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Ultrastructural Studies on Cerebrovascular Permeability in Acute Hypertension

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Summary. Acute hypertension, experimentally induced by intravenous injection of metaraminol in adult rabbits, rapidly induced a damage of the blood-brain barrier in the cerebral cortex, as visualized by Evans-blue-conjugated albumin and horseradish peroxidase. Extravasation of these two exogenous tracers was demonstrated to occur in arterioles, in capillaries and, rarely, in venules. Peroxidase passed the endothelial cell into the nervous tissue in either of three different ways, i. e. through channels, often sigmoidshaped, in the cytoplasm, and transendothelial pinocytosis. The third pathway could, although rarely, be demonstrated between adjacent endothelial cells after cleavage of junctional complexes. The tracer peroxidase was further spread along the blood vessel within the basement membrane and in the extracellular space of the brain. Damaged endothelial cells with diffuse cytoplasmic peroxidase activity and large vesicles were occasionally observed within the areas with blood-brain barrier injury. There were also signs of increased pinocytotic activity in endothelial cells outside the barrier damaged cortical areas. Nerve cells and neuroglial cells could show either a diffuse cytoplasmic peroxidase activity or a vesicular location of the tracer, or sometimes both.

The observations are discussed in relation to previous studies on the mechanism of transendothelial passage of protein tracers at blood-brain barrier damage.

Key words: Blood-Brain Barrier — Hypertension Ultrastructure — Horse Radish Peroxidase — Evans Blue Albumin.

Introduction

Increased cerebrovascular permeability to protein and dyes has been reported to occur in chronic and acute experimental hypertension (Byrom, 1954; Rodda and Denny-Brown, 1966; Giacomelli *et al.*, 1970; Johansson *et al.*, 1970; Johansson and Linder, 1974; Onojama and Omae, 1973). It is a controversial question how the protein leakage takes place. The endothelial cells in the brain are joined with tight junctions which prevents protein tracers to enter the brain (Reese and Karnovsky, 1967). Under pathological conditions the entry could be either through the junctions or through the endothelial cells. The transendothelial passage could theoretically take place by pinocytosis, diffuse entry into the cells, or by focal breaks in the endothelial cells. Earlier electronmicroscopical studies on acute and chronic hypertension are somewhat controversial (for ref. see discussion). The aim of the present experiments was to study cerebrovascular permeability disturbances in acute experimental hypertension by use of peroxidase to trace the protein leakage at the ultrastructural level. Some preliminary results have been reported earlier (Johansson, 1974).

Materials and Methods

11 albino rabbits weighing 1.5—2 kg were anaesthetized with pentobarbital. The blood pressure was recorded electromanometrically through a catheter in the femoral artery. Evans blue, 3 ml 2% solution per kg body weight, was given as a protein tracer (Klatzo and Stein, 1966) before the blood pressure was increased by intravenous injection of metaraminol immediately followed by 300 mg horse radish peroxidase (Sigma Chemical Co., St. Louis, U.S.A. type II). 2.5 or 30 min later the animals were killed by bleeding.

The head was perfused via a canula in the ascending aorta, initially with a solution of one fourth of Karnovsky's fixative (Karnovsky, 1965) in 0.1 M cacodylate buffer for 2—3 min. Thereafter, the undiluted glutaraldehyde-formaldehyde solution of Karnovsky was used. The brain was left *in situ* for 1 hr, and then carefully removed. Small pieces of brain tissue with or without Evans blue extravasation were taken from different areas and sectioned on a Vibratome microtome (Oxford Lab. U.S.A.) set at a thickness of 10 to 20 μm. For comparison some specimens were also sectioned by means of a McIlwains tissue chopper (Mickle Lab. Ing. Comp., England). The sections were incubated in a buffered solution containing diaminobenzidine and hydrogen peroxide (Karnovsky, 1967) rinsed, postfixed in osmium tetroxide, dehydrated and embedded in Epon. Sections, 1 μm thick, were prepared on an Ultrotome ultramicrotome (LKB Instrument AB, Sweden) or a LKB Pyramitome and observed in a phase contrast microscope. Selected areas were further sectioned and observed in a Siemens I A electron microscope, sometimes after double staining with uranylacetate and lead citrate.

Control rabbits were treated as above but not given metaraminol.

Results

The initial mean arterial blood pressure was 85—95 mm Hg, which rapidly increased to 150—170 mm Hg after the metaraminol injection. The rabbits did not show any signs of adverse reaction to the injection of peroxidase. 5—10 small bilaterally scattered blue spots $^1/_2$—2 mm in diameter were seen in the cerebral cortex in acute hypertensive animals. The samples were taken from these spots and from unstained areas. Evans blue and peroxidase showed the same pattern of extravasation.

Light Microscopy. When a McIlwain's tissue chopper, set at 100 μm, was used, the specimens were stained only on the surface but remained unstained in the central parts. The specimens prepared from the Vibratome sections were stained through their whole thickness, and interpretable, stained sections could be obtained from all blocks from the brains and the amount of horseradish peroxidase injected could be kept low in order to avoid adverse reactions towards the injected tracer. The blood vessels, neurons and glial cells remained unstained in the controls, i.e. which did not get any metaraminol.

In blue-stained areas, the basement membrane of blood vessels as well as endothelial cells and a few pericytes were stained already 2 min after metaraminol injection. The tracer substance was observed in arterioles, capillaries and scattered venules. In several areas, nerve cells showed a generally increased electron opacity of their cell bodies, axons and dendrites (Fig. 1). The tracer was also observed, although to a smaller extent, in distinctly outlined vesicles 2, 5 and 30 min after metaraminol injection. The glial cells remained, with a few exceptions, unstained.

Electron Microscopical Findings. Occasionally, pinocytotic vesicles in endothelial cells in the controls contained peoxidase, but there was no extravasation or activity in the basement membrane. In animals made hypertensive, peroxidase passed from the lumen of the small cortical cerebral blood vessels to their basement membranes within 2 min after metaraminol injection by 2 different pathways, either between adjacent endothelial cells, or by transport through the endothelial cells. Scattered junctional complexes were observed to be filled with electron opaque reaction products in arterioles, capillaries and occasionally venules. Several vesicles and scattered larger invaginations, usually filled with electron

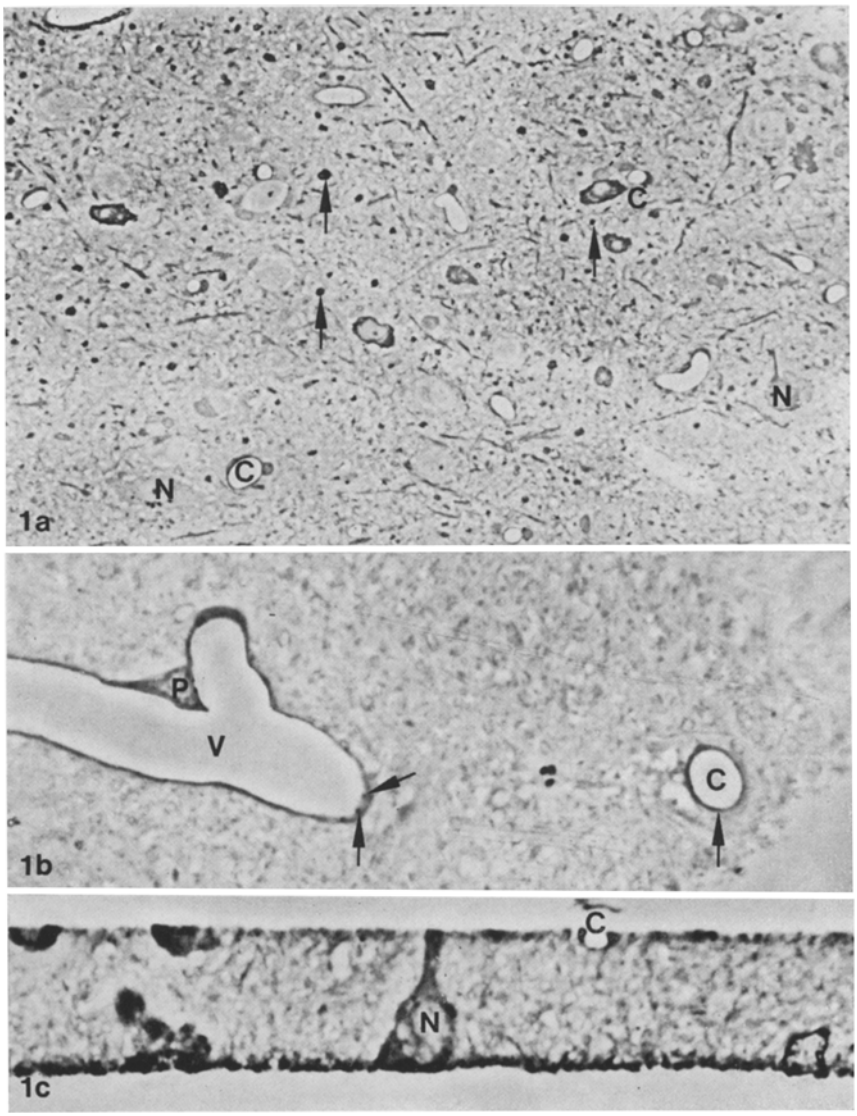

Fig. 1. (a) Survey picture of a 1 μm thick section of a cortical area with Evans-blue and peroxidase extravasation. A few nerve cells are stained dark in their cytoplasm due to diffuse uptake of peroxidase. Their processes are seen as small dark dots in the neuropil (arrow). The endothelial cells contain several dark dots and the basement membranes are distinctly outlined. Light microscopy ×220 (b) Higher magnification of a 1 μm section showing a capillary (*c*) and a venule (*v*) 5 min after injection of metaraminol. Both basement membranes are stained with peroxidase (arrow), Note the dots in the pericyte (*p*). Light microscopy ×1000 (c) Cross section of a Vibratome slice, which is stained all through. The large nerve cell (*N*) is diffusely stained in its cytoplasm. Light microscopy ×1400

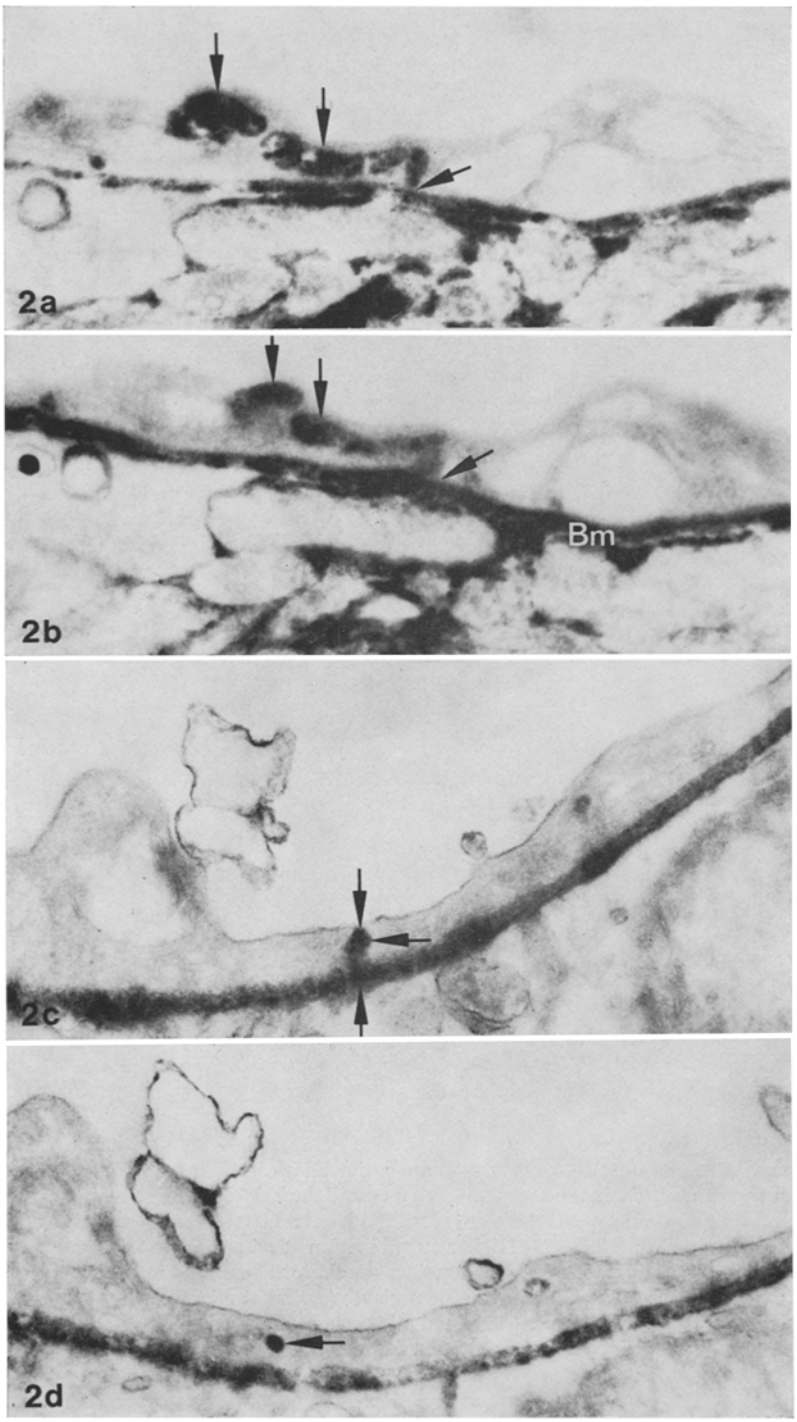

Fig. 2a–d

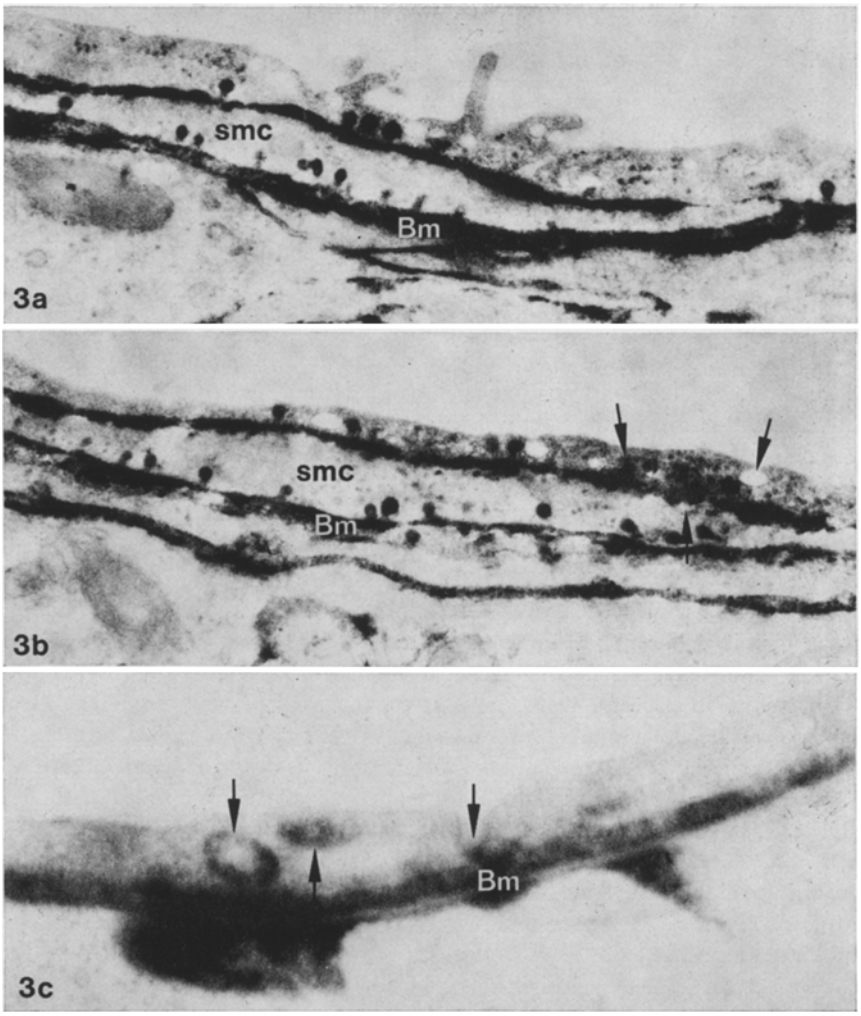

Fig. 3. (a) The endothelial and smooth muscle cells (*smc*) in an arteriole contain an increased number of pinocytotic vesicles 5 min after metaraminol injection. There are many apparently empty vesicles too in this blood vessel. The basement membranes are expanded and heavily stained. ×30500. (b) This arteriol has several pinocytotic vesicles in its endothelial and smooth muscle cells. Note the diffuse staining of the cytoplasm of the endothelial cell and the aggregation of tracer containing vesicles (arrow). ×28000. (c) A channel is apparently connecting the bottom of caveolae (arrow) with the basement membrane. A large vesicle, perhaps part of such a connecting channel, is seen to the left. Note the widening of the basement membrane space and the lack of staining of parts of the basement membrane. ×47000

Fig. 2. a–d The same area in adjacent sections of a capillary 2 min after metaraminol injection. A sigmoid-shaped channel (arrow) is seen to be connecting the vascular lumen with the basement membrane. Note the increased thickness of the basement membrane in Fig. 2b, perhaps reflecting that the tracer and other substances are forced at this point into the extracellular space ×41000. A channel (arrow) is seen in Fig. 2c connecting the lumen of a capillary with is basement membrane. This channel looks like a vesicle in Fig. 2d. That means that vesicles in endothelial cells filled with tracer substance may either be interpreted as a pinocytotic vesicle or regarded as being part of a channel system. ×41000

opaque material, were seen within the cytoplasm of the endothelial cells (Figs. 2 and 3). Some invaginations looked like bent channels with a diameter of 600 Å to 3000 Å penetrating through the hole thickness of the cytoplasm. Only a few endothelial cells were observed in the present study to have diffuse staining of its cytoplasm (Fig. 4). The number of pinocytotic vesicles seemed to be increased in the endothelial cells both in Evans blue stained and unstained areas. However, electron opaque reaction products were only seen in vesicles from the Evans blue stained areas. The basement membrane showed an increased electron opacity. Scattered nerve cells showed a strongly increased electron opacity both of their cytoplasm, nuclei, axons and dendrites. In the neuropil scattered peroxidase activity could occasionally be seen in the intercellular spaces most marked in the synaptic clefts. Occasionally, astrocytes and a few oligodendrocytes could be observed to have electron opaque vesicles in their cytoplasm.

Five minutes after metaraminol injection there was a change in the ultrastructure of the leaking junctional complexes between endothelial cells in extravasation areas compared to the findings after 2 min. Accordingly, the membranes of the 2 adjacent endothelial cells were distinctly separated from each other indicating a cleavage in the gap junction. The intercellular space of the junctional complexes contained a varying amount of peroxidase. The cell membranes and the adjacent parts of the cytoplasm at the junctional complexes showed an increased electron opacity. The intercellular space outside the junctional complex showed less marked activity, particularly close to the basement membrane (Figs. 3 and 4). The basement membrane itself, however, contained much peroxidase. Several stained caveolae and vesicles were observed within the endothelial cytoplasm as well as in the pericytes in the leaking blood vessels, as described above 2 min after the metaraminol injection (Fig. 4). Diffuse staining of the cytoplasm of the endothelial cells, but not in the smooth muscle cells, was rarely observed in arterioles (Fig. 5).

There was no sign of the tracer in the intercellular space in the junctional complex of adjacent endothelial cells in leaking cerebral blood vessels 30 min after metaraminol injection. The endothelial cell membranes had partly lost their initially increased electron opacity within the region of the junctional complex while the basement membranes of the blood vessels remained stained. Rarely, distinctly separated junctional complexes between adjacent endothelial cell membranes could still be observed in capillaries within areas with leaking blood vessels. However, there was little if any peroxidase in the intercellular space. Glial cells commonly showed electron opaque vesicles at this time interval, in contrast to the earlier time intervals studied where such vesicles were seen only occasionally. Several

Fig. 4. (a) The endothelial cell to the right shows a large vacuole in its cytopasm not observed in the control specimens. The junctional complex is distinctly outlined by tracer substances. ×19000 (b) The vascular system occasionally exerts a heavy pressure on the endothelial cells. The endothelial cell to the left contains several peroxidase-filled vesicles and also shows a restricted area with diffuse staining of the cytoplasm (arrow). 5 min after metaraminol injection. ×41000 (c) A large defect in the endothelial cell to the right has been induced by the increased blood pressure. Note the apparent lack of cell membrane between the basement membrane and the lumen. 5 min after metaraminol injection. ×47000

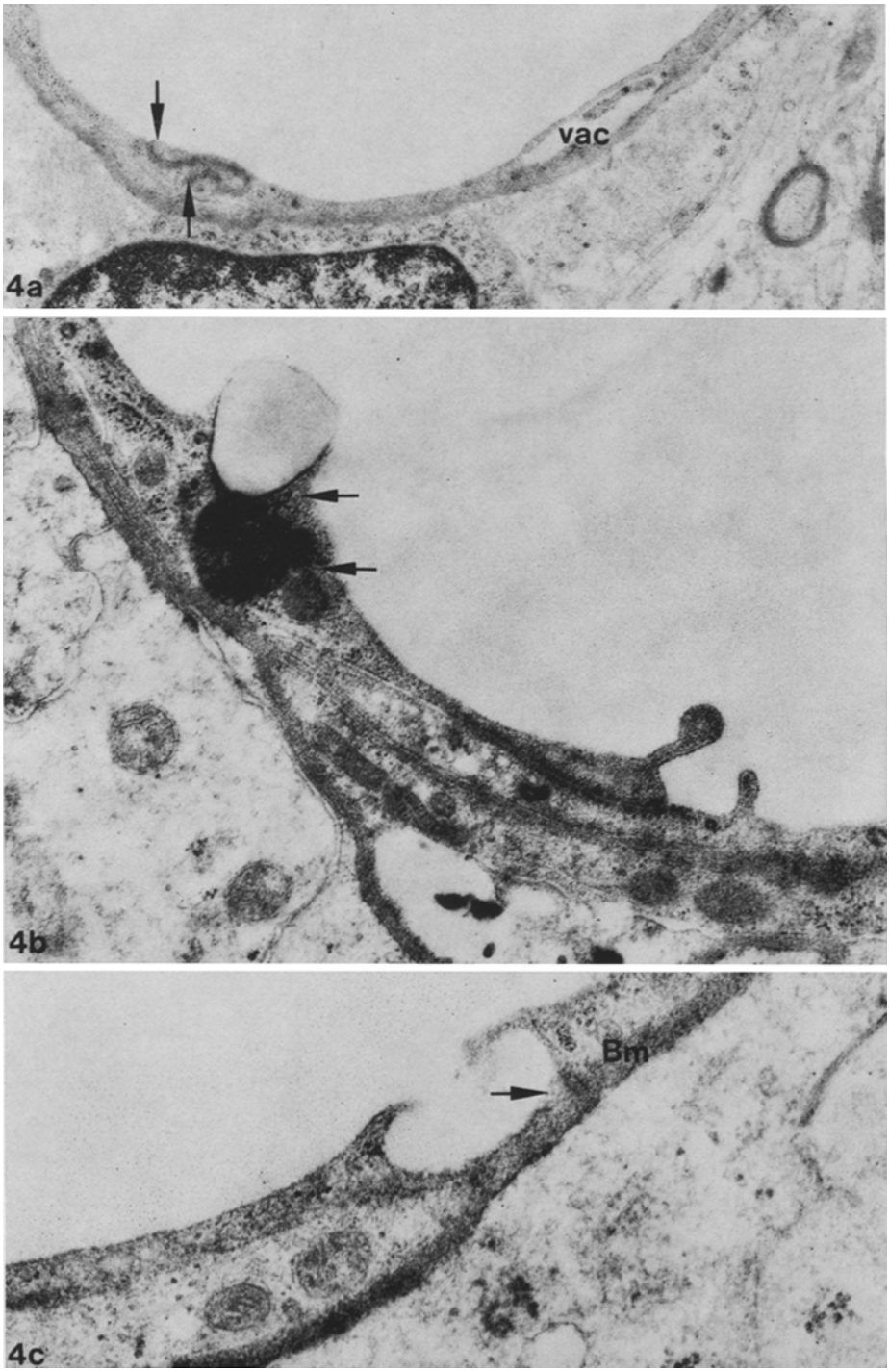

Fig. 4a—c

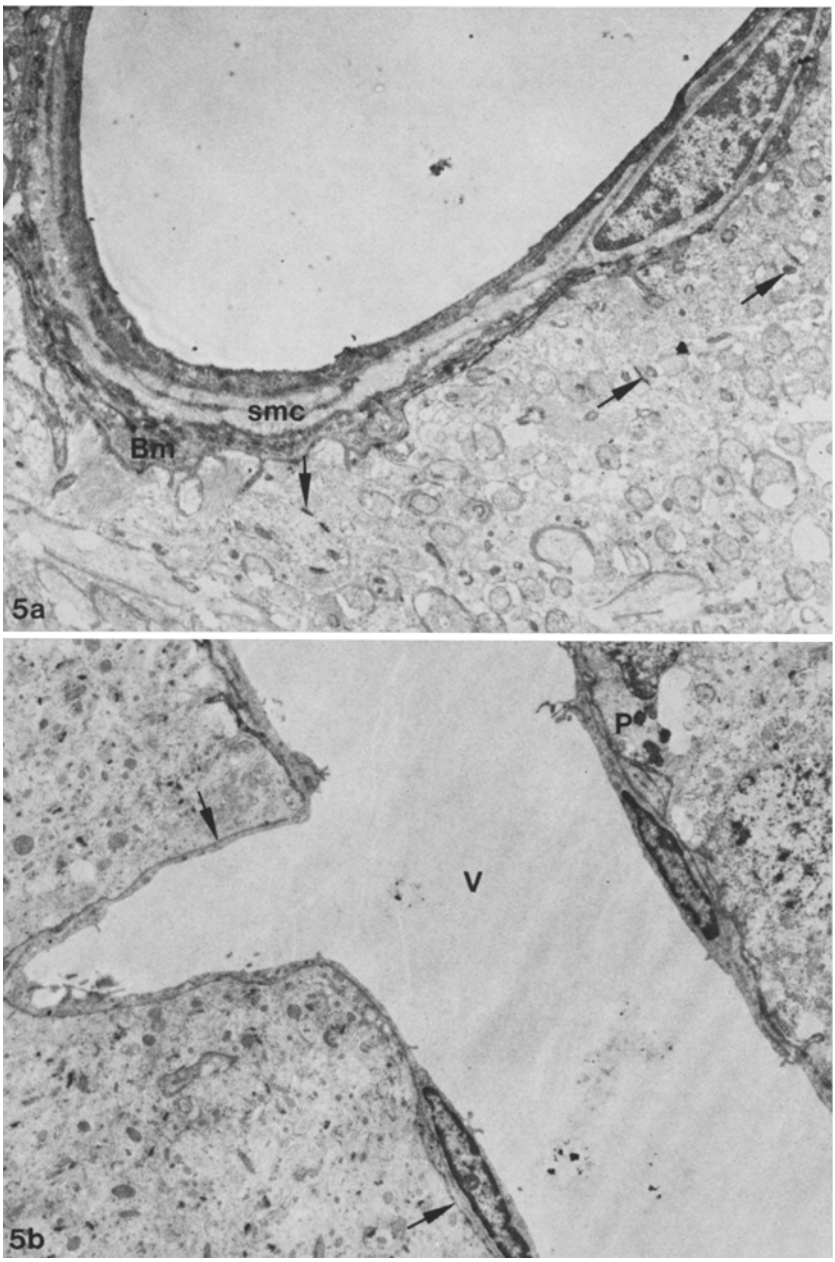

Fig. 5. (a) The cytoplasm of the endothelial cells of the arteriole in the centre shows a diffuse cytoplasmic staining due to peroxidase activity. However, the smooth muscle cells remain unstained. Note the staining of the basement membrane and of the synaptic space 30 min after metaraminol injection. ×31 000 (b) The venule in centre the has got a staining of its basement membrane and of some vesicles in a pericyte in the upper right corner. Scattered vesicles filled with tracer are seen in the cytoplasm

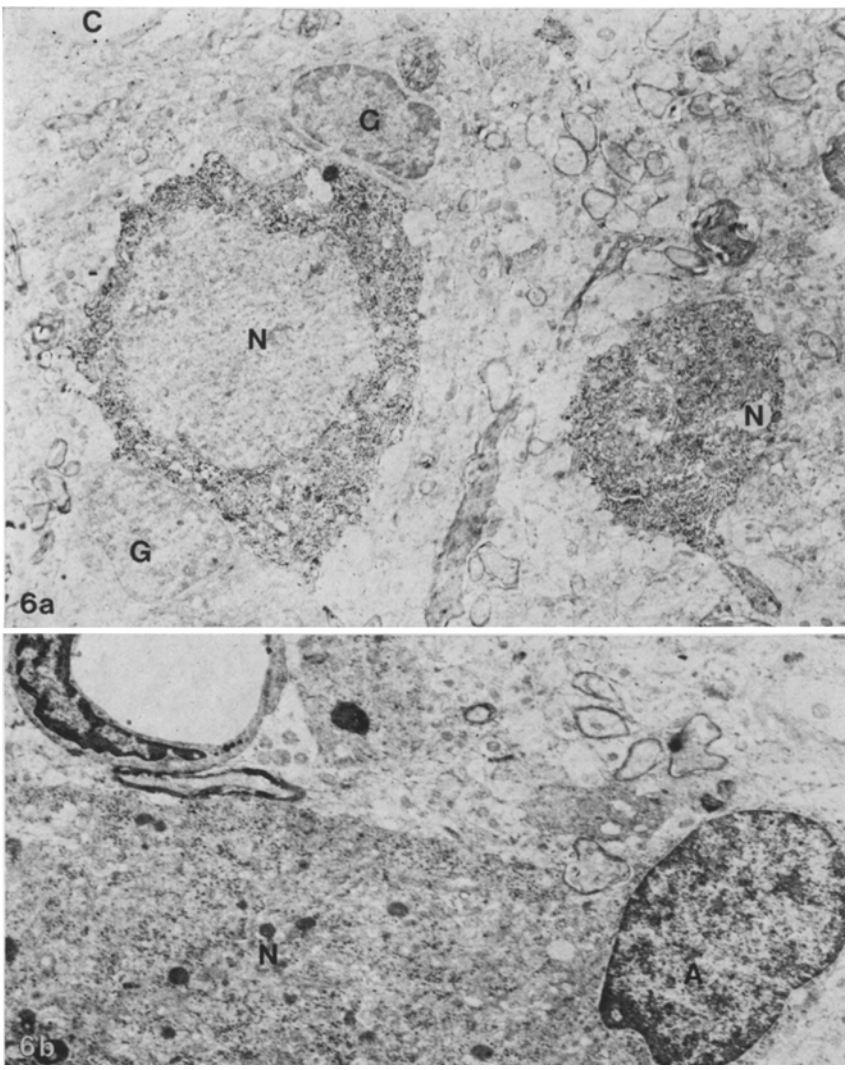

Fig.6. (a) The 2 nerve cells (*N*) both demonstrate an intense, diffuse staining of cytoplasmic organelles reflecting a leakage of the blood-brain barrier, as do the neuronal processes. There is no tracer in the glial cells (*G*). Note the dark dots in an arteriole in the upper left corner. 5 min after metaraminol injection. ×1200 (b) The nerve cell in the lower left part of the picture is diffusely stained in its cytoplasm of peroxidase. This is also true for the astrocyte in the lower right corner. Note the electron opaque vesicles in the capillary in the upper left corner. Treated as in Fig. 6a. ×1900

neurons in the extravasation areas showed a strong diffuse peroxidase activity outlining the cell organelles. Some of these cells also contained an increased number of vesicles filled with electron opaque reaction products as compared to that in the neurons after 2 or 5 min. The heavily loaded neurons (Fig. 6) were usually

seen close to blood vessels showing extravasation of tracer. However, the same staining pattern was also observed in more distant neurons with large processes closely passing a leaking vessel.

Discussion

Peroxidase, which is a protein with a molecular weight of 40000, was demonstrated both in junctional complexes between adjunct endothelial cells and in pinocytotic vesicles and channels in the endothelial cells. A split in some junctional complexes was observed to persist for at least 30 min after metaraminol injection. This change was more obvious during the first few minutes, at which time there was more peroxidase in the intercellular spaces (*cf.* Giacomelli *et al.*, 1970). The pinocytotic vesicles along the luminal border of the endothelial cells of small arteries and arterials contained peroxidase as early as 2 min after the injection. Peroxidase penetrated the gap junctions and could be seen throughout the whole length of the intercellular clefts. 30 min after the injection the pinocytotic vesicles in the luminal portion of the endothelial cells were devoid of peroxidase while those in close proximity to the basal aspects of endothelial cells were filled with peroxidase. A similar type of polarisation was observed by Giacomelli *et al.* (1970), applying renal hypertension, and by Olsson and Hossmann (1970) in acute metaraminol-induced hypertension. Increased number of pinocytotic vesicles has been demonstrated in acute and chronic hypertension (Kung *et al.*, 1968; Eto Omae and Yamamoto, 1972). Thus, the peroxidase seems to penetrate not only the junctional complexes but also the cytoplasm of the endothelial cells in both acute hypertension and in chronical renal hypertension. In the present investigation of acute hypertension peroxidase penetration could be demonstrated through all types of small cerebral blood vessels i.e. arterioles, capillaries and occasionally venules in contrast to the findings of Giacomelli *et al.* (1970). They reported in their study of chronical renal hypertension that the endothelial junctions of capillaries and venules were not penetrated by peroxidase.

In our study and in that of Giacomelli *et al.* (1970) peroxidase was seen in the junction only in animals sacrificed a 5 min after the tracer administration which might explain why it was not seen in the experiments by Olsson and Hossmann (1970) in acute hypertensive cats killed 15—45 min after the injection of peroxidase.

No previous study on the effect of hypertension on cerebral vessels has shown any diffuse uptake of the tracer of the endothelial cells, nor any disruptions comparable to what has been found in the mesenterial or renal vessels (Goldby and Beilin, 1972; Wiener *et al.*, 1969) and in 1 single case in retinal vessels (Ashton, 1972). In Ashton's view the endothelial disruption might be a short lasting phenomenon, and this would explain the difficulty to observe such discontinuity. The results obtained in the present study indicate that damage of endothelial cells may be a significant change in inducing a leakage in the blood-brain barrier. However, the channels observed filled with tracer substance occur only in a fairly limited number of endothelial cells and can thus easily be overlooked if not carefully searched for. Their relation to the canalicles called stigmata in aortic endothelium (Björkerud *et al.*, 1972) is not known.

Peroxidase was concentrated at sites known to be rich in extracellular carbohydrate-containing material, such as caveolae, pinocytotic vesicles, junctional complexes, basement membrane and in extracellular spaces, especially in the synaptic cleft. This explains why peroxidase activity could be demonstrated at these sites while it had disappeared from other parts of the intercellular spaces, as, for example, from the intercellular space between adjacent endothelial cells, between the junctional complex and the basement membrane. This physico-chemical binding of peroxidase limits the possibility to make quantitative evaluations of tracer passage in different routes.

Peroxidase activity could be seen in neurons either in a few small membrane-bound bodies in the periphery of the cytoplasm or freely in the cytoplasm outlining cellular organelles. However, most of the neurons did not contain the tracer. The significance of this finding with regard to the functional state of the neuron is under further investigation.

In conclusion, the protein tracer peroxidase can penetrate the blood-brain barrier in acute hypertension via 2 different routes. i.e. through the endothelial cells and between adjacent endothelial cells, and that the penetration occurs not only at the arteriolar level, but also in capillaries and occasionally in venules. The present method does not allow a quantitative estimation of the mechanism of peroxidase extravasation.

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