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ELECTRON HISTOCHEMICAL STRUCTURE OF CAPILLARIES IN THE RAT BRAIN

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Introduction

In spite of numerous histochemical and ultrastructural investigations, the structural basis of the blood-brain barrier has not been elucidated fully so far. The investigations performed in the last years (Samorajski and McCloud, 1961; Torack and Barrnett, 1964; Joó and Csillik, 1966; Joó, 1968) called, however, our attention to a possibility that the enzymes demonstrated in the cerebral capillaries play a part in regulation of the permeability conditions of the blood-brain barrier.

Firstly the non-specific alcaline phosphatase activity was described in the brain capillaries (Gömöri, 1941; Landow, Kabat and Newman, 1942). The light microscopic investigations concerning the localization of alcaline phosphatase have yielded contradictory results. According to the investigations of Landow, Kabat and Newman, 1942; Bourne, 1958; Bannister and Romanul, 1963 phosphatase activity can be found in the endothelial cells of brain vessels; according to the results of Leduc and Wislocki (1952) and Wislocki and Dempsey (1948) it is, anyway, lacking in the smaller brain and in the walls of arterioles. Where it can be demonstrated, it is localized to the connective-tissue sheath. Adenosintriphosphatase (ATP-ase) activity of capillaries was described by numerous authors (Torack, Besen and Becker, 1961; McIlwain, 1963). Butyrylcholinesterase (BuChE) activity in brain capillaries was first described by Koelle (1954). This enzyme activity had previously been regarded as a "common diffusion artifact" by Gerebtzoff (1959); later on, however, at his experiments with perfusion he got convinced of it being a real enzyme activity of capillaries. Friede and Fleming (1964) have observed an intensive cholnesterase activity in the cerebellum of 12 species.

In this present paper the fine structural localization of the above mentioned enzymes will be described as demonstrated with histochemical

methods in the brain capillaries, and their possible role will be discussed in the regulation of the permeability of the blood-brain barrier for the macromolecules.

Materials and Methods

Our studies were performed on 20 albino rats weighing 150—200 g. The brains were fixed with immersion, resp. in another case perfusion technique, in the mixture of formaldehyde-glutaraldehyde buffered with 0,14 M sodium cacodylate to pH 7,4 for 4—6 hours (Törő and Joó, 1966). In another case we have carried out a perfusion fixation with a fixing solution buffered with 0,14M sodium cacodylate made freshly according to Karnovsky (1965) from paraformaldehyde. After being fixed, our samples were rinsed in a sodium cacodylate buffer containing 0,44 M sucrose in a temperature of + 4°C, for a night. The histochemical reactions were elicited on 50 μ thick sections prepared with a freezing microtome, resp. on thin cuts made by hand with a razor blade.

For demonstrating the non-specific alcaline phosphatase activity we have used Gömöri's post-coupling method (1939), resp. its modification carried out by Fredricsson (1952). For indicating the ATP-ase activity electron histochemically, we have applied the procedure described by Padycula and Herman (1955). For demonstration of the cholinesterase (ChE) activity we have used Karnovsky's "direct colouring method" (1964) and an electron histochemical modification of the thiocholine — method, elaborated by us earlier, so-called "Pb-thiocholine" method (Joó, Sávay and Csillik, 1965; Kása and Csillik, 1966; Csillik, Joó, Kása and Sávay, 1966).

After carrying out the histochemical reactions — except the "direct colouring method" applied for demonstrating the ChE activity — and a short rinsing, we performed a convertation in a 2 p.c. ammoniumpolysulfide solution neutralized with concentrated acetic acid. The samles were postfixed for 30 minutes, in a 1,33 p.c. osmium solution buffered with s-collidine, on +4°C (Benneth and Luft, 1959), and embedded in "Durcupan" (Fluka) after gradual dehydration with alcohol. Thin sections were made in ultramicrotome of types LKB and Porter-Blum, placed on non-coated (300 mesh) grids. Our preparations have been investigated with a table electron microscope Teesla 242D, without being contrasted or, in another case, after Reynolds's (1963) Pb-cirate contrasting.

Results

We found enzyme activity first of all in the cytoplasm of the endothelial cells of brain capillaries, after we had applied Gömöri's method (1939) for demostrating the non-specific alcaline phosphatase activity. The final product of reaction fills in the cytoplasm in the form of an electron dense precipitate of 400—800 Å in diameter, being present here and there in the subendothelial basement membrane, as well (Table 1.A). In another case, an enzyme activity could be observed in the cytoplasm, mainly localized in the pinocytotic vesicles (Table 1.B). With Gömöri's method modified by Fredricsson (1952), — as used generally for light microscopic investigations — the enzyme activity was found in the field of the endothelial cytoplasm in the form of a rough precipitate (Table 1.C). The electron dense precipitate is localized in the surface membrane of the endothelial cell and in the pinocytotic vesicles of cytoplasm. In the basement membrane, there can sometimes be found some precipitate of much weaker density which is, supposedly, an artefact produced in the course of the histochemical reactions.

The adenosintriphosphatase activity was found in the basement membranes of brain capillaries, as we used the post-coupling

method described by Padycula and Herman (1955). The enzyme activity takes place continuously tracing out well the course of basement membrane (Table 2.A,B). In the cytoplasm of the endothelial cell we have not found any enzyme acivity either in the mitochondria or in the pinocytotic vesicles. In the surface membrane of astrocyte end-feet that surround the capillary we have observed ATP-ase activity, as well.

At using the "direct colouring" method, we have found cholinesterase activity with butyrylthiocholineiodide substrate in the capillary wall (Table 3). The final product of reaction can be observed seriatim in the basement membrane, in the form of an electron dense precipitate 800—1500 Å in diameter. In the cytoplasm of endothelial cell with a butyrylthiocholineiodide substrate we have not observed any enzyme activity. Applying, however, acetylthiocholineiodide as a substrate, we have found a ChE activity both in the cytoplasm and in the basement membrane of the endothelial cell. The end-product of reaction can be observed both in the pinocytotic vesicles (Table 4.A) and in the basement membranes (Table 4.B,C). The final product of reaction is indicating the place of acetylcholinesterase activity in a characteristic way and localized seriatim, even with an acetylthiocholineiodide substrate, in the form of an electron dense precipitate of 100—1500 Å diameter.

Applying methods of "Pb-thiocholine", we have found cholinesterase activity in the cytoplasm of endothelial cell, with butyrylthiocholineiodide substrate (Table 5.A). Electron dense precipitate that would refer to a final product of reaction was not found in other parts of capillary, so not in the glial end-feet close to the basement membrane, either (Table 5.B). Butyrylcholinesterase activity was not found in the field of pericytes, either (Table 5.C). In case of an optimal incubation time, enzyme activity can be observed only in the pinocytotic vesicles (Table 5.D). After performing the procedure for indicating the acetylcholinesterase activity, we have not got any final production of reaction in the cytoplasm of endothelial cells, in connection with a structural element. The electron dense precipitate in preparates like these (Table 6.A,B) took place in basement membrane of the capillary wall. With acetylthiocholineiodide susbstrate, first of all in case of a longer incubation, we have found an enzyme activity also bound to the surface membrane of cytoplasm of endothelial cells (Table 6.C)

We want to mention concerning the localization of ChE activity in the pinocytotic vesicles that, in the course of our investigations, we have found both vesicles showing a butyrylcholinesterase activity and those showing no enzyme activity. We do consider as imaginable that in the endothelial cell not every pinocytotic vesicle has BuChE activity; it seems, however, to be more probable that the pinocytotic vesicles that do not show any enzyme activity, develop during the electron histochemical procedure. As it is known, it is demonstrated by Reale and Luciano (1964) about osmium fixation after the application of the histochemical method, and Kalimo and co-workers (1968) about contrasting with Pb-citrate that they can solve a part of the final production of reaction containing heavy metal, being thus capable of resulting in a "false negative" reaction.

Discussion

The results of investigations in the last years are referring to that the enzymes demonstrated in the brain vessels with histochemical methods can play a role in regulating the permeability relations of the blood-brain barrier. Samorajsky and McCloud (1961) investigating the connection between the phosphomonoesterase activity of cerebral capillaries and the permeability state of cerebral vessels have found so that under experimental conditions (brain oedema, meningeoma, meningo-encephalomyelitis, etc.) where the permeability of the hematoencephalic barrier increases, at the same time, in a characteristic way, also the alcaline phosphatase activity of brain vessels intensifies. Alcaline phosphatase activity, according to the results of our electron histochemical investigations, is localized in the cytoplasm of the endothelial cell. The localization of alcaline phosphatase activity in the pinocytotic vesicles could be well observed particularly if a short incubation time was used. The ultrastructural localization of the enzyme is in harmony with several earlier observations concerning the connection between the alcaline phosphatase activity and the transport phenomena in the capillary and the energy supply of that process (Landow, Kabat and Newman, 1942; Wislocki and Dempsey, 1948; Bourne, 1958; Nandy and Bourne, 1963).

According to the observation of Torack and Barrnett (1964), the adenosintriphosphatase activity is localized in the basement membrane and the surface membrane of glial end-feet in capillaries of brain fields where the blood-brain barrier is functioning. On the other hand, in the cerebral structures that are not defended by the blood-brain barrier, as well in the capillaries in other tissues, the ATP-ase activity can be demonstrated with electron histochemical method in the pinocytotic vesicles of the cytoplasm of endothelial cell (Marchesi and Barrnett, 1964). According to the electron histochemical data of Rechardt and Kokko (1967), however, the ATP-ase activity can be found in the capillaries of the medulla oblongata of rats, like a coarse final product, both in the pinocytotic vesicles of endothelial cells and in the basement membrane. According to Hoff's re-investigation (1968), in the intracranial vessels of rabbits an enzyme activity can only be observed if adenosintriphosphate or adenosindiphosphate is used as a substrate. The main part of the final product of reaction localized in the basement membrane from the elements of the capillary wall. According to our own investigations, using the method described by Padycula and Herman (1955), as well, in the brains of rats the ATP-ase activity is localized in the basement membrane of the endothelial cell and in the surface membrane of adjacent glial and-feet; in accordance with the original observation of Torack and Barrnett (1964), applying Wachstein-Meisel's method (1957) in their work. The different result of Rechard and Kokko (1967) may probably be explained so that these authors wanted to demonstrate first of all the ATP-ase activity of mitochondria sensitive to fixatives. Therefore they have carried out a formaldehyde perfusion lasting but a very short time (10 minutes), having visualized even the weak ATP-ase activity of the endo-

thelial cytoplasm. This result calls our attention to the fact that a weak ATP-ase activity can be found in the pinocytotic vesicles of vessels of the central nervous system, too; its function, however, is destroyed by being completely fixed as a consequence of striving after a perfect basic structure. Thus the apparently contradictory result does not change the basic observation described by Torack and Barrnett (1964).

In our earlier investigations about ChE activity of the brain capillaries we have found that the BuChE activity can be demonstrated only in capillaries of the brain fields defended by the blood-brain barrier (Joó and Csillik, 1966). In the course of our ontogenetical investigations, we have found a correlation between the development of the BuChE activity of brain vessels and the time of the blood-brain barrier complexed (Joó, Várkonyi and Csillik, 1967). As to the localization of the fine structure of ChE activity, we have not got an identical result, as using the "direct colouring" method, resp. that of Pb-thiocholine. In case of using the "direct colouring method" founded upon the reduction of ferricyanide, with acetylthiocholineiodide substrate on the surface of the endothelial cell, as well in cytoplasm and the basement membrane, alike, we find some precipitate that refers to the final product of reaction. With a butyrylthiocholineiodide substrate, supporting the data of Shimizu and Ishii (1966), we have found enzyme activity only in the basement membrane of the elements building up the capillary wall. During our investigations performed earlier with Pb-thiocholine methods (Joó and Csillik, 1966; Kása and Csillik, 1966; Joó, 1967), and also we in this study, have observed BuChE activity in the cytoplasm of endothelial cells, as localized mainly in the pinocytotic vesicles, and acetylcholinesterase activity in the basement membrane. At demonstrating Che activity with two different methods, we have obtained two different results concerning the localization of enzyme. As to the real cause of that, we are uncertain at present. It can be imagined that the potassiumferricyanide applied by the "direct colouring" technique has a peculiar affinity to a component of basement membrane, but it is possible, too, that at applying the method of Pb-thiocholine, the differing picture is resulted by the subsequent ammoniumpolysulfide convertation. For recognizing the precise localization of ChE activity in the cerebral capillaries, further investigations are necessary during which we can research the fine structural localization of enzyme with other methods, too, described for demostrating ChE activity in an electron histochemical way. We want to note that Shute and Lewis (according to non-published observations) found BuChE activity in the endothelial cytoplasm and acetylcholinesterase activity in the basement membrane, with the electron histochemical method elaborated by them — and in harmony with our result.

It is clear from the results described above, in which components of capillaries the non-specific alcaline phosphatase, ATP-ase, BuChE and AChE activity were found. The enzymes investigated were localized in the cytoplasm of endothelial cells, in basement membrane and in the surface membrane of glial end-feet close to the capillary. As it is known, in studying the penetration barrier of particular functioning in structures of the central nervous system, some authors held different components

of capillaries responsible for the development of the characteristic permeability relations. On the basis of electron microscopic investigations, some of them (Donahue and Pappas, 1961; Muir and Peters, 1962) consider the close connection of the endothelial cells with the so-called "tight junctions" as a morphological cause of the hemato-encephalic barrier. Others (Friedmann and Elkeles, 1954; Rodriguez, 1955) are seeing the cause of the material supply slowed down in the particular structure of the endothelial layer of brain capillaries. Niessing (1952) is holding responsible the basement membrane surrounding the capillary wall continously, for inhibiting the barrier function. On the other hand, De Robertis and Gerschenfeld (1961) as well Edström (1964) emhasize the importance of the astrocyte end-feet system, that is close fitted to the basement membrane, for the functioning of the blood-brain barrier.

On this basis, therefore, we can not determine, in which degree the different enzymes of the capillary wall are taking part in forming the penetration barrier. At studying the permeability relations of the hematoencephalic barrier we have got to a result by applying some particular enzyme inhibitors. Grieg and Holland (1949) observed an increase of permeability of hematoencephalic barrier after administering eserine, we (Joó and Várkonyi, paper in preparation) observed the increase of the permeability of the blood-brain barrier after administering specific ChE inhibitors (Mipafox and/ or BW284C51) in cause of using a sensitive method founded on the fluorescence of trypane blue. We have observed a similarly increased permeability, as well, in case of investigating the permeability conditions of the hematoencephalic barrier after the ATP-ase activity being inhibited (Várkonyi and Joó, 1968). It is therefore referred to by our investigations that the ChE and ATP-ase activity of the capillaries is — in some way — in a functional connection with the function of the permeability barrier that inhibits the macromolecular transport.

We want to notice that our result according to which the ATP-ase activity of cerebral capillaries is in connection with the barrier function, is demanding a revision of Barrnett's earlier establishement (1964) that had concluded from the localization of ATP-ase activity in the basement membrane to a faster material supply there. A connection existing between ATP-ase activity localized in the surface membrane of astrocyte end-feet round capillaries and in basement membrane and the function of blood-brain barrier is emphasized also by our latter investigations. A fine-structural alteration is namely to be observed after the ATP-ase activity being inhibited that is referring the a possibility that the activity of ATP-ase localized in basement membrane under physiological conditions may have a role in preserving the molecular organization of basement membrane (Joó, 1968).

As to the significance of enzymes, that can be demonstrated in brain capillaries with histochemical methods, in view of inducing the state of an increased permeability in case of the blood-brain being experimentally damaged, we have some further investigations in progress.

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Plate I A—B: Electron histochemical localization of the non-specitic alcaline phosphatase activity in brain capillaries of the rat. The final product of reaction (arrows) can be observed mainly in cytoplasm and basement membrane. Gömöri's method (x 35 000).
C: Electron histochemical localization of the non-specific alcaline phosphatase activity discovered with Gömöri's method modified by Fredricsson. The final product of reaction is localized in the cytoplasm of endothelial cells, first of all in pinocytotic vesicles. (x 35 000).

Plate II A—B: Electron histochemical localization of adenosintriphosphatase activity in brain capillaries, after applying the method described by Padycula and Herman. The final product of reaction (arrows) can be found in the basement membrane and in the surface membrane of glial cells. (A:x 22 000; B:30 000.)

Plate III Electron histochemical localization of butyrylcholinesterase activity in brain capillaries, after carrying out the "direct colouring" method. The final product of reaction (arrows) can be observed in the basement membrane. (x 22 000).

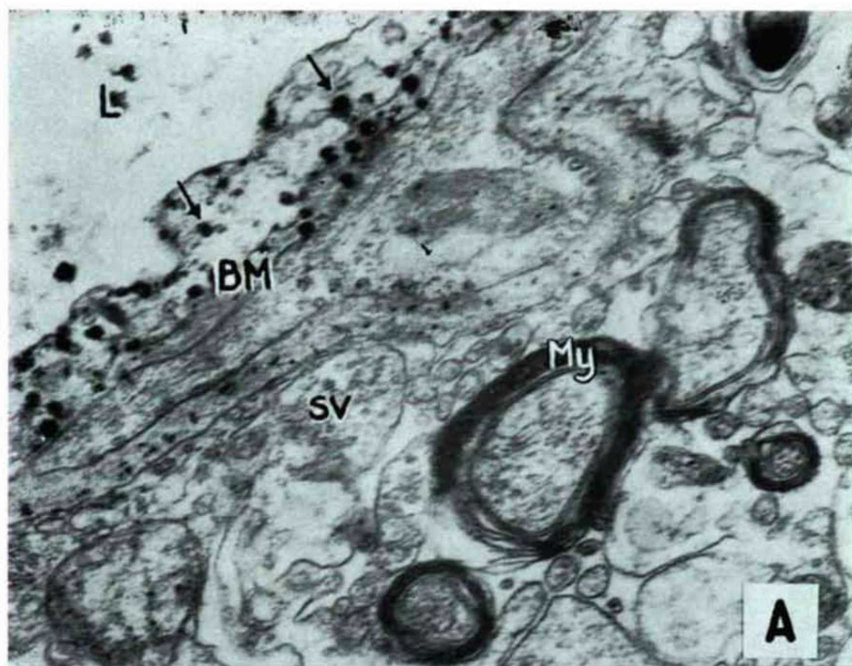
Plate IV A—C: Electron histochemical localization of acetylcholinesterase activity after applying the "direct colouring" method. The final product of reaction (arrows) can be found mainly in basement membrane but here and there also in the cytoplasm of endothelial cells, in pinocytotic vesicles (marked arrow; x 30 000).

Plate V A—D: Electron histochemical localization of butyrylcholinesterase activity, after applying the method of "Pb-thiocholine". The final product of reaction (arrows) is localized only in cytoplasm of endothelial cells, mainly in pinocytotic vesicles. (A and B:x 22 000; C:94 000; D:110 000). Figs. C and D were prepared by an electron microscope JEM 6 C, in the Electron Microscope Laboratory of Central Medical Research Institute, Budapest.

Plate VI A—C: Electron histochemical localization of acetylcholinesterase activity after applying the method of "Pb-thiocholine". The final product of reaction (arrows) can be found mainly in the basement membrane. Enzyme activity can sometimes be observed in the surface membrane of cytoplasm of endothelial cells, as well (marked arrow). A:x 20 000; B:x 25 000; C:x 45 000).

Abbreviations:

L:lumen of capillary
End:cytoplasm of an endothelial cell
BM:basement membrane
My:myelinated axon
sv:synaptic vesicle
M:mitochondrion
pv:pinocytotic vesicle
N:nucleus
Gl:glial elements
RBC:red blood cell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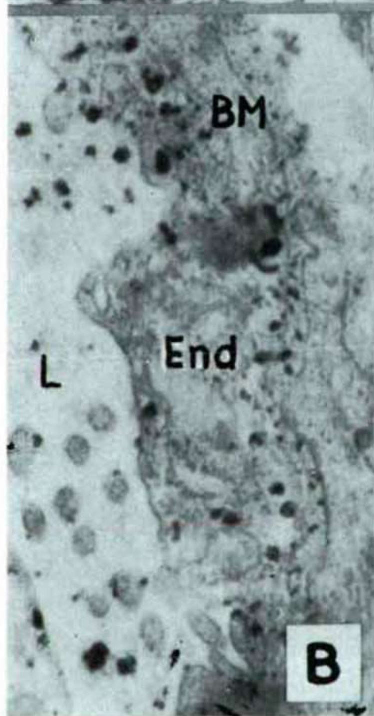
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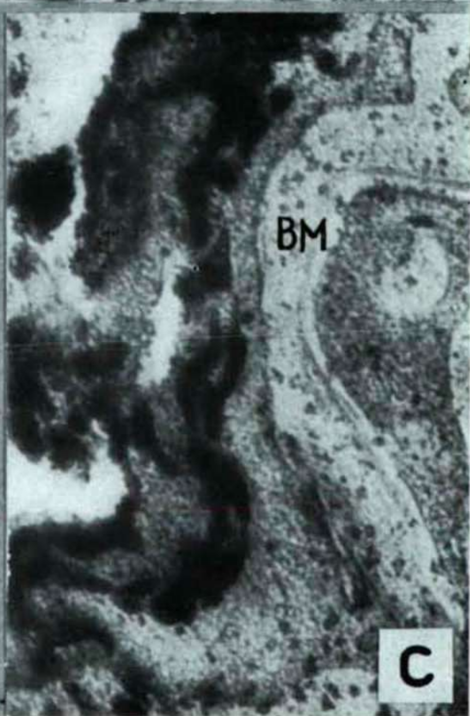
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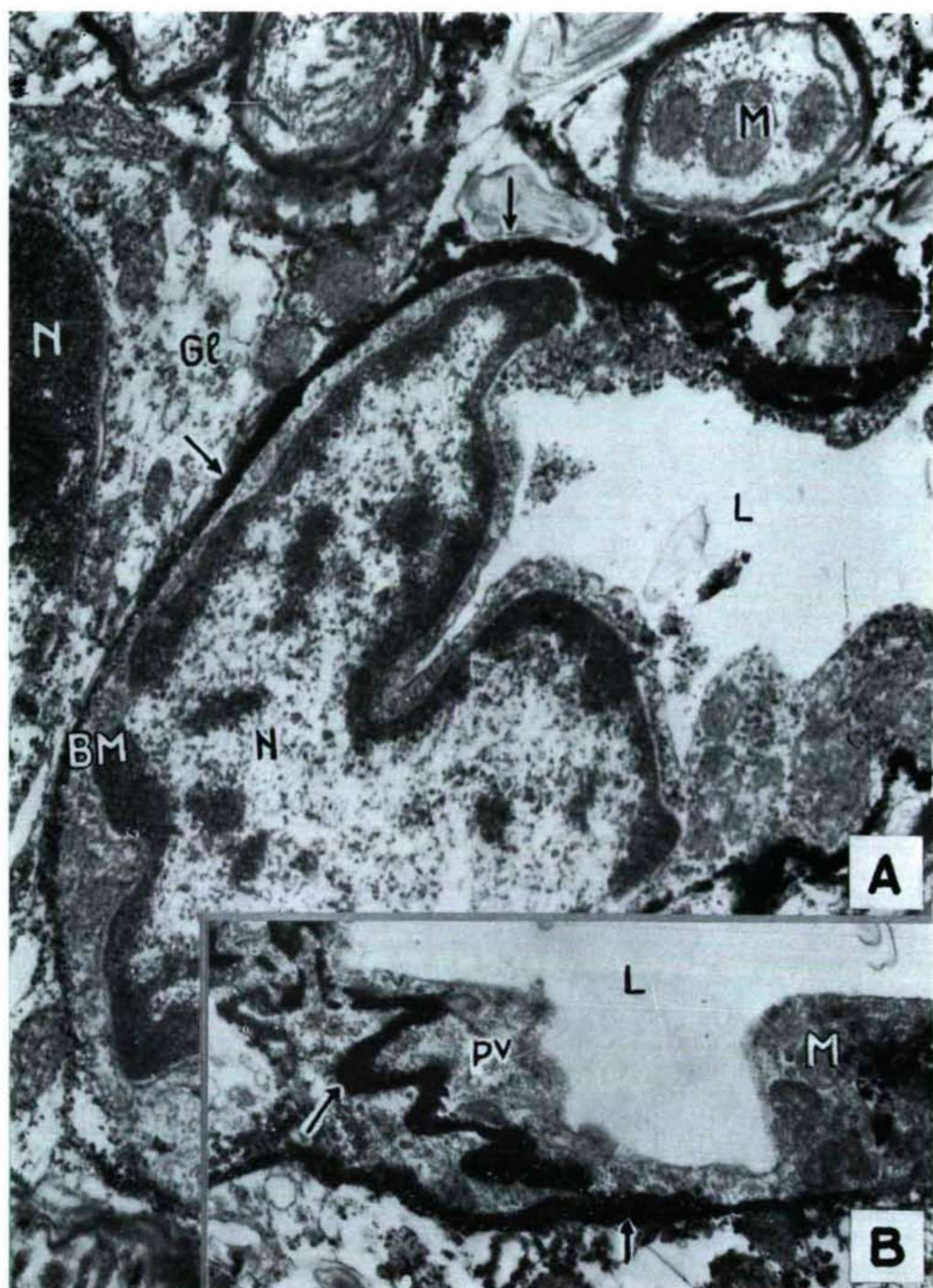
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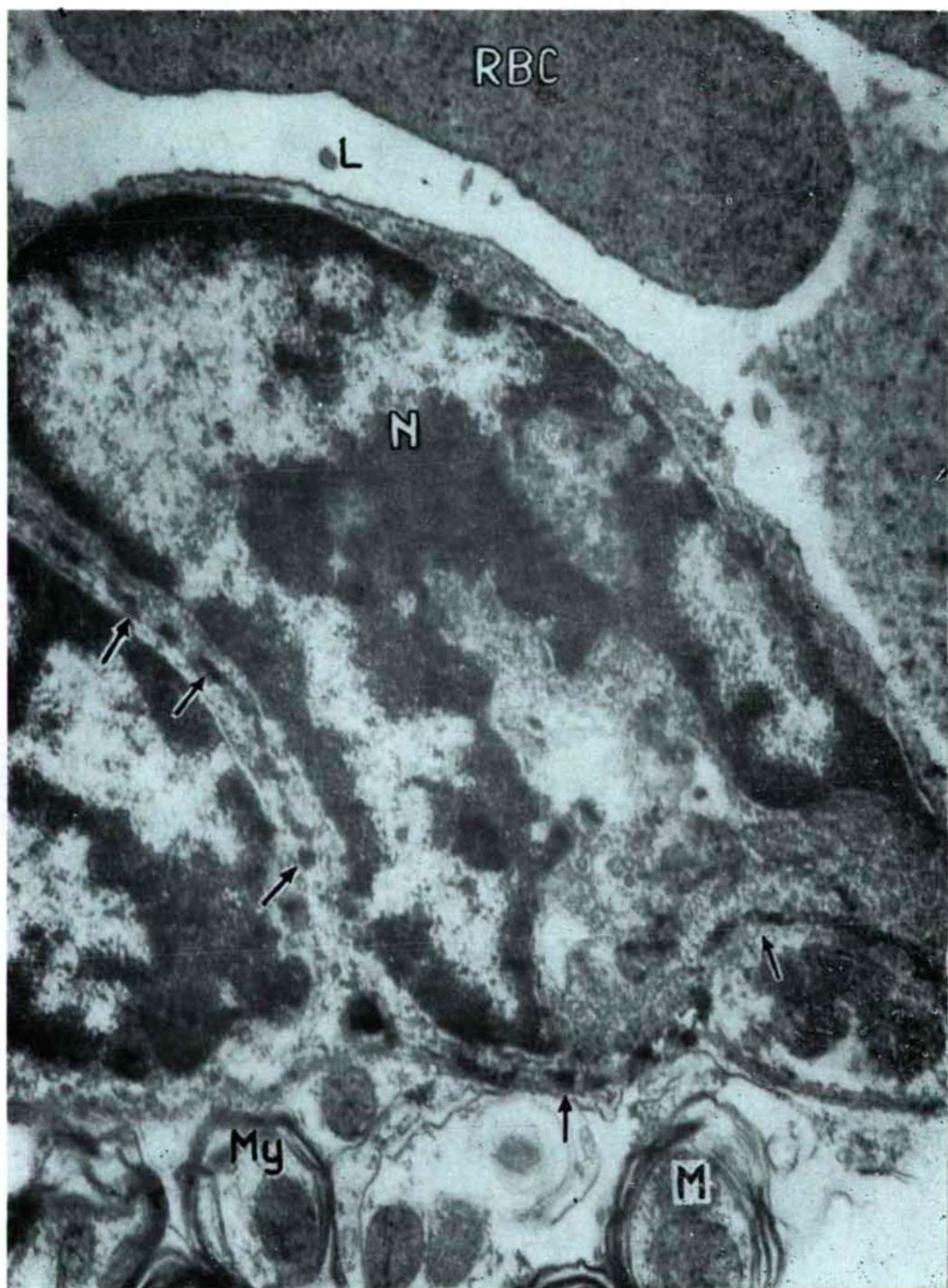
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PLATE IV

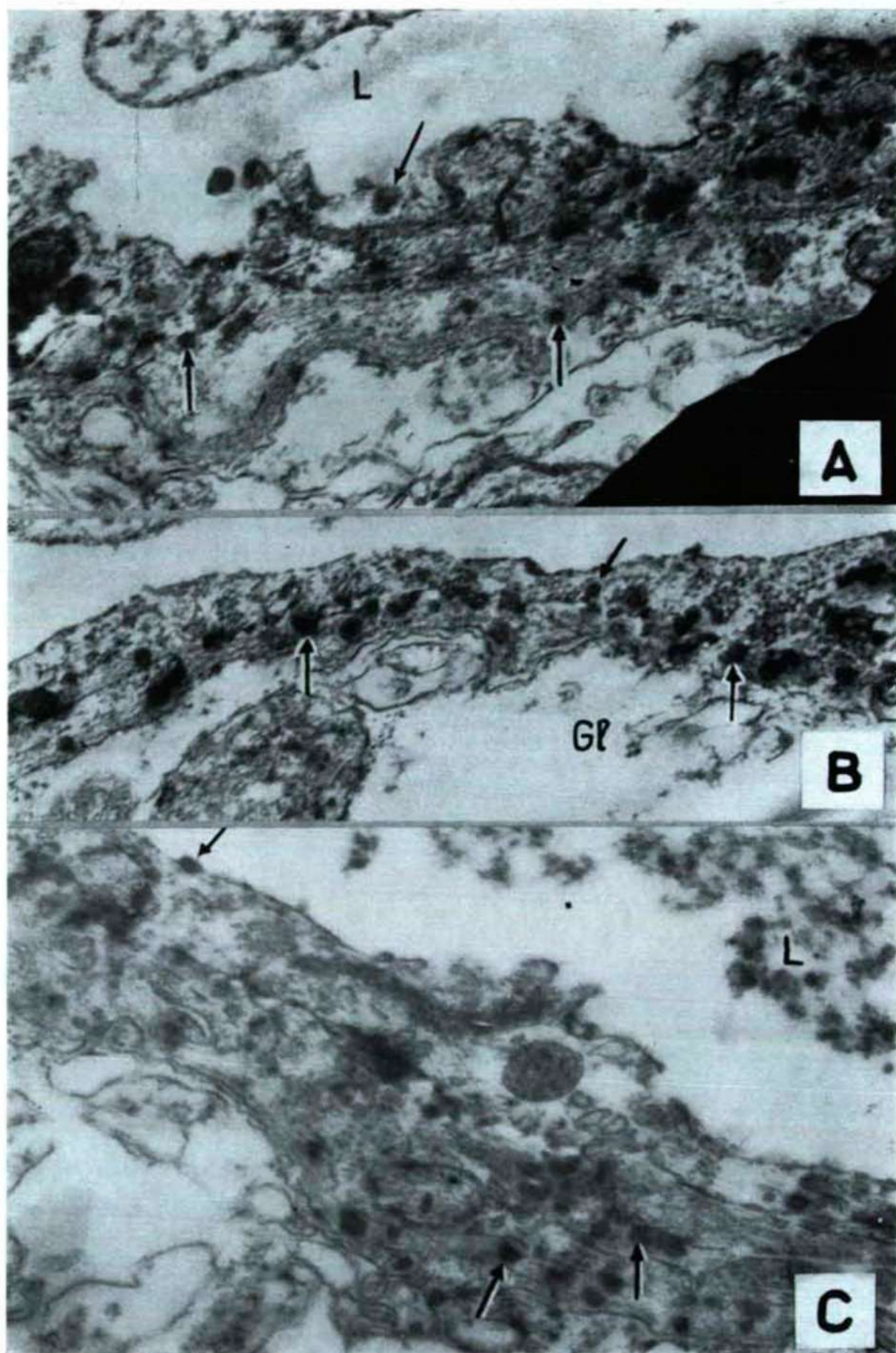
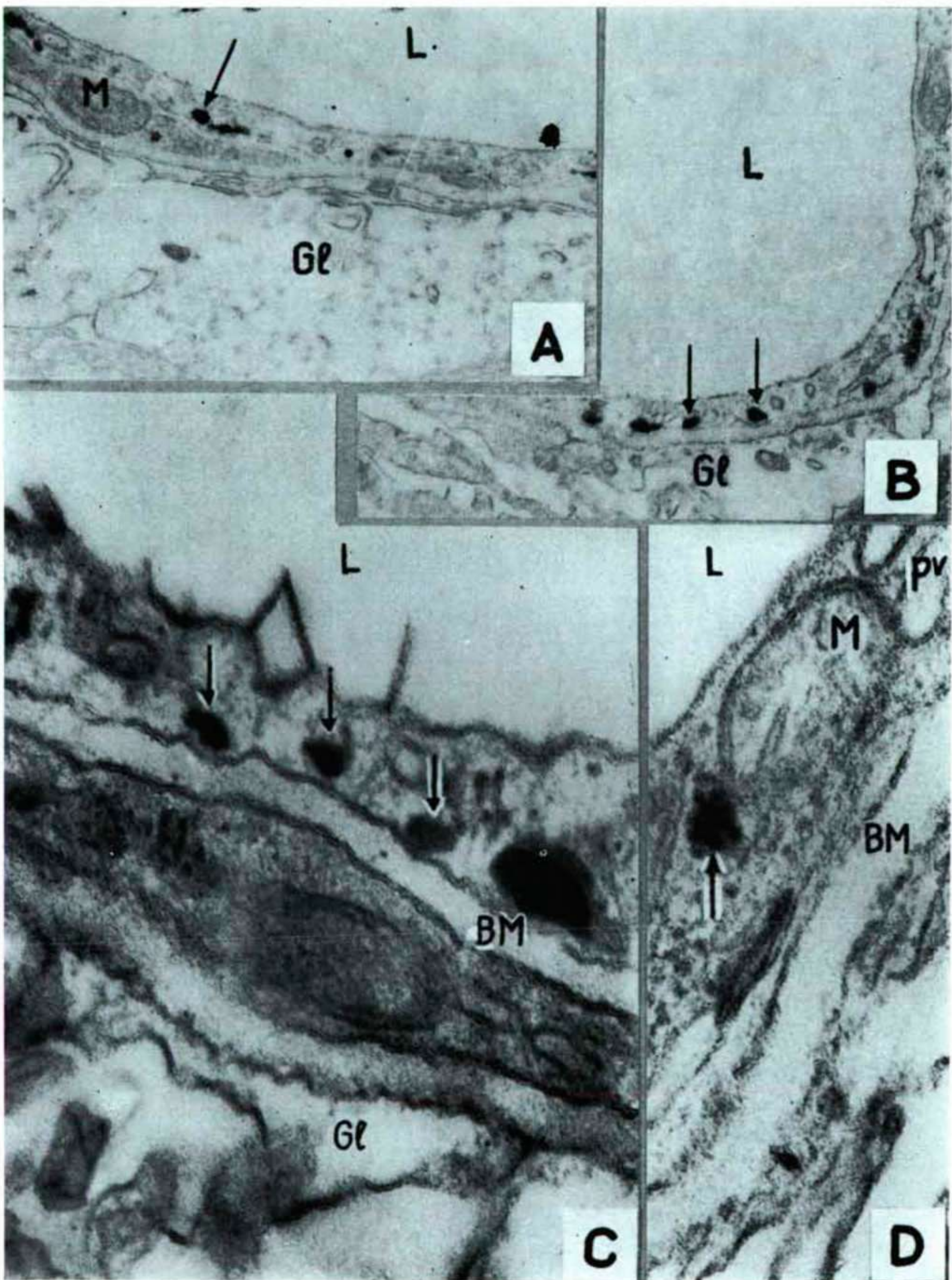
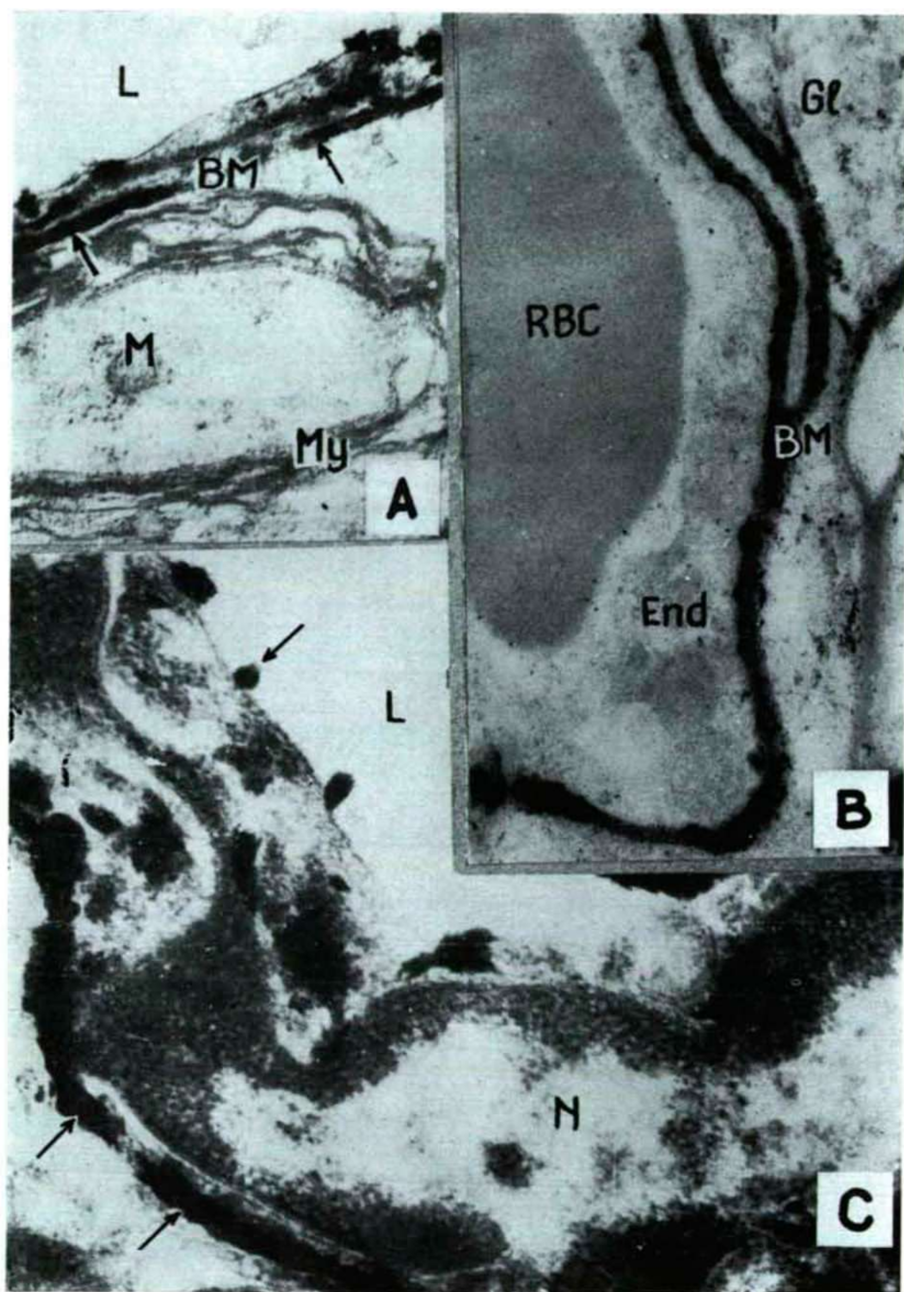


PLATE V

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