THE PATHWAY BETWEEN HYALOID BLOOD AND RETINAL NEURONS IN THE TOAD

Structural Observations and Permeability to Tracer Substances

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ABSTRACT

The hyaloid vessels form a capillary network on the inner surface of the retina. These capillaries are embedded in the vitreous humor, and they lack a glial investment. The intercellular spaces of the retina communicate with the ocular cavity, as can be evidenced by following the penetration of tracer substances. Hence, there is an extracellular diffusion pathway between hyaloid capillaries and retinal neurons, without interposition of glial cells. Trypan blue and ferrocyanide were not detected within the vitreous humor nor the retina after systemic injection. To this extent, at least, the hyaloid capillaries functionally resemble central nervous system capillaries. Intravascular injections of horseradish peroxidase established the absence of vesicular transfer across the endothelium of the hyaloid capillaries. In addition, quintuple-layered junctions between endothelial cells prevented the intercellular passage of the enzyme. It is likely, therefore, that the only pathway across the endothelium of the hyaloid capillaries is through the plasmalemma of the endothelial cells.

INTRODUCTION

The anuran retina is avascular, and it derives its blood supply from the choroidal and hyaloid vessels. The latter originate from the hyaloid artery (27) and form a capillary network adjacent to the inner surface of the retina. It has already been established that the intercellular spaces of the toad retina communicate with the ocular cavity by way of tortuous clefts between Müller cell processes (14). Therefore, if the hyaloid vessels lack a glial investment, the only major obstacle to diffusion which stands between hyaloid blood and retinal neurons is the capillary wall. The possibility that glial cells must be traversed by water and solutes escaping from central nervous system capillaries has been inferred from the finding of quintuple-layered junctions between contiguous astrocytic endfeet (7, 8). It is doubtful, however, that such perivascular glial barriers are either complete or of general occurrence (12).

In this paper, it is shown that the hyaloid capillaries in the toad are not surrounded by neuroglia. Nonetheless, their permeability properties resemble those of central nervous system capillaries.

METHODS

Retinae of toads (*Bufo marinus*) were fixed for 2 hr in cold 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.6). Dehydration was accomplished in graded ethanols and embedding was done in Epon (18).

The sections were stained with lead citrate (24). For observations on the structure of the endothelial junctions, the retinae were fixed for 2 hr in cold 2% glutaraldehyde (25) in 0.1 M phosphate buffer (pH 7.4), washed overnight in the phosphate buffer, and postfixed for 2 hr in 1% osmium tetroxide in phosphate buffer. The blocks were then stained in uranyl acetate (4, 11), embedded in Epon, and the sections stained with uranyl acetate (28) and lead citrate.

EXPERIMENTS WITH TRACER SUBSTANCES: Trypan blue was administered subcutaneously in daily injections (4 ml of a 1% solution in Ringer's fluid to toads weighing about 200 g) for 2 or 3 days. The day after the last injection, the toads were fixed by perfusion with 10% formalin and the retina and vitreous humor examined under a dissecting microscope. Ferrocyanide was given intravenously (1.5 ml of 0.07 M $Na_4Fe(CN)_6$, pH 7.8) under sodium pentobarbital anesthesia. 20–30 min after the injection, the eyes were excised and opened while immersed in 10% formalin containing 1% ferric ammonium sulfate and hydrochloric acid. In one instance, the fixative was perfused. The possible presence of Prussian blue deposits on the inner surface of the retina or within the vitreous humor was explored with a dissecting microscope. Thick histological sections of the posterior hemisphere of the eye and skeletal muscle (pectoralis major, gastrocnemius) were also examined.

Two types of experiments were performed with horseradish peroxidase (Type VI, Sigma Chemical Company, St. Louis). Some toads were injected intravenously with 7 mg of the enzyme dissolved in 0.7 ml of Ringer's solution (NaCl 115.5 mM, KCl 2 mM, $NaCO_3H$ 2.4 mM, $CaCl_2$ 1 mM). The eyes were excised after 15 or 30 min and fixed.

In other instances, peroxidase was applied directly to the retina. The eye was opened in anesthetized toads; the cornea, lens, and part of the vitreous humor were removed; and 2 mg of the enzyme dissolved in 0.2 ml of Ringer's fluid were instilled within the ocular cavity. 1 hr later the eye was fixed. Alternatively, isolated retinae were immersed for 20 min in Ringer's solution containing 0.5 mg of peroxidase per ml. Stirring and aeration were accomplished by bubbling air.

After the peroxidase experiments, the retinae were fixed in glutaraldehyde and washed as described above, then incubated for 15 min at room temperature in a solution of 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide in Tris-HCl buffer, pH 7.6, as described by Graham and Karnovsky (6). The blocks were then washed in phosphate buffer, postfixed in osmium tetroxide as above, and embedded in Epon. The sections were stained with lead citrate.

Control experiments were as follows: incubating glutaraldehyde-fixed retinae in the diaminobenzidine medium without any previous exposure to peroxidase; or performing the histochemical reaction with a retina fixed in the glutaraldehyde fixative containing 0.05 mg of horseradish peroxidase per ml. The enzyme was added to the fixative simultaneously with the tissue.

The electron microscope observations were made with an RCA EMU 3 G.

OBSERVATIONS

STRUCTURAL FINDINGS: The hyaloid capillaries are found overlying the inner surface of the retina, spaced at intervals of about 100 μ from one another. In cross-section, they have an elongated elliptical profile with the long axis parallel to the retinal surface (Fig. 1). The approximate dimensions of the diameters are 13–15 μ and 2 μ. As in other capillaries (19), the wall comprises endothelium, basement membrane, and pericytes.

The endothelium is 0.2–0.6 μ thick and belongs to the type without pores or fenestrations (1). The endothelial cells contain a variable amount of vesicles of irregular shape and diameter (Fig. 3). Invaginations or pits of the plasmalemma are observed infrequently (Fig. 4). This is a distinct departure from what is seen in muscle capillaries, in which the endothelial vesicles are abundant and very often open to the cell surface[1] (21, 22). The spaces between endothelial cells are spanned by quintuple-layered junctions (Fig. 2) (20). These junctions are observed whenever the junctional areas are perpendicularly sectioned, and, therefore, are likely to represent continuous belts around the endothelial cells. Clublike prolongations originate from both surfaces of the endothelial cells and extend towards the lumen and into recesses of the pericytes (Fig. 1).

The pericytes are almost invariably found on the vitreal surface of the capillaries (Figs. 1 and 3), but thin processes of these cells are also sometimes present on the capillary surface facing the retina (Fig. 1). In neither case, however, do the pericytes constitute a continuous investment, and extensive areas of the endothelial cells are exposed to the vitreous humor with only the basement membrane intervening (Figs. 1 and 3). The pericyte coat may also be absent at the level of endothelial junctions (Fig. 3) just as at any other region of the endothelium. Since the degree of impermeability of the

[1] The endothelial vesicles in toad skeletal muscle capillaries are entirely similar to those in mammals with respect to their number and relationship to the cell membrane.

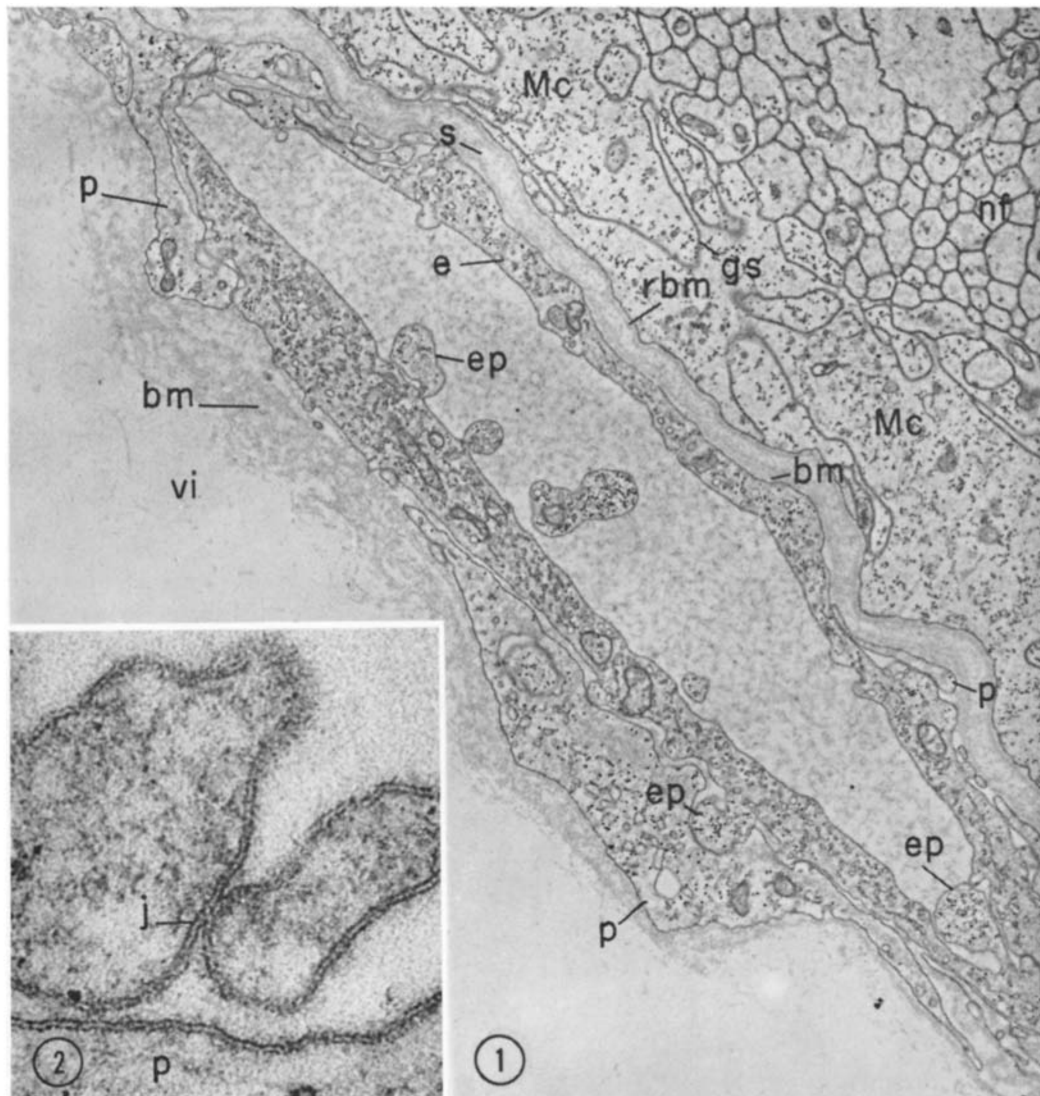


FIGURE 1 Hyaloid capillary and inner layers of the toad retina. The endothelium (*e*) is mostly devoid of any investment other than the basement membrane (*bm*). Pericyte processes (*p*) are observed on the retinal and vitreal surfaces of the capillary, but are more prominent on the latter. On the retinal surface of the capillary, the basement membrane is thin and separated from the inner retinal surface by a narrow space (*s*). On the vitreal surface of the capillary, the basement membrane is thicker and blends gradually with the vitreous humor (*vi*). At the inner surface of the retina a basement membrane (*rbm*) covers the plasmalemma of the end processes of Müller cells (*Mc*). The Müller cell processes are separated from one another by tortuous spaces (*gs*). *ep*, clublike endothelial cell prolongations; *nf*, optic nerve fibers. Fixed in OsO_4. Stained with lead. × 11,000.

FIGURE 2 Quintuple-layered junction (*j*) between two endothelial cells. *p*, pericyte. Fixed in glutaraldehyde and OsO_4. Stained with uranyl (block and section) and lead. × 176,000.

endothelial junctions is still in question (see below), it is worth noting that they are not surrounded by special barriers.

On the retinal surface of the capillaries, the basement membrane is about 0.1 μ thick. A space about 0.1–0.2 μ wide is always seen between the basement membrane of the capillary and the basement membrane covering the inner surface of the retina (Figs. 1 and 3). Fibrillar components of the vitreous humor are observed within this space (Fig. 4). On the vitreal surface of the capillaries, the basement membrane is thicker and the peripheral boundary is somewhat poorly defined (Figs. 1 and 3).

The inner retinal surface is formed by a basement membrane and the plasma membrane of the end processes of the Müller cells (Figs. 1 and 3). Together, these two elements constitute the inner limiting membrane of the light microscopic terminology. The Müller cell processes are separated from one another by meandering clefts (Figs. 1 and 3) which communicate with the spaces between neuronal and glial elements within the retina (14).

EXPERIMENTS WITH TRACER SUBSTANCES: The trypan blue injections did not stain the vitreous humor nor the retina. All the other tissues, with the exception of the central nervous system, were deeply stained. The choroid plexuses and neurohypophysis were also stained. In addition, the presence of the dye in blood was quite conspicuous.

After the ferrocyanide injections, the inner surface of the retina and the vitreous humor were free of Prussian blue deposits. In contrast, the histological sections showed Prussian blue staining of the interstitial tissue in the choroid and skeletal muscle.

When horseradish peroxidase was injected intravenously, the histochemical product was confined to the lumen of the hyaloid capillaries (Fig. 4). No indication of enzymatic activity was observed in the basement membrane of the capillaries, on the inner retinal surface, or within the intercellular spaces of the retina. When peroxidase and the histochemical product were deposited on the inner surface of the retina, they were not removed during fixation and the preparative procedures (Fig. 6). Therefore, enzyme leaking from the capillaries would have been detectable even if it had not penetrated the retina. Occasionally, vesicular profiles containing peroxidase were found in the endothelial cells (Fig. 4). It is not clear, however, whether these images represent vesicles isolated within the cytoplasm or cross-sections of pits of the luminal plasmalemma (Fig. 4). In any case, one never finds vesicles unloading the tracer at the contraluminal surface of the endothelial cells. The tracer did not penetrate between endothelial cells (Fig. 5). This observation indicates that the intercellular spaces of the endothelium are sealed off by the cell junctions (3).

When horseradish peroxidase was applied directly to the retina, it penetrated readily into the intercellular spaces. At the inner layers of the retina, the enzyme moved along the tortuous clefts between Müller cell processes before reaching the perineuronal spaces (Fig. 6). When peroxidase was instilled within the ocular cavity, it diffused across the whole neural retina, through the junctional layer (13), and into the layer of rods and cones (Fig. 7). This penetration occurred entirely through the extracellular spaces (Fig. 7). No clear indication of pinocytotic activity was found throughout the retina,[2] nor was there any peroxidase free within cells.

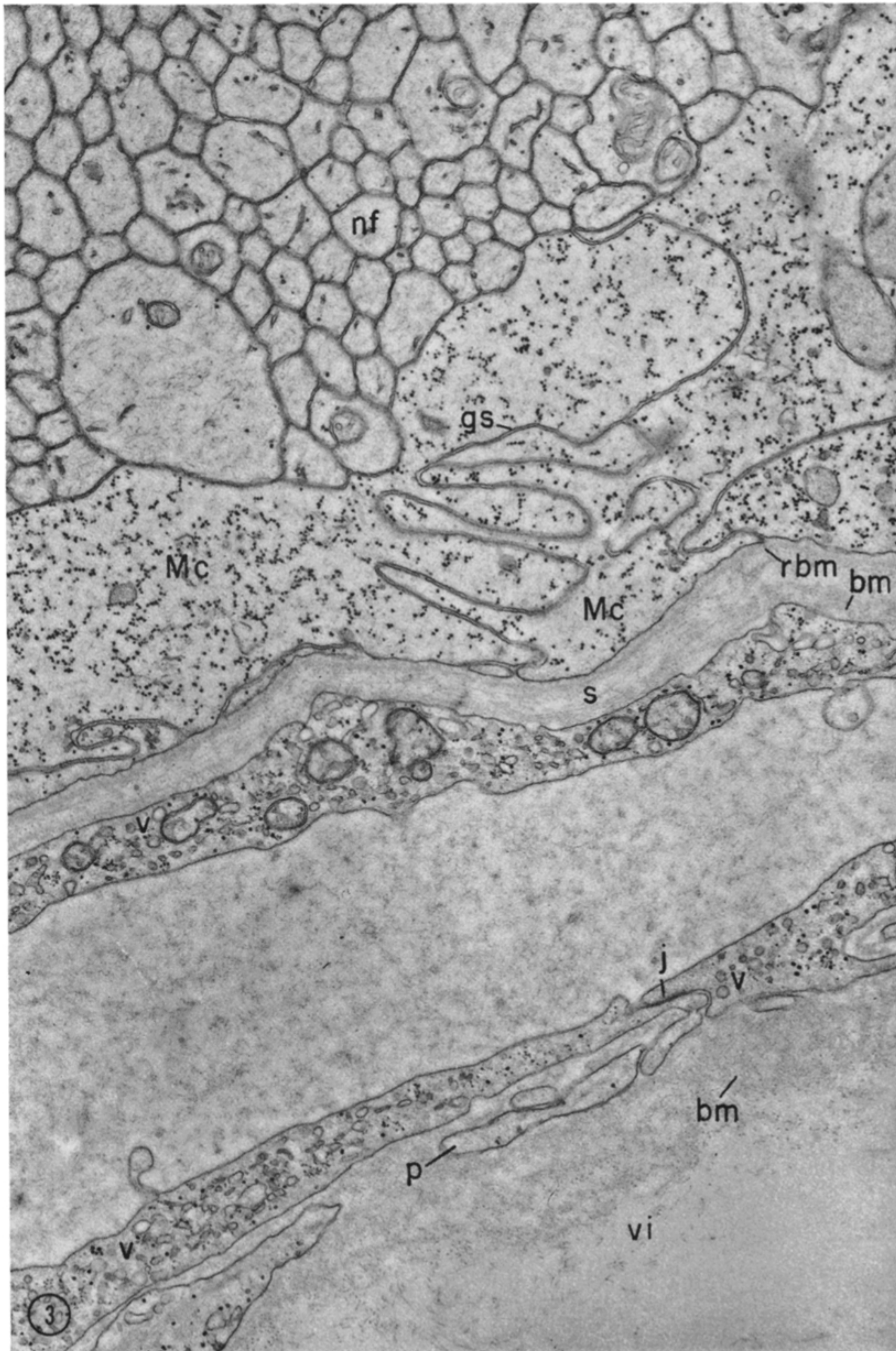
In the control experiments, no deposits of histochemical product were found in the intercellular spaces of the retina.

DISCUSSION

The hyaloid capillaries do not have a glial investment. This implies the existence of an extracellular

[2] The tracer was seen, however, within synaptic vesicles at visual cell endings.

FIGURE 3 Hyaloid capillary and inner layers of the retina. The endothelial cells contain vesicles (*v*) of irregular size and shape. None of the vesicles is open to the cell surface. Only the basement membrane (*bm*) intervenes between an endothelial cell junction (*j*) and the vitreous humor (*vi*). Between the basement membrane of the capillary and the basement membrane covering the inner retinal surface (*rbm*) a narrow space is noticed (*s*). Müller cell processes (*Mc*) are separated by meandering clefts (*gs*) leading to the layer of optic nerve fibers (*nf*). *p*, pericyte processes. Fixed in OsO_4. Stained with lead. × 22,000.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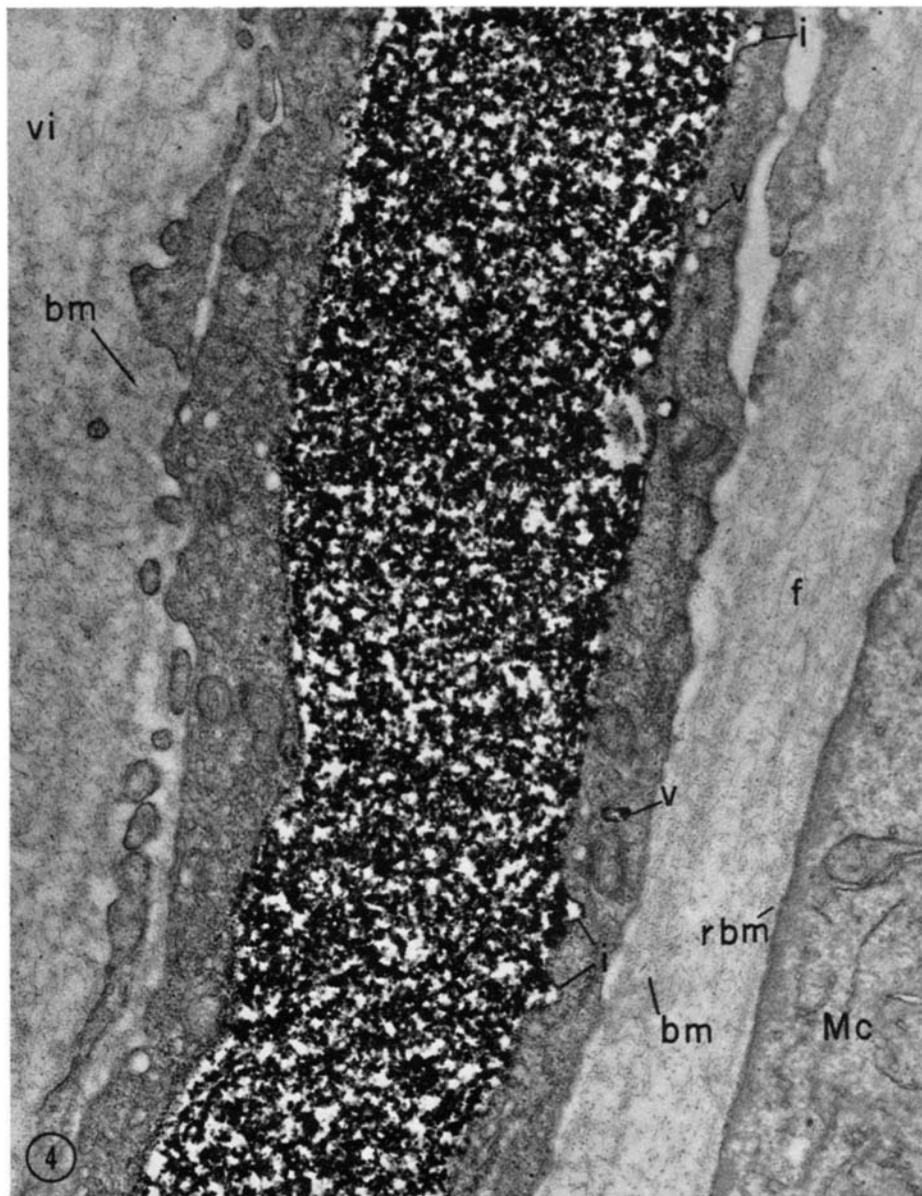

FIGURE 4 Histochemical reaction for peroxidase in a toad injected intravenously with the enzyme. Fixed 30 min after the injection. The histochemical product is confined to the lumen of a hyaloid capillary. Vesicular profiles (*v*) containing peroxidase are seen within the endothelium. They could represent, however, cross-sections of invaginations (*i*) of the luminal plasmalemma. Peroxidase is not detected within the basement membrane of the capillary (*bm*) nor in the space separating the capillary from the inner retinal surface (*rbm*). Within this space, fibrillar components (*f*) of the vitreous humor are observed. *Mc*, Müller cell cytoplasm. *vi*, vitreous humor. Fixed in glutaraldehyde and OsO_4. Stained with lead. × 22,000.

diffusion pathway between hyaloid capillaries and retinal neurons, without interposition of glial cells. At the outer surface of the neural retina, Müller cells can also be bypassed by solutes arriving from the choroidal circulation (13), but the pigment epithelium represents a previous glial barrier regulating ionic movements (15, 16). At the inner surface of the retina, however, the tortuous clefts

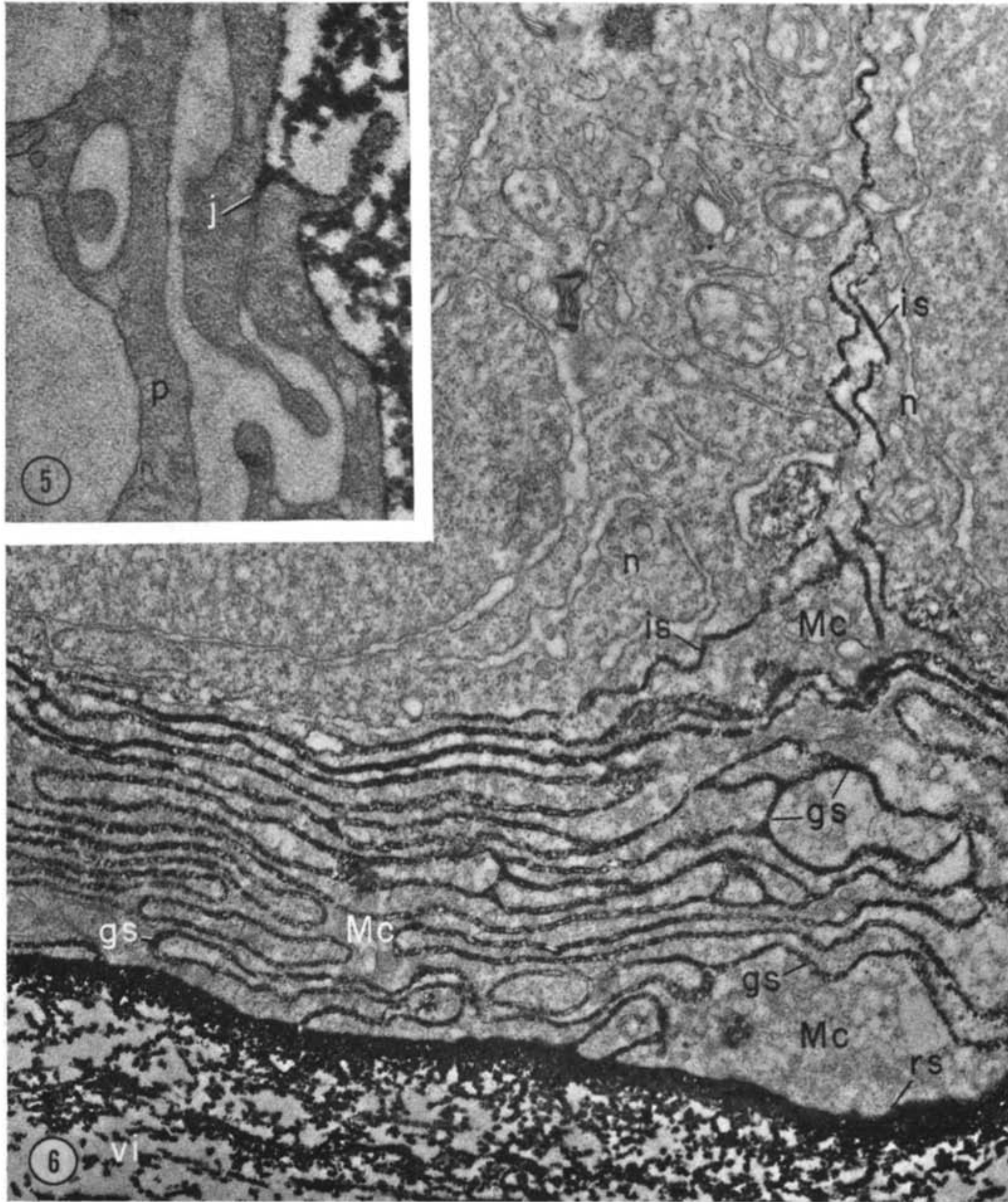

FIGURE 5 An endothelial cell junction (*j*) prevented the diffusion of peroxidase from the lumen of a hyaloid capillary. *p*, pericyte. Fixed, 15 min after the intravenous injection of peroxidase, in glutaraldehyde and, after the histochemical procedure, in OsO_4. Stained with lead. × 44,000.

FIGURE 6 Histochemical reaction for peroxidase in a retina immersed in a solution of the enzyme. Deposits of the histochemical product are seen within the clefts (*gs*) between Müller cell processes (*Mc*) and also within the perineuronal spaces (*is*). The enzyme and histochemical product remained adsorbed, throughout fixation and the preparative procedures, to the inner surface of the retina (*rs*) and to remnants of vitreous humor (*vi*). *n*, ganglion cells. Fixed in glutaraldehyde and OsO_4. Stained with lead. × 22,000.

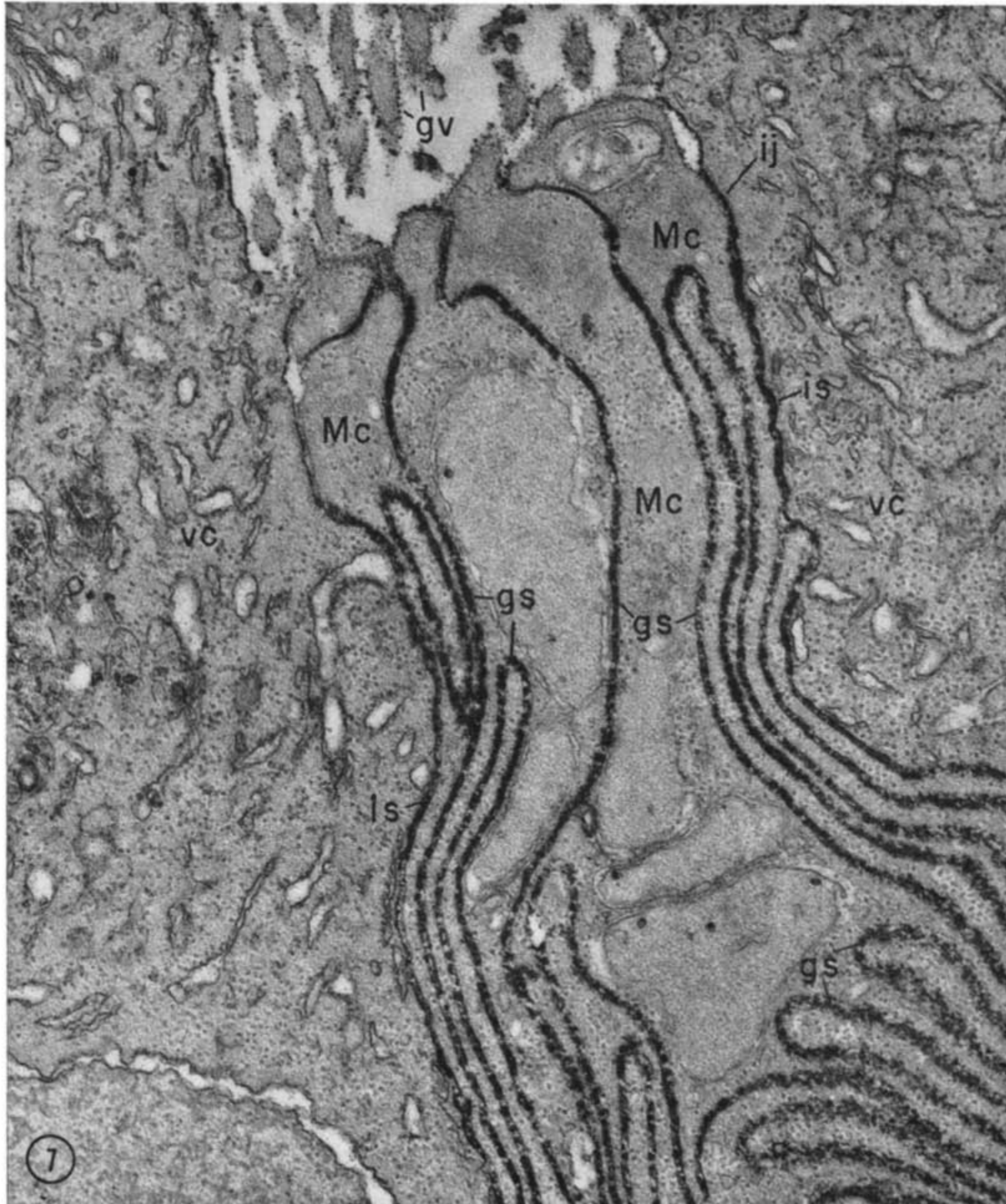

FIGURE 7 Histochemical reaction for peroxidase after the enzyme was instilled into the ocular cavity. At the outer surface of the neural retina, deposits of the histochemical product are found within the spaces (*gs*) between Müller cell processes (*Mc*) and within the spaces (*is*) between visual cells (*vc*) and Müller cell processes. The enzyme moved across the intercellular junctions (*ij*) at the junctional layer of the retina, and is found adsorbed to the surface of the Müller cell villi (*gv*) that form the fiber baskets around rods and cones. Fixed in glutaraldehyde and OsO_4. Stained with lead. × 34,000.

between Müller cell processes provide an open route to the perineuronal spaces as soon as ions and metabolites leave the hyaloid capillaries. This knowledge is derived from the observation that such pathway for penetration into the retina is followed by tracer substances diffusing across the inner retinal surface. Earlier observations on the penetration of ferrocyanide (14) have now proved to be reproducible when peroxidase is used as a tracer.[3] These findings do not imply that the extracellular pathway is the only pathway for movement of materials between hyaloid capillaries and retinal neurons. Metabolites, for instance, might be selectively taken up by Müller cells for eventual delivery to the neurons. This hypothesis has the merit of giving clear meaning to the extensive Müller cell surface provided by the tortuous glial spaces. At present, however, it lacks experimental support.

When the rather conventional structure of hyaloid capillaries is considered, it is noteworthy that the tracer substances are not detected in the retina or vitreous humor after systemic injection. Trypan blue and ferrocyanide are known to be excluded from central nervous tissue (5, 17). It would then seem that the hyaloid capillaries share some of the functional features of central nervous system capillaries. Both tracers, however, have shortcomings that prevent evaluating the closeness of such resemblance. The lack of penetration of trypan blue may simply indicate impermeability to plasma proteins (26). Little indication is, therefore, obtained with respect to the permeability of the hyaloid capillaries to small molecules. On the other hand, the ferrocyanide experiments require a cautious interpretation due to the limited sensitivity of the technique, as judged from a moderate to weak Prussian blue staining observed in skeletal muscle. Hence, the absence of Prussian blue deposits in the vitreous humor does not necessarily connote absolute impermeability of the hyaloid capillaries to ferrocyanide. Because of this uncertainty, the observations with ferrocyanide cannot be said to constitute further evidence of the obliteration of the intercellular spaces in the endothelium of hyaloid capillaries.

Regardless of these objections, the observations with ferrocyanide and trypan blue do indicate functional differences between hyaloid capillaries and muscle capillaries. The intravenous injections of horseradish peroxidase were performed for the purpose of investigating the nature of such differences. This enzyme, which has a molecular weight of about 40,000 (10), has been reported to move across the endothelium in muscle capillaries, both by way of vesicular transport and between endothelial cells (9). In hyaloid capillaries, however, there is no evidence of vesicular transport of peroxidase, and the diffusion of the enzyme is prevented at the intercellular junctions. Similar observations have been reported by Reese and Karnovsky (23) in cerebral capillaries. Therefore, it appears that, in hyaloid capillaries, the only pathway across the endothelium is through the plasma membrane of the endothelial cells. This should place a considerable selectivity on the movement of materials out from and into the capillaries, and it would also give the endothelial cells an important role in regulating exchanges between blood and retina. Nevertheless, such conclusions must be postponed until it is known whether the intercellular junctions are as effective a barrier for small molecules and ions as they are for peroxidase.

[3] Peroxidase has been shown to penetrate also within the intercellular spaces of the brain when injected into the ventricles (2).

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