The Cellular Pathology of Experimental Hypertension

V. *Increased Permeability of Cerebral Arterial Vessels*

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THE EXISTENCE OF THE BLOOD-BRAIN BARRIER has been reaffirmed by the use of an enzymatic vascular tracer and the barrier localized to the endothelium lining cerebral vessels.[1] An increase in brain water and solutes in some instances of cerebral edema is associated with a breach in this barrier (for review see Ref. 2). The occurrence of cerebral manifestations in man and animals with severe hypertension has also been attributed to an increase in brain water content.[3–6]

Since visceral arterial vessels from animals with experimental hypertension exhibit increased permeability,[7–10] the permeability of cerebral cortical vessels from rats with severe renal hypertension has been examined in the present study. Vascular labeling techniques applied at the cellular level indicate an increase in the permeability of endothelial cell junctions of cerebral cortical arterial vessels. Ultrastructural changes in the walls of these vessels are also described.

Materials and Methods

Hypertension was induced in 100-g male Columbia Sherman rats by partially constricting the left renal artery with a silver wire clip. The systolic blood pressure of hypertensive and control animals was determined at weekly intervals.[11] The systolic pressure of the animals before operation, as well as that of unoperated controls, generally ranged from 75 to 125 mmHg. Hypertensive rats with blood pressures exceeding 200 mmHg were selected for study. Many of these animals exhibited weight loss and convulsive episodes.

Animals were sacrificed after periods of hypertension ranging from 2 to 5 weeks. Six control and 10 hypertensive animals were divided into three groups:

Group 1. Horseradish peroxidase Type II, (Sigma Chemical Co., St. Louis, Mo) dissolved in physiologic saline was injected intravenously in a dose of 15 mg/100 g body weight,[1] and the animals were sacrificed after time intervals ranging from 2 to 60 min.

Group 2. Suspensions of colloidal carbon (Pelikan C11/1431a, Gunther

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Supported in part by Grant HE-12741-01 of the US Public Health Service.

Presented in part at the Sixty-Sixth Annual Meeting of the American Association of Pathologists and Bacteriologists, March 9, 1969, San Francisco, Calif.

Accepted for publication Jan 12, 1970.

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Wagner Co., Hanover, Germany) containing approximately 100 mg of carbon per ml were injected intravenously in the amount of 10 mg/100 g body weight [12] 1 hr prior to sacrifice.

Group 3. Both horseradish peroxidase and colloidal carbon were injected intravenously as above. Colloidal carbon was given 10 min after the injection of peroxidase and the animals were sacrificed 5 and 20 min later.[13]

At sacrifice a 0.5 sq cm window was made in the left parietal portion of the skull by an electric drill, exposing the dura mater. Fixation was initiated by dripping a formaldehyde-glutaraldehyde mixture [14] on the dura for about 3 min. The dura was then easily separated from the underlying cortex without hemorrhage. Further fixation of exposed cortex was carried out *in situ* for 5 more min. The parietal cortex was quickly excised and fixed at room temperature for 4–5 hr in a fresh mixture of formaldehyde-glutaraldehyde. The tissue was then washed several times in 0.1 N phosphate buffer (pH 7.6) and stored overnight at 4°C.

Slices of the aldehyde-fixed tissue, approximately 20 and 50 μ thick, were cut on a Smith-Farquhar tissue chopper [15] from all animals receiving peroxidase. The slices were cut perpendicular to the cortical surface in order to obtain a longitudinal orientation of the penetrating vessels. They were then incubated at room temperature for 15 min in a medium containing 10 ml of 0.05 M Tris HCl buffer (pH 7.6), 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chem. Co.) and 0.1 ml of freshly prepared 1% hydrogen peroxide.[13] Substrate controls, in which 3,3′-diaminobenzidine tetrahydrochloride or hydrogen peroxide were omitted from the incubation mixture, were also run. The 20 μ-thick slices were mounted on glass slides and examined by light microscopy to monitor the reaction.

The tissues from all animals were subsequently fixed in phosphate-buffered (pH 7.6) 2% osmium tetroxide, dehydrated in acetone, and embedded in Araldite. Care was taken to maintain the longitudinal orientation of the penetrating cortical vessels. Sections (2 μ thick) were cut from plastic-embedded tissues of both the control animals and those with hypertension and examined under the phase-contrast microscope in order to locate the cerebral cortical arterial vessels as well as to assess the presence of vascular labeling by carbon. Thin sections of selected vessels were then cut with glass knives, stained with lead citrate or uranyl acetate and lead citrate, and examined with a Philips EM 200 electron microscope.

Results

Light-Microscope Observations

The oxidation of 3,3′-diaminobenzidine results in a brown reaction product that is visible with the light microscope in the blood vessels of the 20 μ-thick slices.[1] Light-microscope examination reveals that the penetrating cortical arterial vessels from control animals injected with peroxidase run a relatively straight course (Fig 1A) in contrast to the tortuosity of the corresponding vessels from hypertensive animals (Fig 1B).

Electron-Microscope Observations

Control Animals. The cerebral cortical arterial vessels have an outer diameter of approximately 15–30 μ. The intima consists of a layer of

endothelial cells and an apposed basement membrane (Fig 2–5). The endothelium is separated from the media of the larger arterial vessels, considered here as small arteries, by an internal elastic lamella (Fig 2 and 3). The media consists of either a single or double layer of smooth-muscle cells that is surrounded by a layer of basement membrane (Fig 2–5). The smaller arterial vessels lack elastic tissue, have only a single layer of smooth-muscle cells, and are considered to be arterioles (Fig 4 and 5). The adventitia consists of a thin layer of cells and fibrous connective-tissue elements derived near the surface of the brain from the pia [16] (Fig 2). The adjacent neuropile is intimately apposed to the adventitia.

Colloidal carbon, whether injected alone (Group 2) or in conjunction with peroxidase (Group 3), is not found in the walls of small arteries, arterioles, capillaries, and venules of the parietal cortex. The reaction product in small arteries and arterioles of animals receiving peroxidase (Groups 1 and 3) is mainly confined to their lumens (Fig 2–5). Peroxidase penetrates the luminal portions of the clefts between endothelial cells up to the region of the tight junction (Fig 5). The clefts on the anteluminal side of the tight junctions are devoid of peroxidase reaction product. Horseradish peroxidase is also found in a number of pinocytotic vesicles that are restricted primarily to the luminal aspects of endothelial cell cytoplasm up to 1 hr after injection (Fig 3 and 4). The distribution of reaction product in capillaries and venules of the cerebral cortex is similar to that previously described in normal mice,[1] the product being confined to the lumens and endothelial pinocytotic vesicles.

Hypertensive Animals. Colloidal carbon, whether injected alone (Group 2) or in conjunction with peroxidase (Group 3), is not found in the walls of small arteries, arterioles, capillaries, and venules in the cerebral cortex of hypertensive animals. The lumens and most of the pinocytotic vesicles in the endothelial cells of small arteries and arterioles, including those vesicles opening into lumens and subendothelial spaces, contain peroxidase as early as 2 min after injection (Group 1) (Fig 6 and 7). In contrast to the controls, the intercellular clefts on the luminal side as well as segments of the clefts on the anteluminal side of the tight junctions contain reaction product (Fig 6–8). Peroxidase filling the basal portions of the clefts is sometimes continuous with that staining endothelial basement membranes (Fig 7 and 8). Where a cleft can be examined in a single plane of section from luminal to basal aspects, peroxidase penetrates the tight junctions and may be seen throughout its entire length (Fig 9 and 10). Such junctions are only found in seg-

ments of the arterial vasculature that appear widely patent. The basement membranes surrounding smooth-muscle cells are also heavily stained by the enzyme when reaction product is present in the subendothelial space (Fig 6–10).

Considerably less reaction product is present in the lumens of small arteries and arterioles 10 min after the injection of peroxidase (Group 1) (Fig 11 and 12). The pinocytotic vesicles in the luminal portions of endothelial cells are devoid of peroxidase, while those in close proximity to the basal aspects of endothelial cells or opening into the subendothelial space are filled with peroxidase (Fig 11). In addition, the subendothelial space and basement membranes around the medial smooth-muscle cells are heavily stained. Pinocytotic vesicles in smooth-muscle-cell cytoplasm also contain peroxidase at this time. Furthermore, peroxidase is present extracellularly between cellular processes of the neuropile (Fig 12 and 13). As compared to the controls (Fig 2) there is swelling of cells and processes in the neuropile and expansion of extracellular spaces (Fig 12).

The distribution of reaction product within the capillaries and venules of the parietal cortex of hypertensive animals is similar to that of the controls. The endothelial tight junctions of capillaries and venules are not penetrated by peroxidase.

Variations in luminal diameters are observed in longitudinal sections of some of the small cerebral cortical arteries and arterioles (Fig 14). Many of the vessels have variable degrees of basement membrane thickening and replacement of medial smooth-muscle cells by granular electron-opaque material (hyalin) [17] (Fig 15 and 16). The endothelial cells lining these vessels frequently contain increased amounts of rough-surfaced endoplasmic reticulum (RER) and other organelles (Fig 17). The cisternae of RER are occasionally dilated and filled with finely granular electron-dense material. Cisternae of RER containing similar material are occasionally seen in medial smooth-muscle cells. Increased numbers of free ribosomes are present in the cytoplasm of endothelial cells and tend to be arranged in clusters (Fig 15).

Another feature of the small cerebral cortical arteries and arterioles is the presence in the luminal portions of endothelial cells of bundles of filaments arrayed parallel to the long axis of the vessels (Fig 18). These filaments, in animals injected with peroxidase (Groups 1 and 3), are seen solely in segments of vessels where the subendothelial space contains peroxidase. The individual filaments measure approximately 60–70 Å in diameter and are of an indeterminate length. Electron-dense bands of material measuring approximately 1000 Å in width are distrib-

uted along the long axis of the filament bundles. These electron-opaque bands vary from two to seven in number and have a regular spacing of approximately 0.5 μ.

Discussion

In the present study, small cerebral cortical arteries and arterioles of rats with severe renal hypertension of short duration exhibit increased vascular permeability. Horseradish peroxidase, but not colloidal carbon, escapes from the lumens of these vessels into the surrounding edematous cortical tissue. The fact that peroxidase penetrates the tight junctions of small arteries and arterioles and is also found in endothelial cell clefts on the anteluminal side of the tight junctions is significant. Since the junctions of these vessels are normally impermeable to peroxidase,[1] this observation indicates that they are sites of increased vascular permeability. The difficulty in demonstrating peroxidase throughout the entire length of the clefts from lumen to base in all instances resides in their convoluted course between overlapping endothelial cells. Appropriately oriented sections are therefore infrequent and thus only portions of the clefts are generally visible in any single plane of section. The alteration of tight junctions by the hypertensive state is such as to permit the passage of horseradish peroxidase (50–60 Å estimated diameter)[13] but not particles as large as colloidal carbon (approximately 250 Å in diameter).

The passage of fluid through the walls of the vessels by the process of pinocytosis could also play a role in the pathogenesis of the cerebral edema accompanying the severely hypertensive state. Unlike the controls, where pinocytotic vesicles containing peroxidase are located mainly in the luminal aspects of endothelium as long as 1 hr after injection, there is a change in the location of peroxidase-containing pinocytotic vesicles from luminal to basal aspects of the endothelium with the passage of time. It is not possible to assess the relative contribution of the pinocytotic process to this accumulation of edema fluid. It seems likely, however, in light of recent observations regarding fluid transport through endothelium,[1,13] that pinocytosis plays at best a secondary role in the formation of edema fluid in the cerebral cortex in severe hypertension.

Increased permeability of cerebral cortical arterioles has previously been demonstrated in the hypertensive monkey [18] by the passage of trypan blue into the walls of these vessels. Focal areas of cerebral cortical edema stained by trypan blue have been observed in the hypertensive rat, and the escape of the tracer has been attributed to increased

permeability of capillaries.[6] The present study indicates that the blood-brain barrier at the level of capillaries and venules is intact, for neither horseradish peroxidase nor colloidal carbon escapes from these vessels in the parietal cortex. These observations also exclude the histamine-induced vascular leakage that may occur as a consequence of peroxidase administration.[13]

The increased permeability of renal, pancreatic, and mesenteric arterial vessels of rats with experimental renal hypertension has been shown to be due to endothelial discontinuities that vary in extent from separations of endothelial cell junctions to loss of one to several endothelial cells.[10] The difference between the previous results and the present observations may be related to differences in the ultrastructure of the endothelial cell junctions of arterial vessels. Endothelium lining capillaries and other vessels of the cerebral cortex [1] and retina [19,20] has tight junctions that form continuous belts between adjacent endothelial cells (zonulae occludentes) and that constitute a barrier to the passage of substances measuring an estimated 50–60 Å in diameter (horseradish peroxidase). The endothelial cell junctions of visceral capillaries, in contrast to those in brain and retina, are discontinuous belts (maculae occludentes) with intervening channels measuring about 40 Å in diameter that normally permit the passage of horseradish peroxidase.[13] The ultrastructure of the endothelial cell junctions of visceral arterial vessels has not yet been defined. It appears probable, however, that the identical structure of endothelial cell junctions that is shared by the various types of vessels in brain also holds true for visceral vessels and that the endothelium of visceral arteries, in addition to that of capillaries, is characterized by maculae occludentes. If this is indeed the case, it seems likely that junctions with maculae would be more readily separated by a comparable force than junctions with zonulae occludentes. The former case is exemplified by observations showing separations in the endothelial cell junctions of such an extent as to permit not only the passage of macromolecules such as fibrinogen but also colloidal carbon and cells.[10] Alternatively, the use of larger molecules (colloidal carbon) as a vascular label could have resulted in the detection of more extensive lesions. In this regard the accumulation of colloidal iron (approximately 50 Å in diameter) within the walls of visceral vessels of rats with malignant hypertension has been shown to occur before the accumulation of colloidal carbon particles and to be associated with less severe alterations in the walls of the vessels.[9] These observations are in accord with the present study where the use of a vascular label of low molecular weight permitted the detection of an increase in vessel permeability that is not demonstrable by colloidal carbon.

The mechanisms responsible for the altered permeability of vessels in the hypertensive state are not known. Elevated blood pressure, perhaps mediated by the renin-angiotensin system, as well as nonpressor permeability factors of renal origin [21,22] may be involved. These substances may act by producing contraction of endothelial cells. Endothelial contraction has been offered as an explanation for the increase in vascular permeability and separation of endothelial cell junctions of venules that follow the administration of histamine and serotonin.[23,24] The cross-striated arrays of endothelial filaments that are seen in segments of small arteries and arterioles could represent the structural basis for endothelial contraction.[25] In this regard, actomyosin antigenically similar to that of uterine smooth muscle has recently been demonstrated in vascular endothelium.[26] If the bundles of filaments observed in luminal portions of endothelial cells are contractile elements, they could increase the tension at endothelial cell junctions. The forces of an increased hydrostatic pressure within the lumens of these vessels and/or contraction of the endothelial cells would tend to separate the membranes of the tight junctions in such a manner as to permit the penetration of exogenous peroxidase.

Alternating segments of dilatation and constriction have been observed in both visceral and cerebral arterial vessels of animals with experimental hypertension. [6,8,18,27,28] The dilated segments were found to be not only sites of increased vascular permeability, [8] but also the sole sites of vascular lesions in steroid hypertension. [28] Although it is difficult to assess constriction and dilatation in longitudinal sections of vessels following fixation, the segments of vessels in the present study that are penetrated by peroxidase have widely patent lumens. These observations may indicate a close relationship between the increased permeability of arterial vessels and the occurrence of vascular hyalinosis in hypertension.

There is no general agreement regarding the relation between edema formation and the pathogenesis of hypertensive encephalopathy. An increase in the water content of brain has, however, been found in both man and animals with severe hypertension.[3-6] The animals utilized in the present study exhibit manifestations of hypertensive encephalopathy such as convulsions. Although preservation of the neuropile is difficult to obtain by immersion fixation, there is morphologic evidence of cerebral edema in the parietal cortex of hypertensive animals as compared to the controls. If hypertensive encephalopathy is due to the accumulation of fluid within the brain, then this syndrome has its basis, at least in part, in altered permeability of cerebral cortical small arteries and arterioles. The present study does show that transient crises of

severe hypertension are demonstrably accompanied by increased vascular permeability but are not necessarily associated with severe ultrastructural alterations in the cerebral vasculature.

Summary

The permeability of cerebral cortical arterial vessels of rats with severe experimental renal hypertension has been studied by means of vascular labeling techniques (colloidal carbon and horseradish peroxidase) at the cellular level. Both small arteries and arterioles that exhibit varying degrees of hyalinization have been found to be abnormally permeable to horseradish peroxidase but not to colloidal carbon. Peroxidase penetrates the tight junction of endothelial cell clefts in such vessels and is found in the subendothelial space, the basement membranes around smooth muscle cells, and extracellularly between swollen cells and processes of the neuropile. Peroxidase reaction product is also found within pinocytotic vesicles of both endothelial and smooth-muscle cells. Endothelial contraction has been proposed as the mechanism underlying the increase in vascular permeability. The relationship between the increase in permeability of cerebral cortical arterial vessels and the accumulation of edema fluid in the brain during the severe hypertensive state is discussed. Other ultrastructural alterations in such vessels are also described.

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The authors wish to acknowledge the technical assistance of Miss Elizabeth A. Johnson and Mr. Osmay Yalis, and the critical review of the manuscript by Dr. Alden V. Loud.

Legends for Figures

Key: L Lumen
R Erythrocyte
H Hyalin

Fig 1. Fig 1A *(upper left)* and 1B *(upper right)* are light micrographs of approximately 20-μ-thick sections of cerebral cortex from control and hypertensive animals, respectively, that were injected intravenously with horseradish peroxidase. Sections were incubated in medium appropriate for producing brown reaction product at sites of peroxidase activity. Surface of cortex is at top. Compare relatively straight course of penetrating arterial vessels from control animal (A) with tortuosity of similar vessels from the hypertensive animal. × 130.

Fig 2–5 are electron micrographs from control animals injected intravenously with horseradish peroxidase.

Fig 2. *(bottom)* Lumen of this small cerebral cortical artery contains several erythrocytes and reaction product. Layer of elastic tissue *(single arrows)* is interposed between endothelium and the single layer of medial smooth-muscle cells. Adventitia *(double arrows)* is closely apposed to the media. Lead citrate. × 3000.

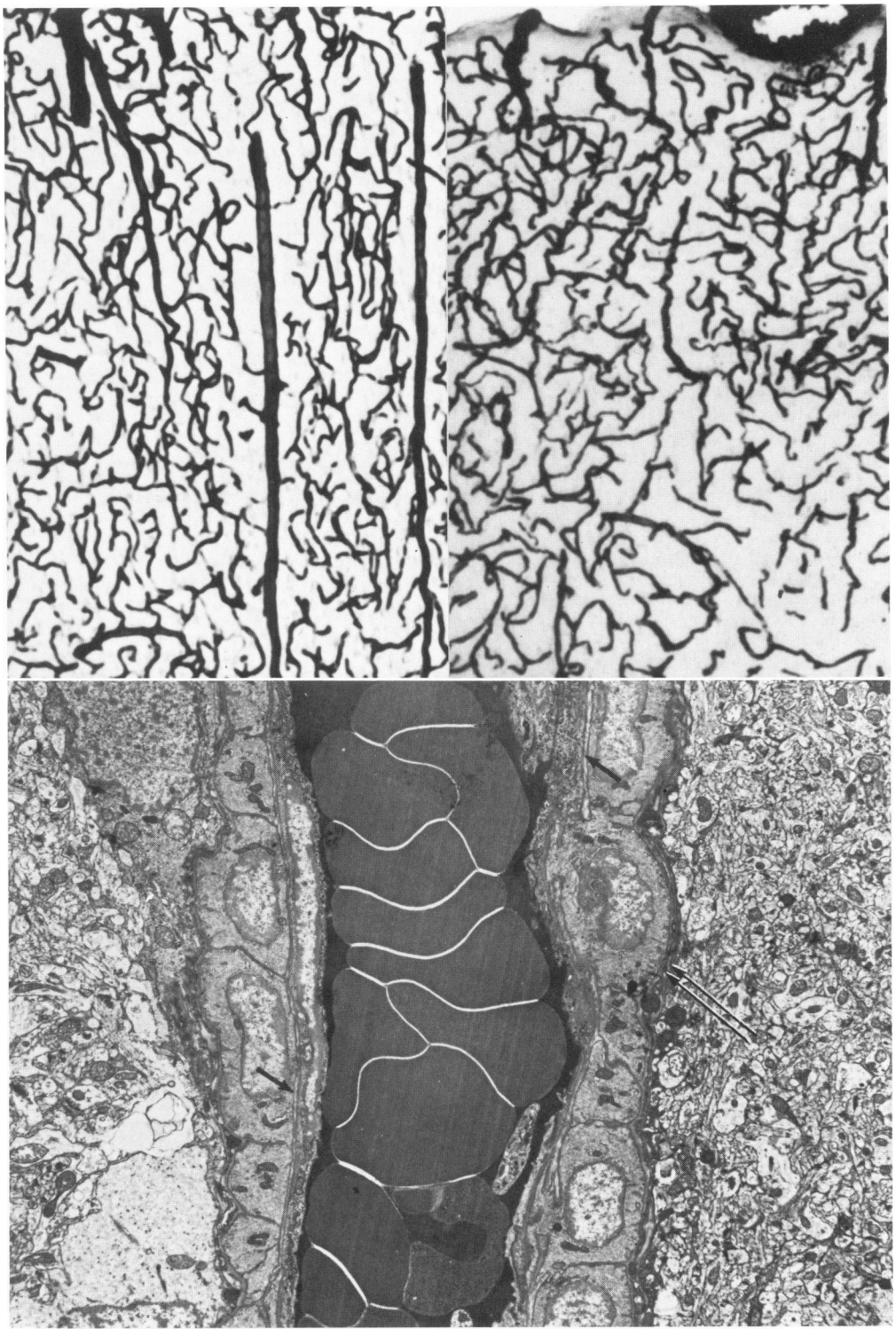

Fig 3. *(upper)* Micrograph from another portion of small artery shown in preceding figure. Peroxidase reaction product is confined to lumen and pinocytotic vesicle in luminal portions of endothelium. Note internal elastic lamella *(arrows)*. Lead citrate. × 24,000.

Fig 4. *(lower)* Micrograph of cerebral cortical arteriole. Horseradish peroxidase is seen both within lumen and in pinocytotic vesicles that lie in luminal aspects of endothelium. Lead citrate. × 20,000.

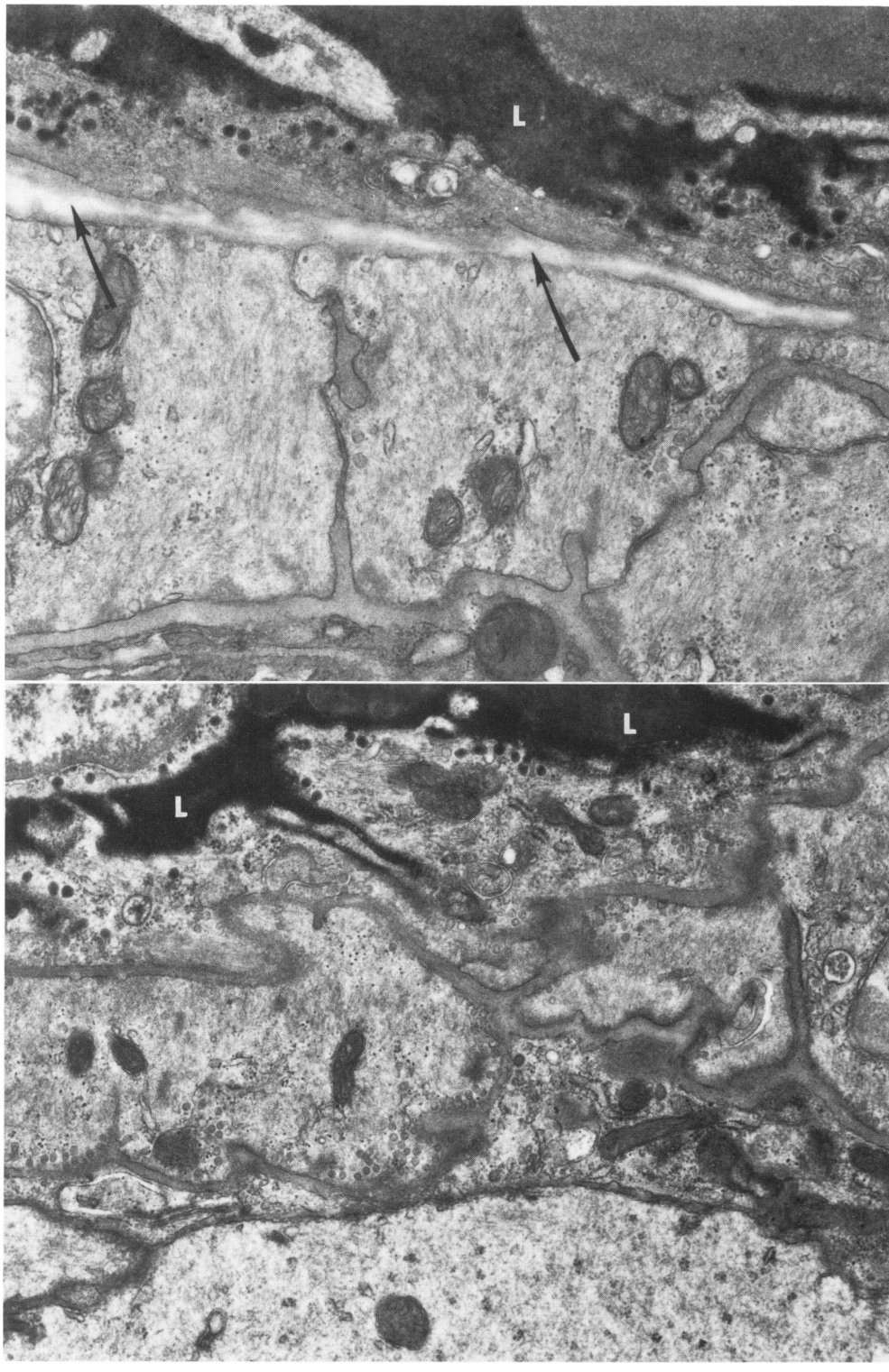
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Fig 5. *(upper)* Reaction product is present within lumen and luminal portion of cleft *(arrows)* between adjacent endothelial cells of arteriole. Remainder of cleft beyond its tight portion is devoid of reaction product. Lead citrate. × 72,000.

Fig 6–14 and 18 are electron micrographs of cerebral cortical arterial vessels from hypertensive animals injected with peroxidase. Fig 15–17 are micrographs of arterial vessels from hypertensive animals injected with colloidal carbon.

Fig 6. *(lower)* Peroxidase reaction product is seen in lumen, in pinocytotic vesicles throughout endothelium, and in subendothelial space of small artery. A few pinocytotic vesicles apposed to surface membrane of a smooth-muscle cell also contain peroxidase *(arrows)*. Two min after peroxidase administration. Lead citrate. × 25,000.

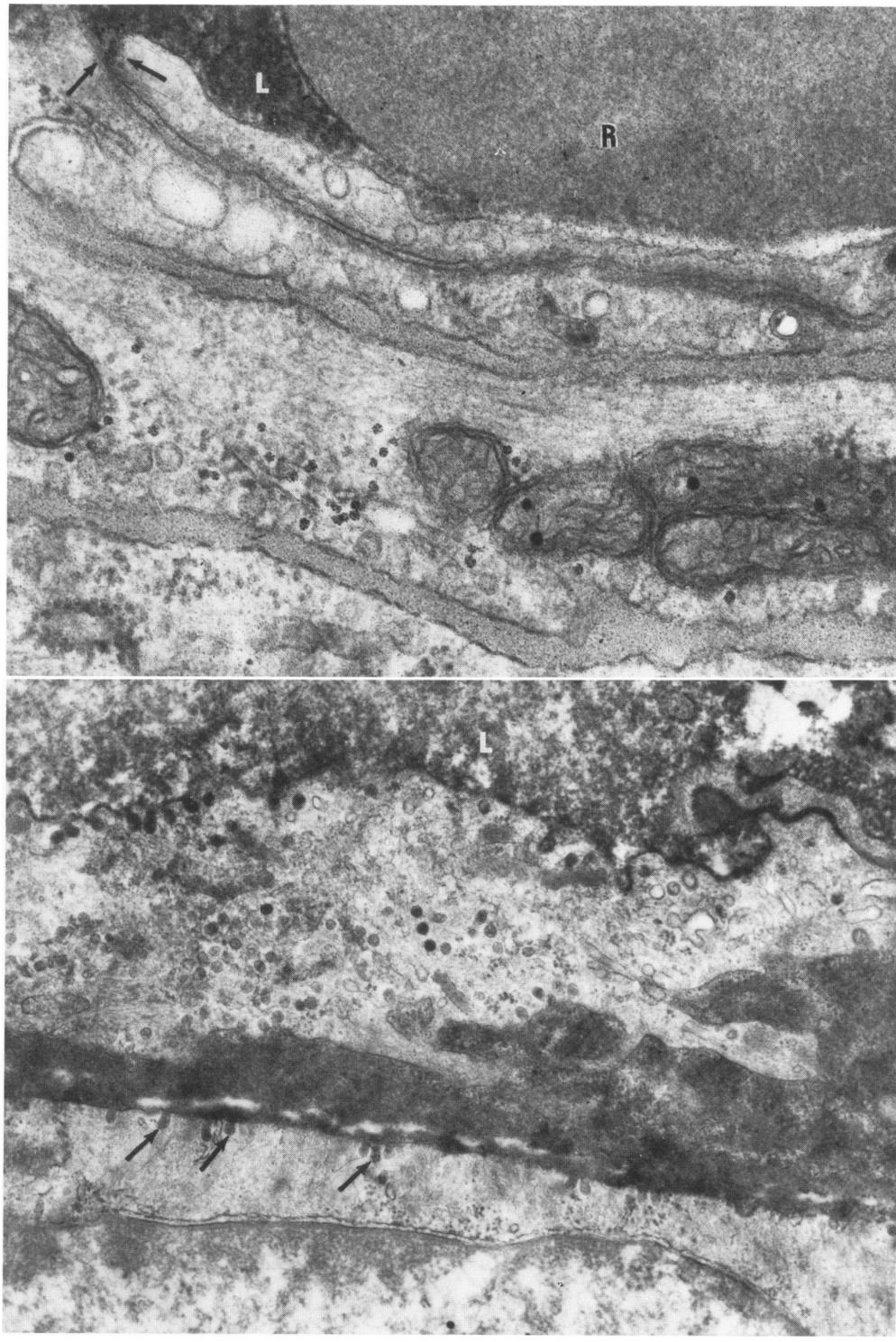
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Fig 7. *(upper)* Similar to Fig 6. Reaction product is seen in both subendothelial space and basal portion of intercellular cleft *(arrow)* that is continuous with this space. Two min after administration of peroxidase. Lead citrate. × 40,000.

Fig 8. *(lower)* Horseradish peroxidase is seen in different segments of endothelial intercellular cleft of small artery *(arrows)*. Similar material is seen in lumen and subendothelial space. Two min after administration of peroxidase. Lead citrate. × 54,000.

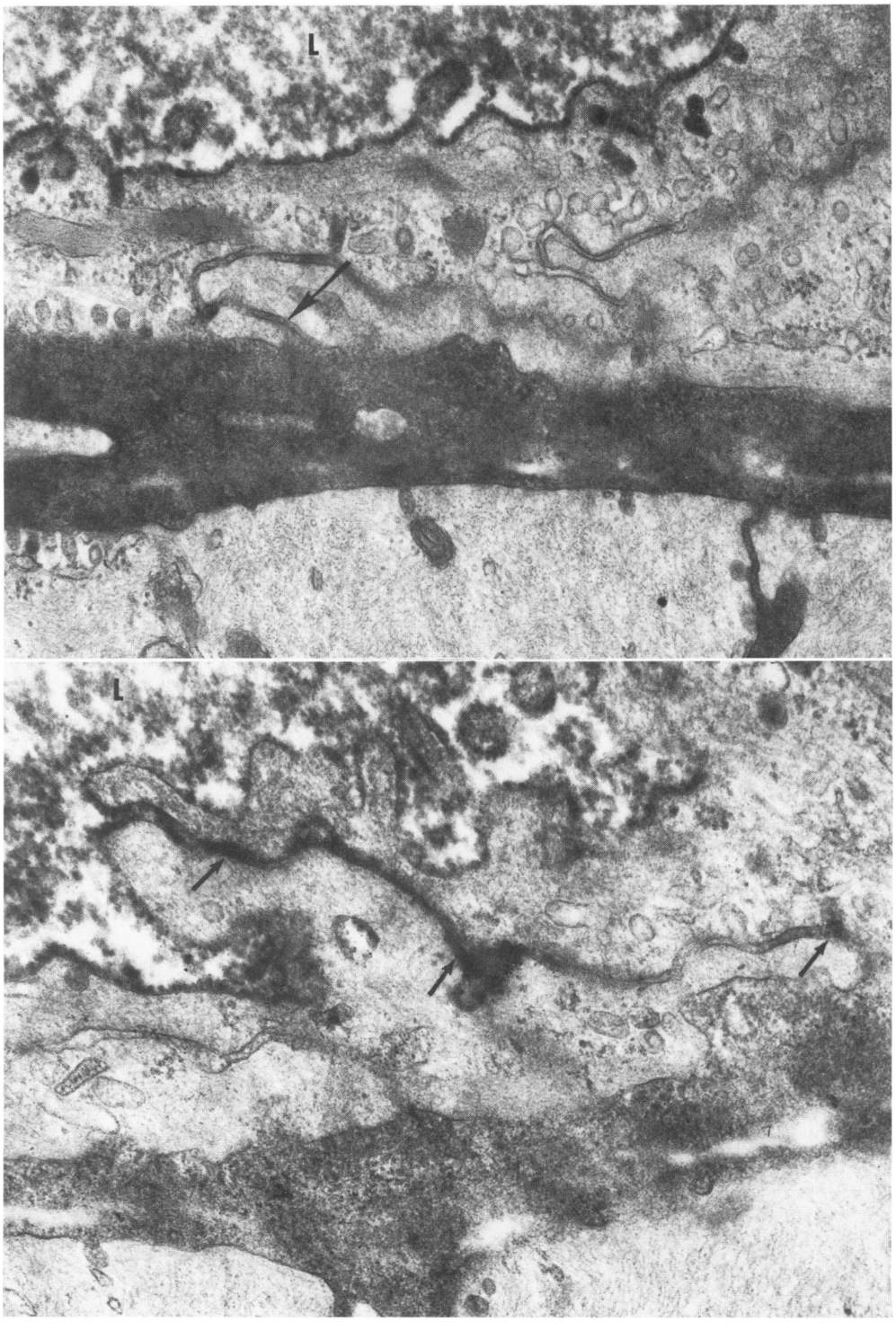
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Fig 9. *(upper)* Reaction product is seen throughout intercellular cleft of arteriole. Reaction product is also present in subendothelial space and in a few pinocytotic vesicles *(arrows)* in basal portions of endothelium. Two min after administration of peroxidase. Lead citrate. × 60,000.

Fig 10. *(lower)* Similar to Fig 9. Basal portion of cleft and subendothelial space are less heavily stained by reaction product than in preceding figure. Lead citrate. × 60,000.

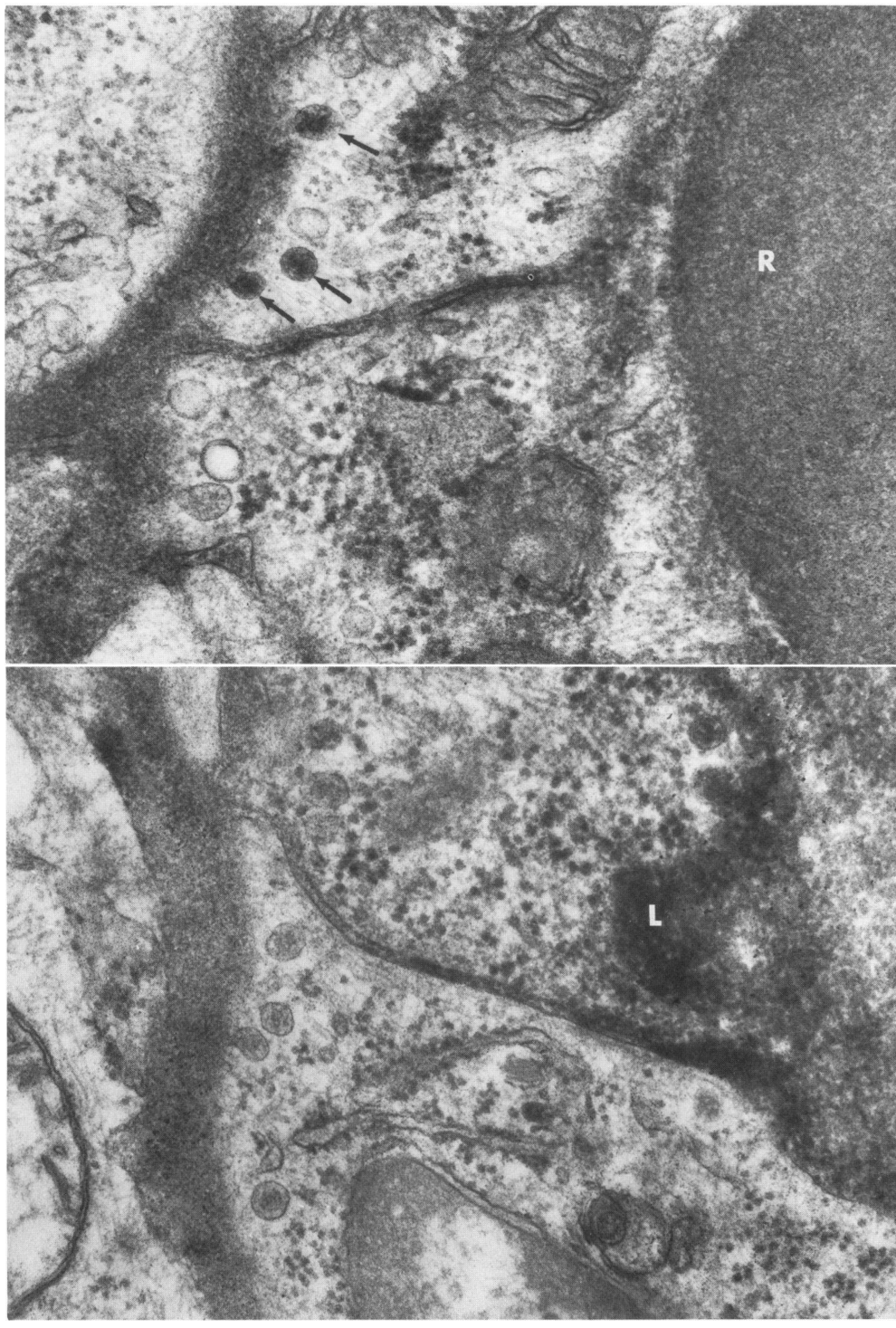
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Fig 11. *(upper)* Reaction product is seen in pinocytotic vesicles in basal aspects of endothelial cell, as well as in vesicles adjacent to surface membrane of smooth-muscle cell *(arrows)*. While reaction product heavily stains subendothelial space, basement membranes adjacent to medial smooth-muscle cells, and adventitia, there is no reaction product in lumen of this arteriole. Peroxidase administered 10 min prior to sacrifice. Lead citrate. × 24,000.

Fig 12. *(lower)* There is swelling of cells and their processes in neuropile adjacent to arteriole. Large deposits of exogenous peroxidase are present between these cellular processes *(rectangle)*. Nature of intracellular dense bodies *(arrows)* is not known. Ten min after the injection of peroxidase. Lead citrate. × 8000.

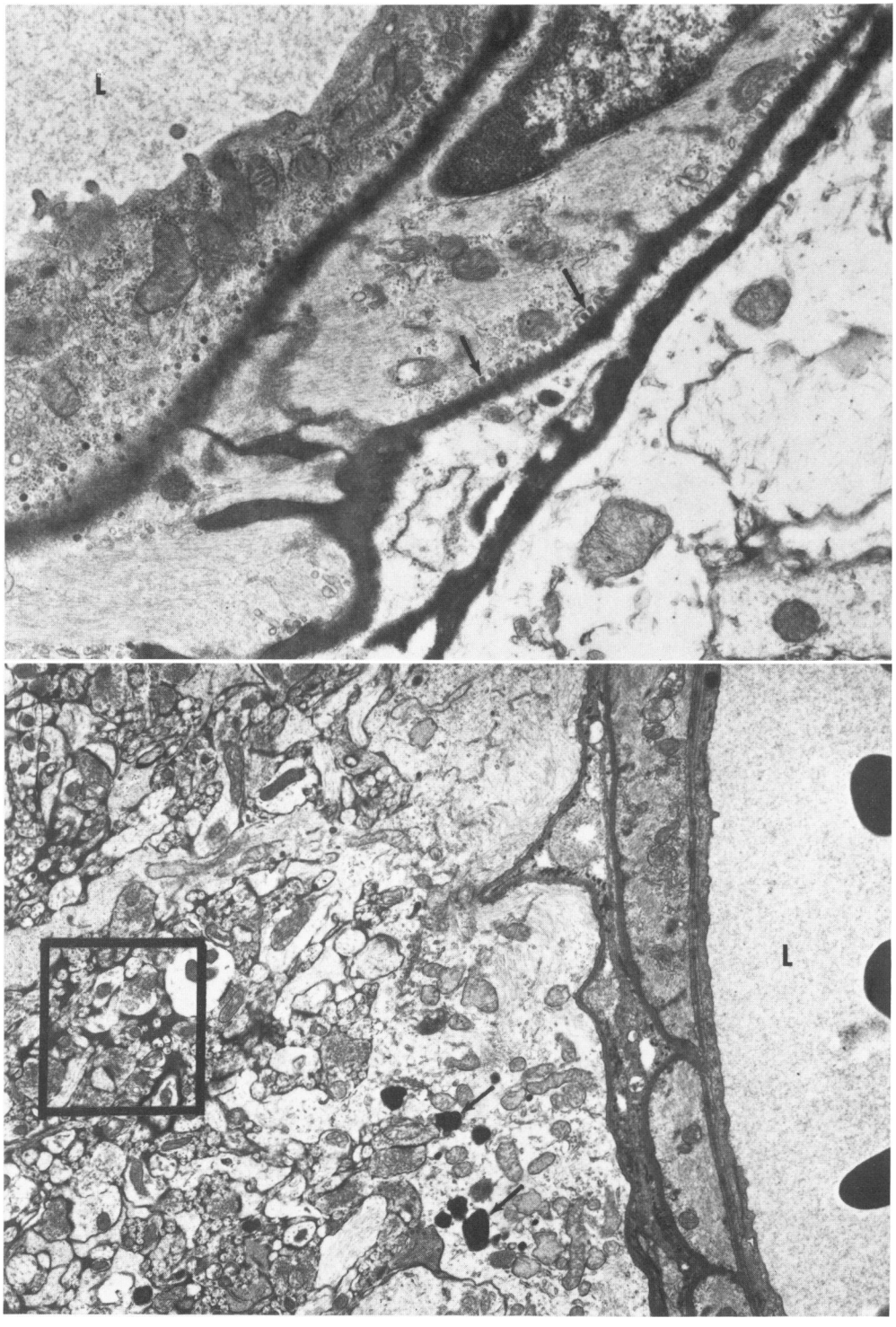
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Fig 13. *(upper)* Micrograph of area similar to that enclosed in rectangle in Fig 12. Exogenous peroxidase is seen extracellularly between cell processes in neuropile. × 48,000.

Fig 14. *(lower)* Longitudinal section of arteriole exhibiting variations in luminal diameter. There is extensive edema and disruption of cellular processes in surrounding neuropile. Lead citrate. × 3400.

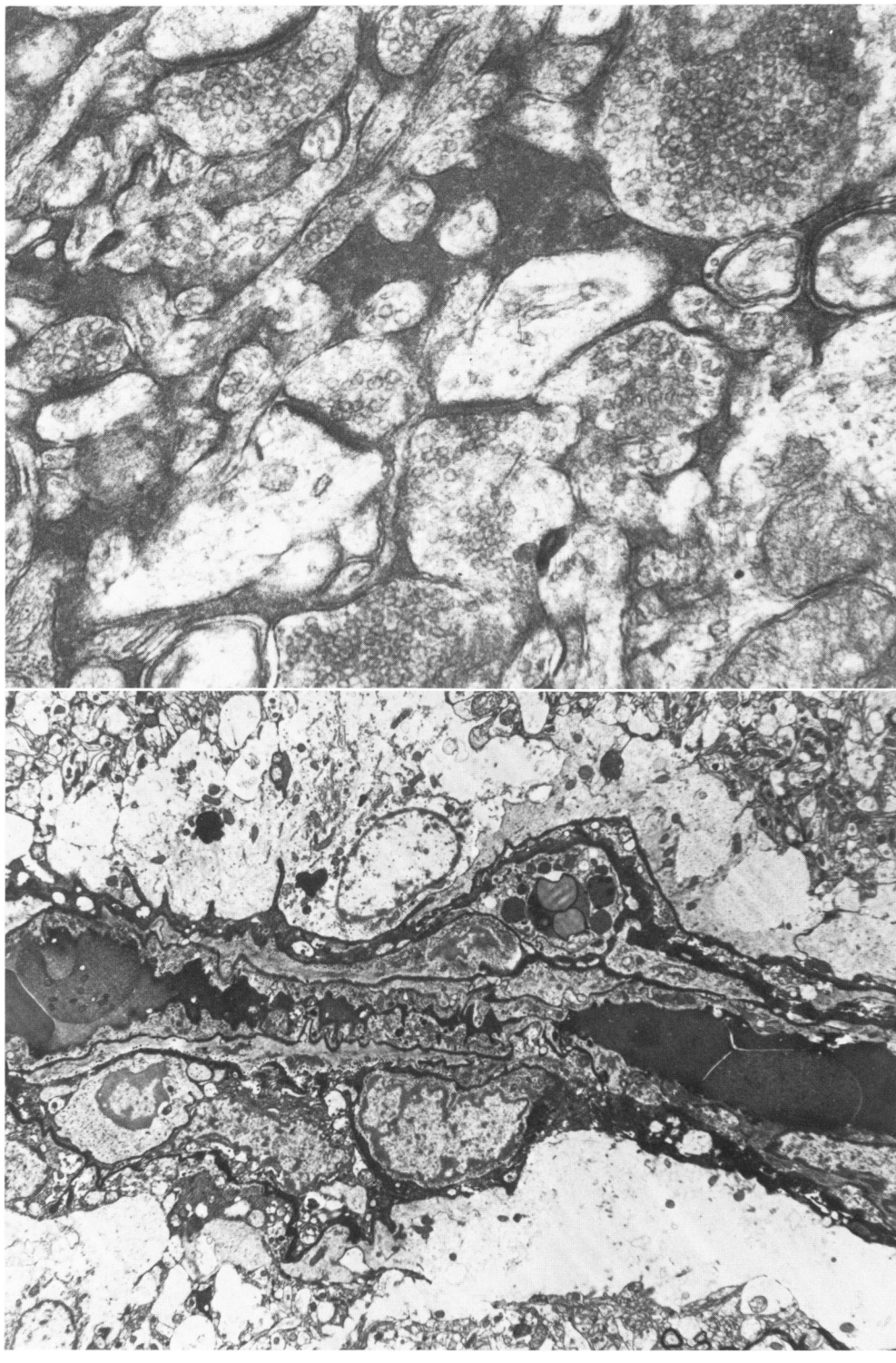

Fig 15. *(upper)* The media of this arteriole is partially hyalinized. Note aggregates of ribosomes in endothelium *(arrows)*. Uranyl acetate and lead citrate. × 20,000.

Fig 16. *(lower)* Media of arteriole contains large aggregates of hyalin. Note compact neuropile. Uranyl acetate and lead citrate. × 4000.

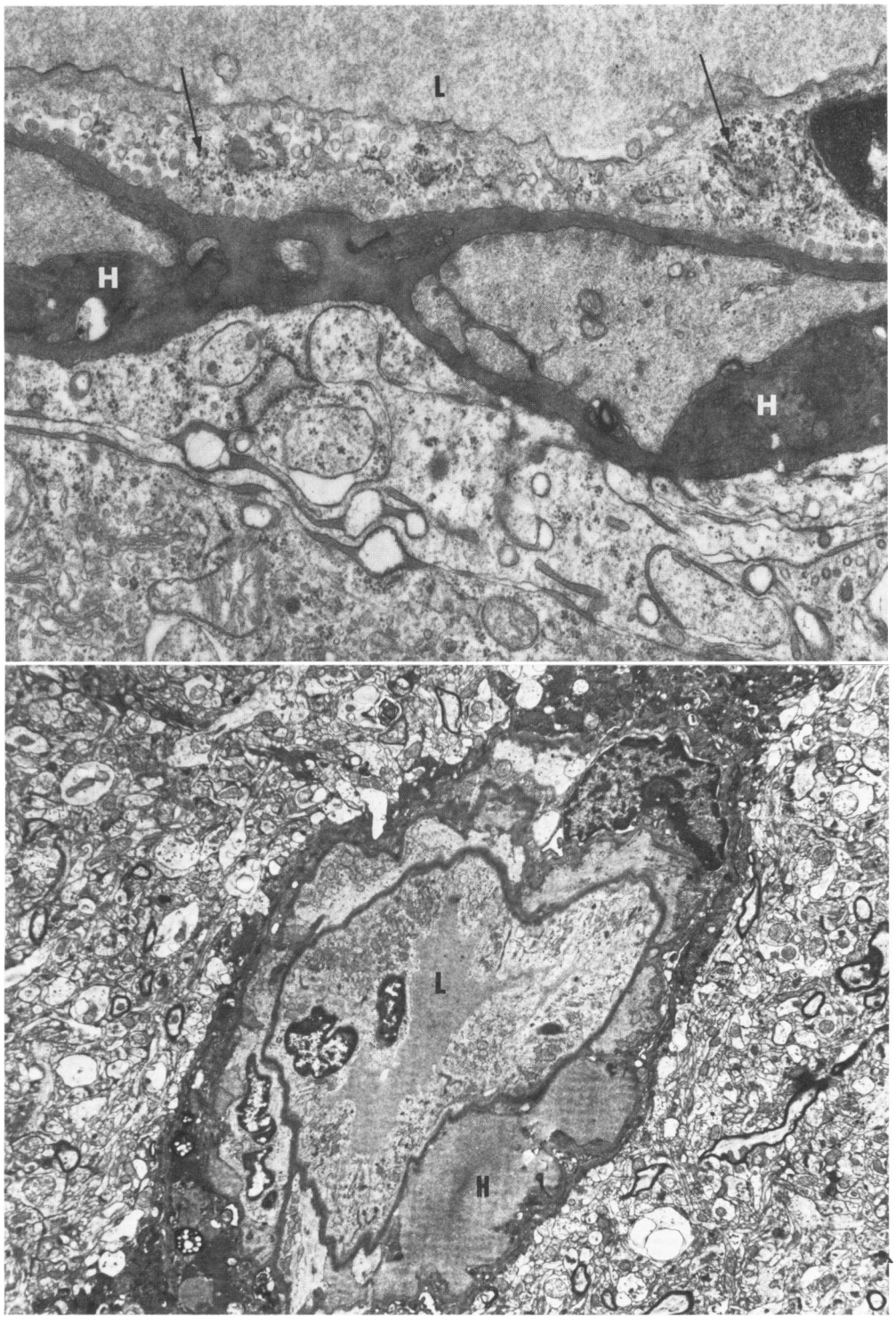
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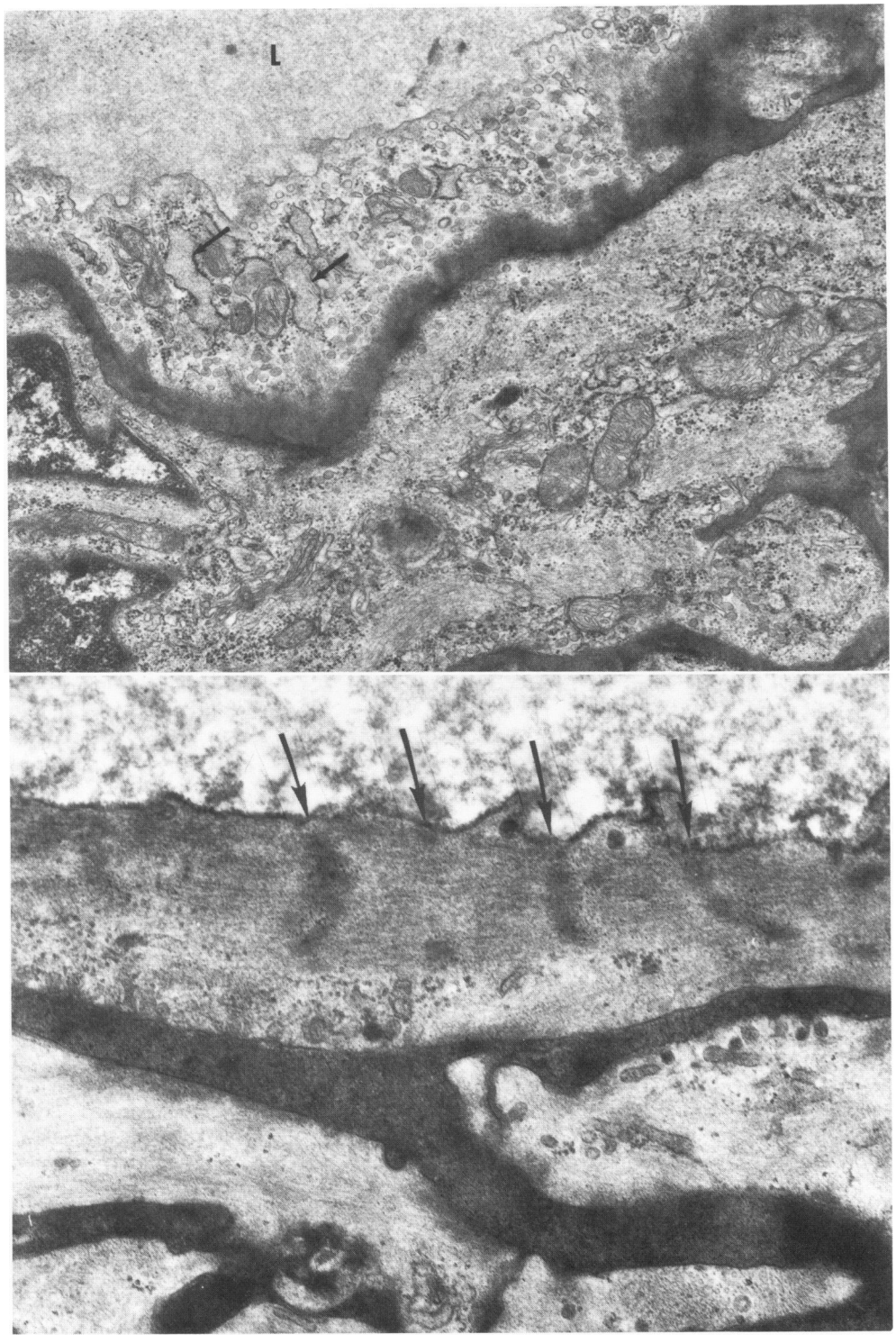
Fig 17. *(upper)* Note electron-opaque contents of cisternae of rough endoplasmic reticulum *(arrows)* in endothelium of arteriole. Uranyl acetate and lead citrate. × 20,000.

Fig 18. *(lower)* Bundles of filaments in luminal aspect of endothelial cell are arrayed parallel to long axis of arteriole. Four electron-dense bands *(arrows)* approximately 1000 Å in width are distributed along long axis of filament bundles at spacing of approximately 0.5 μ. Lumen, subendothelial space, and basement membranes investing the smooth-muscle cells are stained by reaction product. Two min after administration of peroxidase. Lead citrate. × 35,000.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