IMMUNOCYTOCHEMICAL STUDIES OF EXTRACELLULAR MATRIX COMPONENTS IN THE HUMAN EYE

by

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Abstract

The purpose of the present study was to document the fine structural distribution of collagen types I-VI and laminin in various human ocular tissues (cornea, trabecular meshwork, iris, ciliary body, lens capsule and retinal vessels) and relate their distribution to the functional requirements of each tissue.

Twenty-three human eyes (aged 43-82) were suitably prepared for immunocytochemistry. The distribution of the extracellular matrix components studied was determined using the immunogold technique on ultrathin frozen and LR white sections. Two different preparative techniques were employed to consolidate results.

Perhaps the most surprising finding among the many features of great interest, was the presence of type I collagen in all of the vascular basement membranes studied. Other interesting features included the interfibrillar location of type VI collagen in cornea, meshwork and retinal vasculature, the presence of collagens V and VI in corneal keratocyte bodies, and the association of collagen VI and laminin with the corneal basement membrane complex. the identification of collagen IV and laminin in extracellular matrix deposits in the cribriform layer was also of interest as was the association of laminin with linear deposits in the lens capsule and increased labelling of types I and IV collagen associated with pericytes in retinal capillaries.

The presence of the components studied could be related to the functional demands of each tissue.

Chapter 1:
Introduction

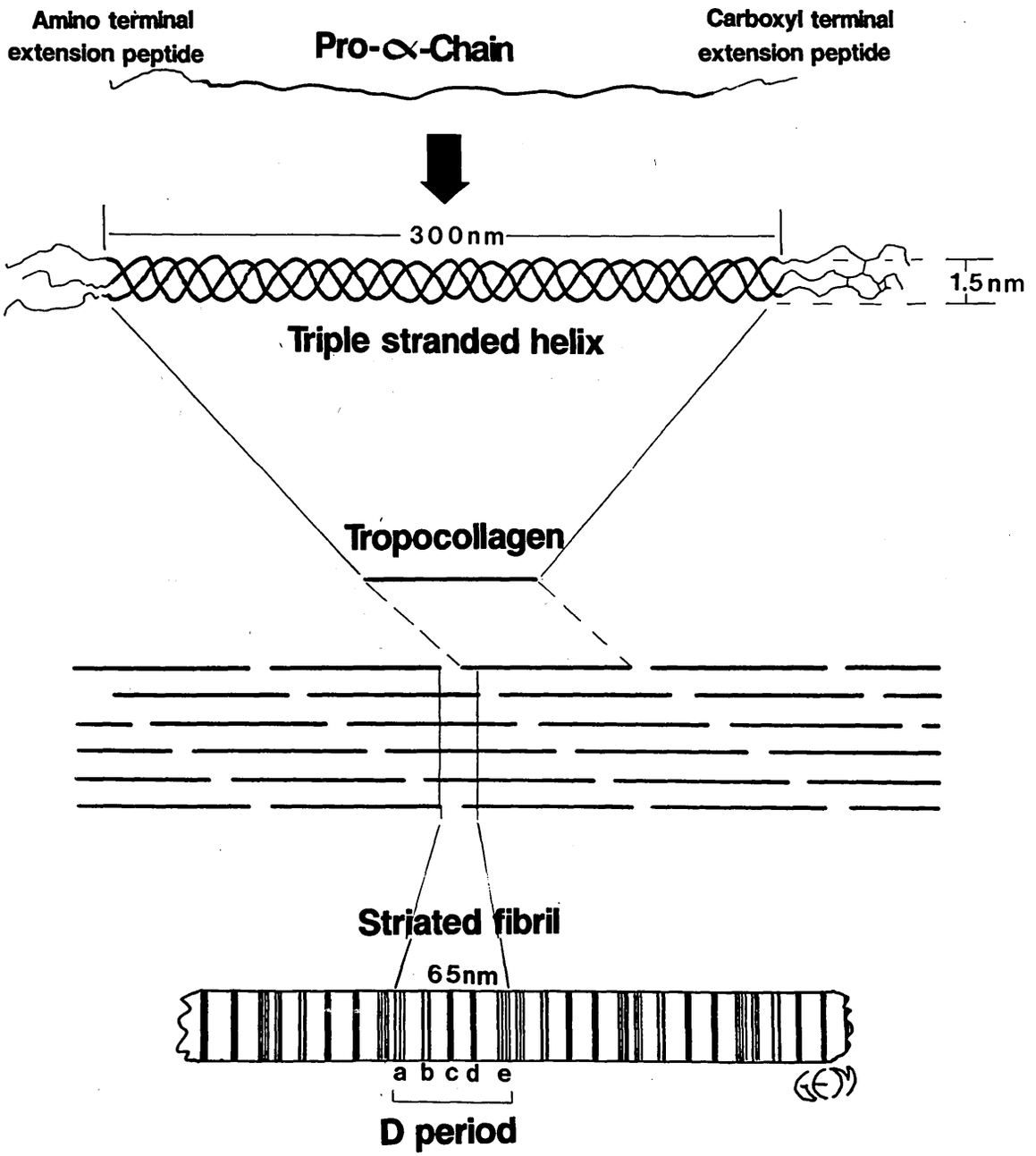
1.1 Collagens

Collagen is responsible for the structural integrity and compartmentation of all major organ systems.[20] The collagens differ from one another in the primary sequence of their constituent polypeptides, in the extent of posttranslational modifications and in their tissue distribution.[20]

1.1.1 The Extracellular Matrix[91]

The extracellular matrix is an intricate meshwork of interacting extracellular molecules. In addition to serving as a universal glue, it forms structures such as cartilage, tendons, basal laminae, corneas, bone and teeth. The macromolecules of the extracellular matrix are secreted by local cells, especially fibroblasts, which are in the matrix. In specialized structures these macromolecules are secreted locally by more specialized cells such as chondroblasts forming cartilage and osteoblasts forming bone. There are two main classes of extracellular macromolecules:- **collagens**, which strengthen and help to organize the matrix and **glycosaminoglycans**, a type of polysaccharides which are usually linked to protein to form proteoglycans.

Collagen and proteoglycans always coexist. Collagen is a structure that gives stiffness and a high degree of tensile strength to the matrix; the highly hydrated proteoglycans confer rigidity to the matrix as well as allowing transport of moving molecules and impeding diffusion of large molecules due to their mesh-like structure. Collagen and proteoglycans also interact by influencing collagen fibril formation and growth as well as


Amino terminal
extension peptide
Pro-α-Chain
Carboxyl terminal
extension peptide
300nm
1.5nm
Triple stranded helix
Tropocollagen
Striated fibril
65nm
a b c d e
D period

1

the assembly of the collagen fibrils and their three-dimensional arrangement.[220]

1.1.2 Collagen Synthesis

Collagens are a family of highly characteristic fibrous proteins found in all multicellular animals and constitute 25% of the total protein in mammals.

Individual collagen polypeptide chains, synthesized by ribosomes, are injected into the lumen of the rough endoplasmic reticulum (rER) as **pro-α-chains.**

Hydroxylation and glycosylation occur before the formation of triple helices. It is not known at which stage in its passage through the cell that triple helical formation occurs. The collagen passes through the Golgi apparatus and into condensing granules and secretory vesicles en route to exocytosis.[114,262] Collagen secretion differs from some secretory proteins in that collagen is secreted continuously by the cell, in a fashion similar to the secretion of immunoglobulins.[190,243] Hormones and some other secretory proteins are synthesized and stored in secretion granules which are released by the cell only upon the recognition of an appropriate signal.

When tropocollagen is secreted by the cell to the exterior, it combines with others to form collagen fibrils (Fig 1). The staggered arrangements of the triple helical

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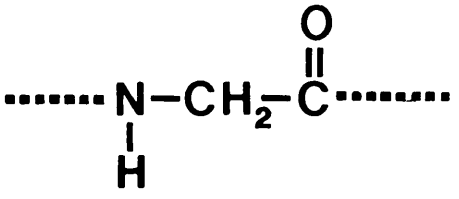
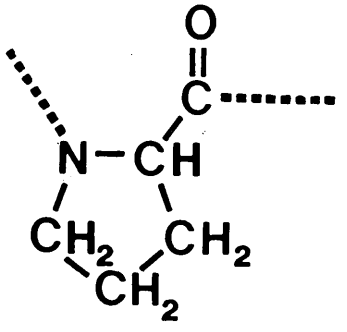
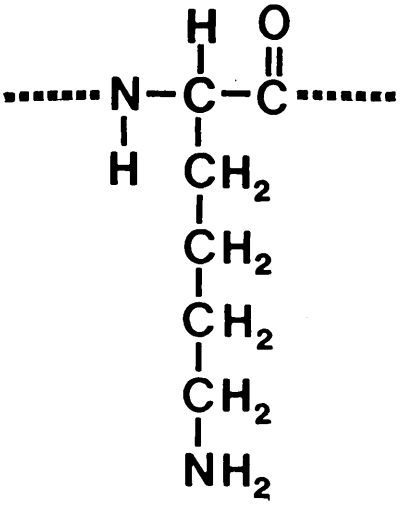
Fig 1: Diagram of collagen synthesis. Collagen chains are initially synthesized in the form of pro-α-chains (top) that contain extension peptides that will later be removed. Note that the carboxy extension peptides of the triple stranded helix are covalently linked together. Tropocollagen molecules are 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.



[a] Glycine



[b] Proline



[c] Lysine

rods with each other is mainly brought about by electrostatic forces determined by the sequence of the basic and acidic amino acids. The 234 amino acid residue long D unit is formed mainly by polar charged and hydrophobic residues. There are four D units per α-chain (Fig 1). Not only the highest polar but also the highest hydrophobic contact occurs when the molecules are shifted against each other by 234 amino acid residues.[100]

1.1.3 Collagen Biochemistry[201]

Collagen polypeptides are rich in glycine and proline, both of which are important in the formation of a stable triple helix. Glycine (Fig 2) is the only amino acid small enough to occupy the crowded interior of the helix and it occurs as every third residue in most regions of the chain. Proline's cyclical structure facilitates the bending of the helical chain (Fig 2).

1.1.4 Classification of Collagens

Collagens can be classified by several criteria: length of molecule, molecular weight, flexibility of the molecule and by ultimate supramolecular structure. On consideration of supramolecular structure collagens can be classified into three groups: the fibrous collagens, the non-fibrous collagens and the filamentous collagens.[7]

Fig 2: Structures of principal amino acids in collagen. **[a] Glycine,** the simplest amino acid which has just a hydrogen atom as its side chain **[b] Proline,** which differs from the other amino acids in having a secondary amino group which produces a kink in an amino acid sequence; **[c] Lysine,** possessing one of the longest side chains of amino acids which causes considerably greater steric hindrance than glycine.

1- Fibrous Collagens

Fibrous collagens are seen in the transmission electron microscope as thick fibres with a characteristic axial repeat pattern of 67nm (Fig 1). There is considerable variation in fibril diameter - 25nm in the cornea to 200nm in the sclera. Size distribution may be uniform within a tissue (e.g. cornea) or highly variable (e.g. sclera). Only types I, II, III and V tropocollagen molecules form striated collagen fibrils visible in the electron microscope. When packed together these collagen fibrils can be seen in the light microscope as collagen fibres. Aging processes affect collagens fibrils in a number of ways: there is an increase in fibril size, tensile strength and mature crosslinks between triple helices and a decrease in solubility, reducible crosslinks and in susceptibility to proteolytic degradation.[7]

2- Non-Fibrous Collagens

Non-fibrous collagens are the principle collagens of non-fibrous membranes separating the fibrous stromal tissue from the cells. Non fibrous membranes vary in thickness from 25nm in capillaries to 200nm in the lens capsule. Only type IV collagen has been classified as a non-fibrous collagen and it has been localised in all basement membranes examined so far.

3- Filamentous Collagens

Filamentous collagens form loosely aggregated fibres with little or no periodicity and can be subdivided into pericellular and matrix collagens. Types VI, VII, IX and X are classified as filamentous collagens.

Collagen type	Molecular formula	Tissue distribution	Ultrastructure	Site of synthesis	Function
I	$[\alpha_1 (I)]_2\alpha_2$	Dermis, bone, tendon, dentin, fasciae, sclera, organ capsules, fibrous cartilage	Densely packed, thick fibrils with marked variation in diameter	Fibroblast, osteoblast, odontoblast, chondroblast	Resistance to tension
II	$[\alpha_1 (II)]_3$	Hyaline and elastic cartilages	No fibers, very thin fibrils embedded in abundant ground substance	Chondroblast	Resistance to intermittent pressure
III	$[\alpha_1 (III)]_3$	Smooth muscle, endoneurium, arteries, uterus, liver, spleen, kidney, lung	Loosely packed, thin fibrils with more uniform diameters	Smooth muscle, fibroblast, reticular cells, Schwann cells, hepatocyte	Structural maintenance in expansible organs
IV	$[\alpha_1 (IV)]_3$	Epithelial and endothelial basement membranes	Neither fibers nor fibrils are detected	Endothelial and epithelial cells	Support and filtration
V	$[\alpha A]_3$ and $[\alpha B]_3$	Placental basement membranes	i.d.	i.d.	i.d.

Table 1: Main characteristics of collagen types I-V. i.d.:insufficient data. Modified from Montes, Bezarra and Junqueira (1984).[170]

1.1.5 Collagen Types

Seven different polypeptide chains have been identified. Although there are at least a hundred different combinations possible when inserting three of these chains into a triple stranded helix, only thirteen have been described to date.

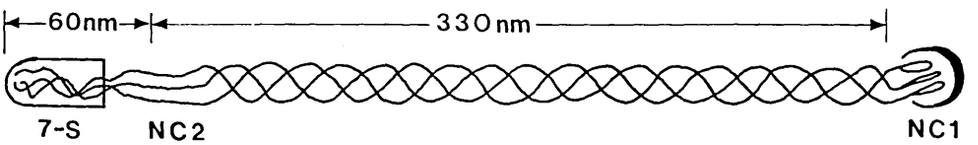
As a new type is discovered it is given the next Roman numeral above the most recently described type. Thus type I collagen was the first to be described, type II was the second, etc. Types I, II and III collagen are the main types of collagen found in connective tissue. Type I constitutes 90% of the collagen in the body. A summary of the main characteristics of the different collagen types is given in Table 1.

1- The Classical Collagens (I, II and III)

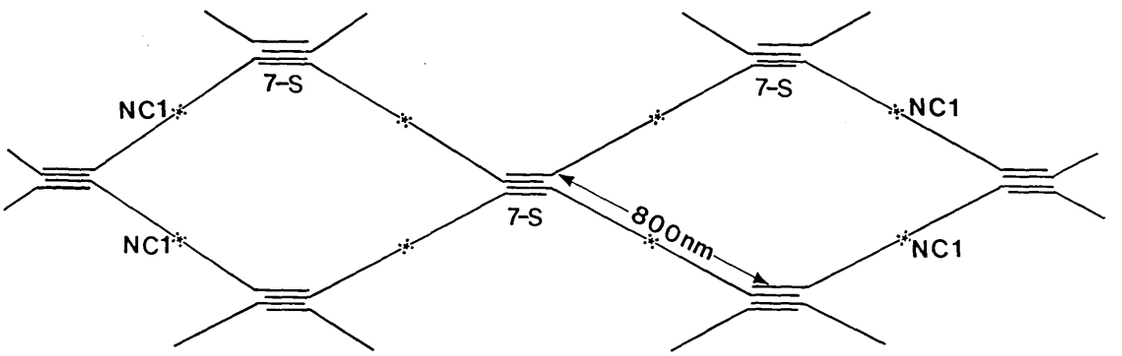
Types I, II and III collagens readily polymerize to form fibrils and fibers and serve primarily in organs requiring transmission of forces, mechanical support and stress control. The final processed form of types I, II and III collagen consist mainly of D period triple helical domains (97%). Type II collagen is more highly glycosylated than type I and generally forms smaller fibrils.[162]

All tissues in which collagen fibers have been described by morphologists, contain type I collagen which forms closely packed, thick collagen fibrils of variable diameters with a clear banding pattern. Collagen type III could be detected in all structures to which reticular fibers (loosely disposed, thin collagen fibrils of more

Type IV Collagen



[a] Single molecule



[b] Chicken wire network

uniform diameters) have been localized.[170]

Type III collagen has only been found in organs that also contain type I collagen, such as uterus, arteries, skin, intestines, lung, spleen, liver and kidney.[170] It is mainly related to smooth muscle cells. Thus it is in the extracellular spaces of the concentric layers of muscle cells of arteries while the adventitial layer and veins are composed mainly of type I. It is always present in tissues and organs that require a motile structural scaffolding.

2- Basement Membrane Collagen

Designated as type IV collagen, this collagen type is significantly longer than interstitial collagens (386nm compared to 300nm)[135] and unlike the interstitial collagens is not substantially processed when deposited in the matrix.[248] However, the triple helical domain is still the major structural element of the molecule.

The type IV collagen triple helix is thought to possess four domains (Fig 3a): two noncollagenous domains (NC1 and NC2) and two triple helical domains (the major triple helix and a small 7S domain).[248] The 7S and NC1 domains are strongly immunogenic, whereas the triple helical domain is only weakly immunogenic.[75] Timpl et al (1981) suggested that type IV collagen triple helices join together to form a chicken wire meshwork (Fig 3b).[248] A

Fig 3: Molecular arrangement of type IV collagen. **[a]** Four domain structure of type IV collagen molecule includes two triple helical segments (discontinuous major triple helix and 7-S domain) and two non-collagenous domains (NC1 and NC2). **[b]** Chicken wire network of type IV collagen. Four molecules attach to each other at the 7-S domain but only two join each other at the NC1 domain. Hence the length between two identical cross-linking sites (800nm) is the length of two type IV collagen molecules.

tertiary structure has also been proposed in which layers of chickenwire lattices may be built up in the plane of the basement membrane in a staggered fashion to allow intermolecular crosslinking.[8]

The direct involvement of type IV collagen has been shown in Goodpastures syndrome, a rare form of glomerulonephritis, which is an autoimmune disease in which the antigen is the NC1 domain of type IV collagen. This syndrome has been produced in mice by injecting NC1 domain.[135]

3- Type V Collagen

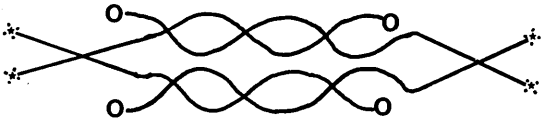
The primary structure of the carboxy propeptide and triple helical domain of type V collagen is homologous to types I, II and III. However, major differences exist in the amino propeptide region which remains part of the molecule when it is incorporated into a fibril.[134]

Immunogold electron microscopy has demonstrated the presence of type V collagen near basement membranes of human amnion but distinctly separate from the locations of laminin and type IV collagen. Labelling for type V included 12nm nonbanded fibrils enmeshed in the type I striated collagen fibrils and it has been postulated that these fibrils have an anchoring function between basement membranes and the stromal matrix.[167] Cultures of smooth muscle cells synthesize and secrete type V collagen over the entire plasma membrane,[167] but within tissue type V tends to be associated with only the stromal aspects of the cell surface.[57] Type V has been reported to promote cell attachment and migration.[235]

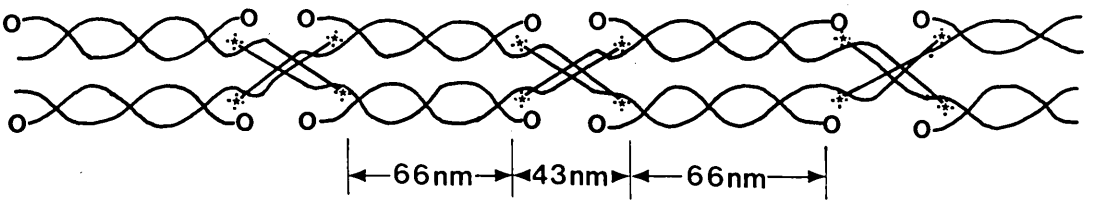
Type VI Collagen

[a] Single molecule

[b] Dimer



[c] Tetramer



[d] Extended aggregation

4- Type VI Collagen

Type VI collagen is quite distinctive from the other collagens in having only a third of its length forming into a triple helix.[31] The trimer molecules, which have the appearance of a dumbbell, assemble into well-defined dimers and tetramers (Fig 4).[246]

Although type VI collagen is located in extracellular spaces which also contain types I and III collagen and fibronectin, differences in staining patterns indicate a lack of co-distribution with these proteins.[246] Basement membranes appear to be negative,[31] nor is type VI collagen directly associated with banded collagen fibrils,[1,261] but with finely fibrillar networks. In skin, type VI filaments are highly concentrated around endothelial basement membranes, forming a loose sheaf around blood vessels, nerves and fat cells and appearing to separate these elements from the surrounding banded fibril network. This network is viewed as a branching array, generally aligned in parallel with the banded collagen fibril axes, but also traversing them. There is no apparent direct connection to the banded fibril network, the elastic microfibrils nor to the basement membranes.[31]

The function of type VI collagen is as yet unknown. Fibroblasts can attach and spread on type VI substrates.[246] Similar interactions may occur **in vivo** as collagen VI filaments are often deposited in close vicinity to cells.[246]

Fig 4: Molecular arrangement of type VI collagen. **[a]** Single type VI molecule, line representing triple helical domain and circle and dot the globular domains. **[b]** type VI dimer; **[c]** type VI tetramer; **[d]** type VI extended aggregation.

Aberrations in the synthesis of type VI collagen may be involved in pathological disorders. Type VI collagen synthesis was found to be greatly increased in cultured fibroblasts from a patient with cutis laxa,[246] a rare, acquired or inherited syndrome characterised by loss of skin elasticity. It is not unlikely that patients with other inherited connective tissue disorders (e.g. Marfans's syndrome) show changes in type VI collagen synthesis.[246]

5- Collagens VII-XII

As collagens VII-XII are outwith the scope of this study, a description of their structure and function cannot here be justified. The reader is therefore referred to review chapters in **Structure and Function of Collagen Types.**[160]

1.1.6 Methods of Studying Collagen Distribution

For the study of collagen type distribution in tissues three methods have been used, namely biochemical, histochemical and immunocytochemical methods.

Biochemical methods are limited in that preferential extraction of one collagen type over another occurs (probably due to differences in cross-linking and in association with other macromolecules), losses result from the purification procedures and dissecting out a pure sample of the particular tissue of interest is fraught with difficulties. However, the most serious disadvantage with biochemical methods is that they give no information as to the precise distribution of collagen types within a tissue.

Collagen molecules, being rich in basic aminoacids, strongly react with acidic dyes. As collagen molecules are

disposed in a parallel orientation they exhibit birefringence. Sirius Red, an elongated strongly acidic dye, enhances collagen birefringence as the long axis of the dye molecules attached to collagen molecules are parallel.[111] This is further enhanced by the Picrosirius-polarization method which is thought to be able to differentiate between collagens I-III by differences in colour. However, colour differences also arise from folds in tissue sections, section thickness and collagen fibre thickness.[112,193]

1.2 Immunocytochemistry

Immunocytochemistry is the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction in which the antibody is tagged with a visible label.

The first label to be employed was the enzyme peroxidase in which the enzymatic product (3,3- diamino-benzidine) was visualized for electron microscopy by osmium tetroxide.[149] Other enzyme labels have been used (alkaline phosphatase and glucose oxidase). Antibodies have also been labelled with radioactive elements and the immunoreaction visualized by autoradiography. Electron dense labels such as ferritin and colloidal gold are also employed to identify immunocyto-chemical reactions at the ultrastructural level.

The original direct labelling of the antigen with tagged antibodies has evolved into an indirect method in which the first or primary antibody is not conjugated, but a second layer is added which consists of an antibody raised against the gamma-globulin (IgG) of the species which donated the primary antibody. This facilitates several advantages:-

[1] Anti-IgG sera are usually hyperimmune and of very high avidity.

[2] Two labelled anti-Ig molecules can bind to each primary antibody molecule, increasing the sensitivity of the reaction.

[3] One tagged second layer antibody can be used to stain any number of first-layer antibodies to different antigens provided all the primaries have

been raised in the same species. Conjugated second-layer antibodies are now widely available. [195]

1.2.1 Cross-Reactivity

Unless an antibody reacts with an antigenic sequence which is unique to one substance, there is always the chance of a reaction to a similar sequence in another substance (cross-reactivity). If the titre (concentration) of antibody in the serum is high enough, the primary antibody can sometimes be diluted to such an extent that most of the unwanted reactions become negligible. The size of the antigenic site affects cross-reactivity. Antigenic sites usually consist of a sequence of 4-7 amino acids. The shorter the sequence, the higher the antibody's chances of finding similar sequences in other substances. The antibody's affinity (i.e. the three dimensional fit of the antibody molecule to its specific antigen) is also affected by the antigen's size. The longer the sequence, the higher will be the affinity and the lower the chances of dislodging the antibody from its antigen during the vigorous washing processes of the immunocytochemical procedure. The most satisfactory way of eliminating unwanted staining is to use affinity-purified or monoclonal antibodies throughout. [194]

1.2.2 Immunocytochemical Markers

For many years the peroxidase-anti-peroxidase method [236] has been the technique of choice for immunocytochemical localisation of tissue bound antigens. Apart from the toxity of DAB, one drawback of the procedure is that the

fine structure of the immunostained organelles is largely obliterated by the homogenous depostion of the final reaction product. Furthermore, conventional counterstaining with heavy metal salts cannot be performed profitably on the immunostained sections,which prejudices the precise interpretation of tissue morphology.[258] This outweighs the practical advantage of enzyme-labelled antibodies over particle-tagged antibodies in the generation of reaction products by enzymatic activity of the tag,leading to an enhancement of antigen site detection.[132]

It has been suggested that the wide acceptance and usefulness of enzyme-labelled antibodies was due to their smaller size relative to particle-tagged antibodies (i.e. ferritin and gold) enabling them to diffuse past cellular barriers.[132] Newell et al exploited this advantage but their technique involved saponin digestion of glutaraldehyde prefixed cells to enhance conjugate penetration which led to serious loss of ultrastructure.[179,180]

Although ferritin-tagged antibodies have been applied to ultrathin plastic sections, the degree of non-specific background due to the interaction of tracer with the embedding matrix has limited the usefulness of this procedure.[240] Ferritin has been successfully used with ultrathin frozen sections but its relatively low electron density is a major difficulty.[71,72,189]

1.2.3 Gold Conjugates

The interest in the use of gold-tagged antibodies as an immunocytochemical marker has greatly increased for a

number of reasons:-

[1] The efficiency with which gold granules can be detected is much higher than that of the enzyme reaction products since it renders possible the localization of antigenic sites with inhomogenous electron density.[219] Gold probes can therefore be used in conjuction with optimally fixed and contrasted thin sections as they do not obscure the ultrastructural details of the labelled structures.[194] This has permitted very fine localisation of antigens, an example being the demonstration of actin's association with several membranes and in the dense content of zymogen granules of rat pancreas.[15]

[2] The particulate nature of the gold probe allows the indirect estimation of the amount of antigenic sites. Individual gold particles are assumed to be in the numbers proportional to the number of antigenic sites: the numbers of particles found over zymogen granules labelled with different enzyme antisera in rat exocrine pancreas, were in good agreement with the respective enzymatic concentrations found by biochemical analysis of rat pancreatic juice.[16]

[3] The use of various monoclonal antibodies linked to gold particles of different sizes has facilitated the performance of double labelling experiments.[13]

[4] Monoclonal antibodies have been shown to provide a first choice material for labelling with colloidal gold. The simplicity and high yield with which monoclonal antibody gold reagents are manufactured has led to the prediction that direct labelling with monoclonal antibody gold probes will become common

practice.[194]

[5] Accumulations of gold-tagged antibodies over antigen-containing sites can be seen in the light microscope, the typical red colour forming without having to incubate with an enzyme substrate.

[6] Gold particles are capable of strong emission of secondary electrons, making them a very useful marker for scanning electron microscopy.

1.2.4 The Fixation Dilemma

The quality of ultrastructure and the preservation of antigenicity is greatly affected by the type of fixative, its concentration, the duration and temperature of fixation. Unfortunately, ultrastructural preservation and the preservation of antigenicity are often in diametrical opposition. Fixation protocols that produce good ultrastructure invariably destroy antigenicity and vice versa. Only the most robust antigens survive the standard fixation regime of 2% glutaraldehyde followed by 1% osmium tetroxide which normally ensures very satisfactory ultrastructural preservation. More sensitive antigens are adequately preserved with low concentrations of paraformaldehyde, but the concomitant loss of ultrastructure is severe.

1- Glutaraldehyde

Although glutaraldehyde is unsurpassed in preserving fine structure it also excels in the destruction of antigenicity, particularly with large protein antigens as their reacivity depends not only on the primary structure, but also on conformational features.[29] Loss of antigenicity is

considered to be primarily caused by the change in tertiary structure of the proteins,[165] which is to be expected since the dialdehyde is an extremely efficient protein crosslinking agent.[94] However, when considering the aldehydes one must also remember that both intermolecular and intramolecular bridges may be formed and that the numerous crosslinkages may cause masking of antigens by steric hindrance. This phenomenon is pronounced when the actual antigen is mixed with high concentrations of other proteins.[211]

For a given concentration of glutaraldehyde, loss of antigenicity is progressive for exposure times up to one hour, but thereafter there is little further loss.[234] In general, inactivation proceeds progressively and markedly from 0% to 0.5% glutaraldehyde, but from 0.5% to 2% antigenicity is not severely depressed any further[132] although the response of individual antigens varies quantitatively. For instance, when 0.5% glutaraldehyde is used for 16hrs at room temperature, 40% of the capacity of bovine trypsinogen to bind to its antibodies is lost,[131] whereas 2% glutaraldehyde used for one hour at 0°C has little effect on antibody binding capacity of bovine ribonuclease.[189]

Concerning ultrastuctural preservation, glutaraldehyde concentrations as low as 0.25% give adequate fine structural preservation in rat liver, but for guinea pig pancreas it is necessary to use at least 0.5% glutaraldehyde to obtain adequate preservation since at lower concentrations obvious extraction of zymogen granules occurs.[132] Glauert (1974) recommended 4°C for glutaraldehyde fixation but has since concluded that

tissues from warm-blooded animals are best fixed by aldehydes at, or just below their physiological temperature, as the increased rate of penetration outweighs the increased rate of enzymatic breakdown (personal communication).

2- Osmium Tetroxide

Although osmium destroys antigenicity, levels of 0.025% osmium tetroxide may well be sufficient to fix proteins in membranes without subsequent extraction; a viewpoint that is gaining considerable support.[29] Pancreatic secretory proteins and insulin have been successfully localised on osmium and glutaraldehyde fixed Epon embedded sections after etching with sodium metaperiodate.[17] The treatment with strong oxidizing agents has been postulated to act by a reoxidizing of the reduced osmium molecules making them into soluble tetroxides, leading to their removal. These therefore might be removed from the crosslinked macromolecules, unmasking the antigenic sites.[17,44]

3- Formaldehyde

Compared to glutaraldehyde, formaldehyde is relatively less destructive to antigenicity but is also less efficient in preserving cellular structure.[94] The deleterious effect of the fixative on antigenic reactivity may be reversed by extensive washing which hydrolyzes the weak crossbridges.[29]

4- Periodate-Lysine-Paraformaldehyde

Another solution may be to devise a fixative that would preserve cellular structure by crosslinking primarily

carbohydrates and lipids rather than proteins. This approach may prove useful because many antigens contain some carbohydrate moieties that could be fixed with little loss of the antigenicity that resides in the accompanying protein moiety. In such a procedure, the hydroxyl groups of carbohydrates are oxidized with periodate to produce aldehydes which in turn can be made to react with diamino compounds (e.g. lysine), resulting in the crosslinking of carbohydrates.[94] Paraformaldehyde is added to achieve some stabilization of proteins and lipids. Basement membrane antigen has been localised in parietal yolk sac cells with this mixture allowing adequate tissue preservation, a task that has not been accomplished by conventional fixatives.[165]

1.2.5 Labelling of Resin Embedded Sections

All the preparatory steps in embedding biological material adversely affect antigenicity. Such embedding procedures elicit conformational changes of proteins by exposing them to denaturing fixatives,[133] organic solvents[34] and high temperatures (during resin polymerization).[218] Cryoenzymology and cryocrystallography have shown that solvent induced conformational changes can be minimized by low temperatures.[34] The influence of the embedding medium on the protein conformation is not well understood but the environment provided should be as polar as possible to minimize disruption of protein conformation.[118,234] These considerations led Carlemalm et al[34] to introduce Lowicryl K4M and the progressive lowering of temperature technique.

A similar rationale lies behind the use of LR white

resin for embedding partially dehydrated tissue (up to 70% alcohol), thereby maintaining hydration shells around antigenic epitopes which results in increased antigenic conservation.[181]

1.2.6 Cryoultramicrotomy

In cryoultramicrotomy immunolabelling is performed prior to heavy metal staining and plastic embedding, but subsequent to fixation and ultrathin sectioning. This technique should have great potential in preserving antigenicity since the only process deleterious to antigenic preservation is fixation.

The infusion of 2.3M sucrose into tissue ensures routine vitrification by immersion in liquid nitrogen.[84] Freezing damage is essentially non-existent in vitrified material.[3,161] Consequently, no improvement in the preservation of the sample can be expected by using more elaborate cooling methods.

Griffiths (1984)[83] reckoned that the two best techniques presently available for localising antigens on ultrathin sections were the Tokuyasu thawed frozen-thin section technique and the low temperature embedding method; a view shared by Roth (personnal communication). A direct quantitative comparison found up to ten times more specific labelling of ultrathin frozen sections than on Lowicryl sections as did a preliminary study by Griffiths.[83]

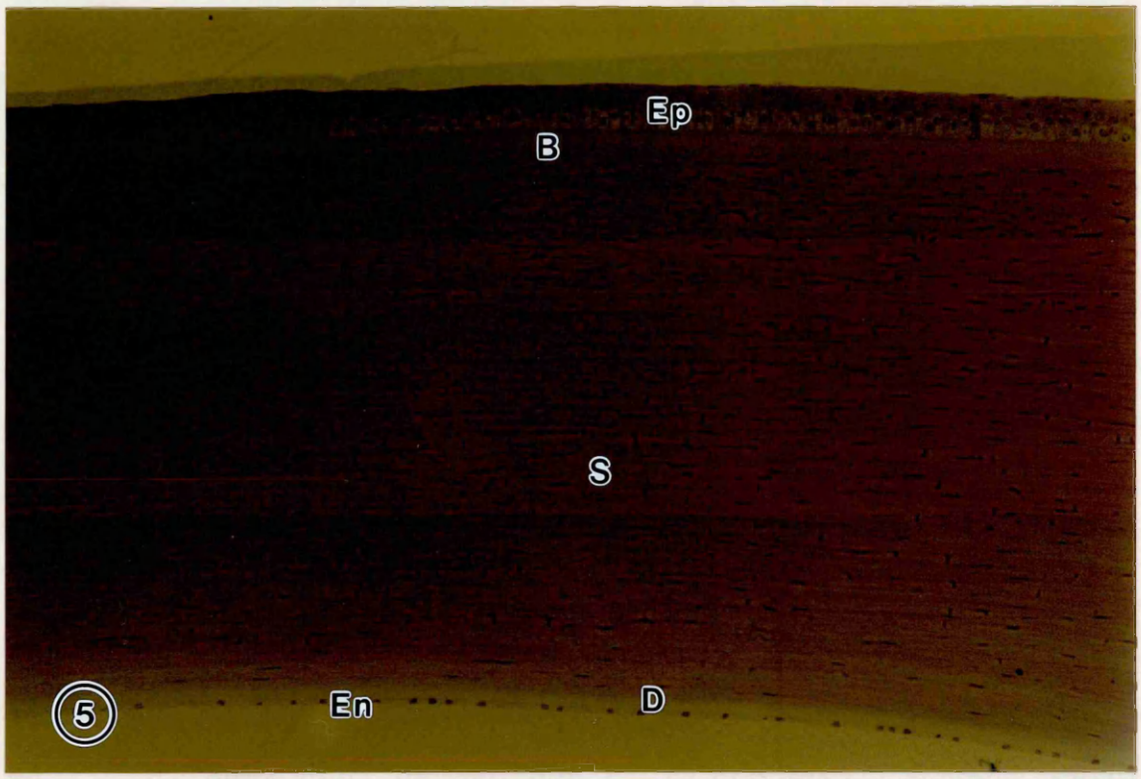
Although the frozen-thin section technique has increased in popularity it is still not universally accepted or appreciated. This is partly because it is considered to be so technically difficult[218] and partly because the fine structural appearance and contrast of the

cells has been poor.

1- Methyl Cellulose Embedding

When the thawed cryosectioning technique was introduced, excellent fine structural preservation was demonstrated on stained, air-dried sections.[249] The negative staining procedures used helped to protect sections against air-drying artifacts, but the resultant high degree of contrast made it incompatable with antibody labelling studies.[84] A compromise of using lower stain concentrations was unsatisfactory because structures could not be clearly visualized and air drying artifacts became a serious problem.[251] The introduction of methyl cellulose to protect thawed frozen sections against surface tension damage caused by air drying was an essential step in the development of the technique.[250] This subtle procedure, together with the initial aldehyde fixation, determines the structural preservation as well as section contrast.

Methyl cellulose was chosen as a hydrophilic embedding material as it is chemically inert, mechanically strong when dried and quite soluble in water near 0°C, but much less so at room temperature. These characteristics make the embedding procedure very simple, with the latter feature contributing to the stability of the embedment against moisture from air.[251]


Ep
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1.3 Cornea

The cornea and the sclera form a tough protective envelope enclosing the more delicate ocular structures. The main function of the cornea lies in its optical properties as it is the principle refracting tissue of the eye: the air/corneal surface interface is the primary refracting surface of the ocular optical system. The almost perfect transparency is therefore an essential feature of the cornea and this is thought to be due to the specific arrangement of the collagen fibrils within it.

1.3.1 Anatomy

Anatomically, the cornea can be divided into five layers (Fig 5), three of which are largely composed of collagen of various types (Bowman's layer, stroma and Descemet's membrane).

1- Basement membrane complex

The corneal epithelium is exposed to shearing stress due to the regular movement of the eyelids across its surface. It therefore has a specialized structure, the basement membrane complex, anchoring the epithelium to Bowman's layer. This basement membrane complex is also present in skin but it is in its most highly developed form in the corneal epithelial/ Bowman's interface:[24] a reflection of

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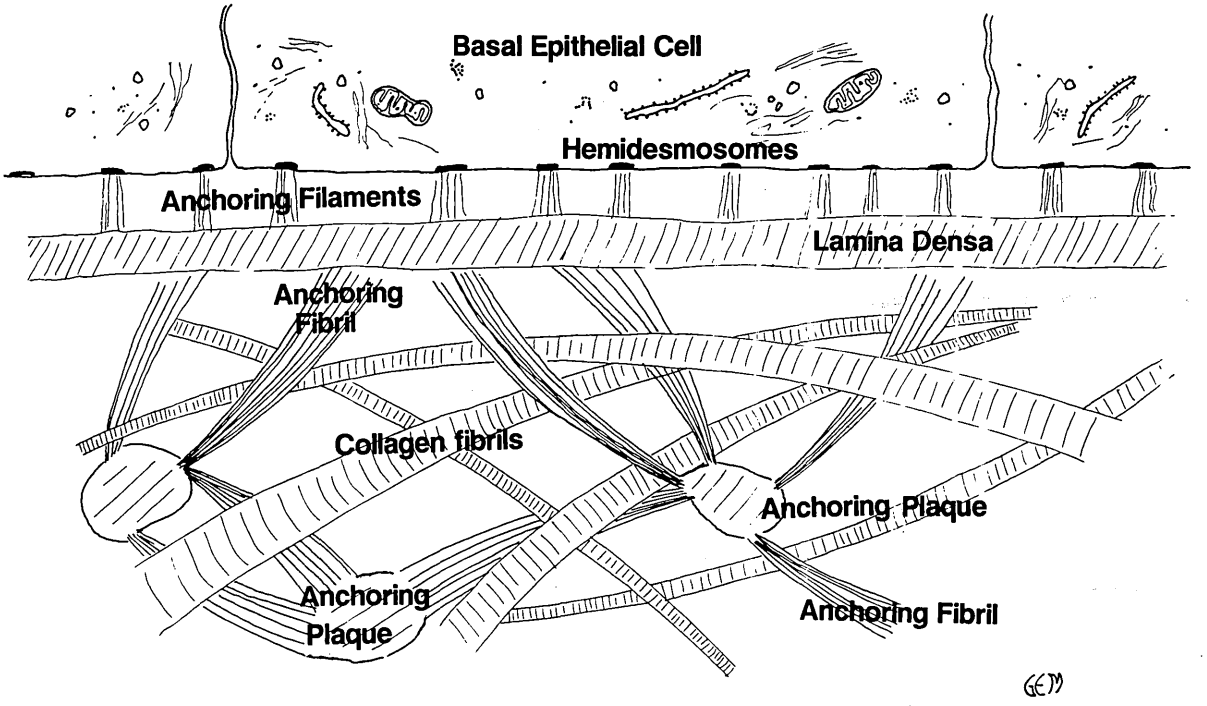
Fig 5: Cornea full thickness. Multilayered epithelium (Ep) is markedly thicker than endothelium (En) but underlying Bowman's layer (B) is not so distinctive as Descemet's membrane (D). Stroma (S) occupies about nine tenths of corneal thickness. Long narrow fibrocyte processes, separating stromal lamellae, lie parallel to corneal surface.



Basement Membrane Complex

the degree of shearing force to which the corneal epithelium is exposed.[30]

Ultrastructural studies have revealed that it is an oversimplification to regard the basement membrane as a single and simple homogeneous layer.[24,116] Examination of the fine structure reveals a basement membrane complex with several components (Fig 6).

2- Bowman's layer

Bowman's layer is a 8-14um thick[101] acellular layer composed of randomly distributed collagen fibrils that form a tightly packed meshwork. This meshwork makes Bowman's layer very resistant to tearing forces, and therefore many consider it to provide considerable protection against penetration of foreign bodies. However, rabbits do not possess a Bowman's layer despite being more at risk to foreign bodies as they don't blink. Although synthesised by the epithelium in the embryonic cornea Bowman's layer cannot be regenerated after injury.

The collagen fibrils of Bowman's layer are uniform in diameter, thinner and less tightly packed than those in the

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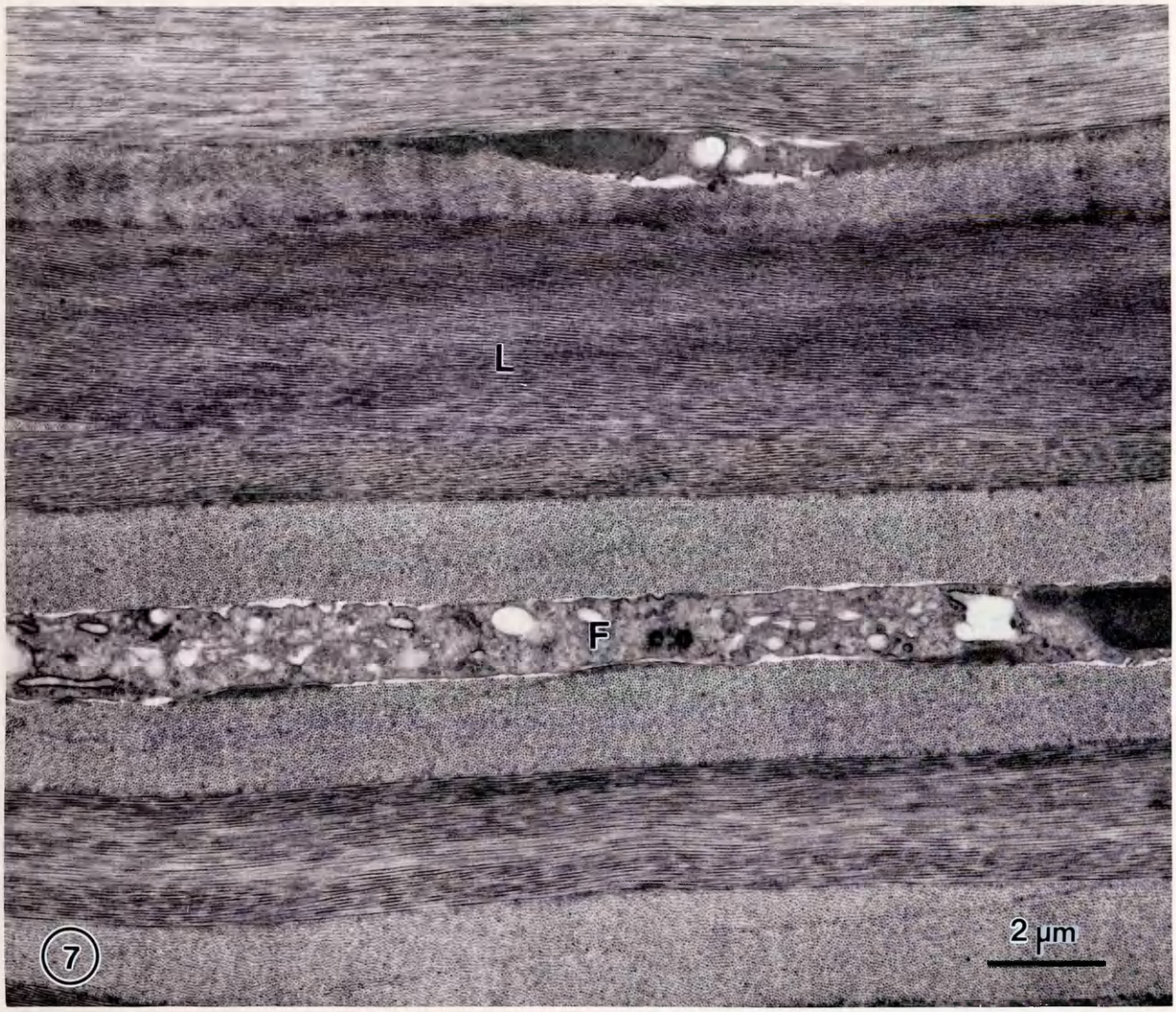
Fig 6: Diagrammatic representation of basement membrane complex. **Hemidesmosomes** (maculae adhaerentes) rivet epithelium to underlying **lamina densa** (basement membrane) by means of **anchoring filaments** which cross the lamina lucida (lamina rara externa) at right angles.[25] **Anchoring fibrils** project into Bowman's layer from the lamina densa, attaching the lamina densa to **anchoring plaques**, which lie in Bowman's layer. Thus these anchoring fibrils form a large scaffold throughout the sub-basal lamina (lamina rara interna), entrapping large numbers of collagen fibrils.[30,116] It is the entrapped collagen fibrils that prevent the anchoring fibril network from being pulled out of Bowman's layer. The anchoring fibril network prevents the lamina densa from separating from Bowman's layer[24] and hemi-desmosomes prevent the epithelium from separating from the lamina densa. Thus is the epithelium anchored to Bowman's layer.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corneal stroma. There is disparity between morphometric studies on the precise diameter of the collagen fibrils in Bowman's layer.[101,108,115] There is no distinct border between Bowman's layer and the stroma as there is a blending of collagen fibrils between the two layers.

3- Stroma

The stroma is the principle component of the cornea, constituting nine tenths of the corneal thickness in the human (500 m).[101] The regular arrangement of collagen fibrils in the stroma is striking (Fig 7). The collagen fibrils are not only uniform in diameter (34-40nm) and equally spaced (20-50nm) but also lie parallel to each other. On light microscopic examination the stroma is seen to be composed of a large number of layers (about 200), termed lamellae. The lamellae are frequently separated from each other by keratocytes. Collagen fibrils within a lamella are parallel to each other, but run in a different direction to fibrils of adjacent lamellae (Fig 7).

The cornea's almost perfect transparency is thought to be due to the regular arrangement and uniform diameter of the collagen fibrils within the stroma.[41]

4- Descemet's Membrane

Descemet's membrane is composed of basement membrane material which exhibits a highly ordered three dimensional lattice pattern when viewed in tangential section. This

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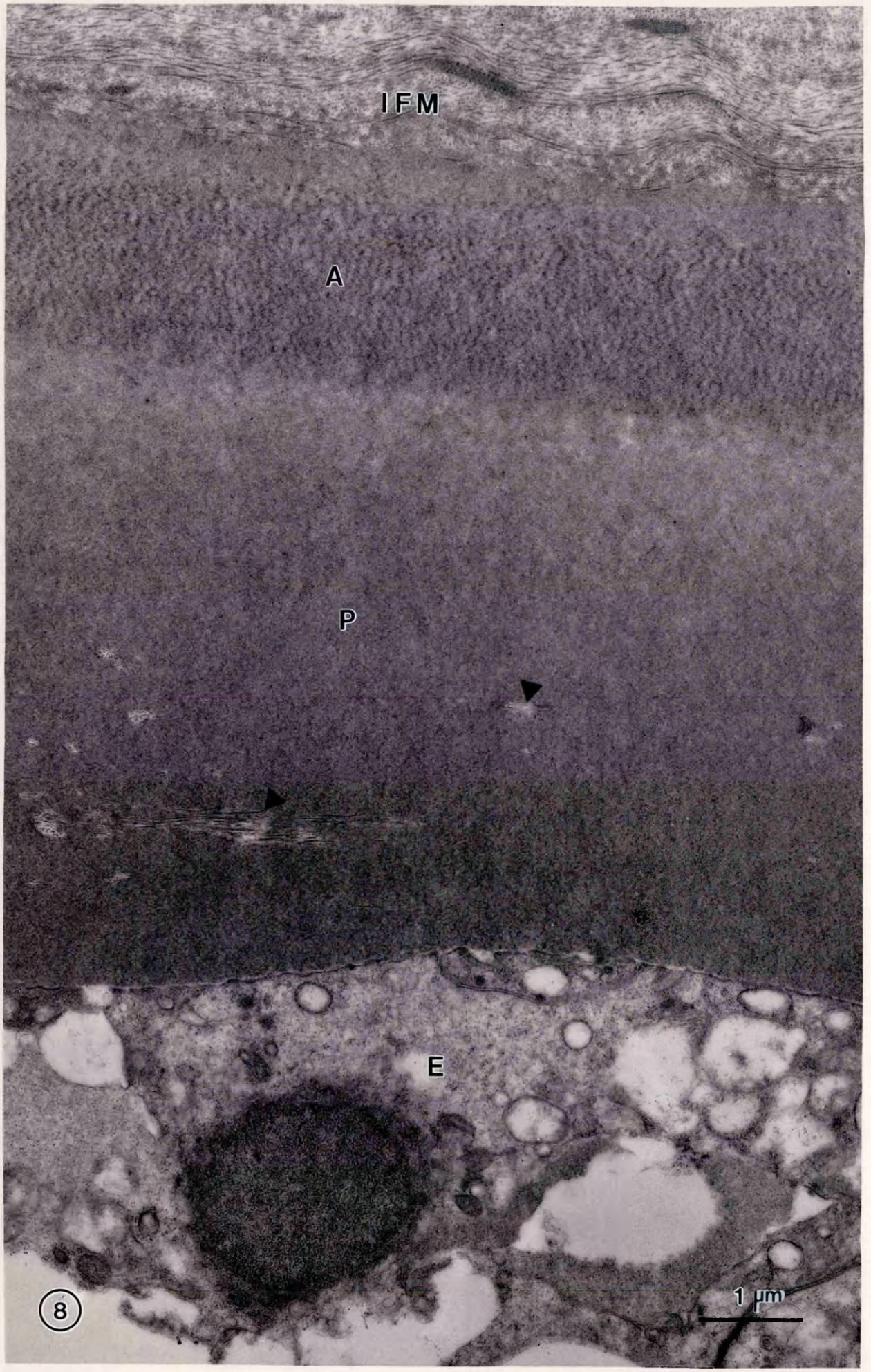
Fig 7: Collagen lamellae (L) in corneal stroma. Collagen fibrils within a lamella are parallel to one another, but fibrils within one lamella run in a different direction to those in adjacent lamellae. Stromal lamellae are frequently separated from one another by flattened fibrocytes (F) sandwiched between them.



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basement membrane of the corneal endothelium thickens with age (3-4 m at birth to 10-12 m in adult) with the appearance of a posterior non-banded region (Fig 8).

1.3.2 Biochemistry

The principle biochemical component of the cornea is collagen (71% dry weight).[123,163] It is widely accepted that type I collagen is the major collagen type of the cornea. Biochemical analysis reveals that the mammalian cornea contains substantial amounts of this collagen type. However, there is a notable species variation; for example type I constitutes 85% of the total collagen in the human cornea,[183] 94% in the calf[49] and a "substantial proportion" in the rabbit.[264]

The most contentious issue concerning collagen types within the cornea centres around the presence of type III collagen. Biochemical analysis has indicated the absence of type III collagen in the rabbit cornea,[264] whereas immunofluorescence studies revealed its presence in Descemet's membrane.[39] Biochemical analysis has shown type III to be present in the human cornea (greater than 10% dry weight).[183,199] Even within the same species, biochemical data obtained from solubilized corneal collagen extracts is irreconcilable. For example, in one biochemical study of bovine cornea, type III collagen could not be detected,[49]

Fig 8: Descemet's membrane of 64 year old female in conventionally processed tissue. A thin zone of non-banded basement membrane is sandwiched between the anterior banded region with a 100nm banding pattern (A) and the most posterior region of the stroma, termed the interfacial matrix (IFM). A number of collagen fibrils (arrowheads) are present in the posterior non-banded region (P) near the endothelium (E). Bar=0.5um.

whereas other studies have demonstrated its presence[199,227] with one recording levels as high as 20% of the total collagen content.[227]

The amount of type V collagen in the mammalian cornea has been estimated by biochemical analysis to be around 7-10%,[63,137,138] and about 5%in the human cornea.[183,199] In this context, it is of interest to note that the ratio of type V to type I collagen is high in the cornea compared to other tissues such as sclera, tendon, bone and dermis.[26, 64,102]

1.3.3 Immunohistochemistry

Attempts to document the distribution of collagen types I-IV in the human cornea using immunofluorescence have not yielded consistent results. Light microscopic studies using immunohistochemistry have demonstrated type I collagen in the corneal stroma of a number of species such as human,[18,177,257] avian,[23,93,95,259] murine[89] and bovine.[138,175] The questionable value of light microscopic immunohistochemistry is illustrated by the fact that type I collagen has been reported as both present in[18,182] and absent from[177,257] Bowman's layer of human cornea.

Most disagreement is related to the presence and distribution of type III collagen. Ben-Zvi et al (1986) described type III as being present in early human life but no longer detectable after 27 weeks gestation. Newsome et al (1981) could only detect type III collagen in Bowman's layer and the anterior stroma, but Nakayasu et al (1986) obtained equally positive labelling for type III as for type I collagen in the human cornea. Tsuchiya et al

(1986) who also localised type III collagen to human corneal stroma suggests that Newsome's negative results were due to the absence of pretreatment to unmask epitopes. The controversy may in part be due to the technical inadequacies of the immunofluorescent technique which yields little information as to the precise localization of these antigens. In some instances, the use of autopsy tissues as controls may have prejudiced the reliability of the findings. It is therefore essential to determine the effects of autolysis on the pattern of collagen localization.

Literature on immunoelectron localization of animal corneal collagens is relatively sparse[23,89,93,95,198] -the majority of the cited studies have been limited to only two collagen types. Nevertheless, one study has revealed valuable information on the co-distribution of types I and V within the same collagen fibril of the avian cornea.[23]

Type VI collagen has a ubiquitous distribution throughout connective tissues[246] and has been shown to be associated with fine lineal aggregates between banded collagen fibrils of rat tail tendon.[117] It has been localized to fine filamentous structures in the avian cornea that are quite distinct from striated collagen fibrils[147] and which are probably located between the striated fibrils.[28] The distribution of type VI collagen has not been documented in the human cornea.

As far as I am aware, only one immunoelectron microscope study[177] has been performed on the distribution of collagen types within the human cornea. This study was limited to types I, III and V collagen and employed the immunoperoxidase technique which is limited as a tracer due

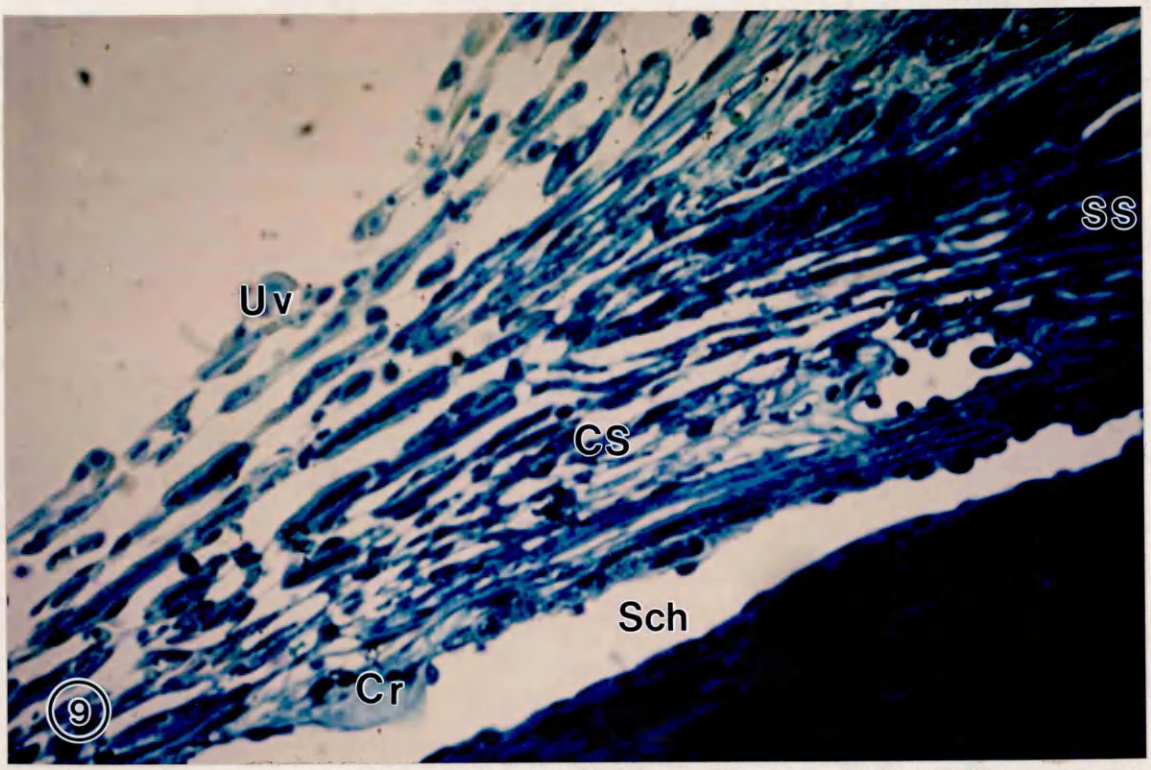
to diffusion of the electron dense tracer molecules. The immunogold technique was used in the present study in an attempt to elucidate the fine structural distribution of six collagen types and laminin in the aged human cornea, in order to provide baseline information for further studies on pathological disturbances. Aged cornea is a particularly relevant normal control as most pathological disturbances occur in aged patients.

1.3.4 Function

It has been suspected that type V collagen may play a vital role in controlling collagen fibril diameter[22] as it has been found to co-distribute with type I collagen within the same striated collagen fibril.[22,23,61]

1.3.5 Conclusion

Disagreements concerning the collagen constituents of the cornea can be explained by the intrinsically unreliable techniques employed for detection, species variation and age related changes. Until the precise localization of extracellular matrix components is established it will not be possible to proceed with meaningful investigations of pathological abnormalities in collagens. As the dividing line between simple ageing changes and the onset of pathology is very indistinct, it is equally important to document changes which occur as part of the ageing process.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1.4.1 Anatomy

Intraocular pressure is determined by the resistance in the aqueous outflow system to the passage of aqueous humour production. The aqueous outflow system is anatomically divided into several regions (Fig 9), some of which will be considered in detail.

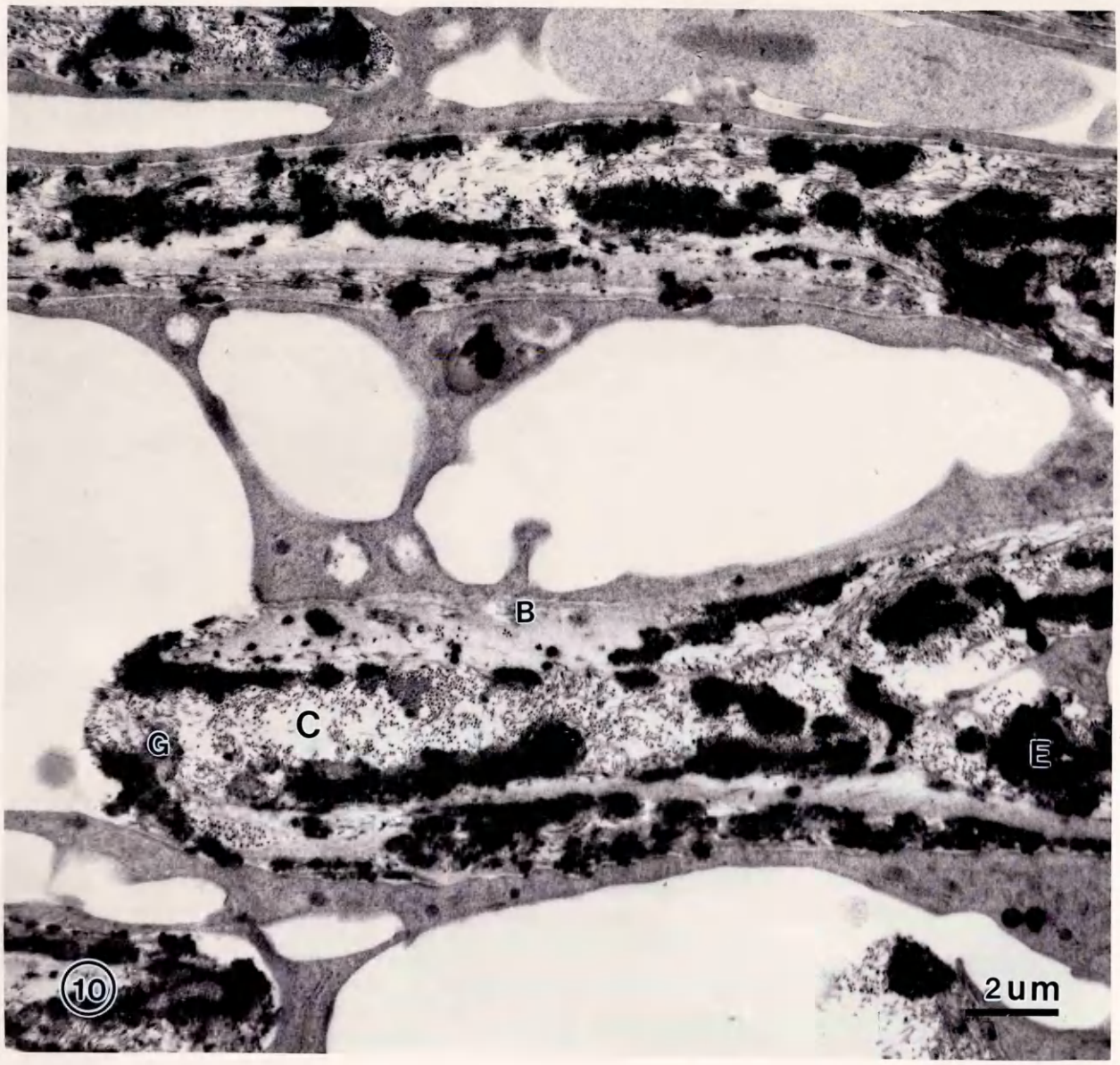
1- Uveal Meshwork

Aqueous humour first traverses the uveal meshwork in its escape from the anterior chamber angle. The uveal meshwork is composed of cords of tissue two to three layers deep. Each cord, which is 4-6um in diameter, comprises a collagenous core and a surrounding layer of endothelial cells. Collagen fibrils of the core run parallel to the long axis of the cord.

2- Corneo-scleral Meshwork

By scanning electron microscopy the corneo-scleral meshwork is seen to differ from the uveal meshwork in that it is composed of a large number of perforated sheets rather than thin cords. These sheets branch and interlace with each other to form a three-dimensional grid within which are intertrabecular spaces (Fig 10). Trabecular sheets consist of a collagenous core surrounded by finely granular basement membrane which is covered by a lining of endothelial cells.

Fig 9: Outflow apparatus. Trabecular meshwork is divided into inner uveal (Uv) and outer corneo-scleral (CS) portion by a line drawn from tip of scleral spur to edge of cornea. Sch=Schlemm's canal; Cr=Cribriform layer; SS=Scleral spur;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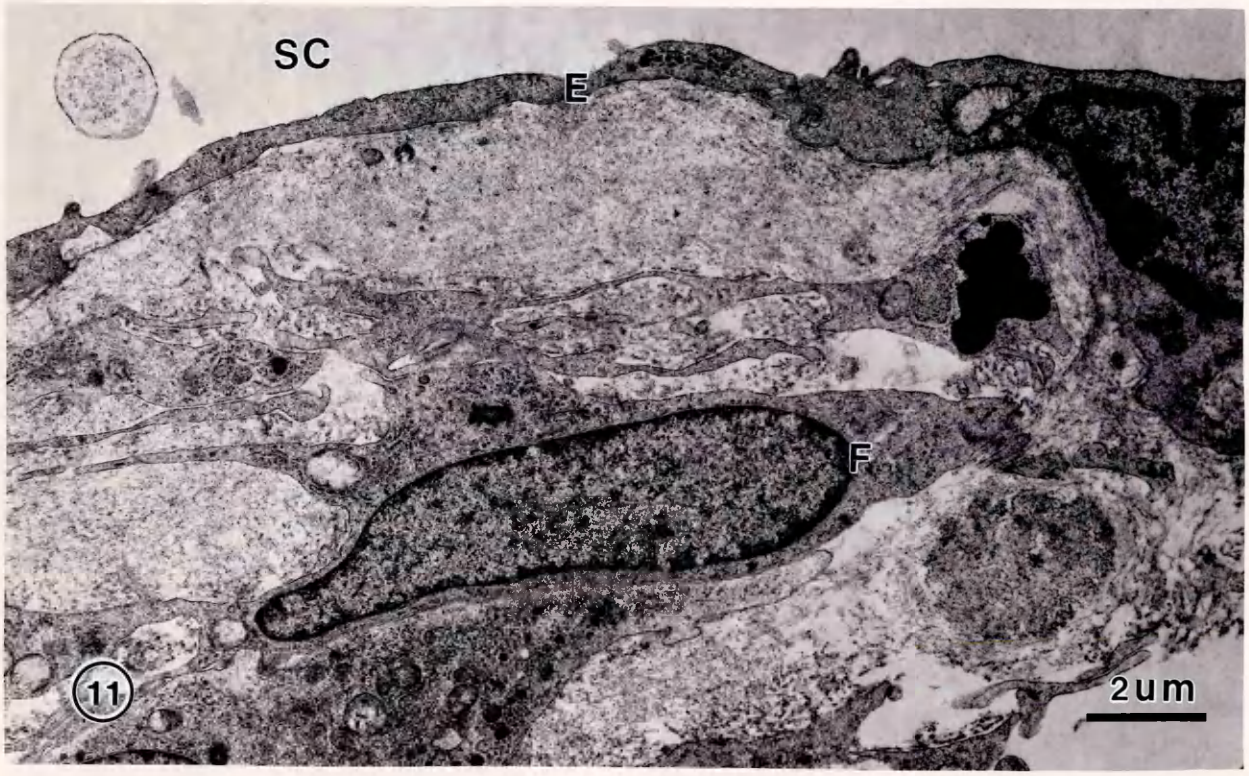
SC
E
F
11
2um

3- Cribriform Layer

The cribriform layer is also referred to as the "inner wall of Schlemm's canal", the "endothelial meshwork" and erroneously as the "juxtacanalicular apparatus"; erroneous in that the juxtacanalicular apparatus includes the entire endothelial lining of Schlemm's canal. The cribriform layer differs significantly from the corneo-scleral meshwork in that it does not possess trabeculae. This layer, measuring 10-20 m in thickness, is composed of several layers of endothelial cells interspersed with collagen fibrils and a variety of extracellular matrix components (Fig 11). Aqueous humour traverses the lining endothelium of Schlemm's canal through giant vacuoles.[4,81,82]

1.4.2 Trabecular Extracellular Matrix Components

In order to ascertain the extent to which pathological accumulation and/or alteration of the extracellular matrix (ECM) within the meshwork are involved in the pathogenesis of primary and secondary glaucoma, careful documentation of ECM components in the normal aged meshwork is necessary. With regard to morphological documentation, numerous investigations using conventional

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Fig 10: Corneo-scleral sheet. Dense elastin-like fibres (type II plaques[E]) with their accompanying fine granular-like material (type III plaques[G]) form the core. Collagen fibrils [C] are cut in transverse section as they run round the circumference of the limbus. B=basal lamina of endothelial cells.

Fig 11: Cribriform layer. Several layers of fibroblast-like cells [F] lie parallel to the lining endothelium [E] of Schlemm's canal [SC]. Intercellular spaces are filled with extracellular matrix material

electron microscopy, have focussed on the important role of the various ECM components in changes in resistance of the outflow system.[80,103,212,213,215,229,244] However, the precise biochemical nature of the structural proteins which provide ultrastructural images of the ECM components is unknown.

Tawara et al[244] divided extracellular matrix components of the trabecular meshwork into two broad categories : fibrous elements and ground substance. The fibrous elements consisted of collagen fibrils and elastin-like fibers. Elastin-like fibers are of high electron density and are present in the trabecular beams, the juxtacanalicular tissue and in the outer wall of Schlemm's canal.

Four types of material were collectively classified as ground substance :- the basal lamina, basal lamina-like material, fine granular-like material and fine fibrillar-like material.

The basal lamina refers to the basement membrane of trabecular endothelial cells and basement membrane-like material closely apposed to endothelial cells in the cribriform layer. Basal lamina-like material exists in the form of a homogenous linear profile not associated with endothelial cells. The fine granular-like material is composed of a homogenous component with electron lucent profiles of granular particulate appearance and is frequently associated with elastin-like fibres. The fine fibrillar-like material, present principally in the juxtacanalicular connective tissue especially near Schlemm's canal, consists of a mixture of short fibrils (10-20nm in diameter) embedded in an electron lucent

homogeneous matrix.

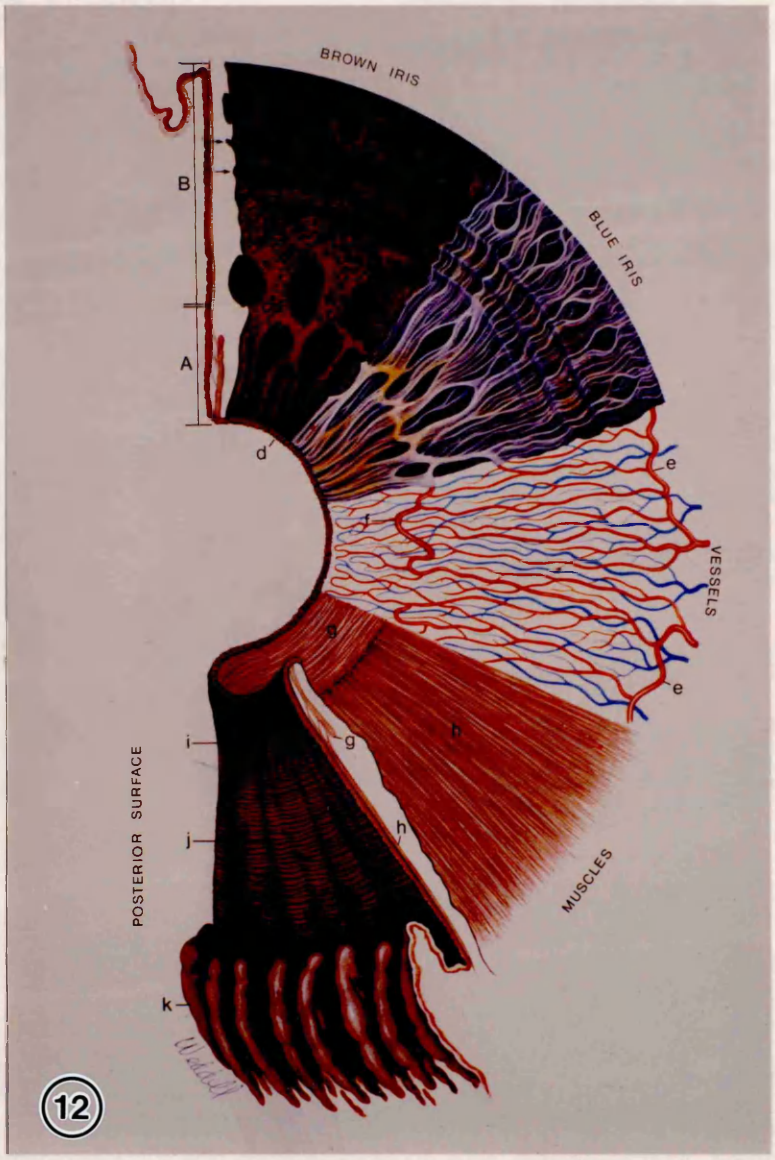
An alternative classification revolves around "plaque-like" structures in the trabecular cores and in the interspaces of the cribriform layer. These electron dense plaques are classified into three distinct subgroups depending on their banding characteristics and the density of the granular component.[77,140,209,215,216] Rohen and Witmer[216] introduced the terms, type I, II and III-plaques, to differentiate between these three subgroups. Type I-plaques are of low electron density forming small patches of various sizes and their morphology indicates that they are remnants of basement membrane-like material.[212] They correspond to the basal lamina-like and fine fibrillar-like material of Tawara et al.[244] Type II-plaques are of high electron density and are distributed throughout the entire cribriform layer. These plaques appear to be elastic-like fibres[151] and having been shown by Rohen et al[215] to form a delicate network underneath the endothelium of Schlemm's inner wall were collectively termed the 'cribriform plexus'. Tawara et al[244] classified them as elastin-like fibres. Type III-plaques are not as electron dense as type II-plaques and were termed fine granular-like material by Tawara et al.[244]

1.4.3 Immunohistochemistry

There is little documentation of the distribution or indeed the presence of collagen types in the human outflow system. Three early light microscopic immunohistochemical studies reported the presence but not the precise location of types I, III and IV collagen in the trabecular meshwork.[127,204,245] Subsequently, a more detailed immuno-

fluorescence study described the distribution of collagens I, III, IV and V in the meshwork.[176] However, this technique lacks resolution, particularly when compared to E.M. immunogold technology which permits precise localization of antigens at the ultrastructural level. Attempts to refine the identification of the extracellular components by means of immunolabelling of the collagen subtypes have not been extensive, presumably because of the lack of resolution at the light microscopic level for immunolabelling. Although one immunohistochemical investigation has been conducted on human trabecular tissue at the electron microscopic level, this study was restricted solely to the presence and distribution of type VI collagen.[152]

With regard to the extracellular matrix of the trabecular core and the interspaces of the cribriform layer, it has been provisionally accepted that finely granular 'basement membrane material' contains type IV collagen and that filamentous strands are derived from types I and III collagen.[176]


BROWN IRIS
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12

1.5 Iris

The iris is the most anterior portion of the uveal tract. It forms a delicate and movable diaphragm between the posterior and anterior chamber. Amongst the ocular structures, the iris is unique in possessing atypical blood vessels, an ability for contractile movement and a stroma which is freely permeable to aqueous humour via crypts along the anterior surface of the iris.

1.5.1 Anatomy

Gross anatomy of the iris is illustrated in Fig 12. The iris can be divided, histologically, into four regions (Fig 13) each of which will be described in some detail.

1- Anterior Border Layer

The anterior border layer consists of a single layer of fibroblasts whose long branching processes interconnect with each other and beneath which is a fairly dense aggregation of melanocytes. This layer forms the anterior

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Fig 12: Diagram of iris gross anatomy.
[I] SURFACE OF BROWN IRIS The thickest part of the iris is in the region of the collarette which is located about 1½mm from the pupillary margin. The collarette divides the iris into two zones -a narrow pupillary zone [A] and a wider ciliary zone [B] which is marked by circular contraction furrows (arrowed). Rounded or irregular openings (Fuch's crypts [c]) are located on the anterior surface which are continuous with spaces in the iris stroma. A pigment ruff [d] is present at the pupillary edge which is a continuation of the posterior iris pigment epithelium.
[II] IRIS VESSELS Radial branches of the major arterial circle [e] of the iris extend towards the pupillary region forming an incomplete minor arterial circle [f] which in turn sends branches towards the pupil in the form of capillary arcades. Venous returns are in blue.
[III] MUSCLES Sphincter muscle [g] is arranged in a circular manner around edge of pupil. Dilator muscle [h] extends from underneath sphincter muscle to root of iris in a radial manner.


ABL
S
DM
APE
PPE
13

surface of the iris (Fig 13) and is frequently interrupted with gaps.

2- Iris Stroma

Striated collagen fibrils are the major component of the iris stroma providing mechanical support for a tissue which undergoes constant movement. The fibrils form small and large bundles which cross at wide angles to form spaces of various sizes. They are more abundant in heavier sheaths around blood vessels and nerves. The spaces are filled with proteoglycans which control fluid movement within the stroma. The cells that are most frequently found in the stroma are fibroblasts and melanocytes but clump cells, mast cells, macrophages and lymphocytes are also present.

3- Iris Pigment Epithelium

The iris pigment epithelium is composed of two layers of pigmented cells that meet at their apices - the anterior and posterior pigment epithelium (Fig 13). Cells of the anterior pigment epithelium possess an apical epithelial portion and a basal muscular portion. The muscular extensions of the basal portion are embedded in basement membrane and are orientated in a radial direction from the iris root towards the pupil and collectively form the dilator muscle.

Fig 13: Iris cross-section. Iris surface consists of a single layer of fibroblasts, below which are several layers of flattened melanocytes (anterior border layer [ABL]). Stroma [S] consists of collagen fibrils amongst which are scattered fibroblasts, melanocytes and capillaries [arrowed]. Iris pigment epithelium has two layers of cells - anterior pigment epithelium [APE] and posterior pigment epithelium [PPE], the anterior portion of the anterior layer forming the dilator muscle [DM]. Bar:50um.

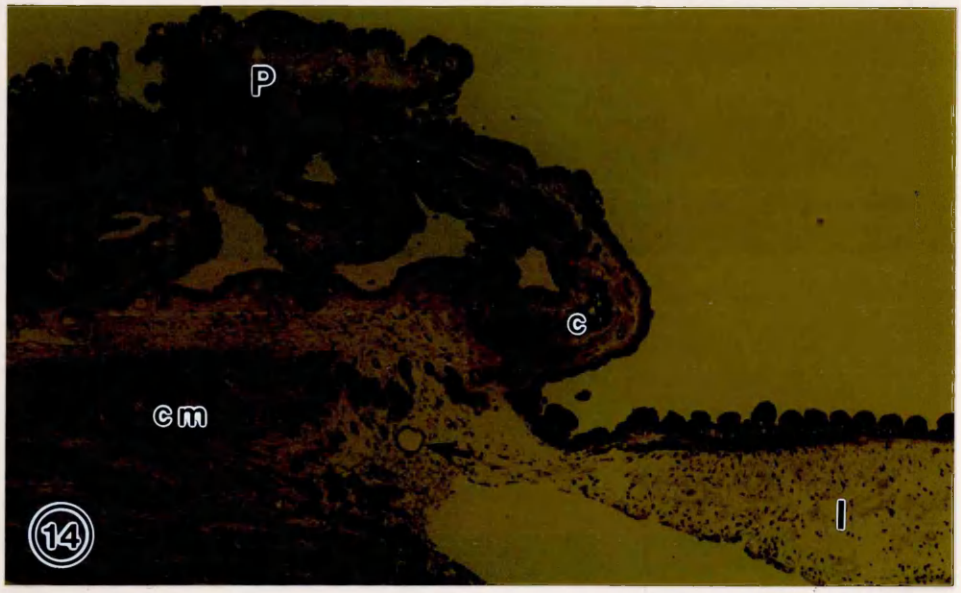
4- Iris Vasculature

Iris vessels arise from the major arterial circle of the iris located at the iris root (Fig 12). This arterial circle is the most incomplete of the three collateral vascular circles formed from the anterior and long posterior ciliary arteries.[173,174] Arteries rapidly diminish in size as they enter the stroma from the major circle and travel in a radial manner toward the pupil forming an incomplete minor arterial circle in the collarette region (Fig 12). Capillary arcades, radiating from the minor arterial circle supply the pupillary zone (Fig 12). Iris blood vessels are believed to have a slight corkscrew shape so that they can accommodate changes in the length of the iris during dilation and contraction. All iris vessels have thick collagenous adventitia with the striated collagen fibrils arranged in a circular manner around the vessel.

Capillaries are commonly very close to the anterior surface of the iris. In iritis and diabetes, vessels increase in number and size producing a condition known as neovascularisation or rubeosis of the iris.

1.5.2 Immunohistochemistry

The precise distribution of the various collagen types had not been documented in either human or animal iris tissue. It is of importance that this information be made available as a baseline for future studies on diseases such as exfoliation glaumoma, neovascular glaucoma and metabolic disorders.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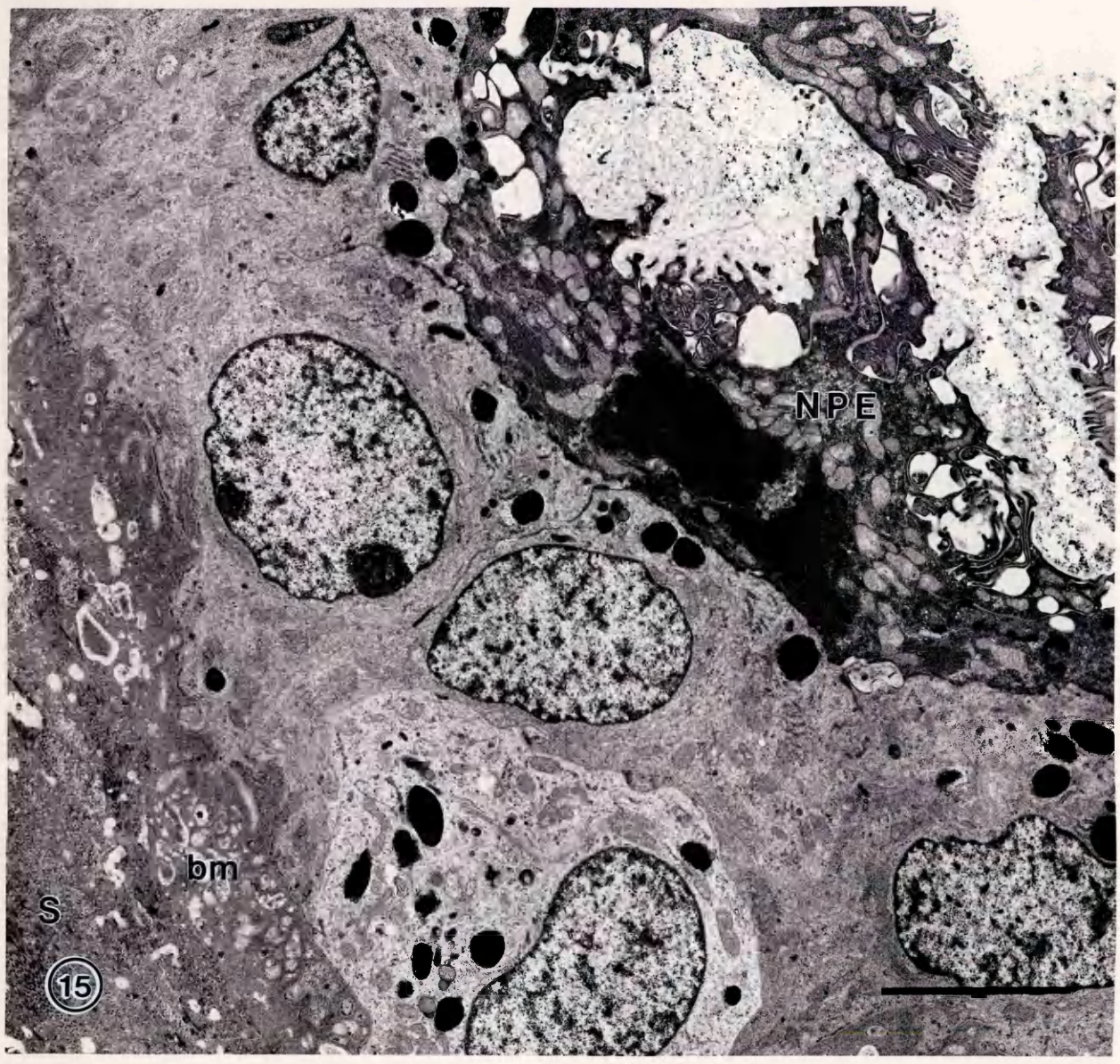
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1.6 Ciliary Body

The ciliary body has been so named due to its resemblance to eyelashes (cilia) as seen by early anatomists. While examined from behind, the ciliary body, being a circular structure, appears as a ring whose inner surface can be divided into two zones: an anterior corrugated pars plicata and a smooth posterior pars plana. The pars plicata is ridged with 70-80 ciliary processes which appear as finger-like projections of the main ciliary body jutting into the posterior chamber.

Histologically, the ciliary body can be divided into four regions (Fig 14): (1) the nonpigmented ciliary epithelium, (2) the pigmented ciliary epithelium, (3) the stroma and (4) ciliary muscle.

1.6.1 Ciliary Epithelium

The ciliary epithelium is composed of a layer of pigmented epithelial cells superimposed apex to apex onto a layer of nonpigmented epithelial cells. The basal aspects of these cells possess numerous infoldings (which is a characteristic of absorption and secretory activity) and are lined with basement membrane (Fig 15). As the cells of

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Fig 14: Meridional section of ciliary body. P: ciliary processes, c:capillary, cm: ciliary muscle, I: iris, arrow: major arterial circle of iris.

Fig 15: Ciliary epithelium of 74 year old female. Non-pigmented epithelial cells (NPE) are highly irregular in shape. The lining basement membrane is highly reticulated with numerous inclusions. Hyalinisation of the stroma (S) is evident as is that of the pigmented epithelial basement basement membrane (bm). Bar:5um.

the ciliary epithelium intercommunicate with each other by means of gap junctions, the entire ciliary epithelium behaves as a syncytium and forms a discrete physiological unit: the blood-aqueous barrier. Aqueous humour is formed by the selective passage of certain blood plasma components across the ciliary epithelium, particularly ascorbate, pyruvate and lactate, by an energy demanding process of active transport. Aqueous humour production is only possible if the transport activity of the ciliary epithelial cells is precisely coordinated and is not dissipated by free diffusion of water and solutes along the intercellular clefts of the epithelium. This ancillary function depends upon gap junctions and zonulae occludentes.[203]

1.6.2 Ciliary Muscle

The ciliary muscle exhibits several pecularities that are not shared by other smooth muscle systems. It is very densely innervated and its muscle cells are enormously rich in mitochondria. Traditionally, the muscle is divided into an outer meridional portion (Brücke's muscle), a radial or reticular component (Iwanoff's muscle) and a circular inner part (Müller's muscle).

The ciliary muscle in human eyes consists of bundles of muscle cells surrounded by flattened fibroblasts. Each bundle generally contains unmyelinated nerve fibers accompanied by Schwann cells and connective tissue components. Single muscle cells are entirely wrapped in a basement membrane except at small areas of contact with other muscle cells. In the spaces between the bundles, collagen, very thin collagen-like (reticular) and elastic

fibers, myelinated and unmyelinated nerve fibers and capillaries are seen.[104]

In the **meridional** part, clearly separated bundles of muscle cells run parallel to each other and the intermediary connective tissue spaces are small. In contrast, in the **radial** part the contour of the bundles is rather irregular and obscure and a dense connective tissue fills the spaces between the bundles.

1.6.3 Extracellular Matrix Components

The role of the extracellular matrix in accommodation and aqueous humour formation merits investigation. This topic is particularly relevant to those changes which occur in the aging process.

The physical barriers in the form of basement membranes of the ciliary epithelium may influence aqueous humour formation and transportation. Specialized basement membranes line the basal surfaces of the nonpigmented and pigmented epithelium. While the conventional fine structure is adequately documented,[101] the nature of the biochemical constituents awaits clarification. In addition, the stromal components of the ciliary processes which surround the fenestrated capillary endothelial cells may influence the fluid exchange between the vessels, and pigmented epithelium of the ciliary processes.

1.6.4 Aging Changes

Marked morphologic changes occur in the aging ciliary body and these can be used to roughly estimate the age of the eye.[178] These changes include (1) the development of a warty appearance on the surface of the pars plicata due to

small nodular proliferations of nonpigmented epithelium cells, (2) thickening of the nonpigmented epithelium basement membrane from a 30nm wide granular layer, characteristic of basement membranes, to a remarkably thickened multilaminar reticulated form containing membrane bound vesicles and granular material, (3) transformation of the PE basement membrane to a thick electron dense multilaminated type containing vesicular, fibrillar and granular inclusions, (4) thickening of the subepithelial region of ciliary processes with collagenous material and (5) the accumulation of dense collagen and granular material (described as hyalin) in the radial (Iwanoff's muscle) and circular inner part (Müller's muscle) of the ciliary muscle.[237]

Collagens are laid down within the circular and oblique components of the ciliary muscle and the stroma of the ciliary processes which progresses to histopathological fibrosis (or hyalinization). In addition, there is an age-related increase in thickness of the basement membranes of the ciliary processes,[205,217,265] and in an advanced form is diagnostic of diabetes. It is not known if the increase of the number of capillary fenestrations with age is a physiological reaction to this abnormality or if the subsequent increase of plasma leakage due to the increase in fenestrations is the cause of the hyalinization.[88] The nonpigmented epithelium of the pars plana often shows retention of hyaluronic acid which leads to the formation of cysts.[58]

Many of these aging features are due to the deposition of collagen and other, as yet unknown, extracellular matrix components. Aging changes have profound effects upon

ciliary body function. An increase in ciliary muscle fibrosis is accompanied by a decrease in the amplitude of accomodation.[178]

1.6.5 Immunohistochemistry and Biochemistry

No attention has been paid to the extracellular matrix components involved in the hyalinization process both in the aged ciliary processes and in the ciliary muscle. Information concerning the extracellular matrix components in the young and aging human ciliary body is restricted to an immunofluorescent study which located laminin and fibronectin to the basement membranes of the ciliary epithelium and ciliary vessels.[126] With regard to collagen composition, a biochemical study has indicated the presence of types I and III collagen in the bovine ciliary body.[228]

A study of collagen types and laminin in the aged normal human ciliary body and zonular apparatus would lead to a better understanding of the aging process and provide background information for future studies on the role of extracellular matrix abnormalities in pathological disorders such as diabetes.

1.7 Lens Capsule

The lens is a transparent living tissue which has no blood supply; metabolic exchange is via the aqueous and vitreous humour. The lens substance is formed by cells which contain crystalline proteins and is completely enclosed by a lens capsule which is a transparent, elastic and unusually thick basement membrane.

The smooth surface and uniform thickness of the lens capsule in the pupillary region contributes to the clarity of the lens, but its main function lies in its elastic properties. The elasticity of the capsule tends to make the lens spherical in accomodation; a tendency which is opposed by tension on the zonular fibres.

Apart from its role in accomodation, the lens capsule has a well-established function as a filter being impermeable to proteins but permitting the passage of smaller molecules such as oxygen, glucose, amino acids, fatty acids and other nutrients required by the lens as well as the metabolic waste products produced by the lens (lactate and CO_2).

1.7.1 Anatomy

1- Lens Capsule

The posterior pole is the thinnest portion of the lens capsule. The anterior capsule is thickest in a circular area 3mm from the anterior pole and the posterior capsule in an area 1mm from the posterior pole. Viewed at low magnification by transmission electron microscopy, the capsule is smooth, homogenous and dense. Linear densities are scattered throughout the anterior and equatorial regions and are composed of fine fibrils which are similar

in appearance to zonules. A zonular lamella or pericapsular membrane at the equator consists of zonular fibres merging with a loose portion of the equatorial capsule.

2- Lens Epithelium

The lens epithelium lies beneath the anterior and equatorial but not the posterior capsule. The basal portion of these cells face the lens capsule forming a smooth capsular-epithelial line of contact. The central cells near the anterior pole are polygonal in flat section and cuboidal in meridional section. Mitoses are normally absent. Cells of the intermediate zone, lying between the central zone and the equator, are smaller, more round and with frequent mitoses. The cells of the equatorial zone divide and become elongated to form the new lens cells.

3- Linear Densities

Linear densities are an interesting feature of the lens capsule,[40,197,202,231] but the significance and biochemical nature of linear densities is unknown. They are thought by some to be part of the zonular apparatus,[40,202,231] but this is a view that is not universally shared.[45,101,230] Linear densities have also been linked with the production of exfoliation material by lens epithelial cells since they are morphologically similar[47] and exhibit similar histochemical staining properties.[46]

1.7.2 Aging Changes of the Lens Capsule

Several physical and morphological changes of the lens capsule have been associated with ageing. These

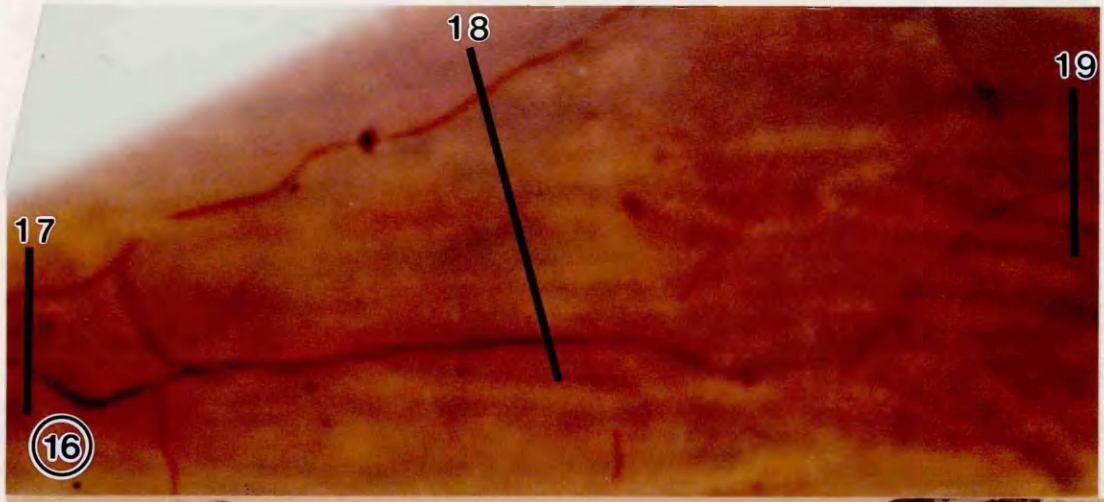
include the lowering of elasticity and breaking point force,[59] thickening of the anterior capsule,[60] the loss of laminations,[231] and an increase in the number of linear densities (formed elements).[40,47,74,231] Many of these changes must be associated with alterations in the tertiary structure and/or synthesis of the extracellular matrix components (ECM) that comprise the lens capsule.

1.7.3 Biochemistry

According to biochemical analysis, lens capsule has a high collagen content (69+8%)[228] comparable to that of cornea (70.2%) and sclera (76%).[196] The biochemical detection of type IV collagen in mammalian lens capsule has been widely reported,[50,70,168,228,242] and is estimated to be about 30-40% of the dry weight of the lens capsule.[9] Barnard et al (1987) suggested that type IV collagen supplies the strength and flexibility of this basement membrane. However, scant attention has been paid to the possible presence of other collagens in the lens capsule.

1.7.4 Immunohistochemistry

Immunofluorescence microscopy has indicated the presence of type IV collagen and laminin in the human lens capsule.[97] However, no immuno-electron microscopical studies on the types of ECM components present in human lens capsule have been found in the literature.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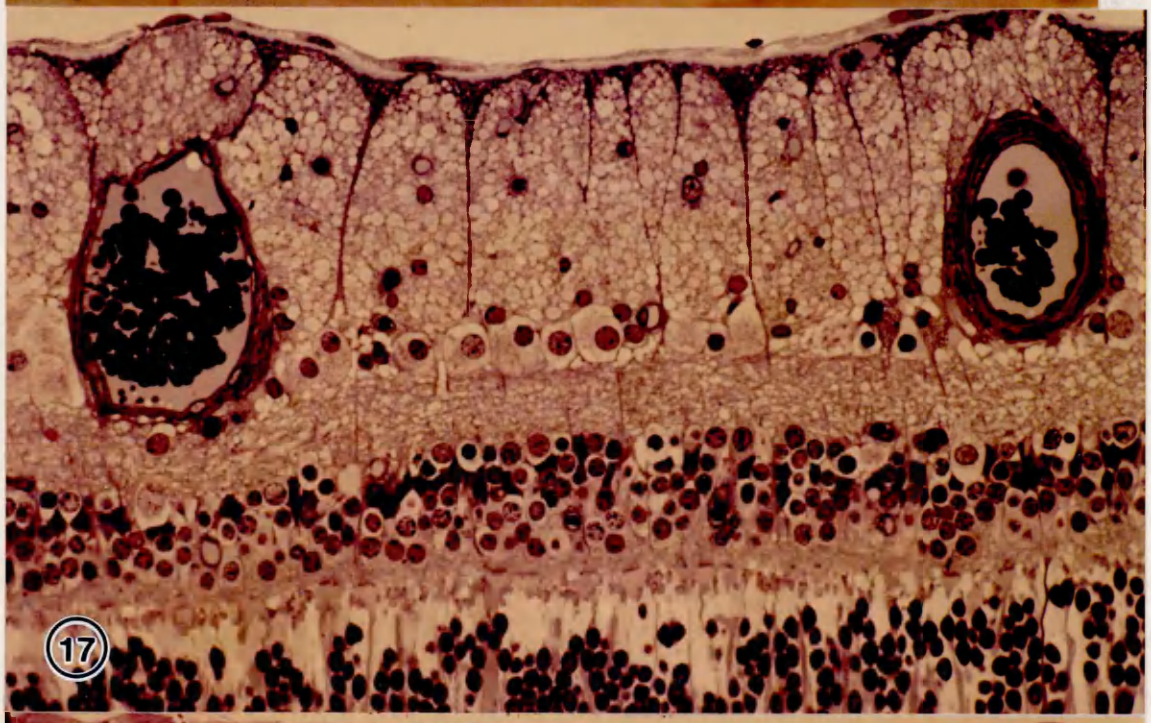
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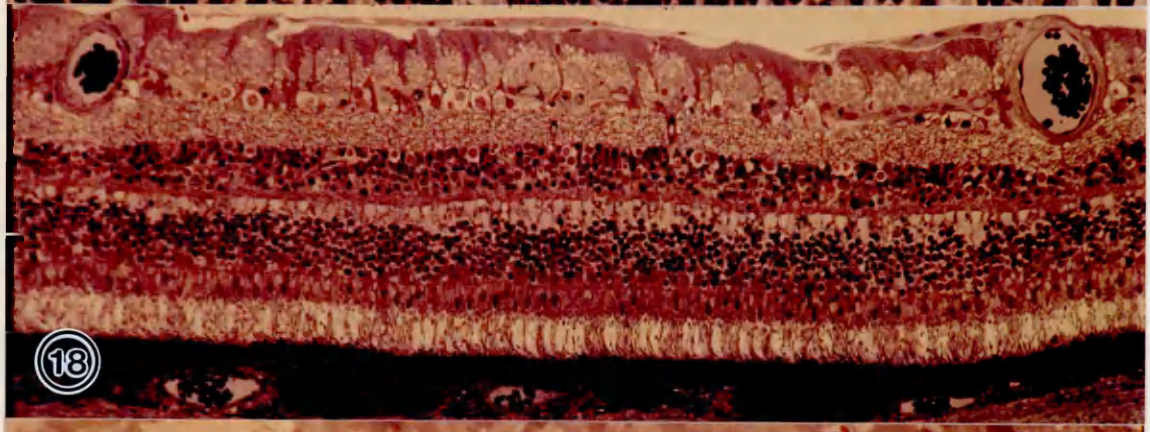
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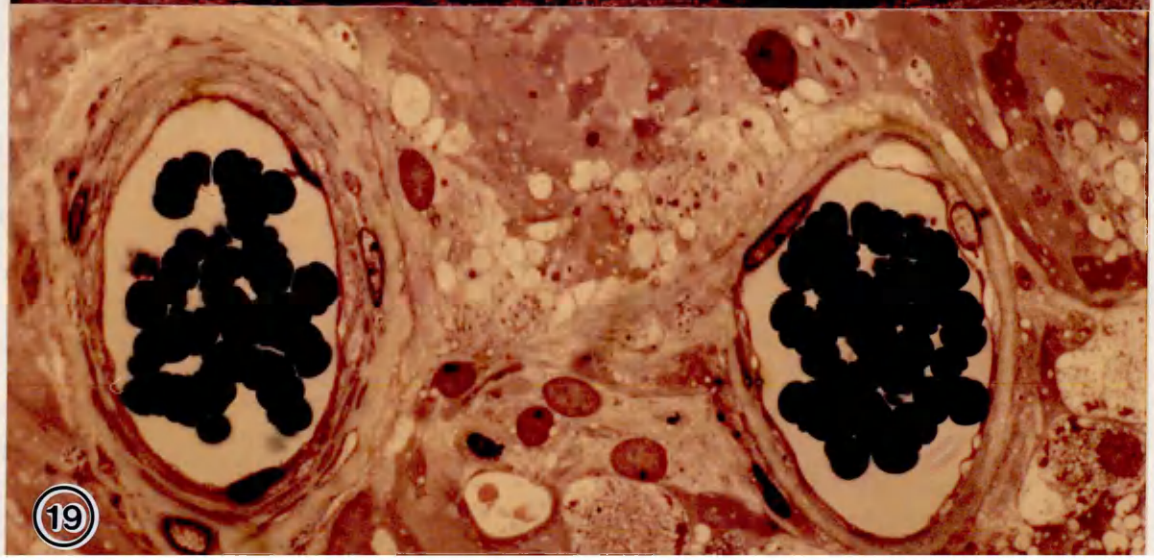
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1.8 Retinal Vessels

1.8.1 Anatomy

The central retinal artery forms two main branches in the prelaminar part of the optic nerve. Each of these branches further divides in the nerve into a superior nasal and temporal, as well as inferior nasal and temporal branches that supply the four quadrants of the retina. Retinal venous branches have roughly the same distribution as the arteries.

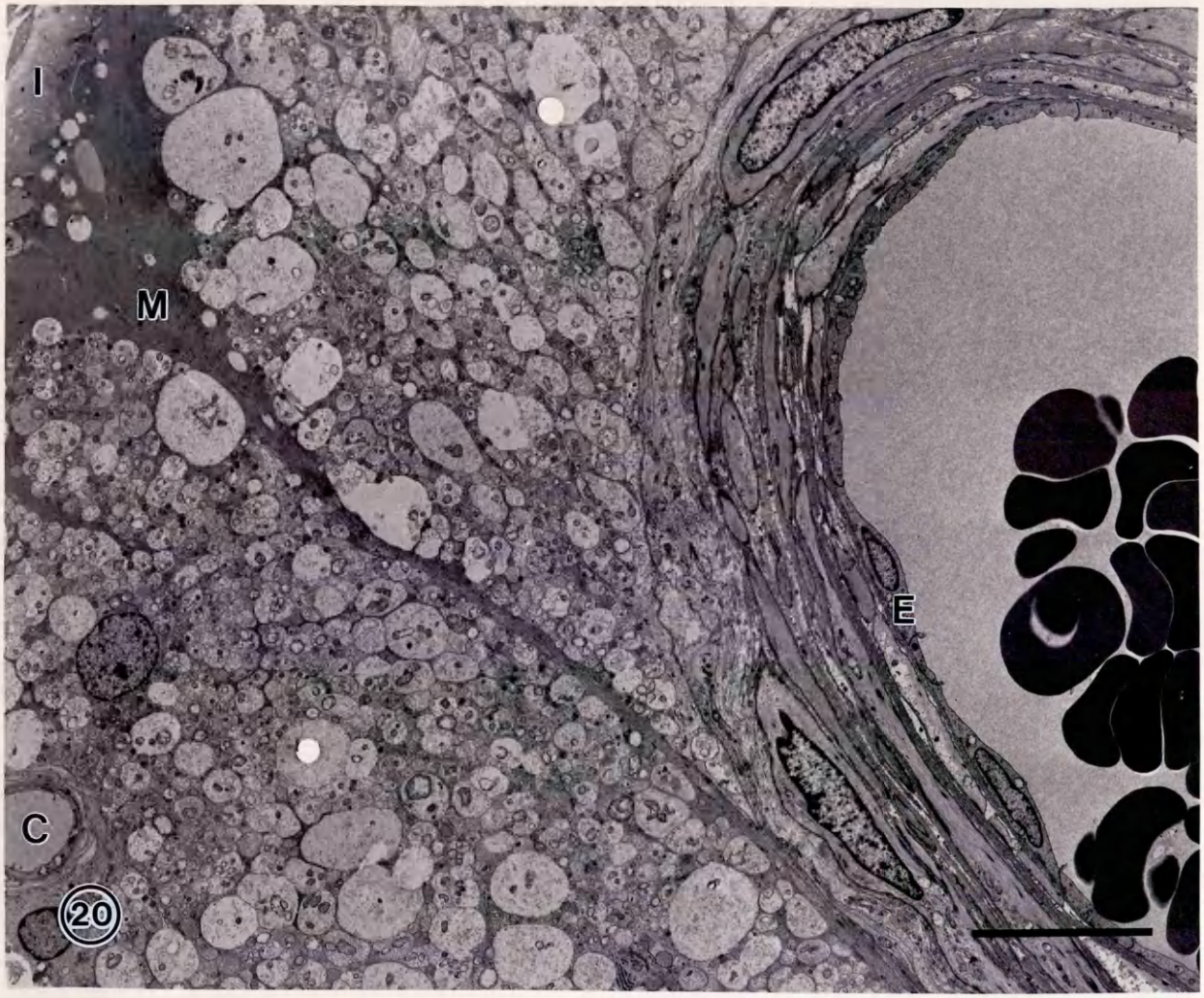
The principle arteriolar and venular branches lie in the nerve fiber layer close to the internal limiting membrane. The retinal arterioles branch either dichotomously or at right angles to the length of the main trunk. The retinal arteriolar system is truly terminal in that there is no connection with other systems of blood vessels. The retinal artery and vein within a quadrant is the sole supply of that quadrant. Thus if the arteriolar supply to that quadrant is interrupted, infarction of the retina occurs.

Fig 16: Macroscopic of retinal arteriole and venule of 63 year old female. Lines indicate sections taken for light microscopy.

Fig 17: Arteriole (LHS) and venule (RHS) at 1mm from optic disc. Lumen of arteriole is smaller than that of venule and vascular wall is thicker. x25, plastic section; toluidine blue.

Fig 18: Arteriole (LHS) and venule (RHS) at 4mm from optic disc. Difference in luminal diameter is more marked. x10, plastic section; toluidine blue.

Fig 19: Arteriole (LHS) and venule (RHS) at 9mm from optic disc. Arteriole contains two to three layers of myocytes within vessel wall but venule exhibits little more than lining endothelium. x63, plastic section; toluidine blue.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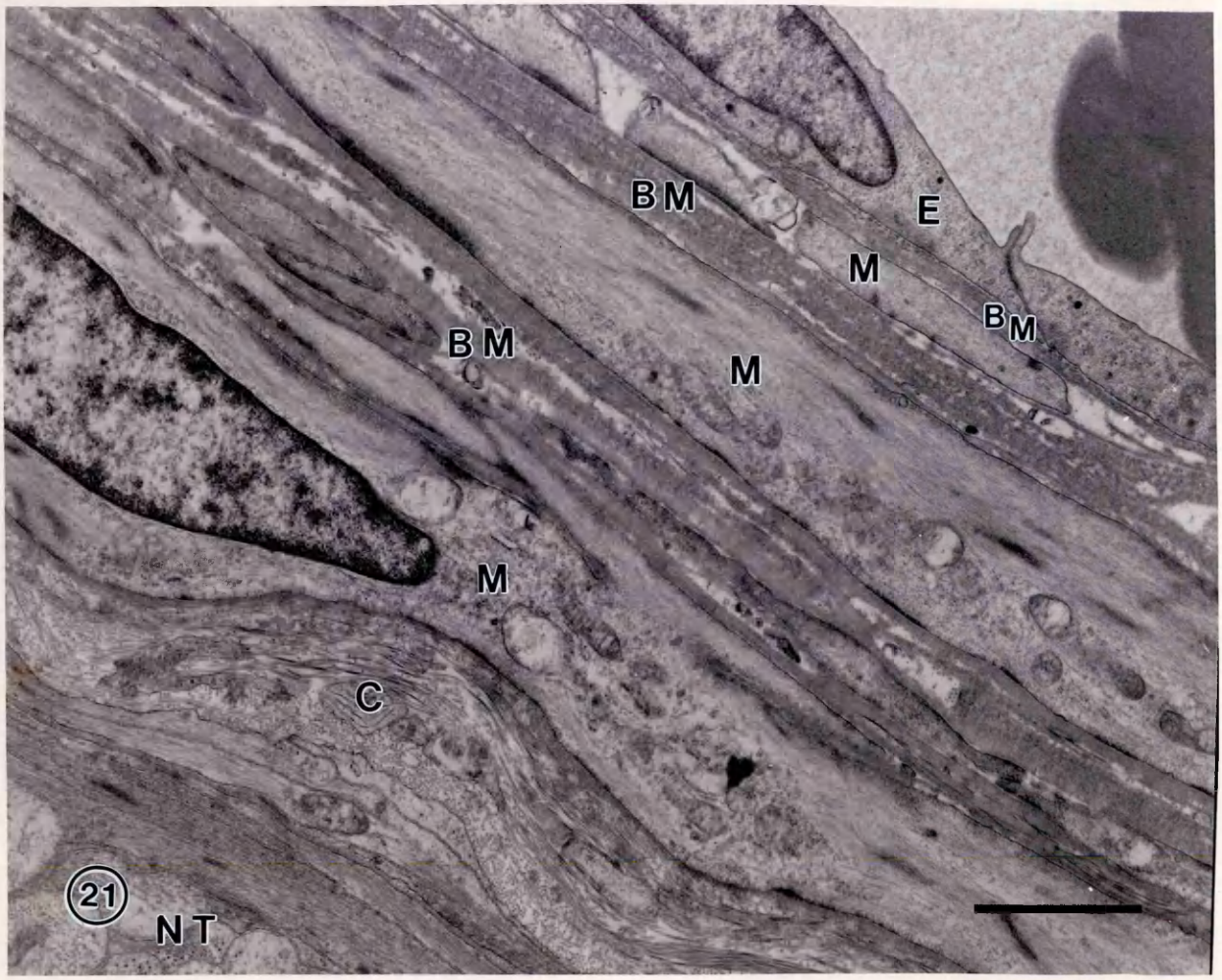
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NT

1- Arterioles

Adjacent to the optic disc the arterioles have a luminal diameter of 100um. Their arteriolar character persists into the peripheral retina. Near the disc the arteriolar wall (Fig 17) has five to seven layers of myocytes (smooth muscle cells) which diminish to two or three layers at the equator (Fig 18) and to one or two peripherally (Fig 19). Retinal arterioles do not possess an internal elastic lamina. The endothelium lining the vessel lumen possesses a basement membrane (about 0.1-0.5um thick) which is continuous with that of adjacent myocytes. Each successive layer of myocytes is enveloped in a basement membrane that contains an increasing percentage of striated collagen fibrils (Figs 20-21). The adventitia contains circularly orientated striated collagen fibrils which are separated from the nerve fibre layer by the basement membranes of Müller and glial cells (Fig 21).

2- Veins

The principle tributaries of the central vein near the disc have a luminal diameter of about 200um. The "veins" in the peripheral retina are venules. The larger veins in the vicinity of the optic disc have three to four layers of myocytes in the vessel wall but these cells are replaced by

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Fig 20: Arteriole of 63 year old female in nerve fibre layer 1mm from optic disc (c.f. Fig 17). Lumen (L) is lined with single layer of endothelial cells (E). Vascular wall contains four to five layers of myocytes (smooth muscle cells). M: Müller cell process; I: internal limiting membrane; C: capillary; Bar: 10um.

Fig 21: Higher power of arteriolar wall. Basement membrane (BM) lies on basal side of endothelial cell (E) and between adjacent myocytes (M). Striated collagen fibrils (C) separate outermost myocyte from neural tissue (NT). Bar: 2um.

fibroblasts within a short distance from the disc (Figs 17-19). The basement membrane of the lining endothelial cells is significantly thinner than that of arterioles (about 0.1um) and the basement membrane surrounding the outermost layer of vascular cells within the venule wall.

3- Capillaries

Capillaries which arise from arterioles in the innermost retinal layers pass directly into the retina often at right angles to the parent vessel and connect with venous capillaries within every level of the inner retina. The basement membrane of the endothelial cell lining is continuous with that of the basement membrane surrounding the intramural pericytes.

1.8.2 Hyalinisation of Retinal Vessels

The changes which occur in retinal vessels as part of normal aging have been documented at the level of light microscopy[141] and essentially consist of progressive replacement of myocytes by collagenous tissue. This process of hyalinisation was explored at the electron microscope level which revealed obvious reduplication of the basement membrane and deposition of 65nm banded collagen.[139] The significance of hyalinisation in blood vessels has been explored in terms of neovascularisation.[155,156,158] It is a reasonable hypothesis that a change in the ground substance may modify endothelial cell proliferation in the presence of retinal ischaemia and modify the subsequent release of vasoformative factors.[139,155,156,158] It is therefore important that the new technology of immunogold labelling be applied to the study of collagen types which are present in aging blood vessels.

1.9 Laminin

Laminin is a large multidomain glycoprotein which together with collagen type IV and heparan sulphate proteoglycan (HSPG) comprises most of the protein found in basement membranes.[2] It was first purified from the basement membrane-like matrix of EHS sarcoma[247] and is one of the two major structural basement components that forms polymers, type IV collagen being the other.[37]

Basement membranes are specialized sheets of extracellular matrix directly involved in a number of important biological processes, such as cell adhesion and orientation.[53,225] It is thought that individual components within basement membranes are responsible for particular functions. Thus collagen type IV forms network structures which provide the structural scaffold onto which the other components are assembled;[225] whereas, heparan sulphate proteoglycan (HSPG) maintains the filtration properties of basement membranes.[2] Laminin promotes the structural integrity of basement membranes through specific binding with collagen type IV and HSPG, but of greater importance is its functional contribution to basement membranes. It has been shown that laminin promotes cell adhesion, migration, and growth and plays an active role in tissue restoration.[53,96] These functions are associated with specific functional domains within the laminin molecule:e.g. the mitogenic and cell attachment activities are two distinct functions controlled by separate laminin domains.[191]

1.10 AIMS AND APPROACH OF CURRENT INVESTIGATION

The eye is composed of highly specialised tissues possessing distinct collagenous structures that exhibit a great degree of functional diversity.[7] Of the techniques available for collagen identification, immunofluorescence and immunoperoxidase have been widely used, but the low resolution at the light microscope level is insufficient to allow their precise localisation. By contrast, immunogold labelling at the transmission electron microscope level allows precise localisation of tissue constituents. However, at the inception of the present study no example of the application of this technique to human ocular collagens was present in the literature. Since the bulk of biochemical and immunohistochemical studies on collagens in ocular tissues have been conducted on the cornea (sections 1.3.2 & 1.3.3), this tissue was used as a point of reference to confirm the reliability of the technique. This technique was then used to document the fine structural distribution of collagen types I-VI and the glycoprotein laminin in as many ocular tissues as possible and to relate these distributions to the specific functional requirements of each tissue.

A critical review of the literature reveals that with immunohistochemistry it is difficult to obtain reproducible and reliable results. Therefore, it is essential to have control systems to consolidate information and the use of two different immunocytochemical techniques in parallel strengthens the validity. The best technique available for localising antigens at the fine-structural level utilises cryoultramicrotomy, the frozen-thin section technique championed by Tokuyasu.[252] Unfortunately, antigenic

preservation is maintained at the expense of architecture. The converse of this is true for the London Resin White (LR white) embedding technique. Therefore, LR white plastic embedding and cryoultramicrotomy were chosen to be used in parallel as the best available methods of comparison.

To eliminate the uncertainty of absence of labelling being due to the genuine absence of the collagen type studied and not to failure of the technique, tissues in which the antigen was known to be present were incorporated into the immunocytochemical procedure as positive controls.

Chapter 2:—
Materials and Methods

2.1 PREVIOUS STUDIES

The choice of approach to the present investigation is the result of two separate projects undertaken before commencement of the present study. Extensive review of the literature highlighted the superiority of the immunogold label to other immunocytochemical markers (1.2.3).[11,12,14,73,219] Attempts were made to localize immunoglobulin subtypes in human myeloma plasma cells with secondary immunogold conjugates on ultrathin sections of conventionally processed Epon embedded bone marrow as a considerable amount of this tissue was already banked. The localisation of immunoglobulins was unsuccessful, even with the use of sodium metaperiodate as an etching agent,[17] the omission of osmium tetroxide (1.2.4.2) and the use of low levels of glutaraldehyde for fixation in subsequently processed tissue (1.2.4.3). However, the extensive background reading has proved invaluable in subsequent immunogold studies.

The immunogold technique was also applied to the interaction of proteoglycans with types I and II collagen. The proteoglycans chondroitin, heparan, dermatan and keratan sulphate were distinguished from one another by the combination of selective enzymatic digestion prior to cupromeronic blue staining on ultrathin frozen sections. Simultaneous labelling of types I and II collagen was successfully performed on these sections. Cryoultramicrotomy had therefore proven itself in my hands to be a reliable technique in the localisation of collagen types.

A dual approach to immunogold labelling of collagens was considered a prerequisite for the consolidation of results. LR white was preferred to Lowicryl K4M for two

reasons:
[1] it is simpler to use in that dehydration and resin infiltration can be conducted in a fridge in contrast to the progressive lowering of temperature technique,
[2] the handling of Lowicryl K4M has produced acute dermatitis with some users (personnal communication).

2.2 MATERIALS

Twenty two surgically enucleated eyes of patients aged 13-81 years were obtained principally from the eye theatre of the Western Infirmary, Glasgow. Reasons for enucleations are indicated in Appendix 1. In addition, five autopsy specimens were obtained from hospitals in the Glasgow Health Board.

2.3 FIXATION

2.3.1 Preliminary Experiments

Optimal concentrations of glutaraldehyde fixative were determined by testing the antigenicity of collagens I and IV in blocks of retinal tissue from the same eye fixed at various concentrations of fixative for 2 hours at room temperature (R.T.). The concentrations tested were 0%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0% and 2.5%. Separate experiments were conducted for each buffer (phosphate and cacodylate). As expected, the intensity of labelling for types I and IV collagen decreased with increasing concentration of glutaraldehyde, with a concomitant increase in the preservation of tissue structure. However, there were differences in antigenic preservation and tissue morphology between the buffers. Tissue preservation was very acceptable with phosphate buffer at concentrations of

0.2% glutaraldehyde whereas tissue preservation was not very high when cacodylate buffer was used even at concentrations up to 0.8% glutaraldehyde. Antigenicity was better preserved with cacodylate buffer. It was therefore decided to use fixative at various concentrations with both buffers to cater for possible differences in antigenic preservation among the collagen types and to keep the options open over the balance between antigenic preservation and tissue morphology.

The potential for utilisation of autopsy material was determined in a separate experiment when unfixed retinal blocks were stored at 4°C for 24 hrs before fixation with the same fixative concentration (0.25% in cacodylate) and the antigenicity of collagens I and IV compared with that of retinal blocks from the same eye that had been fixed immediately after excision. Similar distribution patterns of the two collagen types was observed on comparing freshly fixed to delayed fixed material. However, tissue preservation of delayed fixed material was not as good as that of freshly fixed material. Autopsy material was not therefore extensively used in this study.

2.3.2 Fixation Regime

The concentration of fixative and type of buffer used for each individual eye is listed in Appendix 1. One of two buffers was employed in the fixation regime : 0.15M sodium cacodylate pH 7.2 or 0.1M sodium phosphate pH 7.2. Antigenicity was better preserved with the former buffer but this was at the expense of ultrastructural preservation which was invariably more satisfactory when using phosphate buffer. Increasing the glutaraldehyde fixative

concentration above 0.2% did not significantly improve tissue preservation in LR white embedded tissue when using phosphate buffer. Similarly, improvements in preservation was not noted above 1% glutaraldehyde concentration when using cacodylate buffer.

2.3.3 Preparation of Paraglutaraldehyde

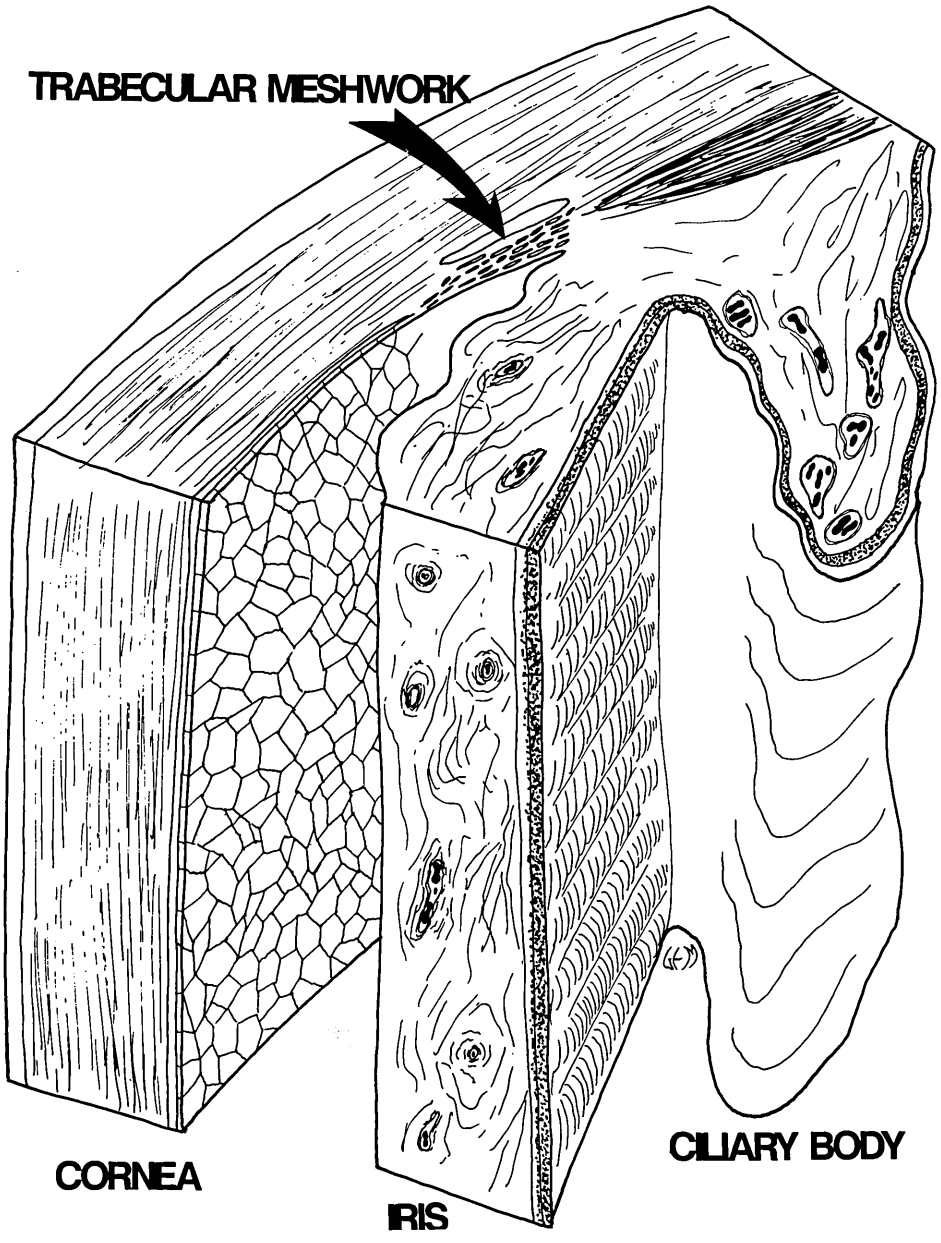
The fixative used for each eye was freshly prepared by adding 4g of paraformaldehyde (PFA) to 100ml of preheated buffer. A few drops of 4M NaOH was added to the phosphate buffer solution to complete dissolution of the PFA. This was not necessary when using cacodylate buffer. The solution was cooled with cold running tap water after complete dissolution and the pH readjusted with 0.1M HCl. The relevant volume of 25% glutaraldehyde was then added to the solution.

2.4 DISSECTION OF SPECIMENS

Eyes were immersed in fixative within a matter of minutes after enucleation and left for 10-15 mins for the sclera to harden: this facilitates the orientation of the calottes. On removal of the relevant portion for pathological examination the remainder of the globe was dissected and the tissue fixed for a total of 2hrs before washing three times in buffer.

2.4.1 Anterior Segment Specimens

The anterior segment was then divided in a radial manner with a razor blade. Eight corneal blocks were dissected out approximately about 3mm anterior to the chamber angle and eight iris tissue blocks at about 1.5mm


TRABECULAR MESHWORK
CORNEA
IRIS
CILIARY BODY

adjacent to the angle thus leaving eight blocks containing trabecular meshwork and ciliary body. As much of the lens cortex which remained attached to the anterior segment blocks was removed, leaving the lens epithelium, capsule and zonular fibres. The pars plana and posterior portion of the pars plicata was then dissected from the remainder of the block. Thus anterior segment blocks routinely contained **trabecular meshwork, peripheral iris, anterior pars plicata and lens capsule** (Fig 22). Two blocks were allocated for conventional processing, four for LR white embedding and four for cryoultramicrotomy.

2.4.2 Retinal Arterioles and Venules

On examination under a Wild M420 dissecting microscope the full length of an artery and accompanying vein from the optic disc was dissected out of the retina with an ample margin on either side. The retina was frequently stripped from the choroid to improve subsequent macroscopic photographic detail. However, the macula was dissected out with the underlying choroid for separate studies.

Segments of retina were photographed onto 3¼ x 4½ inch Polaroid at x8 to x40 magnifications using a Wild MPS 45 Photoautomat camera attachment and an MPs 51 S unit. Duplicate exposures were made onto 6x9cm cut film. Triads of blocks of both arteries and venules were dissected out at measured distances from the optic disc and their location marked on the Polaroid print (Fig 23). Three to

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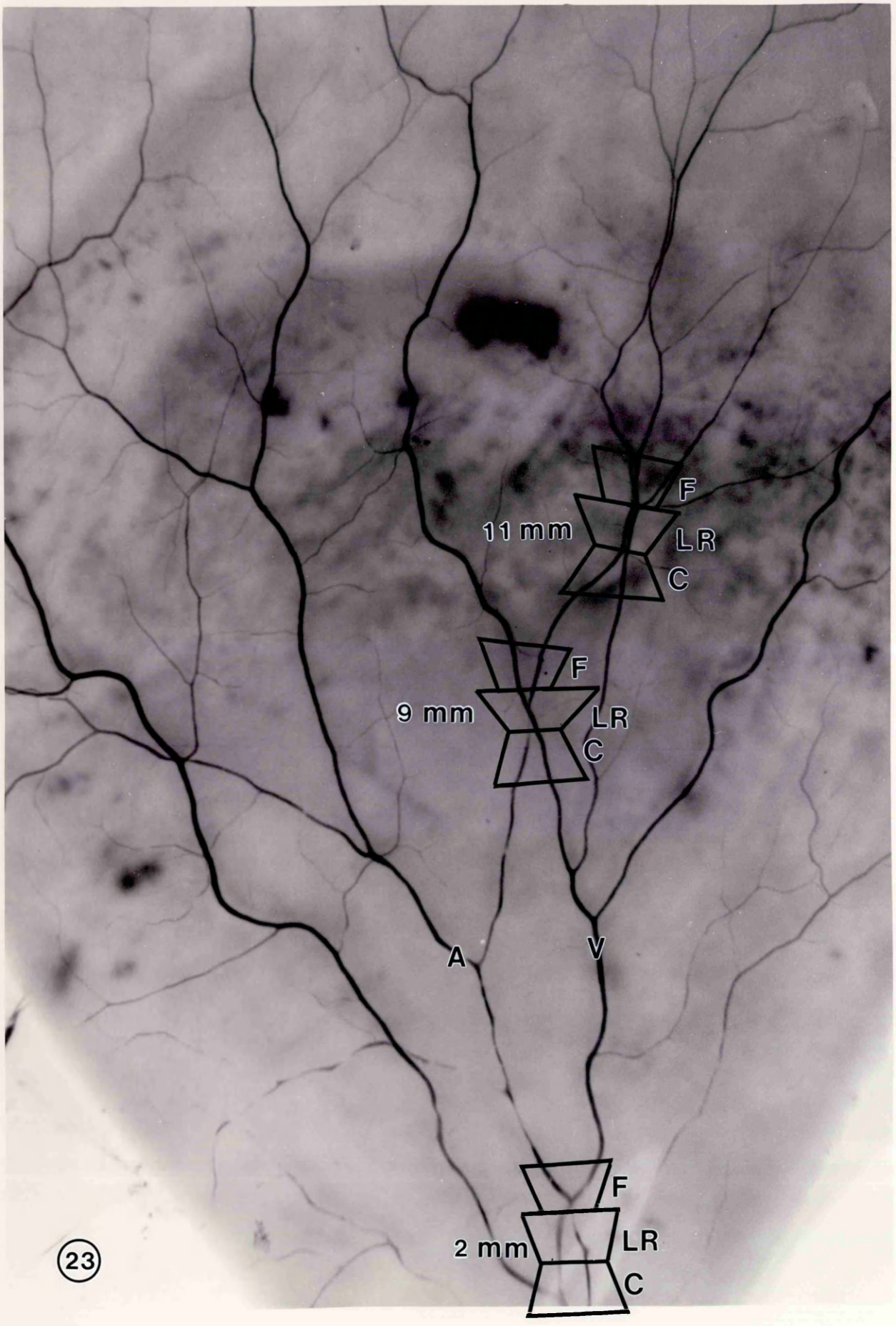
Fig 22: Anterior chamber block containing peripheral cornea, iris, trabecular meshwork and ciliary processes.



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four sets of blocks were dissected out from each vessel pair. Arteriole-venule cross-over points were preferred. Of the three blocks taken from each location one was allocated for conventional transmission electron microscopy, the second for LR White embedding and the third for cryoultramicrotomy.

2.5 TISSUE PROCESSING

2.5.1 Conventional Processing

Blocks allocated for conventional processing were further fixed in 2.5% glutaraldehyde for several days at 4°C. They were then washed in three changes of buffer (5 mins each), post-fixed in 1% osmium tetroxide for one hour at room temperature (R.T.), washed three times in glass distilled water and dehydrated through the following graded series of ethanol:

25% ethanol	2x5 mins
50% ethanol	2x5 mins
75% ethanol	2x5 mins
95% ethanol	2x5 mins
Absolute alcohol (repeated 4 times)	15 mins

The tissue was then cleared with two 5 min changes of propylene oxide and infiltrated overnight with a 1:1 mixture of araldite and propylene oxide. Infiltration was continued with a 2:1 mixture of fresh araldite to propylene

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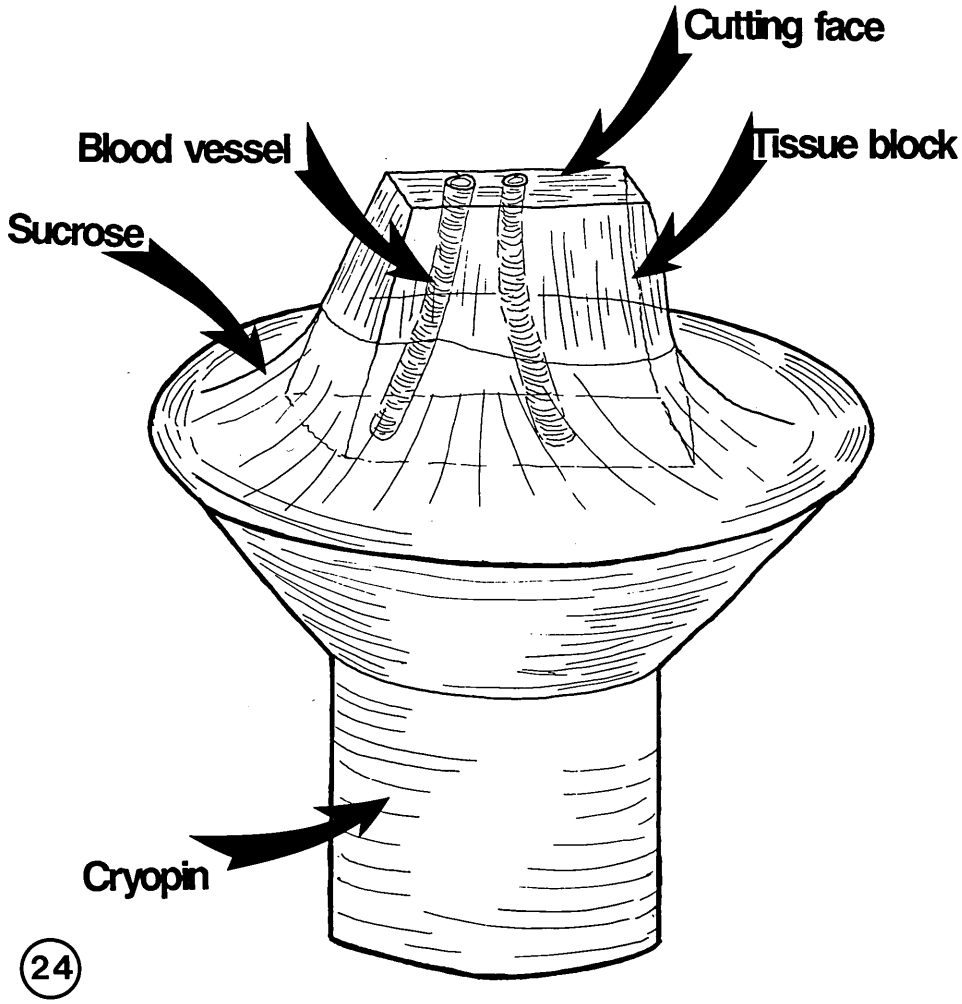
Fig 23: Macroscopic photograph of retinal arteriole (A) and venule (V). Location of blocks for conventional (C), cryo (F) and LR white processing (LR) with distances from optic disc are marked on photograph.

oxide for 6hrs on a rotivator at R.T. before blocking out into labelled rubber moulds; polymerization was for 24-48hrs at 60°C.

The resin blocks were trimmed to form a trapezoidal cutting face and 0.2-0.5 m thick sections were cut with a glass knife mounted on an LKB Ultrotome nova. The sections were transferred to glass slides, dried on a hot plate and stained for 30 secs with a 1% aqueous solution of metachromatic blue containing 2% borax. Ultrathin sections were cut from selected blocks and mounted on 150 mesh or 200 mesh copper grids. Post-staining with freshly made saturated aqueous uranyl acetate (15 mins) and Reynolds lead citrate[255] for 10 mins was performed at R.T.

2.5.2 LR White Plastic Embedding

Blocks allocated for LR white embedding were dehydrated in two 1hr changes of 70% alcohol and infiltrated with LR white hard grade resin (two 1hr changes). Occasionally for convenience, tissue blocks were left overnight in the second LR white change. Dehydration and infiltration were conducted on a modified TAAB type N rotator at 4°C in a domestic fridge. The blocks were then deposited in OO size gelatin capsules filled with LR white resin and the capsules sealed (gelatin capsules are mandatory because plastic capsules are permeable to air and LR white resin will not polymerize in the presence of oxygen). Attempts made to maintain the orientation of the blocks in the capsules by inserting the capsules into a home made capsule holder either in the vertical or horizontal position were unsuccessful. Polymerization was conducted at 48°C for 48hrs in a Mark II TAAB embedding


Cutting face
Blood vessel
Tissue block
Sucrose
Cryopin

oven.

2.5.3 Preparation of Cryo Specimens

Blocks allocated for cryoultramicrotomy were infused with 2.3M sucrose (dissolved in either 0.1M phosphate or 50mM trometamol [TRIS] buffer pH 7.4) overnight at 4°C. After infusion the blocks were trimmed down to a sectionable size and mounted onto silver pins in such a way that sections taken from the cutting face would be of conventional orientation (Fig 24). The pins were then plunged into liquid nitrogen with forceps and deposited into precooled storage containers which were later transferred to a LR40 Union Carbide liquid nitrogen Dewar flask for long-term storage.

2.6 ULTRATHIN SECTIONING

2.6.1 Grid Annotation

It was preferred for various reasons to conduct an immunocytochemical run on a number of tissues and cases as well as using several different antibodies within the same run. This required a means of identifying each individual grid so that grids were not misplaced during the procedure (normally between 40 and 80 grids per immuno-run). Each grid was colour coded on the shiny side with water resistant Staedtler pens, red for the case/tissue, blue for the antibody and yellow for the dilution factor when more than one dilution was used. Grid marking was conducted before sections were mounted on the grids.

Fig 24: Retinal block mounted on cryo pin. Orientation of mounting for transverse sectioning of blood vessels.

2.6.2 LR White Sectioning

Semithin sections (1 and 2 m) were cut on a LKB Ultratome Nova (Cambridge Instruments), mounted on glass slides and stained with toluidine blue. A trapezoid was then cut round the desired area of tissue.

Ultrathin sectioning was more satisfactory at higher cutting speeds than the normal (5mm/sec as compared to 2mm/sec). It was essential that the block face should be kept dry as wetting greatly impaired the sectioning process. Acetone was never added to the distilled water in the trough as it has a deleterious effect on antigenicity. The refractive index of LR white ultrathin sections appeared to be different from that of Araldite or Epon. Interference patterns were therefore higher up the scale to produce sections of comparable thickness. Ultrathin sections were mounted on the matt side of 200 mesh nickel grids. From experience it was discovered that sections mounted on the shiny side were much less liable to survive immunocytochemical manipulations as they sank when placed on the water droplets. In order to ensure adhesion of the sections to the grids, the grids were left to dry for 15 mins in the grid box before rehydration on distilled water. Rehydration was conducted on microtitre plates with one grid per well in the pattern in which they came out of the grid box.

2.6.3 Cryoultramicrotomy

Semithin sections were cut on a Reichert Jung FC4D cryoultramicrotome at -70°C, retrieved from the cryo chamber on a drop of 2.3M sucrose suspended from a wire loop and deposited onto a glass slide. Light microscopic

examination was preceded by toluidine blue staining.

Ultrathin sections were cut at -84°C, retrieved on a sucrose drop and deposited onto carbon coated formvar composite 200 mesh nickel grids. The grids were then placed section side down onto microtitre plate wells of 50mM TRIS buffer until all the sections were cut.

2.7 IMMUNOCYTOCHEMISTRY

2.7.1 Antibodies

The anti-collagen antibodies (types I-V) were raised in goats against human and bovine collagens and were supplied by Southern Biotechnologies (UK distributors, Bionuclear Services Ltd, 24 Westleigh Drive, Sonning Common, Reading RG4 9BL). All were affinity-purified and cross-absorbed against the remaining four purified human collagens. The specificity of these antibodies to human antigens was confirmed by the supplier using indirect enzyme-linked immunosorbent assay.

Polyvalent rabbit antibodies against type VI collagen and laminin were supplied by Heyl (Berlin, Germany) and had been raised in rabbits by multiple injections of human laminin. The serum was tested by the supplier with the following immunological assays: ELISA, immunoblot and immunohistology (fluorescence, APAAP).

The antibody dilutions in TRIS buffer plus 1% BSA, as determined by preliminary experiments, were between 1:30 and 1:150 for types I-IV collagen, type VI collagen antibodies at dilutions between 1:25 and 1:1150 and laminin antibodies at dilutions between 1:20 and 1:80. Types I-V collagen goat antibodies were visualized with 10nm rabbit anti-goat immunogold conjugate. Laminin and type VI

antibodies were visualized with 10nm goat anti-rabbit immunogold (Biocell Laboratories, Cardiff Business Technology Centre, Senghenydd Rd, Cardiff CF24AY).

1- Dilutions

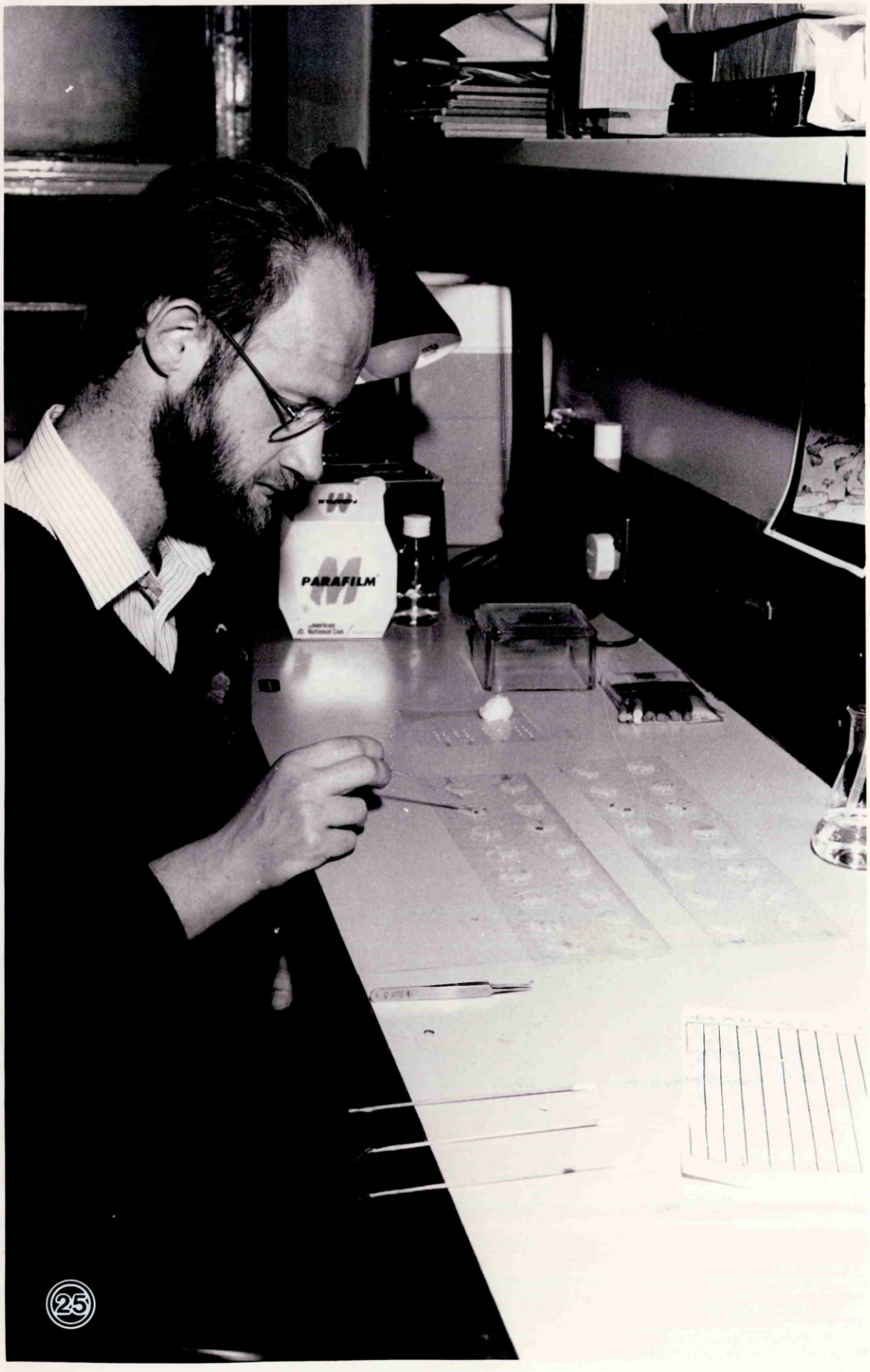
With the delivery of a new batch of primary antibody a range of dilutions was tested in the immunocytochemical procedure to determine the dilution which produced optimal labelling. This dilution was as a rule the minimum dilution of the normal serum control at which there an acceptably low level of nonspecific labelling.

The dilution range was effectively covered by having a factor of two to three between subsequent dilutions with the minimum dilution at 1:20. Dilutions for LR white tissue labelling were generally lower than those of ultrathin frozen tissue sections. A typical dilution range for LR white would be 1:20, 1:60, 1:120, 1:240 whereas a dilution range for ultrathin frozen sections would be 1:60, 1:120, 1:240, 1:480, 1:960, 1:1920. Having determined the preferred dilution it was found that use of that dilution level generally produced satisfactory labelling in all subsequent immunocytochemical runs.

A dilution test was also performed with the immunogold label without the primary antibody. Very little nonspecific labelling was found within the range 1:20 to 1:100. The dilution range was also confirmed by inserting the normal serum before immunogold labelling.

2- Normal Serum in Primary Antibody

Inclusion in the primary antibody solution of 1% bovine serum albumin (BSA), which was essentially immunoglobulin


PARAFILM

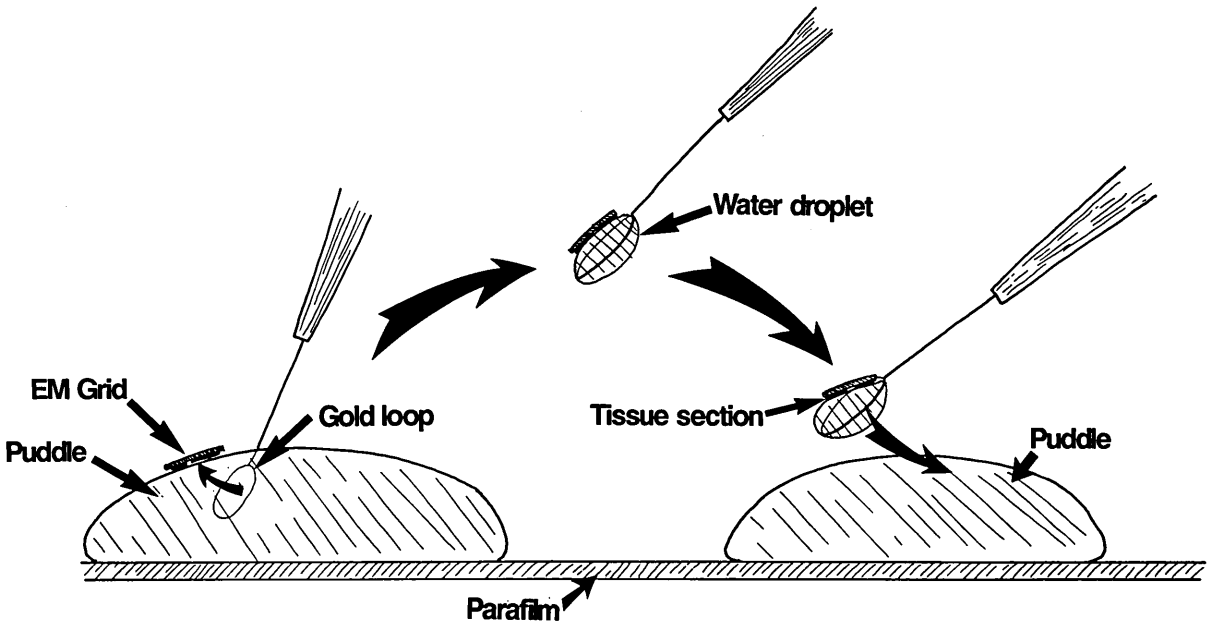
free, helped to prevent non-specific staining by competing with the primary antiserum for non-specific binding sites in the tissue.

2.7.2 Immunocytochemical Buffer

The buffer used throughout the immunocytochemical procedure was 50mM TRIS pH 7.2 with the addition of 0.5M NaCl [80] and 0.05% Tween 20[574] to reduce nonspecific antibody labelling. Tween 20 was omitted from the buffer when labelling ultrathin frozen sections as it causes membrane disruption. The addition of 0.5M NaCl and Tween 20 to the immunocytochemical buffer produced a harsh enviroment for antibody binding. As Tween 20, being a surfactant, was thought to be responsible for the loss of LR white sections from grids, its use was omitted in one immunocytochemical run. Although this resulted in the retention of a larger number of sections, the increase in nonspecific labelling was unacceptable. Carbon Formvar coated grids were also used to promote section retention by increasing the area for adhesion. However, with Carbon Formvar coated grids section retention was promoted at the expense of specific immunogold labelling and with a concomitant increase in the amount of unwanted deposit.

As some nonspecific binding of antibody onto sections is due to ionic forces preventative measures were taken by rinsing after the immunoreaction under high salt concentrations. [80] The conditions should not have been so severe as to dissociate the high affinity specific antigen-antibody bonds.

Fig 25: Immunocytochemical run. Puddles are on Parafilm. Note cotton wool on Parafilm with antibody drops.



Transfer of Grids Between Puddles

2.7.3 Immunocytochemical Techniques

Transfer of grids from one solution to another was accomplished using gold loops (Fig 25) whose diameter was slightly smaller than the outside diameter of the grid. Designated loops were used for each particular antibody. Transfer of fluid via the loop was largely eliminated by making a small break in the loop. The use of forceps to transfer grids was avoided: not only was this technically difficult but also there is greatly increased contamination from one puddle to another due to capillarity between the tips of the forceps.

Buffer and distilled water washes were performed by transferring the grids on loops through a series of 1.5ml puddles on parafilm, depositing the grid onto each puddle for the time indicated. Each antibody had its own separate series of puddle washes.

Antibody labelling was performed on 20ul drops on parafilm. Evaporation of the drops was prevented by covering the group of drops with a glass trough which also enclosed a wad of cotton wool saturated with distilled water.

1- Preincubation with Non-Immune Serum

Preincubation with non-immune serum is an essential step in the procedure and must be applied at the beginning of the procedure. As the non-specific adsorption reaction of the non-immune serum is likely to be of low affinity, the serum is not washed off, but merely drained before the primary antibody is applied and its presence prevents

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Fig 26: Transferring grids of tissue between puddle washes to remove excess antibody.

further non-specific attachments by the primary antiserum. Preincubation was performed with 1%BSA overnight at 4°C.

2.7.4 LR White Immunogold Labelling

On completion of ultrathin sectioning, free aldehyde groups were quenched by incubation on 0.5M NaCl in 50mM TRIS buffer pH 7.4 for 30 mins again using a microtitre plate. The grids were then washed four times in distilled water (2 mins each) and pre-incubated overnight on 1% bovine serum albumin (which is essentially immunoglobulin free) in the buffer at 4°C.

Primary antibody labelling was conducted at R.T. for 2hrs on 20ul drops of antibody diluted in the immuno-buffer with the addition of 1% BSA. Optimal dilution factors were experimentally determined.

The grids were then passed through a series of six buffer puddles (4 mins each) to remove unbound antibody and deposited on 1% BSA dissolved in the buffer for 5 mins. Immunogold labelling was conducted for 1hr at R.T. on 20ul drops of the gold conjugate diluted in the immuno-buffer with the addition of 1% BSA.

Unbound gold conjugate was removed with three washes (4 mins each) of immuno-buffer plus 0.2% BSA followed by three washes (4 mins each) with immuno-buffer. The sections were then fixed in 2.5% glutaraldehyde in 0.15M sodium cacodylate buffer pH 7.2 for 5 mins in a modified grid box used for staining and washed three times (1 min each) in distilled water. Staining was performed with freshly made saturated uranyl acetate in distilled water for 15 mins using the grid box stainer in a light tight container. This was followed by three quick distilled

water washes before viewing in a Philips 301 transmission electron microscope at 80kV.

2.7.5 Immunolabelling of Ultrathin Frozen Sections

Quenching of free aldehyde groups was accomplished with four 2 min changes of 30mM glycine in 50mM TRIS buffer at R.T. The grids were then drained and pre-incubated overnight on 1% BSA in the TRIS buffer at 4°C.

The subsequent immunocytochemical procedure used was identical to that described in section 2.6.3 up to but not including staining.

1- Control of Section Contrast

At the beginning of this study ultrathin frozen sections were then embedded with a 1:1 mixture of methyl cellulose and 2% aqueous uranyl acetate as described by Tokuyasu (1986).[202] Infiltration of the mixture was conducted on ice for 10 mins and excess stain removed by drawing the grid on a loop across filter paper. The grid was then allowed to dry on the loop. The final thickness of the methyl cellulose film was critical to both contrast and fine structural preservation. Optimal films had gold to blue interference colours. As the film got thicker, fine structural preservation improved at the expense of contrast.

Methyl cellulose embedding was later discontinued in favour of polyvinyl alcohol as the latter was not only more convenient to use and prepare but also produced an improved level of contrast. Embedding was performed in a 10:1

Fig 27: Philips 301 transmission electron microscope.

mixture of 3% polyvinyl alcohol and saturated uranyl acetate for 10 mins at R.T. Excess stain was drained and the grid dried on the loop.

2.7.6 Controls

Two types of negative controls were incorporated into the immunocytochemical procedure, (1) omission of the primary antibody, (2) substitution of the primary antibody with non-immune serum from the same species in which the primary was raised. The first negative control was of little significance in that non-specific labelling was always found to be minimal.

The normal goat [Sigma-S 2007] and rabbit sera [Sigma-S 2632] controls were conducted at dilutions identical to those used for the corresponding antibodies. In order to enhance the reliability of the results, comparison was made in immunolabelling between ultrathin frozen sections and LR white embedded tissue sections from adjacent tissue blocks.

2.7.7 Assessment of Results

1- Positive Immunogold Labelling

An antigen was considered to be positively localized if the immunogold particles were restricted to discrete structures. Such labelling, particularly if it was weak, was not considered a positive result if a significant number of immunogold particles were present on what was termed internal negative controls which were the first structures to exhibit nonspecific labelling. These internal negative controls were cell nuclei, mitochondria, pigment granules and red blood cells. The distribution of labelling had to be reproduced in four different cases

before it was considered to be genuine.

Little attention was paid to accumulations of immunogold particles associated either with folds and holes in the sections or with contaminating deposits that were usually homogenous and of low electron density. Such labelling was regarded as spurious.

2- Negative Immunogold Labelling

In order to ensure that the absence of labelling was due to the absence of the antigen and not to failure of the immunocytochemical technique, a tissue in which the antigen was known to be present was included in the immunorun which is refered to as the positive control. With most of the antigens a positive control was normally present within the same tissue section (internal positive control). However, a suitable positive control for type II collagen was not available.

Chapter 3: Results

3.1 Opening Remarks

In this investigation every effort was made in preliminary experiments (2.3.1) to provide an optimum environment for the precise localisation of the extracellular matrix components studied. To minimise the risk of the sources of artifacts surgically excised ocular tissues were used whenever possible. Tissue was taken from surgically removed human eyes in the fifth to seventh decades. Autopsy tissue was only used in the localisation of collagens I-VI and laminin in aged lens capsule. Although the fixation time of two hours was regarded as optimal, the policy is still to use a variety of fixative concentrations and buffers. Considerably better ultrastructural preservation was obtained using phosphate buffer compared to cacodylate, but this was at the expense of antigenic preservation. Tissue fixed in formaldehyde facilitated the highest degree of antigenic preservation but with correspondingly poor ultrastructure. However, consistent results were obtained with all the specimens examined.

At a technical level, LR white processed tissue was considerably more convenient to use for immunocytochemistry than tissue processed for cryoultramicrotomy. Although LR white ultrathin sections were more unstable under the electron beam than conventional ultrathin araldite sections, they were almost always superior in quality to ultrathin frozen sections which were prone to fragmentation, excessive folding and uneven thickness. Ultrathin frozen sections of trabecular meshwork were particulary unsatisfactory in that the cribriform layer was almost invariably folded over on itself which prevented

documentation of collagen distribution in ultrathin frozen sections of this region. Such problems were due to the absence of an embedding medium to support the tissue during the immunocytochemical procedure. However, cryoultramicrotomy was much more satisfactory in the localisation of types V and VI collagen than LR white in that positive labelling for these collagen types was always considerably stronger with the former technique.

Although the addition of 0.5M NaCl and Tween 20 to the immunocytochemical buffer produced a harsh enviroment for antibody binding, it resulted in a minimal degree of nonspecific binding of antibodies to the tissue sections. The specific findings are dealt with in the following pages according to anatomical subcompartments.

3.2 Cornea

In general, types I, III, V and VI collagen shared a similar distribution, in that they were present in Bowman's layer and the corneal stroma. However, type VI collagen differed in its distribution from the others in relation to individual collagen fibrils as labelling for type VI was associated with the matrix between the fibrils rather than with the fibrils themselves. Type IV collagen and laminin were localised to Descemet's membrane. Laminin was also localised beneath the corneal epithelium. Type II collagen was absent from cornea.

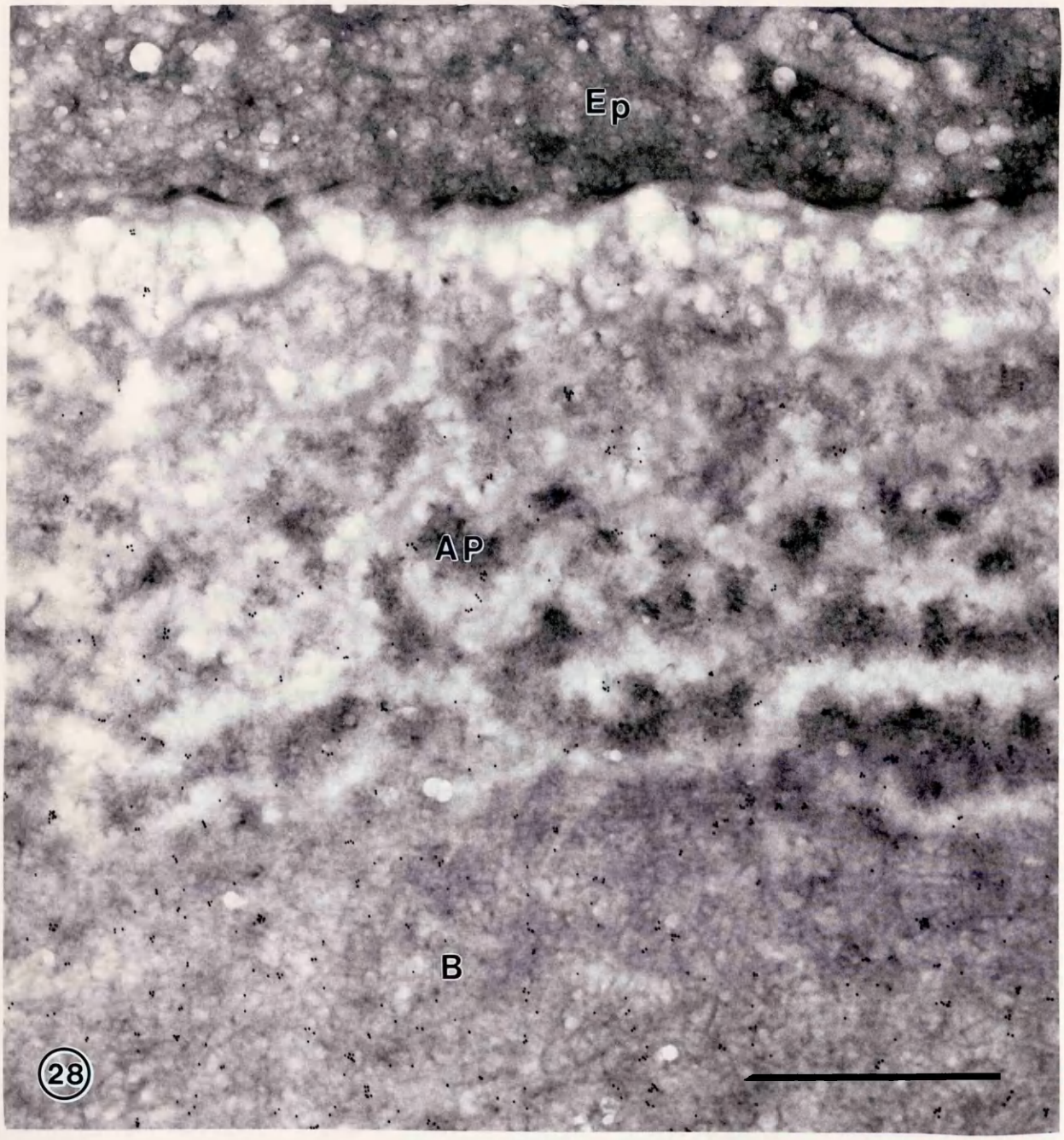
Immunogold localisation of types V and VI collagen was considerably stronger on ultrathin frozen sections when compared to LR white embedded tissue fixed in an identical manner. Type VI collagen exhibited the highest labelling density of all the extracellular matrix components studied.

Of particular interest was labelling of keratocyte bodies (v.i.) for both types V and VI collagen, the perifibrillar location of type VI, its absence from stromal long-spacing collagen, and the localisation of laminin and type VI collagen over anchoring plaques. The detailed analysis is as follows:-

3.2.1 Basement Membrane Complex

Types I, III, IV and V Collagen. Types I, III, IV and V collagen was absent from the basement membrane complex of the corneal epithelium.

Type VI Collagen. Type VI collagen labelling of the basement membrane complex was restricted to discrete plaques of basement membrane-like material lying close to the epithelium (Fig 28). These plaques were considered to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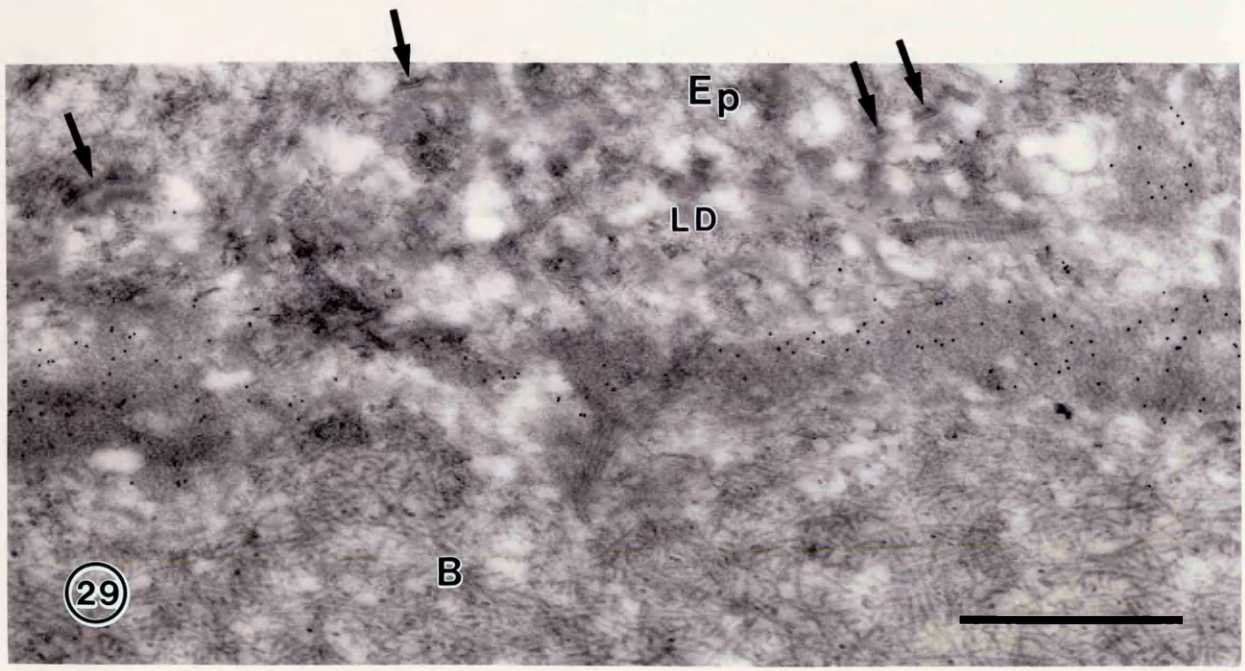
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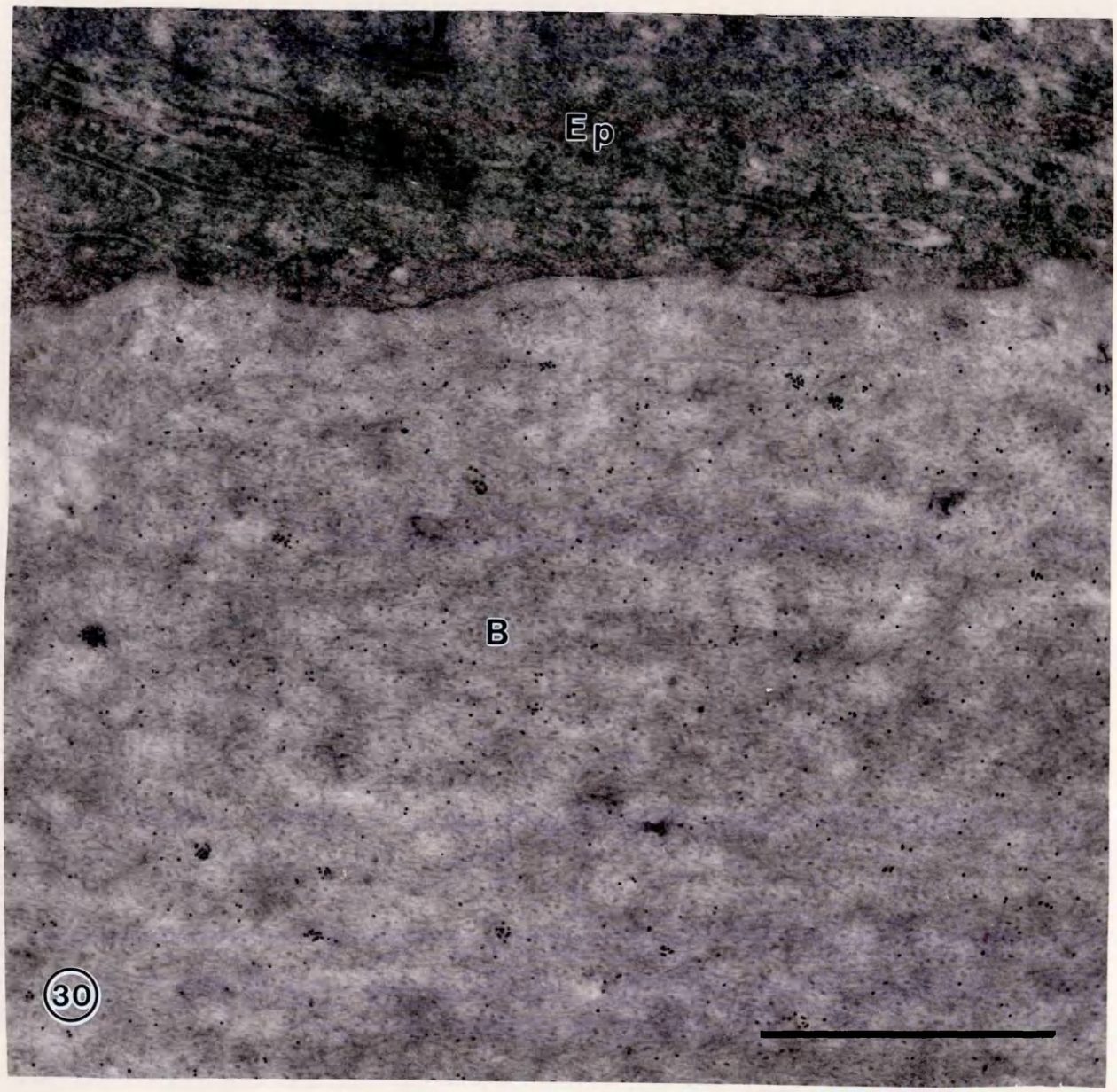
be anchoring plaques on account of three distinguishing features as described by Brewitt and Reale:[24] (1) such plaques consisted of basement membrane-like material, (2) they were distinctly separate from the epithelial basement membrane (3) they were located about 0.5 to 2um beneath the lamina densa. The lamina densa of the epithelium was not labelled with type VI collagen antibodies.

Laminin. Laminin was localised principally to the sub-epithelial region of the cornea. Closer examination revealed that laminin was mainly present within the anchoring plaques of the basement membrane complex (Fig 29). As with type VI collagen, labelling was absent from the lamina densa.

Short striated collagen fibrils were occasionally observed beneath the epithelium. These fibrils had a considerably greater diameter (up to 150nm) than those present either in Bowman's layer or the stroma. No labelling was seen over such fibrils.

Fig 28: Type VI collagen in oblique section of epithelial basement membrane complex and Bowman's layer (BL). Electron dense anchoring plaques (AP) in the basement membrane complex are labelled with immunogold particles as is the interfibrillar matrix of Bowman's layer. Note absence of labelling over basal cell of epithelium (Ep) The circular spaces within and beneath the epithelium are stretching and air drying artifacts that occur when the section is dehydrated at the end of the immunocytochemical procedure. Cryo; Bars represent 1um unless stated otherwise.

Fig 29: Laminin labelling of epithelial basement membrane complex. Hemidesmosomes (arrowed) mark the location of the lamina densa (LD) of the basal lamina. Labelling is restricted to basement membrane-like material beneath the basal lamina. An anchoring fibril is arrowheaded. BL:Bowman's layer, Ep:Epithelium, LR white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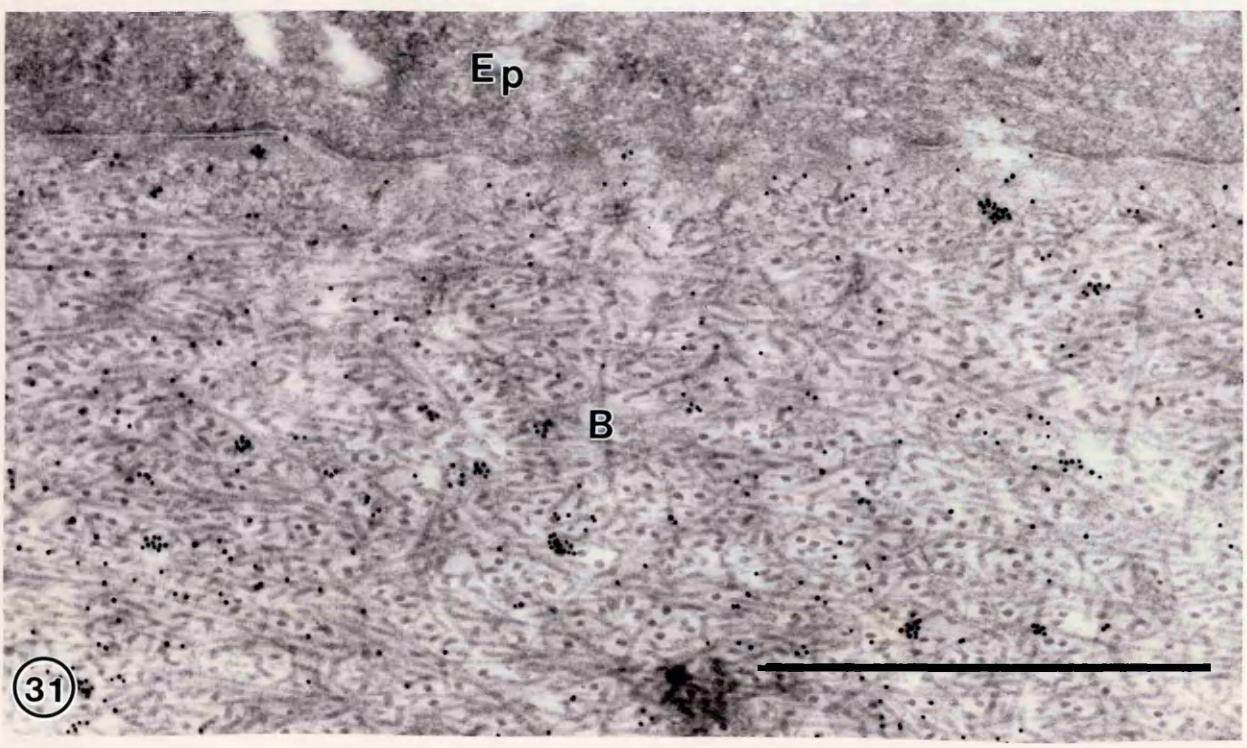
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3.2.2 Bowman's Layer

Bowman's layer exhibited strong labelling for types I, III, V and VI collagen (Figs 28, 30 & 31). No difference in labelling intensity was discerned between Bowman's layer and the stroma (Figs 28 & 30-34).

Immunogold labelling of collagens I, III and V was restricted to the interwoven striated collagen fibrils -the interfibrillar matrix was free of label (Figs 30 & 31). Type VI collagen differed in its distribution from the other collagens in its perifibrillar location - immunogold particles were more frequently observed in the interfibrillar matrix than over individual collagen fibrils (Fig 28).

3.2.3 Stroma

Types I, III, V and VI collagen were observed in the corneal stroma. Labelling for type IV was absent from the stroma.

Type I collagen (Fig 32) Immunogold binding to type I collagen in the stroma was frequently denser on fibrils cut in longitudinal section than fibrils cut in any other plane. The localisation of type I collagen was regarded as extremely specific since keratocytes and corneal epithelial

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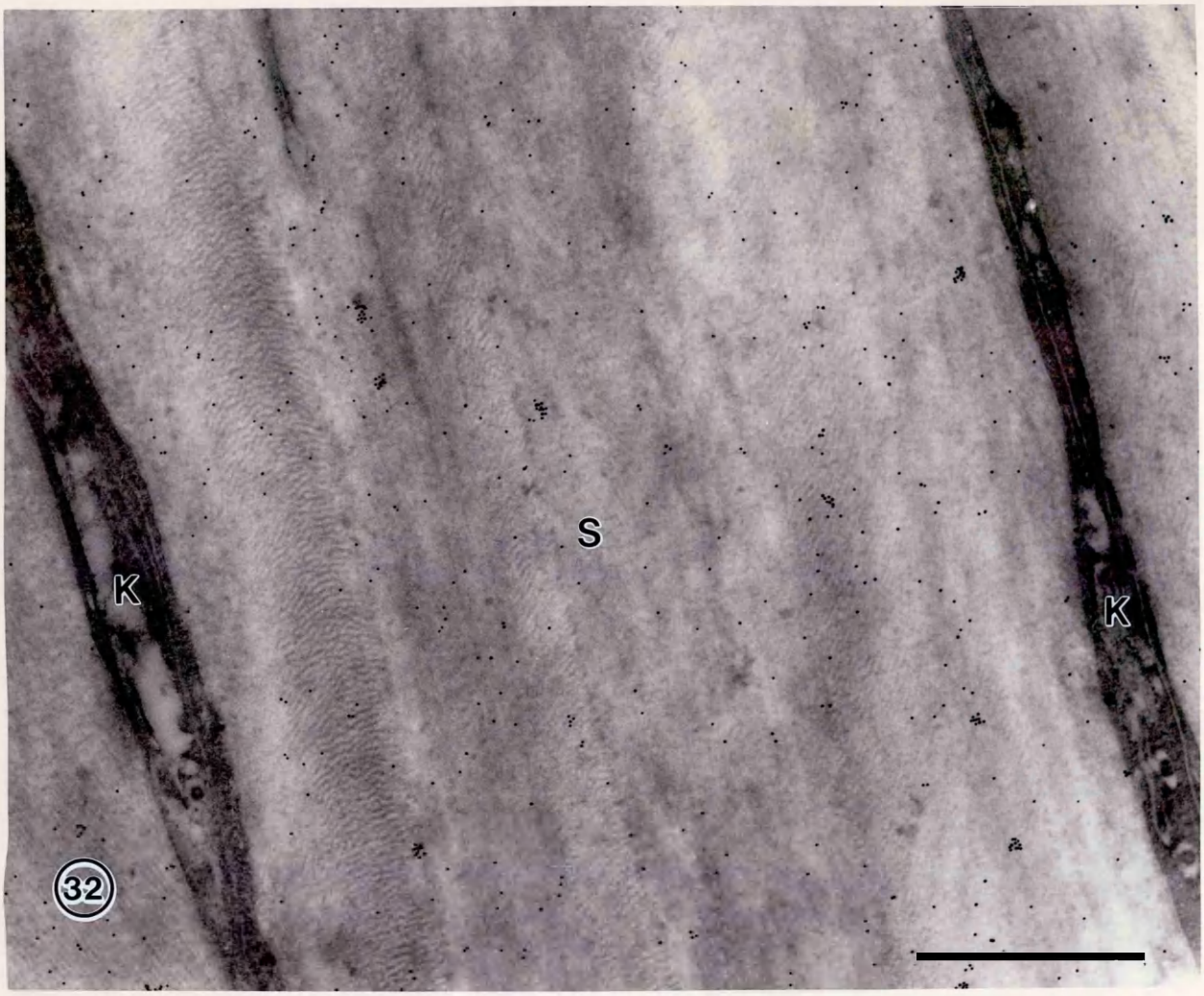
Fig 30: Type I collagen immunogold labelling of collagen fibrils in Bowman's layer (BL). Both the epithelium (Ep) and the interfibrillar matrix (seen as electron lucent areas in Bowman's layer) are free of immunogold particles. Little labelling is present in the region of the epithelial basement membrane complex. LR white

Fig 31: Immunogold labelling of type V collagen in Bowman's layer (BL). Immunogold particles are localised to striated collagen fibrils and are absent from the interfibrillar matrix and epithelium (Ep). The basal lamina of the epithelium is largely free of label. LR white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and endothelial cells were free of label.

Type III collagen Type III collagen labelling was present on stromal fibrils (Fig 33). Unlike type I collagen, labelling in the stroma was not any less intense over collagen fibrils cut in an oblique manner than those seen in almost perfect longitudinal section (Fig 33).

Type V collagen Type V collagen was uniformly distributed throughout the corneal stroma between Bowman's layer and Descemet's membrane. Immunogold particles were more frequently seen directly on top of the stromal fibrils rather than over the interfibrillar matrix. There were no obvious variations in the intensity of labelling linked to the orientation in which the fibrils had been sectioned.

Type VI collagen Intense immunolabelling for type VI collagen was present throughout the corneal stroma (Fig 34). Immunogold particles were more frequently seen in the interfibrillar matrix rather than directly over the collagen fibrils. This was in contrast to labelling for collagens I, III and V in which immunogold particles were localised to collagen fibrils. Immunogold particles were frequently distributed in the form of short chains running at an angle to the striated collagen fibrils (Fig34insert). This suggested that type VI collagen formed filamentous structures closely associated with the striated fibrils. Type VI collagen labelling was reduced in regions where the striated collagen fibrils of the corneal stroma were packed

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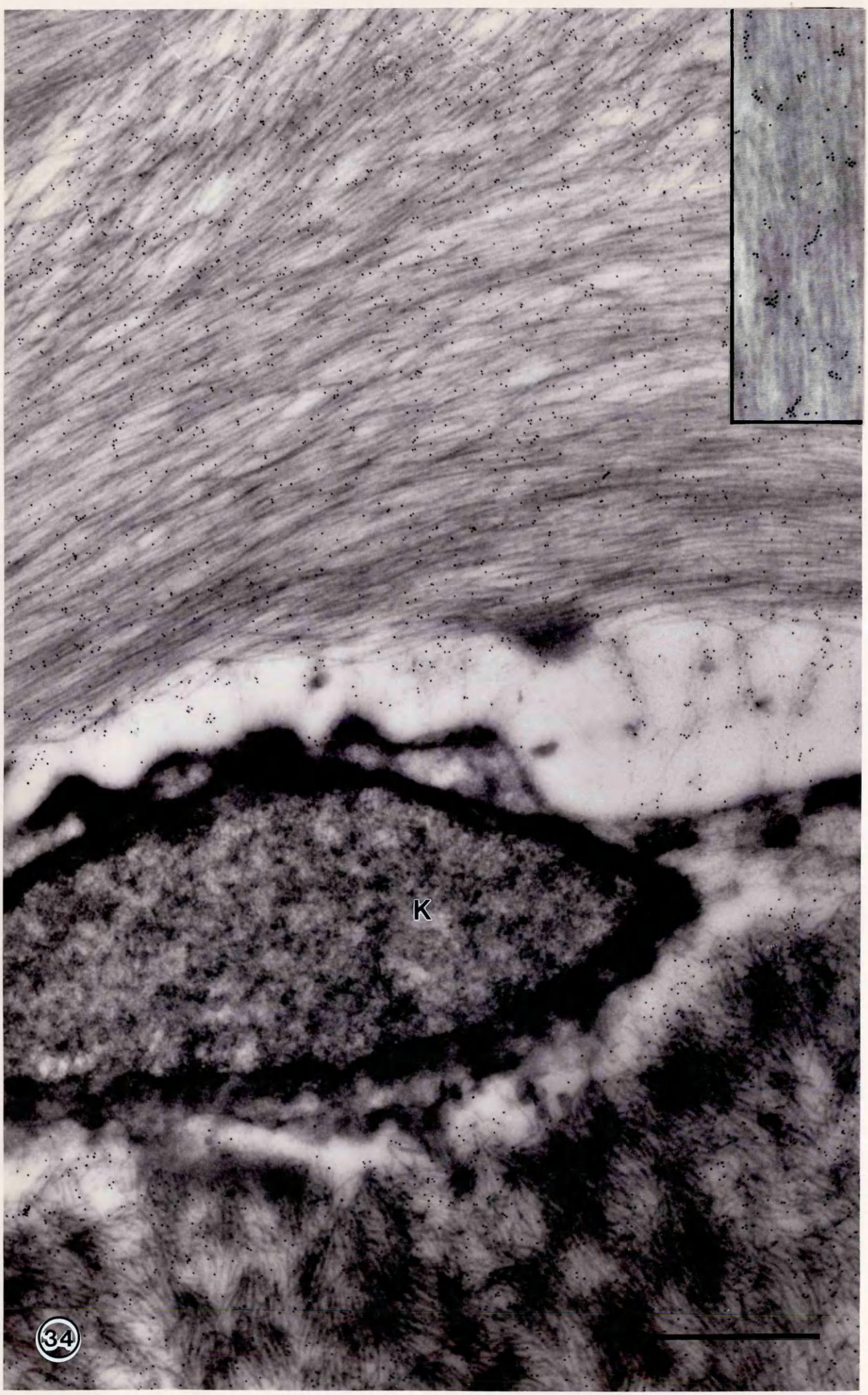
Fig 32: Type I collagen labelling of corneal stroma (S). Labelling is of a similar intensity to that of Bowman's layer (Fig 30). Note absence of labelling from keratocytes (K). LR white.

Fig 33: Type III collagen labelling of corneal stroma. Keratocyte (K) is free of label. LR white.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in perfect parallel arrays. Labelling was more intense in regions where the normal arrangement was slightly disturbed.

In ultrathin frozen sections immunogold particles were also observed on filamentous structures seen wherever keratocytes had separated from the stromal lamellae (Fig 34).

3.2.4 Keratocyte Bodies

The frequent presence of large deposits of extracellular matrix-like material within or adjacent to the cytoplasm of keratocytes was an impressive feature of the stroma (Figs 35-37). These electron dense bodies were either sandwiched with keratocytes between the stromal lamellae or were actually within the keratocytes themselves. In conventionally processed material, the bodies located within the keratocytes were surrounded by a unit membrane which was fused with the plasmalemma to form pores or fenestrations (Fig 35). Fine filamentous strands passed between the inclusion bodies and the exterior (Fig 35). These fine filaments bore a strong resemblance to the filaments between collagen fibrils of the stromal lamellae (c.f. Fig 7). Similar connections were present between the extracellular deposits adjacent to the keratocyte cytoplasm

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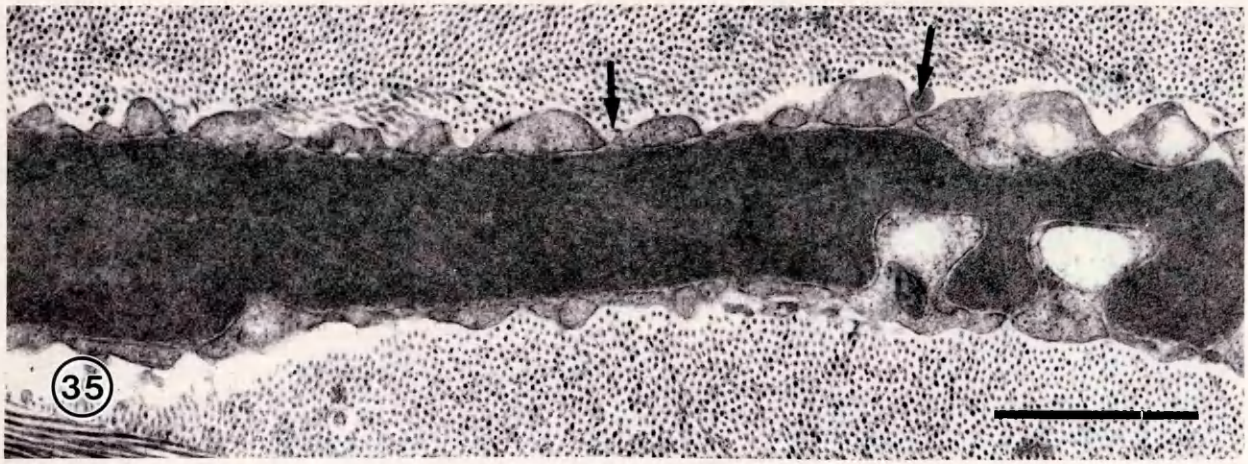
Fig 34: Type VI collagen in corneal stroma. Labelling is intense with immunogold particles normally localised on either side of the collagen fibrils. Labelling is also present over a loose network of filaments where the lamella has separated from a keratocyte (K). Nu = nucleus of keratocyte. Cryo.

Insert: Higher magnification of stromal labelling for type VI collagen. Occasional threads of gold particles among the striated collagen fibrils indicate the filamentous nature of type VI collagen. Cryo; Bar indicates 0.25um.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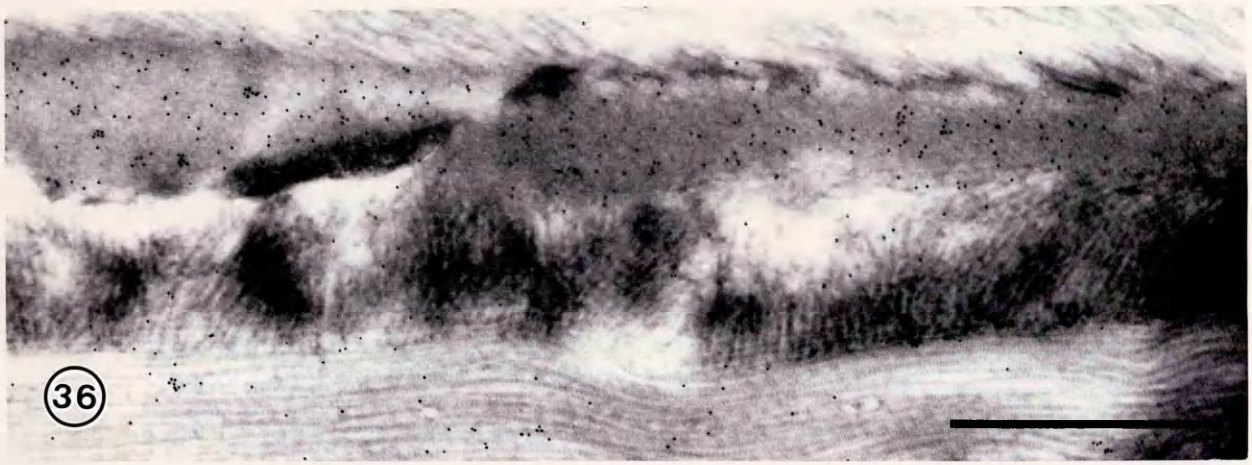
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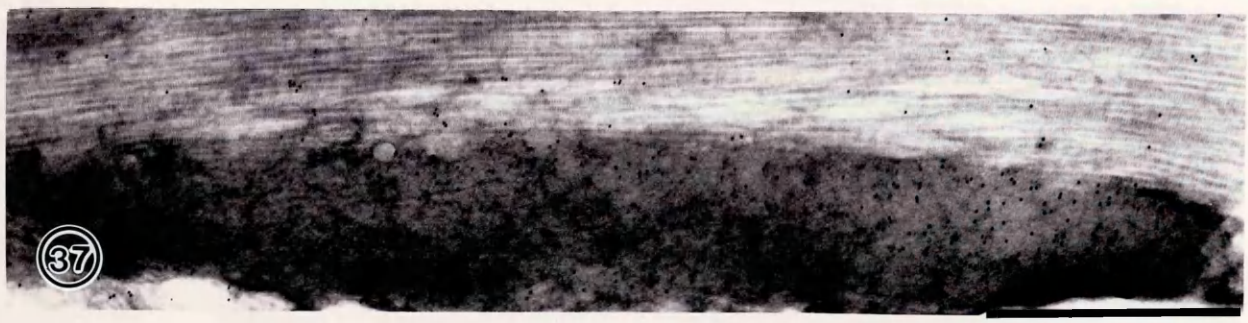
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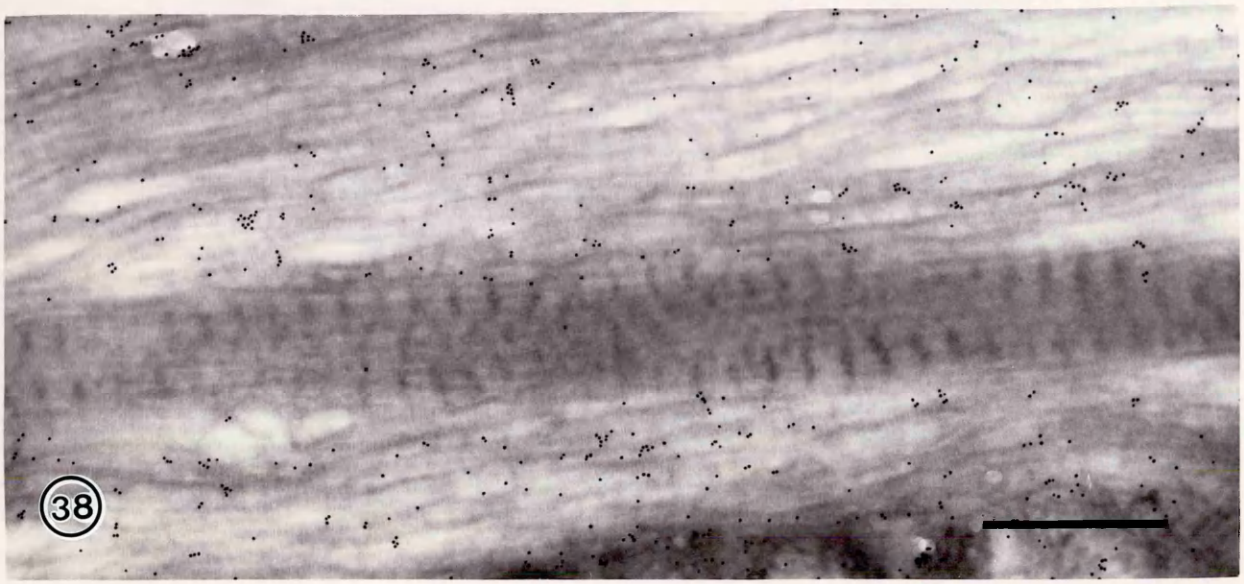
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and the lamellar fibrils. These keratocyte bodies were strongly labelled for both type V (Fig 36) and type VI collagen (Fig 37). Weak labelling was also observed with type III collagen.

3.2.5 Long-spacing collagen

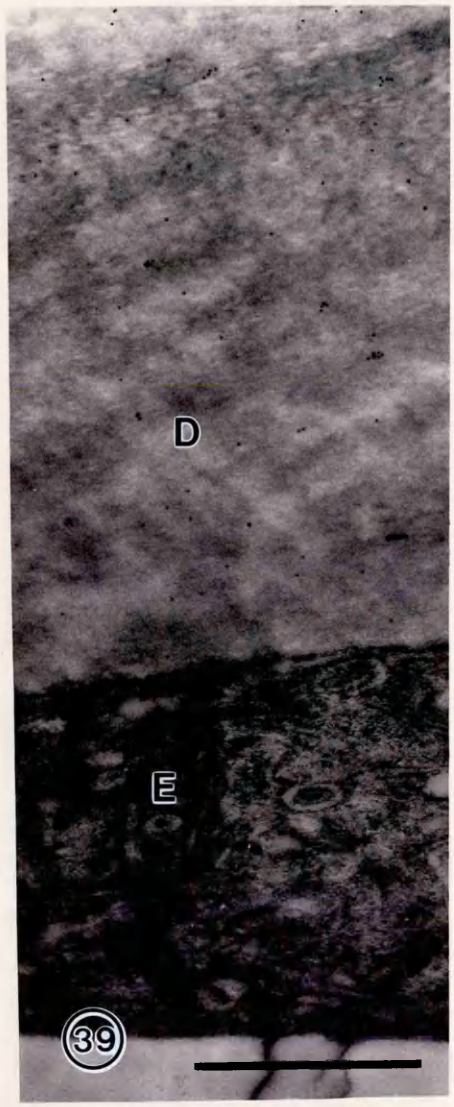
Another intriguing observation was the frequent presence of long-spacing collagen (100-110nm banding) in the corneal stroma (Fig 38). This extracellular matrix deposit was either located between the stromal lamellae or embedded within the substance of individual lamellae, with its banding pattern at right angles to the striated fibrils of the stroma. Striated collagen fibrils were frequently seen to lie within this deposit running parallel to its longitudinal axis. Labelling of such structures was not observed with any of the antibodies used in the present investigation.

Fig 35: In conventionally prepared tissue the intracytoplasmic bodies of keratocytes appear electron dense and granular. Fine filaments project through fenestrations (arrowed) to the adjacent lamellar fibrils. The keratocyte cytoplasm also contains rough endoplasmic reticulum and numerous vesicles. Araldite

Fig 36: Ultrathin frozen section of keratocyte intracytoplasmic body labelled for type V collagen. Type V collagen labelling is also present on stromal collagen fibrils. Cryo.

Fig 37: The electron dense keratocyte body in this ultrathin frozen section is intensely labelled with type VI collagen. In this preparation the body appears to have a filamentous substrate. Cryo.

Fig 38: Long-spacing collagen in corneal stroma. Immunogold labelling for type VI collagen is largely absent from the long-spacing collagen and is preferentially localised to the interfibrillar matrix between the striated collagen fibrils of the lamella. Cryo; bar indicates 0.5um.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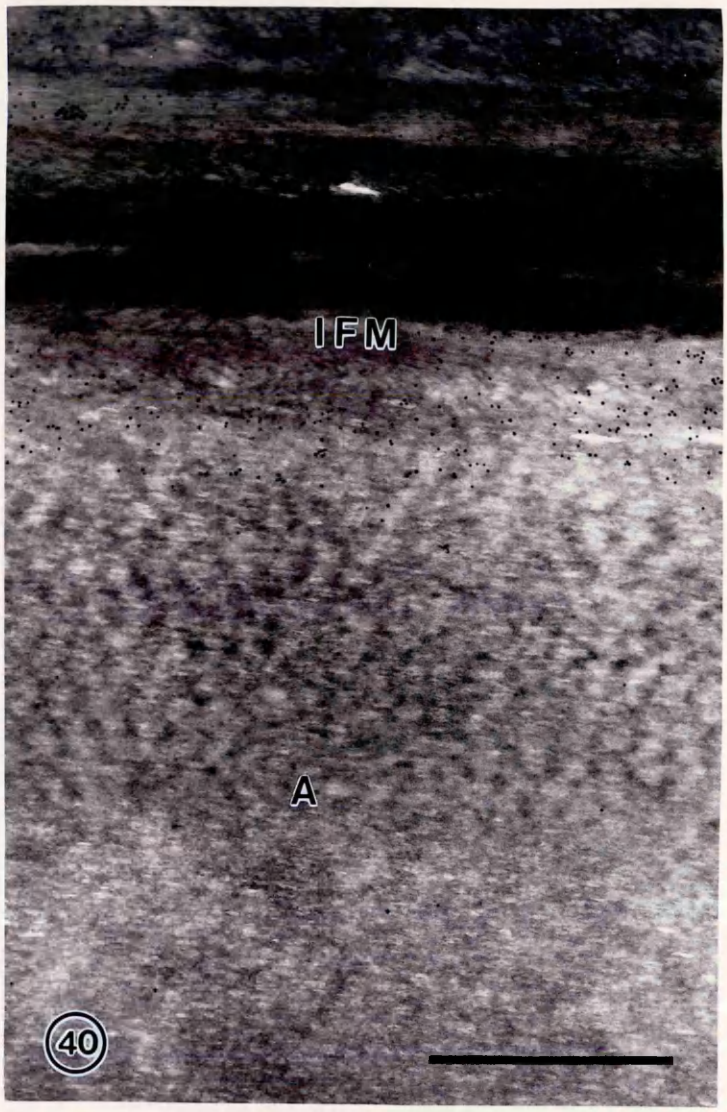
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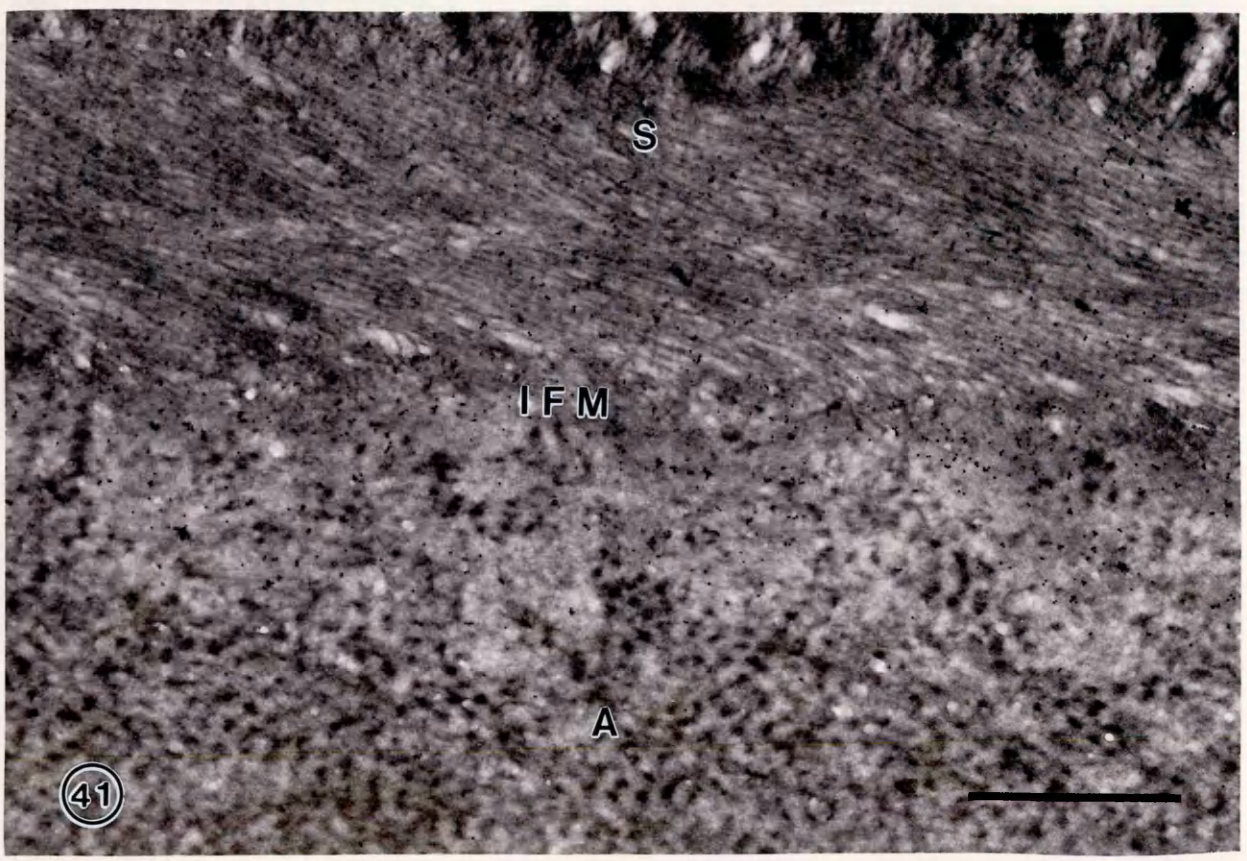
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3.2.6 Descemet's Membrane

Types I and III collagen Type I collagen was absent from Descemet's membrane. Although labelling for type III collagen was observed in the posterior non-banded region, the signal was weak and was restricted to a zone adjacent to the endothelium.

Type IV collagen Type IV collagen was demonstrated both on LR white sections and ultrathin frozen sections in the posterior non-banded region of Descemet's membrane (Fig 39). Although weak labelling was also noted in the anterior banded region on ultrathin frozen sections, no such labelling was observed on LR white sections. No labelling was observed with either technique over a thin non-banded zone of basement membrane lying anterior to the anterior banded region, but still within Descemet's membrane.

Type V collagen Although labelling of the stroma was comparable to that of Bowman's layer, a significantly greater density of labelling for type V collagen was observed over the interfacial matrix of the corneal stroma bordering Descemet's membrane (Fig 40). Type V collagen

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Fig 39: Type IV collagen labelling of posterior non-banded region of Descemet's membrane (D). Labelling is absent from the endothelium (E). LR white

Fig 40: Type V collagen labelling of interfacial matrix (IFM) of the stroma and Descemet's membrane. Labelling of the interfacial matrix is intense and is also present in the most anterior zone of Descemet's membrane in a thin non-banded region of basement membrane. Immunogold particles in this region appear not to be associated with striated collagen fibrils. The anterior banded region (A) of Descemet's membrane is free of label. Cryo.

Fig 41: Type VI collagen labelling of the interfacial matrix (IFM) extends further into Descemet's than does type V collagen. Note weak labelling of the anterior banded region (A). S: posterior lamella of stroma;


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was also present at the junction of Descemet's membrane with the stroma. More specifically, labelling was observed over a thin non-banded zone of Descemet's membrane sandwiched between the anterior banded region of Descemet's membrane and the interfacial matrix of the corneal stroma (Fig 40).

Type VI collagen As with type V, labelling for type VI collagen was observed over a thin non-banded zone of Descemet's membrane sandwiched between the anterior banded region and the most posterior corneal lamella (Fig 41). Scant labelling for type VI collagen was present over the anterior portion of Descemet's membrane. The posterior non-banded region of Descemet's membrane (c.f. Fig 8, section 1.3.1.4) was free of label.

Laminin. A weak patchy signal for laminin was also observed at the endothelial face of Descemet's membrane.

3.2.7 Controls

Immunogold particles were rarely observed in rabbit anti-goat and goat anti-rabbit immunogold control sections (Fig 42). Although more immunogold particles were observed in the negative normal goat and rabbit serum control sections, the particles were distributed in a random manner and were also present on the nuclei and cytoplasm of the stromal keratocytes, corneal epithelial and endothelial cells.

Fig 42: Normal goat serum control (1:30 dilution) in corneal stroma.

3.3 Aqueous Outflow System

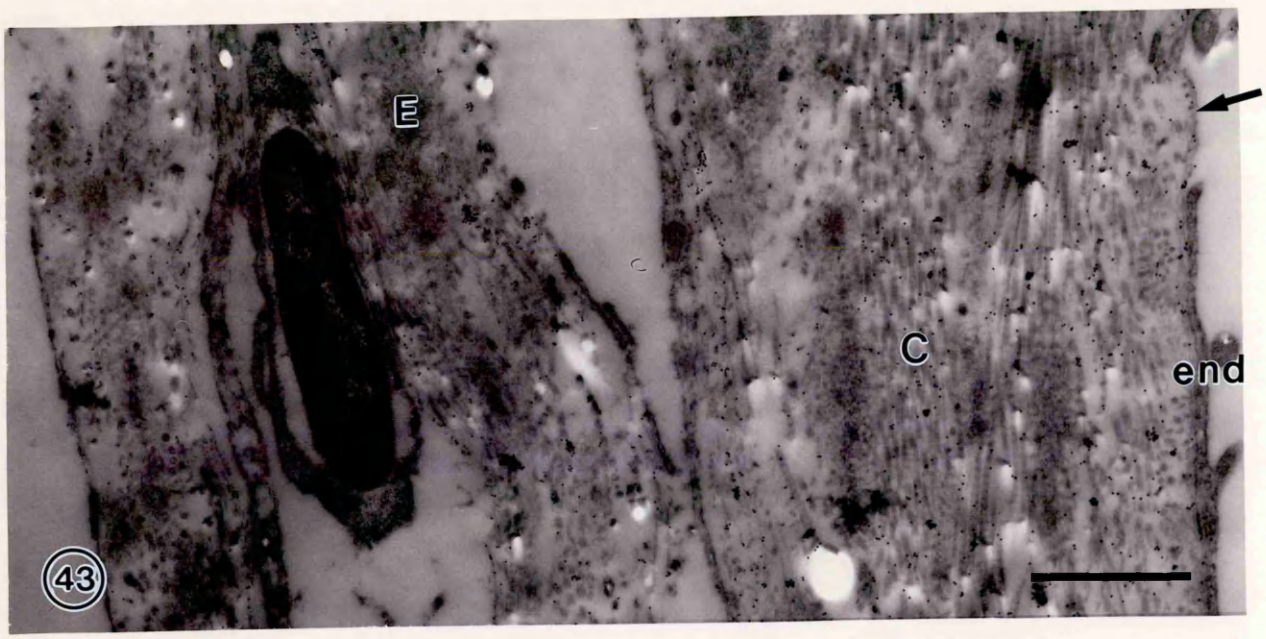
All eleven trabecular specimens used in this study were considered normal by light microscopic examination of toluidine blue stained semi-thin plastic sections and by paraffin histology of the blocks used for the pathological report. No attempt was made to analyze changes specific to aging as this was a relatively small sample and the intensity of labelling varied slightly according to the fixative used. Tissue fixed in PFA only, provided stronger staining than tissue fixed in a mixture of PFA and glutaraldehyde.

The collagen distribution in the aqueous outflow system was qualitatively uniform and was studied in three morphologically distinct zones : uveal and corneo-scleral meshwork, juxtacanalicular tissues (cribriform layer) and outer wall of Schlemm's canal.

Types I, III, IV, V and VI collagen were present in all of the zones investigated and type II collagen was absent throughout. Laminin was localised mainly to the cribriform layer. Cryoultramicrotomy was considered more sensitive in the fine structural localization of types V and VI collagen. Unfortunately, lack of support for the cribriform layer during cryoultramicrotomy prevented the use of ultrathin frozen sections in the study of this region. However, studies on the distribution of collagens I, III and IV and the glycoprotein laminin within the cribriform layer were performed on LR white ultrathin sections.

3.3.1 Uveal and Corneo-scleral Meshwork

No differences in either the intensity or pattern of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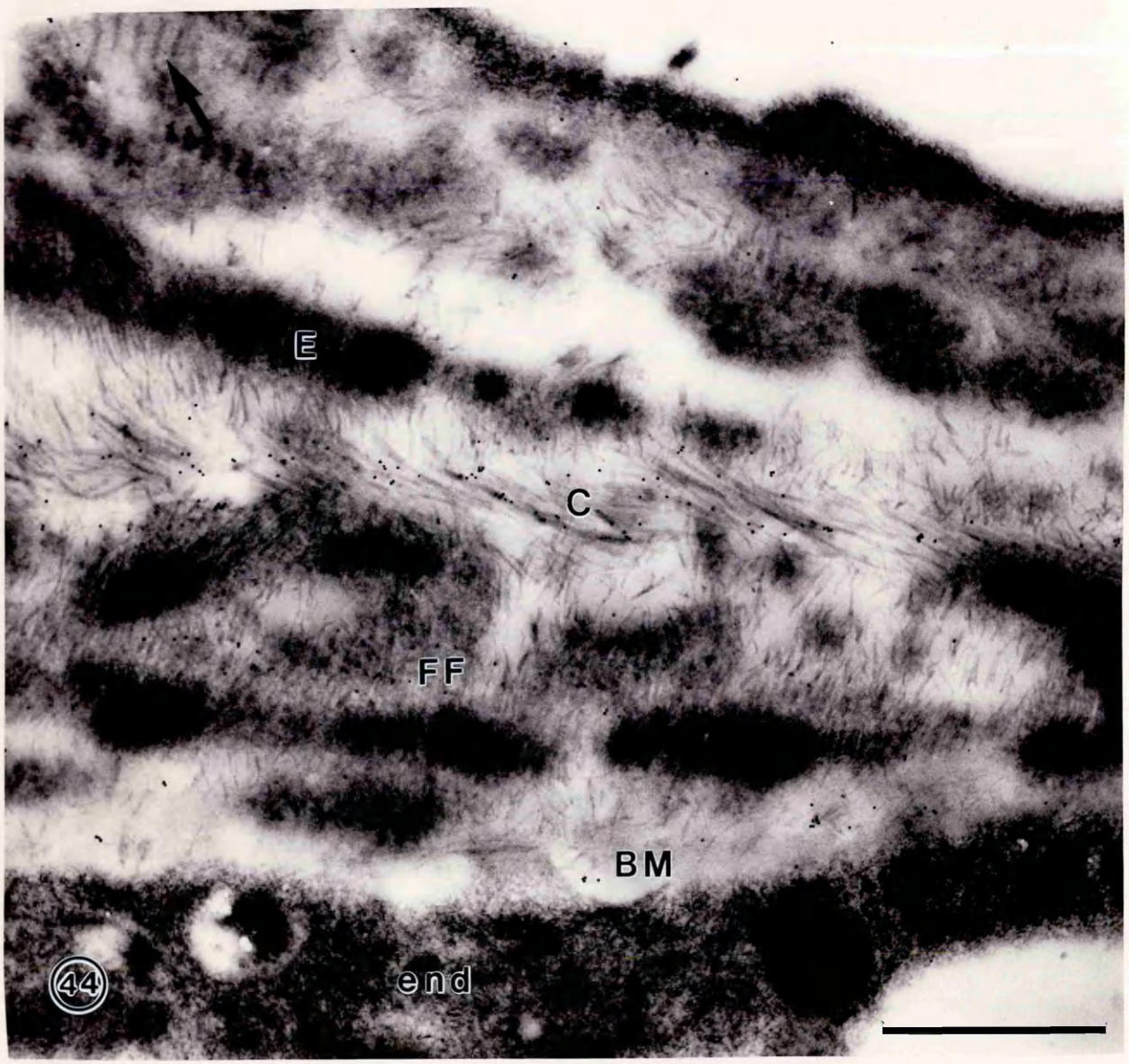

E
C
FF
BM
end
44

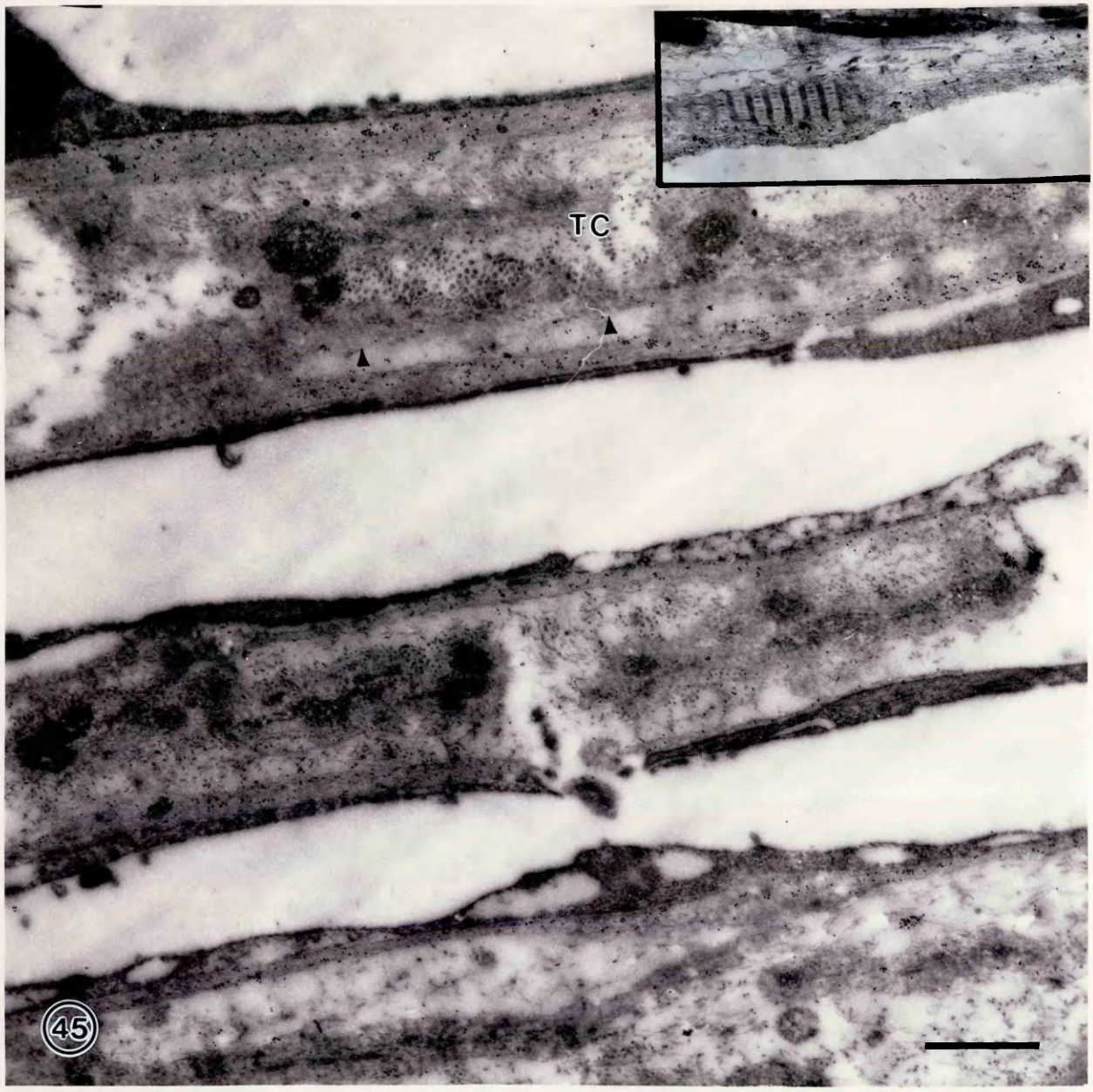
No differences in either the intensity or pattern of antibody labelling for collagens I, III, V and VI were noted between the uveal and corneo-scleral meshwork within each individual eye.

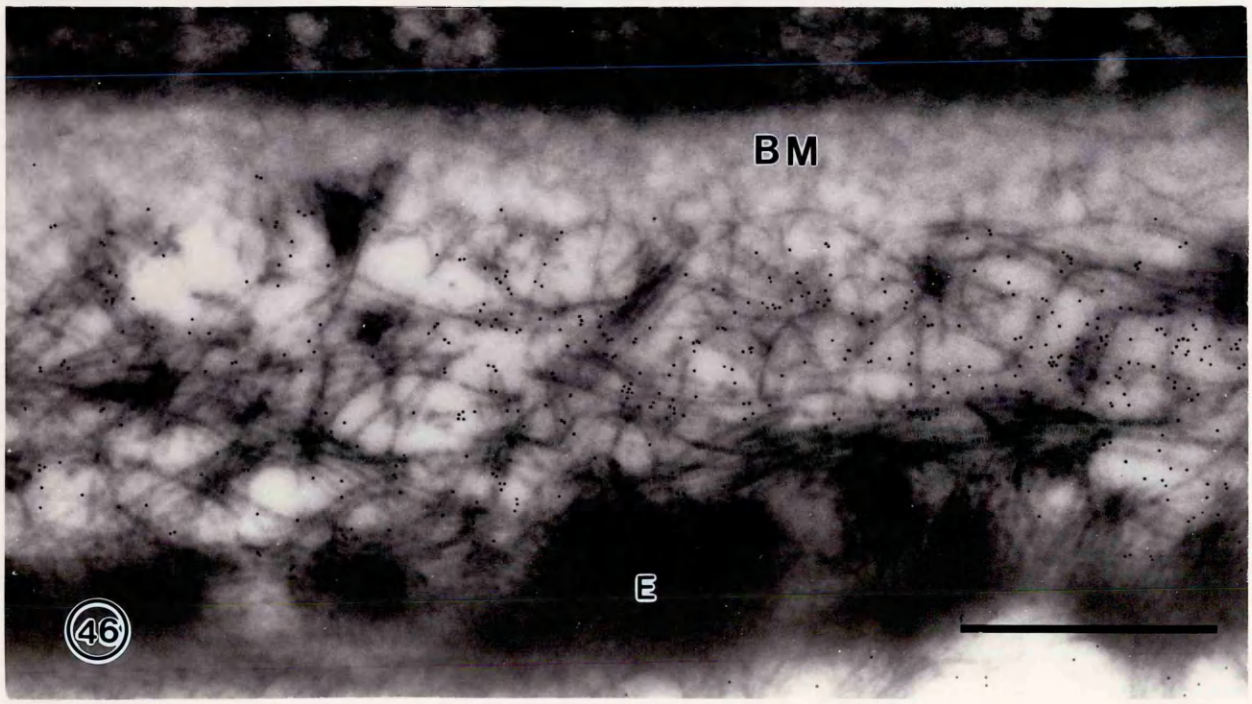
Type I collagen. The presence of type I collagen was demonstrated in the collagenous core, basal laminae (basement membranes) and fine fibrillar-like material of the uveal and corneo-scleral layers (Fig 43). Labelling of the core was restricted to striated collagen fibrils: such labelling was absent from non-striated fibrils of smaller diameter. No labelling was observed over bundles of elastin-like fibers present in the core (Fig 43); neither was labelling observed on the sheath of fine granular-like material surrounding these fibers nor over bundles of long-spacing collagen that were embedded in the basement membrane.

Type III collagen. The striated collagen fibrils of the trabecular cores were positively labelled with type III collagen antibodies (Fig 44). As with type I collagen, labelling was absent from bundles of elastin-like fibers and associated fine granular-like material.

Fig 43: Type I collagen labelling of corneo-scleral meshwork. Striated collagen fibrils [C] of the trabecular beams are labelled with immunogold particles. Electron dense elastin-like fibers [E] are free of label. The thin basement membrane [arrowed] above the endothelial cell [end] contains immunogold particles. LR white; bars represent 1um unless stated otherwise.

Fig 44: Immunogold localization of type III collagen in corneo-scleral beam. Both the striated collagen fibrils [C] and fine fibrillar-like material [FF] of the core are labelled with immunogold particles. Labelling of the fine fibrillar-like material is restricted to the fibrillar component. A small degree of labelling is also present over basement membrane [BM] in the periphery of the beam. Both long-spacing collagen [arrows] and elastin-like fibres [E] are free


TC
45


BM
E
46

Labelling of the basement membrane lining the trabecular endothelial cells was sparse and unconvincing. On no occasion was labelling for type III collagen observed over long-spacing collagen sandwiched in the basement membrane of the endothelial cells (Fig 44).

Type IV collagen. Labelling for type IV collagen was noticeably stronger in the uveal meshwork than in the corneo-scleral meshwork and was largely restricted to the basement membranes of the endothelial cells lining the trabecular beams (Fig 45). Type IV collagen localization on these basement membranes frequently included coarse filamentous strands embedded in the amorphous lamina densa (fine filamentous-like material) as well in loose irregular filamentous arrays of the lamina fibroreticularis. Type IV collagen labelling was absent from the collagenous cores of the beams. Less prominent but observable labelling was present on basal lamina-like material lining the cores (Fig 45). Long-spacing collagen was negative for type IV but the sheath of basal lamina-like material in which it was normally enclosed exhibited a positive signal (Fig 45 insert).

Type V collagen. The collagenous core of the trabecular beams labelled intensely with type V collagen antibodies (Fig 46). Loose bundles of striated collagen fibrils,

Fig 45: Type IV collagen immunogold labelling in corneoscleral trabecular beam. Immunogold labelling is present on thickened basal laminae of endothelial cells and at margin (arrowheaded) of trabecular core (TC). **Insert;** type IV labelling absent from long-spacing collagen but present in adjacent basal lamina LR White (insert bar = 0.2um).

Fig 46: Core of trabecular beam labelled for type V. Immunogold particles are not lying directly on top of the striated collagen fibrils but tend to straddle or lie in close proximity to them. Both basement membrane [BM] and elastin-like fibrils [E] are free of label. end = endothelial cell; Cryo.


E
BM
FG
47


47 b

occasionally observed within the basement membrane of the trabecular endothelial cells, were also labelled. However, the labelling pattern indicated that type V collagen was not a component of striated fibrils as labelling was rarely observed to be directly on top of the fibrils. The basement membranes were free of type V collagen labelling (Fig 46) as were the elastin-like fibers and the fine granular-like sheaves of material enclosing these fibers.

Type VI collagen. Labelling for type VI collagen was almost exclusively restricted to the core of the trabecular beams, being absent over the basal laminae lining the trabecular endothelial cells and from long-spacing collagen (Fig 47). Although labelling for type VI collagen was associated with the striated collagen fibrils of the core, the immunogold particles were not observed to be directly over individual fibrils. The pattern of immunogold labelling suggested that type VI consisted of a fine filamentous network, in that immunogold particles frequently occurred in long chains whose path did not follow that of the striated fibrils (Fig 47). Most significantly, type VI labelled non-striated fibrils were found in the region of the basal lamina. Labelling of the

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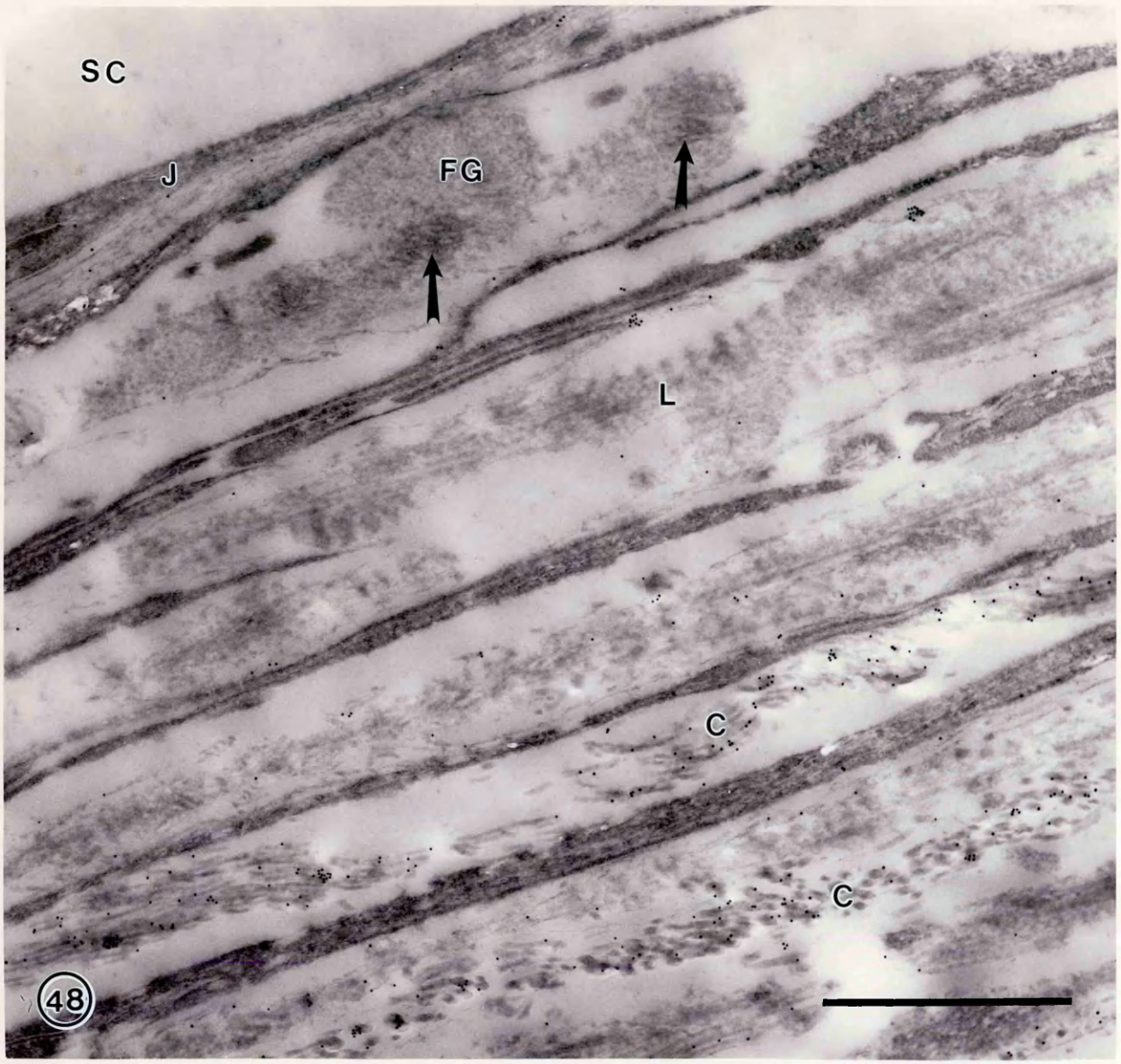
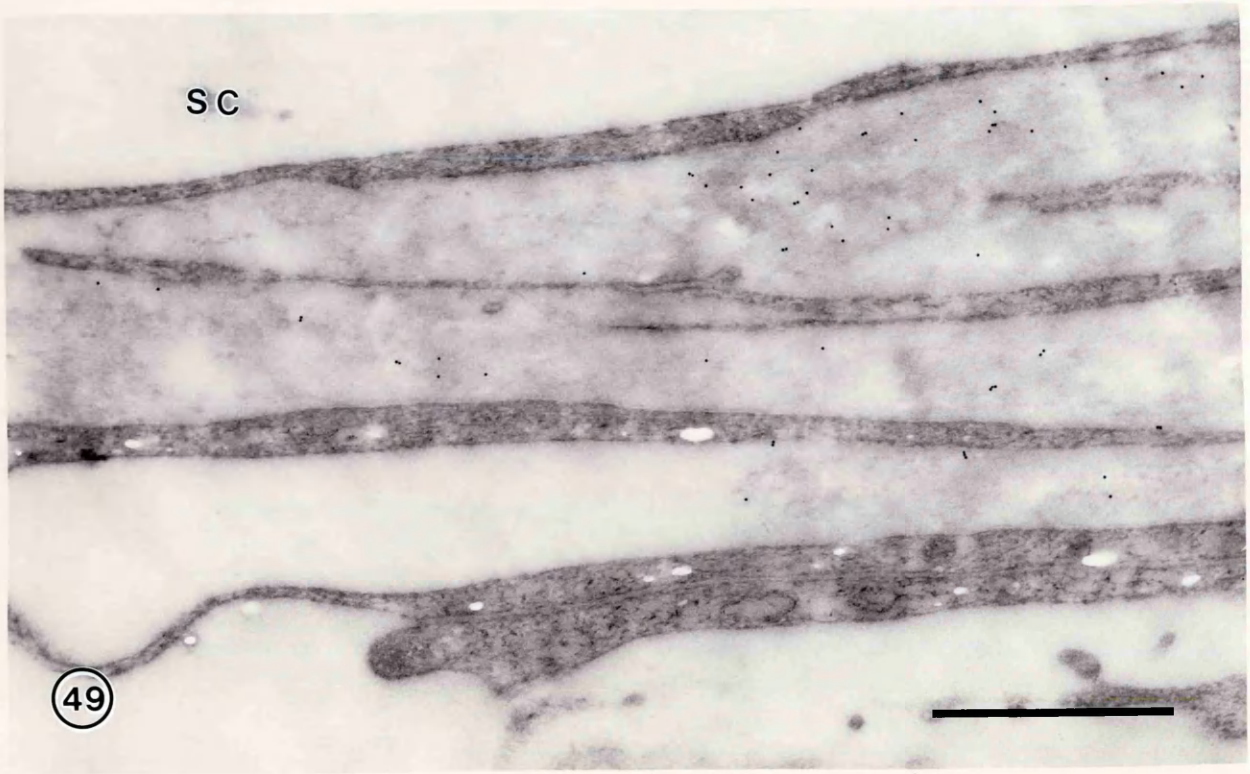
Fig 47: Type VI labelling of corneo-scleral meshwork. Immunogold particles are located over collagen fibrils of core in a similar arrangement to type V. Type VI also localized to fibrils [small arrows] closely apposed to the basement membrane [BM]. Labelling is absent from long-spacing collagen [curved arrows], fine granular-like material [FG] and elastin-like material [E]. Cryo

Fig 47b: Higher magnification of collagen fibril labelling in the core. Immunogold particles are frequently arranged in strings [arrowed] indicating the filamentous nature of type VI collagen as distinct from its incorporation in striated collagen fibrils. Cryo; bar indicates 0.5μm.


SC
J
FG
L
C
C
48


SC
49

trabecular core was associated only with striated collagen fibrils and not with elastin-like fibers within the core or with the sheaves of fine granular-like material surrounding these fibers (Fig 47).

Laminin. Laminin was sparse in both the corneo-scleral and the uveal meshwork apart from very occasional pockets of basement membrane-like material. The weak signal immediately apposed to the extracellular side of trabecular endothelial cells was not of sufficient intensity to be regarded as positive for laminin by our criteria.

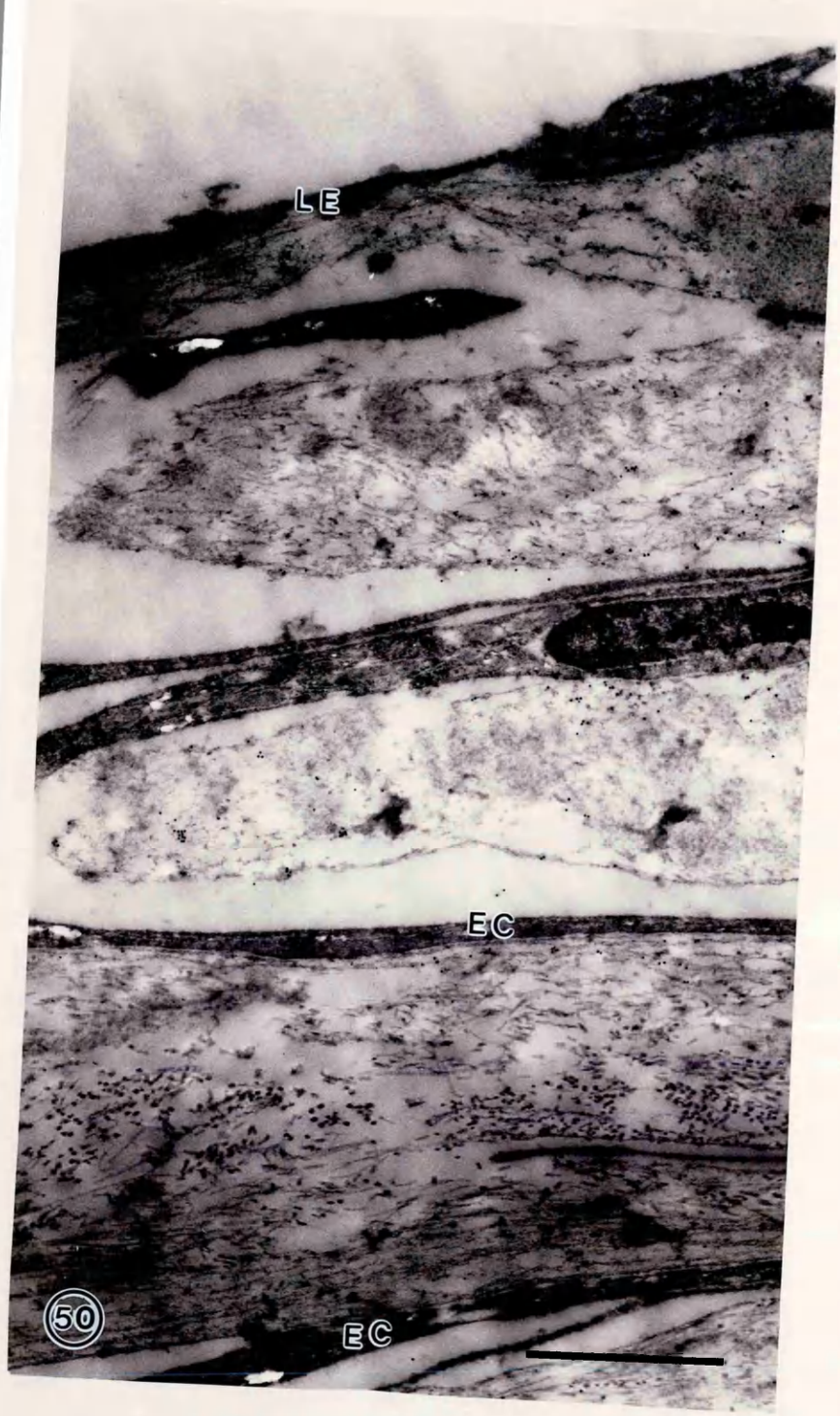
3.3.2 Cribriform Layer

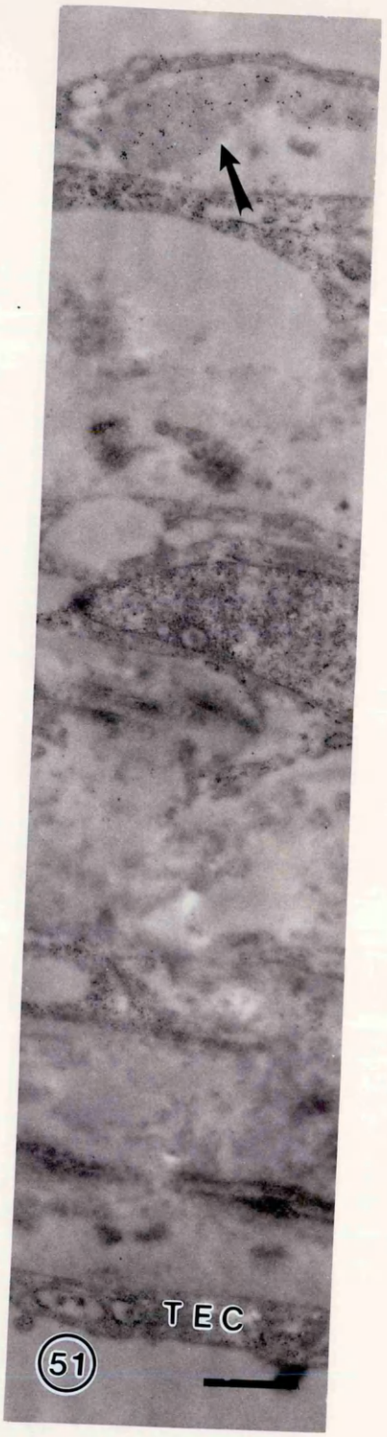
Type I collagen. Type I collagen labelling was sparse but was observed in the fine fibrillar-like material and basal laminae of the cribriform layer (Fig 48). Striated fibrils seen in the trabecular aspect of the cribriform layer were also positively labelled with type I collagen antibodies (Fig 48). Immunolabelling was absent from elastin-like fibers, fine granular-like material and long-spacing collagen.

Type III collagen. Both the fine fibrillar-like material and basal lamina of the cribriform tissue were positively stained with type III collagen antibodies (Fig 49).

Fig 48: Type I distribution in the juxtacanalicular tissue is restricted to striated collagen fibrils [C] in the subendothelial zone and to fine filaments in the basement membrane of the elongated juxtacanalicular cells [J]. Plaques of long-spacing collagen [L], fine granular-like material [FG] and elastin-like material [arrows] are free of label. SC: Schlemm's canal; LR white

Fig 49: Type III collagen in juxtacanalicular tissue. Immunogold particles are present over indistinct fine fibrillar material beneath the lining endothelium of Schlemm's canal [SC]. LR White.


LE
EC
EC
50


TEC
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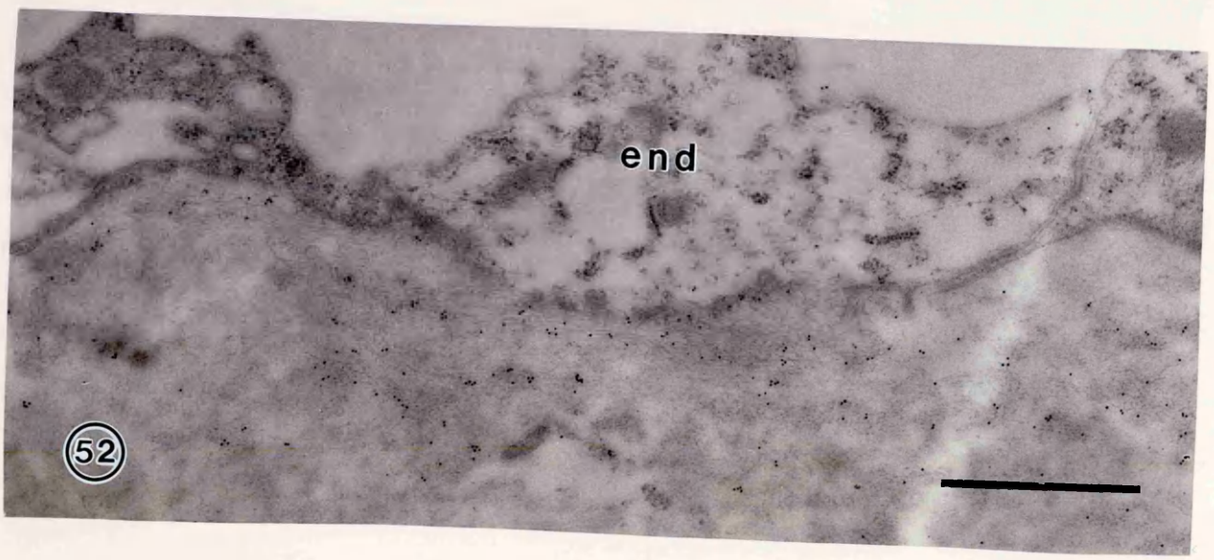

end
52

However, labelling of the basal lamina was patchy. In the case of fine fibrillar-like material the immunogold label was more frequently localized to fine fibrils than to the fine homogenous component. No labelling was observed over fine granular-like material or elastin-like fibers.

Type IV collagen. Type IV collagen labelling was present throughout the entire thickness of the cribriform layer including the basement membranes of the endothelial cells (Fig 50). In particular labelling for type IV collagen was present on the basal lamina-like material.

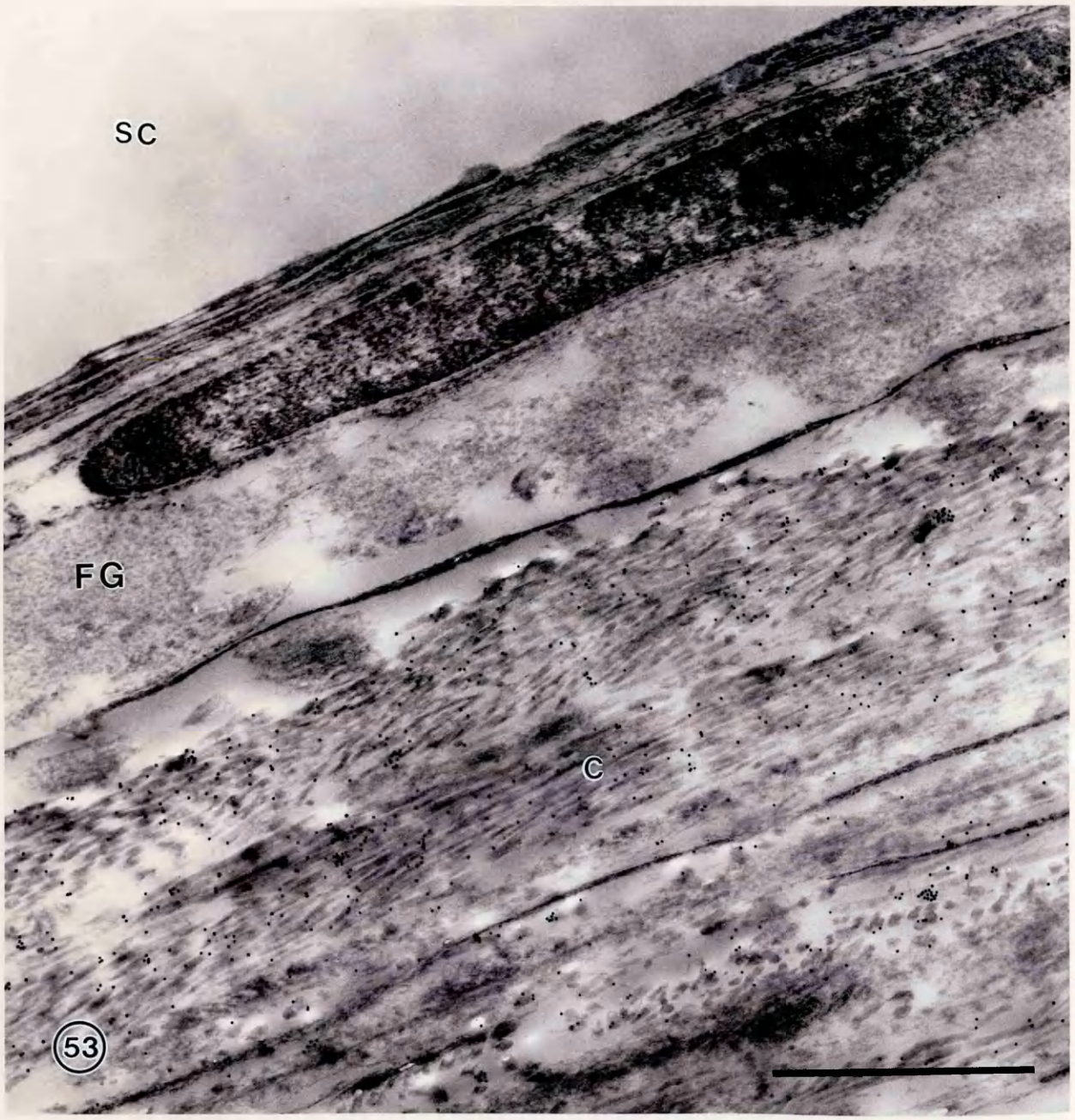
Types V and VI collagen. Labelling for types V and VI was associated with collagen fibrils within the cribriform tissue. The labelling pattern of the collagen fibrils was similar to that of collagen fibrils in the corneo-scleral meshwork in that immunogold particles did not lie directly on top of the fibrils but formed a filamentous network among the fibrils. No labelling was present on the basal lamina.

Laminin. In the cribriform layer the localization of laminin was restricted to the subendothelial layer (Fig 51,52). Labelling was occasionally observed on fine

Fig 50: Type IV collagen immunogold labelling in cribriform layer. Type IV collagen is present throughout the entire thickness of the cribriform layer including the basal lamina-like material in the subendothelial region and on the basal laminae of the endothelial cells (EC). LE: lining endothelial cell of the inner wall of Schlemm's canal; LR White.

Fig 51: Laminin distribution in the cribriform layer. Full thickness of cribriform layer with endothelial cell lining the first intertrabecular space at the bottom (TEC). Immunogold localization of laminin is restricted to the subendothelial region (arrowed); LR white.

Fig 52: Higher power of endothelial lining of the inner wall of Schlemm's canal with laminin localised on the underlying fine filamentous-like material and on the basal surface of the lining endothelial cell [end]. LR white.


SC
FG
C
53


54

fibrillar-like material but never on elastin-like material.

3.3.3 Outer Wall of Schlemm's Canal

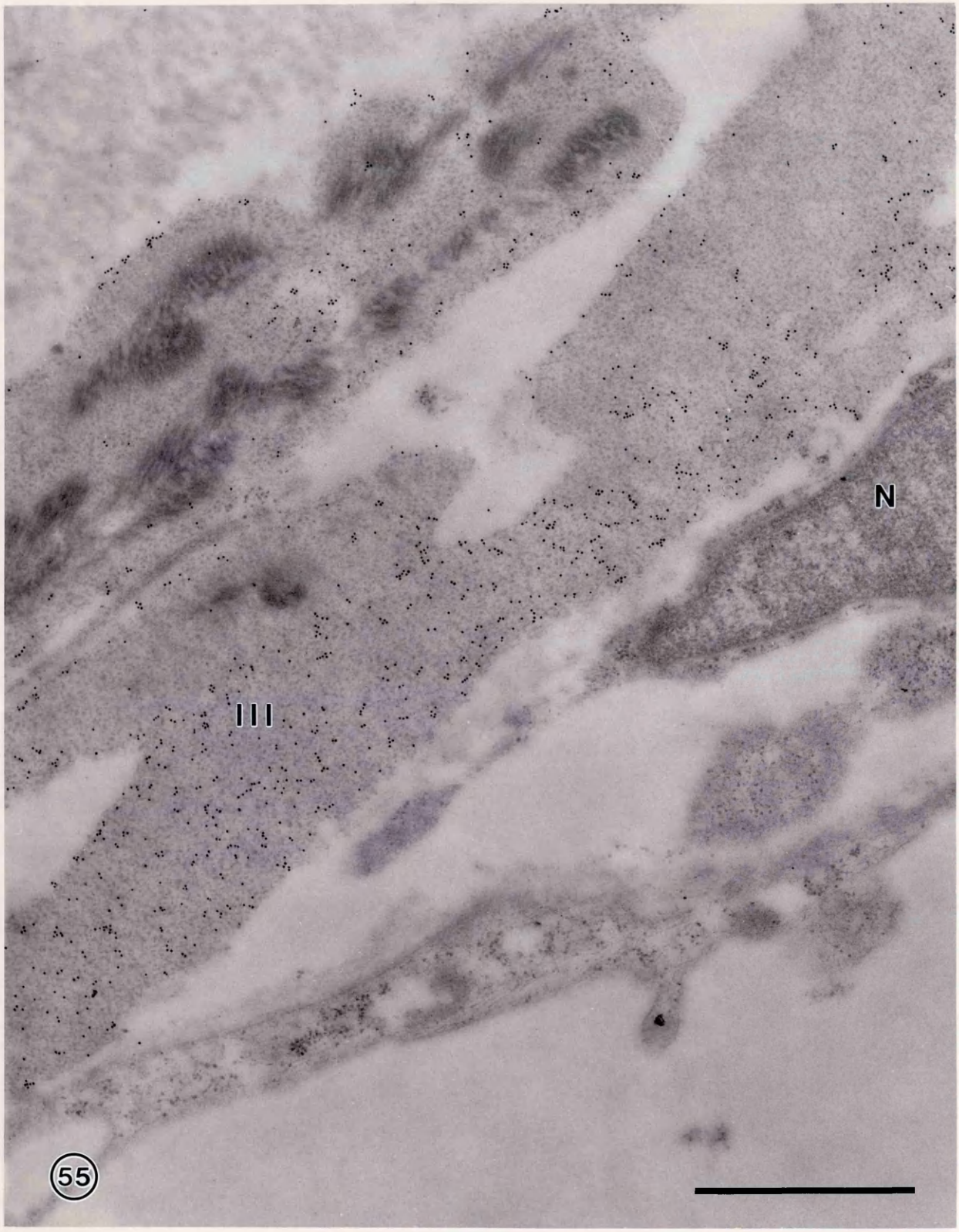
Type I collagen. Striated collagen fibrils in the outer wall of Schlemm's canal stained intensely for type I collagen (Fig 53). The degree of labelling seen over this component was greater than that seen elsewhere in the trabecular meshwork. Immunogold labelling for type I collagen was absent from fine granular-like material which lay above the endothelial cells lining the outer wall of Schlemm's canal.

Type III collagen. The distribution of type III collagen in the outer wall of Schlemm's canal was similar to that of type I collagen. Striated fibrils cut in tranverse and longitudinal section were labelled equally for type III collagen. Immunogold particles were absent both from the basal lamina of the endothelial lining and adjacent fine granular-like material (Fig 54).

Type IV collagen. Collagen fibrils of the sclera and scleral spur were free of label for type IV. However, type IV collagen was observed in the basement membranes of the outer wall of Schlemm's canal. A variable amount of signal was noted in the walls of the aqueous veins and veins of the deep scleral plexus.

Fig 53: In the outer wall of Schlemm's canal [SC], the labelling for type I collagen is intense over the striated collagen fibrils [C]. The fine granular material [FG] of the subendothelial zone is free of immunogold label. LR white X28,500 Bar=1 m.

Fig 54: Labelling for type III collagen in outer wall of Schlemm's canal is restricted to striated collagen fibrils. LR White.



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56

Laminin. Deposits in the outer wall of Schlemm's canal, similar to fine fibrillar-like material of the cribriform layer, were strongly positive for laminin (Fig 55). Such fine fibrillar-like material deposits often enclosed electron dense deposits which resembled elastin-like material. The elastin-like material was negative for laminin (Fig 55). Such labelling in the outer wall was present on tissue fixed in paraformaldehyde only. The presence of laminin was also demonstrated in the walls of aqueous veins (Fig 56).

3.3.4 Limbal collagen

Since it was difficult to preserve the lining layers of the outer wall of Schlemm's canal, the following observations refer to the distribution of collagens V and VI in limbal tissue immediately adjacent to Schlemm's canal. Immunogold labelling for types V and VI collagen was superficially similar to that of types I and III collagen in that striated collagen fibrils were intensely labelled, whilst the fine granular-like material was void of immunogold particles. However, closer examination revealed subtle differences in collagen fibril labelling: immunogold particles tended to lie on the margins of fibrils rather than directly on top of them. These particles were associated with amorphous regions of slightly increased

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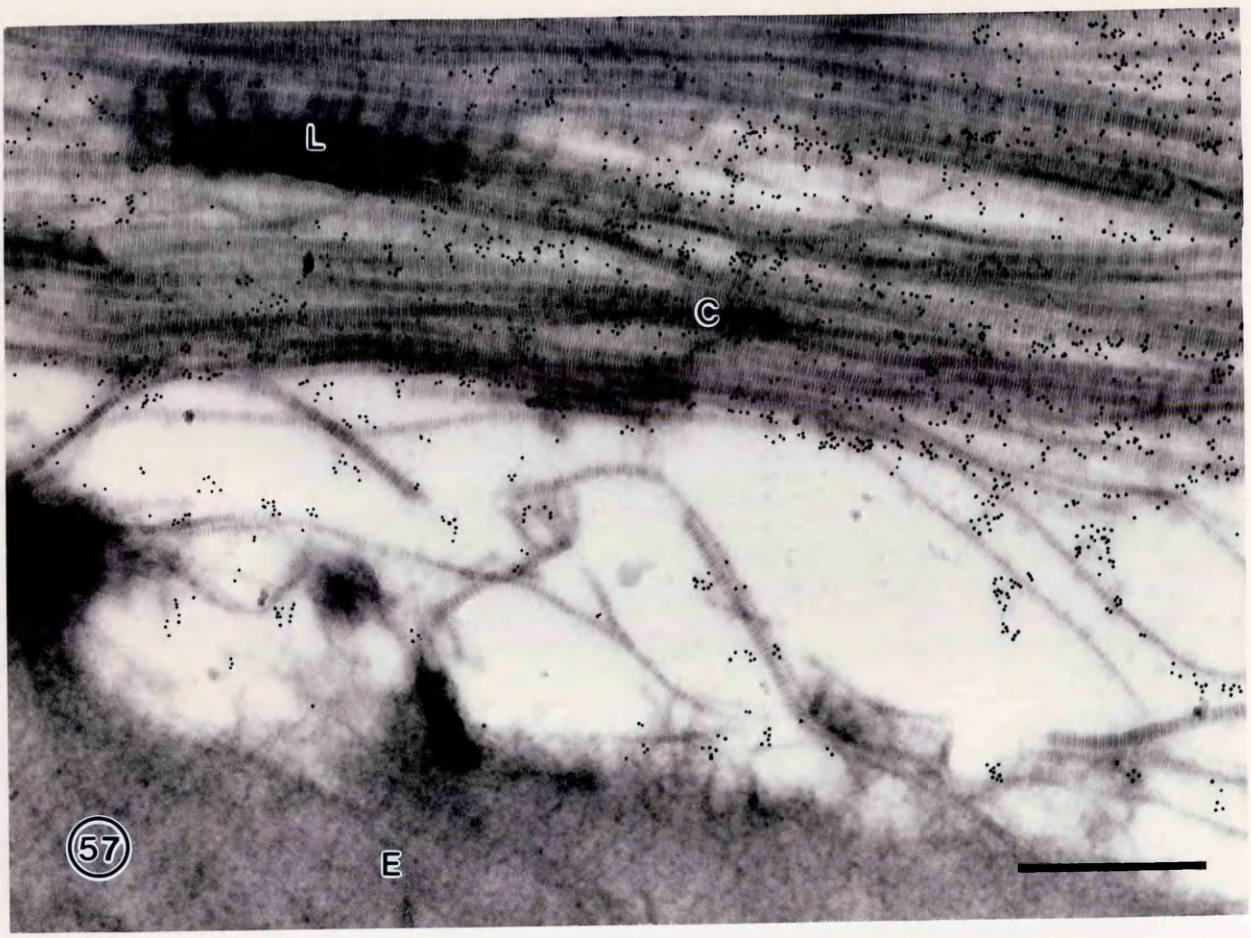
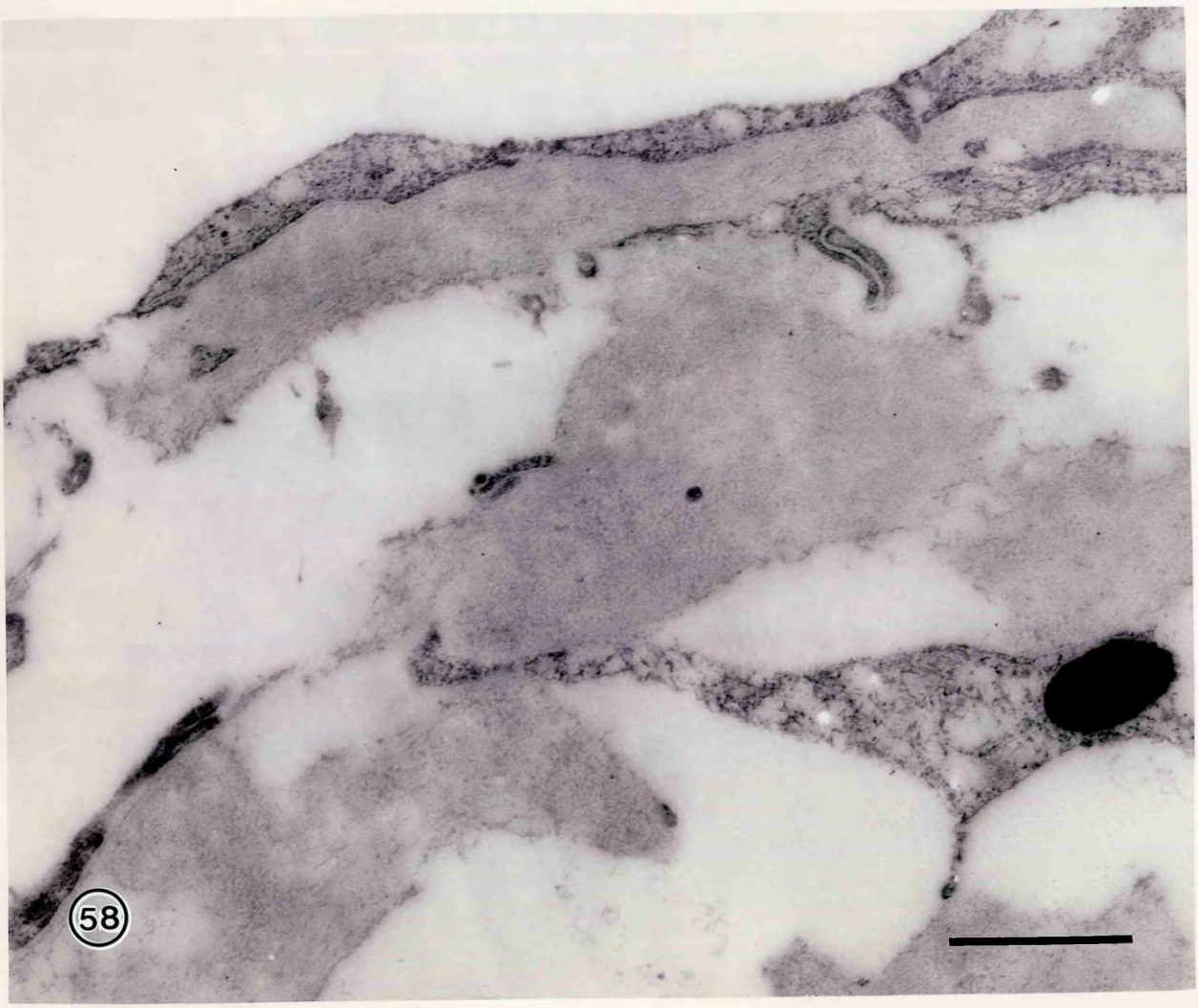
Fig 55: Laminin distribution in the outer wall of Schlemm's canal. Type III-plaques (III) exhibit a strong signal for laminin whereas elastin-like fibres are free of label. The low level of background labelling is indicated by the absence of immunogold particles over the nucleus (N). LR White

Fig 56: Laminin beneath the endothelial cell of an aqueous vein: the nucleus (N) is free of label. LR White


L
C
57
E


58

electron density (Fig 57).

3.3.5 Controls

Immunogold particles were rarely observed in rabbit anti-goat and goat anti-rabbit immunogold control sections. A slightly higher level of nonspecific labelling was observed in the negative normal goat and rabbit serum control sections (Fig 58). In the positive corneal controls specific labelling for collagen types I, III, V and VI was demonstrated in the corneal stroma and Bowman's layer. Laminin was demonstrated immediately underneath the corneal epithelium. Specific labelling was also demonstrated for types I and III collagen in the iris internal positive control (i.e. iris tissue within the same section): types I and III were localized to the striated fibrils in the stroma and types I and IV were localized to the basement membranes.

3.3.6 LR white Embedding

We compared the effect of LR White processing with that of conventionally processed tissue from the same eye. It was of interest that a larger amount of extracellular matrix material was retained with LR White processing. There were fewer and smaller electron lucent spaces in the cribriform layer and giant vacuoles which were empty in

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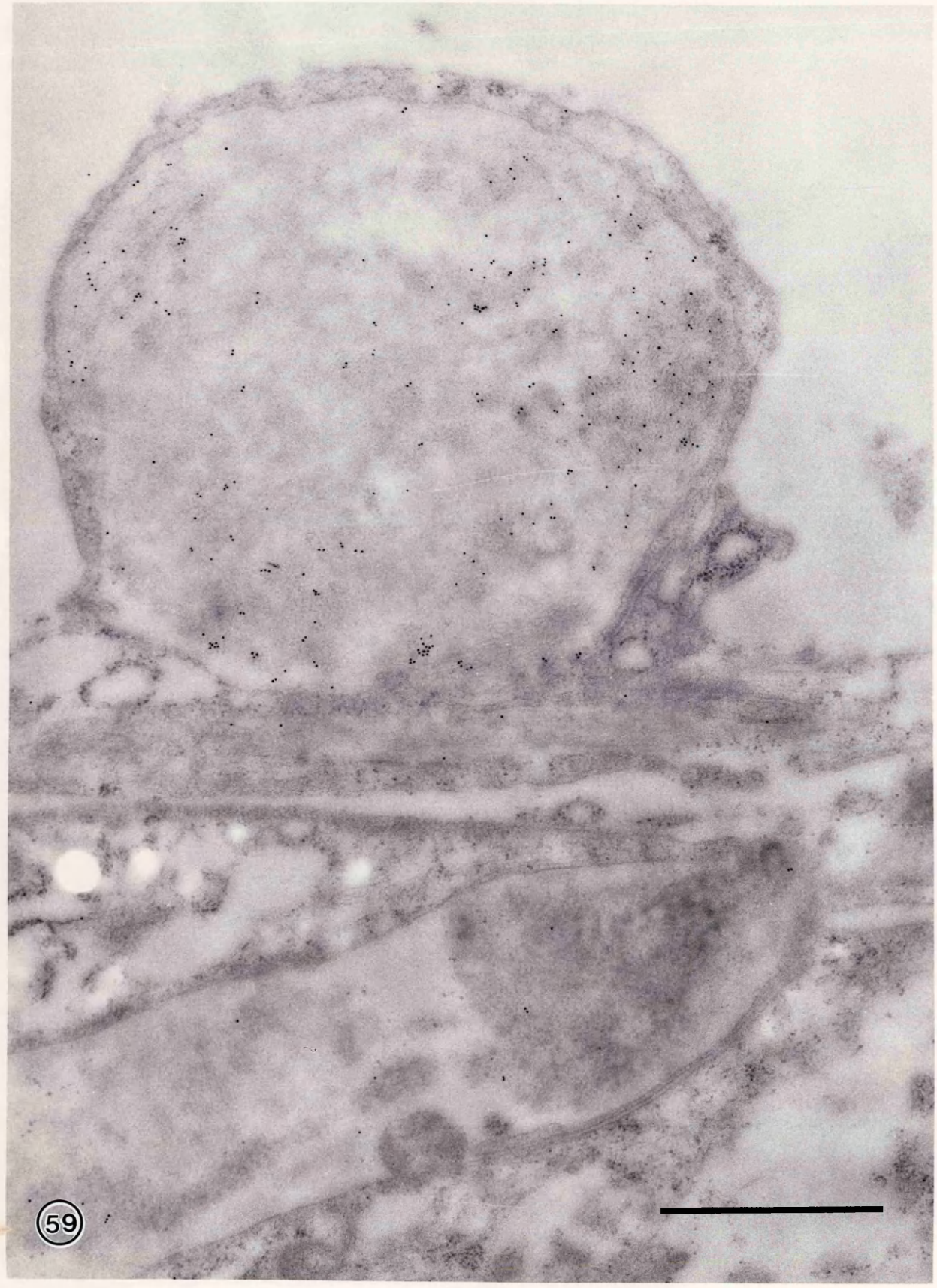
Fig 57: Artefactual separation of striated fibrils [C] from elastin-like material [E] reveals the perifibrillar location of type VI collagen. Note absence of label over long-spacing collagen [L] and elastin-like material. Cryo

Fig 58: 1:20 rabbit serum negative control for laminin. The subendothelial region of the cribriform layer is devoid of immunogold particles. LR White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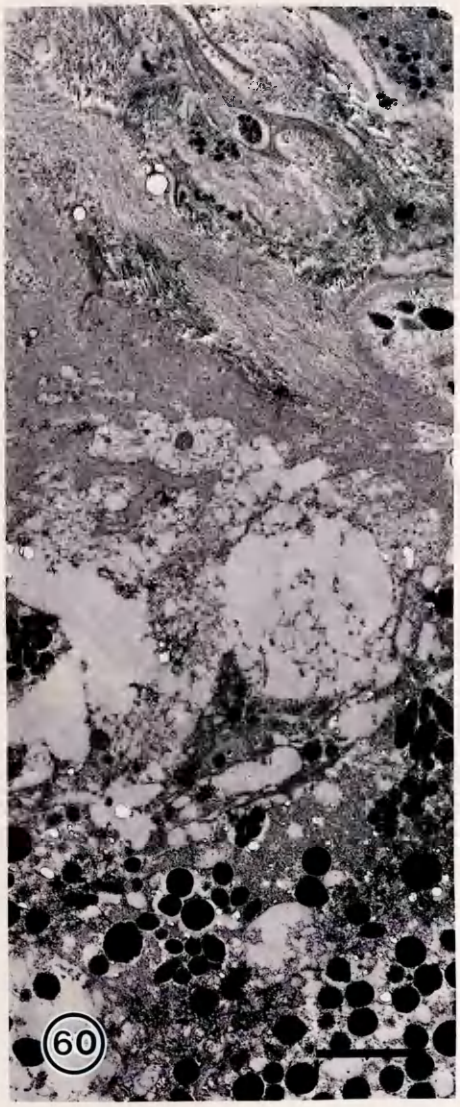
conventional sections contained basement membrane-like material of variable electron density which labelled for laminin (Fig 59).

Fig 59: A giant vacuole in a lining endothelial cell containing extracellular matrix material: labelling for laminin is confined to more electron dense filamentous regions of the matrix material. LR White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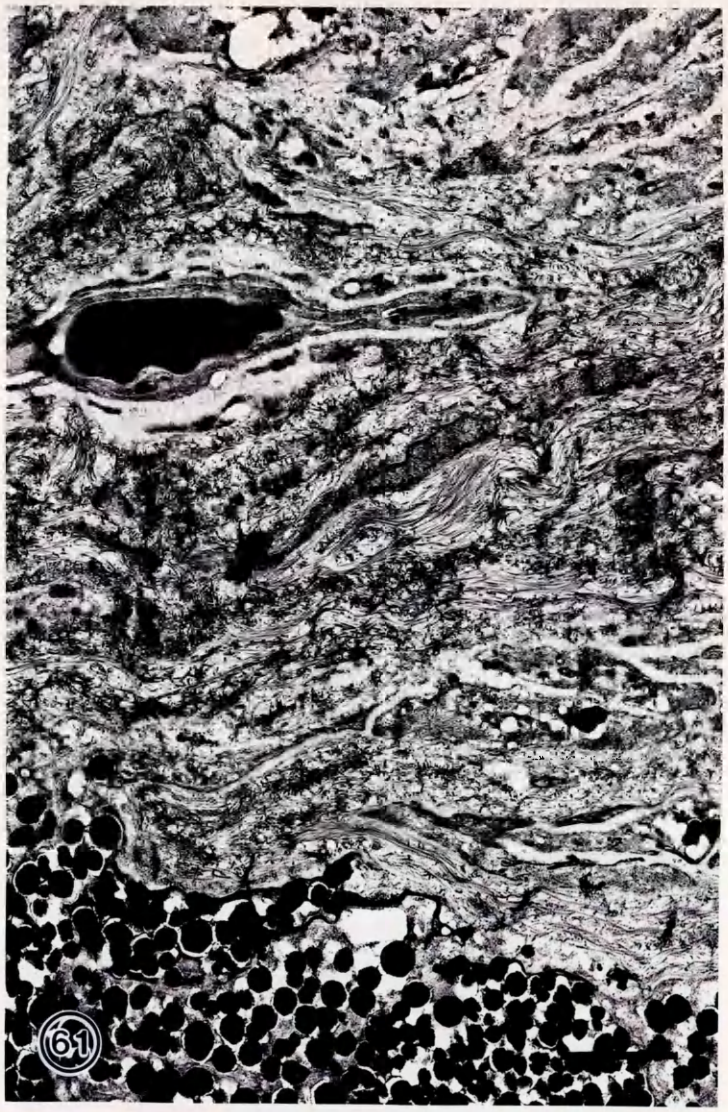
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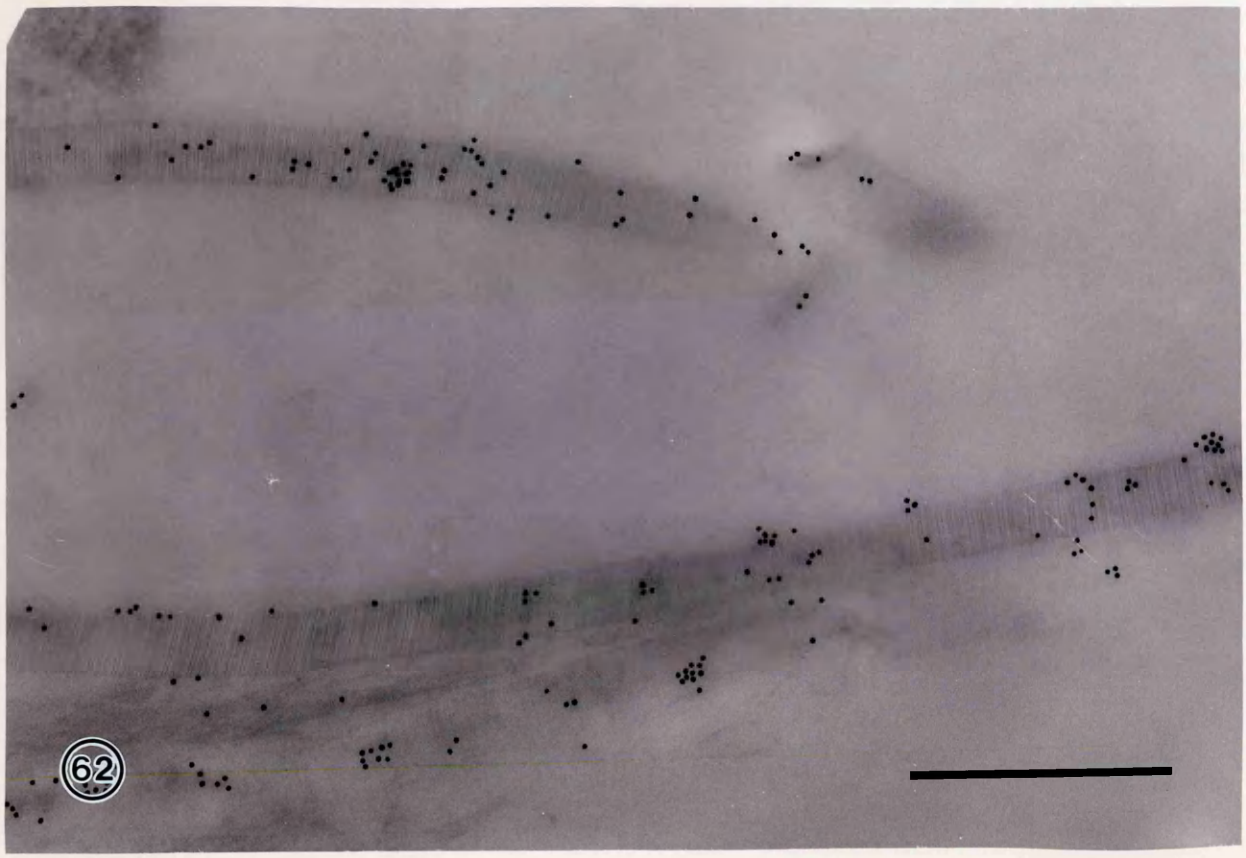
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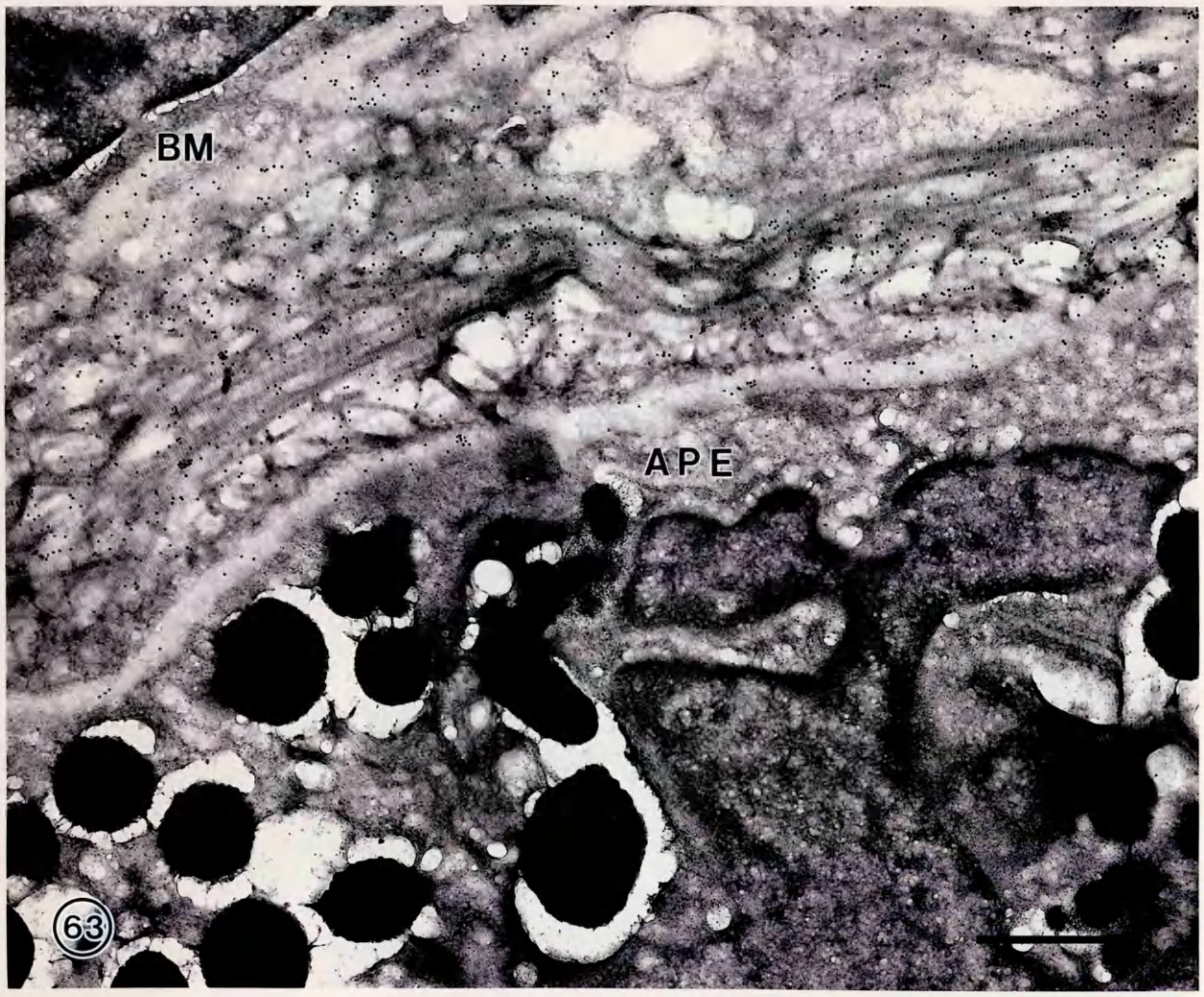
3.4 Iris

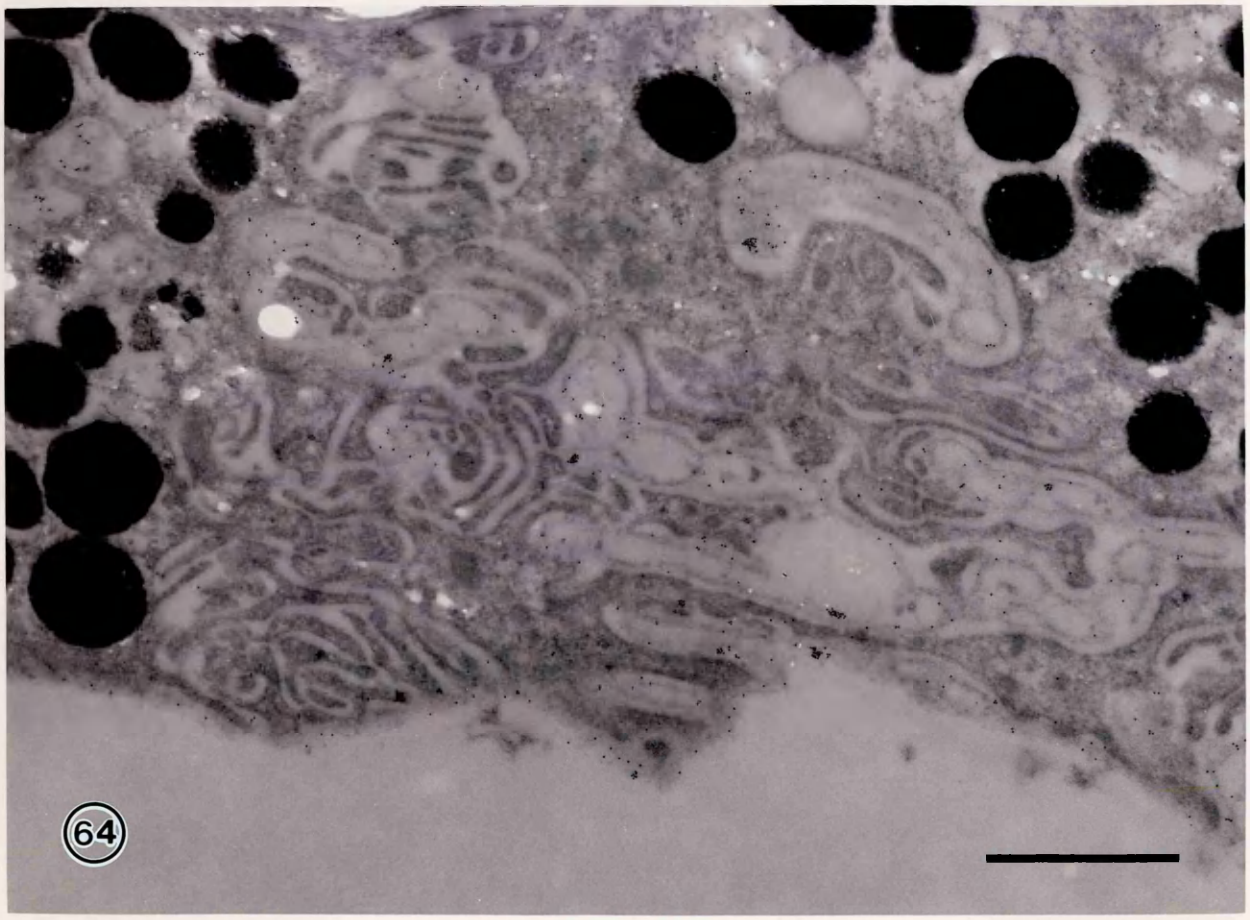
An intense specific signal was demonstrated for collagen types I, III and IV and laminin in the human iris. The immunocytochemical localisation of collagens in ultrathin frozen and LR white embedded sections were similar in all six specimens examined. Examples of the ultrastructure obtained with both techniques in shown in Figs 60-61. Subtle differences were observed between the two techniques. Cryoultramicrotomy provided a more intense signal for type III compared with LR white at optimum primary antibody dilution. The reverse occurred with type I, whose intensity was greater with LR white. Type IV collagen exhibited a signal of similar intensity with either cryo or plastic embedding. Types II and V did not demonstrate a signal fulfilling the criteria for intensity and specificity and were therefore considered to be negative. An attempt to use vitreous collagen as a positive control for type II collagen was unsuccessful. Nuclei, red blood cells, pigment granules and mitochondria, used as markers for non-specific staining, were devoid of the immunogold label.

Fig 60: Examples of variation in appearance with two forms of tissue preservation. With cryoultramicrotomy, there is atendency towards tissue shrinkage, but in this rare example, cell morphology is preserved. Bar indicates 3um.

Fig 61: a more typical example of LR White embedded tissue with cytoplasmic rarefaction. IPE, iris pigment epithelium; BV, blood vessel. Bar indicates 3um.

Fig 62: Labelling of striated collagen fibrils in stroma with type III collagen antibodies. LR White; bar indicates 0.5um.


BM
APE
63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Five LR white embedded iris specimens were used for studying the distribution of laminin. Laminin was specifically localised to the basement membranes within the walls of blood vessels and to the extracellular matrix in the dilator muscle region.

3.4.1 Anterior Border Layer and Iris Stroma

Types I and III Collagen Immunogold labelling for collagens I and III (Fig 62) was present upon striated collagen fibrils within the iris stroma. An interesting, but not uniform, finding was the more intense labelling for type III collagen present on large collagen fibrils.

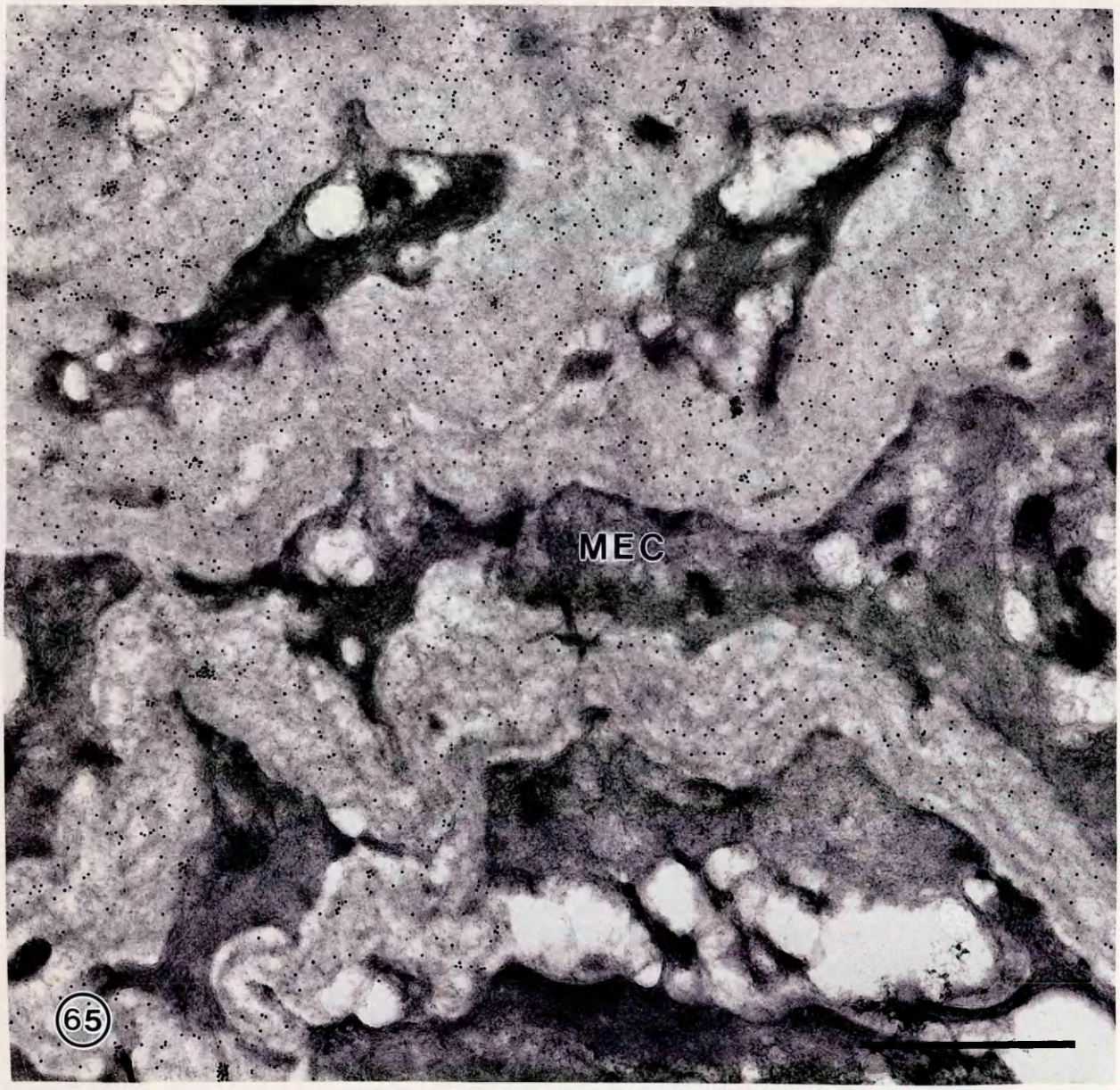
Laminin Gold particles were absent from the iris stroma and associated cells (fibrocytes, melanocytes, and mast cells). However, a discrete localised signal for laminin was present on small clusters of filaments associated with multilayered fibrocytes of the anterior border layer. Such labelling was generally absent from the outermost layer of fibrocytes.

3.4.2 Pigment Epithelium

Type I Collagen This collagen type was present within the basement membrane of the muscular extensions of the anterior pigmented myoepithelial cells [dilator muscle] (Fig 63) and the basement membrane of the posterior iris pigment epithelium (Fig 64).

Fig 63: Type I collagen labelling of striated collagen fibrils immediately apposed to anterior iris pigment epithelium (APE). Note labelling in basement membrane (BM) of myoepithelium. Cryo; bars indicate 1um unless stated otherwise.

Fig 64: Basement membrane of posterior pigment epithelial infoldings labelled with type I collagen. LR White.


MEC
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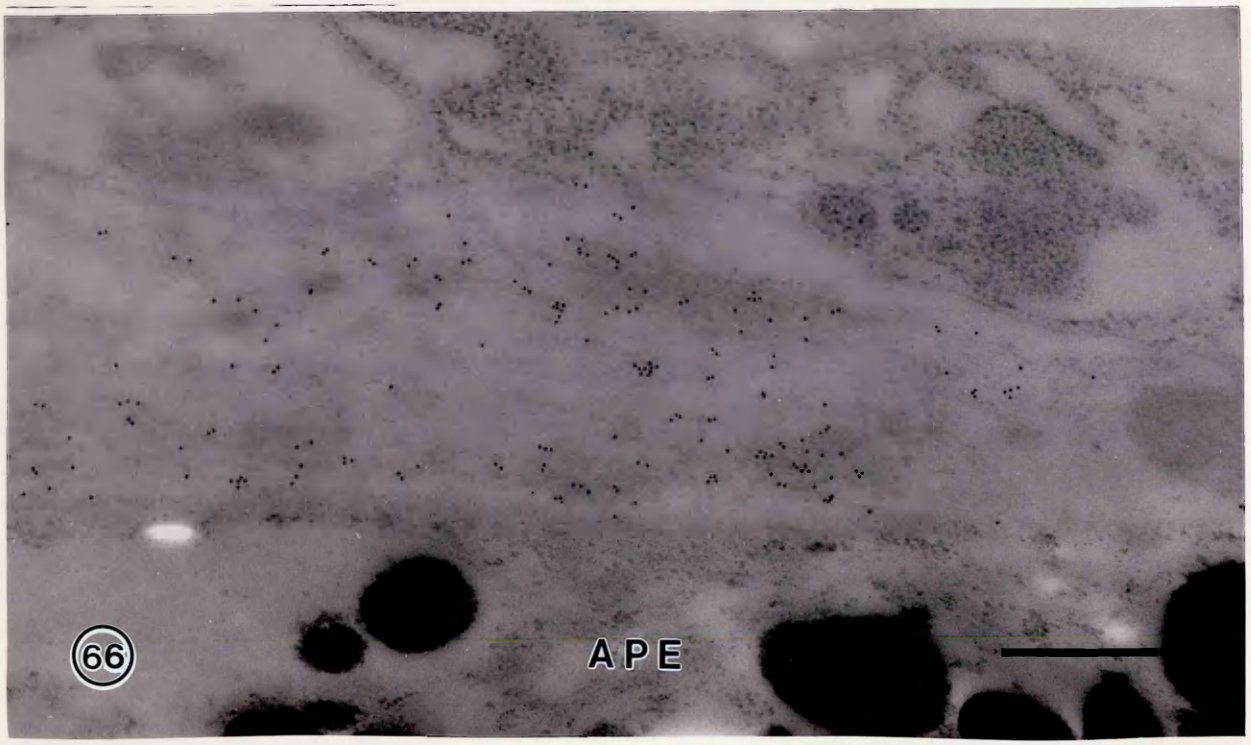
APE
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Type IV Collagen A positive signal for collagen type IV was observed in the loose array of non-fibrous collagen around the muscular extensions of the anterior pigmented myoepithelial cells (Fig 65).

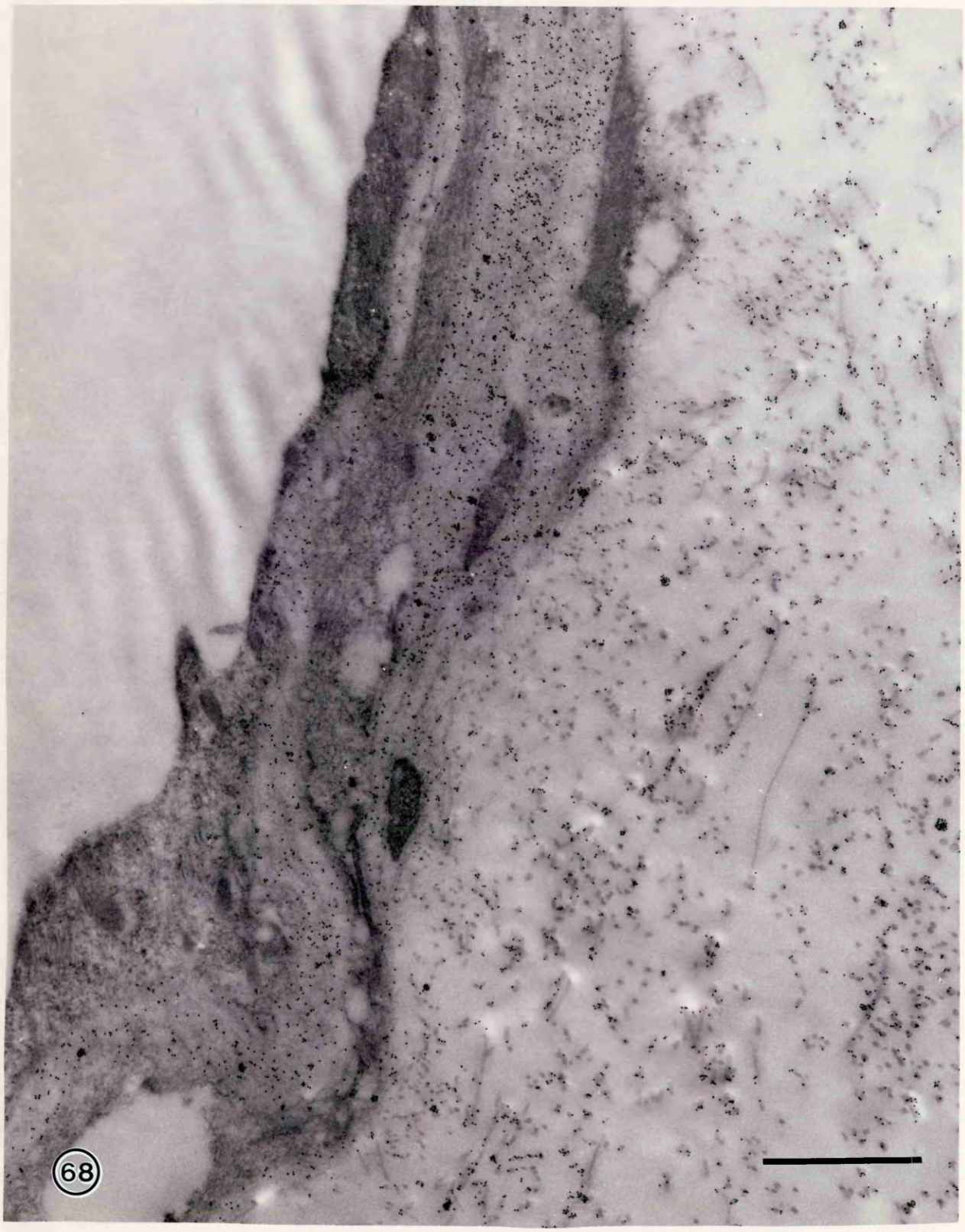
Laminin Laminin was localised to the extracellular matrix in which the basal muscular portion of the anterior iris pigment epithelium was embedded (Fig 66). Immunolabelling was mainly concentrated on dense bands of filaments within an amorphous granular substance filling the intercellular spaces. Labelling intensity increased in proportion to the quantity and density of the filamentous bands and was mainly restricted to those electron dense areas in all of the normal iris specimens studied. The thin basal membrane of the posterior pigment epithelium of the iris exhibited weak but specific labelling.

Of particular interest was the strong signal for laminin associated with iris fibrocytes which form the boundary between the dilator muscle region and the iris stroma. Labelling was intense on filamentous bodies adjacent to these fibrocytes (Fig 67). Amongst the five normal iris specimens studied the strongest signal in the dilator muscle region was in the youngest patient.

Fig 65: Type IV collagen labelling of basement membrane in which muscular extensions of myoepithelial cells (MEC) of anterior iris pigment epithelium are embedded. Cryo.

Fig 66: Laminin is localised to the clumps of filamentous electron dense material in the basement membrane of the anterior pigment myoepithelial cells (APE). LR White.

67



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3.4.3 Vasculature

Type I Collagen Collagen type I was present in the basement membranes of iris vessels (Fig 68) and within striated collagen fibrils of the vascular adventitia (Fig 68). The signal intensity increased proportionally with the size of the vessel, i.e. larger vessels exhibited a much stronger signal.

Type III Collagen In the case of collagen type III (Fig 69), immunolabelling was absent from the basement membrane of the vessels, but the signal was intense upon striated collagen fibrils which formed the perivascular coat.

Type IV Collagen Collagen type IV was strongly positive for all basement membranes with the most intense signal on the lamina densa and lamina fibroreticularis of the iris vessels (Fig 70). The lamina lucida appeared not to contain collagen type IV. It is of interest to note that the signal within the basement membrane of the iris vessels did not depend on the size of the vessel, but labelling intensity increased with increasing endothelial cell thickness.

Laminin The label was most intense on the matrix surrounding the myocytes of the larger iris vessels (Fig 71). Labelling for laminin was prominent in large and medium sized vessels, and greatly reduced or absent in

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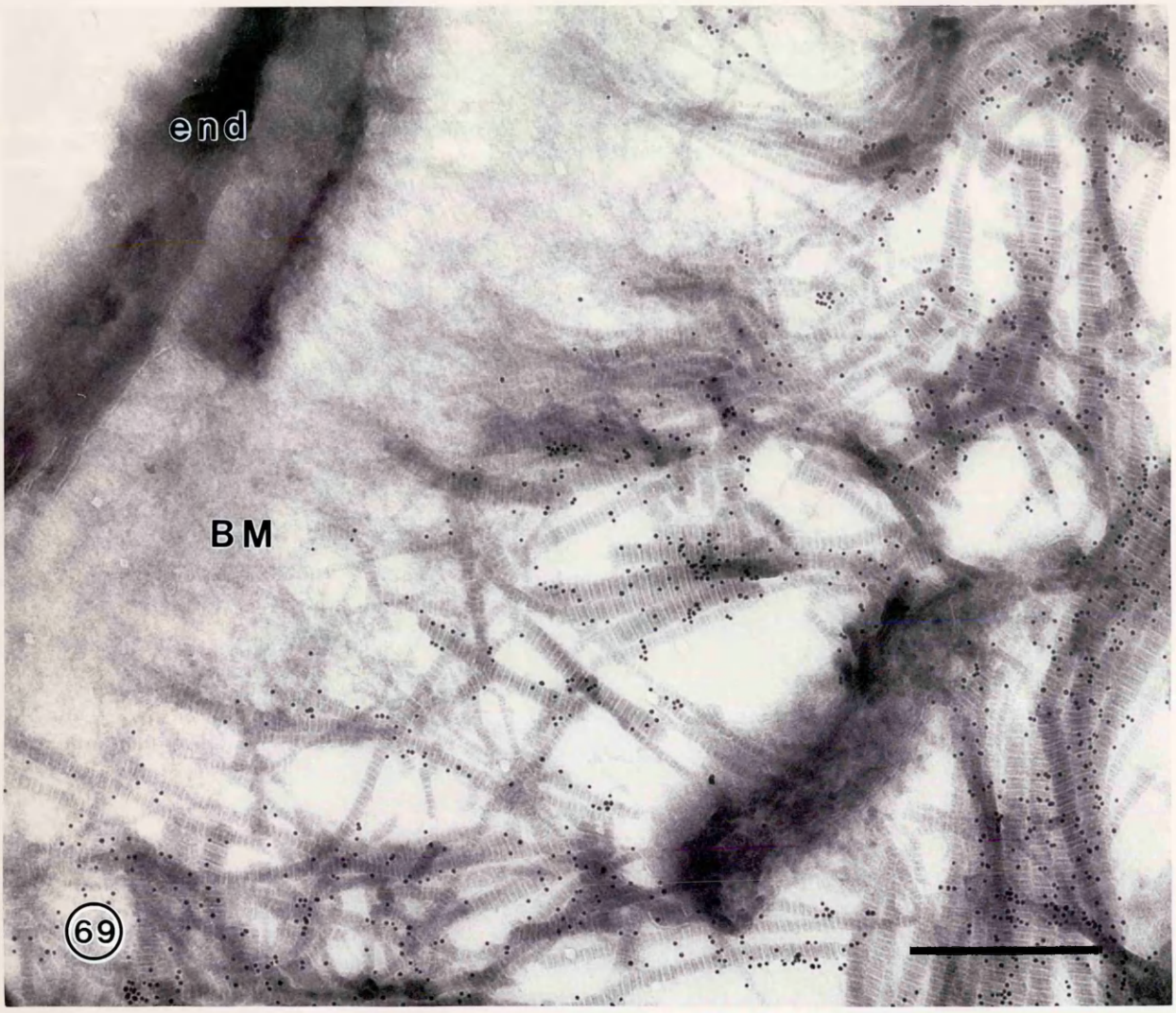
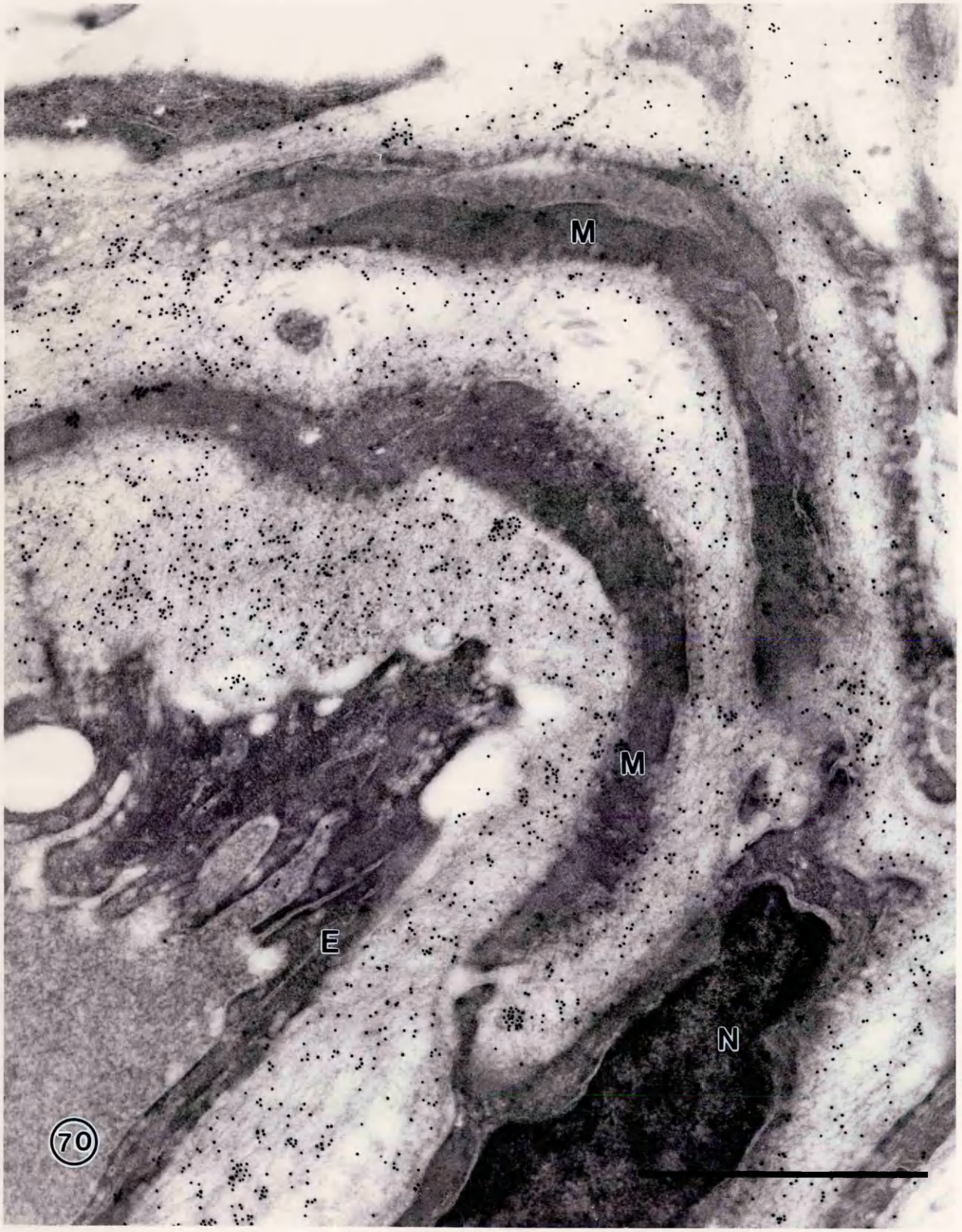
Fig 67: Laminin associated with extracellular matrix in the form of parallel filamentous bundles adjacent to a layer of stromal fibroblast immediately anterior to anterior pigment epithelium. LR White.

Fig 68: Collagen type I in a vessel wall is localised to the basement membrane of the endothelial cell and supporting cell. Striated collagen fibrils of the adventitia are also labelled. LR White.


end
BM
69

small vessels. When present in small vessels the signal was seen to be more intense on the outer side of pericytes. Of the five cases studied the highest intensity of labelling around small vessels was observed in the youngest individual: in this particular specimen laminin was demonstrated throughout the whole of the iris vasculature. In contrast, laminin was markedly reduced in iris vessels from the oldest patient.

Fig 69: Type III collagen in the adventitia of a blood vessel is restricted to striated collagen fibrils. Note absence of gold particles from endothelial cell (end) and basement membrane (BM). Cryo; bar indicates 0.5um.



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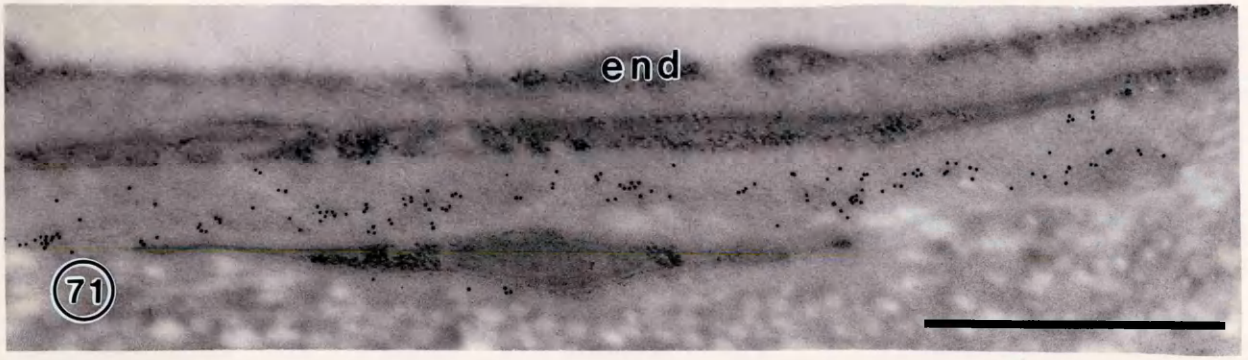

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Fig 70: Basement membranes of iris vessel strongly labelled with type IV collagen antibodies. E: endothelial cell; M: myocyte; N: nucleus of myocyte. LR White.

Fig 71: Laminin distribution in wall of iris vessel. Immunogold particles are absent from basement membrane of endothelial cell (end) lining the vessel lumen and vessel adventitia, but strongly label basement membrane of subendothelial myocyte. LR White.

3.5 Ciliary Body

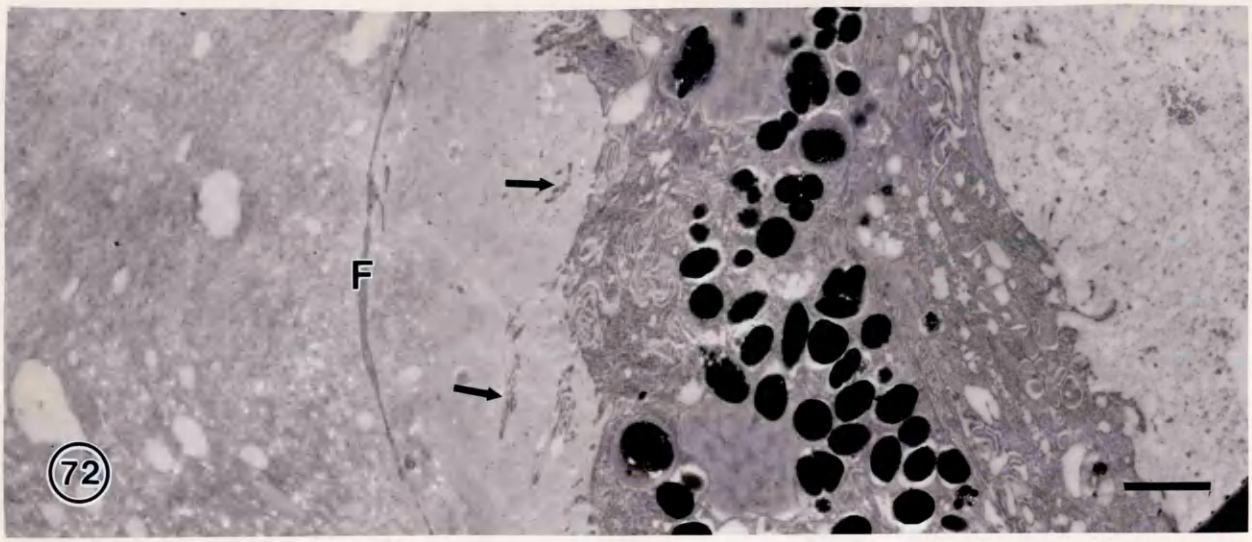
The distribution of extracellular matrix components in the pars plicata was studied in five morphologically distinct zones: (1) basement membrane of the nonpigmented ciliary epithelium, (2) basement membrane of pigmented ciliary epithelium, (74) stroma, (4) ciliary muscle, and (5) vascular system including the capillary bed of the ciliary processes.

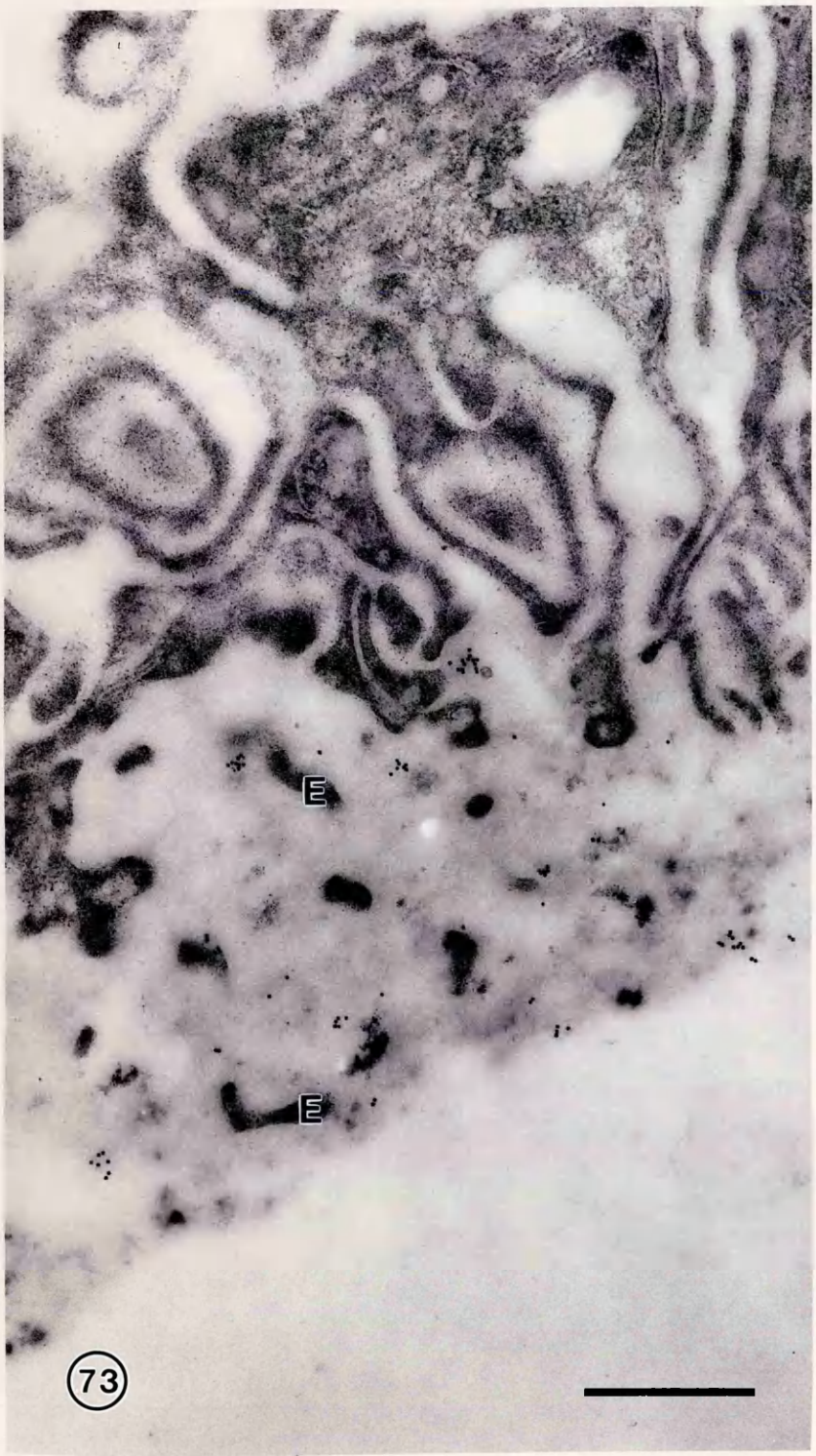
Some authors have employed the term, "internal limiting membrane", to refer to the basement membrane of the nonpigmented epithelium. In this study, the term "internal limiting membrane" has been avoided, following the recommendation of Gärtner[67] who pointed out that this term refers to an entity visible in the light microscope which consists of a basement membrane-fibrillar complex. The basement membrane itself is visible only in the electron microscope.

Collagen types I, III, and IV, and laminin were consistently demonstrated in a number of the specified zones (1-5) of the pars plicata. Type II collagen was absent from all the zones studied. Labelling for types V, and VI collagen was not of sufficient strength to confirm the presence of these collagen types within the ciliary body. Therefore, no further reference will be made to collagens V and VI in this section. The detailed findings for collagens I-IV and laminin are as follows:

3.5.1 Basement Membrane of Nonpigmented Ciliary Epithelium

The basement membrane of the nonpigmented ciliary epithelium was found to contain mainly types I, and IV collagen, laminin, and a small but significant amount of


F
72


E
E
73


74

type III collagen.

Aging Changes: A significant number of potential age-related changes were noted. In all of the specimens, the basement membrane was thickened (Figs 72-76) by comparison with illustrations provided by standard works,[58,67,101,217] and in some cases was about twice the thickness of the nonpigmented epithelial monolayer (Fig 72). The basement membrane consisted of a thick, highly reticulated zone. The network was formed by fine strips of electron dense material resembling lamina densa. The basal infoldings were free of lamina densa. Numerous granules of varying electron density, shape, and size were present both within the reticular spaces, and more compact regions of the basement membrane. The basement membrane was compact over the surface of individual cells which projected into this layer (Figs 73-74).

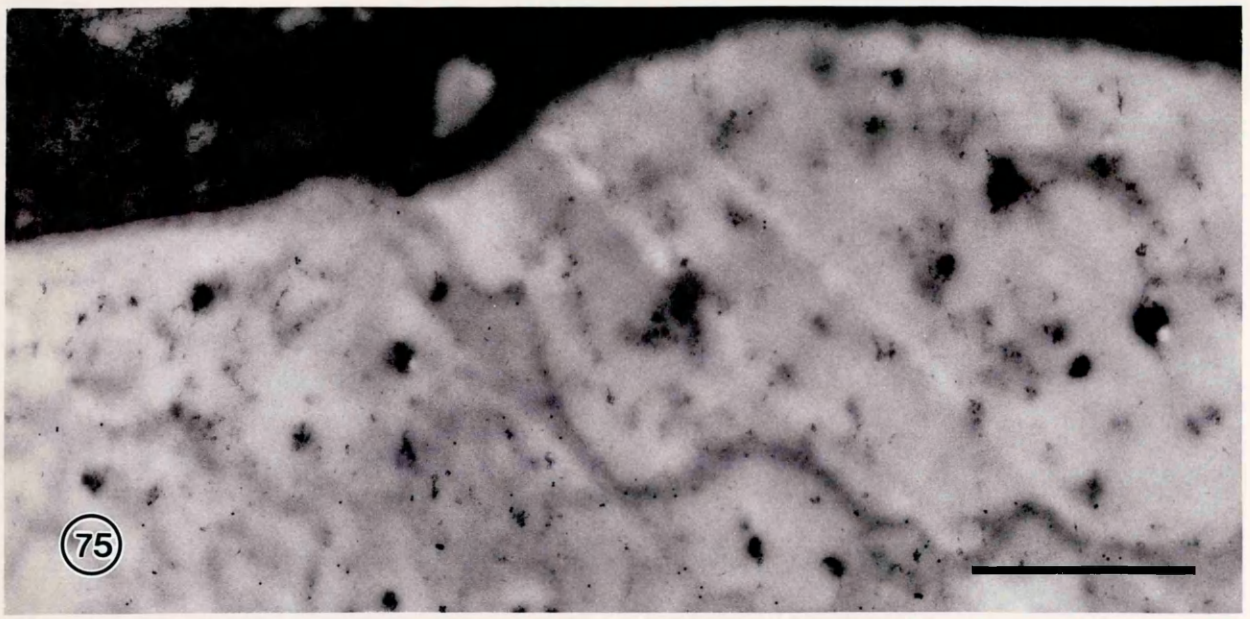
Zonules were frequently present on the surface of the basement membrane as were nodular proliferations of nonpigmented epithelial cells. Zonular penetration of the basement membrane was also noted.

Immunocytochemistry: Collagen types I, III, and IV were localized to the strips of lamina densa-like material in the network (Figs 73-75). The labelling pattern for

Fig 72: Ciliary epithelium of 75 year old. Both epithelial basement membranes are grossly thickened; that of the pigmented epithelium is bounded by a single layer of fibrocytes (F) and also contains cytoplasmic extensions of the epithelial cells (arrowed). LR white: bar indicates 2μm.

Fig 73: Type I collagen labelling of nonpigmented epithelial basement membrane. The basement membrane is thickened and of uneven electron density. The larger electron dense bodies are epithelial processes (E). LR white; bar indicates 0.5μm.

Fig 74: Nonpigmented epithelial basement membrane labelled with type III collagen antibodies. LR white; bar indicates 0.5μm.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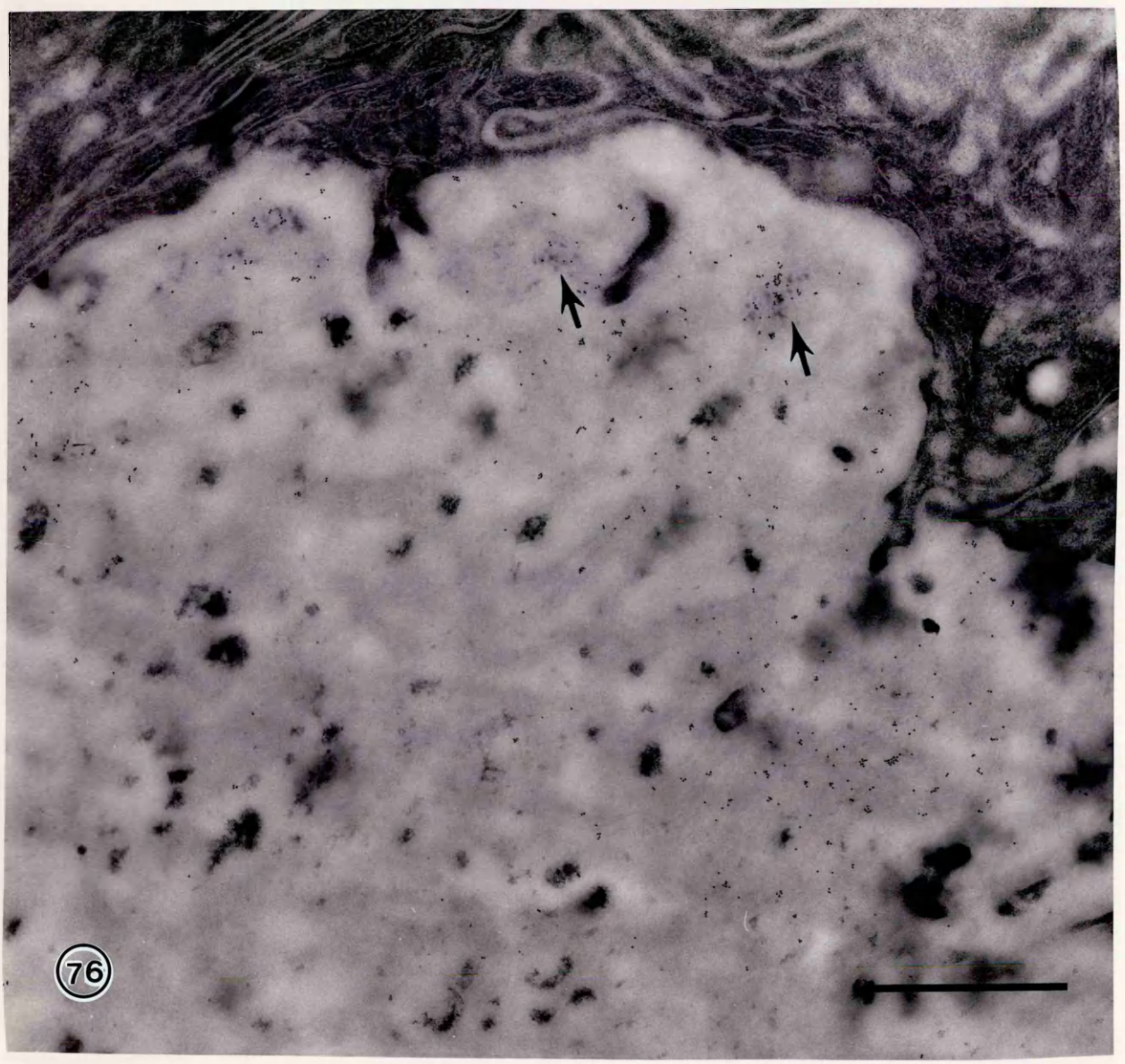
76

types I, and III collagen was similar, in that it was more intense in regions in which the basement membrane was compact as opposed to reticulate (Figs 73-74). However, the distribution patterns differed in that labelling for type I showed focal aggregation whereas type III was more diffuse. In general, labelling for type III collagen was less intense than type I (Fig 74). Type IV collagen labelling was the most intense of the collagens localized in the nonpigmented epithelial basement membrane (Fig 75): immunolabelling was located over strips of basement membrane material in reticulate regions, and over the whole width of the compact basement membranes. No difference in labelling intensity for type IV collagen was distinguished between the two regions, i.e. compact and reticulate.

There were a number of interesting features concerning the pattern of laminin distribution in the basement membrane of the nonpigmented epithelium. Immunogold particles were more prevalent near the surface of the nonpigmented epithelial cells with a conspicuous laminin free zone immediately adjacent to the epithelial cell surface (Fig 76).

This labelling pattern was also observed in the vicinity of nodular proliferations. The labelling intensity decreased in proportion to the distance from the cell membrane. Labelling was increased in the core of the

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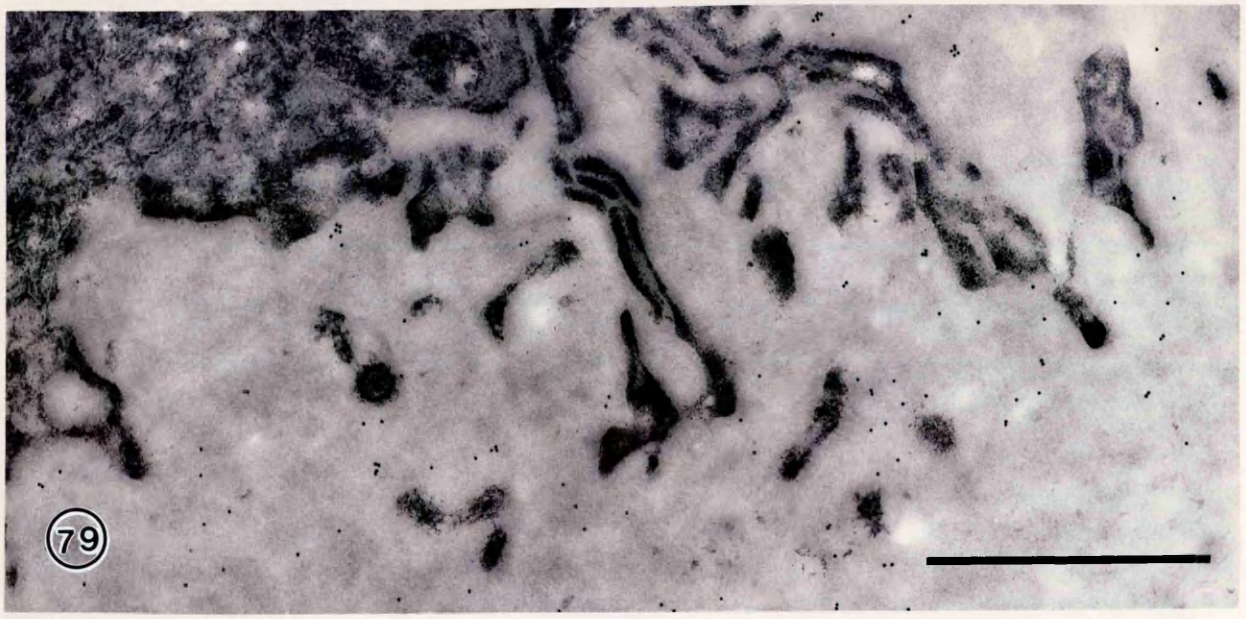
Fig 75: Type IV collagen labelling is restricted to electron dense strips of basement membrane in the nonpigmented epithelial basement membrane region. LR white; bars indicate 1μm unless stated otherwise.

Fig 76: Nonpigmented epithelial basement membrane. The immunogold distribution with laminin antibodies is concentrated in regions near the epithelial surface particularly with more electron dense flocculant material (arrowed). LR white.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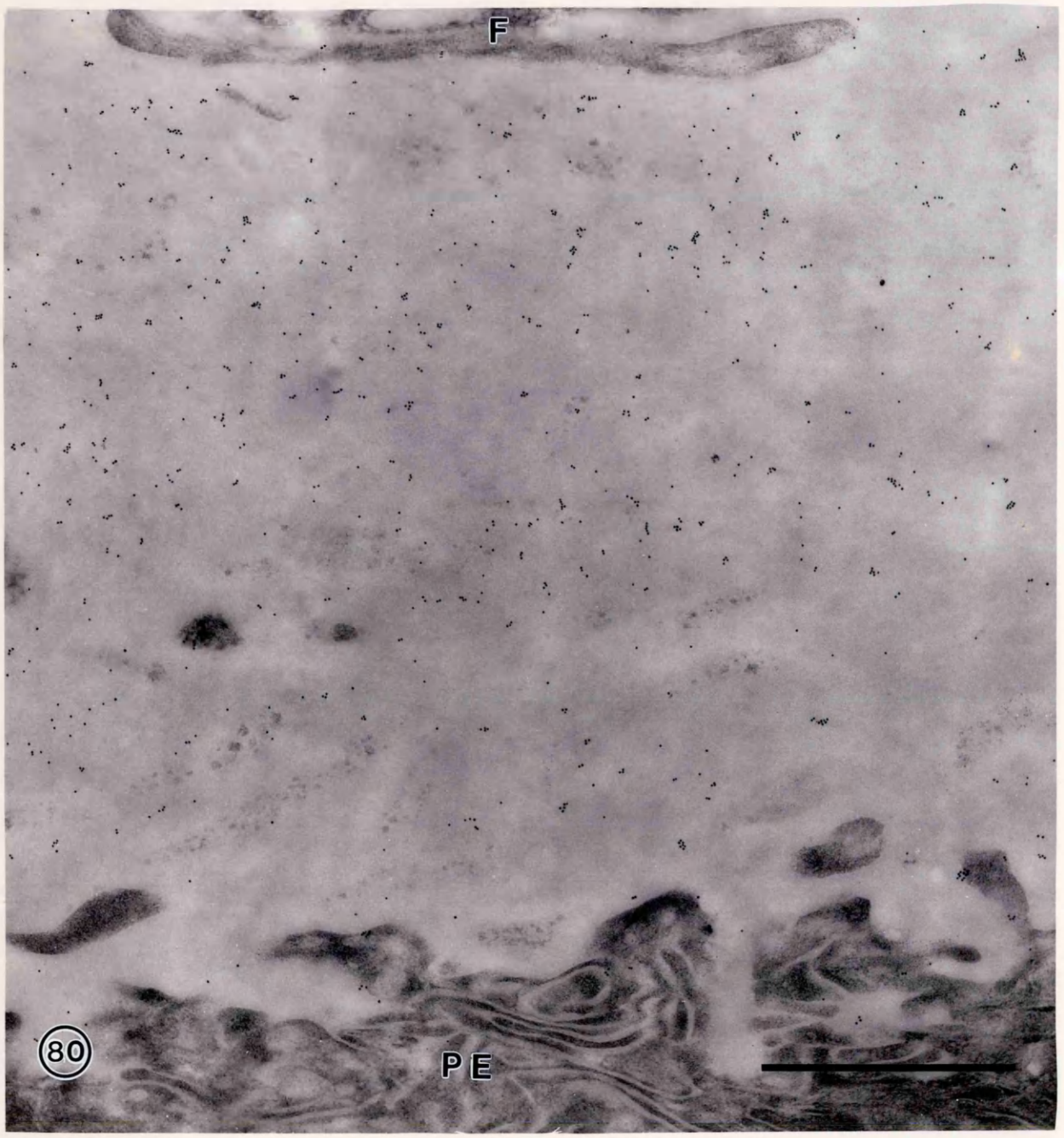
F
80
PE

basement membrane in regions where a nodular proliferation projected over the surface of the nonpigmented epithelial monolayer. Moreover, an association with clumps of diffuse granular-like material was apparent within the basement membrane (Fig 76). The electron density of this granular material was slightly higher than that of lamina densa-like material but was significantly lower than that of other types of inclusions within this zone. Thus the distribution of laminin was quite distinct from that of the collagens.

3.5.2 Basement Membrane of Pigmented Ciliary Epithelium

The presence of types I, III, and IV, and laminin was demonstrated throughout the basement membrane of the pigmented ciliary epithelium (Figs 77-80).

Aging Changes: The basement membrane zone external to the pigmented layer exhibited the following features. A large degree of infolding was observed in the basal parts of the cells, but there were frequent attenuated extensions of the cells into the adjacent matrix; these took the form of long thin cytoplasmic processes (Figs 72 and 77-79).

However, the indentations were fewer, and smaller than those seen in the nonpigmented epithelium. The basement membrane appeared to be thickened approximately to 30μm in all specimens (Fig 72). In comparison with the

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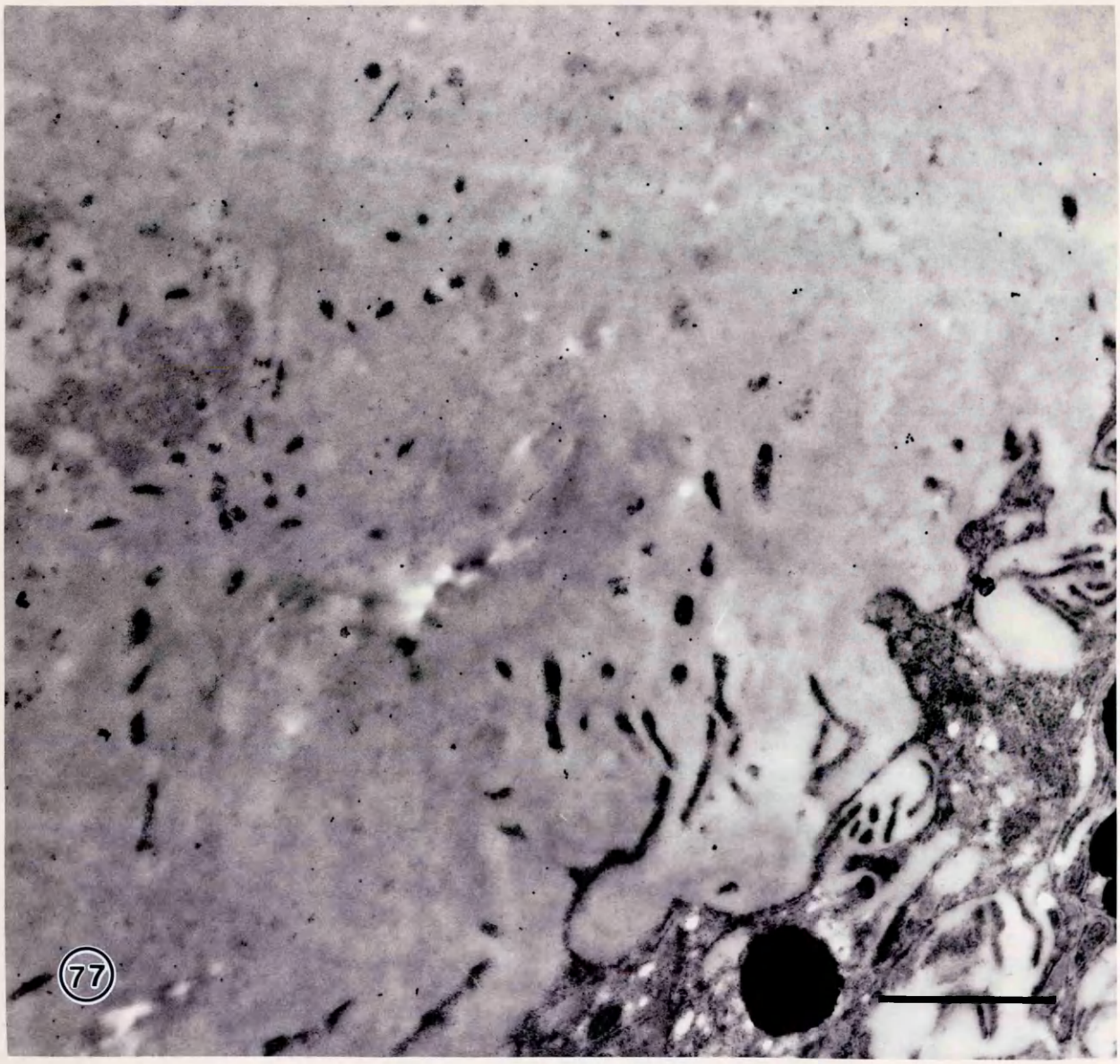
Fig 77: Type I collagen is evenly distributed throughout the pigmented epithelial basement membrane. Note cytoplasmic extensions of epithelium into basement membrane layer. LR white.

Fig 79: Type III collagen labelling of pigmented epithelial basement membrane (BM) and stroma (S) which are separated by a single layer of fibrocytes (F). Note epithelial cytoplasmic extensions. LR white.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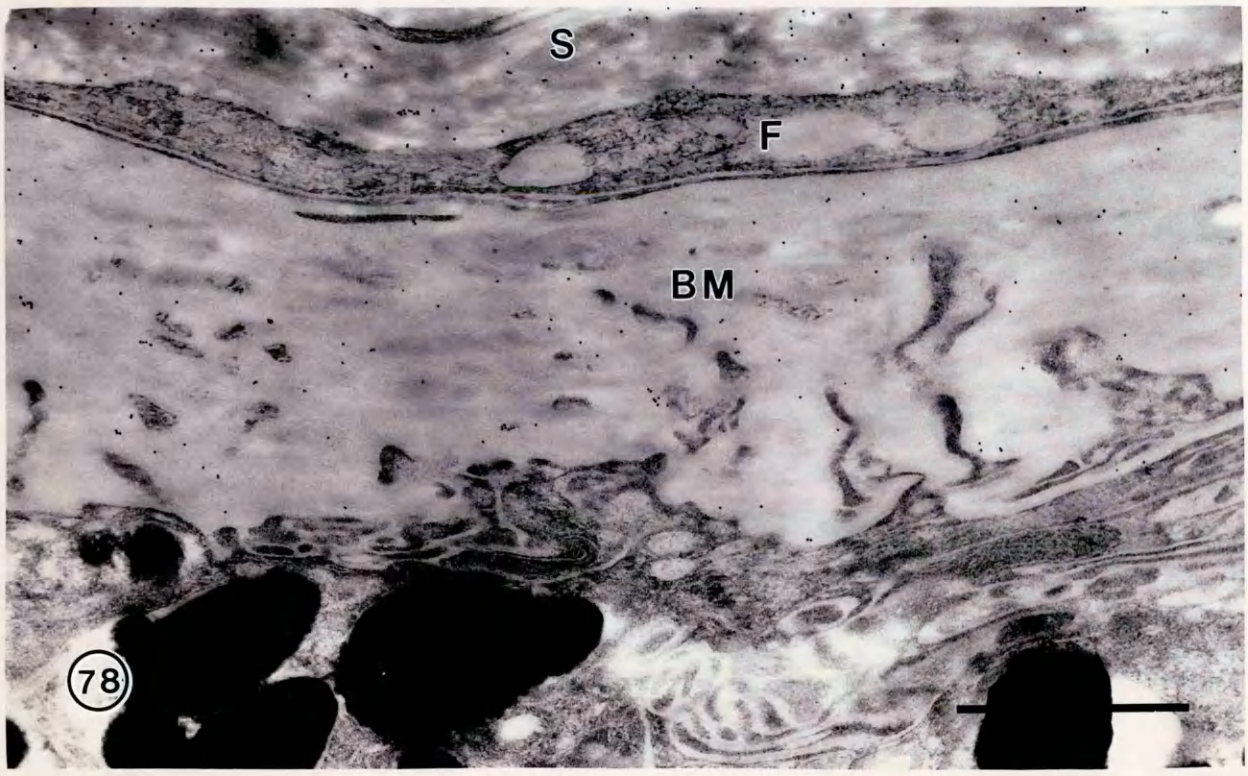

S
F
BM
78

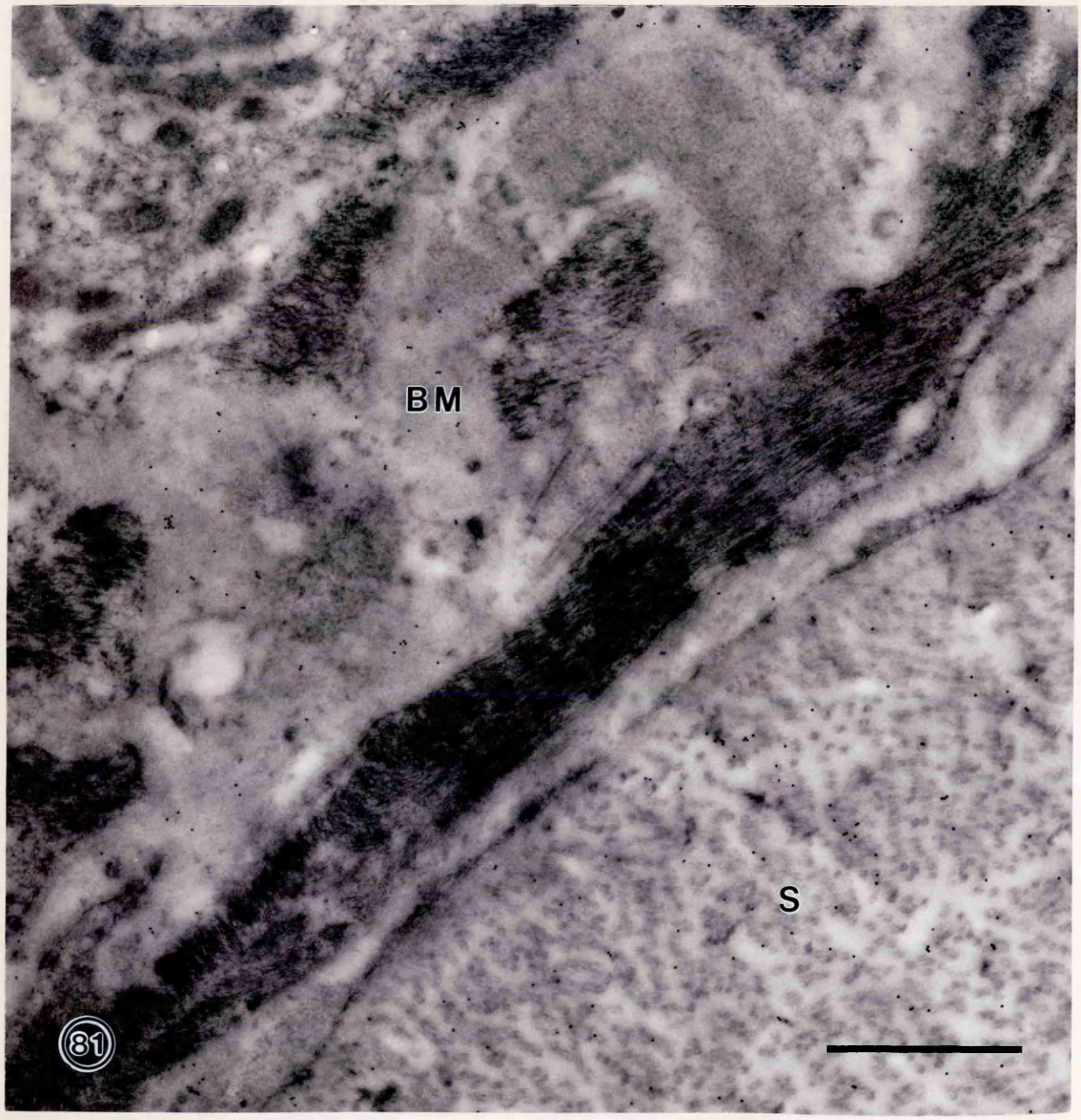
inner basement membrane, the outer basement membrane was more electron dense, and homogeneous in nature. Reticulations were absent, and granular inclusions less frequent. Some of the inclusions were considered to be cross-sections of the cytoplasmic processes arising from the pigment epithelium. The pigment epithelial basement membrane was separated from the stroma either by wall of a blood vessel, or by a lining of very slender processes of uveal fibroblasts.

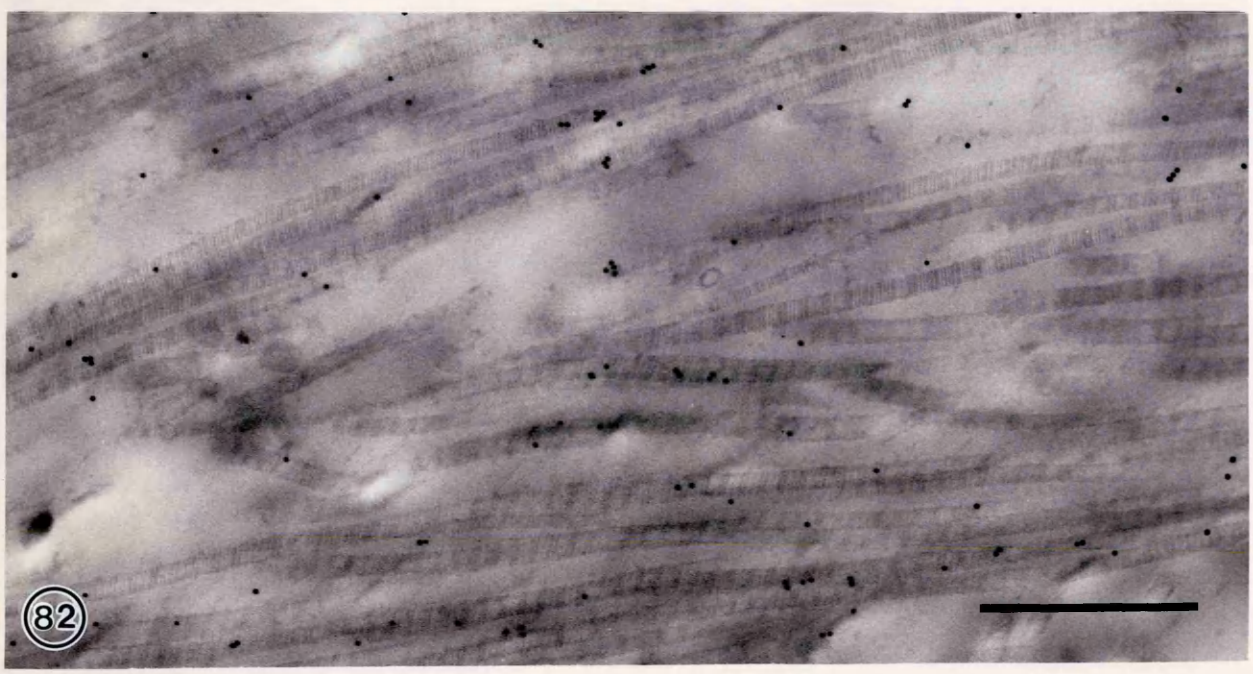
Immunocytochemistry: Although labelling for collagen types I, III, and IV was uniformly distributed within the basement membrane of the pigmented epithelium, the degree of labelling was significantly less than that for laminin (Figs 6-9). Of the three collagen types localized, immunogold labelling was weakest for type III collagen (Fig 78). There were no obvious concentrations of immunogold particles close to cytoplasmic processes nor was there an association of immunogold particles with the various granules present in the basement membrane.

Immunogold staining for laminin was both intense, and uniformly spread throughout the width of the pigment epithelial basement membrane (Fig 80). In contrast to the nonpigmented epithelial basement membrane, laminin was not associated with granular inclusions, nor was labelling concentrated close to the epithelial surface.

Fig 79: Type IV collagen labelling of nonpigmented epithelial basement membrane. LR white.

Fig 80: Laminin is uniformly distributed throughout the entire thickness of the nonpigmented epithelial basement membrane. F: lining fibrocyte; PE: pigmented epithelium. LR white.


BM
S
81


82

3.5.3 Stroma

The stroma of the pars plicata was defined in the present study as the connective tissue which lay between the processes, and the ciliary muscle. This consisted of loosely arranged striated collagen fibrils surrounding cells (fibroblasts, and melanocytes), and blood vessels of varying size.

The stromal collagens labelled positively with antibodies against both types I, and III collagens (Figs 81-82). More specifically, immunogold labelling was localized to striated collagen fibrils that possessed the characteristic 65nm banding pattern (Fig 82). The density of immunolabelling was similar for both collagen types, with immunogold particles being present on most fibrils irrespective of fibril diameter. There was no selective labelling of the margins or the core of the collagen fibrils (Fig 82). Collagen types I, and III were not present on finer filaments observed in the vicinity of striated collagen fibril bundles. No labelling for type IV collagen, and laminin was observed.

3.5.4 Ciliary Muscle

Aging Changes: All of the specimens exhibited a significant degree of atrophy of myocytes in the oblique

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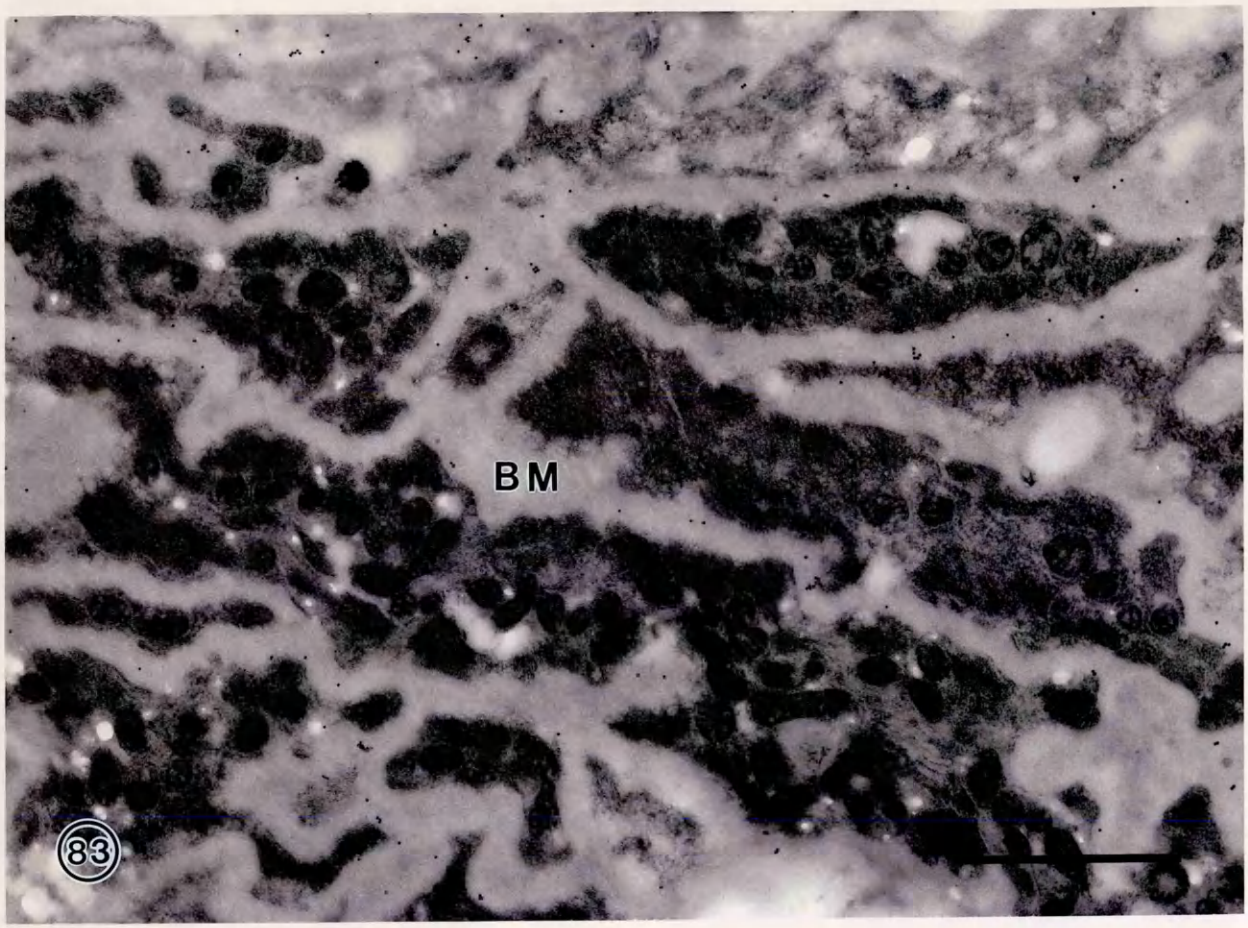
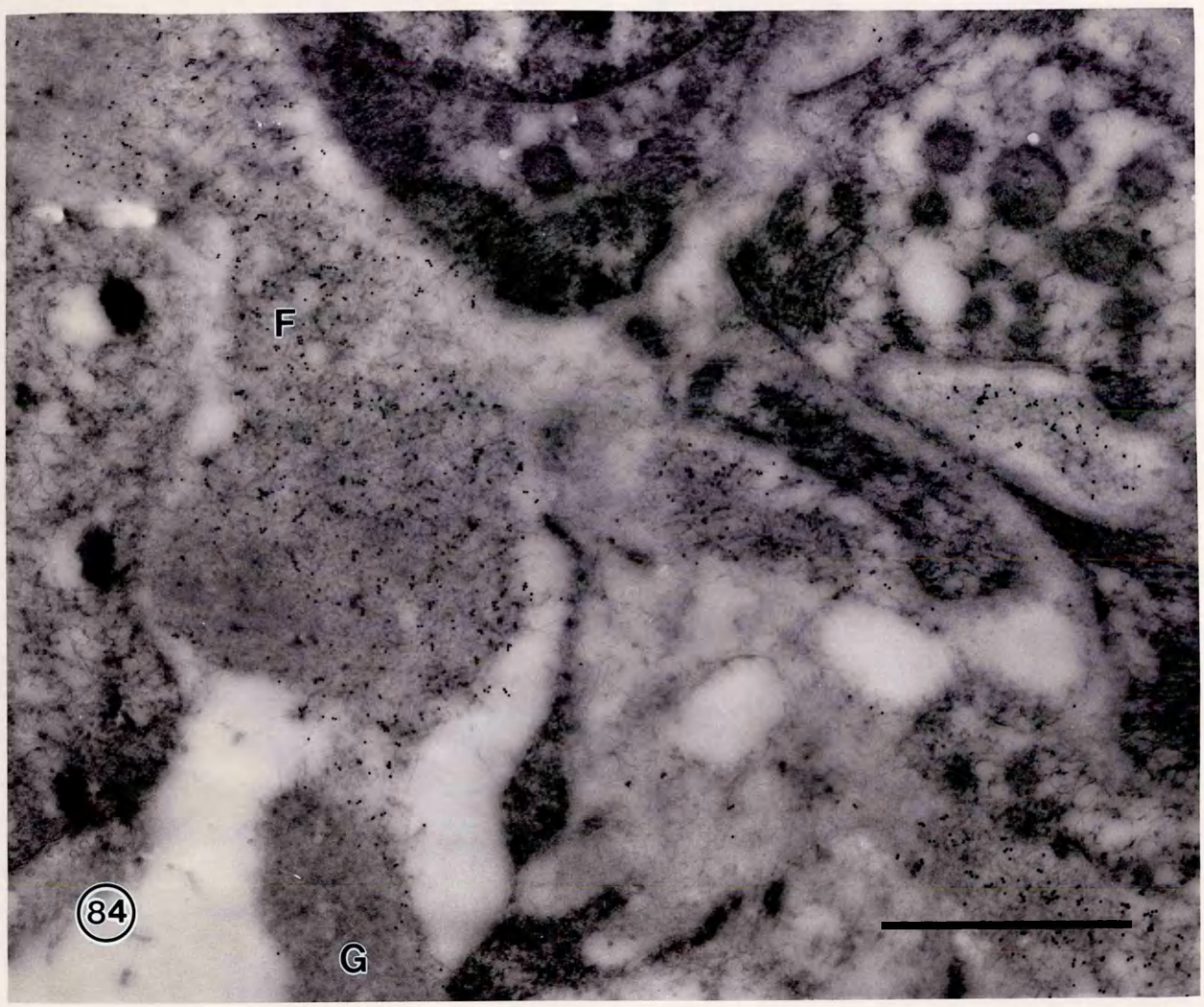
Fig 81: Type I collagen in ciliary body stroma (S) and ciliary muscle. Immunogold particles in the stroma are restricted to collagen fibrils which are seen in transverse section. Type I collagen also localised to the basement membrane (BM) of the ciliary muscle. LR white.

Fig 82: Labelling of type III collagen in ciliary body stroma is localised over striated collagen fibrils. LR white.


BM
83


F
84
G

(radial), and circular components with hyalinization characterized by a prominent fibrous pericellular matrix in the ciliary muscle region. This included thickening of the basement membranes between myocytes, and a accumulation of extracellular matrix material between bundles of myocytes. The spaces between individual myocytes were completely filled with uniform electron dense material which appeared to be an extension and fusion of the lamina densa of adjacent myocytes. With regard to the accumulation of extracellular matrix material surrounding the muscle bundles, unusual lobulated discrete structures were a prominent feature. On the basis of their composition three types of matrix deposits could be distinguished. These plaque-like structures were either filamentous, granular or amorphous in appearance. However, mixtures of plaque material were frequently seen, and will be refered to as composite plaques. All three types of plaque-like structures were more abundant, and considerably larger in the region of the ciliary muscle tips.

Immunocytochemistry: Labelling for types I, III, and IV collagen was present in the basement membrane surrounding individual myocytes of the ciliary muscle (Figs 81 and 83). No appreciable difference was noted in signal intensity amongst the three collagen types. Laminin was

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Fig 83: Basement membrane (BM) of ciliary muscle labelled with type III collagen antibodies. Particularly good preservation of mitochondria is with phosphate buffered fixative. LR white.

Fig 84: Laminin localised to extracellular matrix deposits between ciliary muscle myocytes. Intense labelling is present over matrix deposits that contain a fibrillar component (F) but is absent from finely granular deposits (G). LR white.

considered, by the criteria used in this study, to be absent from the basement membrane enveloping individual myocytes.

Very intense immunolabelling for laminin was present on both granular, and filamentous plaque-like structures (Fig 84). Amorphous plaque-like structures exhibited weak labelling particularly around their margins (Fig 84). However, labelling was stronger over amorphous material intermingled with other fibrillar or granular material in composite plaques (Fig 84). None of the three types of plaque-like structures contained any of the collagens under investigation.

3.5.5 Vascular System of the Ciliary Body

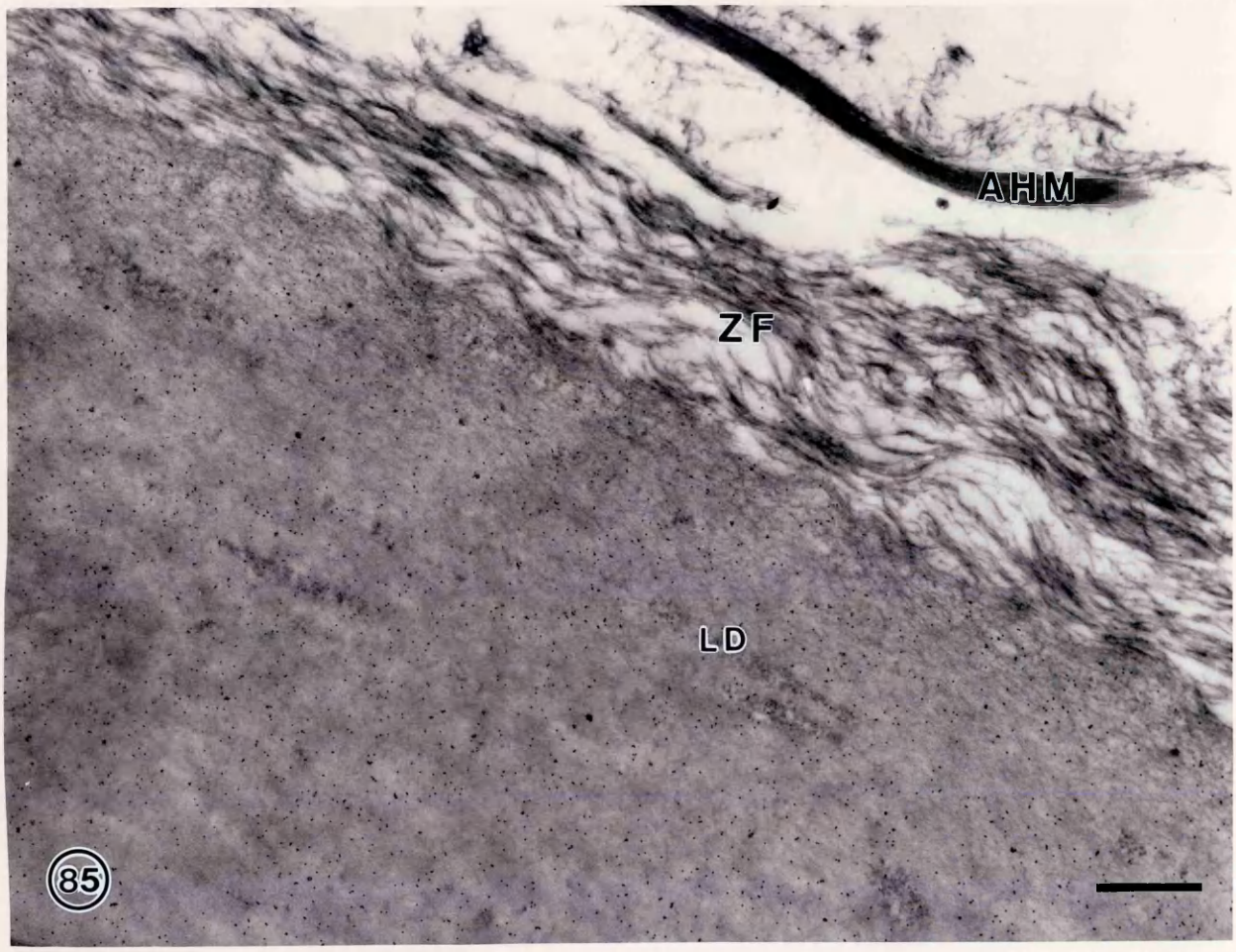
In the regions examined the blood vessels were thin walled with inconspicuous periendothelial contractile cells embedded in a prominent striated collagen matrix.

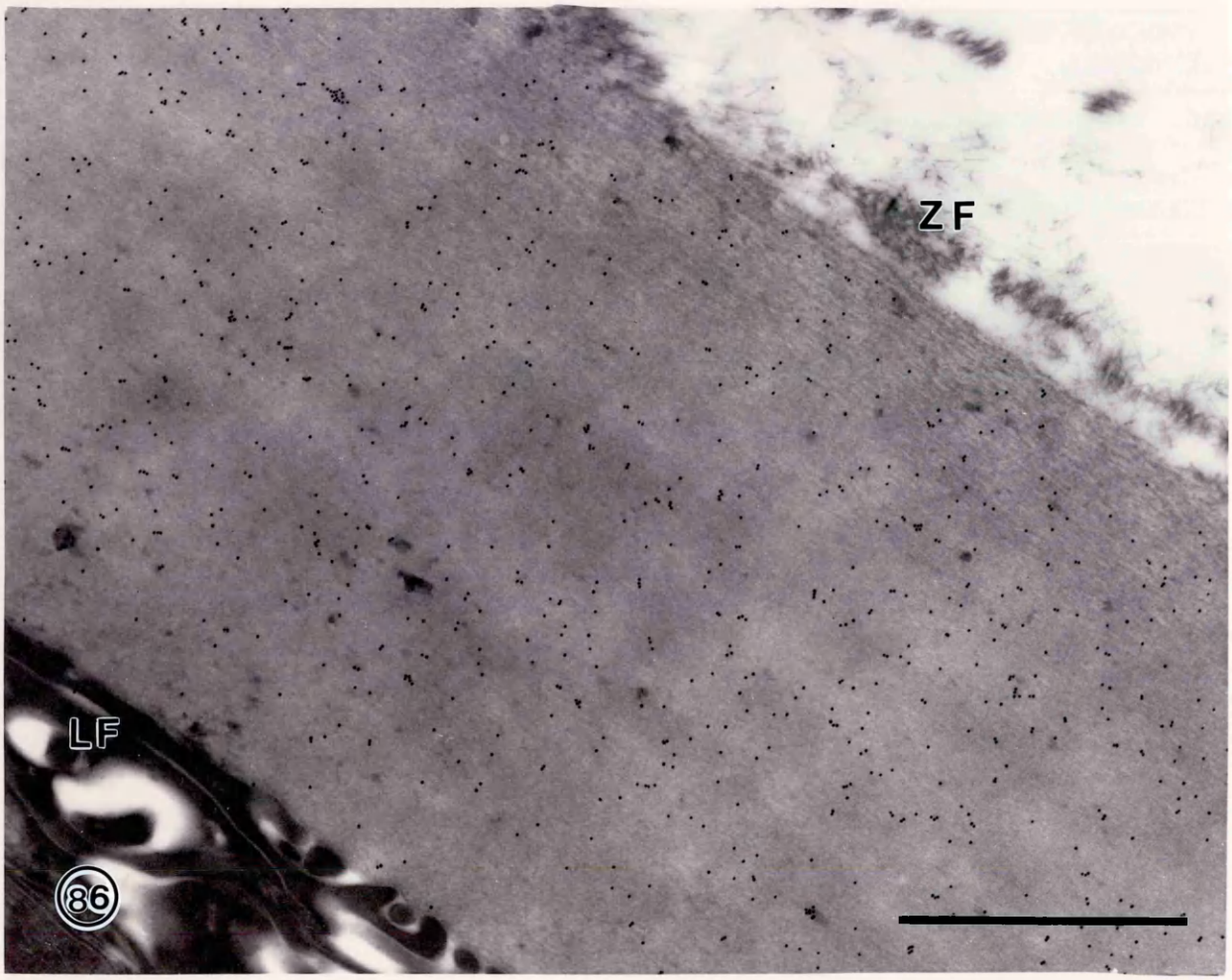
Types I, and IV collagen were present in the basement membranes of arterioles, capillaries, and venules in the ciliary processes, and vessels located in the stroma of the ciliary body. Labelling was notably stronger on the outer basement membrane of the supporting cells than on the basement membrane of the endothelial cells. Surprisingly, labelling for laminin was absent from the basement membrane of the endothelium although a strong reaction was observed in adjacent plaque-like structures. Labelling of the vascular basement membrane was not seen with type III collagen antibodies. The adventitia merged imperceptibly with the matrix, and the constituents included striated collagen fibrils labelled for types I and III.

3.5.6 Controls

Immunogold particles were rarely observed in rabbit anti-goat, and goat anti-rabbit immunogold control sections. A higher level of nonspecific labelling was observed in the negative normal goat and rabbit serum control sections.

Specific labelling was demonstrated for types I, III, and IV collagen and laminin in the iris internal positive control (i.e. iris tissue within the same section): types I and III were localized to the striated fibrils in the stroma, and types I, and IV were localized to the basement membranes. Laminin was demonstrated in the basement membrane of iris vessels. Trabecular meshwork, when present, was also used as an internal positive control. The presence of collagen types I, and IV collagen was demonstrated in the basement membranes of trabecular beams. In addition, types I, and III collagen were localized to the striated fibrils of the beams, and the sclera. Laminin was distributed over plaques of extracellular matrix in the cribriform layer.


AHM
ZF
LD
85


ZF
LF
86

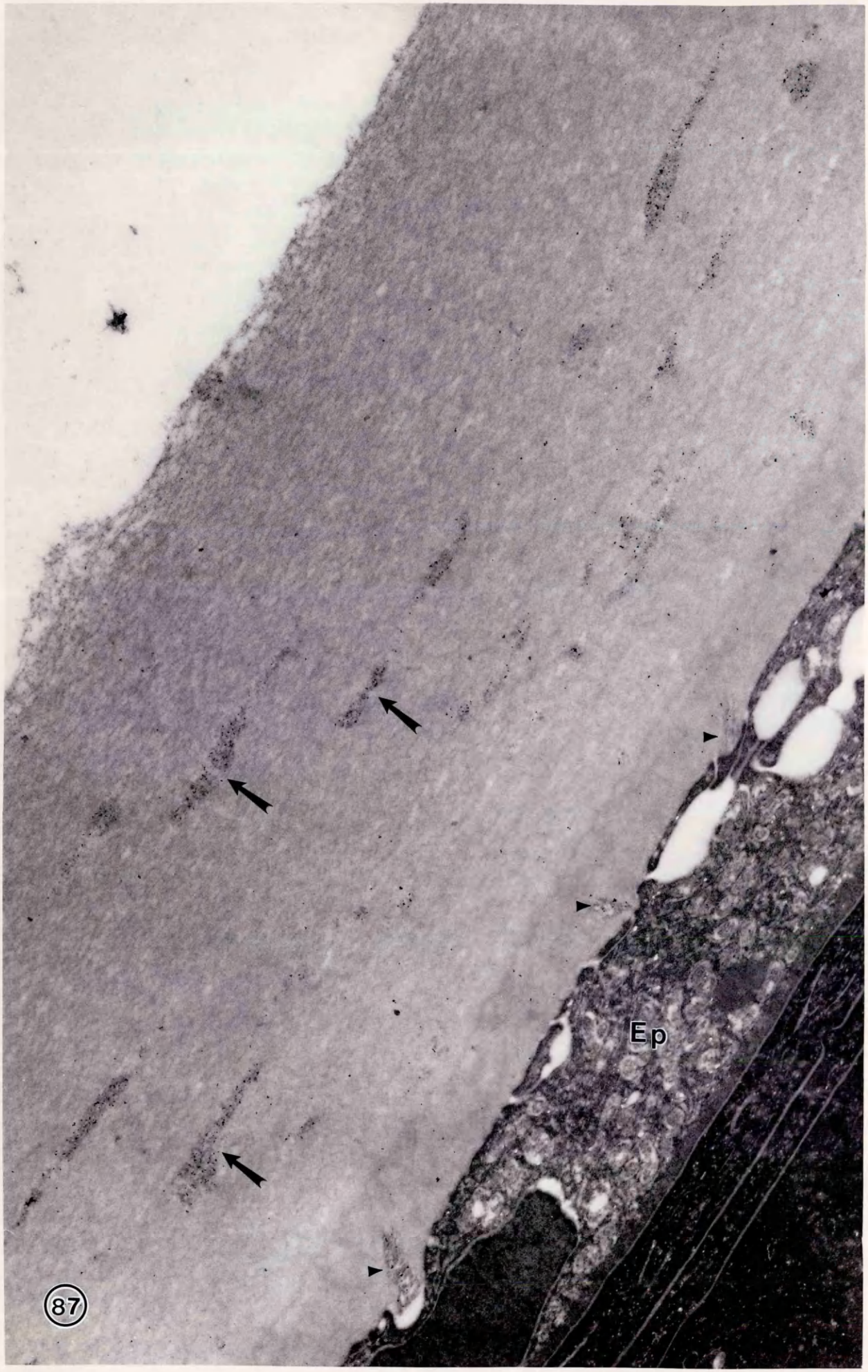
3.6 Lens Capsule

The peripheral lens capsule was arbitrarily divided into anterior, equatorial and posterior zones by reference to the underlying lens epithelial cells and lens fibres. For convenience the results are divided according to morphological distinct entities that comprise the lens capsule.

Labelling of the anterior, equatorial and posterior regions of the lens capsule proper (i.e. the basement membrane portion of the capsule) was observed with antibodies against types I, III and IV collagen (Figs 85 and 86). The zonular fibres did not contain these collagens (Figs 85 and 86). No appreciable difference in labelling density could be detected on comparing the labelling intensity between these three collagen types nor could a difference be detected between the anterior, equatorial and posterior zones. A density gradient was observed in one specimen for type III collagen with the density of immunogold particles being least at the interface between the lens capsule and the superficial lens epithelial cells. This was an exception to general observations which emphasised the homogeneity of labelling throughout the thickness of the capsule. The findings in autopsy specimens were similar in intensity and distribution to those of lens capsules from freshly enucleated eyes.

Fig 85: Labelling with type I collagen is evenly distributed in the posterior capsule. The linear densities (LD), zonular fibres (ZF) and the anterior hyaloid membrane (AHM) are free of label. LR White; bars indicate 1um unless stated otherwise.

Fig 86: The even distribution of type III collagen as seen in the posterior capsule. The lens fibres (LF) and the posterior zonular fibres (ZF) are negative. LR White.


Ep
87

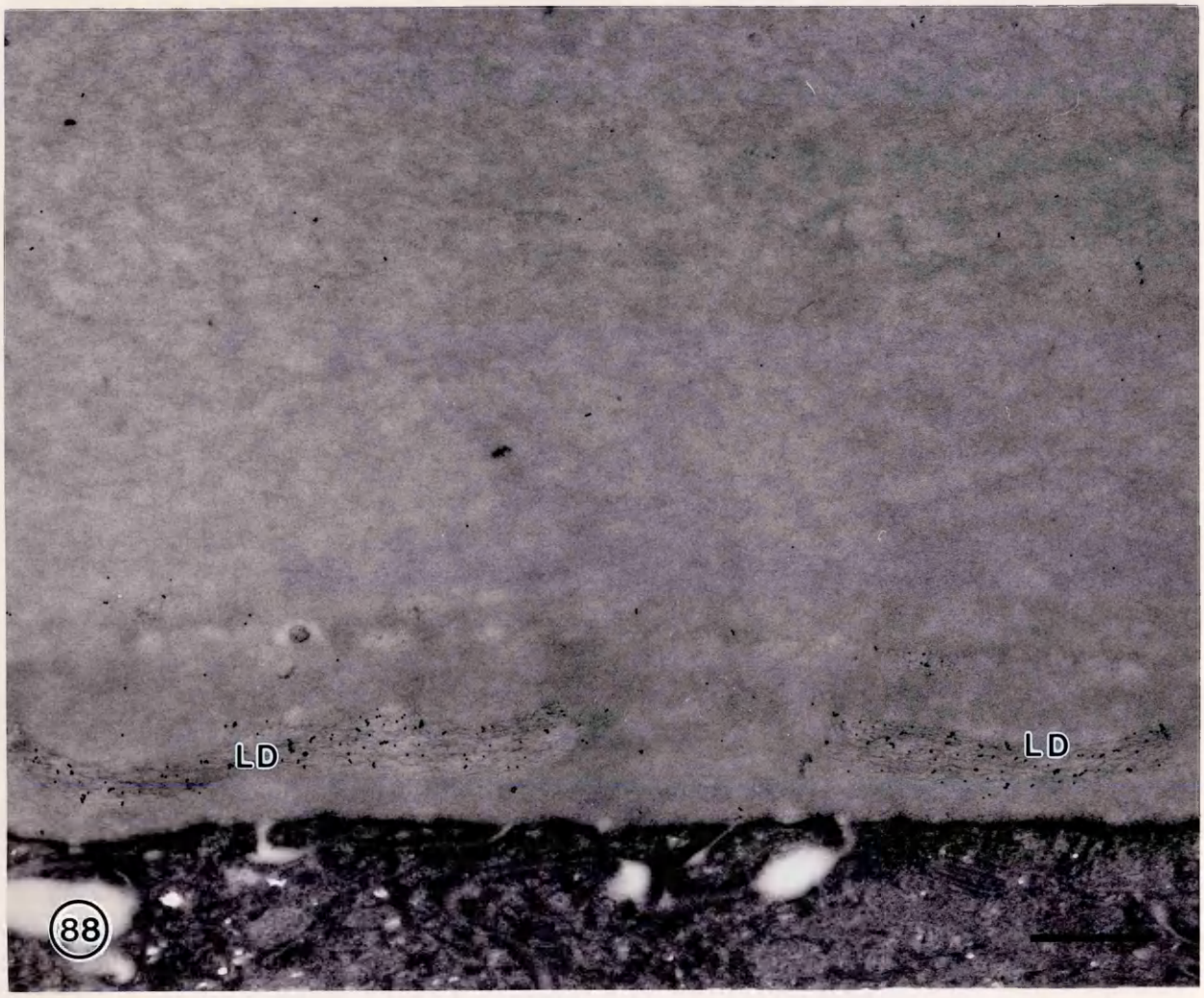
No immunolabelling of the basement membrane portion of the lens capsule was observed with either laminin (Figs 87-90) or type II collagen antibodies.

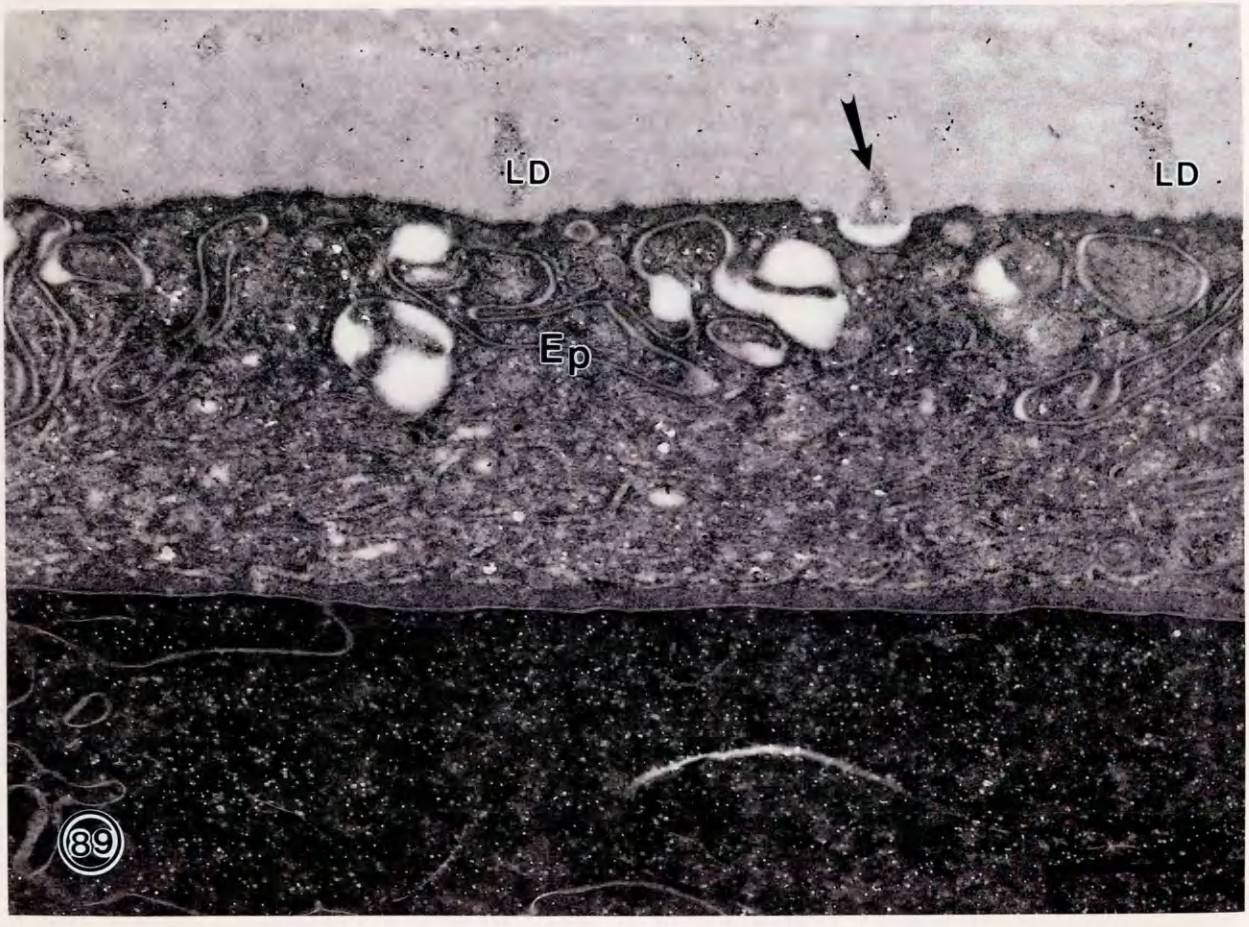
Laminations were generally absent from the anterior, equatorial and posterior zones. The innermost portion of the lens capsule was the most homogenous in appearance with the fibrillar nature of the capsule becoming more apparent in the more superficial layers, particularly in the equatorial and posterior zones (Fig 86).

3.6.1 Linear Densities

A strong signal for laminin was restricted exclusively to linear densities (formed elements) within the lens capsule (Figs 87-90). Labelling for collagen types I-IV was absent from such densities (Fig 85). Linear densities were observed throughout the thickness of the lens capsule and could be categorized into two morphologically distinct types: those having a granular appearance (Fig 87) and those consisting of coarse filaments running parallel to the capsule surface (Fig 88). Granular-like linear densities were distributed throughout the entire thickness of the lens capsule whereas the filamentous type were restricted to the deeper regions of the capsule. Granular-like linear densities were either roughly spherical or elongated, with the long axis running parallel to the surface of the capsule. Linear densities were frequently observed close to the superficial lens epithelial cells and were sometimes

Fig 87: Granular-like linear densities (arrowed), distributed throughout the full thickness of the lens capsule, are labelled with laminin. Note close proximity of linear densities (arrowheaded) to indentations of lens epithelium (Ep). LR White.


LD
LD
88


LD
LD
Ep
89

lying within grooves on the surface of the epithelial cells (Figs 87 and 89). Labelling with laminin was evenly distributed on both types of linear densities irrespective of their appearance or location within the lens capsule.

3.6.2 Pericapsular Membrane

The pericapsular membrane (zonular lamella) was positively labelled only for laminin. However, this zone was not easily distinguished when zonular insertions were present and was occasionally absent from autopsy specimens. Although labelling with collagens I, III and IV was present in the superficial region of the capsule (which was much less homogenous in its electron density than the deeper regions of the capsule (Fig 86), no labelling was observed in the filamentous portion that constitutes the pericapsular membrane (Fig 85).

3.6.3 Zonular Insertions

Zonular insertions were strongly labelled for laminin (Fig 90) and very occasionally for type III collagen. Labelling for types I, II and IV was absent from these structures (Fig 85). Two types of morphologically distinct zonular insertions were observed:-those consisting of dense aggregations of coarse wavy filaments (Figs 85 and 90) and those that consisted of fine filaments which formed

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Fig 88: Appearance of fibrillar-like linear densities (LD) in longitudinal section after labelling with laminin antibodies. LR White.

Fig 89: Laminin labelling of linear densities (LD) close to the lens epithelium (Ep). Note linear density lying over groove in epithelial surface (arrowed). LR White.


LC
90

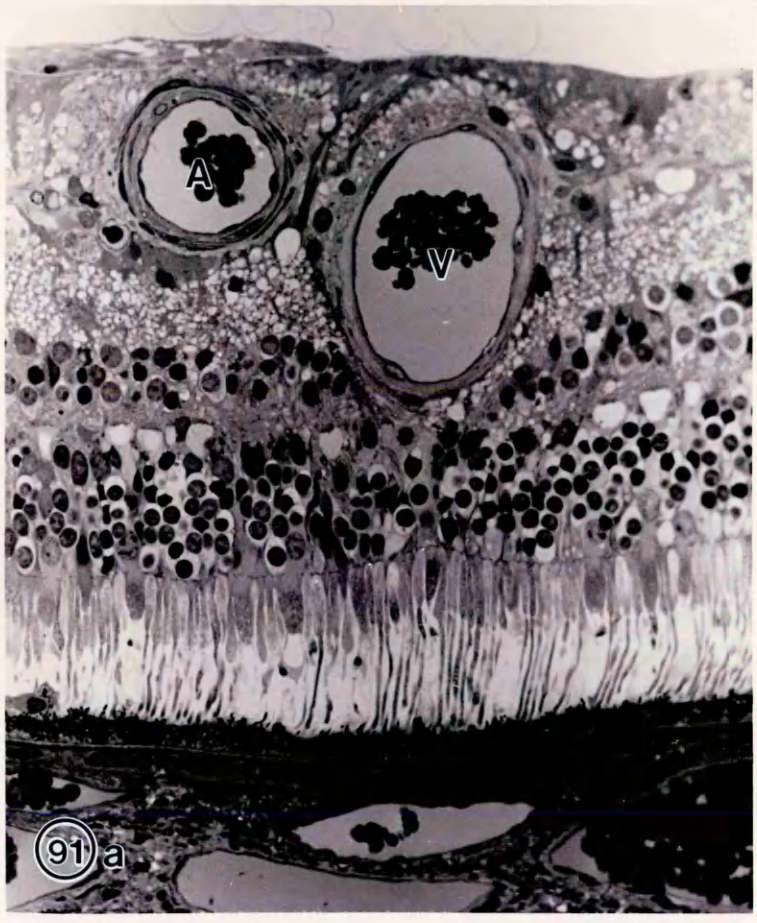
a less ordered and less electron dense structure. The former structures were comparable in appearance to the filamentous type of linear densities (Fig 88).

3.6.4 Controls

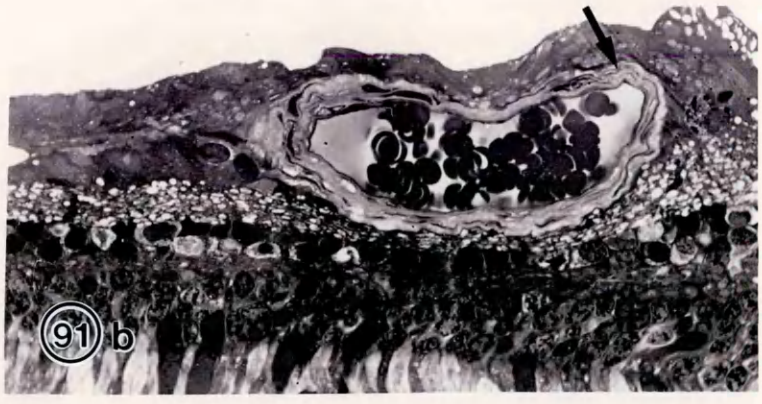
Although immunogold particles were largely absent from negative serum controls some degree of labelling was observed in autolysed regions of the lens cortex in autopsy specimens.

Labelling of negative rabbit serum controls was higher than that seen with negative goat serum controls.

Fig 90: A coarse fibrillar zonular fibre attachment to the lens capsule (LC) is strongly labelled with laminin antibodies. Labelling is absent from the capsule. LR White.



91 a



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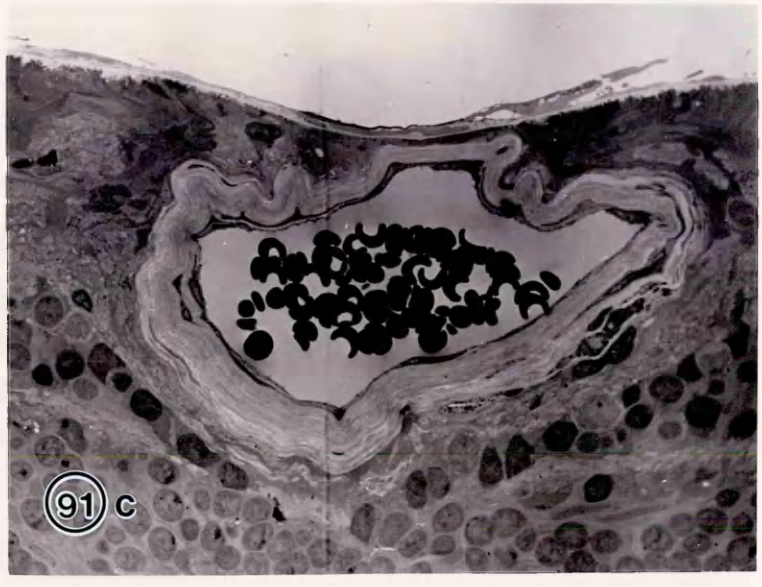


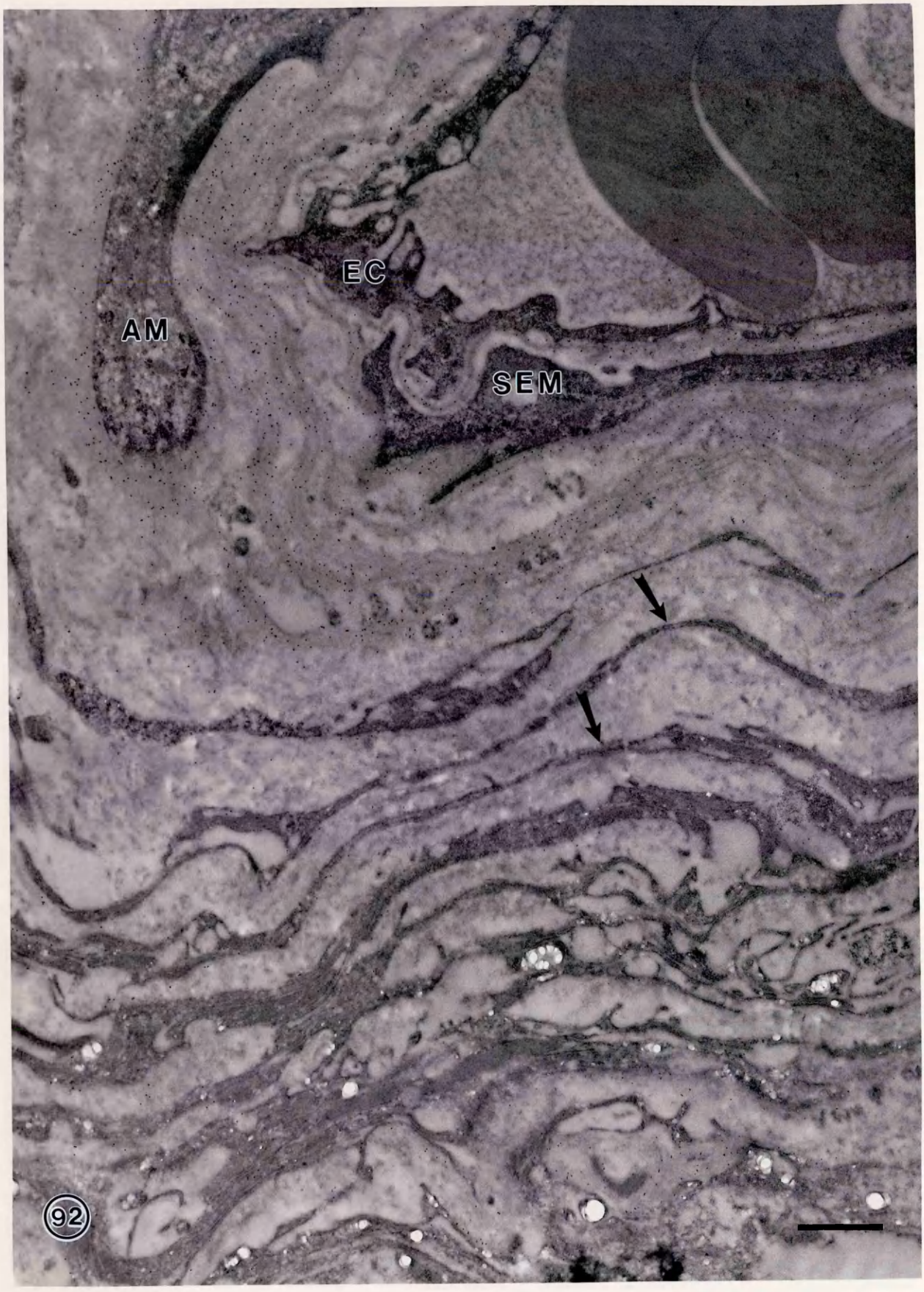
91 c

3.7 Retinal Vessels

An arbitrary classification was used to distinguish the degree of hyalinisation in venules and arterioles. Owing to considerable variation within an individual eye, retinal vessels were placed into one of three grades of the hyalinisation process by light microscopic examination of toluidine blue stained semithin plastic sections. Category A (Fig 91a) consisted of what was thought to be ostensibly normal vessels in which normal myocytes were identified. In Category B (Fig 91b) the walls were significantly thickened but still possessed several layers of myocytes, whereas Category C (Fig 91c) consisted of grossly thickened walls with very few myocytes. Five of the cases studies were placed into category A, four into category B and three into category C.

In all three categories into which the vessel wall was categorised, major vessels were found to contain collagen types I, III, IV, I and VI. Smaller branch vessels contained types I, IV and V collagen, with diminished amounts of types III and VI. The connective tissue round capillaries contained types I, IV and V only. Types III and V collagen were found in normal and hyalinised vessels. However, the thickening of hyalinised vessel walls was found to be due to excessive amounts of type I, IV and VI. Type II collagen was consistently absent at all levels of the retinal vasculature.

Fig 91: Retinal vessels in various stages of hyalinisation. **[a]** Artery (A) and venule (V) of normal vasculature (category A). **[b]** Hyalinised artery with thickening of vessel wall (category B). Some myocytes (arrowed) are atrophic. **[c]** Grossly Hyalinised artery (category C). Few myocytes are present and the vessel is surrounded by a thick layer of hyalin like material. Toluidine Blue, a x24; b x60; c x60.


EC
AM
SEM
92

3.7.1 Large Vessels

Type IV Collagen: Type IV collagen labelling was restricted to basement membranes round myocytes and lining the basal aspect of endothelial cells in larger vessels (Fig 92). There was no detectable difference in the intensity of labelling of basement membranes sandwiched between endothelial cells and subendothelial myocytes with basement membranes in the outer part of the vessel wall. The basement membranes of retinal cells that were in contact with blood vessels such as astrocytes, perivascular cells, glial cells, Müller cells were also labelled for type IV. In diseased vessels marked labelling surrounded activated myocytes but was largely absent from atrophic myocytes (Fig 92). Thickened basement membranes were evenly labelled with type IV, as were the multilayered basement membranes typical of hyalinised vessels (Fig 92). The inner half to three-quarters of hyalinised vessels contained type IV collagen, but the outermost region excluding the basement membranes of surrounding glial tissues was negative (Fig 92).

Other Collagens: It is noteworthy that the collagens in the outer half of the hyalinised vessel walls were identified as types I, III, V and VI and the distribution of types I and V in retinal vessels other than capillaries was identical to that of type IV, except that striated fibrils were also positively labelled and that both

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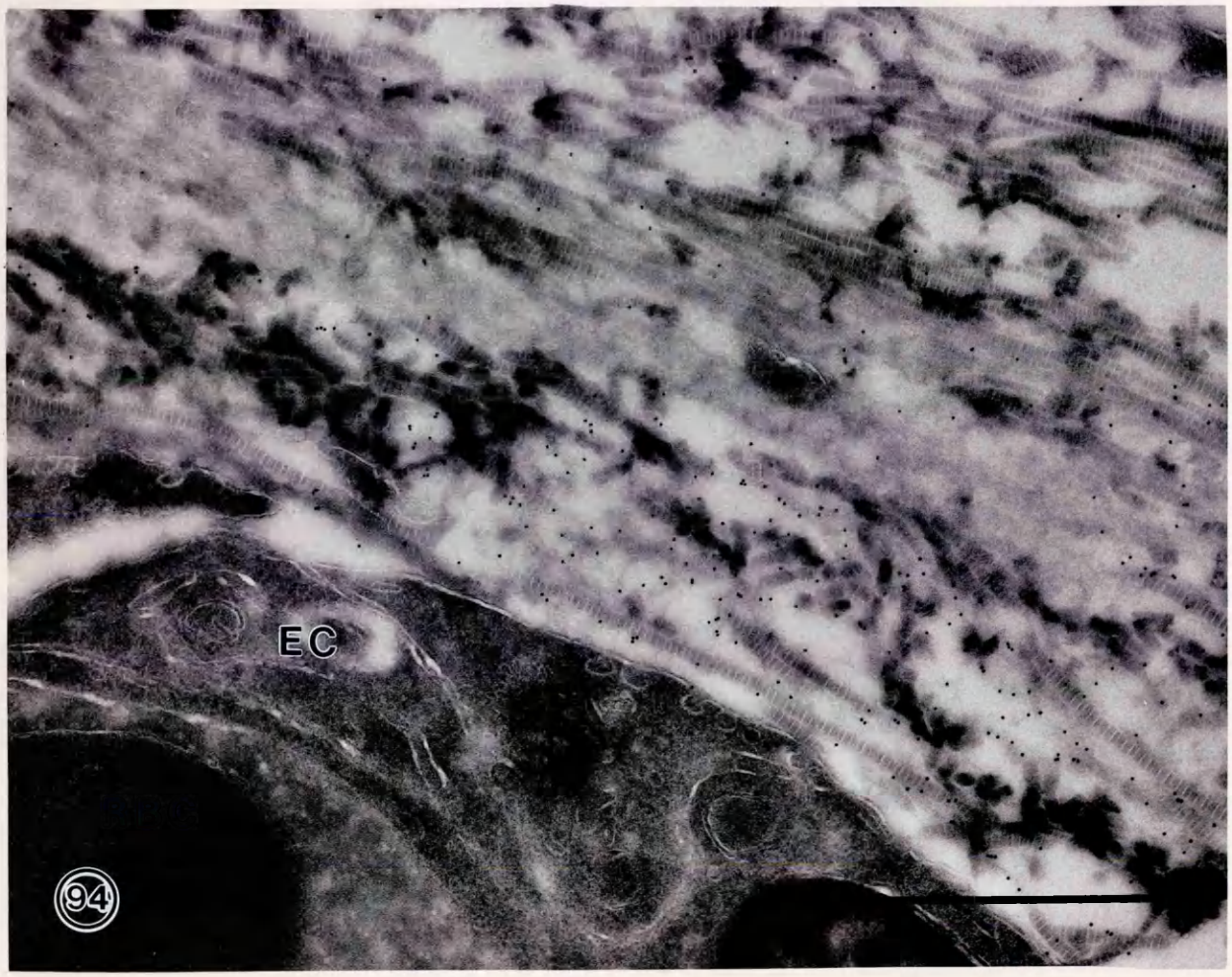
Fig 92: Type IV collagen distribution in hyalinsed artery (category B). The basement membrane of the endothelial cells (EC) and that of the subendothelial myocyte (SEM) are labelled for type IV collagen, as is the multilayered basement membrane (MBM) and and activated myocyte (AM). Atrophic myocytes are void of label (arrowed). LR White; bars indicate 1um unless stated otherwise.


NT
93


EC
94

collagen types were present thoughout the entire thickness of hyalinised vessel walls.

Type III Collagen: Type III collagen was restricted to striated collagen fibrils of large vessels (Fig 93) being completely absent from basement membranes.

Type VI Collagen: Type VI labelling was observed on striated fibrils but was also noted in areas where striated fibrils were absent (Fig 94). Basement membranes sandwiched between endothelial cells and subendothelial myocytes were consistently negative for type VI as well as capillaries and small vessels. In larger vessels immunogold labelling for type VI was present on loose aggregations of collagen fibrils but was largely absent over areas where the fibrils lay parallel to one another in tight bundles.

3.7.2 Capillaries

Types I and IV Collagen: In capillaries labelling for type IV was consistently stronger than that for type I (Fig 95) but there was no detectable difference in the pattern of distribution of the two collagen types. Endothelial cell basement membrane staining for types I and IV was not as intense as that on the outer side of intramural pericyte

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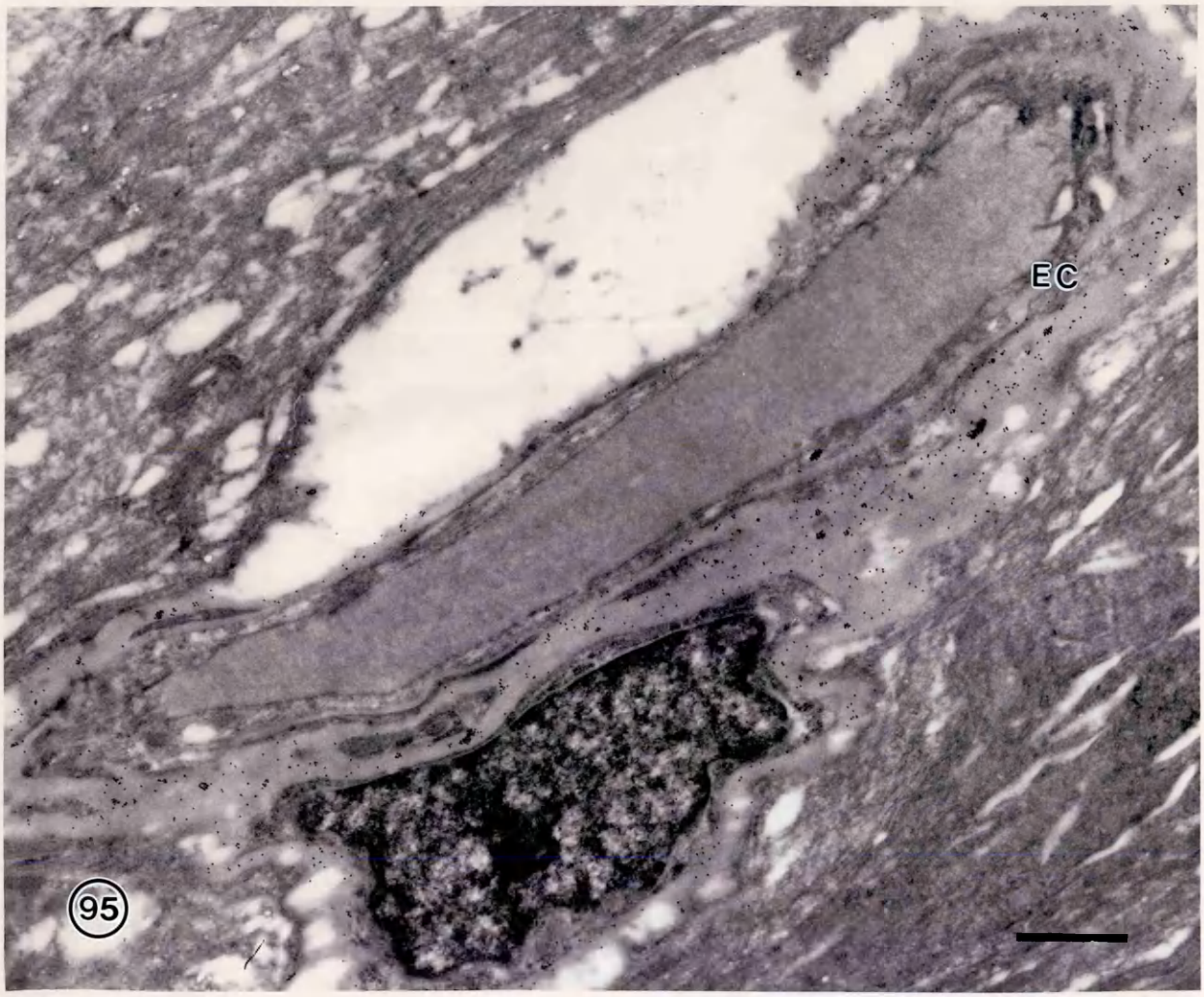
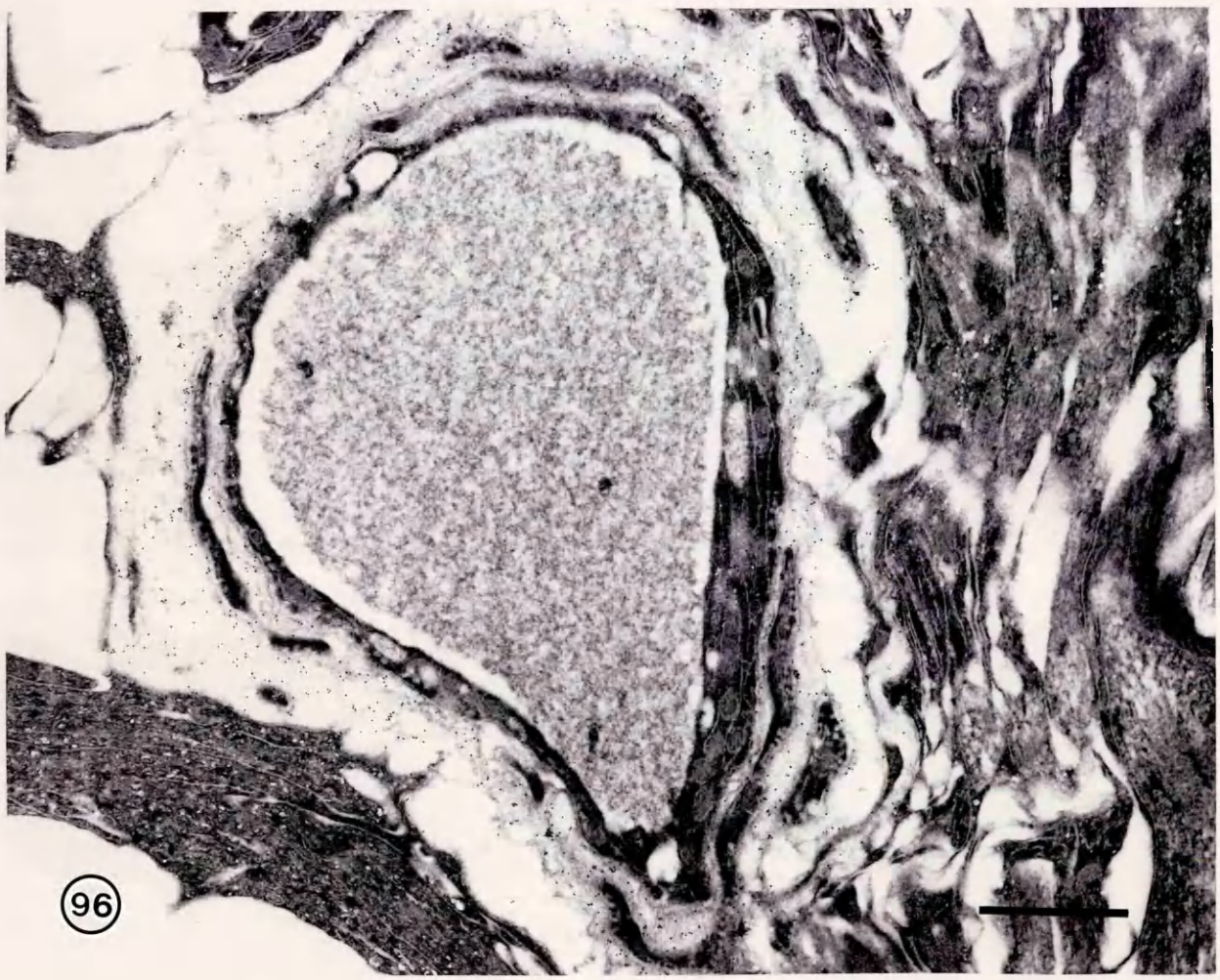
Fig 93: Labelling of striated fibrils at outer boundary of vessel wall with type III collagen antibodies. Neural tissue (NT) is free of label. LR White.

Fig 94: Immunogold localisation of type VI collagen in medium sized blood vessel. Labelling is associated with areas of striated collagen fibril aggregations. Note that immunogold particles do not lie directly on top of collagen fibrils but either directly beside the fibrils or in the adjacent extracellular matrix. Particles are absent from red blood cell (RBC) and endothelial cell (EC). Preservation of fine structure is particularly good as this specimen was fixed in 1% glutaraldehyde. Cryo.


EC
95


96

processes. There was no obvious increase in staining intensity with increasing endothelial cell or pericyte thickness. Capillaries in categories B and C contained a uniform distribution of both types I and IV throughout the width of the thickened basement membrane. Striated fibrils, more clearly visualised in ultrathin frozen sections, were absent.

Fig 95: Type I collagen in retinal capillary. Labelling is localised to basement membranes surrounding endothelial cell (EC) and pericyte. LR White.

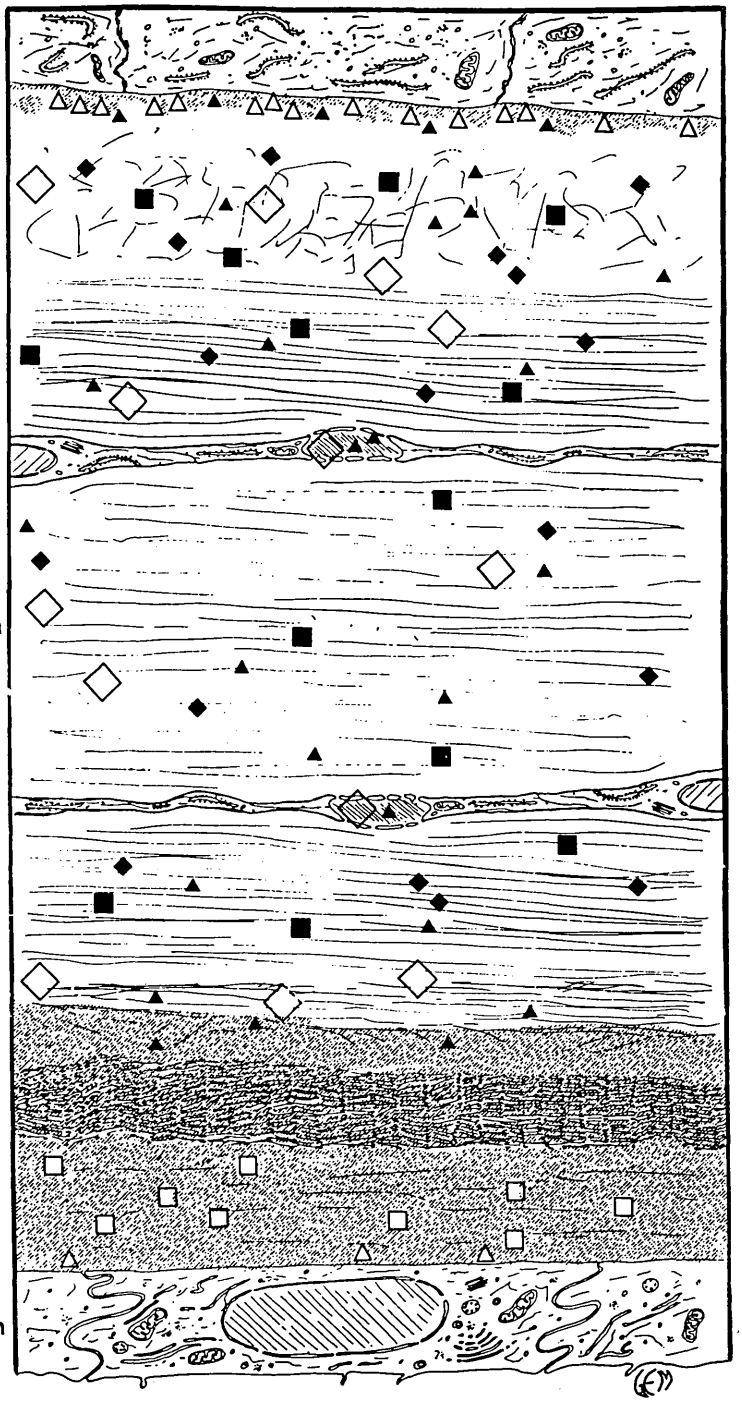
Fig 96: Type IV collagen labelling of retinal capillary is present over the basement membrane. Cryo.

Chapter 4:
Discussion

4.1 General Remarks

The recent development of hydrophilic embedding resins such as LR White and Lowicryl K4M for electron microscopic immunocytochemical studies combined with the immunogold localization technique has made possible the fine structural localization of antigens on significantly better preserved tissue. The greatest benefit of the immunogold technique is the precision with which antigens can be located in anatomical subcompartments.

It is now accepted that the use of colloidal gold in immuno-electron microscopy is a reliable technique for the identification of various tissue components.[11,12,14,73,219] The methodology is well established but the value of two complementary techniques (LR white embedding and cryoultramicrotomy) is to be re-emphasized. In the present study, the immunogold technique (transmission immunoelectron microscopy) was employed to document the presence of several extracellular matrix components (collagen types I-VI and laminin) in a number of ocular tissues.


Epithelium
Basement Membrane Complex
Bowman's Layer
Stroma
Interfibrillar Matrix
Anterior Banded Region
Descemet's Membrane
Posterior Nonbanded Region
Endothelium

4.2 CORNEA

A study of the literature led to the expectation of localising collagens I, V and VI in the corneal stroma, collagen IV to the epithelial basement membrane and Descemet's membrane and laminin to the epithelial basement membrane. The exact distribution of collagen IV in Descemet's membrane was open to question as was the presence or absence of type III collagen in the corneal stroma. It was considered likely that type II collagen would be absent.

The presence of collagens I, V and VI in the corneal stroma was confirmed in the present study as was the absence of type II collagen. However, the localising of type VI collagen to anchoring plaques of the basement membrane complex was surprising, as was the localising of collagens V and VI to keratocyte bodies in the corneal stroma. Equally surprising was the complete absence of type IV collagen from the epithelial basement membrane and the paucity of immunogold labelling for type IV collagen in Descemet's membrane which leads me to suggest that type IV collagen is not the major component of Descemet's membrane. The distribution of laminin also proved to be of great interest in that it was almost exclusively restricted to anchoring plaques.

Fig 97: Summary diagram of the distribution of collagens I-VI and laminin in the aged human cornea as seen the present study. Collagens I [◆], III [■], V [◇] and VI [▲] are evenly distributed throughout Bowman's layer and the corneal stroma. Types V [◇] and VI [▲] are also present in the interfibrillar matrix, type VI being also located in the most anterior portion of Descemet's membrane and in the basement membrane complex. Type IV collagen [□] is present in Descemet's membrane. Laminin [△] is localised to the basement membrane complex and immediately adjacent to the basal surface of endothelial cells.

The functional significance of the collagens present in the cornea can be summarily conjectured as follows. Types I and III collagen enable the cornea to resist tension exerted by the intraocular pressure. Type III collagen has the additional role of elasticity which could cater for changes in intraocular pressure. Type V collagen may control fibril diameter,[26,233] an essential mechanism for corneal transparency if it is dependent on the uniform diameter of its constituent collagen fibrils. Type VI collagen may be essential for the maintainance of regular interfibrillar distance between collagen fibrils (which is also considered essential for corneal transparency) as it is the only collagen located in matrix surrounding the fibrils.

4.2.1 Basement Membrane Complex

The corneal epithelium is attached to Bowman's layer by means of anchoring fibrils which are attached to anchoring plaques. As laminin is an adhesive glycoprotein it may play a major role in the adhesion of the anchoring fibrils to the anchoring plaques. The anchoring plaques are thought to be attached to Bowman's layer by being trapped in its meshwork of collagen fibrils. Having localised type VI collagen to anchoring plaques in addition to a network which is separate from the collagen fibrils, I suggest that type VI collagen may ensure the attachment of anchoring plaques to Bowman's layer by linking them with the type VI collagen network present throughout Bowman's layer and the corneal stroma.

Some immunofluorescent studies report the presence of type IV collagen in the lamina densa of the corneal

epithelium,[128,177,257] - a surprising claim considering that the lamina densa cannot be resolved by light microscopy. This preferential localisation was not confirmed in the present study of aged cornea. It is conceivable that a diminution of type IV collagen in the corneal epithelial basement membrane may be a feature of the aging process. Type IV collagen has also been seen to be associated with nerves in the avian stroma[10] and with microfibrils of the mouse stroma.[198]

Of major interest was the presence of type VI collagen in the anchoring plaques of the epithelial basement membrane complex. These electron dense patches of basement membrane material lie in the sub-basal lamina and as they have been shown by serial sectioning to have no continuity with the lamina densa, they are considered as distinctly separate entities.[116] Anchoring plaques have been graphically portrayed as structures entrapped in the superficial region of Bowman's layer by several connective tissue elements, particularly banded collagen fibrils.[227] As the presence of type VI collagen was demonstrated both in the anchoring plaques and Bowman's layer, it is possible that this collagen type may directly attach anchoring plaques to the surrounding matrix of Bowman's layer.

An immunofluorescent study described laminin as being present in the epithelial basement membrane and Descemet's membrane of the developing human cornea, although it was absent in the latter region at an early stage of growth.[18] High resolution immunoelectron microscopy employed in this study permitted more detailed localisation of laminin to the anchoring plaques.

Being an adhesive glycoprotein, laminin may also play

a role in attaching anchoring plaques to the matrix of Bowman's layer. Laminin has previously been shown to have profound effects on corneal epithelial cell growth and adhesion in vitro[253] and significantly increases the rate of corneal epithelial cell proliferation.[253] The principal role of laminin appears to be in the adhesion of corneal epithelial cells to their underlying basement membrane as it was the only extracellular matrix protein examined that produced adhesion sites in the cultured cells.[253]

4.2.2 Bowman's Layer

The presence of types I, III and V collagen were conclusively demonstrated in Bowman's layer of aged human cornea on both ultrathin frozen and LR white embedded tissue sections. In addition, these techniques permitted the localisation of these collagen types to the fibrils which form a meshwork in Bowman's layer. Labelling was absent from spaces between the fibrils and the anchoring fibril network.

The localisation of type V collagen to the fibrils in Bowman's layer in the present study amplifies the information obtained by immunofluorescence.[260] In the present study the intensity of labelling in Bowman's layer equalled that of the stromal collagen. This differs from previous studies in which although type V was demonstrated in Bowman's layer, labelling of the stroma was virtually absent when fibril disruption was not employed.[144,145,260] That the labelling of Bowman's layer should be equal to that of the corneal stroma indicates the efficiency with which the tissue preparation techniques employed in the present study unmask the stromal epitopes.

Type VI collagen labelling differed from that of the other collagen types in that the label had a closer association with the extrafibrillar matrix. The significance of this distribution pattern is discussed in section 4.2.3.

4.2.3 Stroma

Immunogold labelling of the corneal stroma revealed the presence of types I, III, V and VI collagen. The occurrence of type III collagen in this region appears to be unique to the human eye.[144,145,260] The majority of reports indicate that type III is present in the corneal stroma.[144,145,260] However, Newsome et al[182] in an immunofluorescence study illustrated immunolabelling only over Bowman's layer and over scar tissue in keratoconus, which could reasonably be regarded as an adequate positive control. However, the control tissue was obtained at autopsy and the significance of autolysis has yet to be determined. In a similar study of keratoconus, Nakayasu et al[177] reported immunostaining of equal intensity when type I and type III immunolabels were compared.

The potential importance of species variation may be derived from the present study in that 8-11nm fibrils seen in conventionally stained tissue did not react with type III collagen antibodies. This is in sharp contrast to the study of Pratt and Madri[198] which drew attention to the fact that such microfibrils (which are quite distinct from 65nm banded striated collagen[38,113,188]), stain positively for collagen type III in the murine cornea.

Various authors have stressed the difficulty of immunocytochemical localization of type V collagen in the

corneal stroma[144,145,260] and successful localization has required fibril disruption before labelling.[144,145,260] In the present study the intensity of labelling for both types V and VI collagen was significantly greater on ultrathin frozen sections than on LR white embedded tissue sections. Cryoultramicrotomy is therefore regarded as a more satisfactory technique for the localization of these two collagen types. It is thought that type V collagen is located in the centre of the fibrils. Presumably the technique of cryoultramicrotomy opens up the fibrils thereby exposing the type V collagen epitopes to the respective antibody.

Type V collagen may control fibril diameter in the corneal stroma through its longer helix[233] and/or the presence of its terminal globular domain within the assembled fibrils.[26] Type V collagen is distinctive from collagen types I, II and III in that its amino propeptide region remains part of the molecule when it is incorporated into a fibril.[134] It is thought that uncontrolled accretion of type I collagen would be prevented by incompatability with the "nonfitting" type V molecule already present in the fibril. The uniform distribution of types I, III and V collagen throughout Bowman's layer and the corneal stroma is indirect evidence of the co-distribution of these three collagen types within the same fibrils. Co-distribution of type I and type V collagen has been demonstrated in chick cornea using a double labelling technique.[23] Further evidence on the distribution of these two collagen types in chick cornea has been derived from enzyme digestion studies of Fitch et al.[61]

It has been concluded from the present study that

type VI collagen is not incorporated into the striated collagen fibrils but is present in the interfibrillar matrix. Type VI collagen has been located in non-ocular extracellular spaces which also contain types I and III collagen and fibronectin but differences in light microscopic staining patterns indicate a lack of co-distribution of these proteins.[246] Linsenmayer et al[147] localized type VI collagen to thin filaments in developing chick cornea and not to the striated fibrils. However, such localisation required extensive disruption of the fibril matrix in unfixed material. The separation of keratocytes from the lamellae in our cryo preparations was a fortuitous artifact for it enabled us to conclusively demonstrate the perifibrillar location of type VI collagen in association with filamentous components of the stroma.

Although types I, III and IV are present in the primary stroma of chick embryos, types V and VI only appear at the point of fibroblast invasion.[92,147] As type VI collagen appears very rapidly at this stage in embryogenesis[28] it is thought that type VI may be involved in cell attachment of invading fibroblasts.[36]

4.2.4 Keratocyte Bodies

The significance of keratocyte bodies is not known, but they may be a feature of aging. Whether or not the intracytoplasmic bodies contain newly synthesized collagen or represent phagocytosed collagen that is being degraded is open to conjecture. It is also debatable if these bodies are truly intracytoplasmic or if in fact they are merely surrounded by cytoplasmic processes.

From morphological studies, those bodies sandwiched

between the keratocytes and the adjacent lamellae have traditionally been regarded as either mucoprotein, glycoprotein or collagen precursors.[101] Iwamoto and DeVoe [106] noted an increase in the amount of this material in the stroma in Fuch's combined dystrophy when compared to normal controls.

The labelling of keratocyte bodies with types III, V and VI collagen was somewhat unexpected. As labelling for type III collagen was considerably less than that for types V and VI it would be reasonable to assume that types V and VI are the major components of these bodies. Keratocyte bodies appeared to contain a mixture of types III, V and VI collagen. This is regarded as one of the most intriguing features of the present study on collagens I-VI and laminin in the aged human cornea.

4.2.5 Long-Spacing Collagen

Long-spacing collagen has not been previously reported in the stroma of normal or aged human cornea. It was first described in Descemet's membrane;[107] this was closely followed by documention in trabecular meshwork[36] and Bruch's membrane.[66] The accumulation of long-spacing collagen is regarded as an aging phenomenon, as it does not appear in Bruch's membrane until the fourth decade of life [101] and as it also increases in quantity with increasing age in the trabecular meshwork.[152] Long-spacing collagen has been observed in Descemet's membrane in Fuch's corneal dystrophy,[105] but was not observed in Descemet's membrane of any of specimens examined in this investigation. The nature of long-spacing collagen is conjectural but it is of interest that Hirano et al[99] induced the formation of long-

spacing collagen in mouse corneal stroma and trabecular meshwork by incubating the tissue with 20mM ATP in acidic conditions at 37°C. The nature of long-spacing collagen is considered in the discussion concerning the aqueous outflow system (Section 4.3.4) but it is relevant to note in the present discussion that long-spacing collagen present in the corneal stroma did not exhibit labelling for any of the collagens studied in the present investigation (collagens I-VI).

4.2.6 Descemet's Membrane

The current investigation demonstrated both the presence of types III and IV collagen in, and the absence of types I, V and VI from Descemet's membrane.

Although a single study describes the location of type I collagen in Descemet's membrane of the embryonic human cornea,[18] other studies fail to mention whether or not type I collagen is present in this region.[177,182,257] The demonstration of type III collagen in the posterior non-banded region of Descemet's membrane could be regarded as an early manifestation of ageing.

Type IV collagen has been located in Descemet's membrane both by immunofluorescence[177,182] and by immunoelectron microscopy used in this study. Agreement is not forthcoming, however, on the precise distribution within Descemet's membrane. In two studies[177,257] labelling was restricted to the stromal interface, but subsequently Newsome et al[182] observed two discrete layers of labelling, one at the stromal face and one at the endothelial face. This led to the suggestion that the unstained middle portion corresponded to the region of

wide-spacing collagen (anterior banded region), described in conventional electron microscopic studies. In our investigation labelling for type IV collagen was present in the anterior banded region but was significantly less intense than the level of labelling present in the posterior portion of Descemet's membrane.

Types V and VI collagen were also located in the interfacial matrix. As these collagens also encroached into a narrow margin of Descemet's membrane it is suggested that they may play a role in the welding of Descemet's membrane to the corneal stroma.

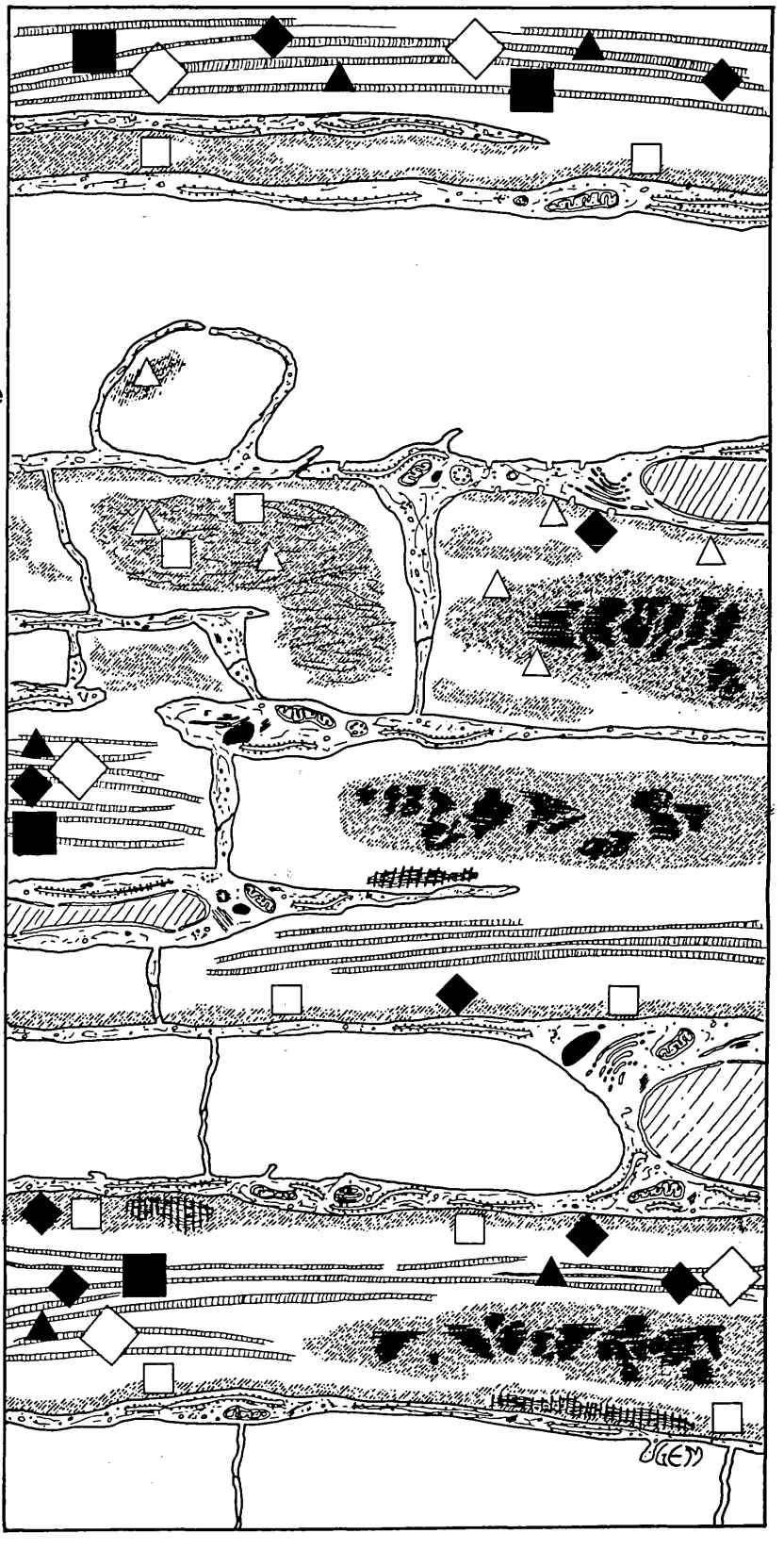
The pronounced increase of labelling intensity for type V collagen in the interfacial matrix (i.e. the most posterior lamellae of the stroma) can be related to anatomic differences as this region is supposed to consist of 10nm diameter fibrils, resembling tropocollagen or immature collagen.[210] In this regard, Fitch et al[61] noted that in chicken cornea, the interfacial matrix contained especially dense deposits of collagen types I, II, V and VI (**sic**, preliminary observations). It is also relevant to note that in avian embryonic cornea, digestion of this region with type I collagen degrading enzymes proceeds considerably more slowly and that the collagen in this region has a higher thermal stability than elsewhere in the cornea.[148]

The node-to-node distance of 110nm in the anterior banded hexagonal matrix of Descemet's membrane is similar to the distance between the globular domains of type VI collagen fibrils.[30] However, immunofluorescence studies have not located type VI collagen in Descemet's membrane,[261,268] not even after treatment with hyaluronidase or

acetic acid.[224] In the present study, labelling for type VI collagen was very weak in the hexagonal matrix of the juvenile (anterior banded) portion of Descemet's membrane. This supports the view that type VI collagen is not responsible for the hexagonal matrix. The hexagonal matrix is probably due to the presence of type VIII collagen which has recently been biochemically demonstrated to be a major component of Descemet's membrane.[222] Type VIII collagen is secreted by most endothelial cells in culture and was thus originally termed endothelial collagen.[221]

4.2.7 Type II Collagen

The presence or absence of type II collagen within the cornea depends on the species being considered. It has been reported as absent from human[18,177,183,257] and bovine cornea[49] but present in murine[89] and avian cornea.[93,95,259] In the latter species it appears to be restricted to the primary acellular stroma, but persists in later life in Bowman's layer[259] and Descemet's membrane.[93,95,259] The present investigation confirms those previous studies[18,177 257,259] which have failed to demonstrate the presence of type II in the human cornea.


Outer Wall
Schlemm's Canal
Giant Vacuole
Cribriform Layer
Intertrabecular Space
Corneoscleral Meshwork

4.3 AQUEOUS OUTLFOW SYSTEM

The morphology of the human outflow system, as studied by light microscopy and conventional transmission electron microscopy, is now comprehensively documented and there is, in general, agreement concerning the age related changes which occur in the various layers of the trabecular meshwork (reviewed by Tripathi and Tripathi,[254] McMenamin et al,[166] Grierson and Howes[79]). In attempting to elucidate the pathogenesis of primary open angle glaucoma, some authors have placed emphasis on age-related cell depopulation,[5,79] while other groups have concentrated investigations on the various electron dense collagenous materials which accumulate within the trabeculae and the cribriform (or juxtacanalicular) layer.[166,215]

It was established by Bill and Svedberg[21] that the site of at least two thirds of outflow resistance lies beneath the endothelial lining of Schlemm's inner wall and not in the endothelial lining itself. Rohen[212] suggested that the areas of electron lucent spaces located immediately underneath the endothelial lining of Schlemm's canal were preferential aqueous pathways which he termed cribriform pathways. In this context the use of LR White embedding

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Fig 98: Diagramatic summary of distribution of collagens I-VI and laminin in aged human trabecular meshwork as seen in this study. Type I collagen [◆] is present in striated collagen fibrils in trabecular beams and the outer wall of Schlemm's canal as well as in basement membranes. Type III collagen [■] is restricted to striated collagen fibrils. Type IV collagen [□] is localised over endothelial cell basement membranes and basal lamina-like material. Types V [◇] and VI collagen [▲] share a similar distribution to type III collagen except that they are not directly associated with the collagen fibrils. Laminin [Δ] is restricted to the cribriform being present on basal laminae, basal lamina-like material and fine fibrillar-like material.

medium has suggested that there may be extracellular components within what was previously considered to be empty flow channels. Morphometric analysis of the cribriform layer by McMenamin et al[166] demonstrated an age-related change in the character of the extracellular materials with an increase in the deposition of electron dense plaques and a decrease in the ground substances from the fifth decade onwards. In this study no attempt was made to relate the findings to the aging process. However, this is a potential avenue for future investigation.

The following discussion is based on the anatomical divisions of the meshwork.

4.3.1 Trabecular Beams

1- Types I and III collagen

Immunofluorescence has indicated the presence of type I in the extreme centre of the beam and patchy staining for type III in the core[176] but in the present study types I and III were localised specifically to the striated fibrils in the cores of the trabecular beams. As the pattern of gold labelling of these two collagen types was similar, it is likely that they were co-distributed within in the same fibril. While this suggestion should be confirmed by double-labelling, it is noteworthy that types I and III collagen are co-distributed in extensible tissues such as skin, blood vessels, lung and uterus. [for reviews see 263] The importance is that these two collagen types impart tensile strength (type I) and resilience (type III) to the trabecular "skeleton."[170] These physical characteristics are obviously an essential requirement for the biomechanical demands made on the trabecular beams.

2- Collagens associated with basement membranes

Type IV collagen was located both within the basement membrane of the trabecular endothelial cells and at the margins of the trabecular cores. An earlier report described the presence of type IV collagen in electron dense granular deposits "in the peripheral portion of the trabecular beams"(sic).[208] However, due to technical problems in obtaining good orientation of the frozen sections used in the study, no elaboration could be made on the precise localization of this collagen type. Another disadvantage of the frozen section technique was that there was no preservation of fine structure and endothelial cells were lost in the processing. It has subsequently been shown that the pre-embedding immunoperoxidase technique used is unreliable due to diffusion of the label from the site of antigen location.[2] Immunofluorescence has indicated the absence of type I collagen and the presence of types III and V collagen in the basement of the trabecular beams.[176] The present study is at variance with such findings. It is to be stressed that labelling for type V collagen was only present in areas where collagen fibrils had penetrated the basement membrane, labelling being absent from the basement membrane proper. In contrast to this, the presence of type I collagen was demonstrated in the basement membranes of endothelial cells lining the trabecular beams.

The presence of type I collagen in trabecular endothelial cell basement membranes was surprising as this collagen type, along with types II and III has been classified as a fibrous collagen forming the characteristic 65nm periodic striated collagen fibrils.[134]

Labelling of the basement membrane for type III collagen was sparse and unconvincing. This fibrous collagen is assumed to be absent from basement membranes in general.[134] However, human trabecular meshwork cell culture studies have shown that trabecular cells synthesize collagens I, III, IV, V, and VI.[267] Therefore the process of subendothelial deposition of collagens merits further investigation as this is an important part of the age-related cellular remodelling process.[6,166]

3- Type V collagen

The location of type V collagen within the trabecular beams was of great interest. Although type V collagen was seen to be associated with the striated collagen fibrils, it did not appear to be localized within the fibrils nor was it present in the basement membranes. Observation of type V labelling of unbanded fibrils enmeshed in striated collagen fibrils of human amnion led to the postulation of an anchoring function of type V between basement membranes and the stromal matrix.[57] The labelling pattern of type V in the trabecular core suggests the existence of a fine network of type V collagen which maintains the structural integrity of the striated fibril core which contains I and III. This can be contrasted with the distribution and function of type V collagen in the cornea. In the latter tissue, type V is localized within the striated collagen fibrils[22,23,61] and is thought to control fibril diameter [22]:a function which is critical to corneal transparency.[41] There is no reason to suspect that control of fibril diameter is as necessary in the trabecular meshwork.

4- Type VI collagen

Type VI collagen had a similar distribution to type V in the trabecular core. It is therefore suggested that type VI also forms a network and presumably has a similar role in maintaining the structural integrity of the collagenous core of the trabecular beams. The important difference in distribution between types V and VI collagen was that type VI labelling occurred in band-like regions within the basement membranes. This suggests that, as in the cornea, type VI collagen is the important component in an anchoring system between the endothelial cell basement membranes and the trabecular core.

The localisation of type VI collagen within the core of the trabecular beams is in agreement with a light microscopic study,[152] in which weak knot-like staining was also noted within the basement membranes underneath the trabecular endothelium. This knot-like staining can be attributed to type VI collagen labelling of occasional loose bundles of striated collagen fibrils lying outside the core and within the basement membrane zone of the endothelium as seen in the present study (Fig 47).

4.3.2 Cribriform Layer

Because it was necessary to fix the globes by division at the equator prior to immersion at atmospheric pressure, the cribriform layer was compressed and in the blocks available for study, the lining endothelium did not contain giant vacuoles. This form of fixation had an advantage over perfusion fixation,[4] in that there was less risk that the extracellular components would be washed into Schlemm's canal and therefore be unavailable for study. As

a consequence of hypotonic fixation, the collagenous elements appeared to be more compact than usual and this enhanced identification of the immunogold label.

1- Type I collagen

This feature of the material was important in the appearance of the immunolabel for type I collagen which was apparently rather sparsely distributed in the basal lamina beneath the endothelium of Schlemm's canal and in the underlying fine fibrillar plaques. By contrast the striated collagens in the trabecular cores took up clumps of label. It is not surprising therefore that a previous immunofluorescence study failed to detect type I collagen [176] in the cribriform tissue but gave a positive reaction in the trabecular cores. The label did not attach either to electron dense plaques of elastin-like material or to plaques of long-spacing collagen.

2- Type III collagen

The patchy staining of type III collagen in the cribriform tissue with immmunofluorescence[176] is also confirmed by the present finding of labelling over clumps of basal lamina and fine fibrillar-like material. Type III collagen was localized to both the basal lamina beneath the lining endothelium of the inner wall of Schlemm's canal and to the fine fibrillar-like material scattered throughout this layer (initially described by Rohen and Witmer as type III plaques[216]). Labelling of the basal lamina occurred in discrete patches whilst labelling of the fine fibrillar-like material was localized more to the fibrils than to the homogeneous matrix.

3- Type IV collagen and laminin

In this investigation it was found that basal lamina-like and fine granular-like material labelled for both type IV collagen and for laminin. Elastin-like fibers were negative for both extracellular matrix components and the fine fibrillar-like material labelled for laminin but only occasionally. While with immunofluorescence Murphy et al [176] identified type IV collagen within the periphery of trabecular beams it was not possible using their technique to demonstrate the presence of type IV collagen in the basal lamina-like material of the subendothelial layer.

Type IV collagen and laminin along with heparan sulphate proteoglycan are the major components of basement membranes.[124,136,169,223,247] It can therefore be concluded that basal lamina-like material is composed of basement membrane material and is most likely of a different origin from both elastic-like fibers and fine fibrillar-like material.

Laminin is a large multidomain glycoprotein that is synthesized by a variety of cell types viz: epithelial, endothelial, myocytic and neural (Schwann) cells (reviews by Martin and Timpl,[159] Engel and Furthmayer,[54]). This ubiquitous glycoprotein has been demonstrated to have potent action on cells, e.g. stimulating cell adhesion, growth, differentiation and migration. It has been shown that the laminin domains influence the behaviour of overlying cells.[53,225]

The present study shows that laminin is co-distributed with type IV collagen in the basement membranes of the endothelial lining of the inner wall of Schlemm's canal. By contrast only type IV collagen was present in the

basement membranes of the endothelial cells lining the trabecular beams. Generally speaking there is a higher proportion of laminin in thick basement membranes compared to thin ones.[77] In the case of the trabecular beams it would be reasonable to expect that the amount of laminin present would be small, due to the thinness of the basement membranes. Since laminin is thought to be a better immunogen than type IV collagen,[77] it can be concluded that the proportion of laminin to type IV collagen is considerably less in trabecular basement membranes than in the basal lamina-like material beneath the endothelial lining of Schlemm's canal. It is suggested that this indicates a functional difference between the two cell populations. Aqueous humour passes **through** the lining endothelium of the canal and **over** the surface of the trabecular beam endothelial cells. The present study stresses the importance of laminin in promoting adhesion of the canal lining cells to the underlying cribriform layer. Cell adhesion and stability is particularly important in the cribriform layer because the endothelial monolayer utilizes a transcellular giant vacuole system for aqueous transport.[254] It is tempting to speculate that mobility of the trabecular endothelial cells[79] is facilitated by a relatively low concentration of laminin in the substratum of the trabecular beams.

The functional and synthetic capabilities of the two cell populations viz : Schlemm's canal endothelium and trabecular endothelium, could be attributed to their different embryonic origin. By immunohistochemically localizing neuron-specific enolase (NSE), a marker believed to indicate cells differentiated from neuroectoderm,[226]

Tripathi and Tripathi[255] suggested the likelihood that trabecular endothelial cells are of neuroectodermal origin whereas it is generally accepted that the cells of the endothelial lining of Schlemm's canal are derived from mesoderm.[256]

4- Types V and VI collagen

It was disappointing that more detailed information could not be given concerning the distribution of types V and VI collagen in the cribriform layer. As was stated previously, repeatable results concerning collagens V and VI could be obtained only by cryoultramicrotomy, but the ultrathin frozen section technique did not maintain the structural integrity of the cribriform layer. However, it can be said with reasonable confidence that collagens V and VI were present in the cribriform layer and a limited description has been provided. Technical problems prohibit a more detailed description of their distribution.

4.3.3 Giant vacuoles

Giant vacuoles are not found in the lining endothelium in the canal when the fixation technique is performed at atmospheric pressure prior to immersion.[4] We are therefore unable to comment on the possible washout of collagens from the outflow system via giant vacuoles.

The unique observation of laminin within a giant vacuole is subject to two interpretations. The first is that laminin adjacent to the invaginated cytoplasmic membrane is incorporated within the giant vacuole. The second is that laminin deposits are washed into the giant vacuole after its formation. The distribution of laminin

within the giant vacuole favours the second suggestion.

4.3.4 Long-spacing Collagen

Long-spacing collagen was devoid of immunolabel for all the collagens studied, although the envelope around the long-spacing collagen contained type IV. The present study does not confirm a recent report that long-spacing collagen contains type VI collagen.[152] This study[152] was based on tangential sections through the cribriform layer in the region of the scleral spur. Cryoultramicrotomy facilitated particularly convincing labelling of filamentous collagen in the trabecular cores with type VI collagen antibodies and this was regarded as a valid positive control which substantiated the negative finding for long-spacing collagen.

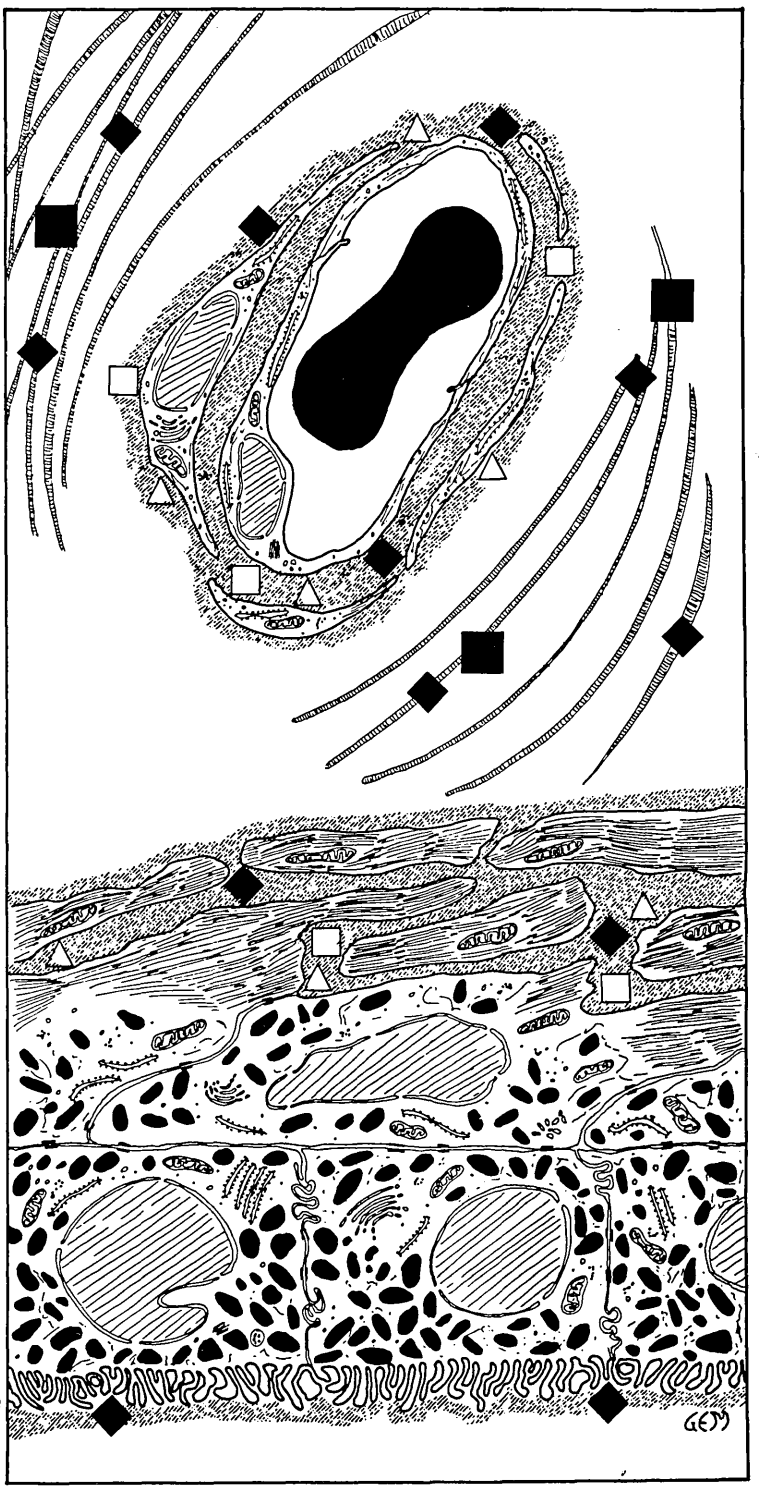
Such results are strongly supported by the study on corneal collagens in which type VI collagen was again absent from long-spacing collagen in the aged corneal stroma. Strong perifibrillar labelling of the corneal stroma with type VI collagen strengthened the validity of such results. These findings are treated with confidence, but confirmatory studies by independent groups will obviously be required.

The application of the immunogold labelling technique for collagen types VII -XIII would be justified in an attempt to unravel the core structure of this morphologically intriguing lattice-like matrix component. Nevertheless, on theoretical grounds, it is unlikely that the higher collagens form a major constituent of long-spacing collagen as these collagens are particularly sensitive to collagenase [for review chapters see 160]

which has been shown to be ineffective in destroying the wide electron dense bands.[151] Thus there is as yet no justification for classifying this material as a collagen. The significance of long-spacing "collagen" is that it increases with age and forms part of the so-called plaque material underneath the inner wall of Schlemm's canal and thickenings within the basement membranes of the trabecular beams.[153] This contrasts with the apparent lack of age-related changes in the diameter of striated collagen fibrils in the trabecular meshwork[214] and it suggests that striated collagen fibrils do not undergo metamorphosis to the long-spacing component. On teleological grounds, an increase in collagen fibril diameter with age would be positively disadvantageous in that it would increase the rigidity of the trabecular tissues.

4.3.5 Conclusion

In conclusion the concept now emerges that each collagen type may have an individual functional role and there are also complex interrelationships between the collagen types. Such collagen linkages must be essential to the structural integrity of the outflow system and its responses to physiological stress. Certainly an improved understanding of the biomechanical properties in terms of the endogenous constituents appear to be an encouraging approach to the phenomenon of outflow resistance.


Iris Stroma
Blood Vessel
Basal Myo Portion of Anterior
Iris Pigment Epithelium
Basement Membrane of Posterior

4.4 IRIS

In this study it was possible to precisely document the distribution of fibrous collagen types I and III, non-fibrous collagen type IV and laminin in the human iris. Previously published information on the distribution of collagen types in the iris at either the electron microscope or the light microscope level could not be found.

It is surprising that so little attention has been paid to the distribution of collagens and laminin in the normal iris considering that the iris has many unique structural and functional properties. This information is obviously necessary in order to better comprehend the functional role of these extracellular matrix components in the iris.

In all six cases (age range 28-68 years) the iris stroma and the pigment epithelium were histologically normal and qualitatively there was no apparent age-related change.

4.4.1 Iris Stroma

1- Type I Collagen

The present findings indicate that type I is the most prevalent collagen in iris tissue. In the iris stroma, the signal intensity for type I collagen did not increase with the diameter of the fibrils, which is in contrast to the

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Fig 99: Diagrammatic summary of distribution of collagens I-IV and laminin in aged human iris as seen in the present study. Striated collagen fibrils in the stroma are labelled with collagens I [◆] and III [■] antibodies. Type I collagen is also present over basement membranes of vascular, myoepithelial and posterior pigment epithelial cells. Both type IV collagen [□] and laminin [△] are present in vascular and myoepithelial cell basement membranes.

results obtained with type III collagen. This would indicate that an increase in fibril diameter is due to the deposition of type III rather than type I.

2- Type III Collagen

It is of interest that collagen type III was localised within the striated collagen fibrils throughout the iris, but was lacking from the basement membranes of the cellular constituents. It is likely that type III co-exists with type I, and this would be in agreement with previous evidence that uniform banded collagen fibres often contain more than one collagen type.[7,23,31] Although the function of type III is as yet unknown, it has been suggested that it may increase the plasticity of tissues; whenever the collagen ratio shifts in favour of type III, the tissue becomes more flexible.[200] The main difference in distribution between type I and type III is the absence of type III from the basement membranes. It is possible that the proportion of type III changes throughout life as maturation of the collagen ensues and this change may occur more rapidly or markedly in certain diseases (e.g. Marfan's syndrome). Quantitative immunolocalisation of type III in various disease processes will test this hypothesis.

4.4.2 Iris Pigment Epithelium

1- Type I Collagen

The basement membranes around the myoepithelial cells in the iris showed an intense signal, which suggests that these contractile cells synthesise type I collagen. The basement membrane of the posterior iris pigment epithelium also exhibited immunogold labelling within the basal

infoldings of the posterior pigment epithelial cells. This also implies a synthetic capability of this monolayer.

2- Laminin

With reference to the label exhibited for laminin in the matrix in which the dilator muscle is embedded we suggest that in this instance laminin may be acting as an adhesive factor. Its presence in the matrix may be instrumental for the orderly contraction of the dilator muscle. Changes in its distribution within this matrix could conceivably interfere with the dilatation of the iris. The intense labelling associated with the filamentous bundles bordering iris stromal fibrocytes is more difficult to interpret as the functional role of these cells remains unclear.

4.4.3 Iris Vasculature

1- Type I Collagen

Although collagen type I is the most abundant of all collagens in ocular tissues[31] it has not been shown to be present in the basement membrane of blood vessels. Identification of type I collagen at this site was unexpected. When the basement membrane of the iris vessels was examined carefully in high magnification, it became evident that the immunogold delineated fine strands within the lamina densa and the signal was stronger in the lamina densa of pericytes. That small vessels in the iris exhibit a weaker signal may be a reflection of the smaller concentration of accompanying myocytes.

2- Type IV Collagen

Collagen type IV has been identified in a series of

basement membranes of various animal tissues[77,119] and has been shown to be a major component in the basement membrane of retinal vessels in cats.[55] What was particularly impressive in the present study was the intense signal obtained in the lamina densa of the iris vessels and particularly in the loose non-fibrous elements around the myoepithelium. As expected, type IV was absent from the stroma of the iris. The impression was gained that there was increased labelling in association with the perinuclear zone of the endothelial cells. This indicates that type IV, like other components present in basement membranes, may be secreted by endothelial cells.

3- Laminin

It was of interest to note the distribution of laminin in the normal iris vasculature. Labelling for laminin was restricted principally to the extracellular matrix enclosing the myocytes within the walls of the larger iris vessels. A similar pattern was also observed in smaller vessels when labelling was observed around contractile cells, but not beneath the vascular endothelium (Fig 71). The same localisation pattern for laminin was reported in a recent immunocytochemical study of cat retinal vessels.[55] This pattern of laminin distribution may indicate that within the iris vasculature the synthesis of laminin is a responsibility of the myocytes and pericytes rather than the endothelial cells. This conclusion is based on the assumption that cells attached to basement membranes secrete most if not all the extracellular matrix components present within that basement membrane. By contrast there was more intense labelling for collagen type IV in the

subendothelial basement membrane of iris vessels in comparison with the matrix around vessel supporting cells. Consequently, it is considered that in iris vessels endothelial cells assemble primarily collagen type IV whilst laminin is mainly produced by myoepithelial cells and pericytes. This is important as differences in the production and utilization of laminin may contribute to variations in cell behaviour. It is relevant that a recent **in vitro** study demonstrated that aortic endothelial cell adhesion is preferentially promoted by collagen type IV when compared to laminin.[18]

It has recently been emphasized that the chemical composition of basement membranes may vary according to the demands of particular tissues.[2] The observation that in the normal iris, laminin seems to be mainly present in the walls of thicker vessels indicates heterogeneity in the components of thick and thin basement membranes within the iris. The present findings are in accord with an immunogold quantitative study performed on a variety of animal basement membranes.[77]

4.4.4 Type II Collagen

Collagen type II was negative in iris, cornea and vitreous with both techniques employed. Thus we were unable to confirm biochemical findings in vitreous concerning type II collagen;[200] this might be explained in terms of antigenic sensitvity of type II to fixation. The question of presence of absence of type II collagen in the iris must therefore also remain open.

4.4.5 Type V Collagen

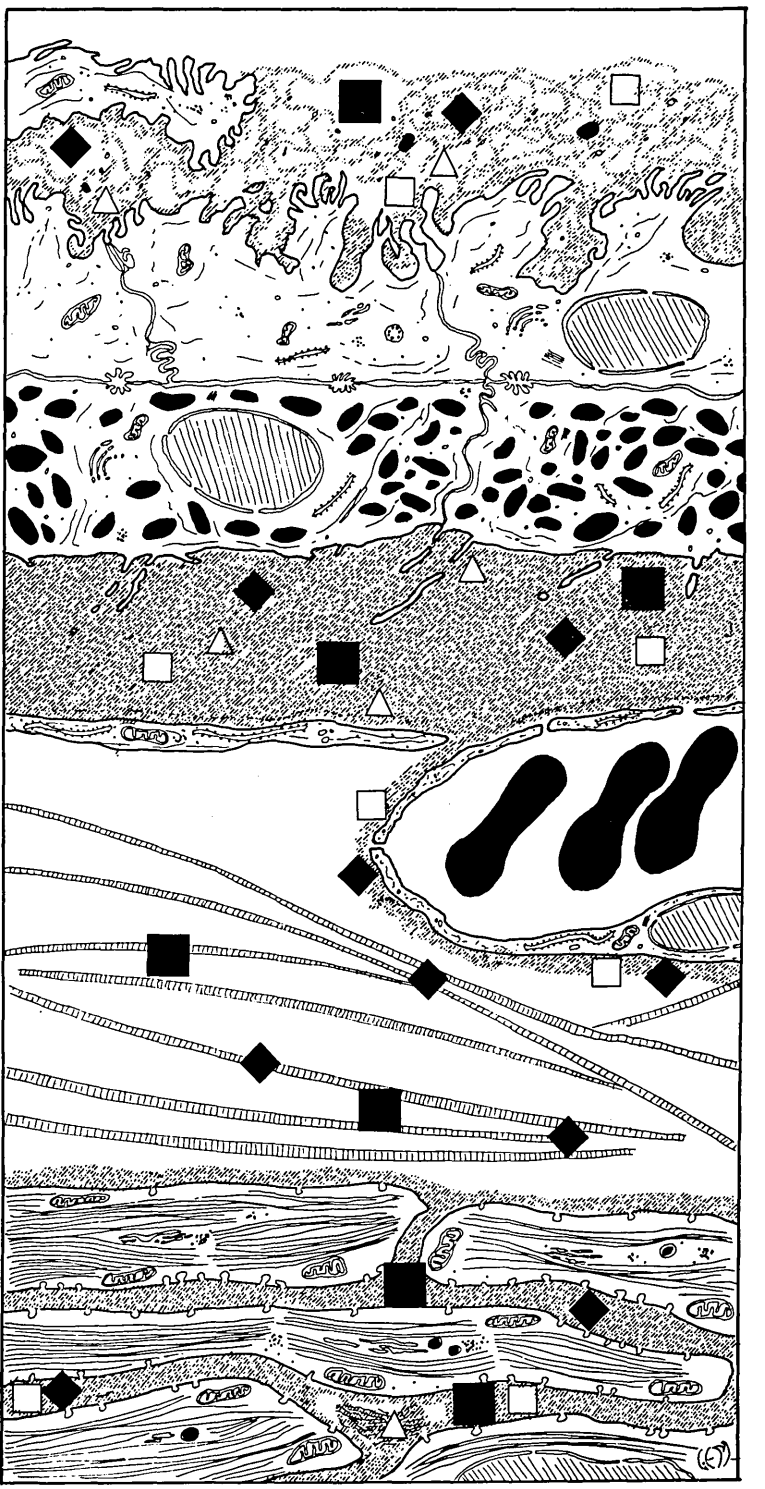
The absence of type V collagen from the iris was not completely convincing for although labelling was of a very low intensity, some gold particles were observed on striatedfibrils and on basement membranes. Other workers have had to resort to various permeabilisation techniques in pre-embedding immuno-labelling to demonstrate this fibrous collagen.[23,146] This was not necessary with using the techniques used in this study since in postembedding immunolabelling the fibrils are opened by ultrathin sectioning, thereby exposing the type V collagen epitopes in the cornea. Nevertheless, future technical refinements may be required to produce a definitive answer to the question of presence or absence of this fibrous collagen in the iris.

4.4.6 Relevance to Pathological Disorders

It is clear that abnormalities in basement membrane biosynthesis and metabolism are major characteristics of several pathological disorders.[200] Errors in the biosynthesis or metabolism of basement membrane components can lead to an overproduction of matrix or a reduction of turnover rate. In some diseases, such as diabetes,[2] the end result is thickening of basement membranes, whilst in others, e.g. exfoliation glaucoma, the basement membrane becomes thinner and may lose its structural integrity.

The study of the distribution of various collagens in ocular structures should enhance our understanding of the role that collagens play in normal or accelerated aging and in the pathogenesis of those ocular diseases which are

associated with aberrant collagen production.


Basement Membrane
Nonpigmented Ciliary Epithelium
Pigmented Ciliary Epithelium
Basement Membrane
Stroma
Ciliary Muscle

4.5 CILIARY BODY

This is the first immunoelectron microscopic study conducted on the distribution of collagens I-VI and laminin in the aged human ciliary body. To the best of my knowledge no data is available as to the distribution of collagens in the human ciliary body. A summary of the distribution of collagen types I-IV and laminin in the ciliary body as seen in the present study is shown diagrammatically in Fig 100.

4.5.1 Ciliary Epithelium

Collagen types I, III and IV in the basement membranes of the nonpigmented and pigmented epithelium differed only in quantity: pigmented epithelial basement membrane exhibited more intense labelling. Although the thickness of both basement membrane layers was comparable, the actual quantity of basement membrane present in the nonpigmented epithelium was considerably less than that of the pigmented epithelium as the former layer was highly reticulated with large empty spaces between strips of lamina densa material.

Fig 100: Diagrammatic summary of the distribution of collagens I-IV and laminin in aged human ciliary body as seen in the present study. Collagens I [◆] and III [■] are present in the basement membranes of the pigmented and nonpigmented ciliary epithelium and ciliary muscle and over striated collagen fibrils in the ciliary body stroma. Type I collagen is also present in vascular basement membranes. Type IV collagen [□] was localised to all the basement membranes whereas laminin [Δ], although localised to most basement membranes was absent from those of vascular cells.

1- Type III Collagen

Collagen type III imparts resilience and plasticity to the extracellular matrix in which it is present.[23,170] The presence of this collagen in the epithelial basement membranes was of considerable surprise. Type III collagen has been classified as a fibrous collagen[134] and has not been considered to be a component of basement membranes. Studies on collagen distribution in other aged ocular tissues have not localized this collagen type in basement membranes of the iris, retina, cornea and trabecular meshwork.

However, in the present study type III collagen was demonstrated in the normal aged lens capsule (section 3.6), a basement membrane that is subject to an exceptional degree of mechanical stress and distortion. Similarly, mechanical stress has been suggested as the underlying cause for the morphological features (i.e. multilamination) observed in the aged human nonpigmented epithelial basement membrane.[67] These biomechanical forces are exerted by zonular fibers during contraction of the ciliary muscle. This speculation has arisen from the observation that no such aging changes are observed in the rodent eye which has little accomodation.[67] It is therefore hypothesized that exposure to biomechanical forces accounts for the presence of type III collagen in the ciliary body epithelial basement membranes in which resilience and plasticity are required. These observations require further confirmation. However, it is noteworthy that type III collagen was absent from vascular basement membranes.

2- Type I Collagen

The detection of type I collagen in the basement membranes of the ciliary epithelium was more predictable. This collagen type has been localized to basement membranes of retinal vessels.[56,235,274] In addition, type I collagen was localized to the basement membranes of iris vessels, the anterior iris pigment epithelium, the trabecular beams and the lens capsule. It should be stressed that types I and III collagen in ciliary epithelial basement membranes were not localized to striated collagen fibrils. In the stroma, the striated collagen fibrils exhibited strong labelling for types I and III, a labelling pattern which is characteristic of extensible tissues.[263]

3- Laminin

Another finding of interest was the difference in the distribution of laminin between the two basement membranes of the pigment epithelium. Laminin was present throughout the thickness of the pigmented epithelial basement membrane but was preferentially localized to the proximity of the nonpigmented epithelial cells and to diffuse granular-like deposits scattered within the basement membrane. This was in contrast to collagens I, III and IV which were uniformly distributed throughout the thickness of the basement membrane. The distribution of laminin may be due to a leaching process in which laminin is washed out of the basement membrane with the bulk flow of aqueous humor through the nonpigmented epithelium. It is reasonable to assume that basement membrane material closest to the nonpigmented epithelium is the most recently synthesized and therefore of relatively normal composition. This would account for the preferential localization of laminin

described above. The uniform distribution of collagens implies that they are not suceptible to this leaching process. Laminin is more likely to be washed out than the collagenous components of the basement membrane for collagens become less soluble, more stable and resistant to proteolytic degradation with age.[7] No similar stabilizing process has been described for laminin.

4.5.2 Zonular Apparatus

Zonular insertions were intensely labelled with laminin antibodies. Zonules are described as consisting of 10-12nm diameter fibrils with a 12-14nm microperiodicity and an electron-dense tubular profile when cut in transverse section.

Where the fibrils aggregate, a macroperiodicity of 40-45nm has been noted. [202 238] Speculation as to their nature has included the possibility of either mature collagen[228] or a form of immature or embryonic type of collagen.[228] However, zonules are not affected by collagenase, but they can be completely digested by alpha-chymotrypsin and elastase.[202] It has therefore been suggested that zonular fibrils are connected with the elastic system as they are morphologically, biochemically and immunologically similar to the microfibrils of the elastic system.[236] From these considerations it is clear that the precise biochemical composition of the zonular apparatus awaits further elucidation.

The localization of laminin to zonular insertions confirms studies on aged human lens capsule in which strong laminin labelling of both zonular insertions, and linear densities was noted. The adhesive properties of laminin

have been well documented, and may be responsible for the close aggregation of adjacent zonular fibrils.[53,96]

4.5.3 Ciliary Muscle

Collagen types I, III, and IV were uniformly distributed in the basement membrane of the myocytes of the ciliary muscle. By contrast, laminin was restricted to fibrillar aggregations, and plaque-like material, particularly at the tendinous insertions. The most notable aging change associated with the ciliary muscle, as seen in this study, was the formation of plaque-like structures in the ciliary muscle tips in the region of the scleral spur. Three types of plaque-like structures were observed: filamentous, granular, and amorphous. Both filamentous and granular plaque-like structures labelled intensely with laminin antibodies. The amorphous plaque-like structures did not contain any of the collagens studied; laminin was also absent. In addition, labelling for the collagens was absent from the filamentous and granular plaque-like structures.

Speculation on the nature of the dense granular and fibrillar material present around the tendinous insertions of the longitudinal muscle (Brücke's muscle) at the scleral spur has led to the suggestion that they may represent a mixture of basement membrane and elastotic degenerative products.[237] This suggestion is supported by the demonstration in the present study of laminin within the plaque-like structures. It is interesting that type IV collagen, a major component of basement membranes, was absent from these plaque-like structures.

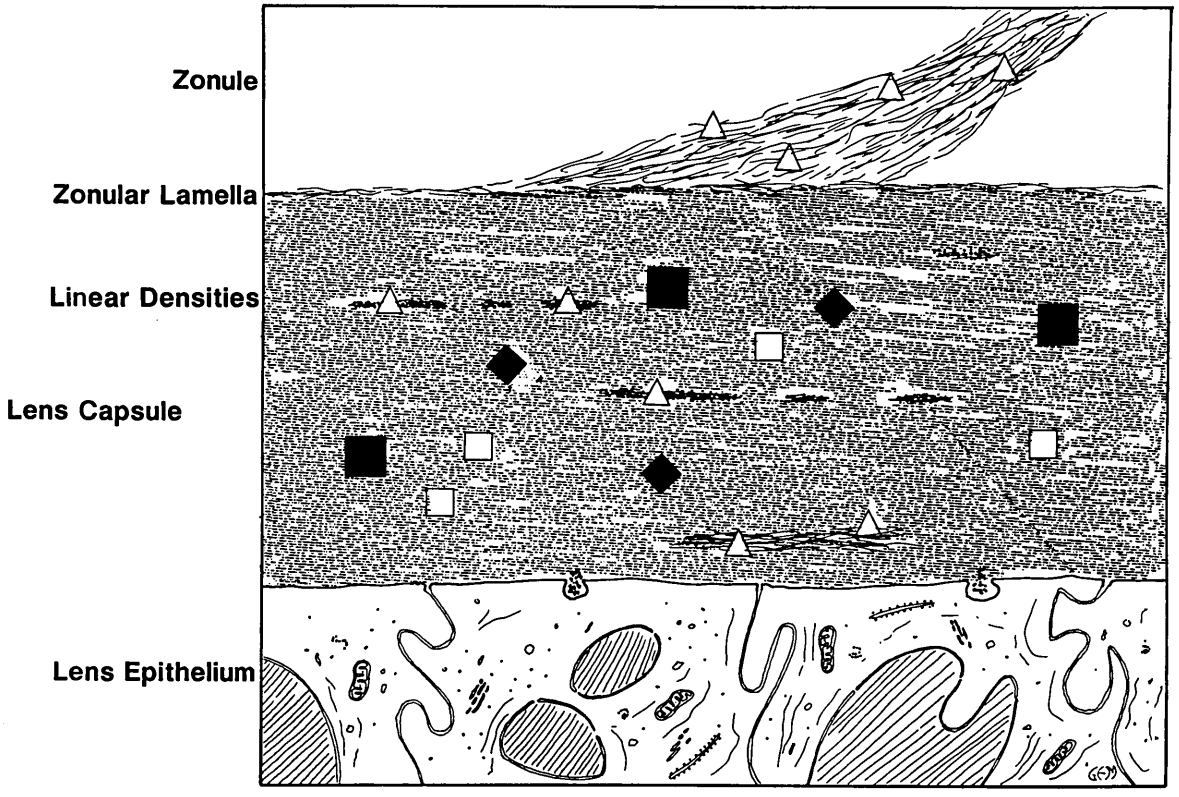
Three types of plaque-like structures have been

described: (1) the sheaths of elastic-like fibers running from the trabecular meshwork, (2) plaques derived from the anterior elastic-like tendons of the ciliary muscle, and (3) plaques which have the characteristic of hyalinised basement membrane of trabecular beams.[153] These plaques, which increase with age, have been collectively termed by Lutjen-Drecoll et al as ciliary muscle plaques.[153] Their origin is unknown. Certain substances could penetrate the interstitial spaces and become incorporated into the sheaths of the elastic-like fibres or basement membrane, or a common factor could stimulate both the trabecular endothelial cells, and fibroblasts of the ciliary muscle tips to produce specific substances which then are deposited into the sheaths of elastic-like fibers or basement membrane.[153]

4.5.4 Ciliary Vasculature

Although type I collagen is classed as a fibrous collagen,[134] and therefore not expected to be present in basement membranes, several studies have documented its presence in vascular basement membranes.[56,109,119] Type I collagen was also localized in the present series of studies to the vascular basement membranes of retinal and iris vessels. The presence of type IV collagen in the basement membranes of ciliary body vasculature was of no surprise as this collagen type is regarded as a classic basement membrane collagen.[37] Although the presence of laminin was clearly demonstrated in the basement membranes of the nonpigmented and pigmented epithelium and over zonules, labelling of basement membranes associated with

the vascular cells throughout the ciliary body was very weak. The label was not considered to be of sufficient intensity to clearly demonstrate the presence of laminin in this location. The present findings therefore differ from an immunofluorescent study of young and aged human ciliary body.[126] Staining for laminin was observed in the basement membranes of the nonpigmented and pigmented epithelium but not over zonules.[126] However, Kohno et al (1987) concede that, as the immunofluorescent study was performed on unfixed post-mortem tissue there was poor preservation of morphology. Nevertheless, this interesting discrepancy requires further investigation.


Zonule
Zonular Lamella
Linear Densities
Lens Capsule
Lens Epithelium

101

4.6 LENS CAPSULE

4.6.1 Type IV Collagen

The presence of type IV collagen in human lens capsule was confirmed using the gold-labelled antibody technique combined with transmission electron microscopy. Biochemical analysis, enzymatic digestion and rotary shadowing of type IV collagen isolated from tumor basement membrane have indicated that it forms a very open structure with strands only a single molecule in thickness.[248] However, X-ray diffraction studies of bovine lens capsule by Barnard et al (1987) revealed a pattern incompatible with this "chicken wire" network, but indicated the presence of microfibrils with a 10nm periodicity. They therefore suggested that some other component of the lens capsule may exist in a fibrillar form with an axial repeat of 10nm, or that other components could be regularly associated with the collagen, giving a repeat distance of 10nm. The present work indicates that two of the other components whose presence was suggested by Barnard et al (1987) are types I and III collagen.

4.6.2 Types I and III Collagen

The presence of types I and III collagen in the aged human lens capsule was noted with considerable surprise. The possibility cannot be excluded that this phenomenon is intrinsic to aging and that these collagens may not be present in the human capsule in the early decades. The

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Fig 101: Diagrammatic summary of the distribution of collagens I-IV and laminin in aged human lens capsule as seen in the present study. Collagens I [◆], III [■] and IV [□] are evenly distributed throughout the lens capsule thickness. Laminin [Δ] is restricted to linear densities, and zonules.

occurrence of type III collagen in ocular basement membranes is peculiarly unique to the lens capsule. Type III collagen has not been previously reported to be present within ocular basement membranes. In the present study, labelling for types I, III and IV collagen was evenly distributed throughout the thickness of the lens capsule. It is therefore suggested that these collagens are co-distributed within the capsule and that their association with each other may give rise to the 10nm banded microfibrils observed in the X-ray diffraction studies of Barnard et al (1987). It is relevant to note in this connection that incubation of the human lens with 6% potassium chloride solution changes the lens capsule from a transparent, amorphous membrane into a fibrillar substance resembling collagen in ultrastructure.[87]

Collagen type I fibrils are present where tensile strength is necessary and thus possess a very important structural function [sic].[170] Collagen type III is always present in tissues and organs that require a motile structural scaffolding, such as arteries, muscular layers of the intestine, uterus, etc, and its function is thought to be structural maintenance in expansible organs [sic].[170] It is reasonable to assume that these two collagen types play a similar role in the lens capsule and we suggest that type III collagen imparts elasticity, an essential property of the lens capsule.

The juvenile lens capsule has been described by Seland (1974) as consisting of numerous parallel 70nm wide laminae whose width decreases towards the capsule surface. The age-related loss of laminations appears to be due to a disappearance of the electron-lucent zones between the

laminae leaving a more homogenous capsular structure comprised of 5-7nm diameter filaments embedded in an amorphous ground substance. The arrangement of the filaments is controversal. These filaments have been described as being randomly orientated by Seland (1974) and distributed in parallel alignment by Cammarata et al (1986). The present study confirms the absence of laminations in the aged capsule. In addition to this, clearly discernable filaments were only seen in the more superficial regions of the capsule, the deeper regions consisting of an almost completely homogenous matrix interrupted by linear densities (formed elements).

4.6.4 Linear Densities

Of great interest was the labelling pattern for laminin which was exclusively localized to linear densities, the zonular lamella (pericapsular membrane) and zonular insertions. Seland (1974) described the location of linear densities as initially in the anterior equatorial and anterior peripheral regions, sometimes indenting or even being partially enclosed by epithelial cells, an observation also made in our studies. Granular-like linear densities, the most common form, consist of randomly placed 7-10nm dots arranged in clumps up to 3.5um long and whose long axis lie parallel to the capsular surface.[231] Filamentous linear densities have been described as consisting of multiple parallel strands about 12nm thick whose long axis lie parallel to the epithelial surface. They sometimes exhibit cross-striations with a band periodicity between 50 and 60nm[231] and are more commonly located in the inner third of the capsule.[232] This is in

agreement with our observations, the diameter of the strands being estimated by using the 10nm diameter gold particles as a scale marker.

4.6.5 Relation of Linear Densities to Zonules

Dark (1961) demonstrated a close similarity in histochemical staining reactions between linear densities and zonular fibres. Both showed slight affinity for toluidine blue and other basic dyes; both exhibited increased affinity for certain basic dyes afer preliminary oxidation with periodic acid or potassium permanganate; both were coloured by the aniline blue component of Mallory's connective tissue stain and both gave a positive reaction for polysaccharide with the periodic acid-Schiff technique. He was, however, unable to detect any connections between the two on examining human lens capsules by light microscopy. A subsequent electron microscopic study showed that certain zonular fibrils penetrate deeply into the lens capsule, far enough to come in contact with the surface of the epithelial cells [**sic**].[197] This coupled with the morphological similarity of filamentous linear densities to zonular fibres has led to the suggestion that they are part of the zonular apparatus.[197] An alternative suggestion, arising from their proximity with the zona germinativa, is that linear densities are excretion products from the metabolically most active epithelial cells.[231] Having demonstrated the presence of the glycoprotein, laminin, in both zonules and linear densities, we would support the former suggestion, although the paucity of linear densities in the young capsule[231] could be used as a counter arguement. The supposed

relationship to atypical collagen (based on the morphology of their cross-striations[40]) is not substantiated by observations arising from the present investigation: no labelling of linear densities was observed with collagens I-IV or with collagens V-VI.

4.6.6 Relation of Linear Densities to Exfoliation

The clinical and pathological relevance of this study is related to the exfoliation syndrome. The suggestion that linear densities are associated with exfoliation material has arisen from morphological similarities[610] and histochemical staining properties.[46]

We have recently demonstrated that laminin, a glycoprotein, is an integral component of exfoliation material[129,130] and the present study has shown that laminin is a component of linear densities. Streeten and colleagues[142,239] have demonstrated the presence of an elastic microfibrillar protein in exfoliation material. The possibility of coexistance of elastin and laminin is entirely acceptable and an intriguing topic for further investigation.

4.6.7 Conclusion

Perhaps the most intriguing feature of this investigation is that lens capsule increases in thickness with age and this accretion process is due to completely uniform assembly of three types of collagen (I, III and IV). This regulated process contrasts markedly with the atypical products of metaplastic lens epithelium as seen in anterior subcapsular cataract. This is a pathological phenomenon which would lend itself to further

immunocytochemical studies.

4.7 RETINAL VESSELS

A detailed search of the literature revealed the absence of studies concerning the fine structural localisation of collagen types in human retinal vessels. Moreover, in animal studies such investigations of the retinal vasculature have been limited as to the collagen types examined.

Collagens I, IV and V have been localised by immunofluorescence in vessels of human,[109,184] bovine[125] and rat [76] retinas. Jerdan and Glaser (1986) found that a broad spectrum of retinal vessels stained for types I and IV, with types III and V primarily staining the walls of larger vessels.

4.7.1 Intramural Neovascularization

Although immunofluorescence can be used to document the presence or absence of collagen types, the precise distribution within the retinal vessel wall is elusive. Information on the ultrastructural distribution of collagen types, made available only through immunocytochemical electron microscopical studies, may be valuable in understanding such phenomena as matrix involvement in intramural neovascularisation. Manschot and Lee[155,156] have emphasised the frequency of neovascularisation within and around the walls of hyalinised blood vessels and there is considerable evidence that changes in extracellular matrix components, including collagens, can have profound effects on endothelial cell behaviour. For example, type IV collagen was shown by Roberts and Forrester (1990) to inhibit retinal vessel endothelial cell migration and proliferation <u>in vitro,</u> whereas type I collagen stimulated

cell migration *in vitro*.

4.7.2 Type IV Collagen in Retinal Capillaries

The advantage of fine structural localisation of the collagen types is underlined in the case of type IV collagen distribution in retinal capillaries. In the present study type IV appeared to be more abundant on the outer basal lamina of the intramural pericytes than the basal lamina of the endothelial cells, an observation which could not have been made from the poor resolution immunofluorescent technology. This subtlety in type IV distribution was also noted by Essner and Lin (1988) in rats but had been attributed to failure of antibody penetration. The results of the present investigation confirm that this is in fact a difference in antigen concentration, since the techniques employed eliminate the problem of antibody penetration. This difference in antigen concentration may be extremely significant, since if pericytes produce more type IV collagen than endothelial cells, as this circumstantial evidence would indicate, then such a difference might be of significance in the proliferative stage of diabetic retinopathy. Pericyte loss is an early abnormality in diabetes and this could presumably lead to a decrease in the amount of type IV collagen, thus removing the inhibiting influence that this collagen has on endothelial cell migration and proliferation.

4.7.3 Advantages of the Immunogold Technique

In immunocytochemistry the use of immunogold is preferred to immunoperoxidase, since with the latter

technique oxidised diamino benzidine (DAB) can diffuse from one site to another. This drawback can be illustrated by the report of Abrahamson[2] who found that the localisation of laminin obtained with a post-embedding technique differed from that seen with the immunoperoxidase technique in glomerular basement membranes in which the label had diffused from the site of antigen location. Immunogold also lends itself to quantitation by image analysis, which has already been performed by other workers.[77] This may be important in the study of vascular disease, since the relative quantities of various collagen types are probably more significant than documentation of the actual types occurring in the area concerned.

In an immunomicroscope study of extracellular matrix of the monkey optic nerve head using LR white embedding combined with the immunogold technique, Morrison et al[171] noted the presence of types I, III, IV and V within the walls of the central retinal artery with only the latter two labelling the basement membranes. Using antibodies supplied from the same source we noted that in addition to types IV and V, type I was also found to be present in the basement membranes of retinal vessels.

4.7.4 Type I Collagen in Basement Membranes

It is already known that basement membranes typically contain types IV and V collagen, with several workers demonstrating the presence of type IV in retinal microvessels.[109,119] The association of type I with basement membranes as seen in our studies was surprising, as this fibrous collagen is not usually found in capillary basement membranes. Nevertheless, microvessel preparations

from rabbit cerebrum as well as bovine retinal microvessels have been shown to contain type I collagen.[56,119] On finding this collagen type in retinal vessels, including capillaries, Jerdan and Glaser (1986) suggested that it is the presence of type I collagen that makes the human retinal vasculature so uniquely resistant to trypsin digestion. The extensive alpha-helical structure of type I collagen results in an increased stability to digestion by this protease.[164]

In the present study the fine structural distributions of collagens types I-VI have been documented in normal and aging retinal vessels. The technique of immunogold localisation appears to be of great value in future study of the changes which occur in the extracellular matrix components in ocular vascular disease.

Final Comments and Future Studies

The fine structural localisation of collagen types I-VI and laminin has been documented in normal ocular tissues (cornea, trabecular meshwork, iris, ciliary body, lens capsule and retinal vessels) as this is essential for the subsequent study of pathological changes of extracellular matrix components. These findings have been related to the body of biochemical information concerning the constituents of the normal human eye. Progression has been made from studying normal aged tissue to studying the diseased state in the form of exfoliative vasculopathy in which clear secondary ECM changes were demonstrated.[129,130] The immunogold technique is eminently suitable in the study of ECM components involved in a large number of pathological disorders of the eye with associated ECM changes.

The use of the immunogold technique should permit the identification the matrix consitiuents in the vessel walls and retina which are essential to the process of neovascularisation in degenerative (aging and central retinal vein occlusion CRVO) and diabetic vasculopathy. One could document the matirx constituents which are located in Bruch's membrane and beneath the retinal pigment as part of the aging process, senile macular degeneration and disciform degeneration amd thus identify the cells and the metabolic products intrinsic to the evolution of subretinal neovascularisation. This would provide information which will establish more accurate in vitro models for therapeutic modifications of the aberrant cellular reactions.

Extracellular matrix components can have profound effects on cell behaviour. The immediate intention is to determine the role played by the extracellular matrix in

retinal neovascularization (diabetes) and age-related macular degeneration by establishing changes in extracellular matrix composition related to macrophage infiltration and endothelial cell behaviour. Changes will be detected by studying individual matrix components (collagens I-VI, laminin and sulphated proteoglycans) with the immunogold technique and related to individual cells by high resolution localization of these components (electron microscopy). Such cell-matrix associated changes will be related to in vitro studies of cell-matrix interactions which should help in understanding the mechanism of neovascularization in the above diseases.

Retinal Vasculopathy

Studies of non-diabetic and diabetic degenerative disease in human retinal blood vessels have revealed extensive deposition of extracellular matrix within the vessel wall (hyalinisation) which leads to luminal narrowing and reduced blood flow to the tissues.[35,65,139,155-158] As neovascularization frequently commences within hyalinized walls the speculation arose as to the role played by extracellular matrix accumulations in this process.[110,139,155-158]

The mechanism of intramural neovascularization is complex as not only have in vitro studies demonstrated that the extracellular matrix of blood vessel walls influences endothelial cell proliferation,[52,62,120,121,187,207] but also that endothelial cells and transformed myocytes are capable of altering the composition of the extracellular matrix.[19,120] In addition, degeneration and fragmentation

of myocytes leads to the migration of macrophages which are capable of releasing factors which stimulate endothelial proliferation and dermatan sulphate production in myocytes.[86,139,158] Myocytes, when present, inhibit proliferation of other cells, so that atrophy or absence of these cells in hyalinisation might contribute to endothelial cell invasion.[89]

Macular Degeneration

While there is a considerable volume of descriptive pathological documentation of the variants of senile macular degeneration at the light microscopic level (see 266 for review) information is required concerning the precise biochemical nature of the materials which are deposited within Bruch's membrane[32,48,122,192] and beneath the retinal pigment epithelium (e.g. soft drusen,[68] hard drusen[78,185,186] and basal linear deposits[150]). As in vasculopathies, the behaviour of cells involved is modulated by the underlying matrix.[51] The most important problem is the role which the matrix components of these extracellular deposits play in the attraction of macrophages,[123,150] endothelial cells and cohort cells into the space between the pigment epithelium and Bruch's membrane.[123]

Appendix 1: Details of patients, fixatives, and tissues used in study.

EM No	Sex	Age	Diagnosis	Fixative (% glut + 4% PFA)	Buffer	Corr LRW	rabecular LRW	Cryo	Iris LRW	Cryo	Ciliary Body	Lens Capsule	Retina LRW	Cryo
86/88	m	68	CM	0.2-1%	Ph	+	+		+		+		+	+
87/88	f	64	CM	0.2-0.8%	Ph	+	+							+
92/88	m	36	CM	0.2-1%	Ph								+	
100/88	m	52	CM	0.1%	Ph	+	+		+					
101/88	m	28	CM	0.1%	Ph		+	+	+		+		+	+
145/88	m	66	CM	0.2%	Ph									
151/88	m	68	CBM	0.2%	Cc		+							+
6/89	m	64	CM	0.5%	Cc	+	+		+	+	+		+	+
24/89	f	81	Sec. glauc.	1%	Cc	+			+				+	+
34/89	m	43		1%	Cc									+
35/89	m	46	Alkali burn	1%	Cc									+
67/89	f	45		1%	Cc									
79/89	m	68	Corneal degen.	1%	Cc									
97/89	m	63	CBM	1%	Cc									
107/89	f	64	Metast. carc.	1%	Cc		+	+			+			
112/89	m	76	CM	1%	Cc						+			
120/89	f	60	CM	1%	Cc									
145/89	m	months	RB	1%	Cc									
157/89	f	63	CM	0%,0.5%	Cc	+	+		+		+	+	+	
160/89	m	78	CM	1%	Cc	+					+			
161/89	f	months	RB	1%	Cc									
167/89	m	76	CM	1%	Cc						+			
224/89	m	13		formalin					+					
233/89	m	31	CBM	0.2%	Cc									
260/89	m	16	Sturge Weber	0.5%	Cc									
282/89	m	28	suicide	formalin										
317/89	f	74	CM	0.2%	Ph		+		+					
318/89	f	64		0.5%	Cc									
322/89	f	44		0.3%	Ph		+							
18/90	f	75	CBM	0.2%	Ph		+		+		+	+		
21/90	m	41		0.25%	Ph									
24/90	m	73	CM	0.25%	Ph	+					+			
39/90	m	64		0.4%	Ph									+
42/90	m	55	autopsy	0.4%	Ph									
44/90	f	50		0.4%	Ph									
52/90	m	82	bullous kerat.	0.4%	Cc									+
54/90	m	66	autopsy	0%	Cc									+
55/90	m	66	autopsy	0.25%	Cc									+
173/90	f	41	autopsy	0.25%	Cc									+
193/90	f	69	autopsy	formalin										+

Key: **m**:male; **f**:female; **CM**:choroidal melanoma; **CBM**:ciliar melanoma; **Ph**:phosphate buffer; **Cc**:cacodylate buffer; **LRW** white embedded tissue; **Cryo**:ultrathin frozen sections.

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Fig 33.	43273	Type III in Stroma.
Fig 34.	45224	Type VI in stroma.
Fig 35.	45753	High power keratocyte body.
Fig 36.	45231	Type V in keratocyte body.
Fig 37.	45221	Type VI in keratocyte body.
Fig 38.	45345	Type VI long-spacing collagen.
Fig 39.	43230	Type IV in Descemet's Membrane.
Fig 40.	45226	Type V in interfacial matrix.
Fig 41.	45341	Type VI in interfacial matrix.
Fig 42.	45716	NGS control
Fig 43.	42689	Type I corneo-scleral meshwork.
Fig 44.	46439	Type III corneo-scleral beam.
Fig 45.	42612	Type IV corneo-scleral beam.
Fig 46.	46943	Type V corneo-scleral beam.
Fig 47.	46585	Type VI corneo-scleral beam.
Fig 48.	43030	Type I cribriform layer.
Fig 49.	45933	Type III cribriform layer.
Fig 50.	43024	Type IV cribriform layer.
Fig 51.	44243	Laminin cribriform layer
Fig 52.	44236	Laminin Schlemm's inner wall
Fig 53.	43032	Type I outer wall of Schlemm's canal.
Fig 54.	43043	Type III outer wall of Schlemm's canal.
Fig 55.	44238	Laminin outer wall
Fig 56.	44534	Laminin aqueous vein.

Fig 57.	46600	Type VI limbal collagen.
Fig 58.	44697	Normal rabbit serum control
Fig 59.	44244	Laminin Giant vacuole
Fig 60.	42836	Low power iris stroma
Fig 61.	42234	Low power iris stroma
Fig 62.	42465	Type III iris stroma
Fig 63	42205	Type I myoepithelium
Fig 64.	42455	Type I posterior pigment epithelium
Fig 65.	42233	Type IV anterior pigment epithelium
Fig 66.	44816	Laminin pigment epithelium
Fig 67.	44240	Laminin fibrocyte
Fig 68.	42452	Type I, iris vessel
Fig 69.	42439	Type III adventitia of blood vessel
Fig 70.	42603	Type IV iris vessel
Fig 71.	44322	Laminin blood vessel
Fig 72.	45504	Ciliary epithelium
Fig 73.	45533	Type I BM NPE
Fig 74.	45543	Type III BM NPE
Fig 75.	45524	Type IV BM NPE
Fig 76.	45515	Laminin BM NPE
Fig 77.	45530	Type I BM PE
Fig 78.	45544	Type III BM PE
Fig 79.	45524	Type IV BM PE
Fig 80.	45512	Laminin BM PE
Fig 81.	47772	Type I stroma
Fig 82.	47746	Type III stroma
Fig 83.	45549	Type III ciliary muscle
Fig 84.	47725	Laminin ciliary muscle
Fig 85.	47042	Type I, lens capsule
Fig 86.	47073	Type III, lens capsule
Fig 87.	47200	Laminin, lens capsule
Fig 88.	47199	Laminin, lens capsule
Fig 89.	47203	Laminin, lens capsule
Fig 90.	47066	Laminin, lens capsule
Fig 91a.	LM	Normal artery and vein. (223/89B4)
Fig 91b.	LM	Hyalinized artery (107/89B2)
Fig 91c.	LM	Grossly hyalinized vessel (79/89)
Fig 92.	41393	Type IV, retinal vessel LRW
Fig 93.	42890	Type III, large vessel LRW
Fig 94.	41625	Type VI, retinal vessel cryo
Fig 95.	43288	Type I capillary
Fig 96.	42867	Type IV capillary
Fig 97.	Diagram	Summary cornea
Fig 98.	Diagram	Summary meshwork
Fig 99.	Diagram	Summary iris
Fig 100.	Diagram	Summary ciliary body
Fig 101.	Diagram	Summary lens capsule