Endocytosis by Vascular Smooth Muscle Cells *In Vivo* and *In Vitro*

Roles of Vesicles and Lysosomes

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Overloading of lysosomes of smooth muscle cells with excess substrate may be a key event in the development of hypertensive and atherosclerotic vascular disease. Cellular uptake of materials and its relation to lysosomal function were studied by ultrastructural cytochemistry in aortic smooth muscle cells grown *in vitro* and in the intact animal. Injection of horseradish peroxidase (HRP) into hypertensive rats resulted in rapid insudation of the material into the environs of medial smooth muscle cells, entrance into surface pinocytic vesicles, and transport via vesicles into the cell interior where material was seen to accumulate within lysosomes. *In vitro* exposure of calf aortic cells to HRP in the medium resulted in a similar sequence of events. Pinocytic vesicles, seen both *in vitro* and *in vivo*, ranged in diameter from 650–1000 Å. These dimensions are adequate to permit incorporation of intact lipoproteins of all classes, except the larger chylomicrons. (Am J Pathol 83:45–60, 1976)

RECENTLY, ATTENTION HAS BEEN DIRECTED to the mechanism by which circulating plasma elements gain entrance into the vessel wall. Endothelial permeability varies regionally in the vascular tree [1] and is greatly increased when hypertension [2] or atherosclerosis [3] is present. The fate of plasma components that pass through the intima and bathe the medial smooth muscle cells of the vessel wall has not been described. Vascular smooth muscle cells are abundantly equipped with coated surface vesicles, and these serve as one of the morphologic criteria for their identification. Although these vesicles resemble the pinocytic vesicles in other cell types, their functions have not been defined in vascular smooth muscle. Intralysosomal lipid accumulation in smooth muscle cells of the early atherosclerotic lesion appears to be a significant pathogenic event.[4] Our understanding of the relationship between altered vessel wall permeability and cellular accumulations of the lipid and other materials would be greatly enhanced if the mechanisms by which these cells inter-

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nalize circulating plasma components were known. The present study was intended to examine the roles of vesicles and lysosomes in the uptake and storage of materials by vascular smooth muscle cells.

Materials and Methods

In Vitro

Cultures of aortic cells were prepared as follows: the arch and descending thoracic portion of male calf aortas were obtained within 15 minutes of death at a local slaughterhouse. After transfer to the laboratory in plastic bags, vessels were opened longitudinally and the intimal surface was rubbed vigorously with sterile gauze to remove endothelium. From the intimal aspect a superficial layer of media, approximately 1 mm thick, was removed with tweezers. Deeper portions of wall were avoided because of the presence of vasa vasorum and their adventitial sheaths in the outer portion of the calf aortic media. The sheets of media were then diced into 1- to 2-mm pieces which were placed in 35-mm Petri dishes. These pieces were placed in 0.5 ml of Dulbecco's modified minimal essential medium supplemented with 1% nonessential amino acids and 10% fetal calf serum (Grand Island Biological Company, Grand Island, N.Y.) buffered with 40 mM Tris acetate, pH 7.4. Explants were left undisturbed for 4 days in a 37 C incubator (95% air–5% CO_2), after which medium was changed every 2 days. After 3 weeks, sufficient cells were present to allow subculturing.

Cells were removed for subculturing with 0.05% trypsin–0.5 mM EDTA in Puck's saline and were plated into 75 sq cm flasks at a density of 2.5×10^6 cells/flask. Cells were fed three times per week; confluency was reached within 1 week. For these experiments, 35-mm Petri dishes were seeded with 1×10^6 cells/dish; at confluency, dishes contained 2 to 2.5×10^6 cells. In some cases, coverslips were placed in the bottom of dishes prior to seeding and were overgrown by cells. Cells used were in the second or third generation.

Horseradish Peroxidase

On the day of the experiment, medium was changed in all 35-mm plastic Petri dishes. In experimental dishes, 2 ml of a solution of horseradish peroxidase (1 mg ml: HRP Type II, Sigma Chemical Co., St. Louis, Mo.) in tissue culture medium was added; to control dishes a like volume of fresh tissue culture medium was added. Dishes were then replaced in the incubator (95% air–5% CO_2) at 37 C for 1 hour. Then all dishes were removed, the medium was poured off, and cells were immediately covered with a 3% glutaraldehyde solution which was then poured off to remove excess HRP and was replaced with fresh fixative for 2 hours at 4 C. This was followed by an 18-hour period in 7.5% sucrose-cacodylate buffer (0.2 M, pH 7.4).

Coverslips were incubated for light microscopic study in modified Gomori medium with β-glycerophosphatase as substrate for 45 minutes at 37 C for acid phosphatase activity,[5] and in a modification[6] of the Karnovsky–diaminobenzidine (DAB) medium for 30 minutes at room temperature for the demonstration of HRP.[7] The coverslips were thoroughly rinsed and mounted on slides.

The remaining dishes were prepared for electron microscopic cytochemistry by first incubating in the above media for acid phosphatase or HRP, then rinsing four times in cacodylate–sucrose buffer (0.2 M, pH 7.4) before fixation in 1% osmic acid in phosphate buffer (0.1 M, pH 7.2) for 45 minutes. An 18-hour soak in cacodylate–sucrose buffer followed. Then the buffer was replaced with uranyl acetate (0.5%) in veronal acetate buffer (0.05 M, pH 5) for 1 hour. After several rinses with 7.5% sucrose, the cells were carried through alcohol dehydration and embedded in Epon (Lufts B). After polymerization at

60 C for 60 hours, the plastic dishes were cracked away, and the Epon layer was cut into approximately 1-mm-square chips from areas with observable cells. These chips were mounted on blank pellets and further polymerized at 80 C for 18 hours. The blocks were sectioned on the LKB ultramicrotome and poststained with lead citrate for 15 minutes. Sections were examined in a Siemens Elmiskop at 80 kV.

Polystyrene Particles

To test dishes containing similar cultures of calf aortic smooth muscle cells, 100 μl of a 10% suspension of either 0.312- or 1.101-μ polystyrene particles in distilled water (Uniform Latex Particles, Dow Diagnostics, Dow Chemical Company, Indianapolis, Ind.) was added to the tissue culture medium; a like amount of distilled water was added to the tissue culture medium; a like amount of distilled water was added to medium in control dishes. After 30 minutes of incubation at 37 C, the dishes were removed, and the cells were fixed and embedded as described above.

In Vivo

Horseradish Peroxidase

Male CF-N rats (Carworth Farms, New City, N.Y.) were made hypertensive by means of a renal artery clip. Into femoral veins of 3 animals weighing 300 to 350 g, 30 mg of HRP in 1.5 cu cm of normal saline was injected over a 2-minute period. Twenty minutes later, the animals were killed by exsanguination, and rings of ascending thoracic aorta were quickly removed, placed in 3% glutaraldehyde for 2 hours, kept in sucrose–cacodylate buffer for 18 hours (see *in vitro* methods) and were prepared exactly as described for the cultured cells to demonstrate acid phosphatase or HRP activity.

Polystyrene Particles

One milliliter of a 10% suspension of polystyrene particles (0.312 μ) was injected intravenously via a femoral vein into 2 hypertensive male rats. Animals were killed after 3 hours, and ascending aortic rings were removed. Processing of tissues was identical to that described above.

After embedding and sectioning the cell layer or tissue, electron microscopic studies were carried out with a Siemens Elmiskop, using magnifications calibrated against the known sizes of uniform polystyrene particles.

Results

In Vitro

Horseradish peroxidase, demonstrated by reaction with DAB, was found on the surfaces of smooth muscle cells. In addition, small vesicles indenting the surface and free in the cytoplasm were filled with reaction product (Figure 1A). Vesicles close to or in continuity with the surface were quite uniform in size (Figure 1B). Vesicles were bordered by a unit membrane and "rosette forms" and other complexes of vesicles were common (Figures 1 and 2). Most single vesicles ranged in size from 650 to 1000 Å, a size distribution which was also seen in *in vivo* studies. These vesicles were easily seen as "empty" structures in cells exposed to HRP

and not incubated in the DAB medium (Figure 2). They were unit-membrane bounded and a surface "coat" was often observed. Beneath the surface of the cell, HRP was also seen in large vacuoles ranging from 0.5 to 2 μ in diameter. Deeper in the cell, these also fused and formed even larger structures (Figure 3).

Cytochemical incubation for acid phosphatase activity showed reaction product in the larger vacuoles (Figure 4A). Their enzymatic activity and delimiting unit membranes identified them as lysosomes (Figure 4B). Small vesicles similar in dimension to those derived from the plasma membrane were in close association with the surface of the lysosomes.

Exposure of cells *in vitro* to polystyrene particles, either 1.101 μ (Figure 5) or 0.312 μ in diameter also resulted in their internalization. Cell processes appeared to engulf single particles. Some cells were "stuffed" with particles without evidence of cell degeneration. Each particle near the cell surface was bounded by a unit membrane. Deeper in the cell interior, clusters of particles were seen within larger vacuoles.

In Vivo

Endothelial cells showed the transcytoplasmic HRP-containing vesicles previously described.[8] Horseradish peroxidase was frequently demonstrable in the inner few layers of the wall media; however, its presence was spotty, with very densely positive zones often adjacent to nonreactive areas. Correspondence to gaps in the internal elastic lamina could not be demonstrated. By light microscopy (Figure 6), it also appeared that staining of the outer media was occurring, presumably by entrance of HRP from adventitial capillaries. Horseradish peroxidase was present in invaginations of the plasma membrane of medial smooth muscle cells (Figure 7A). Reactive vesicles of similar size were also seen in the cell interior (Figure 7B). Surface vesicles were found in all stages of detachment from the cell surface; note the stalk-like attenuations of some vesicles and the presence of similarly sized "empty" vesicles (Figure 7C). Again, vesicles in the size range of 650 to 1000 Å were most prominent in the region of the plasmalemma whether they contained reaction product or not (Figure 7). Deeper in the cell interior, huge accumulations of much larger vacuoles rimmed by reaction product were seen to practically fill portions of cells (Figure 8). These "empty" appearing vacuoles resemble those described in fibroblasts given HRP in culture.[9] Reaction product was usually seen only on the periphery of the vacuoles; however, some vacuoles were filled with product (Figure 9). Despite the large area of cytoplasm filled with swollen lysosomes, cell integrity was remarkably well maintained, with preservation of mitochondria and other structures. In these cells the pinocytic

vesicles at or near the surface did not contain HRP reaction product (Figure 8).

Cytochemical staining for lysosomal acid phosphatase[10] showed reactive bodies, at the periphery of large vacuoles, that were similar to those shown to contain HRP (Figure 10A and B). These frequent images suggest fusion of lysosomes with vacuoles containing HRP.

Phagocytosis of injected polystyrene particles could not be demonstrated in the vessel wall interstices or in vascular smooth muscle after 3 hours.

Discussion

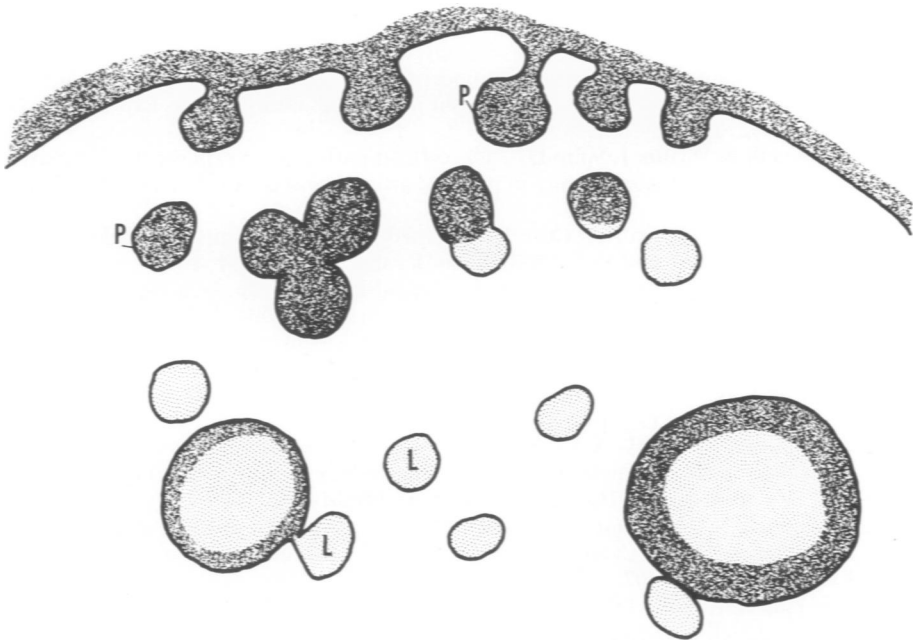
One of the distinctive morphologic features of vascular smooth muscle cells is an array of surface vesicles so extensive that portions of the plasma membrane assume a "ruffle-like" configuration. The size of these vesicles have generally been reported to be 100 to 300 Å, but some mention has been made of a larger species (1000 to 4000 Å) in smooth muscle of the carotid artery.[11] Indeed, measurements made by us on published electron micrographs of aortic smooth muscle cells [12,13] indicate that values of 500 to 600 Å diameters are not uncommon. A pinocytic function for the smaller (100 to 300 Å) vesicles has been suggested.[14,15] If these dimensions reflected a static condition, an important role for these vesicles in the internalization of macromolecules would be precluded. For example, the sizes of most lipoproteins (chylomicrons, 750 to 10,000 Å; very low density lipoproteins, 300 to 500 Å; low density lipoproteins, 200 to 220 Å; high density lipoproteins, 75 to 100 Å [16,17]) would be too large for them to be taken up appreciably by these structures. For the hepatic cell, an alternative suggestion has been made that uptake of low-density lipoproteins (mainly cholesterol ester) might take place through a hydrolysis-reesterification sequence occurring at the plasma membrane.[18] The present study of smooth muscle cells demonstrates a population of vesicles 650 to 1000 Å in diameter which are actively engaged in pinocytosis. These dimensions are compatible with the suggestion that all lipoprotein classes, except perhaps for the largest chylomicrons, enter the cell by endocytosis. Though such a mechanism of entry of those complexes is not proved in smooth muscle cells, it has been established for the macrophage by Werb and Cohn.[19] The existence of high-affinity and low-affinity lipoprotein uptake processes by human fibroblasts and vascular smooth muscle cells *in vitro* has been described using labeled lipoproteins as markers.[20,21] The possible role of specific low-density lipoprotein receptor sites on the cell surface has been invoked to explain the high-affinity saturable process. Establishment of endocytosis as a major mechanism for internalization is

compatible with the presence of specific surface receptor sites. In fact, this mechanism may account for the low-affinity, nonsaturable uptake of materials by these cells, since it has recently been shown that uptake of HRP by cultured fibroblasts is linear over a large concentration range.[9] However, clear distinctions between surface binding and internalization of materials by smooth muscle cells may be enormously complicated by the presence of their extensively convoluted, vesiculated surface. In any case, knowledge that this extensive surface is actively engaged in endocytosis, emphasizes the potential for these cells to internalize materials in their environment. Their capacity for this relative to other cell types, such as the fibroblast, needs further study and quantitation and may provide an explanation for the intracellular accumulation of materials, such as lipid, by mural cells as a special feature leading to vascular disease.

The ability of vascular smooth muscle *in vitro* to internalize very large particles, such as red blood cells, latex particles, and even other cells,[22] raises the possibility that they may also serve a phagocytic function in damaged vessels. A scavenger role would be attractive as an aspect of vessel wall repair, but extrapolations made from *in vitro* studies alone should be cautiously drawn. Virtually all cells grown *in vitro*—including epithelial, HeLa, and thyroid cells—phagocytize latex particles introduced into the medium.[23,24] This may be an artifact of adaptation to *in vitro* conditions since some of these cell types rarely, if ever, demonstrate this capability *in vivo*.

The sequence of events following endocytosis by vascular smooth muscle cells *in vivo* and *in vitro* appears to be similar to that seen in other cell types (Text-figure 1).[25] Endocytic vacuoles may fuse to form complex rosettes or progressively larger vacuoles after internalization and may fuse with lysosomes. Noteworthy in our cells, *in vivo*, was the formation of huge lysosomes after 20 minutes which, in some instances, filled most of the cell cytoplasm. Despite the presence of these structures, however, cells did not show degenerative changes, and other organelles, such as mitochondria, remained remarkably intact. Inactivation of HRP and a return to normal appearance occurs with a half-life of 7 hours in fibroblasts;[9] the time sequence in smooth muscle cells is unknown.

Smooth muscle cell lysosomes in the intact vessel wall are usually sparse. However, a striking increase in number and size variation occurs in the presence of hypertension;[10] we have attributed this to the marked increase in permeability of these vessels.[2] Lysosomal activity and vascular permeability are also markedly increased in atherosclerotic vessels.[26] Entry of circulating HRP into the hypertensive rat aortic wall resulted in a marked lysosomal response comparable to that seen *in vitro*, where, of



TEXT-FIGURE 1—A diagrammatic scheme of the process of smooth muscle cell endocytosis. Material on the outside of the cell is brought into the cell by pinocytic vesicles (*P*) which form at the surface. These vesicles fuse with each other to form large vacuoles and with lysosomes (*L*) where the material is degraded or stored.

course, no endothelial barrier is present. In view of the ability of many stimuli to disrupt the endothelial barrier, it seems that breaches in its integrity must be continually present in vulnerable portions of the arterial tree. Endocytosis by smooth muscle cells of plasma components that enter the vessel wall probably occurs continuously. Chronic sustained or repeated intermittent exposures of the underlying cellular elements of the wall to increased amounts of circulating plasma components could accelerate the sequence of intracellular events described here under experimental conditions. The relative ability of vascular smooth muscle cells to metabolize and degrade many complex macromolecules, including lipoproteins, could determine which of those "substrates," or their by-products, accumulates intracellularly. A seemingly trivial imbalance between influx of substrate and efflux of product could, over the course of decades, result in massive overloading of cells with undigested substrate,[4,27,28] with the accompanying cell proliferation and connective tissue accumulation in the vessel wall leading to plaque development, compromise of the lumen, and subsequent clinical signs of vascular insufficiency.

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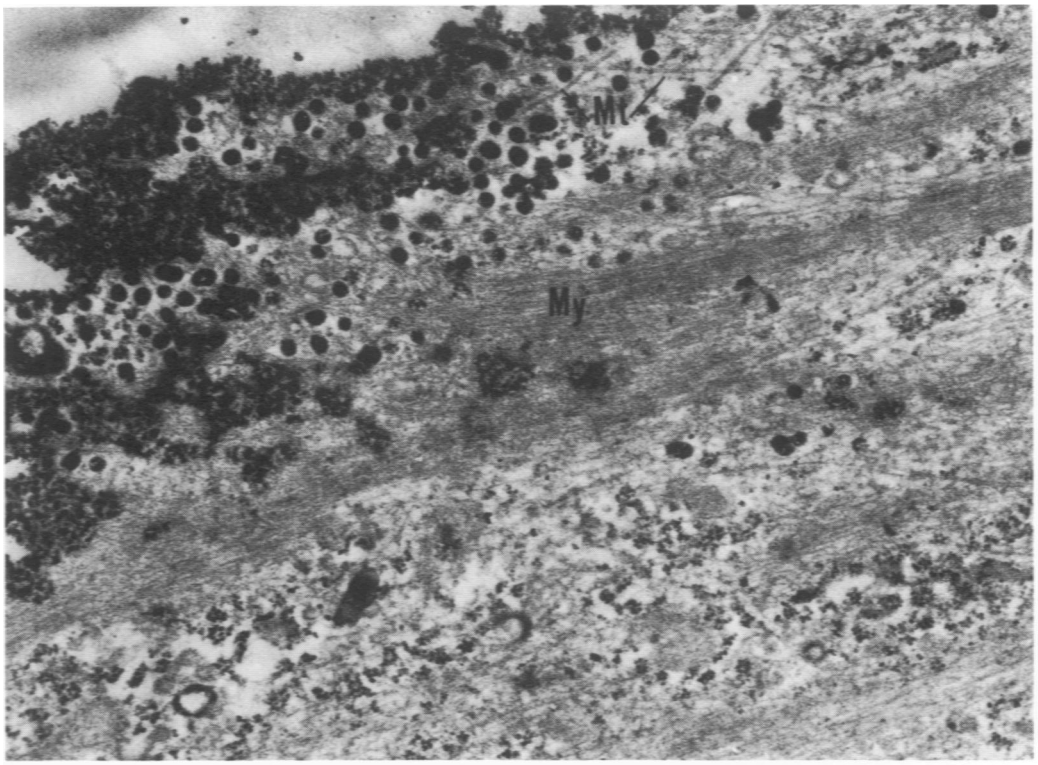
[*Illustrations follow*]

Figures 1–4 are of calf aortic smooth muscle cells grown in a culture medium to which HRP was added.



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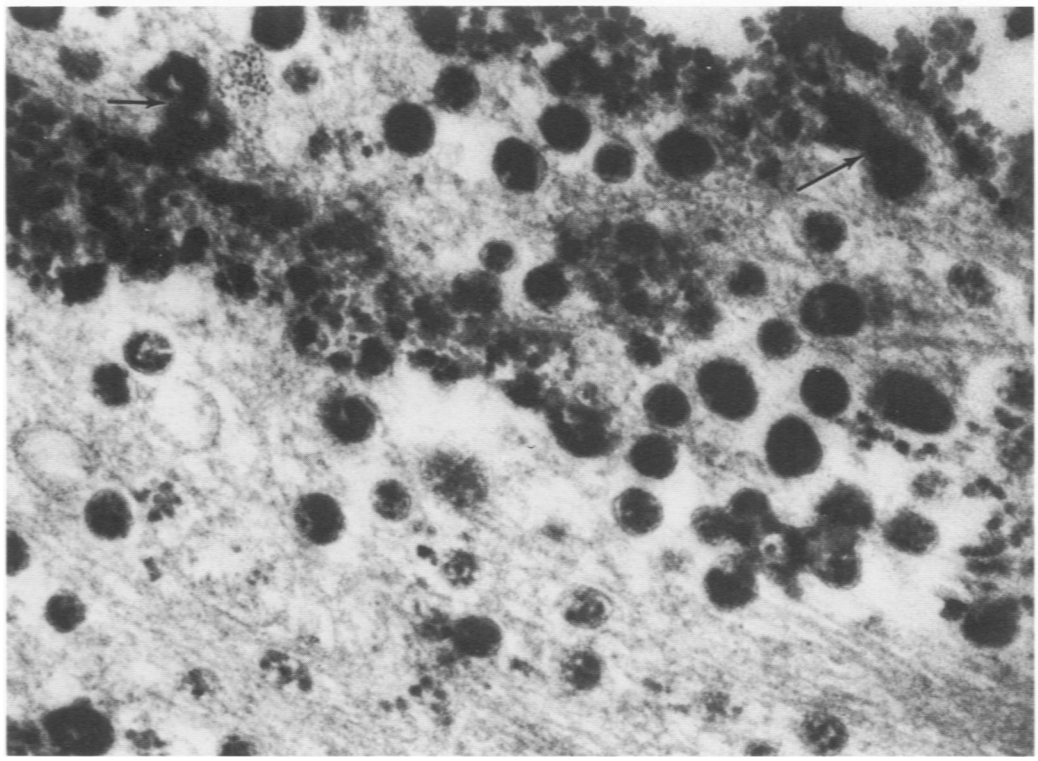

B

Figure 1A—Smooth muscle cell incubated in a DAB medium. Pinocytic vesicles, fairly uniform in size, are filled with electron-dense, osmiophilic reaction product. An array of myofilaments *(My)* and microtubules *(Mt)* typical of smooth muscle cells is evident. (× 18,000) **B**—Higher magnification shows pinocytic vesicles bordered by a unit membrane. Complexes of vesicles are seen *(arrows)*. (× 66,000)

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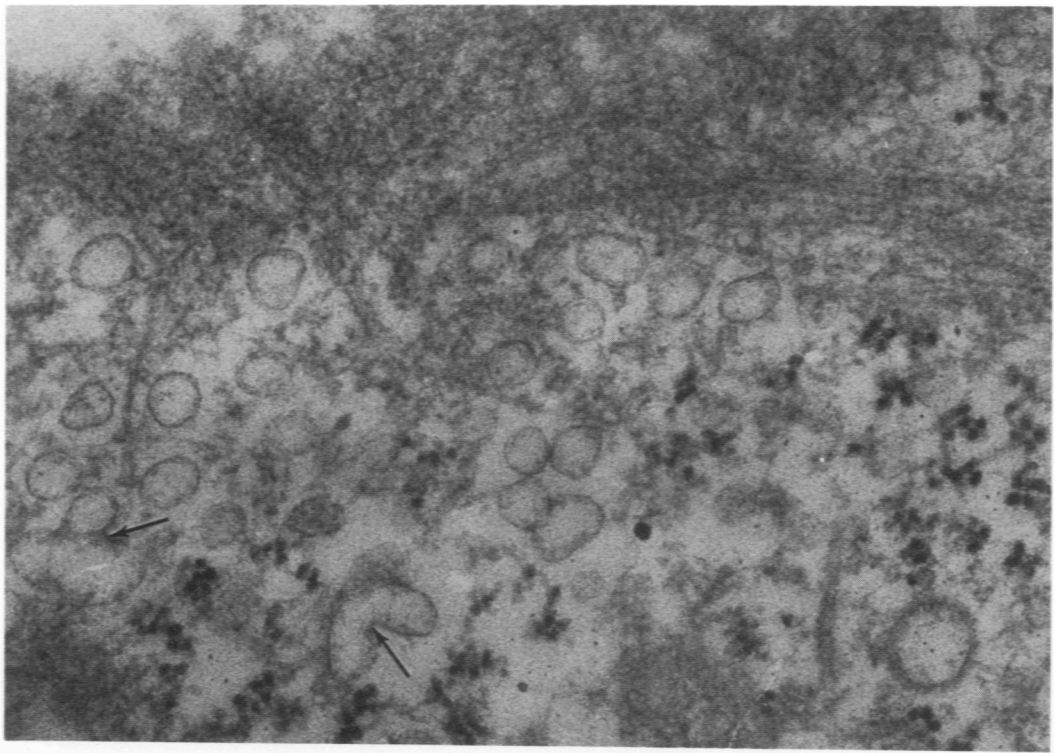

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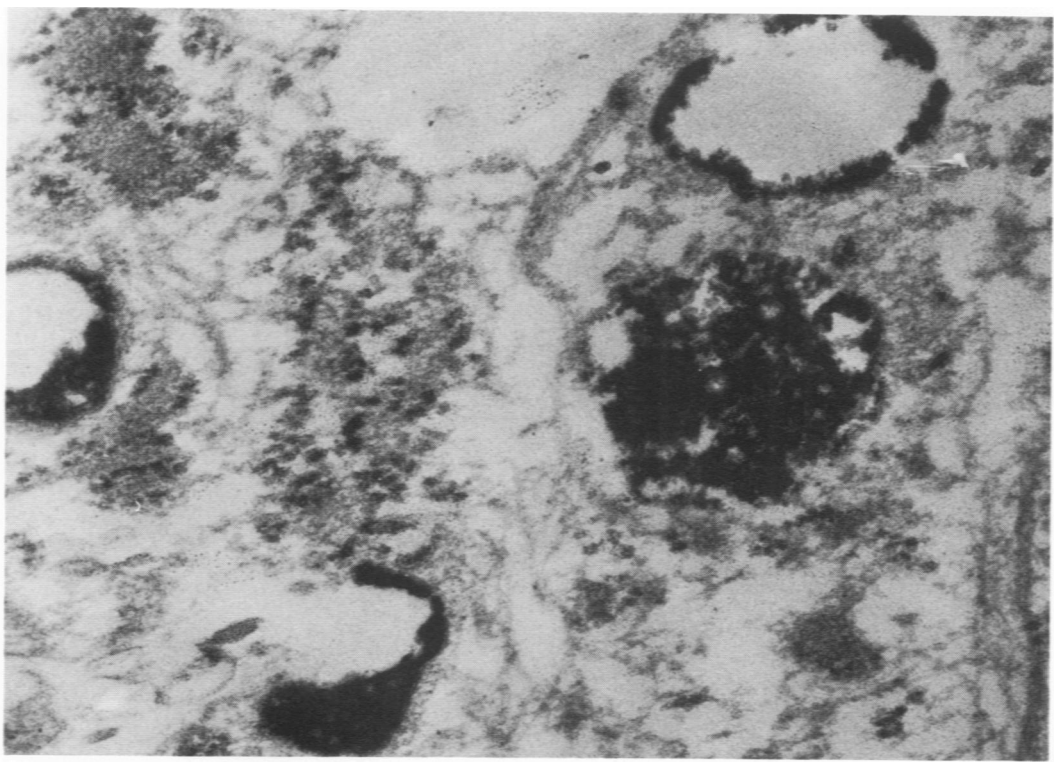


Figure 2—This smooth muscle cell has not been incubated, and the vesicles appear to be empty. On some a surface coat is preserved. Complex forms resulting from fusion of vesicles are seen *(arrows)*. (× 66,000) **Figure 3**—Smooth muscle cells incubated in the DAB medium. Large vacuoles, some completely filled with HRP, and others with product at their periphery are seen deep in the cell. (× 60,000)

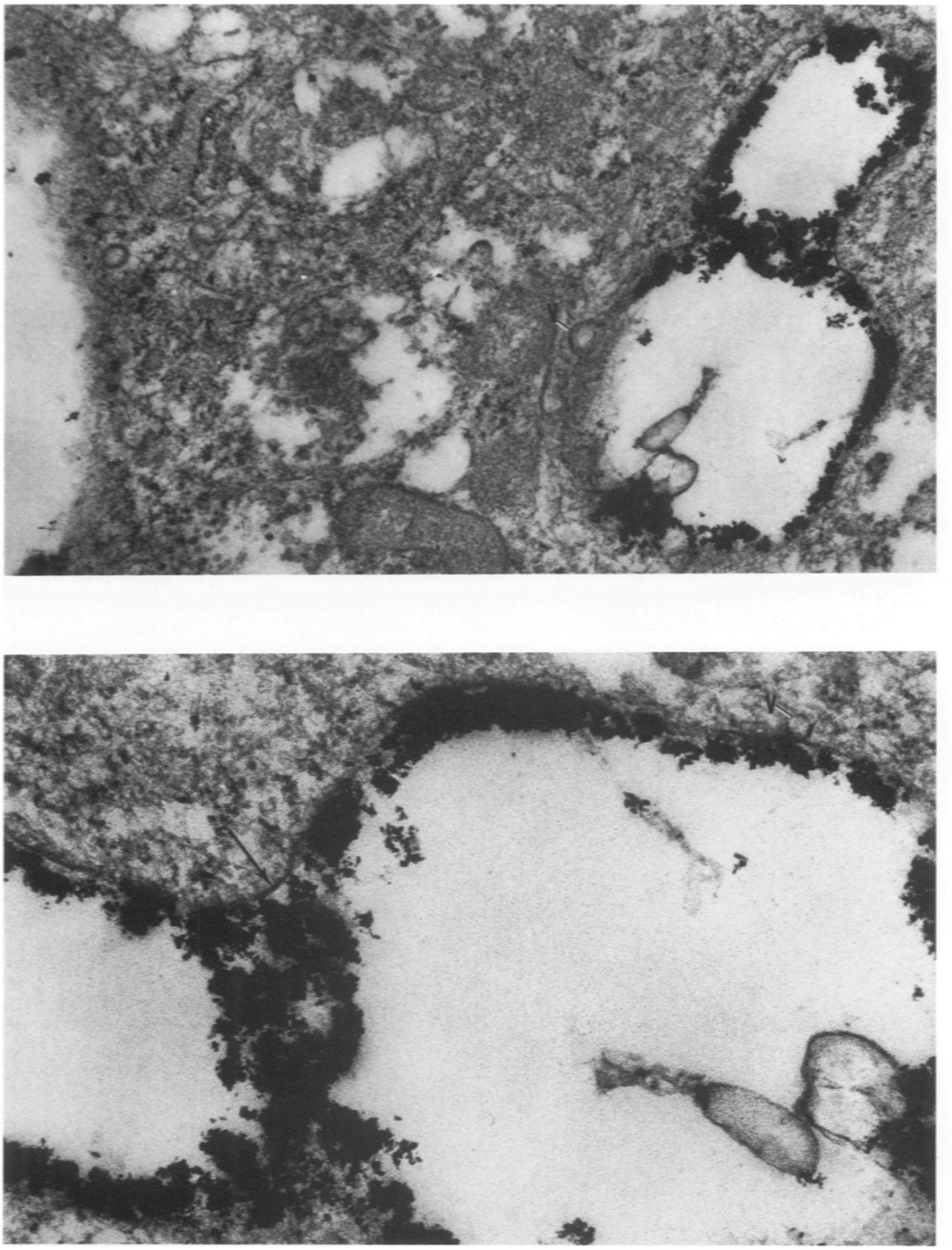


Figure 4A—Smooth muscle cell incubated in a β-glycerophosphate medium for acid phosphatase activity. Two large vacuoles are identified as lysosomes by the presence of lead phosphate reaction product at their periphery. A vesicle *(V)* is adjacent to the lysosome. (× 24,000) **B**—Enlargement of **A** shows that the two vacuoles are fused and are bordered by a unit membrane *(arrow)*. A small vesicle *(V)*, similar in size to those derived from the plasma membrane, is seen in close association with the surface of a lysosome. (× 60,000)

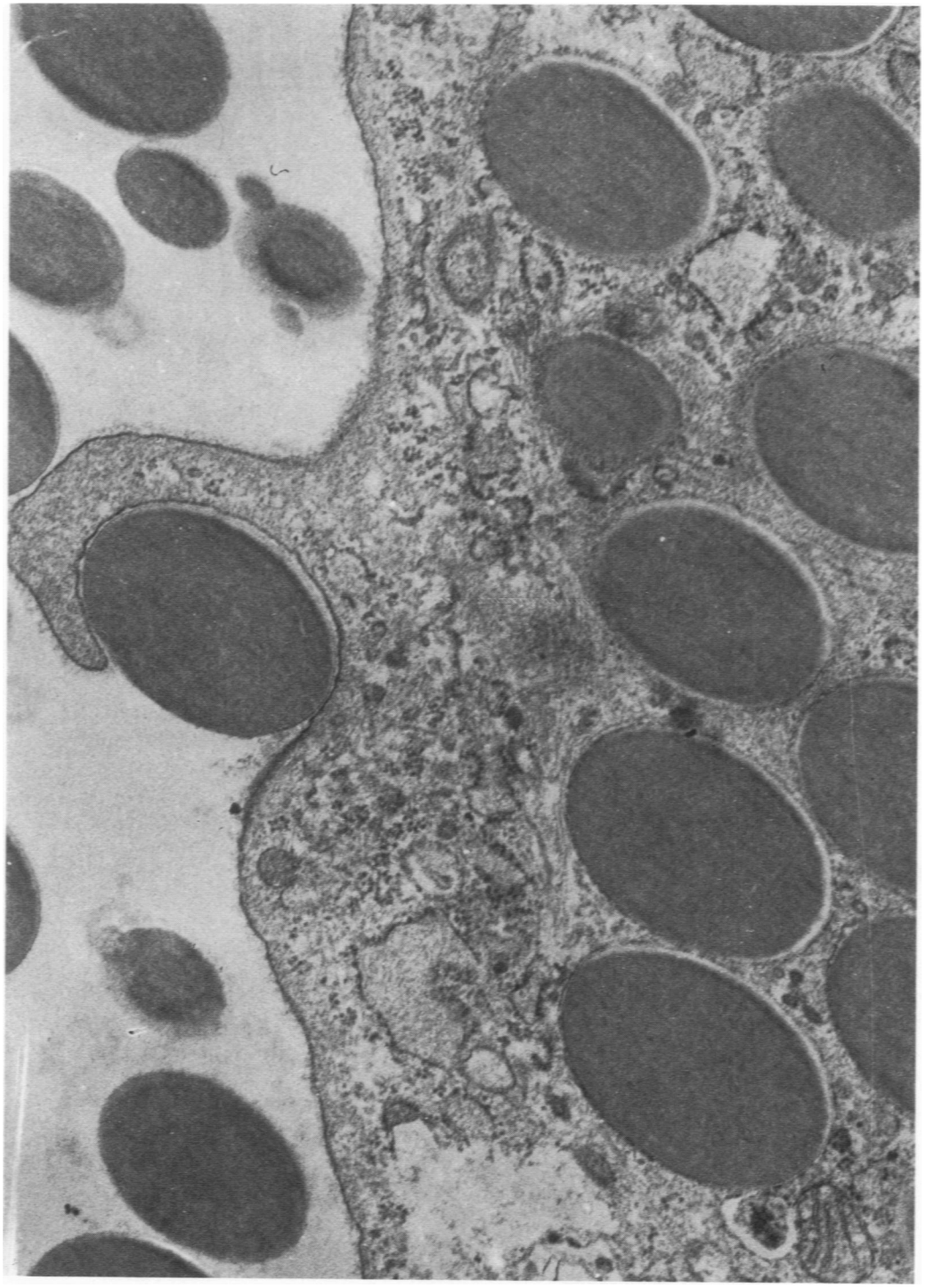


Figure 5—Cultured smooth muscle cell grown in a medium to which polystyrene particles were added. A cell process appears to be engulfing a particle and numerous particles are present inside the cell. (× 30,000)

Figures 6-10 are of smooth muscle cells of a rat aorta from an animal injected with HRP.

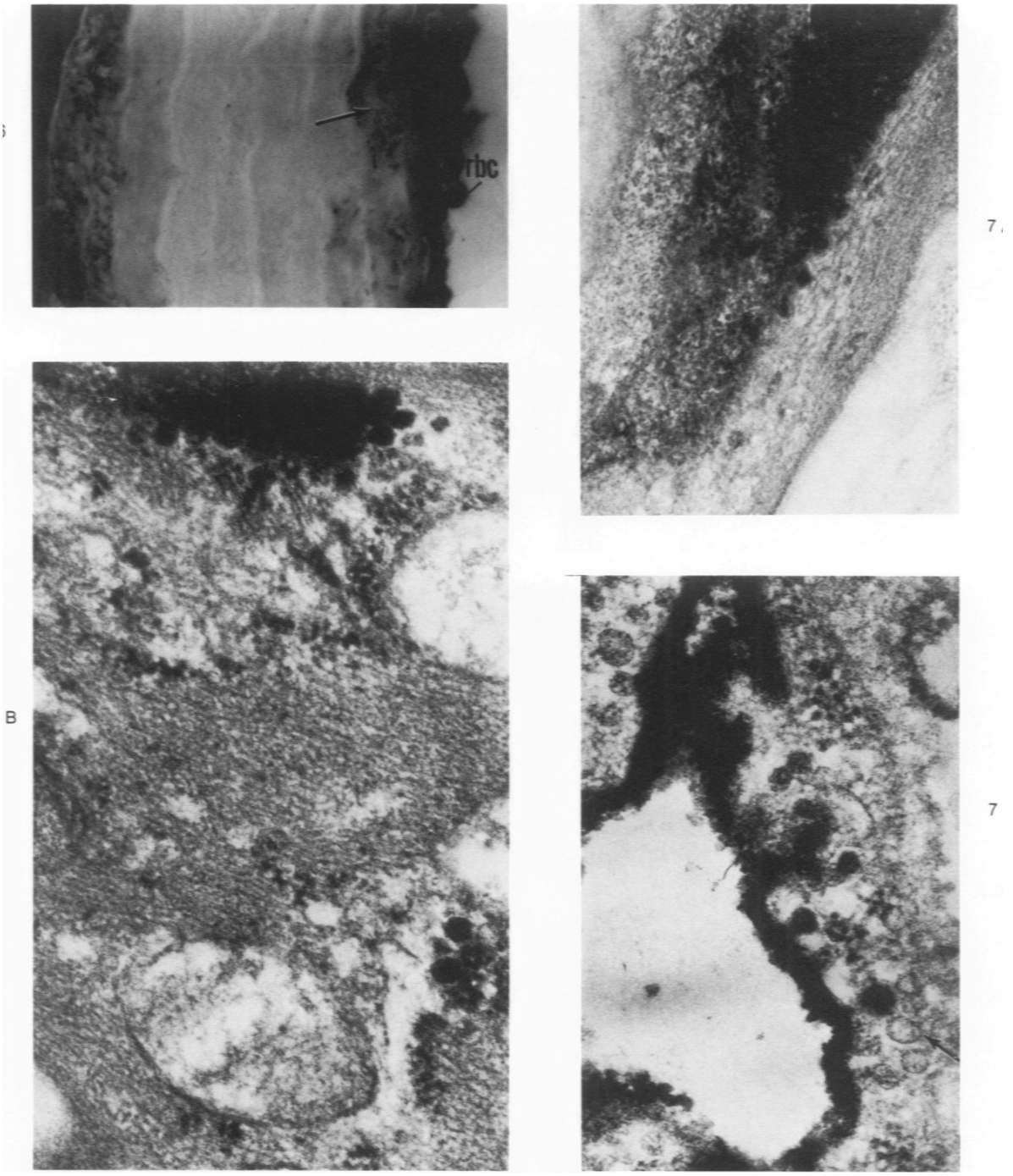
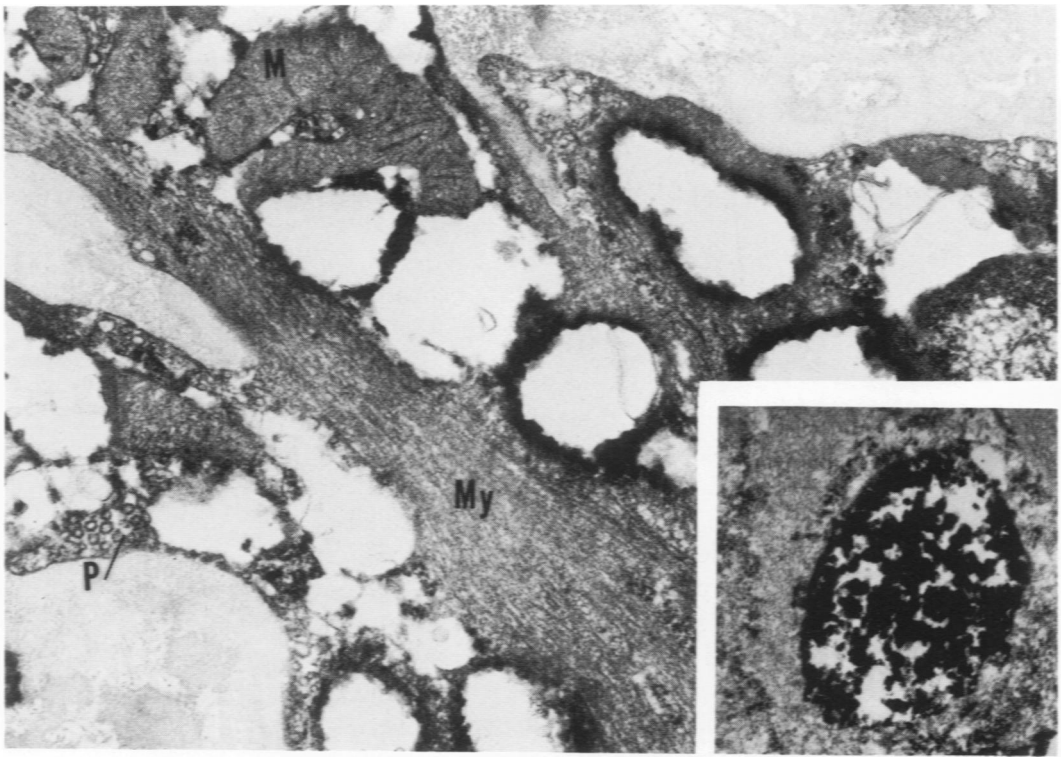


Figure 6—Light micrograph of a section of aorta incubated in the DAB medium. Red blood cells *(rbc)* in the lumen are reactive because of the peroxidatic activity of hemoglobin. HRP is not only present in the subintimal portion of the medial wall *(arrow)* but also in the outer layers close to adventitial capillaries. (× 400) **Figure 7A**—Aortic smooth muscle cells from a section incubated in the DAB medium. Reaction product is located outside the cell and in invaginations of the plasma membrane. (× 48,000) **B**—HRP is present in small vesicles near the cell surface and deep in the cell (× 60,000). **C**—Reaction product in pinocytic vesicles in various stages of detachment. Vesicles without reaction product *(arrow)* are seen just beneath the reactive vesicles. (× 54,000)



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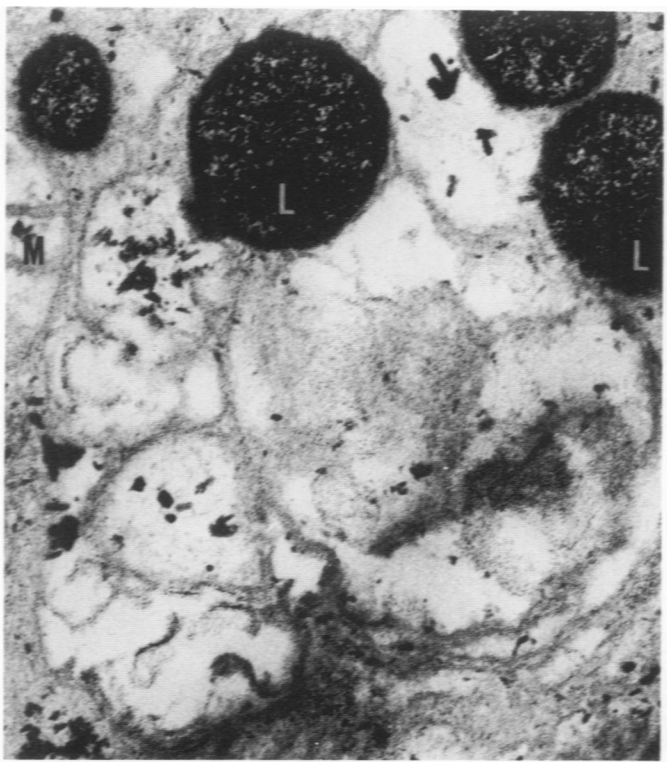

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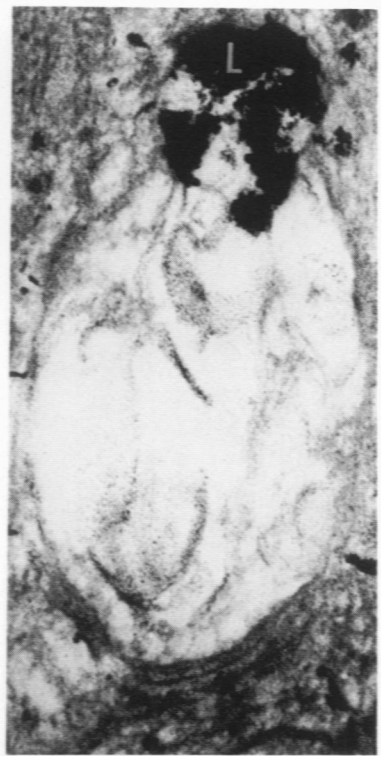

10 B

Figure 8—Aortic smooth muscle cell of a section incubated in the DAB medium. The cell contains many large vacuoles with HRP reaction product at their periphery. Surface vesicles *(P)* formed at this stage no longer contain HRP. The mitochondria *(M)* and myofilaments *(My)* are well preserved. (× 24,000) **Figure 9**—A large vacuole filled with DAB reaction product (× 24,000). **Figure 10A**—Aortic smooth muscle cell from a section incubated for acid phosphatase activity. Lysosomes *(L)* darkly stained with lead phosphate reaction product are in close association with large vacuoles. Mitochondrion *(M)* is seen at the left. (× 66,000) **B**—A lysosome *(L)* fused with a large vacuole (× 66,000).