# FINE STRUCTURAL LOCALIZATION OF A BLOOD-BRAIN BARRIER TO EXOGENOUS PEROXIDASE

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## ABSTRACT

Horseradish peroxidase was administered to mice by intravenous injection, and its distribution in cerebral cortex studied with a recently available technique for localizing peroxidase with the electron microscope. Brains were fixed by either immersion or vascular perfusion 10–60 min after administration of various doses of peroxidase. Exogenous peroxidase was localized in the lumina of blood vessels and in some micropinocytotic vesicles within endothelial cells; none was found beyond the vascular endothelium. Micropinocytotic vesicles were few in number and did not appear to transport peroxidase while tight junctions between endothelial cells were probably responsible for preventing its intercellular passage. Our findings therefore localize, at a fine structural level, a "barrier" to the passage of peroxidase at the endothelium of vessels in the cerebral cortex. The significance of these findings is discussed, particularly with reference to a recent study in which similar techniques were applied to capillaries in heart and skeletal muscle.

## INTRODUCTION

Experiments demonstrating the failure of intravenously administered dyes to stain all but certain regions of the brain have given rise to the concept of a blood-brain "barrier," although this interpretation of these experiments has been questioned (4). Other experiments with intravenously administered tracers that can be localized with the electron microscope appear to show that a barrier exists at the level of the vascular endothelium (3, 5, 18), although this barrier is apparently not unique to brain because the same tracers fail to penetrate the walls of many peripheral capillaries (12). The concept of a barrier between the blood and the brain has also been used to describe the apparent low permeability of cerebral vessels to a wide range of solutes (16), and it has been postulated that either the capillary endothelium or the perivascular astrocytic foot processes are the anatomical counterpart of this barrier (11).

Peripheral vessels, unlike those in the central nervous system, are permeable to a wide range of water-soluble substances with molecular sizes below a certain limit, and this permeability has been attributed to hypothetical pores penetrating the surface of the endothelium (23). Horseradish peroxidase has recently been employed as an enzymatic tracer to localize these pores at an ultrastructural level and has been found to pass from the circulation to the extravascular spaces of cardiac and skeletal muscle by diffusing through gaps between endothelial cells (15), as well as by transport in micropinocytotic vesicles (13). Although these gaps have been claimed to be sealed by tight junctions (20), doubt has been cast upon their functional tightness by these studies. Indeed, they may well correspond to the hypothetical endothelial pores mentioned above (15, 23).

The use of horseradish peroxidase as a tracer in

studies of this type has the advantages that it is a relatively small protein (mol wt 43,000) and that very sensitive localization is obtained by virtue of the amplification of visible marker through enzymatic activity; thus, a few molecules of enzyme yield a large number of molecules of electron-opaque reaction product (10). Since previous quantitative measurements of the passage of peroxidase from the circulation into various tissues have shown that passage of this tracer into brain is much slower than into any other tissue (28), it was of interest to use this technique to study the permeability of the cerebral vessels. Evidence will be presented in this paper that vascular endothelium in brain is distinctly less permeable to peroxidase than vascular endothelium in heart and skeletal muscle and that this difference in permeability can be explained primarily on the basis of structural differences in the junctions between endothelial cells.

## MATERIALS AND METHODS

Methods for visualization of peroxidase with the electron microscope as well as pertinent control experiments have been previously described in detail (10). In the present experiments, 5–10 mg of horseradish peroxidase (Sigma Chemical Company, St. Louis, type II) were dissolved in less than 0.4 ml of balanced salt solution and injected into the tail veins of adult mice 10–60 min before fixation of the brain. In one experiment, two injections of 5 mg of peroxidase were spaced 30 min apart, the last being given 30 min before fixation. Fixation was either by perfusion of warm (34–36°C) fixative through the aorta or by immersion of a slab of cerebral cortex in fixative at room temperature. The best results with the latter mode of fixation were obtained with a sodium cacodylate–buffered mixture of glutaraldehyde (Baker Chemical Company, North Phillipsburg, N. J.) and formaldehyde (14). For perfusion, one part of this fixative was added to four parts of 0.1 M sodium cacodylate buffer; blocks of cerebral cortex from perfused brains were fixed for 2–4 hr longer in the full-strength fixative. After fixation, the blocks of tissue were washed overnight in 0.1 M sodium cacodylate buffer and then sliced without freezing at approximately 50 $\mu$ (27). The slices were incubated for 15 min at room temperature in 0.05 M Tris-HCl buffer, at pH 7.6, containing 5.0 mg of 3-3′ diaminobenzidine (Sigma Chemical Company) per 10 ml of buffer and a final concentration of 0.01% hydrogen peroxide. Oxidation of 3-3′ diaminobenzidine results in a brown reaction product which was examined with the light microscope prior to postfixing the sections in 2% osmium tetroxide in sodium cacodylate buffer. The final electron opacity of this reaction product is due, in part, to its avid reaction with osmium tetroxide (10). Subsequent steps in preparation for electron microscopy were routine; all sections were stained with lead citrate (30). Only the gray matter of cerebral cortex was examined in this study.

Control animals received no peroxidase, but brain slices were exposed to the incubation medium. Reaction product in the absence of exogenous peroxidase was found only in erythrocytes and within lipofuscin granules in neurons (Fig. 6). The latter localization is probably attributable to intrinsic peroxidase or peroxidase-like activity (9).

For the examination of junctions between endothelial cells, blocks of tissue were stained for 2 hr in 0.5 or 2.0% uranyl acetate (7) in 0.2 M sodium maleate buffer at pH 5.2[1]. Blocks were washed in maleate buffer at the same pH for approximately ½ hr before and after staining. Good staining was achieved after perfusion with either the aldehyde fixative described above or with 2% osmium tetroxide in 0.15 M sodium cacodylate or phosphate buffer at pH 7.0. All embedding was in Araldite.

## RESULTS

In sections examined with the light microscope, peroxidase activity was apparent as a brown reaction product that appeared to be confined within blood vessels in brains that were fixed by immersion (Fig. 1). A small amount of reaction product was also present at edges of the sections where blocks of tissue were initially separated from the cortex, but these areas were avoided in trimming blocks for electron microscopy. With this exception, visible reaction product was completely absent in animals that were fixed by perfusion because the perfusion washed the peroxidase out of the vessels. In electron micrographs of cerebral cortex fixed by immersion, peroxidase activity was apparent as an opaque, noncrystalline reaction product that filled the lumina of vessels (Figs. 3–5). This reaction product was absent from most of the vessels in cortex fixed by perfusion (Fig. 7), but occasionally a vessel that was not well perfused still contained blood cells and reaction product (Fig. 2). None of these results varied with exposures to peroxidase ranging between 10 and 60 min.

The intravascular confinement of peroxidase was not dependent on the type or location of vessels. Vessels in the subarachnoid space as well as vessels of all sizes in the parenchyma, with or without

[1] Karnovsky, M. J. In preparation.

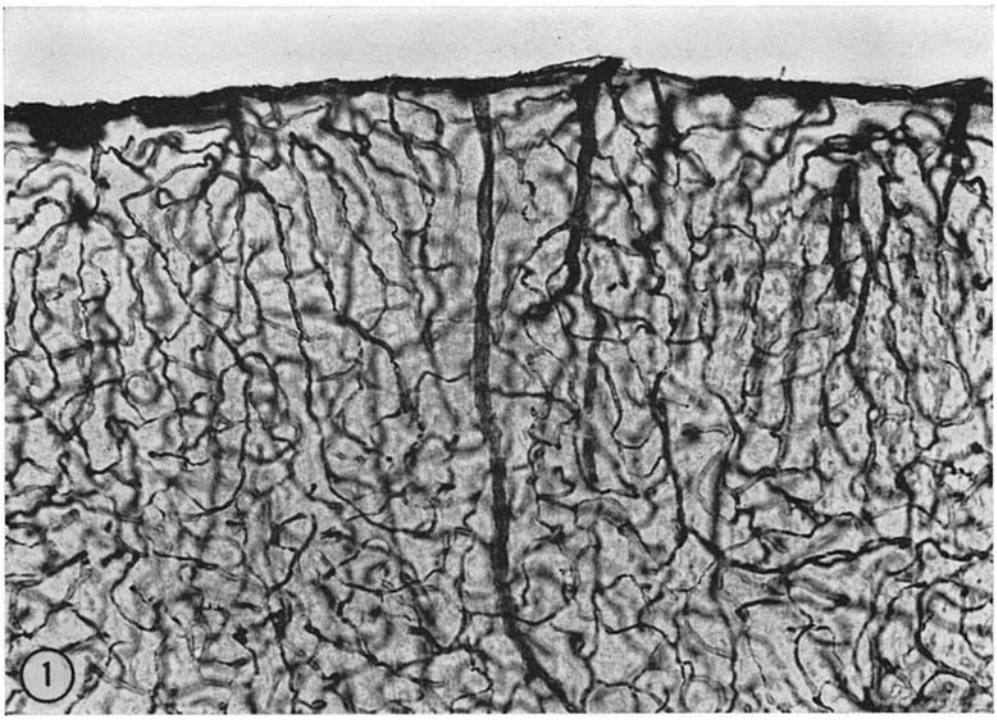


FIGURE 1 Cerebral cortex showing distribution of intravenously administered peroxidase. This section is about 50 $\mu$ thick and was incubated in an appropriate medium to produce a brown reaction product at sites of peroxidase activity. Reaction product is found only in association with blood vessels. Surface of cortex is at top. × 175.

surrounding smooth muscle cells (Fig. 7), confined peroxidase within their lumina. In some animals, in which fixation was delayed, many vessels were found with the endothelial cytoplasm, basement membrane, and perivascular extracellular space permeated by reaction product. This distribution was attributed to artifactual spread of peroxidase during fixation, because free intracellular or perivascular reaction product was not found in well fixed cortex. This artifactual spread of peroxidase shows that it is detectable in the perivascular spaces of the brain; furthermore, peroxidase has been found readily to enter extracellular spaces in the brain after it is injected into the cerebral ventricles (2).

The concentration of reaction product was uniform throughout the lumen of each vessel except adjacent to the plasma membranes of endothelial cells; these were coated with reaction product as if they were slightly sticky (Figs. 2, 3). Although this apparent binding to the plasma membrane may be genuine, the possibility that peroxidase may be artifactually bound during fixation cannot be excluded at this stage (10), particularly since this coating is washed away in animals fixed by perfusion (Fig. 7).

In untreated as well as experimental animals, endothelial cells of cerebral cortical vessels contained vesicles in their cytoplasm, but these were noticeably few in number and more variable in size and shape than those in vascular endothelial cells of cardiac or skeletal muscle (13). A few of these vesicles were "coated" (25), having a rim of dense material applied to the cytoplasmic side of their limiting membrane. Invaginations of the luminal plasma membrane of endothelial cells appeared to be precursors of the vesicles since in brains fixed by immersion the invaginations as well as some of the vesicles were filled with reaction product (Figs. 3, 8) while in brains fixed by perfusion, reaction product was present within vesicles (Figs. 7), but not within invaginations. Invagina-

tions of the plasma membrane were also found on the contraluminal side of the endothelial cells, but no reaction product was found in these invaginations or anywhere else around the vessel wall, regardless of the method of fixation.

Along its lateral edge, each endothelial cell overlapped its neighbor for a variable distance and reaction product penetrated these regions of overlap for only very short distances at the luminal surfaces (Figs. 3–5). The failure of peroxidase to penetrate these intercellular spaces was attributed to tight junctions that were present in every region of overlap that could be examined in a favorable plane of section (Figs. 4, 5, 9–11). At tight junctions, the outer leaflets of adjacent cell membranes appeared to fuse or touch, obliterating the intercellular space. This occurred in immersion-fixed cortex from animals treated with peroxidase and incubated for peroxidase activity (Figs. 4, 5), but was studied primarily in normal animals fixed by perfusion with either buffered osmium tetroxide or the aldehyde fixative (Figs. 9–11). The ratio of the total width of a tight junction to the average total width of immediately adjacent single cell membranes seemed an appropriate way to characterize morphological tightness (Fig. 11, inset). For tight junctions between endothelial cells in cerebral cortex, the ratio of the two measurements was 1.6–1.8, regardless of the type of fixative used, suggesting actual fusion of the cell membranes. Tight junctions might extend radially through much of the region of overlap between endothelial cells (Figs. 9, 11), or short tight junctions might be present at only a few places (Fig. 10). However, a tight junction was always present, and it was concluded that they form continuous belts or zonulae occludentes (6) around every endothelial cell, although a series of overlapping shelves is also consistent with their being present in every section.

Distinct from tight junctions and not always present were other regions of close apposition of neighboring endothelial membranes in which the intercellular gap was 30–60 A instead of the 100-A or larger gap characteristic of the remaining intercellular spaces between endothelial cells or the intercellular spaces in the surrounding neuropil (Figs. 9, 13). At many places along regions of overlap between adjacent endothelial cells, a rim of amorphous dense material extended from the inner leaflet of the cell membrane for variable distances into the cytoplasm (Figs. 4, 5, 9–11). This dense material was usually found in regions of either close apposition or tight junction, and it was often symmetrically distributed on the two adjacent plasma membranes.

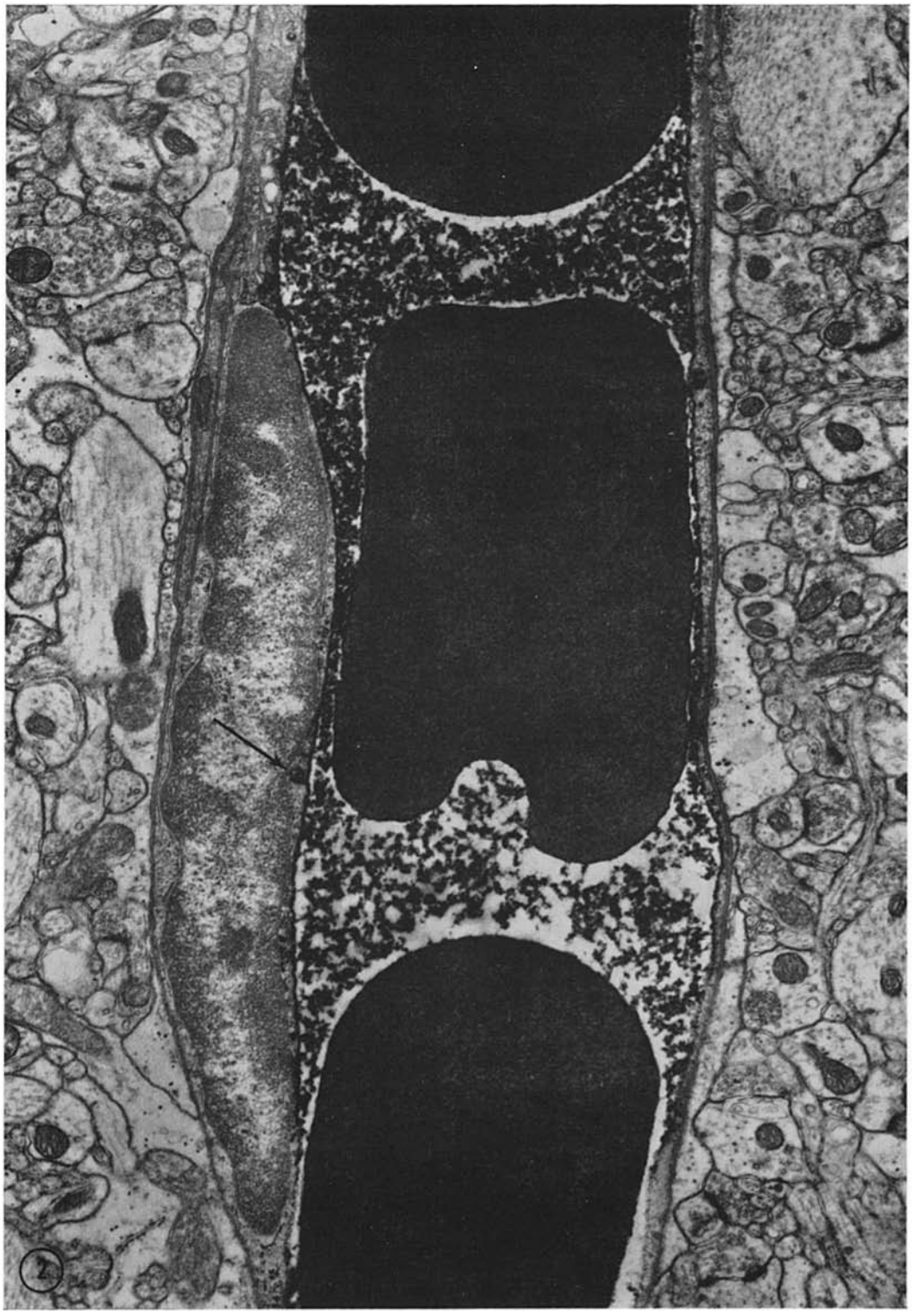
Completely enclosing each cortical vessel were overlapping glial sheets. Along the regions of overlap, channels 30–70 A wide at their narrowest points could be traced from the basement lamina of the vascular endothelium to the surrounding neuropil (Figs. 12, 13); these channels appear to be a general feature of perivascular astroglia (17). Their widths in our material were usually somewhat less than the widths of the intercellular spaces in neuropil surrounding the vessels.

## DISCUSSION

Since no extravascular peroxidase was detected by means of the histochemical technique used for this study, or by direct quantitative determination of the peroxidase content of perfused whole brains (28) after administration of intravenous peroxidase, it is apparent that appreciable amounts of this enzyme do not pass through or around cerebral endothelial cells. The endothelial cells must maintain a considerable gradient of this enzyme because the doses used in this study were, in most instances, three times the amount sufficient to permeate extracellular spaces in heart or skeletal muscle of mice after an intravenous injection.[1] Two features of cerebral endothelium may be important in establishing this gradient.

The first feature is the tight junction between endothelial cells. Similar junctions are present in epithelia (6), and their presence between endothelial cells in cerebral (20) and retinal (26) vessels

FIGURE 2 Intravenously administered peroxidase is localized in electron micrographs by a dense reaction product that is seen here in the lumen of a cerebral vessel. Dark staining of three erythrocytes is due to endogenous peroxidase activity of hemoglobin. The reaction product characteristically coats the luminal plasma membranes of endothelial cells. Within an endothelial cell is a micropinocytotic vesicle filled with reaction product (arrow), but no reaction product is found beyond the endothelium. Fixation was by perfusion which did not flush out this vessel. × 20,000.



has been previously noted. Our observations emphasize that they probably form an unbroken belt between cerebral endothelial cells. Since peroxidase readily penetrates between cardiac endothelial cells, which are also believed to be joined by tight junctions (20), its failure to pass between cerebral endothelial cells may be attributed to some distinctive differences between cerebral and cardiac endothelial junctions. Pertinent features of the former when compared to cardiac endothelial junctions are that they occupy a larger proportion of the radial length of the intercellular cleft and that they actually appear to represent an apposition or fusion of the external leaflets of the cell membranes. Between the cell membranes of cardiac endothelial cells, there are merely close approximations of the external leaflets so that they sometimes appear to be separated by small spaces. Further details of the cardiac junctions will be given in a subsequent publication.[1]

The difference in "tightness" between cardiac and cerebral endothelial junctions can be characterized by comparing the over-all width of the junction with the average widths of single cell membranes adjacent to the region of junction. In heart, fixed by either immersion or perfusion of aldehydes, this ratio is usually greater than 2.0, averaging about 2.4 whereas in brain the ratio is less than 2.0, averaging about 1.7. Thus, the so called tight junctions between cardiac endothelial cells could be characterized as "close," whereas those in brain are "closed." This distinction may well apply to iridic and retinal vessels because junctions between endothelial cells in the former can be disrupted by histamine or ocular paracentesis while tight junctions in the retinal vessels remain intact (26). Since the tight junctions in cerebral vessels are apparently continuous and are structurally tighter than the junctions in cardiac vessels, it is probably these junctions that block the passage of peroxidase through the clefts between the endothelial cells.

A second characteristic feature of the endothelium of cerebral vessels is the low frequency of vesicles of the type that have been found to be associated with transport of materials across endothelia elsewhere (21). Both the proportion and actual number of vesicles filled with peroxidase are low in cerebral endothelial cells in comparison to cardiac endothelial cells. Furthermore, no evidence was found of discharge of peroxidase on the contraluminal side of endothelium of cerebral vessels. Since there is evidence that vesicles in the vascular endothelia in cardiac and skeletal muscle transport materials by filling on the luminal side of the endothelial cells and discharging their contents into the perivascular spaces (22), the paucity of similar vesicles in brain and their failure to discharge their peroxidase on the contraluminal side of the vessels could be regarded as another manifestation of a blood-brain barrier. Uptake in vesicles could also be regarded simply as micropinocytosis (8), serving some nutritional or other need of the endothelial cell.

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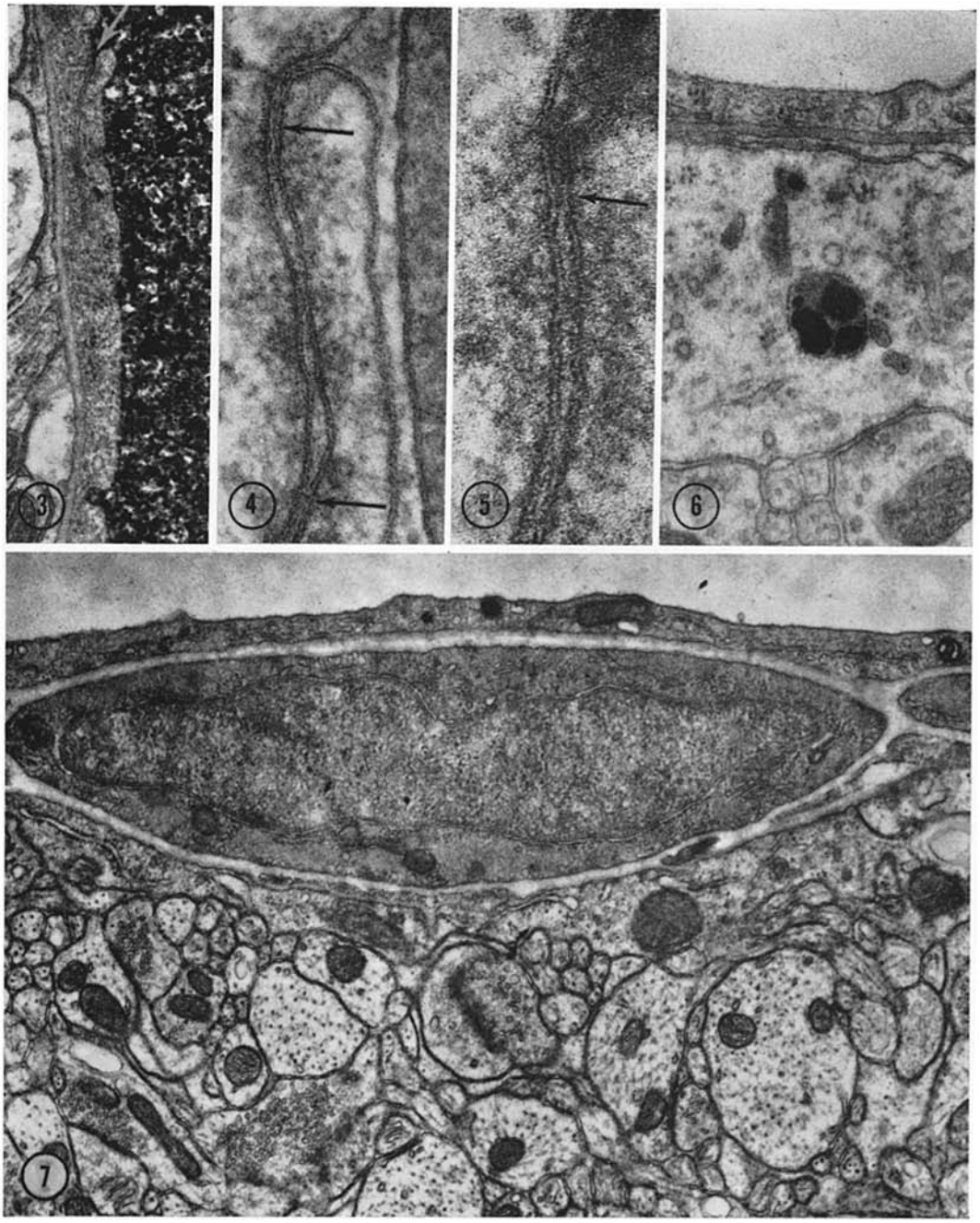
FIGURE 3 Peroxidase has entered an invagination in the cell membrane of an endothelial cell (below) but does not pass through the cleft between neighboring endothelial cells (arrow). From cerebral cortex immersed in fixative. × 40,000.

FIGURES 4 and 5 Tight junctions (arrows) within clefts between neighboring endothelial cells in mice injected with peroxidase. In Fig. 4, a small amount of reaction product lies between the endothelium and an erythrocyte. In Fig. 5, where a larger amount of reaction product is present within the lumen of the vessel (top), it is seen that the entry of peroxidase into the cleft is stopped at the tight junction. From cerebral cortex immersed in fixative. Uranyl acetate block stain. Fig. 4, × 110,000. Fig. 5, × 210,000.

FIGURE 6 Vessel and perivascular neuropil from control animal. Dense body in neuron (center) contains areas made more dense by exposure to peroxidase incubation medium. Normal mouse; treated with incubation medium. × 30,000.

FIGURE 7 Intravascular perfusion of fixative has flushed peroxidase out of vessels, but reaction product is present in vesicles within the endothelium. Peroxidase does not pass beyond the endothelium of this larger vessel with a muscular wall. From mouse injected with peroxidase. × 20,000.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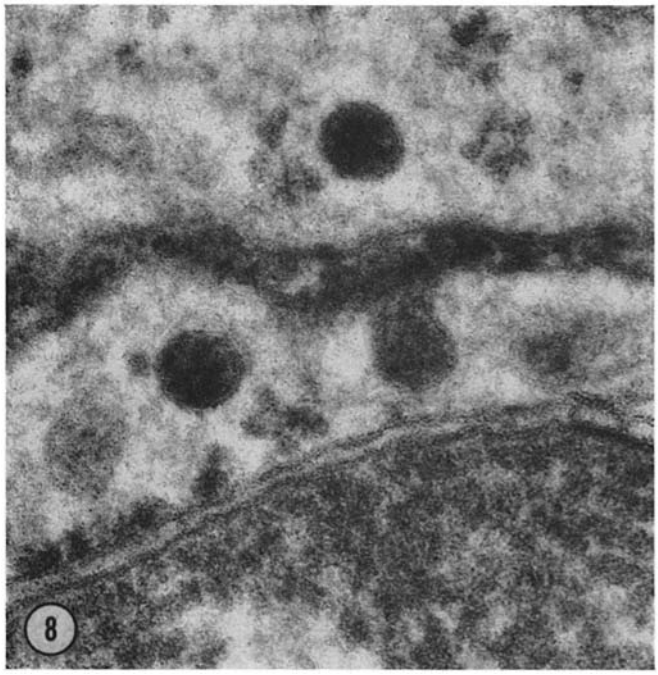


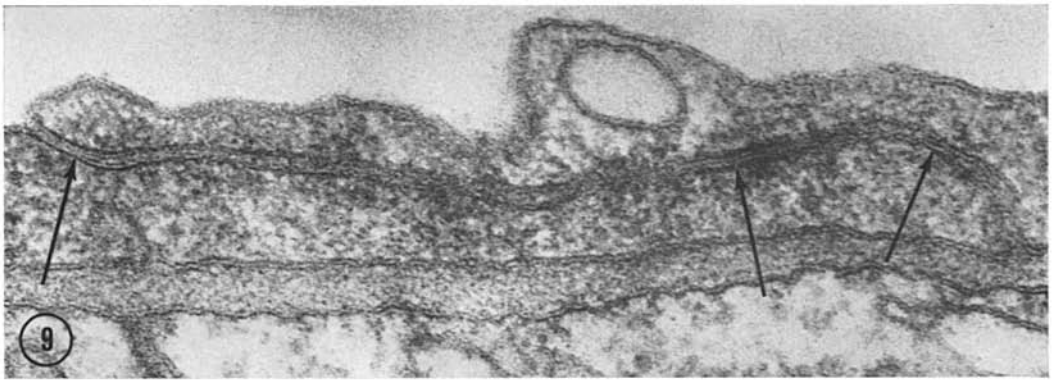

FIGURE 8 Peroxidase has entered an invagination in the cell membrane of an endothelial cell. Invaginations such as these appear to be the precursors of vesicles filled with peroxidase like the two shown here lying within endothelial cells. Opposite endothelial surfaces are closely apposed because vessel collapsed during fixation. From cerebral cortex immersed in fixative. × 160,000.

Convincing physiological evidence is recently available that extracellular spaces in brain serve as channels for exchange of various ions between neurons and the cerebral circulation (17). That these spaces are present in the mammalian brain has been demonstrated by the observation that ferritin diffuses into them (1) and by the preparation of the brain by freeze-substitution, which presumably does not alter existing spaces (29). Since various ions which move into and out of the brain do not move through glial cells but between them, it is not surprising to find that these open spaces are also present between astrocytic end feet (17). The vascular endothelium is, therefore, the only continuous cellular layer between the circulation and the extracellular spaces in the brain.

According to our evidence, horseradish peroxidase is, to a large degree, prevented from passing the endothelium of cerebral vessels which could, therefore, be characterized as a "barrier." While

FIGURES 9–11 Regions of overlap between neighboring endothelial cells, illustrating range of variation in structure of the intercellular cleft. In Fig. 11, the cleft is obliterated by a tight junction (arrow) extending throughout most of its length, while in Fig. 10 a short tight junction (arrow) occupies only a small part of the cleft. Fig. 9 is most typical; several tight junctions (arrows) are present along the cleft, and much of the remaining cleft is very narrow. The region of the intercellular cleft indicated by the arrow in Fig. 11 is shown in inset at higher magnification to illustrate that the total width of the tight junction (between arrows at right) is less than twice the width of the adjacent plasma membrane (between arrows at left). Normal mouse; uranyl acetate block stain. Fig. 9, × 135,000. Fig. 10, × 190,000. Fig. 11, × 170,000. Fig. 11 inset, × 420,000.

FIGURE 12 Astrocytic end feet (*A*) lying between a myelinated axon and the basement lamina of the vascular endothelium. A cleft between the astrocytic end feet extends from the basement lamina to a perivascular myelinated axon and appears open except near the blood vessel where it is invaded by some basement lamina material. Normal mouse; uranyl acetate block stain. × 150,000.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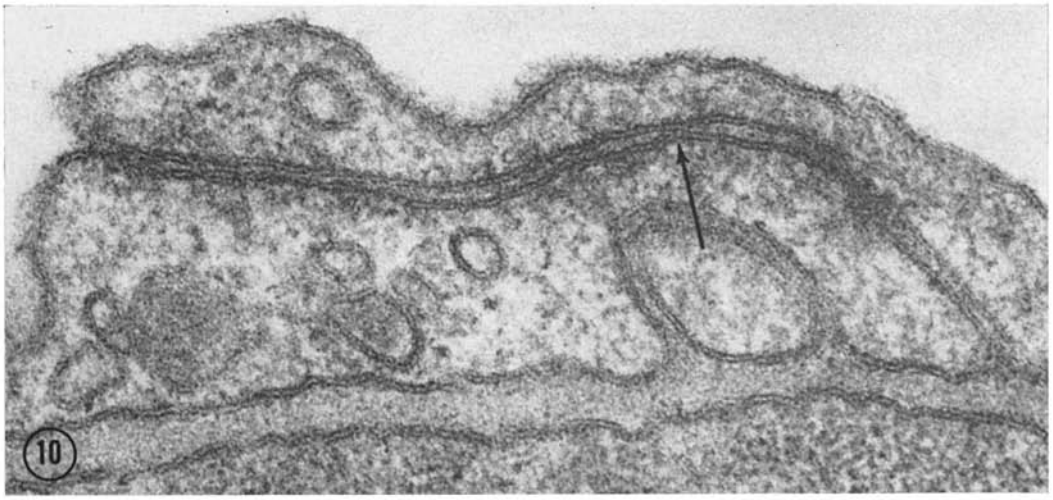
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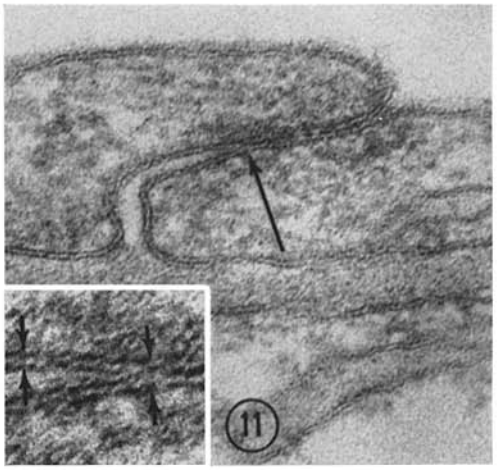
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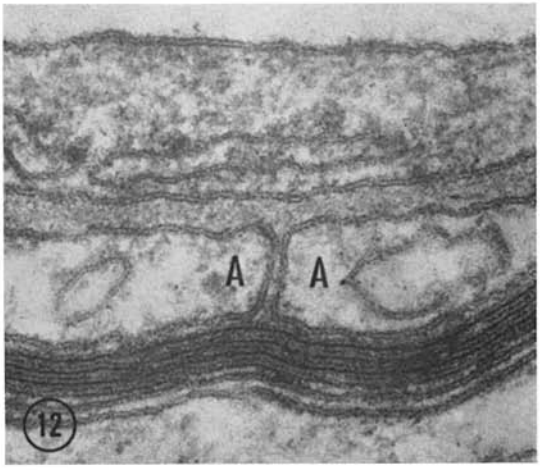
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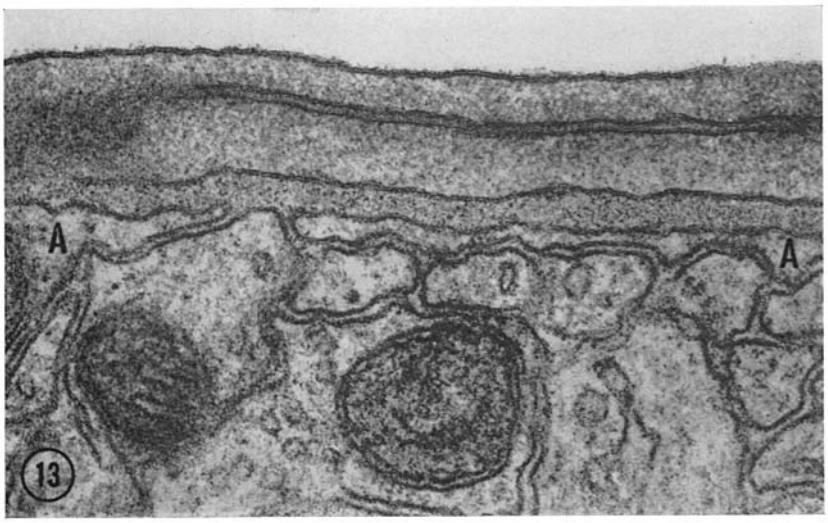


FIGURE 13 Astrocytic end feet (*A*) lying between the basement lamina of the vascular endothelium and the perivascular neuropil which here contains many small axons. Two clefts between astrocytic end feet connect the basement lamina of the endothelium with the continuous extracellular space around the axons. Normal mouse; uranyl acetate block stain. × 100,000.

it is likely that other proteins and larger molecules might be similarly impeded at this barrier, this may not necessarily be true for smaller substances. Certain substances, such as glucose, must certainly pass the vascular wall in large quantities in order to fill the metabolic needs of the brain (19). If the tight junctions between endothelial cells are relatively impermeable, these substances would enter the brain by passing through endothelial cells by special transport mechanisms. It is also possible that these tight junctions are permeable to some extent to small molecules and that other mechanisms such as metabolic "pumps" are necessary to maintain a blood-brain concentration gradient. Such a mechanism has been postulated for maintaining hydrogen ion gradients between blood and brain (24). These pumps may well be located at the endothelium, which is structurally organized as a continuous "surface" across which the pumps could act.

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