential orientation in the middle. Transect 4 corresponds to the middle of the wound with a high intensity centring on 0° and 180°, with none at 90° i.e. large amount of tangentially orientated fibrils, at either side of the wound. Transect 5 shows the return of an intensity peak moving from 0/180° through 90° back to 0/180° across the wound, with the wound edges having the highest degree of fibrillar orientation at 0/180°. Transects 6 and 7 both show an even spread of orientated fibrils, similar to transect 2 and 3 except in the opposite direction so as to bring the orientation of the fibrils around into a complete circle. Transects 8 and 9 continue to indicate the circular orientation of fibrils within the wound.

If the intensity and orientation of the aligned material is measured at approximately 1 mm along each transect of Figure 3.12, a picture can be built up displaying the amount of fibrils in any one direction and their relationship to the shape of the wound. With a resolution of 3.6mm x 4.0mm, the result of this is shown in Figure 3.13. From Figure 3.13 it can be clearly seen that the fibrils appear to be at different angles in and around the wounded area forming a pattern of concentric circles. By assigning arbitrary values to the intensity measurements i.e. 3 (high), 2 (medium) and 1 (low), and then summing the values around each circle, a higher total is achieved towards the edge of the wound. This indicates an increase in the number of fibrils towards the edge of the wound compared to the centre.
3.1 FULL THICKNESS WOUNDS

3.1.3 Swelling of Tissue

After the addition of measured amounts of de-ionised water to both the 5 month (22 OS) and 12 month (12 Aug OS) corneas, and measuring any change in thickness with the pachymeter it was found that the wounded tissue did not swell, whereas the tissue surrounding the wounds swelled considerably (Figure 3.14).

Figure 3.13: Orientation and intensity of collagen molecules along each transect (x) at positions across the wound (y). Intensity: High (≡), Medium (—) and Low (''').
3.1 FULL THICKNESS WOUNDS

3.1.4 Electron Microscopy

3.1.4.1 Standard Fixation

At 5 months (23OS and 23OD), the newly formed collagen fibrils differ greatly from the control (Figure 3.15). Instead of forming normal lamellae with the fibrils running parallel to each other with a regular spacing between them, the fibrils appear to be entwined with each other, irregularly spaced and non-parallel, forming a type of mesh (Figure 3.16). The fibrils also vary in diameter to a large degree (ranging from approximately 20 to 70 nm) (Figure 3.17). At 12 months, there were still large differences in fibril diameter but the fibrils were becoming more axially aligned and starting to form lamellae (Figure 3.18). At 16 months, the diameter spread was still acute, and lamellae were no better formed and the collagen fibrils were no more regularly aligned than at 12 months.

The observed differences between fibril diameters in electron micrographs supports the large spread of fibril diameters measured from XRD results (Figure 3.7 page 95).
Figure 3.15: Normal corneal stroma. The fibrils are gathered within lamellae, regularly aligned and of uniform diameter. Scale bar = 200nm.

Figure 3.16: 5 month wound. The fibrils have no regular axial alignment. Scale bar = 200nm.
Figure 3.17: 5 month wound. There is a wide distribution of fibril diameters and no obvious structuring of fibrils into lamellae. Scale bar = 200nm.

Figure 3.18: 12 month wound. The distribution of fibril diameters is still visibly wide but the grouping of similarly orientated fibrils into lamellae is present. Scale bar = 200nm.
3.1.4.2 Cuprolinic Blue Staining

Using Cuprolinic blue stain, larger GAG precipitates than normal could be seen in the mid-stroma up to 16 months after wounding (Figures 3.19 and 3.20), these precipitates maintained a normal shape. Very large GAG precipitates of abnormal shape were constantly visible near to the Descemet's membrane throughout the healing period. These precipitates could be seen alongside more normally shaped and sized GAGs (Figure 3.21). These two distinct populations of GAGs were more clearly seen in Cuprolininc blue stained tissue which had not been post-stained with uranyl acetate and lead citrate (Figure 3.22).

![Figure 3.19: Cuprolinic blue staining of normal corneal mid-stroma. The fine filaments of normal GAGs are visible in-between and perpendicular to the collagen fibrils (arrows). Scale bar = 200nm.]
3.1 FULL THICKNESS WOUNDS

Figure 3.20: In the disorganised mid-stroma of a 12 month wound the GAGs appear slightly thicker and occasionally longer than normal. Scale bar = 200nm.

Figure 3.21: Adjacent to the Descemet’s membrane very large GAGs are visible alongside a separate population of more normally sized GAGs as seen in the mid-stroma. From a 16 month wound. Scale bar = 200nm.
3.1 FULL THICKNESS WOUNDS

3.1.4.3 Image Analysis

Image analysis of the anterior and mid-stroma of 5 and 16 month old full thickness wounds showed that, there was no significant difference between the 5 and 16 month wounded GAG area fractions (similar to the thickness measurement results seen in Figure 3.11). However, when these results were pooled together the combined 5 and 16 month wounds from anterior and mid-stroma, had a smaller and highly significant total glycosaminoglycan area fraction than normal cornea (Figure 3.23).

Figure 3.22: A micrograph of tissue near to the Descemet's membrane stained with Cuprolinic blue but not counter stained with uranyl acetate and lead citrate, clearly showing the different populations of GAG precipitates in a 16 month wound. Scale bar = 200nm.
3.1 FULL THICKNESS WOUNDS

After 5 to 16 months of wound healing, twelve of the sixteen wounded corneas showed no significant increase in either the total amount of collagen, pachymetry readings of \textit{in vivo} thickness or GAG area fractions of the wound, despite these values being significantly lower than in normal unwounded tissue. Although none of these parameters increased or returned to normal other parameters changed with time. The transparency of the wounds (judged by haze measurements) significantly improved over time and during this same period electron micrographs indicated an increase in the formation of lamellae and a more ordered arrangement of the collagen fibrils. During the healing period x-ray diffraction patterns of the wounds indicated a decrease in fibril diameter spread, an increase in interfibrillar Bragg spacing and, in support of the findings of Rawe et al. (1994), a decrease in the spread of interfibrillar Bragg spacings (although unlike the results in Rawe et al. the spread did not return to normal). XRD patterns also indicated that the amount of aligned fibrillar material was significantly reduced while the amount of non-aligned material was increased proportionally.
3.1 FULL THICKNESS WOUNDS

The shape of the cornea reflects the balance between intraocular pressure, corneal thickness and the tissues 'stiffness' which would depend on the amount and arrangement of collagen, which acts as a scaffold, (Philips and Quick, 1960; Buzzard, 1992). The shape of the wound could also be described in the same terms. Therefore, if it is assumed that the newly synthesised collagen fibrils try to follow exactly the same arrangement as the fibrils before the wound occurred, that is, in parallel straight lines across the cornea spread evenly over 360° with no obvious preferred orientation, then as the new fibrils attempt to span the wound the intraocular pressure behind the cornea pressing against the thinner, less-stiff tissue of the wound would push the fibrils towards the wound edge. This would explain the high degree of circular fibril alignment at the wound edge when compared to the wound centre. It would be difficult for a fibril to cross the centre of the wound because such a process faces resistance. The resistance would be caused by a necessary change in the relative orientation between adjacent fibrils in order to support the adaptation of the wounded corneal shape to the biomechanical requirements for reduced corneal thickness (as measured by fibril scattering intensities) and given intraocular pressure. This reasoning would also explain the observed decrease in aligned material with the proportional increase in non-aligned material and interfibrillar Bragg spacing as the wound healed, since as the wound becomes 'stiffer' with time (due possibly to increased lamellae formation) there is less force being exerted on the fibrils pushing them to the side of the wound (aligned). This in turn would allow the fibrils to straighten becoming more evenly distributed across the wound (non-aligned) and with an increased but normal interfibrillar Bragg spacing. However, it is not possible to determine the degree to which the fibrils are either curling around or are straight and tangential to
the wound edge because of the relatively large beam size, making it impossible to track individual fibrils. Therefore, the proposed reasoning as to what is happening to the fibrils at the wound edge, as described above, can not be proven from the study of high or low-angle x-ray diffraction patterns.

In pathological conditions such as keratoconus in which the apex of the cornea is much thinner than normal, collagen fibril orientation has been shown to change dramatically (Daxer and Fratzl, 1997) and a visibly poor lamella structure within this area has been suggested as a contributory factor.

The population of large GAG precipitates seen near Descemet’s membrane has previously been shown to be of a dermatan sulphate PG (Hassel et al., 1983; Cintron et al., 1990) although they have not previously, to the best of the author’s knowledge, been reported in wounds of this age (up to 16 months). As in the developing cornea, DSPG is the major PG in opaque wounded tissue and has a higher charge density in comparison with the smaller quantities and low-sulphation of KSPG in this tissue (Cintron, 1989). Therefore, wounded corneas with smaller quantities of KSPG and less collagen, but with a similar corneal thickness to normal cornea may explain the lower area fractions of Cuprolinic blue stained GAGs seen in 5 and 16 month wounds. PGs play an important role in corneal hydration (Scott, 1985; Hodson, 1997) and if it is considered that KSPGs from adult corneas have more swelling ability (potential) but less water-retentive power than DSPGs (Cintron, 1989) then wounded tissue which contains an increased DSPG concentration and a decreased KSPG concentration may be swollen to its maximum whereas the tissue surrounding the wound which contains the normal concentration of
these PGs can swell freely. This would explain the lack of swelling within the wound as measured by pachymetry after the addition of measured amounts of water.

It has been assumed that GAGs within wounded tissue are being fixed and stained in situ by Cuprolinic blue and glutaraldehyde to the same extent as GAGs found in normal tissue i.e. GAGs within wounded and normal tissue have the same affinity to their extracellular matrix. However, the lower GAG content, as measured by image analysis, could be attributed to some of the GAGs being moved from their original place either by being washed from the cornea during fixation or during storage following excision. A corollary to this would be large aggregates of dissolved GAGs accumulating and precipitating near to the endothelium. This would not, however, readily explain the larger but normally shaped GAGs found in the anterior and mid-stroma of the wounded corneas.

In conclusion, the ability of full thickness 2mm diameter wounded tissue to heal was proven by the increase in transparency with time (as measured by a decrease in back-scattered light). Transparency has previously been shown to depend on the regular arrangement of the collagen fibrils within the cornea (Maurice 1957; Benedek 1971; Twersky, 1975; Freund et al., 1986). Transparency in wounded corneas has also been shown to increase with a concurrent increase in the regular arrangement of the collagen fibril spacing (Rawe et al., 1994). In this experiment, a method has been postulated whereby the regular alignment of collagen fibrils within healing corneas comes about. That is, with time lamellae start to form and the newly formed fibrils move from the edge of the wound and spread out attempting to cover the wound evenly, leading to an increase in transparency.
These wounds do not swell appreciably above their in vivo hydration due to the wound containing less collagen and GAGs. The possibility of abnormal levels of dermatan sulphate, which have high water avidity but low swelling pressure (Cintron, 1989), may also contribute to the lack of increased swelling.

3.2 PRK Wound Response in Old and Young Rabbits

3.2.1 Haze

The haze values for the old PRK-treated corneas were consistently higher than the young PRK-treated corneas throughout the experiment, apart from the very last data points. Neither the young nor the old rabbits returned to their original haze values before PRK. The haze measurements showed that both the old and young rabbits peaked after 24 days. At 24 days post-PRK, haze value in the old rabbits was higher at 135.35 ± 28.13 than that in the young rabbits, which was 103.71 ± 21.24 (see Figure 3.24).

Figure 3.24: Haze values for both young and old rabbits peak around 1 month. Despite a higher value for old rabbits compared to young, the difference is on the negative side of significant (p=0.06 student t-test).
3.2.2 Electron Microscopy and Image Analysis

3.2.2.1 Standard Fixation

Electron micrographs were taken of the central anterior section of the rabbit cornea, just below the epithelium. The PRK-treated corneas of young rabbits (Y3 and Y8) and old rabbits (O3 and O8) showed a discontinuous basal lamina of differing thickness, a large amount of irregularly orientated collagen fibrils and no distinct lamellae within the first 5μm. Further into the rabbit stroma (15μm) the tissue gradually started to return to normal (Figures 3.25 and 3.26). This differed sharply with the controls (both old and young), which showed a continuous basal lamina of an even thickness, fibrils with a well ordered spatial arrangement and a high degree of axial alignment, separated into defined lamellae (Figure 3.27).

Figure 3.25: Young rabbit with 8 month wound. The basal lamina (arrow) is wavy and of irregular thickness, it is discontinuous in places (not shown). Scale bar = 500nm.
Figure 3.26: Old rabbit with 8 month wound. Similar to figure 3.25 the basal lamina shows a wavy membrane of differing thickness and discontinuity (arrow). Large interfibrillar spaces are also visible (Δ). Scale bar = 500nm.

Figure 3.27: Control cornea. The basal lamina is continuous, smooth and of a constant thickness (arrow). The fibrils have regular arrangement and are ordered into lamellae. Scale bar = 500nm.
The fibril radii in both old and young PRK-treated corneas appeared similar in diameter to the control fibrils. This observation was supported by results gained by image analysis (Table 3.3 and Figure 3.28).

<table>
<thead>
<tr>
<th>Cornea</th>
<th>N</th>
<th>Mean Diameter (nm)</th>
<th>Confidence Interval (95%)</th>
</tr>
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<td>Y8_{PRK}</td>
<td>905</td>
<td>39.22</td>
<td>±2.43</td>
</tr>
<tr>
<td>Y8_{CONT}</td>
<td>955</td>
<td>39.51</td>
<td>±2.58</td>
</tr>
<tr>
<td>O8_{PRK}</td>
<td>356</td>
<td>39.80</td>
<td>±3.38</td>
</tr>
<tr>
<td>O8_{CONT}</td>
<td>821</td>
<td>40.43</td>
<td>±2.41</td>
</tr>
</tbody>
</table>

Table 3.3: No significant difference was measured between the mean fibril diameters of PRK and control corneas from rabbits Y8 and O8.

Figure 3.28: The fitted curves for each sample peak around 40nm, the area under each curve represents the respective sample number (N).
The most obvious age related difference in the electron micrographs was the presence of amorphous, granular bodies found throughout the stroma of old rabbits (Figure 3.29). These deposits were similar in size and amount in both PRK-treated corneas and their controls but were present to a much lower extent in the young.

Figure 3.29: Old rabbit control. Large patches of amorphous, heavily stained bodies are interspersed within the stroma, between the collagen fibrils (arrows). Scale bar = 500nm.

3.2.2.2 Cuprolinic Blue Staining

The anterior stromas of young (Y8 and Y20) and old (O8 and O20) rabbits following PRK were stained using Cuprolinic blue and the differences in the staining of GAG amount and size were quantified using image analysis as described in the methods section. The results are shown below in Table 3.4.
The image analysis results indicated that there was no significant difference in the percentage of area covered by filaments, P, between each of the control and PRK-treated corneas measured, except O20, and likewise between the young and the old. Despite this, P, increased steadily over time for both control and PRK-treated corneas, (until rabbit O8 at approximately 32 months) (Figure 3.30). There was also a general increase in mean particle size after PRK (Figures 3.31 and 3.32), except in rabbit O20, although the measurement errors were larger than any difference found (except Y20). Mean particle size was also seen to increase as age increased when comparing Y8 with Y20 and O8 with O20.
3.2 PRK WOUND RESPONSE IN OLD AND YOUNG RABBITS

Figure 3.30: The percentage area of GAGs (as measured by image analysis) increases until 32 months, in both control (normal) and PRK-treated corneas.

Figure 3.31: Old rabbit control stained with Cuprolinic blue. GAGs are visible between fibrils, and all have a similar shape. Scale bar = 200nm.
3.2.3 X-ray Diffraction

3.2.3.1 Low-angle

Data from the low-angle camera gave information on the average interfibrillar Bragg spacing. The control and PRK-treated corneas of all the rabbits were included in the x-ray study except rabbits Y20 and O20 which were clinically clear at the time of enucleation and were therefore not included.

The corneas from rabbits Y3, and O3 did not yield x-ray diffraction patterns strong enough to draw any meaningful conclusions from because they were too dry at the time of exposure to the x-ray beam. However, diffraction patterns were obtained from the anterior stroma of the remaining rabbits Y8, O8 and Y12.
The lattice interference function peaks from the intensity profiles of Y8, O8 and Y12 showed an increase in the interfibrillar Bragg spacing for each of the PRK-treated corneas when compared to their respective controls (Y8\text{CONT} = 57\text{nm} \pm 1.71, Y8\text{PRK} = 61\text{nm} \pm 1.83; O8\text{CONT} = 51\text{nm} \pm 1.53, O8\text{PRK} = 55\text{nm} \pm 1.65; Y12\text{CONT} = 52\text{nm} \pm 1.56, Y12\text{PRK} = 59\text{nm} \pm 1.77). The spread of the lattice interference function peaks was also measured by dividing the full height of each peak by its full width at half height. For Y8 this ratio equalled 1.53\text{CONT} and 1.28\text{PRK} (Figure 3.33), for O8 it equalled 1.65\text{CONT} and 0.96\text{PRK} (Figure 3.34) and for Y12 it equalled 2.39\text{CONT} and 1.5\text{PRK} (Figure 3.35).

![Graph](image.png)

Figure 3.33: Low-angle x-ray diffraction profiles from the central anterior stroma of Y8\text{PRK} and Y8\text{CONT} (full thickness).
3.2 PRK WOUND RESPONSE IN OLD AND YOUNG RABBITS

Figure 3.34: Low-angle x-ray diffraction profiles from the central anterior stroma of O8_{PRK} and O8_{CONT} (full thickness).

Figure 3.35: Low-angle x-ray diffraction profiles from the central anterior stroma of Y12_{PRK} and Y12_{CONT} (full thickness).
3.2.3.2 High-angle

Data from the high-angle camera gave information on the average intermolecular Bragg spacing within the collagen fibrils. The intermolecular spacing of the PRK-treated and control corneas were compared but no difference was noted in either the young or the old rabbits. Data from Y8 (Y8\textsubscript{PRK} = 1.42nm ±0.17; Y8\textsubscript{CONT} = 1.36nm ±0.15) and O8 (O8\textsubscript{PRK} = 1.21nm ±0.14; O8\textsubscript{CONT} = 1.25nm ±0.14) are shown as an example, (Figures 3.36 and 3.37).

![Figure 3.36: High-angle x-ray diffraction profiles from the central anterior stroma of Y8\textsubscript{PRK} and Y8\textsubscript{CONT}.](image-url)
3.2 PRK WOUND RESPONSE IN OLD AND YOUNG RABBITS

3.2.4 Radial Distribution Function (RDF)

Radial distribution functions were calculated from multiple TEM images that were taken from the sub-epithelial region (between 5-20μm below epithelium) where the more aligned collagen fibrils could be found. Each radial distribution function was normalised by dividing $g(r)$ by the bulk fibril number density, $\rho$, for the whole area covered. Therefore, the heights of the primary peaks above zero could be compared. These values equalled 1.69 and 1.73 for Y8_cont and O8_cont respectively whereas the equivalent peaks from Y8_prk and O8_prk had an averaged height of 1.31 and 1.37 (Figure 3.38). The interfibrillar spacings, as measured from the peak position of the RDFs, are larger in the PRK-treated corneas compared to their controls, similar to the results gained by the low-angle x-ray patterns.

Figure 3.37: High-angle x-ray diffraction profiles from the central anterior stroma of O8_prk and O8_cont.

![Graph showing x-ray diffraction profiles](image-url)
3.2 PRK WOUND RESPONSE IN OLD AND YOUNG RABBITS

Figure 3.38: Normalised radial distribution functions of PRK-treated and control corneas for old and young rabbits.

3.2.5 Transparency

The effect of 8-month-old PRK wounds on the transparency through the cornea was assessed using the direct summation of fields light scattering model. The positions and radii of the collagen fibrils were determined from each of the TEM images used for the RDF calculations. These images were re-scaled by comparing the interfibrillar spacing from XRD to the position of the g(r) peaks, before normalisation, as described in the methods. Before re-scaling, the radial symmetry of each TEM image used for the RDF calculations was assessed by plotting the relative displacements of each fibril from every other fibril around a common origin. The results shown in Figure 3.39 suggest that the PRK-treated corneas do contain some radial asymmetry, as measured by circularity.
(Y8_{PRK}=1.2, \ O8_{PRK}=1.25) whereas the control anterior stromas appear radially symmetrical (circularity = 1 for Y8 and 1.06 for O8).

![Graphs showing radial symmetry plots](image)

**Figure 3.39:** Radial symmetry plots calculated from micrographs of collagen fibrils in cross section from the anterior stroma of control and PRK-treated corneas. By plotting the relative displacements of each fibril from every other fibril around a common origin (0.0, 0.0) any radial asymmetry (a greater interfibrillar spacing in one direction than in another) is made apparent by an elliptical zone in the centre of the plot and circularity measurements.

The direct summation of fields method was then used to calculate the percentage transmission assuming a tissue thickness of 0.1\,mm (the approximated depth of ablation for PRK-treated stroma and therefore the assumed maximum thickness for newly
synthesised collagen). The results (Figures 3.40 and 3.41) showed that the average percentage transmission through the anterior of the young and the old PRK-treated rabbit corneas at 500nm is 96 % and 95 % respectively. These values represent an average decrease in the percentage transmission of visible light through the PRK-treated corneas of both young and old rabbits, of only 1-2 % (controls both registering 97 %). Therefore, no significant difference was predicted in percentage transparency between either young and old PRK-treated rabbits or PRK-treated and control corneas, using the direct summation of fields method.

![Graph showing the predicted transmission of visible light through the anterior of Y8 control and PRK-treated corneas.](image)

**Figure 3.40:** The predicted transmission of visible light through the anterior of Y8 control and PRK-treated corneas.
3.2 PRK WOUND RESPONSE IN OLD AND YOUNG RABBITS

Figure 3.41: The predicted transmission of visible light through the anterior of O8 control and PRK-treated corneas.

3.2.6 Discussion

The results from the study of haze in PRK-treated corneas showed a clear trend, with both the old and the young rabbits displaying a similarly marked response that peaked after 24 days. At this same point in time (1 month post-PRK) previous studies have shown that the sub-epithelial layer is highly vacuolated and the influx of cells to the wounded area is at a maximum, also the newly formed tissue reaches its maximum thickness (Tuft et al., 1989; Rawe et al., 1992). It was also noted in the present work that, at this point, the difference in haze values between young and old was at its greatest although, the difference was not significant (p=0.06). However, even after 19 months the haze values of both young and old PRK-treated corneas were 60% higher than before PRK. Haze of this kind, which remains many months after surgery is termed persistent haze.
The results indicate that PRK induces an irregular fibril order in the anterior stroma. The mean interfibrillar spacing is increased and within the first 5µm the fibrils are neither axially aligned nor parallel, nor bundled into discrete lamellae. The low-angle x-ray results confirm the presence of a less ordered fibril packing, since the interference function profiles showed a decrease in the ratio of full height to width at half height for the PRK-treated corneas; a low value indicating a wider variation of nearest neighbour interfibrillar spacing (Fratzl et al., 1993). This data also provides supporting evidence for long term stromal remodelling already reported by confocal microscopy (Bohnke et al., 1997; Lipshitz et al., 1997) since the variation of interfibrillar spacing decreases between Y8 and Y12.

Further, the decreased height of the normalised RDF primary peaks could also indicate a decrease in fibril order in PRK-treated corneas because a smaller proportion of the total amount of measured fibrils are at a similar distance from their respective neighbours. Micrographs of PRK-treated cornea demonstrated the presence of localised interfibrillar spaces of approximately 0.1µm², which are not present in the control corneas (Figures 3.26 and 3.32). These localised gaps may contribute to the shorter peaks seen in the RDF plots for PRK-treated eyes although the shorter peaks may have also been caused, at least in part, by the radial asymmetry also shown to exist in the PRK-treated corneas. This radial asymmetry is probably a real effect of PRK wound healing caused by stresses placed between the fibrils within the healing stroma and not an artefact caused by processing for electron microscopy since the control stromas appear radially symmetrical. Radial asymmetry of the fibril positions would cause both a widening and lowering of the RDF peaks.
The control of interfibrillar spacing within the stroma has been theorised to be due to PGs acting as spacers between the fibrils (Scott, 1995). Therefore, one could assume that any difference noted in interfibrillar spacing between two corneas would be accompanied by a similar difference in PG size. The results from this study appear to support this theory since there is a concurrent increase in mean particle size and interfibrillar spacing between both control and PRK-treated corneas, and old and young corneas, as shown by low-angle XRD, RDF and GAG image analysis. However, this is clearly an oversimplification as the differences were not proportional to the changes in fibril spacing.

If PGs are thought of as physical spacers between the fibrils, the expected in vivo relationship between the length (or size as measured by image analysis) of the GAG part of PGs and interfibrillar spacing might be expected to be proportional. The difference between this hypothetical relationship and the one actually measured may be due to each of the corneas drying to differing amounts during transportation, dissection or examination by XRD. The effects of drying during transportation were minimised by keeping the tissue frozen or wrapped tightly in cling film and kept on ice, and during examination by XRD drying was limited by reducing the tissues exposure to air by placing it in an air tight low volume cell. During dissection of the tissue, especially when trying to remove semi-thin sections from the anterior of the corneas, drying of the corneal tissue is inevitable due to the length of time it is exposed to the air. An undisturbed cornea will be less susceptible to dehydration because its epithelium and endothelium offers some protection to the hydrated stroma. No attempt was made to re-hydrate the tissue at the synchrotron because the correct amount of water to add would not have been
accurately known. Therefore dissection was completed in as little time as possible and it was assumed that any drying that occurred was similar in each case.

A further possible cause for the disproportional increase between interfibrillar spaces and GAG size, could be the insensitivity of the method used for measuring mean GAG size. For rabbits Y8 and O8 the error associated with the mean GAG filament size is approximately 15% compared to only 3% for the interfibrillar Bragg spacing. Measurement of the mean GAG filament size is the most inaccurate of the parameters measured by image analysis. This is because the individual GAG filaments have, relatively, a smaller area than that of either the fibrils or the total area covered by GAGs. Each of these three types of measurements all share the same degree of error inherent to the image analysis process (Chapter 2.1.6.1 page 40) and therefore the ratio between the inherent errors and the object area measured is much higher in small objects such as GAG filaments.

XRD and GAG measurements suggest a possible difference in the wound healing response of old and young rabbits. The interfibrillar spacings (as measured by XRD and RDF) are larger in Y8_{PRK} than O8_{PRK} and the GAG percentage area covered is larger in PRK-treated old rabbits. Also, the GAG percentage area covered increased, as the rabbits (both young and old) became older until the age of 32 months. Mean GAG filament size also increased between 8 and 20 months following PRK, in both old and young corneas. The clearest difference in morphology between old and young rabbits is demonstrated by electron microscopy. Micrographs of the old rabbit corneas contained a large amount of heavily stained material in between the fibrils. This material was either stained in situ or possibly, was a coagulation of precipitates formed during the fixation protocol. It is
unlikely to be due to section staining since the material was never seen on the section surface, which would obscure collagen fibrils in cross-section, it was only found surrounding them. The presence of amorphous material is a normal finding in rabbit cornea (Hanna et al., 1989), but the more acute amount found in old corneas has, to the author's knowledge, not been remarked upon before. The effect of this stained material, if any, on the transmission of light through the cornea was not assessed.

The refractive indices of the hydrated fibrils were assumed to have remained constant in the PRK-treated/control corneas since no changes in the intermolecular Bragg spacings were measured and the mean diameter appeared normal. On the assumption that the refractive index of the interfibrillar matrix was also unchanged, it was investigated what change, if any, the altered fibril packing and the presence of varied interfibrillar spaces should have on the transmission of light. A small but insignificant increase in light scattering was predicted by the direct summation of fields method.

This small increase in light scatter is unlikely to be responsible for the 60% increase in haze after 8 months, i.e. the fibril disorder within the newly formed anterior stroma of PRK-treated corneas, does not significantly contribute to persistent haze. Therefore, another contributor to the cause of persistent haze must be looked for. One such contributor might be the scattered light from the cut collagen fibres at the wound surface which persist throughout the healing process as seen by electron microscopy (Cintron et al., 1978; Tuft et al., 1989) and have been measured objectively by confocal microscopy, (Moller-Pederson et al., 1998). Another contributor could be the wavy basal lamina reported above, these irregular waves are much larger than half the wavelength of light incident upon them and would in theory cause a diffuse scatter (assuming shrinking of the
tissue during TEM preparation does not cause these waves). Yet, another possible contributor to corneal haze was proposed recently by Jester et al. (1998). Jester et al. suggested the keratocytes play a pivotal role in defining the results of refractive surgery. Normal rabbit corneal keratocytes have little reflection from confocal microscopy (Moller-Pederson et al., 1998) and express high levels of putative enzyme-crystallins, which appear to play a structural function in regulating cellular refractive index and transparency (Jester et al., 1999). Whereas there are increased reflections from, and a decreased expression of putative crystallins in, activated and transformed keratocytes in the cornea following PRK.

In conclusion, these results indicate that following PRK-treatment, anterior corneal collagen fibrils are more widely spaced and misaligned than normal cornea (up to 12 months post-PRK), particularly those immediately below the epithelium. Young rabbits have also been shown to differ with respect to old rabbits in their response to PRK-treatment, by displaying less measurable haze, less measurable GAG content and by exhibiting a greater degree of fibrillar disorder in the anterior stroma. Therefore there exists an age dependent response to PRK-treatment in rabbit corneas, old rabbits showing a more virulent response producing more haze at one month and a greater ability to reassemble collagen fibrils into a more normal ordered arrangement. These results suggest that conclusions drawn from the study of PRK-wound healing in young rabbits (3 months old) are not necessarily applicable to the wound healing in adult human corneas, and that a more accurate comparison could be obtained from the study of old rabbits (24 months old). It has also been observed that there is a measurable increase in both mean size and number of GAG filaments as the rabbit ages.
The causes of persistent haze have yet to be determined, but it seems that this haze is not due to changes in the packing of the collagen fibrils, but more possibly caused by an increase in light scatter by uneven surfaces within the corneal tissue such as the reported 'wavy' basal lamina, the original wound surface and/or a decreased keratocyte transparency.

3.3 Application of Mannose-6-Phosphate (M6P) to PRK Wounds

3.3.1 Haze

No difference in haze was noted between the M6P-treated, and the untreated, PRK-wounded eyes. Curves fitted to the haze data for each treatment, (Figure 3.42) were similar in shape and position and showed the haze values peaking at one month post-PRK for both the M6P-treated, and the untreated, PRK-wounded eyes before returning to a value similar to before PRK treatment.

![Figure 3.42: Haze values from M6P-treated and untreated PRK-wounds. Negative exponentially-weighted smoothed curves were fitted, assuming the influence of individual points decreases exponentially with the horizontal distance from the respective points on the curve, due to a decline in subject number and variability in wound response.](image-url)
3.3.2 Hydration

Hydration values increased after 2 weeks in the wounded (M6P-treated and untreated) corneas compared to the controls (the control’s value was the averaged value from the left eyes of rabbits A1 and A2 (H=2.25)) assuming any dehydration of the wounded and control corneas during manipulation of the tissue occurred equally. At 4 and 6 weeks post-PRK, the M6P-treated corneas had a measurably lower hydration value than the untreated corneas, the largest difference occurring at 4 weeks (Table 3.5, Figure 3.43). The hydration values of the wounded corneas appeared to be returning to the control value after 8 weeks.

![Figure 3.43: The relative hydration values (H) of the wounded and control corneas over time.](image-url)
3.3.3 Image Analysis of GAGs

The image analysis results of anterior GAG content in the wounded corneas (Table 3.5) showed that at 4 weeks the M6P-treated corneas had less GAG filaments per μm² and less percentage area covered by GAG filaments than the untreated corneas in electron micrographs. By 8 weeks, no differences between the M6P-treated and untreated wounded corneas were found. However, an approximate increase of 80% in both the percentage area covered and mean area per GAG filament was found at 4 and 8 weeks in the M6P-treated and untreated PRK wounds when compared to the control.

<table>
<thead>
<tr>
<th>Rabbit / eye</th>
<th>Weeks between PRK and death</th>
<th>Treatment (Untreated, M6P or Control)</th>
<th>Hydration = (wet weight-dry weight) / wet weight</th>
<th>Number of filaments per μm²</th>
<th>Percentage of area covered by filaments (P) ±0.6 %</th>
<th>Mean area per filament / 95% confidence (nm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7/R</td>
<td>2</td>
<td>Untreated</td>
<td>2.19 ± 0.14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A8/R</td>
<td>2</td>
<td>M6P</td>
<td>2.24 ± 0.13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A5/R</td>
<td>4</td>
<td>M6P</td>
<td>2.63 ± 0.32</td>
<td>361</td>
<td>9.2</td>
<td>258.5 / ±15.6</td>
</tr>
<tr>
<td>A6/R</td>
<td>4</td>
<td>Untreated</td>
<td>4.44 ± 0.40</td>
<td>472</td>
<td>10.9</td>
<td>231.2 / ±15.0</td>
</tr>
<tr>
<td>A1/R</td>
<td>6</td>
<td>Untreated</td>
<td>3.80 ± 0.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A1/L</td>
<td>-</td>
<td>Control</td>
<td>2.33 ± 0.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A4/R</td>
<td>6</td>
<td>M6P</td>
<td>2.94 ± 0.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A3/R</td>
<td>8</td>
<td>M6P</td>
<td>2.62 ± 0.19</td>
<td>362</td>
<td>8.6</td>
<td>237.6 / ±15.0</td>
</tr>
<tr>
<td>A2/R</td>
<td>8</td>
<td>Untreated</td>
<td>2.50 ± 0.13</td>
<td>352</td>
<td>7.4</td>
<td>209.5 / ±14.6</td>
</tr>
<tr>
<td>A2/L</td>
<td>-</td>
<td>Control</td>
<td>2.20 ± 0.04</td>
<td>430</td>
<td>4.9</td>
<td>118.9 / ±14.7</td>
</tr>
</tbody>
</table>

Table 3.5: Details of rabbit M6P treatments and image analysis results of GAG content.

3.3.4 Discussion

Previously Sutton et al. (1996) reported a successful modulation of the corneal wound healing response by M6P and a significant decrease in the level of haze at four weeks. In this repeated study, haze results indicated no significant difference in haze at any time
3.3 APPLICATION OF MANNOSE-6-PHOSPHATE TO PRK WOUNDS

during the healing process. The reason for this inconsistency is unclear since apart from a theoretically increased contact time between the M6P and the wounded cornea, by the use of a more viscous solution, the same instruments and materials were used and a similar depth of ablation was attained. The rabbits in this study appeared to heal normally and re-epithelialization occurred at a similar rate in each rabbit, no abnormalities were seen via slit-lamp examination and the haze peaked in both treatments at approximately the same time.

At four weeks following PRK the haze values peaked and the largest difference in hydration between M6P-treated and untreated wounds was recorded, a 40% decrease for the M6P-treated cornea. This difference in hydration between the two treatments is unlikely to be an osmotic effect i.e. the high salt concentration within the M6P solution drawing water out of the cornea, since the M6P is applied for the first few days only. The difference in hydration appears to be more likely a consequence of a change in GAG content, since the M6P-treated cornea at 4 weeks also has a decrease both in the number of stained GAG filaments (23%) and the area covered by GAGs (15%). The hydration of the corneal stroma is due, in part to GAG content (Hodson, 1997). GAGs common to the normal corneal stroma such as keratan sulphate and dermatan sulphate contribute to corneal hydration by virtue of their charged side groups, whereas hyaluronan does not (to any significance) because of its lack of charged side groups. However, this would not have precluded the staining of hyaluronan by Cuprolinic blue, due to the MgCl₂ concentration specified in the methods, Chapter 2.1.2.2 page 33, which permitted the staining of every known GAG within the corneal tissue (Scott, 1985). Therefore, the GAG(s) specifically affected by the application of M6P is not known. Keratan sulphate
PG is unlikely to be responsible for the 80% increase in stained GAG content (filament size and percentage of area covered) noted between PRK-wounds and the control. This is because previous studies have concluded that there is a decrease in the amount of keratan sulphate PG within the first few weeks of healing wounds (Hassell et al., 1983; Sundarraj et al., 1998). Therefore if it is assumed that the difference in hydration between wounded and the control corneas is due to a GAG with the potential to swell, the dermatan sulphate PG may be responsible. This would be the case for two reasons, one it has been shown to have an increased charge density in tissue adjacent to a wound (Cintron et al., 1990) and two it is present in unusually large sized filaments within wounds (Hassell et al., 1983; Cintron et al., 1990). This would explain both the increase in hydration and mean area per filament within the M6P and untreated PRK-wounds compared to the controls, up to eight weeks, following PRK.

In conclusion, following PRK, the corneal stroma of the wound and the region below it become hydrated. Within this stroma, the anterior GAG percentage of area covered increases, as does the mean GAG filament size, possibly due to a type of dermatan sulphate PG. Furthermore, the application of M6P appears to suppress this increase in GAG content (assuming the staining potential of GAGs by Cuprolinic blue is not affected by M6P) and also stromal hydration. The level of haze remains unaltered. The positive effects of increasing the M6P contact time by using a more viscous solution were inconclusive.
3.4 Chemical Alteration of Corneal Curvature

3.4.1 Haze

No difference in the amount of observable back-scatter was noted within the corneas or their anterior segments by slit-lamp examinations up to 7 days post-operative, and both the control and enzyme-treated corneas were clinically clear at their time of removal.

3.4.2 Electron Microscopy and X-ray Diffraction

The paraformaldehyde and freeze-preserved corneas (rabbits 1 to 4) were analysed by low and high-angle cameras using synchrotron x-rays as described in the Methods section. Figure 3.44 shows the profiles of the Bragg spacing from the innermost low-angle, equatorial reflections of both the enzyme-treated and control corneas from freeze-preserved tissue. Figure 3.45 shows the low-angle equatorial results from paraformaldehyde-preserved corneas. Figure 3.46 shows the results from the frozen tissues that were subsequently re-equilibrated. These re-equilibrated tissues had an average hydration (H) of 3.8 for the control tissue and 2.3 for the enzyme-treated tissue. The two enzyme-treated corneas in Figure 3.44 are seen to have a reduced Bragg spacing (38.0 ± 1.9nm and 36.5 ± 1.4nm) compared to the untreated or BSS-treated control corneas (47.0 ± 2.3nm and 43.7 ± 2.2nm). The small difference between the two control corneas was not regarded to be significant and therefore a mean control value was calculated by averaging the Bragg spacings. The mean Bragg spacing of the enzyme-treated corneas was 18% lower than the mean Bragg spacing from the controls. Similar differences of 15% and 21% respectively were found between the enzyme-treated and
untreated corneas following paraformaldehyde preservation (Figure 3.45) and re-equilibration of the freeze-preserved tissue (Figure 3.46).

Figure 3.44: The intensity profiles across the diffraction patterns of freeze preserved corneas, converted into real space.

Figure 3.45: The intensity profiles across the diffraction patterns of paraformaldehyde-preserved corneas, converted into real space.
Figure 3.46: The intensity profiles across the diffraction patterns of freeze-preserved and then re-equilibrated corneas, converted into real space.

A similar decrease in mean interfibrillar spacing (18%) was observed from the radial distribution data obtained from the electron-optical images of freeze-preserved cornea, via image analysis (Figures 3.47 and 3.48) otherwise the enzyme-treated stroma appeared normal.

Figure 3.47: TEM images of control 4/L (left) and enzyme-treated 3/L (right) corneal mid-stroma in cross-section (Scale bars = 250nm).
The normal appearance of the enzyme-treated corneal mid-stroma is also supported by comparing the heights of the primary peaks from the RDF of enzyme-treated and control corneas. Each radial distribution function was normalised by dividing $g(r)$ by the bulk fibril number density, $\rho$, for the whole area covered. Therefore, the heights of the primary peaks measured at the nearest neighbour distance could be compared and were found to be 1.53 and 1.62 for the controls (4R and 4L), 1.55 and 1.51 for the enzyme-treated (3L and 3R).

![Figure 3.48](image)

**Figure 3.48:** The normalised radial distribution functions of freeze-preserved enzyme-treated and control corneas.

The radial symmetry results from TEM images suggest little or no differences between enzyme-treated and control corneas, both have a similar circularity; a mean value 1.08 for the control (4L+4R) and a mean value of 1.06 for the enzyme treated (3L+3R) (Figure
3.4 CHEMICAL ALTERATION OF CORNEAL CURVATURE

3.49). This indicates that the compression within the enzyme-treated corneas occurred equally in all directions.

Figure 3.49: Radial symmetry plots calculated from micrographs of collagen fibrils in cross section from the mid-stroma of rabbits 3 and 4. By plotting the relative displacements of each fibril from every other fibril around a common origin (0.0, 0.0) any radial asymmetry (a greater interfibrillar spacing in one direction than in another) is made apparent by an elliptical zone in the centre of the plot.

Figure 3.50 shows the predicted transmission in the visible light range for the enzyme-treated corneas compared to a normal rabbit cornea with no injection. It can be seen that there is only a small reduction in expected transmission, following removal of
3.4 CHEMICAL ALTERATION OF CORNEAL CURVATURE

hyaluronidase-sensitive proteoglycans, which may not be experimentally significant. Even if it is, with 90% of the original transmission maintained at 500nm (specimen 3/L), no appreciable loss of vision would be expected.

![Graph showing percentage transmission of visible light through control and two enzyme-treated corneas.](image)

**Figure 3.50: Percentage transmission of visible light through control and two enzyme-treated corneas.**

In theory any increase in collagen fibril diameters and/or intermolecular spacing, following the removal of hyaluronidase-sensitive proteoglycans would be expected to change the refractive index, \( n_f \) which would increase \( m \), used in the summation of fields model (see equation 2.5 page 56). To examine this, mean fibril diameters were measured from over 3500 fibril cross-sections in TEM images of 3/L, 3/R, 4/L and 5/L (Figure 3.51). The control diameters had a mean value of 37.93nm and the enzyme-treated had a mean value of 40.93nm. The 95% confidence interval for the control diameters was ±2.33nm and for the enzyme-treated diameters ±2.35nm. These results infer a small
difference between the control and the enzyme-treated fibril diameters, but the difference is not significant at the 95% confidence intervals.

Figure 3.51: Histograms of fibril diameters, fitted with curves assuming normal distribution.

The packing of the collagen molecules within the fibrils was determined from the high-angle x-ray pattern. The Bragg spacing equalled 1.57 ±0.15nm in all the tissues and the reflection had a similar profile in each case. This suggests that the mean intermolecular spacing, and hence the fibril refractive index \( n_f \), was normal in the enzyme-treated corneas.

3.4.2 Discussion

The use of ACS-005 hyaluronidase to soften the cornea for re-shaping, by breaking up the sugars in the glycosaminoglycan chain of the proteoglycans, has the effect of
decreasing the interfibrillar Bragg spacing by about 18%. This was shown by synchrotron x-ray diffraction and by radial distribution functions from TEM images of enzyme-treated corneas and was found in all samples, regardless of the method of preservation used. This decrease was also show to be radially symmetrical.

Other than the decrease in interfibrillar spacing, the fibrils of the enzyme-treated corneas remained similar to the control. TEM images depicted the fibrils as axially aligned and areas devoid of collagen, 'lakes', were not present. Similar heights for the RDF primary peaks indicate a similar degree of fibril order between the control and enzyme-treated corneas because a similar proportion of the total amount of measured fibrils are at a similar distance from their respective neighbours.

It can be inferred from the low-angle Bragg spacings that the enzyme-treated corneas reached a lower final hydration than the controls (Goodfellow et al., 1978). This is consistent with the fact that the wet weights of the corneas measured immediately after excision were significantly lower in the enzyme-treated corneas (Table 2.3 page 84).

It is accepted that proteoglycans present in the stroma are essential for swelling to take place to any extent (Hodson, 1997) and that this swelling is essentially uniform. Collagen fibrils in the cornea do not swell appreciably above physiological hydration (Meek et al., 1991). The swelling forces between the fibrils, however, are due to fixed charges (Donnan potentials) and the fixed charge concentration in the stroma is due, in part, to the glycosaminoglycans on the proteoglycan molecules and to transient mobile ion binding to proteins (Hodson, 1997). GAGs such as dermatan sulphate and keratan sulphate contribute to the fixed charge by virtue of their sulphonic acid groups (and the carboxyl groups in dermatan sulphate). The reduced tissue hydration suggests that the enzyme-
3.4 CHEMICAL ALTERATION OF CORNEAL CURVATURE  

Treated corneas have a lower overall fixed charge than the control corneas. This is supported by the observation that in the re-equilibrated corneas, identical osmotic conditions produced a lower final hydration in the enzyme-treated cornea. Since the enzyme hyaluronidase breaks up GAGs, the loss of fixed charge could be attributed to the action of hyaluronidase on the stromal GAGs. A reduced swelling potential caused by this loss of fixed charge would be expected to lead to a decrease in the interfibrillar spacing, as was observed.

Unfortunately, attempts to stain either the control or enzyme-treated tissue for GAGs using Cuprolinic blue did not work, possibly due to the sensitive nature of PGs to their storage media whilst in transit from California, USA to Oxford, UK. It was therefore not possible to compare the size and amount of GAGs present in the enzyme-treated and control corneas or to support the personal observation of Dr Karageozian, (Advanced Corneal Systems, Inc., Irvine, California) that no GAGs are visible for up to a week after the corneas are treated with the enzyme ACS-005 hyaluronidase.

There are a number of possible consequences of a decrease in the interfibrillar spacing. Clearly, it will cause an increase in the bulk fibril number density and affect the interference between light photons scattered by different fibrils. These changes are taken into account in the direct summation of fields model. However, the other assumptions mentioned earlier also need to be reviewed. (i) The reduction in fibril spacing might not occur uniformly throughout the full depth of the stroma, which could lead to a refractive index gradient within the tissue. Corneal refractive index is known to vary slightly with depth in the normal cornea (Patel et al., 1995) but the structural implications of this, and in particular any association with changes in the fibril packing, have not yet been
investigated. (ii) The high-angle data implies that the refractive index of the collagen fibrils themselves does not change. (iii) The reduction in the interfibrillar spacing implies that the tissue thins following corneoplasty. From equation 2.3 (page 54), a reduction in corneal thickness would have the effect of reducing light scattering. A recent study has found a measurable decrease in total corneal thickness following corneoplasty (Karageozian et al., 1998) (iv) The reduction in the interfibrillar spacing might lead to an increase in the refractive index of the interfibrillar matrix. Theoretically, this would also have the effect of reducing light scattering but, in this case, the refractive index of the cornea as a whole would increase. Unfortunately, attempts to measure the refractive index of treated corneas have not provided reproducible results.

The experimental results and the theoretical considerations above suggest that the important factor with respect to possible increased light scattering following corneoplasty is the change in the arrangement and spacing (bulk fibril number density) of the collagen fibrils. In order to see the effect of uniformly compressing the collagen fibrils, assuming that everything except the bulk fibril number density remains constant, a transmission of visible light through the enzyme-treated corneas was predicted. This was accomplished by artificially reducing the measured interfibrillar spacing from the TEM images of a normal cornea (specimen 5/L) by 15 and 20% (Figure 3.52). This resulted in two curves, which gave a similar reduction in transmission to the enzyme-treated tissues in Figure 3.50. This similarity indicates that the predicted small reduction in transmission following corneoplasty may be a real effect related directly to the decrease in interfibrillar spacing.
Despite the exact mechanisms by which corneoplasty might alter refractive error remaining unclear, it can be assumed that the reduction in interfibrillar spacing in enzyme-treated corneas was a direct effect of hyaluronidase on the tissue’s hydration and potential to swell, as neither control showed any change in interfibrillar spacings.

The lack of post-operative haze appears to be because the stroma can tolerate a small compression of its constituent collagen fibrils; without this ability there would be an increase in light scattering. The combination of ACS-005 hyaluronidase treatment, specially designed forming lenses and the use of topically applied stabilising solution, therefore, seems to offer an alternative procedure to surgery for the improvement of visual acuity, with little or no disruption to fibril organisation and no post-operative haze.

Figure 3.52: Theoretical decrease in the transmission of visible light after an artificial compression of the interfibrillar spacing by 15 and 20%.
'Phase I Safety Studies' have been completed in the United States and offshore. In addition, 'Phase IIa Safety and Efficacy Studies' are being conducted offshore in several dozen patients with improvement of visual acuity in all cases although the long-term implications have not yet been assessed.
4 Conclusions

4.1 Overview

The work described in this thesis is loosely divided into four subjects: 1. The study of ultrastructural changes during the healing of full thickness wounds; 2. The study of ultrastructural changes during the healing of partial thickness wounds in old and young rabbits caused by PRK, with subsequent changes in transparency predicted using the direct summation of fields method; 3. The study of hydration and glycosaminoglycan content during the healing of PRK wounds in young rabbits treated with mannose-6-phosphate; 4. The study of chemically altered stromal ultrastructure by the ophthalmic procedure corneaplasty and its affect on transparency (as assessed by direct summation of fields method).

Therefore a comparison of the results can be made between the ultrastructure of full thickness wounds and partial thickness anterior wounds over a period of 8 to 20 months, and between the ultrastructure and resulting transparency from two methods of refractive correction i.e. photorefractive keratectomy and corneaplasty.

4.1.1 Full thickness vs. Partial thickness Wounds

Use of electron microscopy demonstrated that at 8 months the newly synthesised tissue in the partial thickness wounds had similarly sized fibril diameters to the control, but at 16 months the full thickness wounds still had a large variation in fibril diameters. Interestingly during the same periods, normal lamellae were present in all but the first 5μm of the partial thickness wounds and not at all in the full thickness wounds.
In both types of wound fibrillar order (fibril packing or interfibrillar spacing) moved towards a more normal value with time.

The mean glycosaminoglycan filament size increased in response to both types of wound, this increase was probably due to an increase in dermatan sulphate proteoglycan (Hassel et al., 1983; Cintron, 1989; Cintron et al., 1990b; Sundarraj et al., 1998). Glycosaminoglycan content was more affected by full thickness wounds than by partial thickness wounds. A marked difference in GAG size was still visible after 8 months and very large GAG precipitates near to the endothelium, which were not present in partial thickness wounds, were present throughout the entire wound healing process of full thickness wounds and were possibly a consequence of endothelial damage.

Despite both types of wound failing to return to their previous and normal haze values after 16 months (full thickness) or 20 months (partial thickness) they both showed an increase in stromal order, indicating that healing and its associated processes were taking place. These processes within the stroma were shown to be remarkably similar in both types of wound but on different time scales, probably due to the differences in wound depth and endothelium integrity and as a result the partial thickness wounds healed in less time. However, the extent to which endothelium damage contributed to a delay in the healing of full thickness wounds would have presumably been slight since only a small percentage of the endothelium was damaged (1 to 3 %) and rabbits are able to renew these cells (Van Horne et al., 1977).

The difference in the spread of fibril diameters between the two types of wound was probably due to the difference in wound thickness. Intraocular pressure applied more stress to full thickness wounds than to partial thickness wounds. This was because,
following a partial thickness wound, two thirds of the original collagen fibrils remained intact which supported the remaining cornea, and lessened the amount of stress applied to the wound. Consequently, full thickness wounds displayed a large spread of fibril diameters whereas partial thickness wounds contained fibrils of a similar diameter. Tissues that contain a mixture of small and large fibril diameters can endure tensile stress and inhibit creep (non-elastic stretching of fibrils) to a greater degree than those with fibrils of a similar diameter. This is because the percentage of possible covalent links, between collagen molecules, increases with fibril diameter. Therefore, fibrils with large diameters have a high cross-link density, and consequently a high tensile strength. Similarly, as the fibril diameter decreases the surface area of fibrils per unit mass of collagen increases. This, in turn, allows for the possible number of interactions between the matrix and the fibrils to be maximised. A maximisation of fibril-matrix interactions helps to inhibit permanent creep (Parry et al., 1978).

Therefore, circular central corneal wounds, with respect to the deposition of stromal matrix, share some common wound healing responses, and the rate of wound healing appears to be primarily related to wound depth. However, although remodelling of the corneal wound tissue is evident, neither wound returns completely to its normal form. This may indicate either that the wound never obtains the tensile strength of the original tissue or that the functional demand of tensile strength is satisfied by the deposition of a modified structure.

4.1.2 Photorefractive Keratoplasty (PRK) vs. Chemical Alteration (Corneaplasty)

During the few months after PRK, epithelial and stromal wound healing typically results in replacement of the lost tissue (Tuft et al., 1989, Moller-Pederson et al., 1998).
However, tissue remodelling, particularly extracellular matrix composition and the orientation of the collagen fibrils, continues for years after the initial wounding (as shown in the full thickness and partial thickness wound results). In some cases, this stromal remodelling may result in myopic regression a condition that is most common in highly myopic patients and usually results in 0.64 to 9.1% of operated eyes requiring retreatment. The exact moment when this regression may occur is difficult to predict since the postoperative time required for a stable refractive endpoint may vary according to the patient's age, the amount of attempted myopic correction and the type of laser used (Seiler and McDonnell, 1995).

The formation of early haze is another significant feature of PRK (Tuft et al., 1993) but in this condition stromal remodelling is beneficial. As the disorganised collagen fibrils gradually re-organise themselves into their previously ordered arrangement over a period of months or years, at which point interfibrillar spacing returns to normal (Rawe et al., 1992; Quantock et al., 1994) and parallel layers of lamellae are recreated across the region where ablation had interrupted them (Davison and Galbevy, 1986), early haze dissipates as normal transparency returns. There is currently no method for predicting which patients will develop an exaggerated postoperative wound healing response such as the type of haze which clears with the remodelling of the collagen fibrils, or persistent haze of which the cause(s) is (are) presently unknown. Thus while clinical results seem promising, questions concerning predictability and safety remains a concern.

Therefore it can be stated that the complex healing processes which involve cells, cytokines, digestive enzymes and extracellular matrix, following laser ablation of the central anterior stroma cause the associated problems of PRK i.e. myopic regression and
the formation of subepithelial haze. This represents a major problem because, while PRK offers the promise to correct visual refractive errors permanently and predictably, variability and complications continue to hinder widespread acceptance. Therefore, it has become evermore important that refractive surgical procedures are devised free of the associated haze and myopic regression of PRK. One such method is corneaplasty.

Corneaplasty is a new method for correcting the refractive error of the cornea and offers a fresh approach to the problems of haze. Cutting or ablating the corneal stroma results in damaged lamellae and keratocyte death which invokes a healing response, corneaplasty causes minimal stromal damage, effectively side stepping the problems of associated haze by not invoking a strong healing response and with only a small and inconsequential decrease in transparency. As to the second problem associated with surgical refractive correction, myopic regression, it is too soon to tell, although preliminary results are encouraging (Karageozian et al., 1996).

In the last decade there have been major advances in characterising the events involved in corneal wound healing, but maintaining long-term refraction and preventing haze formation with consistent success has not been achieved. Thus, it is foreseeable that at a time in the near future pharmacological rather than physical intervention may be preferred. Corneaplasty may be seen as the first step to a solely pharmacological method for correcting corneal refractive error. For example if a more sensitive method for manipulating corneal proteoglycans were found, the amount of proteoglycans at specific sites within the cornea could be adjusted therefore changing the hydration hence the shape and curvature of the cornea, ultimately affecting its refraction.
4.2 Further Work

Following on from the results reported in this thesis, further work could be attempted. Questions to address may include: i) does the wound scattering intensity return to normal in transparent full thickness wounds? ii) is there a fibrillar orientation at the edge of healing partial thickness wounds? and iii) is there any statistical difference between fibril diameters, spacings or glycosaminoglycans as a function of depth through the normal cornea? The reasoning behind each of these follow-up studies is described below.

Scattering intensity from high-angle x-ray diffraction patterns was related to the total amount of collagen through the cornea and was found to be reduced in healing full thickness wounds. Further work studying fully healed i.e. transparent, full thickness wounds is required to elucidate whether or not a return to a normal amount of collagen is required for corneal transparency. In theory, less collagen should not limit transparency since it would mean fewer scatterers and a higher transmission of light. Furthermore, it may also be possible to measure how much of the collagen within the healing wound is newly synthesised or merely old collagen that has been moved from the wound edge into the wound. This could be done by measuring the relative amount of scatter (collagen) outside the wound compared to what is inside as the wound heals. If it were the case that some of the collagen filling the wound was not newly synthesised but came from outside the wound, the average interfibrillar spacings across the cornea may increase to compensate, this could also be measured by x-ray diffraction.

The study into the organisation of fibrils surrounding the periphery of healing full thickness wounds resulted in the finding of a distinct and a previously not remarked upon fibrillar orientation. This study could be extended to other types of wound such as partial
thickness wounds caused by a laser or a knife, the results of which may further illuminate the ultrastructural processes of corneal wound healing or provide a method to increase the rate of wound healing.

The image analysis methods developed for this thesis could be further employed to study the change in the corneal stroma as a function of corneal depth. Information regarding the amount of glycosaminoglycans, fibril diameters and fibril positions (to calculate interfibrillar spacings) with respect to depth through the cornea could all be attained via image analysis of transmission electron images. The curvature of the cornea differs between the epithelium and the endothelium and is known to cause a refractive gradient (Patel et al., 1995). The relationship between the collagen fibrils and this gradient is unknown but a change in either fibril diameter or fibril spacing would not be unexpected. Using the image analysis methods established in this thesis, a large amount of data could be gained in a relatively short period and a statistically significant conclusion attained.
5 Publications


6 References


Fullwood NJ. *Ultrastructural studies of cornea and sclera.* Ph.D. 1994. Open University, Milton Keynes, UK.


Hong B-S, Davison PF, Cannon DJ. Isolation and characterization of a distinct type of collagen from bovine fetal membranes and other tissues. *Biochemistry.* 1979;18:4278-4282.


Appendix: Nomenclature

Symbols

\( \rho \)  
bulk number density of collagen fibrils in the stroma

\( \sigma \)  
scattering cross section

\( \lambda \)  
wavelength (of x-rays or light)

\( \theta \)  
scattering angle

\( \Delta \)  
corneal thickness (either wounded or unwounded)

\( a \)  
collagen fibril radius

\( D \)  
collagen fibril diameter

\( F_T \)  
transmittance (incident light intensity / transmitted light intensity)

\( g(r) \)  
radial distribution function

\( H \)  
hydration of the cornea \(((\text{wet weight } \times \text{ dry weight}) / \text{dry weight})\)

\( J_n \)  
\( n^{\text{th}} \) order Bessel function of the first kind

\( m \)  
ratio of the refractive indices of the fibrils and ground substance \((n_f / n_g)\)

\( N \)  
number of fibril diameters

\( n_f \)  
refractive index of hydrated collagen fibrils

\( n_g \)  
refractive index of hydrated stroma

\( P \)  
total percentage area covered by GAGs

\( R \)  
reciprocal space co-ordinate

Abbreviations

CCD  
charge-coupled device

GAGs  
glycosaminoglycans
APPENDIX: NOMENCLATURE

M6P  mannose-6-phosphate
PGs  proteoglycans
PRK  photorefractive keratectomy
RDF  radial distribution function
SRS  synchrotron radiation source
TEM  transmission electron microscopy
XRD  x-ray diffraction