Electron microscopic studies of nucleoside phosphatase activity in blood vessels and glia of the retina

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Nucleoside phosphatase activity was localized to the basement membranes of blood vessels and to glial cell plasma membranes in the retina with the electron microscope. The endothelial cells lining the small blood vessels were nonreactive in contrast to the endothelium of capillaries in heart and lung. Extensive enzymatic activity was found associated with the interspaces between adjacent glial elements and glial and neuronal elements. These findings are discussed in terms of the possible relationship of this enzymatic activity to permeability phenomena.

Studies on the appearance of small blood vessels of the retina with the electron microscope have shown that the endothelium of these vessels forms a continuous cell lining and has many of the fine structural characteristics of endothelium lining the vessels of nonneural tissue. It is generally considered, however, that the retinal vessels are similar to those in the central nervous system in that their permeability properties differ from those in nonneural tissue. While it has been suggested that the peculiar permeability properties of small blood vessels of the central nervous system may reside in the endothelium which lines the vessels, others have attributed this to the continuous basement membrane and to the glial sheath which surrounds all the small blood vessels in these tissues.

Recent studies on permeability and transport in other biologic systems have provided evidence which suggests that nucleoside phosphatase activity (ATPase) is involved in cation transport across cell membranes. A comparative study of the localization of nucleoside phosphatase activity in blood vessels of different morphologic types with different known or alleged permeability properties suggested that this enzymatic activity might also be involved in the regulation of vascular...
permeability.\textsuperscript{17,18} The present work is a continuation of these studies on the localization of nucleoside phosphatase activity to fine structural elements of blood capillaries and attempts to characterize the small vessels and the supporting tissue of the retina.

**Methods**

Eyes from 200 gram albino rats, killed by ether, were quickly removed, transected, and fixed in 4 per cent glutaraldehyde\textsuperscript{19} buffered to pH 7.4 with 0.05M cacodylate for 1 hour at 4°C. At the end of fixation the retinas were teased from the choroid and washed and stored in cold cacodylate buffer containing sucrose (0.4M) for periods ranging from overnight to several days. After storage, some of the retinal tissues were incubated as small strips in the Wachstein-Meisel\textsuperscript{20} adenosinetriphosphatase (ATPase) medium at pH 7.1 to 7.2, containing lead nitrate as the capture reagent. Incubation was carried out at room temperature for periods of 10 to 30 minutes. No change in pH occurred as a result of the incubation.

When pieces of the retina were to be examined with the light microscope, strips were removed from the incubating medium, washed in buffer, and treated with ammonium sulfide to develop a visible black precipitate (lead sulfide) from the reaction product, lead phosphate. Tissues processed for electron microscopy were not treated with ammonium sulfide since lead phosphate is electron dense.

The same procedures were followed in other experiments in which the adenosinetriphosphate (ATP) in the incubating medium was substituted for by \(\beta\)-glycerophosphate (GP) or adenosine-5'-monophosphate (AMP) at equimolar concentration. Control experiments included the incubation of tissue initially fixed in 1 per cent osmium tetroxide for 10 minutes in the above ATP medium and the incubation of glutaraldehyde-fixed tissue in media that lacked only substrate. Eyes taken for the study of morphology alone were fixed directly in buffered osium tetroxide for 2 hours at 4°C.

After incubation in the media described above, tissues were washed in buffer and post-fixed in buffered 1 per cent osmium tetroxide for 1 hour at 4°C. All tissues processed for electron microscopy were dehydrated in a graded series of ethanol and embedded in Maraglas.\textsuperscript{21} The polymerized material was trimmed under a dissecting microscope and oriented so that only the retinal surfaces were sectioned. Thin sections were cut on an LKB microtome, and these were mounted on formvar-coated copper grids, and examined without any additional staining in an RCA EMU 3F electron microscope.

**Results**

**The structure of retinal capillaries.** Small blood vessels of the retina are lined by a continuous layer of endothelial cells. Individual endothelial cells are closely joined together at intercellular junction sites and no gaps, pores, or fenestrations are seen extending across the endothelial cytoplasm (Fig. 1). In addition to the usual cell organelles the endothelial cells of these vessels contain only a small number of cytoplasmic vesicles which correspond to the pinocytic vesicles found in capillaries of heat, lung, and skeletal muscle. In some capillaries these vesicles are continuous with the cell membranes which border both the luminal and extravascular surfaces (Fig. 2).

A continuous basement membrane which is similar in appearance to that surrounding other types of small vessels is directly applied to the exterior of the endothelial cells (Figs. 1 and 2). In addition to this layer, a sheath of periendothelial cell processes (mural cell)\textsuperscript{4} also surrounds the endothelium. In these cases the basement membrane of the endothelial cell splits to enclose the periendothelial cell (Figs. 1 and 2). This arrangement is also seen in other types of small blood vessels.\textsuperscript{22,23,24}

One feature peculiar to the structure of both the small vessels in the retina and those of the central nervous system is the scant extracellular space surrounding the vessel wall. Glial cells abut directly on the vessel wall, either on the basement membrane of the endothelium or that of the periendothelial cell. The apparent extracellular space around the capillaries consists of a 100 to 200 A gap between the apposed glial cell membranes or a slightly larger space at the glial cell membrane-basement membrane interface.

**Nucleoside phosphatase activity of blood vessels.** When full-thickness slices of retina were incubated with ATP as substrate, the reaction product was localized to the blood
Fig. 1. Cross section view of a blood capillary in the retina fixed directly in osmium tetroxide. The vessel wall is lined by two endothelial cells (E) which are closely joined at tight intercellular junctions (J). The endothelium is surrounded by a continuous layer of basement membrane (B) and a discontinuous layer of periendothelial cell processes (P). The surrounding glial cells (G) are closely applied to the basement membranes. (x22,500.)
Fig. 2. Part of a capillary in the retina fixed in osmium tetraoxide directly. Several pinocytic vesicles (arrows) are present in the endothelial cells of this vessel. Note how the basement membrane (B) of the endothelium splits to enclose the periendothelial cell processes (P). (x32,000.)
Figs. 3 and 4. Light photomicrographs of flat preparations of retina following incubation with ATP. Dense reaction product is localized in the small blood vessels. (Both ×320.)

Figs. 5 and 6. Light photomicrographs of flat preparations of retina. Fig. 5 shows the appearance of retina following incubation with AMP (and also glycerophosphate), and Fig. 6 shows the reaction product which outlines the blood vessels following overincubation with ATP. (Both ×125.)
Fig. 7. Cross section of a blood capillary in retina incubated with ATP. Reaction product lines the membranes of the glial cells (G) surrounding the blood vessel and precipitate also appears in the region of the basement membrane (B). Reaction product is not present within the endothelial cells (E). L marks the vessel lumen. (×32,000.)

Fig. 8. Part of a blood vessel in retina incubated with ATP. While reaction product lines the surface membranes of the glial cells (G) which surround the vessel, both the basement membrane and endothelium (E) are nonreactive. (×22,500.)
Fig. 9. Part of a vessel from the same preparation shown in Fig. 8. Dense reaction product fills the region of the basement membrane of this vessel. (×32,000.)
Fig. 10. Section of retina taken from an area adjacent to the vitreous cavity (V) following incubation with ATP. A dense precipitate which outlines membranous elements occurring as linear stacks and whorls is seen immediately surrounding synaptic terminals (S). These sites of reaction product correspond to the folded and spiral formations of glial cells described by previous workers.\textsuperscript{25, 27} Reaction product is not present at the interfaces between neuronal elements (arrows) or on the membranes of the cells lining the vitreous cavity, but reaction product is seen at the interfaces between neuronal and glial membranes or between adjacent glial membranes. (×22,500.)
Fig. 11. Higher magnification of a whorl of reactive membranes which presumably represents extensive infoldings of glial membranes. The reaction product is present in the space between the apposed membranes. (x32,000.)
Fig. 12. Section of retina taken from the outer nuclear layer following incubation with ATP. Dense reaction product is present at the interfaces between the glial cell processes and the receptor cells and their processes. N marks some of the nuclei of the receptor cells. (x22,500.)
Fig. 13. Section of retina taken from an area external to that shown in Fig. 12, but still internal to the external limiting membrane. Reaction product is precipitated at the interfaces between glial cell processes (G) and receptor cell projections. (×22,500.)
Figs. 14 and 15. Light photomicrographs of retina incubated with ATP. The microscope was focused at approximately the level of the external limiting membrane. Dense reaction product outlines the unreactive rod and cone segments. (Fig. 14, ×320; Fig. 15, ×1,600.)
Fig. 16. Electron micrograph of an area which corresponds to that seen in the two preceding light photomicrographs. The dense bands of reaction product seen with the light microscope have been resolved to a delicate meshwork of reaction product which appears at the interfaces between glial cells and rod and cone inner segments. (×22,500.)
Fig. 17. The reaction is present on the interfaces surrounding each glial process, but it is not present at the interfaces between rod segments or rods and cones (arrows). (×44,000.)

Fig. 18. A section from the same preparation shown in Figs. 16 and 17 but cut external to the external limiting membrane and beyond the extension of glial elements. No reaction product is seen on the surface membranes of the rods and cones. (×32,000.)
Fig. 19. Part of retina incubated with ATP and cut in a plane perpendicular to that shown in Figs. 16 to 18. Reaction product outlines the process of glial cells which accompany the rod and cone segments. (x16,000.)
Figs. 20 and 21. Control preparations. Sections of retina incubated with β-glycerophosphate. No reaction product is seen on the glial cell membranes surrounding the blood vessel (Fig. 20), the basement membrane (Fig. 20), or at the interfaces between glial cells and receptor cells in the outer nuclear layer (Fig. 21). (Fig. 20, ×25,000; Fig. 21, ×22,500.)
vessels with the light microscope (Figs. 3, 4, and 6). The reaction product appeared to be associated with the walls of the vessels, but the resolution was inadequate to determine the exact site of deposition. When β-glycerophosphate or AMP (Fig. 5) was substituted for ATP in the reaction media, and incubated under the same conditions, activity was not present in the retinal blood vessels.

In control electron microscopic observations, brief preincubation fixation of tissue in osmium tetroxide inhibited all enzymatic activity so that no reaction product was deposited. When β-glycerophosphate (Fig. 20) or AMP was used as substrate, blood vessels were also unreactive. However, with ATP as substrate a dense reaction product was found on the surface membrane of glial processes abutting on the blood vessel (Figs. 7 and 8) and in some instances reaction product was seen in the basement membrane region (Figs. 7 and 9). In others, however, the basement membrane was unreactive, while only the glial membranes showed activity (Fig. 8). No activity was found within the endothelial cells or on their surface membranes. The surfaces of erythrocytes in the lumen of the vessels were usually intensely reactive.

Activity on glial cell membranes. In the layers of the retina proximal to the vitreous space a striking finding was the presence of reaction product on or between various aggregates of membrane which occurred either in close linear stacks (Fig. 10) or in tight spiral whorls (Figs. 10 and 11). The interpretation of these electron micrographs has been facilitated greatly by reference to several recent articles on the fine structural morphology of the retina, especially those of Fine and Lasansky and Wald, who described these folds as being associated with the surface of Müller cells.

In addition to the activity on the glial cell surfaces (Fig. 8) surrounding the vessels described above, activity appeared at the interfaces of the apposed membranes of glial cells or of these and adjacent neuronal elements (Figs. 10 and 11). Since the deposits filled the interspaces of these apposed membranes, it was not possible to decide whether both or only one of the membranes contributed to the reaction product, or, indeed, whether the reaction product was located in the space between the cells. However, when only neuronal elements were apposed (as in the region of axon terminals and synaptic clefts) no reaction product was deposited at the interfaces of the apposing neural plasma membranes (Fig. 10).

In the outer nuclear layer, activity with ATP as substrate was also associated with the interfaces of glial process with each other and with neuronal elements. Although these findings were apparent in observations made with the light microscope (Figs. 14 and 15), they were more precisely defined by electron microscopy. Figs. 12 and 13 are sections cut roughly perpendicular to the receptor cell nuclei and their projections to the rod and cone segments. Dense reaction product fills the interspaces between the glial membranes and the adjacent receptor cell segments. Again it was not possible to determine whether the site of the reaction was associated with glial membranes, neural cell membranes, or both. Figs. 16 to 18 are approximately perpendicular sections which are cut external to the external limiting membrane. At the level shown in Fig. 16 the glial processes which surround the rod and cone elements are fingerlike extensions of cytoplasm which form the so-called "fibre baskets" of Schulze. These fingerlike extensions of glial cytoplasm have been described by Fine and Zimmerman, and Cohen in material fixed in osmium tetroxide. In Figs. 16 and 17 they are clearly outlined by dense reaction product as they abut against each other or against rod or cone elements. Fig. 17 demonstrates that the rods and cones do not have reaction product precipitated where their own cell membranes are apposed, and Fig. 18, taken from a section just external to the areas seen in Figs. 16 and 17, confirms the finding that the rod and cone surface mem-

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branes alone are nonreactive. A section cut roughly parallel to the long axis of the rods and cones (Fig. 19) shows the extent of the long fingerlike extensions of glial cells lined by dense reaction product. The sites of activity between glial cells or between glial and neuronal cells that contained final products when ATP was used as substrate showed no deposits when incubated with glycerophosphate (Fig. 21) or AMP.

Discussion

The unfenestrated endothelial cells of retinal capillaries in the continuous layer are similar to those found in the heart, lung, skeletal muscle, and other sites. However, there are certain structural differences between small retinal vessels and unfenestrated peripheral capillaries. In particular, the endothelial cells of the retina have comparatively few pinocytic vesicles and a close investment of glial tissue surrounds the vessels. These features might relate to the alleged difference in permeability of retinal and cerebral capillaries in comparison with peripheral ones. Although studies with the light microscope suggested that nucleoside phosphatase activity was associated with retinal and cerebral capillaries, the present electron microscopic studies showed that the activity was present on the plasma membrane of glial cells which surround the blood vessels, and, in some cases, activity was present in the basement membrane region of some of the endothelial cells. No activity was found within the endothelial cells of the retinal vessels. Similar observations were made on the capillaries of the central nervous system with the same techniques, and both observations are in sharp contrast to the localization of the same activity in blood vessels of other tissues.

Small blood vessels of heart and lung, which also have a continuous endothelium but which contain numerous pinocytic vesicles, show nucleoside phosphatase activity confined to these vesicles. Activity was not seen on the basement membrane or on any of the surrounding structures of these vessels. The endothelial cells of small blood vessels in the kidney, intestine, and some endocrine glands which have fenestrations extending across their cytoplasm did not contain this enzymatic activity. In the light of these comparative studies, the localization of nucleoside phosphatase activity in this study shows a distribution unique for small blood vessels found in the retina and central nervous system.

It was suggested earlier that the nucleoside phosphatase activity in pinocytic vesicles of heart capillaries might be involved in the transport of substances across the endothelium. The absence of this activity in the endothelium of the retinal vessels and in similar vessels of the central nervous system may be significant in terms of the apparent differences in the permeability of these vessels from peripheral ones. On the basis of the present observations, we suggest that the permeability properties peculiar to retinal and cerebral vessels may be due to lack of pinocytic vesicles and their associated nucleoside phosphatase activity.

The presence of nucleoside phosphatase activity on the membranes of the glial cells abutting on the capillary bed is consistent with the assumed role of the glial cells as cellular intermediates between the bloodstream and the neurons. Previous workers have shown that the glial cells have high concentrations of oxidative enzymes and glycogen, and ATPase activity has been localized to the glial cells of the central nervous system by biochemical and histochemical methods. Many workers have supposed on morphologic grounds that the basement membrane and the adjacent glial cell membrane may be part of the barrier in retinal and central nervous system vessels. We suggest, on the other hand, that, since these structures contain a nucleoside phosphatase similar to that found at other sites implicated in transport (pinocytic vesicles in some endothelial cells, erythrocyte membranes, basal infoldings of tubular epithelium of the kidney...
and of the choroid plexus), these regions may actually facilitate transport of some materials rather than serving solely a "barrier function."

On the basis of the arrangement of glia in both vessels and neurons, it has been suggested that the retina and central nervous system do not have an extracellular space of dimensions comparable to that found in other tissues. Recent experimental evidence suggests that the small 100 to 200 Å gaps between the apposed membranes of glial cells and other cells represents an extracellular space. Since there is extensive infolding of glial cell membranes especially near the vitreous surface and between membranes of glial cells and other cells, this space between the apposed membranes might be more extensive in the retina than was previously supposed. On the assumption held previously by many workers that there was no true extracellular space which might permit diffusion of substances in both directions between blood and neural tissues, it was suggested that the transport of substances took place across the cytoplasm of the glial cells. However, in the present study as well as in the central nervous system proper, nucleoside phosphatase activity was localized not only to the membranes of the glial cells which surround the walls of small blood vessels but also to the interfaces of glial cells and neurons. The extensive infoldings of glial cell membrane alone, described earlier by Lasansky and Wald, were also highly reactive when incubated with ATP.

If the interspaces between apposed glial cell membranes or glial-neuronal membranes represent extracellular space, it is possible that the nucleoside phosphatase activity associated with the membranes serves to maintain electrolyte gradients across the membranes, perhaps through a mechanism similar to the Na-K ATPase of other systems. Although there is no evidence to indicate that the enzymatic activity demonstrated in this study is activated by Na⁺ or K⁺, such enzymatic activity has been found in glial tissue of the central nervous system. The present finding of the enzymatic activity of glial-glial and glial-neuronal interfaces also suggests a mechanism for the concentration of ions in a limited extracellular space.

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REFERENCES


*Although the enzymatic activity demonstrated in this work hydrolyzed ATP but not AMP or glycerophosphate, we hesitate to name the enzyme an ATPase and prefer to call it a nucleoside phosphatase on the basis of previous studies of the hydrolysis of nucleoside phosphates by various tissues including capillaries. Other sites of ATPase or nucleoside phosphatase activity such as mitochondria or the outer segments of retinal rods were probably inhibited by pronase fixation in glutaraldehyde.
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