An ultrastructural study of the complex carbohydrates of the mouse posterior vitreoretinal juncture

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The content and distribution of complex carbohydrates of the posterior vitreoretinal juncture of the mouse eye were examined by electron microscopy. Eyes were fixed 24 hr or 192 hr in glutaraldehyde or glutaraldehyde and cetylpyridinium chloride (CPC) and then block-stained with cationic dyes. Globular material of intermediate electron density was found in the basement membrane of the retina and on collagen fibrils in the vitreous cortex with CPC fixation and disappeared after Streptomyces hyaluronidase digestion. More of this material was found at the juncture of the basement membrane and the vitreous body with alcian blue than with the other cationic dyes after the shorter fixation period. After prolonged fixation, all of the cationic dyes revealed a thick layer of globular material on the basement membrane. A finely filamentous network associated with the globular material was revealed by glutaraldehyde fixation and alcian blue staining. Some laminated bodies were found at the vitreoretinal juncture after block-staining. Neither the finely filamentous material nor the laminated material was sensitive to the hyaluronidase. It is suggested that the globular material is hyaluronic acid, which is more labile along the basement membrane than toward the inner vitreous cortex. The finely filamentous network may be formed of oligosaccharide chains associated with vitreous proteins. The laminated bodies may be formed of lipid and complex carbohydrates of an otherwise uncharacterized mixture. The various complex carbohydrates form parts of a vitreoretinal-juncture layer that may participate in the known chemical, cellular, and mechanical barrier functions of this region. (INVEST OPHTHALMOL VIS SCI 22:460-477, 1982.)

Key words: vitreous body, internal limiting membrane, basement membrane, complex carbohydrates, hyaluronic acid, cationic dye, ultrastructure

The retina and vitreous body are adherent to a different extent at various points of attachment. The strongest attachments are at the vitreous base and the optic disc. The attachment in the posterior fundus is tenuous and posterior detachments of the vitreous body are a problem of advancing age and of medical and surgical conditions. The vitreous body is bounded anteriorly by a delicate limiting network or membrane, but no definitive posterior "hyaloid membrane" seems to exist along the inner surface of the retina. Rather, the basement membrane of the inner retina and the outer vitreous cortex form extracellular structures, which are the internal limiting membrane (ILM) of the light microscopist. The retinal basement membrane and the extracellular matrix of the outer vitreous cor-

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Fig. 1. Region of posterior vitreous body and retina. The tissue block was fixed with glutaraldehyde for 24 hr and postfixed with OsO₄. The thin section was not stained. The basement membrane has a lamina rara externa (LRE), a lamina densa (LD), and a lamina rara interna (LRI). MC, Müller cell; VC, vitreous cortex. (×75,000.)

tex comprise a functional region for the physiologic flow of solutes⁶,⁷ and for the localization of inflammatory and healing processes.⁸,⁹ Lesions of the posterior vitreous body must involve its collagen fibrils and acid polysaccharides.¹⁰⁻¹² Knowledge of the normal and deranged structure of the posterior vitreous body therefore must include details of the structure of its complex carbohydrates. A study of the normal vitreoretinal juncture may aid in the experimental approach toward a better understanding of this region and ultimately in the clinical management of various vitreous and retinal problems.

The nature of the attachment region of the posterior retina to the vitreous body is investigated here with fixation and staining methods that allow complex carbohydrates to be localized with the electron microscope. Toluidine blue O (TBO) has been compared to ruthenium red (RR) and alcian blue (AB) in the localization of proteoglycans, and all of these cationic dyes seem to give a similar localization of complex carbohydrates in tissue, but reveal morphologic variations depending on the particular agent and on the use of counterions in the staining method.¹³ Staining with these dyes has been enhanced by the use of cetylpyridinium chloride (CPC) in the fixative.¹⁴ CPC is a precipitant of glycosaminoglycans (GAG) that can be exchanged for cationic dyes in histochemical studies,¹⁴,¹⁵ so CPC was used here in the primary fixation step to allow complex carbohydrates to be retained in the tissue and stained by one of several cationic dyes in a subsequent step. This strategy allows the complex carbohydrates to be localized at the same time that any finding with one dye that differs from the findings with the others can be evaluated.

Materials and methods

Fixation. Young adult male Charles River albino mice (25 to 31 gm) were sacrificed by an overdose of intraperitoneal pentobarbital. The eyes were enucleated and immersed in fixative.
about 15 min before the cornea was removed and the lens was gently freed. The eyes then were dissected meridionally into six or eight blocks. Fixation was for 24 hr or 192 hr with 4% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.4 or with 4% glutaraldehyde and 0.1% CPC in cacodylate buffer.

Staining en bloc. The blocks of ocular wall and attached vitreous body were rinsed in cacodylate buffer. Some blocks were dehydrated directly with 2,2-dimethoxypropane and embedded in an Epon-Araldite resin mixture. Other blocks were treated with Luft's mixture of RR (Pelco) and OsO₄ (RR-OsO₄) for 3 hr, dehydrated, and embedded. Most of the ocular tissue was block-stained with other cationic dyes in cacodylate buffer at pH 7.4 for 24 hr prior to postfixation in 2% OsO₄, dehydration, and embedding. These block-staining procedures included either 0.5% AB SCX (Matheson, Coleman and Bell), 0.1% TBO (Pelco) as modified from Shepard and Mitchell, 1% lanthanum nitrate (LN; Pelco) as modified from Shea, or the critical electrolyte concentration (CEC) method of Scott and Dorling as modified from its ultrastructural application by others.

Enzyme digestion. A few blocks were fixed with glutaraldehyde and CPC for 2 hr. These blocks were rinsed in cacodylate buffer and then in 0.1M sodium formate buffer at pH 7.0, incubated 1 hr
Figs. 5a and 5b. Glutaraldehyde and CPC fixation for 24 hr and postfixation with OsO₄.

Fig. 5a. The basement membrane shows its greatest osmiophilia along the inner side of the lamina densa (arrowhead). The thin section was not stained. (×75,000.)

at 37°C in 50 TRU/ml of Streptomyces hyaluronidase (Miles Laboratories, Inc., Elkhart, Ind.) in formate buffer or incubated in formate buffer alone, rinsed sequentially in formate and cacodylate buffers, block-stained with AB, postfixed with OsO₄, dehydrated, and embedded.

Staining hyaluronic acid in vitro. Purified hyaluronic acid (bovine vitreous humor, grade IV; Sigma Chemical Co., St. Louis, Mo.) was precipitated in a glutaraldehyde and CPC fixative (1 mg/ml) and fixed for 24 hr. Rinsed precipitate was further processed with all of the staining methods described above, dehydrated, osmicated when necessary, and embedded.

Staining thin sections. Semithin sections of the posterior pole were cut perpendicular to the ILM for orientation and stained when necessary with a TBO solution. Thin sections were collected on copper grids and viewed on a Philips 200 or a Zeiss 10C electron microscope. Most grids were studied further after staining with uranyl acetate and usually with lead citrate. Thin sections collected on nylon or gold grids from nonsmicated tissue blocks were stained with uranyl acetate or a tannic acid–uranyl acetate (TA-U) sequence to demonstrate complex carbohydrates.²³

Fig. 5b. Staining the thin section with uranyl acetate demonstrates densities more clearly in the vitreous cortex (arrows). Posts (arrowheads) are seen in the lamina rara interna. (×75,000.)

Results

Comparison of fixation and block-staining methods. Fixation with glutaraldehyde for 24 hr followed by postfixation in OsO₄ revealed the typical structure of a basement membrane underlying the Müller-cell plasma membranes. There was a 15 nm thick lamina rara interna, a 10 to 30 nm thick lamina densa, and a thin region of sparse densities 5 or 10 nm thick corresponding to the lamina rara externa (Fig. 1). Block-staining with AB between the fixation and postfixation steps showed many minute particulate and flocculent densities in both laminae rarae, some
very dense focal material that slightly distended the lamina rara interna (especially over junctions of adjacent Müller-cell processes), and electron-dense ovoid laminated bodies up to 800 nm in diameter extending inward from the lamina rara externa. Filamentous material in strands and loops and a finely filamentous network up to 800 nm in thickness were associated with the lamina rara externa and the outer vitreous cortex (Fig. 2). Glutaraldehyde fixation for 24 hr or 192 hr followed by treatment in RR-OsO₄ revealed a very dense, occasionally fibrillar basement membrane in which laminae rarae were difficult to recognize. Müller-cell plasma membranes and intercellular material or cellular coats were dense in the inner retina. Membranes of mitochondria and of agranular cisternae had minute punctate densities in Müller-cell inner processes after fixation for 192 hr. Adjacent ganglion cells had very little membranous stain (Fig. 3).

Tissue fixed with glutaraldehyde and CPC and prepared without osmication revealed dense staining of the basement membrane and some cytoplasmic membranes when the TA-U sequence was carried out on thin sections (Fig. 4). Staining with uranyl acetate alone provided only some electron density to collagen fibrils. Fixation with glutaraldehyde and CPC for 24 hr and postfixation with OsO₄ showed particulate or flocculent densities and 10 to 40 nm thick posts within the lamina rara interna. The posts connected the lamina densa to the Müller-cell plasma membranes.

The entire inner aspect of the lamina densa showed a thin density that obscured or replaced the lamina rara externa and was itself obscured after the thin sections were stained. Some dense material covered the collagen fibrils in the vitreous cortex, particularly in the inner cortex (Figs. 5a and 5b). Primary fixation with glutaraldehyde and CPC for 192 hr and subsequent postfixation yielded a basement membrane of thin dense anastomotic fibrillar structure and a homogeneous layer of globular material of intermediate electron density extending from the basement membrane into the vitreous cortex (Fig. 6).

A homogeneous layer of intermediate density also was found after 192 hr of fixation with glutaraldehyde and CPC when this was followed by treatment in RR-OsO₄, although the thick layer of intermediate density was lacking in tissue fixed only for 24 hr prior to RR-OsO₄ treatment (Fig. 7). A few small breaks observed in the basement membrane were bridged by the material of intermediate electron density (Fig. 7b).

Fixation with glutaraldehyde and CPC for 24 hr prior to block-staining in AB and postfixation revealed an incomplete layer of intermediate-density globules and a finely filamentous network on the basement membrane and similar material around vitreous collagen fibrils. The basement membrane was a series of inclined anastomotic posts or fibrils, and the lamina densa, where discernible, was up to 50 nm thick (Fig. 8). Fixation with glutaraldehyde and CPC for 192 hr prior to AB block-staining and postfixation revealed a complete layer of material of intermediate density and a thin, incomplete dense layer on the outer aspect of the lamina densa (Fig. 9).

Tissue fixed with glutaraldehyde and CPC and subjected to the CEC method showed different features as the salt concentration was increased during block-staining with AB.
Fig. 7. Glutaraldehyde and CPC fixation and RR-OsO₄ treatment; no thin-section staining. a, Fixation for 24 hr shows the same distribution of extracellular reaction product as that after fixation with glutaraldehyde alone (cf. Fig. 3). b, Fixation for 192 hr shows a layer of material of intermediate electron density (asterisk) on the basement membrane. Note that this layer is found after prolonged fixation with glutaraldehyde and CPC. A mechanical break in the basement membrane is covered by the layer of intermediate-density material (arrowhead). MC, Müller cell. (a, ×75,000; b, ×30,000.)

At the lower ionic strengths (0.05M and 0.1M MgCl₂), the lamina rara interna contained some minute densities and the lamina densa was composed of a thin layer of moderate electron density with a region of higher density on its inner edge. An irregular layer of flocculent material extended a short distance into the outermost vitreous cortex from the lamina rara externa, particularly at 0.05M. Rare small globules of intermediate electron density were found. At higher ionic strengths more globular material of intermediate density appeared, very little scattered along the lamina rara externa at 0.5M and a fairly complete layer within and internal to the basement membrane at 1.0M. Thin inclined posts in the lamina rara interna were seen only after block staining at 1.0M (Figs. 10a to 10c).

Tissue fixed with glutaraldehyde and CPC for 24 hr and block-stained with LN or TBO had little observable globular material of intermediate and higher densities. Moderately dense material in the lamina rara externa comprised a thin incomplete layer in some areas. The longer period of fixation allowed a complete globular layer of intermediate density to be seen (Figs. 11a and 11b). These results are summarized in Figs. 12 and 13.

Enzyme digestion. The network of globular and finely filamentous material on the basement membrane observed after AB block-staining was somewhat collapsed in formate-buffer control tissue. The filamentous net-
work remained intact after incubation in \textit{Streptomyces} hyaluronidase, but almost all of the globular material on the basement membrane and on the collagen fibrils disappeared. The ovoid laminated bodies did not appear to be affected by the digestion procedure (Fig. 14).

\textit{Stained hyaluronic acid in vitro.} Purified hyaluronic acid precipitated with fixative, block-stained, and postfixed or treated with RR-OsO$_4$ showed clumped material with little substructure. Some clumps were outlined with small globules (Fig. 15).

\textbf{Discussion}

\textit{Basement membrane.} Basement membranes have a very regular structure that probably changes with environmental shifts.$^{24}$ The ultrastructural appearance of a basement membrane depends on preparative techniques, with the method of fixation$^{25}$ as well as subsequent block-staining steps such as those used here being critical. Most basement membranes are about 50 nm thick and are thought to function as filters and as part of a cellular microskeleton.$^{25,26}$ The constituents of basement membranes seem to be relatively few and ubiquitous and include procollagen-like peptides, type IV collagen, oligosaccharide chains associated with collagen, noncollagen glycoproteins, and GAG.$^{26}$ Hyaluronic acid is well represented as a nonsulfated GAG in embryonic basement membrane,$^{27}$ and sulfated GAG is found in developing$^{27-29}$ and adult basement membranes.$^{30-32}$ The constituents of a particular basement membrane probably are dependent on genetic as well as environmental factors, although some major differences between them involve changes in complex carbohydrates.$^{33}$

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Glutaraldehyde and CPC fixation for 24 hr, block-staining with AB, and postfixation with OsO$_4$, thin-section staining with uranyl and lead salts. A finely filamentous network (FFN) covers globular material of intermediate density (asterisks) on the basement membrane (BM) and on collagen fibrils. The basement membrane appears to be distended by material of intermediate electron density. MC, Müller cell. (x75,000.)}
\end{figure}
Fig. 9. Glutaraldehyde and CPC fixation for 192 hr, block-staining with AB, and postfixation with OsO₄; thin-section staining with uranyl and lead salts. The layer of intermediate density (asterisk) on the basement membrane is complete after prolonged fixation. Intermediate-density material and inclined posts or fibrils form the basement membrane (between arrowheads). (×75,000.)

The basement membrane of the mouse inner retina has a lamina densa in its center bounded by internal and external laminae rarae. The lamina rara interna is anatomically external to the other laminae because of the inversion of the retina during the embryologic formation of the optic cup. The thickness of the basement membrane and the clear demarcation of the layers change with the various preparation sequences used. Glutaraldehyde and CPC might fix more material in situ than glutaraldehyde alone, thus allowing thicker layers to be found. The presence of more material in situ also should increase the number of dye-binding sites, although each dye might vary in its avidity for these sites and residual CPC can block these sites, resulting in some staining differences with each preparation sequence used. The sensitivity of the intermediate-density material in and adjacent to the basement membrane to Streptomyces hyaluronidase indicates that this material is hyaluronic acid. Except for the presence of hyaluronic acid in the laminae rarae in our specimens, the general findings and the approximate dimensions of the posts and intervening gaps resemble the findings in other basement membranes studied with cationic dyes. The inclined angles of the posts after some fixation and block-staining protocols and the apparent thinning of the lamina densa could be manifestations of the elasticity of basement-membrane fibrils, in this case affected by an inclusion of dye-hyaluronic acid complexes. Hyaluronic acid reported in embryonic basement membranes has not been shown in the form in which it is observed here, possibly because of differences in tissue preparation. There is some evidence that the inner retinal basement membrane differs in its chemical composition from other basement membranes, so that further comparisons must be made carefully with a quantitative or immunochemical approach.

The changing appearance of the posts and a thickening of the lamina rara interna with hyaluronic acid observed after block-staining are particularly evident in the series of variations of the CEC method. The CEC method demonstrates hyaluronic acid at 0.1M in light-microscopic studies. Staining at a
higher CEC generally is a function of a higher molecular weight of a particular GAG or may indicate a weak physicochemical binding of AB to a polycarboxylate such as hyaluronic acid. The chain lengths of vitreous hyaluronic acid vary as a group, so the findings with the CEC method that vary with counterion concentration may be partially dependent on the binding properties of AB with the hyaluronic acid. Dye binding may have occurred as the ionic strength increased because this removes CPC from anionic sites. Increased concentrations of salt also may decrease dye-dye repulsive forces that would tend to prevent adequate dye-binding to most of the available polyanionic sites on the hyaluronic acid fixed in situ.

There may be sulfated GAG in the basement membrane represented in part by dense staining between the lamina densa and the lamina rara externa. This dense layer corresponds to a layer of fixed negative charges demonstrated with AB in this part of the glomerular basement membrane and recently ascribed to a basement membrane-specific heparan-sulfate proteoglycan. The dense staining with the TA-U sequence serves as a separate indication of the presence of complex carbohydrates in the basement membrane.

Vitreoretinal juncture as extracellular matrix. The close connection of basement membranes to subepithelial connective tissue generally is formed by many thick collagen fibrils and a network of proteoglycans. This connection is represented at the mouse vitreoretinal juncture by thin sparse collagen fibrils but by none of the filaments and matrix granules of a proteoglycan network. Proteoglycans could play some role at the mouse vitreoretinal juncture, since there is a minor component of sulfated GAG in the mammalian vitreous and probably in the basement membrane of the retina. The complex carbohydrates in the vitreous do not hold the collagen fibrils in register or in any obvious and regular orientation, as occurs at other basement membrane-connective tissue junctions. Loose spacing and hydration promoted by hyaluronic acid may
Figs. 11a and 11b. Glutaraldehyde and CPC fixation, block-staining with LN and postfixation with OsO₄.

Fig. 11a. Less globular material is associated with the basement membrane than with the vitreous collagen fibrils after fixation for 24 hr. The thin section was not stained. (×75,000.)

be aided by the relatively wide spacing and great hydration of the type II collagen fibrils found in the vitreous.

Intermediate-density (hyaluronic acid) and finely filamentous material. The relatively thick and continuous layer of complex carbohydrates along the retinal basement membrane has not been reported previously. The layer is formed of hyaluronic acid of intermediate electron density and of a finely filamentous network. Foulds probably described components of this layer as amorphous colloidal iron-positive material on the basement membrane after removal of the vitreous. Ashton and Tripathi showed RR deposits within the basement membrane and on vitreous collagen fibrils but not in a layer connecting these structures. This layer is best demonstrated with AB after prolonged fixation with glutaraldehyde and CPC. The hyaluronic-acid component appears to be labile, since it is difficult to demonstrate without prolonged fixation, and this may be explained by the high degree of solubility of vitreous hyaluronic acid. Rinse steps may allow it to be solubilized, even after precipitation by CPC, if it has not been sufficiently crosslinked by glutaraldehyde, whereas it is demonstrated to some extent by OsO₄ alone after prolonged fixation. The partial demonstration of this labile layer of hyaluronic acid with AB after even short periods of fixation could indicate special fixation or binding properties of that dye compound. The lability (solubility) of hyaluronic acid in this layer may be due to a short chain length or a paucity of lateral aggregating associations. Hyaluronic acid may be able to become more stable with time, allowing its accumulation on collagen fibrils in a less soluble form toward the inner vitreous cortex. Schwarz has observed an accumulation of hyaluronic acid on collagen fibrils toward the center of the ox vitreous body. The origin of vitreous hyaluronic acid in the adult is unknown, but it could be derived from the retina. The topographic relationships of the Müller cells, the labile layer of hyaluronic acid on the base-
ment membrane, and the less soluble hyaluronic acid of the inner vitreous cortex suggest that a synthetic relationship between retina and vitreous should be explored.

A layer of hyaluronic acid that requires prolonged fixation may not be unique in this location. Trelstad et al., using AB and RR in the fixative, but with a very short fixation time, noted an unexpected absence of histochemically demonstrable hyaluronic acid where it is known to exist in association with developing basement membranes. Labile hy-
aluronic acid might be a special form required to fill the proposed roles of space-occupation and tissue hydration in developing connective tissue\textsuperscript{29} as well as in the vitreous cortex.

The finely filamentous network must be present in all of the blocks of tissue studied even when it is not visualized. It is found after glutaraldehyde fixation following block-staining with AB but not with the other cationic-dye methods. AB block-staining at low MgCl\textsubscript{2} concentrations shows some flocculent material on the basement membrane that may correspond to the finely filamentous network observed with AB alone. The weaker ionic solutions in the CEC method should permit oligosaccharide side-chains of glycoproteins to be complexed with AB, whereas binding to glycoproteins is suppressed at higher ionic concentration.\textsuperscript{19}

The stability of the finely filamentous network with glutaraldehyde fixation alone,\textsuperscript{56} the staining characteristics with AB, and the close association of the network with collagen fibrils suggest that it is a series of oligosaccharide chains extending from the collagen fibrils,\textsuperscript{12, 51} a network of noncollagen glycoproteins,\textsuperscript{50} or both. Carboxylate moieties may affect the form or function of proteins,\textsuperscript{57, 58} so the close association of this network with collagen fibrils in the vitreous cortex is likely to be of significance. It is not known if the thin filaments interconnecting collagen fibrils in the rabbit vitreous are part of a similar network, since their chemical composition has not been studied.\textsuperscript{50} However, the homogeneity of the structures considered here argues for their indigenous nature.

The thickness of the layer of complex carbohydrates of the vitreoretinal juncture probably is underestimated because of the great diminution in the size of the molecular do-

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**Fig. 12.** Schematic representation of ultrastructure of vitreoretinal-juncture layer following various fixation and staining protocols. Fixation in glutaraldehyde (G) or glutaraldehyde plus CPC (GC) is indicated in an area representing a Müller-cell inner process, along with the period of primary fixation. Also indicated is the block-staining protocol, or the use of grid-staining as noted. Postfixation with OsO\textsubscript{4} or treatment with RR-OsO\textsubscript{4} is indicated. a, Basement membrane has a lamina densa and some small densities in the laminae rarae. b, Fixation that includes CPC enhances the basic structural arrangement of the basement membrane, with the lamina rara externa and lamina densa appearing as several fibrillar horizontal arrays and the lamina rara interna as a series of vertical posts and flocculent densities. c, TA-U grid-staining sequence shows complex carbohydrates in the basement membrane and in intracytoplasmic cisternae. d, Prolonged fixation prevents any separation of the basement membrane into laminae. The intermediate-density material, or hyaluronic acid (see Discussion), appears after prolonged GC fixation. e to g, The shorter period of fixation, using G or GC, and subsequent treatment with RR-OsO\textsubscript{4} shows only solid or occasionally fibrillar basement membrane staining. Prolonged fixation with G, as shown in f, reveals a thicker basement membrane and punctate staining of intracytoplasmic cisternae of the same size and distribution as that seen in c. h, Prolonged fixation with GC and RR-OsO\textsubscript{4} treatment show the layer of hyaluronic acid, a thick basement membrane, and cisternal densities. i, Block-staining with LN or TBO reveals the fibrils or posts of the basement membrane after GC fixation and some hyaluronic acid on the basement membrane. j, Prolonged fixation with GC and block-staining with LN or TBO show a complete layer of hyaluronic acid. k, Block-staining with AB shows some distension of the lamina rara interna with complex carbohydrate, and a finely filamentous layer is found on the basement membrane. l, GC fixation and AB block-staining reveal an incomplete layer of hyaluronic acid and posts or anastomotic fibrils in the basement membrane. m to o, CEC method shows flocculent material at low ionic strength, while the layer of hyaluronic acid and the appearance of posts in the basement membrane are seen as the ionic strength increases. p, Block-staining with AB after prolonged fixation with GC shows a thick, complete layer of hyaluronic acid compared with that found with the shorter fixation time as depicted in i.
Fig. 13. Schematic representation of ultrastructure of vitreoretinal-juncture layer after fixation with glutaraldehyde and CPC, block-staining with AB, and postfixation with OsO₄. Components of the vitreous cortex also are shown. The finely filamentous network is shown within the labile layer of hyaluronic acid even though this layer obscures the network in electron micrographs. See text for further details.

main of hyaluronic acid after fixation with CPC⁶ as well as the tremendous shrinkage caused by routine electron-microscopic preparative techniques.⁴ The "solid" appearance of the hyaluronic acid precipitated in vitro is probably the result of similar effects. There is no appreciable collapse of the collagen fibrils or of the finely filamentous network on the basement membrane in vitreous cortex fixed with glutaraldehyde and CPC as opposed to glutaraldehyde alone, so it is unlikely that the layer forms by collapse of all of the components of the vitreous cortex upon exposure to CPC. It should be noted that after primary fixation, exposure to formate buffer at neutral pH does cause some collapse of the layer. Therefore the demonstration of complex carbohydrates requires care in the selection of buffer systems.

Ovoid laminated bodies. The ovoid laminated bodies on the lamina rara externa may be extracellular glycolipid or lipophilic glycoprotein, since they are demonstrated with cationic agents.⁶¹ However, lipoidal material generally is observed after osmication without the use of other cationic agents,⁶² suggesting that the ovoid laminated bodies contain a labile component that is precipitated and preserved along with a lipid component by bound cationic dyes. The vitreous body is
known to contain lipid with a variation among
species. The lipid content of the mouse vitreous body has not been investigated.

**Intracellular complex carbohydrates.** RR stains complex carbohydrates in or on plasma membranes and in organelar membranes in Müller cells and in lesser amounts in ganglion cells. The decrease in staining away from the ILM region may be a function of poor tissue penetration of the dye compounds. CPC is not needed to fix this material, but prolonged fixation with glutaraldehyde allows its demonstration with RR. Staining with the TA-U sequence also demonstrates this material and indicates that it is a form of complex carbohydrate.

**Vitreoretinal-juncture layer.** The vitreoretinal juncture includes the inner retinal basement membrane, a labile layer of hyaluronic acid, a finely filamentous oligosaccha-
Fig. 15. Purified hyaluronic acid fixed with glutaraldehyde and CPC and treated with RR-OsO₄. The thin section was not stained. Clumped hyaluronic acid is partially surrounded by globular forms. (×75,000.)

The appearing network, collagen fibrils, and ovoid laminated bodies. These elements constitute the vitreoretinal-juncture layer that corresponds to the posterior "hyaloid membrane" or ILM region. This also is part of the cortical barrier that acts as a negatively charged lipidal membrane and that helps to sequester the vitreous body from the general circulation.⁴¹ ⁶⁴ ⁶⁵

The appearance of the structures of the vitreoretinal-juncture layer suggests the presence of both a physical and a chemical barrier. The slowing of cellular migration through the vitreous cortex might be particularly affected by this layer.⁴¹ Relatively concentrated hyaluronic acid can immobilize cells,⁶⁶ probably by barring their migrating processes from contact and adhesion to a substrate.⁶⁷ It would be expected that a concentration of hyaluronic acid on the basement membrane would interfere with cellular migration across or along the vitreoretinal juncture and thereby influence local cellular inflammatory reactions. Components of the vitreoretinal-juncture layer also could function as a filter for immunoglobulins⁶⁴ or in the vitreous-retina-blood route of solutes.⁶

A mechanical break in the retinal basement membrane is thought to decrease flow resistance and allow the retina to be detached easily.¹¹ The layer of complex carbohydrates along the basement membrane may be the site of much of the retinal flow resistance, so that this layer should offer significant protection against a retinal detachment.¹¹ Surgical procedures can disrupt the retinal basement membrane,⁸ and although small mechanical breaks seem to be filled with the hyaluronic acid of the vitreoretinal-juncture layer in the mouse, procedures resulting in large breaks and removal of the vitreous cortex cause abnormal vitreoretinal adhesions⁹ and permanent detachments of the retina.⁶⁸

**Summary.** Ultrastructural staining with cationic dyes reveals hyaluronic acid forming a labile layer at the attachment site of the posterior retina to the vitreous body and in the inner retinal basement membrane itself. It is speculated that this labile hyaluronic acid may have some features in common with embryonic hyaluronic acid found in and adjacent to developing basement membranes. Oligosaccharides form a separate component in the same anatomical layer of the vitreous cortex. The morphologic details of this layer and of the retinal basement membrane vary according to the methods of fixation and staining. The reasons for such variations, aside from the absence of some of the constituents with inadequate fixation methods, probably include the availability of dye-binding sites (possible blocking by CPC), changes in dye-dye repulsive forces or dye-substrate avidity as the counterion concentration is varied, and the varying avidity of the several cationic dyes to the dye-binding sites under similar conditions. These methods have helped to identify the components of the vitreoretinal-juncture layer more precisely than has been possible before.

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