THE FINE STRUCTURE OF THE AORTIC ENDOTHELIAL LESIONS IN EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS OF RABBITS *

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Lesions of arteries induced in the rabbit by feeding a high cholesterol diet have been the subject of many investigations from the time of their discovery by Anitschkow and Chalatow.[1] An excellent review of the subject was given by Duff and McMillan.[2] With the naked eye the aortic lining shows pale raised lesions, especially in the upper part, and after prolonged feeding of the cholesterol-rich diet, most of the aorta, as well as parts of other arteries, may become affected. With the light microscope the lesions are characterized by the development of multilayered cushions of lipid-filled cells ("foam cells"), a variable amount of necrosis, collagen fibrillogenesis, production of acid mucopolysaccharide and calcification. The present communication describes the fine structure of the endothelial lesions.

Methods

Male rabbits of 2 to 3 kg. body weight were fed a diet containing 1.5 per cent of cholesterol (U.S.P.) intimately mixed with the rabbit food pellets (Master Feeds) by dissolving it in ether which was then evaporated. Rabbits were kept on this diet for periods of 14 days, 1, 2, 3, 5 and 7 months. Each of 3 rabbits, sacrificed after 2, 3 and 5 months on the diet, was given an intravenous injection of 5 ml. of colloidal thorium dioxide (Thorotrast; Testagar and Co.) 24 hours before sacrifice. Thirteen rabbits were given this diet. Three other rabbits received the same amount of cholesterol in their diets, but 5 per cent corn oil was added; the animals were sacrificed after 6 months. Although the fine structure of normal rabbit arterial endothelium has been reported elsewhere,[3] some of its important features are reviewed here, by way of introduction to the observations in atheromatous vessels.

The thoracic aorta and sometimes the femoral artery were fixed in osmium tetroxide, dehydrated, and embedded in methacrylate as described previously.[3] Sections were cut on a Porter-Blum microtome

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and examined with a Philips EM 100 A electron microscope. Frozen sections and paraffin sections of formalin-fixed material were observed with the light microscope.

Observations

Endothelium of Normal Rabbits

The endothelial cells often protruded into the lumen as a consequence of contraction of the artery (Fig. 1). They lay either directly on the internal elastic lamina or on a delicate network of collagen fibrils. The endothelium formed a continuous cellular layer although, in places, it was extremely thin. The shape of the nuclei was determined by the amount of contraction of the vessel and varied from flattened to spherical configuration. The cytoplasm contained relatively few mitochondria, some membranes of a Golgi apparatus, and numerous vesicular or tubular structures of great variety in size. These were thought to be a part of the endoplasmic reticulum. A few dense bodies, about the size of mitochondria, were present and were shown to incorporate intravenously injected particles of thorium dioxide. A striking feature was the very great number of tiny invaginations of the plasma membrane on all surfaces of the cells (Fig. 2). Both Palade[4] and Moore and Ruska,[5] who observed these structures in capillaries and small arteries, considered that these might represent pinocytic vesicles, and might be of importance in the movement of fluid substances through the cell.

Endothelial Lesions in Rabbits Receiving a 1 Per Cent Cholesterol Diet

Animals sacrificed after 14 or 30 days on the diet had no lesions of the aorta visible to the naked eye or the light microscope. The fine structure of the aorta also showed no consistent change from that of normal endothelium. At the end of the second month on the diet, however, lesions were found, and all animals sacrificed after 3, 5, and 7 months showed well-developed experimental atherosclerosis.

The atherosclerotic plaques were always covered by a continuous unicellular layer. The cells were frequently of pyramidal shape, with the base towards the lumen (Figs. 3 and 5). In places, this cell layer was extremely thin, reduced almost to the width of the two cytoplasmic membranes. The nuclei were oval or circular in outline. The cell membrane was folded at the lateral intercellular boundaries (Fig. 4) and a light gap 100 to 200 Å wide was enclosed between the membranes of the cells in this layer. Each cell appeared closely united and interdigitated with those on either side of it, but its deep margin was separated from the inner cells of the plaque by a large and irregular

extracellular space (Figs. 3, 4 and 5). Adjacent to the cytoplasmic membrane of all surfaces were small vesicles or invaginations (Fig. 6) of the type found in greater numbers in normal arterial endothelium. They measured up to 600 Å across.

The cytoplasmic matrix was somewhat more dense than that of normal endothelium. It contained large cisternas of endoplasmic reticulum, normal mitochondria, and a much hypertrophied Golgi apparatus. Occasionally, relatively large light spaces and dense granular inclusions were observed. The endoplasmic reticulum (Figs. 4, 5 and 6) was represented by numerous and frequently distended cisternas and zones measuring up to 1 μ^2, filled with moderately dense material. The outer surface of its membranes was studded with particles of about 150 Å size, the Palade granules (Fig. 6).

Parts of the Golgi apparatus were usually seen at several points in a section of each cell (Figs. 4 and 6). The structure was much more developed than in normal endothelium, where it was relatively inconspicuous. It consisted of circular profiles 300 to 700 Å in diameter, parallel membranes which appeared to be oblique, and longitudinal sections through tubules or cisternas. The membrane bounding these structures had a smooth surface, in contrast to the endoplasmic reticulum.

Cytoplasmic inclusions were of two types. The commonest, which was also found in normal endothelium, consisted of relatively dense material bounded by a single membrane (Fig. 4). Another type of inclusion, seen only occasionally, was larger and appeared as an empty space, often having no definite limiting membrane.

Twenty-four hours after the intravenous injection of Thorotrast, some of this material could be seen in the first type of inclusion described above. In some inclusions only a few dense particles were present, but in others so many were taken up that it was not possible to see whether they were, in fact, lying in the dense matrix of this inclusion or whether they had formed a different type of vacuole, containing only the thorium particles (Fig. 8).

The interior of the lesions contained cells, relatively large extracellular spaces, and fibrils of collagen. The cells were of two types. Those making up the covering layer were occasionally found close to the surface of the lesions (Fig. 3). This type of cell, which was characterized by prominent cisternas of endoplasmic reticulum and the virtual absence of vacuoles, was much less numerous than the other. The latter appeared to correspond to what conventional microscopists have called "foam cells" (Figs. 5 and 7). The cytoplasm in these contained many light spherical vacuoles, usually having definite limiting

membranes (Fig. 5). Typically, these cells were more or less spherical, measuring 15 to 40 μ in diameter. Sometimes they contained more than one nucleus. The cytoplasm between the light vacuoles contained mitochondria, irregularly oval profiles of endoplasmic reticulum and, occasionally, numerous very dense granules. The endoplasmic reticulum did not show the large cisternas of the surface cells. The very dense granules usually appeared to be associated with the light vacuoles (Fig. 5).

Parts of the margins of these cells did not have contact with other cells, so that extracellular spaces of irregular shape were formed (Figs. 3 and 7). Such spaces contained fibrils assumed to be collagen and much finely granular material. The substance in which fibrils and granules were embedded was of low electron density. The proportion of fibrils and other extracellular material to cells appeared to increase with increasing time on the diet.

Single-layered endothelium was found between the atherosclerotic plaques. With the naked eye, the conventional microscope, and the electron microscope, many portions of this endothelium retained the normal structure, even after 7 months on the diet. Other regions showed definite pathologic alterations in the form of slight increase in prominence of the endoplasmic reticulum and Golgi apparatus and the appearance of irregular dense masses in the cytoplasm. The small invaginations of the surface membrane were present, as were larger pockets projecting into the cytoplasm. These features of the cell membrane were found also in normal endothelium.

Endothelial Lesions of Rabbits Receiving a Diet of 1 Per Cent Cholesterol and 5 Per Cent Corn Oil

Vacuoles (Fig. 9) were seen more frequently in the surface layer of cells than in the corresponding cells of the rabbits on cholesterol diet only. The vacuoles now appeared to contain very dense material. Similarly, in the cells in the interior of the lesion (Fig. 9), many dense bodies and a smaller number of light ones were present. The former were interpreted to be osmiophilic lipid vacuoles and the latter to represent either non-osmiophilic lipid or some other material. In other respects the lesions in these arteries resembled those already described.

Discussion

The chemical nature of substances contained in the various inclusions could not be defined by electron microscopy. The lesions of cholesterol-fed rabbits have been shown to contain lipids by Sudan staining[6] and by chemical analysis.[7] Moreover, minerals,[8] acid muco-

polysaccharides[9] and collagen[8] have also been found in them. It is usually assumed that the lesions develop as a result of the incorporation into endothelial cells of substances from the blood in the lumen, although Leary[10] proposed that they might be formed by the deposition of "foam cells," as such, on the endothelial surface. A high level of blood lipids is associated with the development of the lesions, and the lipid has been shown to be combined with protein.[11]

This background of earlier work on experimental cholesterol atherosclerosis is of limited value in assessing the significance of the observations by electron microscopy. The problem is, of course, to localize, in the lesion, these various substances which have been identified by chemical methods. As far as I am aware, no study of the cytochemistry (except for Sudan staining) of these lesions has yet been published.

Lipoidal materials may appear as dense granules after osmium fixation (corn oil in intestinal cells[12]) or may be dissolved out in the preparation of the sections (sebaceous gland cells[13]), giving the cells a "foamy" or vacuolated appearance. Which of these two forms the lipid assumes in the sections appears to depend largely on the degree of saturation; unsaturated lipids generally become blackened by osmium.[14] Lipoprotein usually has a high density in electron micrographs (plasma membrane, mitochondria, myelin sheath). A paper by Wigglesworth[15] should be consulted for a more detailed account of osmium in fixation of lipids.

In the present study, frozen sections of formalin-fixed tissue were stained with Sudan black B in an attempt to correlate the electron micrographs with the features observed with the light microscope. The staining was diffuse, and a clear distinction could not be made between the surface covering cells and those in the interior of the plaque. Both the cells and the extracellular material were stained. The impression was gained, however, that the vacuolated cells noted in the interior of the plaques in the electron micrographs were probably "foam cells" and that the "empty" areas were spaces from which lipid had been dissolved. It was to test this idea that the rabbits were given the added corn oil, an unsaturated lipid, rather than cholesterol alone, on the assumption that osmiophilic lipid would then appear in the lesions. In these animals, many of the vacuoles were found to be filled with dense material, interpreted to be osmiophilic lipid. It was thus concluded that the vacuolated cells in the interior of the lesions in animals receiving cholesterol without corn oil were, in fact, "foam cells" and that probably the majority of the vacuoles contained lipid in life. The particular lipid mixture in these cells appeared to have been largely dissolved in the preparation of the sections, although the dense granules

associated with the otherwise "empty" vacuoles (Fig. 5) could be a highly osmiophilic residue of the vacuole contents. The view that these granules might represent early mineralization is considered with favor although there is no very good evidence for this.

The striking feature in the covering layer of cells was the scarcity of lipid vacuoles of the type seen in the "foam cells." Instead, the dilated cisternas of the endoplasmic reticulum were filled with a moderately dense material. The nature of this material is a matter for speculation at the present time. Since these cells were diffusely stained by Sudan black B, they probably contained some lipoidal material. As a working hypothesis, it is suggested that the substance staining in this manner corresponds to the dense material observed in the electron micrographs and that this is lipoprotein—more specifically, lipoprotein from the plasma.

It is suggested that the layer of cells covering the lesions is actively engaged in the taking up of lipoprotein from the plasma. The obvious hypertrophy of the endoplasmic reticulum and Golgi apparatus, as compared with the normal, speaks for an increased metabolic activity in these cells. The scarcity of lipid vacuoles in them suggests that, in cholesterol-fed rabbits, this layer of cells is presented with plasma lipids in a particle size too small to be recognized in the electron micrographs; that is, in the form of lipoprotein molecules, rather than chylomicrons. This idea is in accord with the observations obtained by ultracentrifugal investigations of plasma lipids in cholesterol-fed rabbits.[11] Possibly, if the cells had larger lipid particles available to them, these would also be taken in. The lining cells are capable of phagocytosis, as seen by their uptake of intravenously administered colloidal thorium dioxide. The conventional microscopic observations of Duff, McMillan and Lautsch[16] also established the phagocytic property of endothelial cells.

A hypothesis to cover the present findings is suggested. Lipoprotein enters the surface cells from the lumen. Possibly this occurs by way of the small vesicles under the plasma membrane, although these structures are less numerous than in normal endothelial cells. Lipoprotein is taken into the large cisternas of the endoplasmic reticulum. Here the protein component survives the treatment of embedding in methacrylate and appears in relatively dense fashion in electron micrographs. As the covering layer of cells is only poorly united with those below it (although its cells fit sufficiently closely to form a continuous layer), lipid is passed through the outer cytoplasmic membrane and comes to lie in the relatively wide extracellular spaces. From here it is taken up through a process of phagocytosis by the cells ("foam cells") com-

prising the greater part of the lesion. The location of the lipid is represented by "empty" spaces or vacuoles as the protein previously combined with the lipid has now been separated. Whether these phagocytic cells arise from endothelium or from the macrophage system is not known.

Endothelial lesions produced in the aortas of rabbits by feeding a diet containing 1.5 per cent cholesterol for various periods of time were studied in thin sections with the electron microscope.

Summary

Two morphologically distinct types of cells were observed. The cells of one type formed a continuous layer over the surface of the lesions. This layer was apparently rather loosely bound to the underlying lesion. Peculiar features of these cells were the prominent Golgi apparatus and the dilated cisternas of the endoplasmic reticulum filled with moderately dense material. Only very occasionally were vacuoles seen in the cytoplasm. The other cell type, found in the interior of the lesion, was highly vacuolated.

The electron microscopic picture is interpreted as suggesting the incorporation of lipoprotein into the endoplasmic reticulum of the surface cell layers. It is proposed that this layer of cells may play a particularly important role in the metabolism of lipid in the lesion. The other cell type appears to have the less specific role of phagocytosis of lipid from the extracellular spaces into cytoplasmic vacuoles.

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LEGENDS FOR FIGURES

Key:

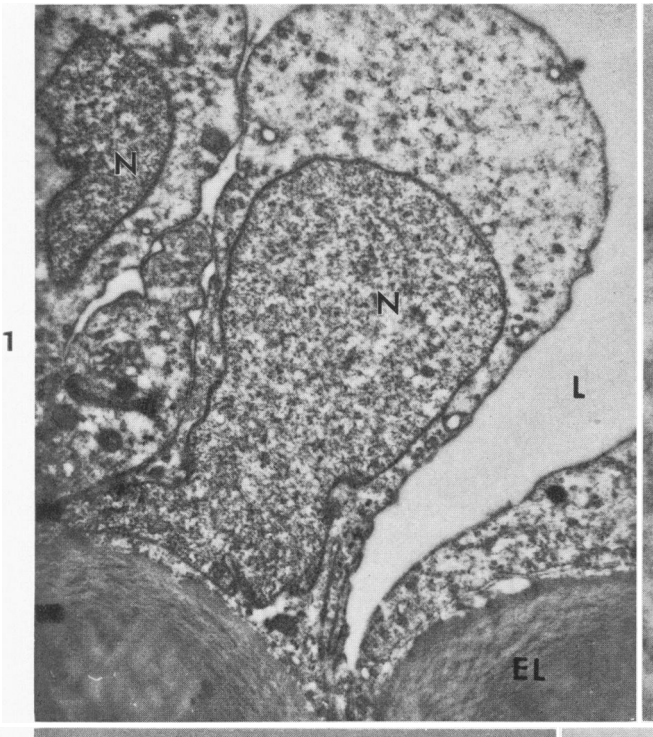
L, lumen of artery
N, nucleus
EL, internal elastic lamina
I, inclusion
V, vesicle or small invagination of cell membrane
GA, Golgi apparatus
ER, endoplasmic reticulum
M, mitochondrion
G, granule
Va, vacuole
ECS, extracellular space
PM, cytoplasmic membrane

FIG. 1. Normal endothelium of rabbit femoral artery. The cells protrude into the lumen as a result of contraction of the artery. They may rest directly on the internal elastic lamina, as seen here, or on a network of a few collagen fibrils. × 13,000.

FIG. 2. In the normal endothelial cell, the most characteristic feature of the cytoplasm is the presence of numerous tiny vesicles which are invaginations of the cytoplasmic membrane. × 41,000.

FIG. 3. Survey micrograph of a part of an intimal lesion from the aorta of a rabbit on a high cholesterol diet for 7 months. This section shows the characteristic pyramidal surface cell and part of the cytoplasm of two cells deeper in the lesion. One cell (a) is the common type in this location, having many light cytoplasmic vacuoles. The other (b) resembles the surface-type cell in having few vacuoles but a prominent endoplasmic reticulum. A large part of the lesion consists of extracellular material. × 9,000.

FIG. 4. A small area at the surface, showing the junction between two covering cells and a small part of one of the underlying cells (lower right quadrant). The covering cells are closely fitted at their lateral margins but not on their deep surfaces. The cytoplasmic membranes between adjoining cells of the surface layer may be quite convoluted, as seen here. The Golgi apparatus of these cells, consisting of smooth surfaced membranes, is prominent and here is seen cut across at two places. × 23,000.



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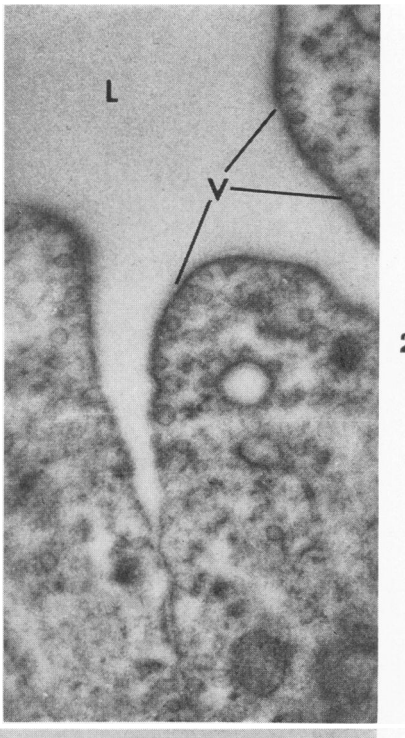

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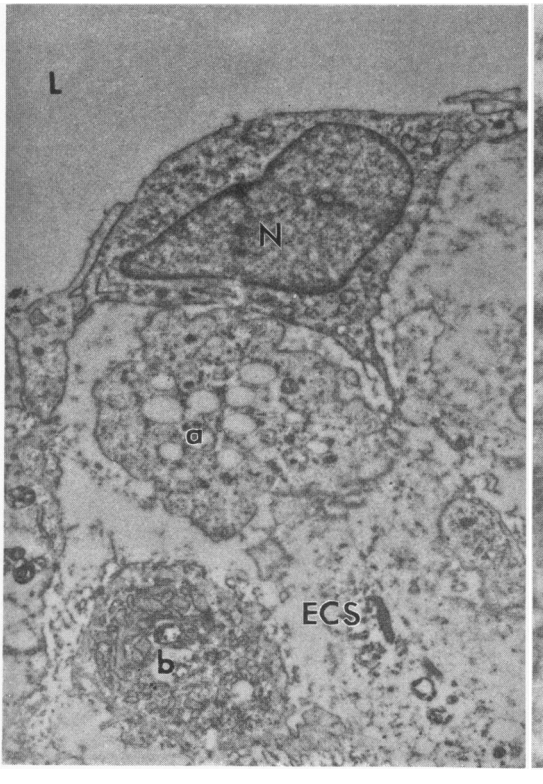

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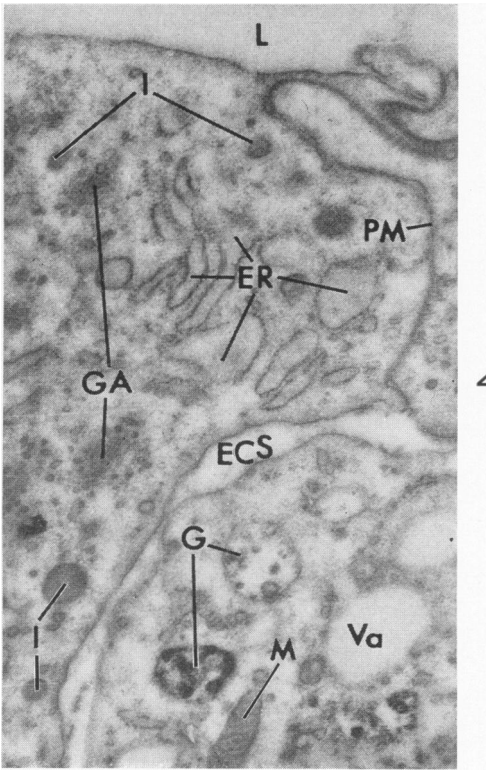

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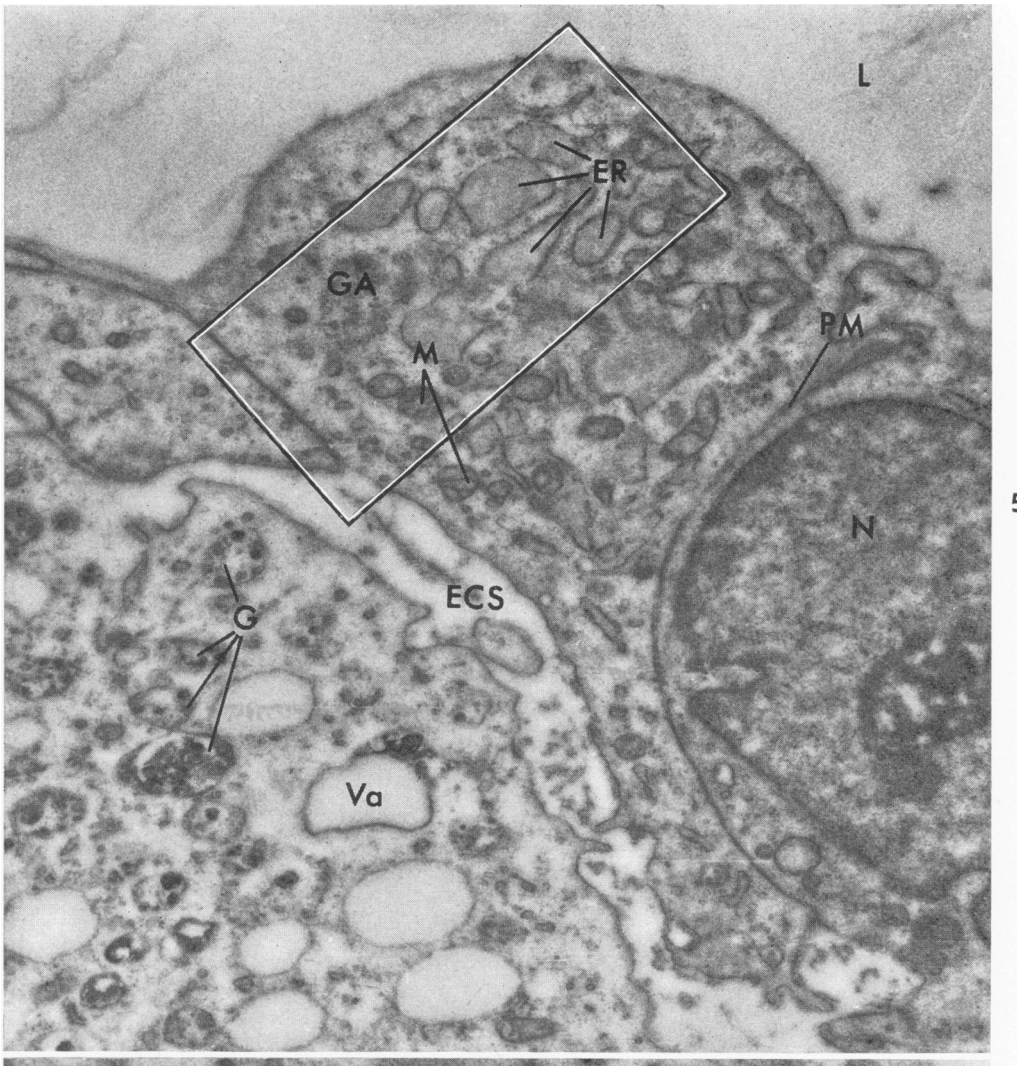
FIGS. 5 and 6. Figure 6 is the area outlined in Figure 5. The layer of covering cells containing prominent Golgi apparatuses and the dilated cisternas of the endoplasmic reticulum are covered on their outer surfaces by many fine granules (Fig. 6). Small invaginations of the cell membrane are seen at the surface and at intercellular boundaries. The cytoplasmic membranes between cells of the covering layer are separated by a relatively uniform gap, which enlarges to a wide and irregular extracellular space under this layer. This space continues into the interior of the lesion. A portion of one of the cells of the type found in the interior of the lesion is seen in the lower left quadrant of Figure 5. It contains many clear vacuoles, some of which are associated with very dense granules. Rabbit on cholesterol-rich diet for 7 months. Fig. 5: × 18,000; Fig. 6: × 39,000.



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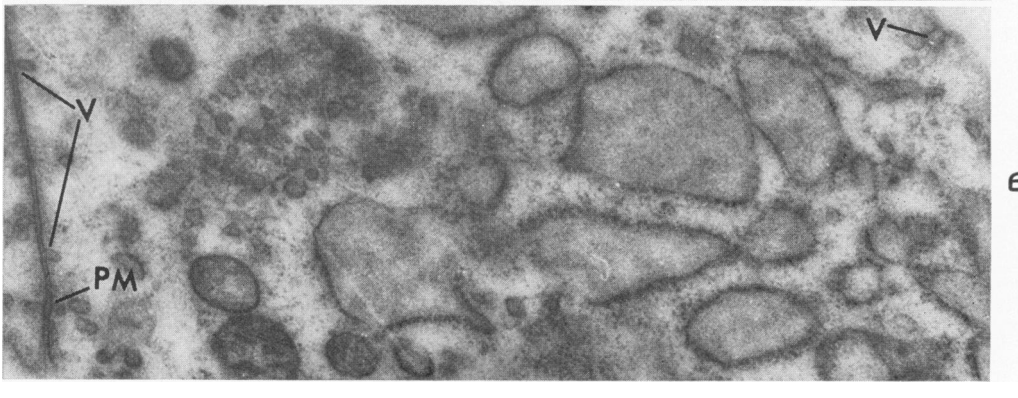

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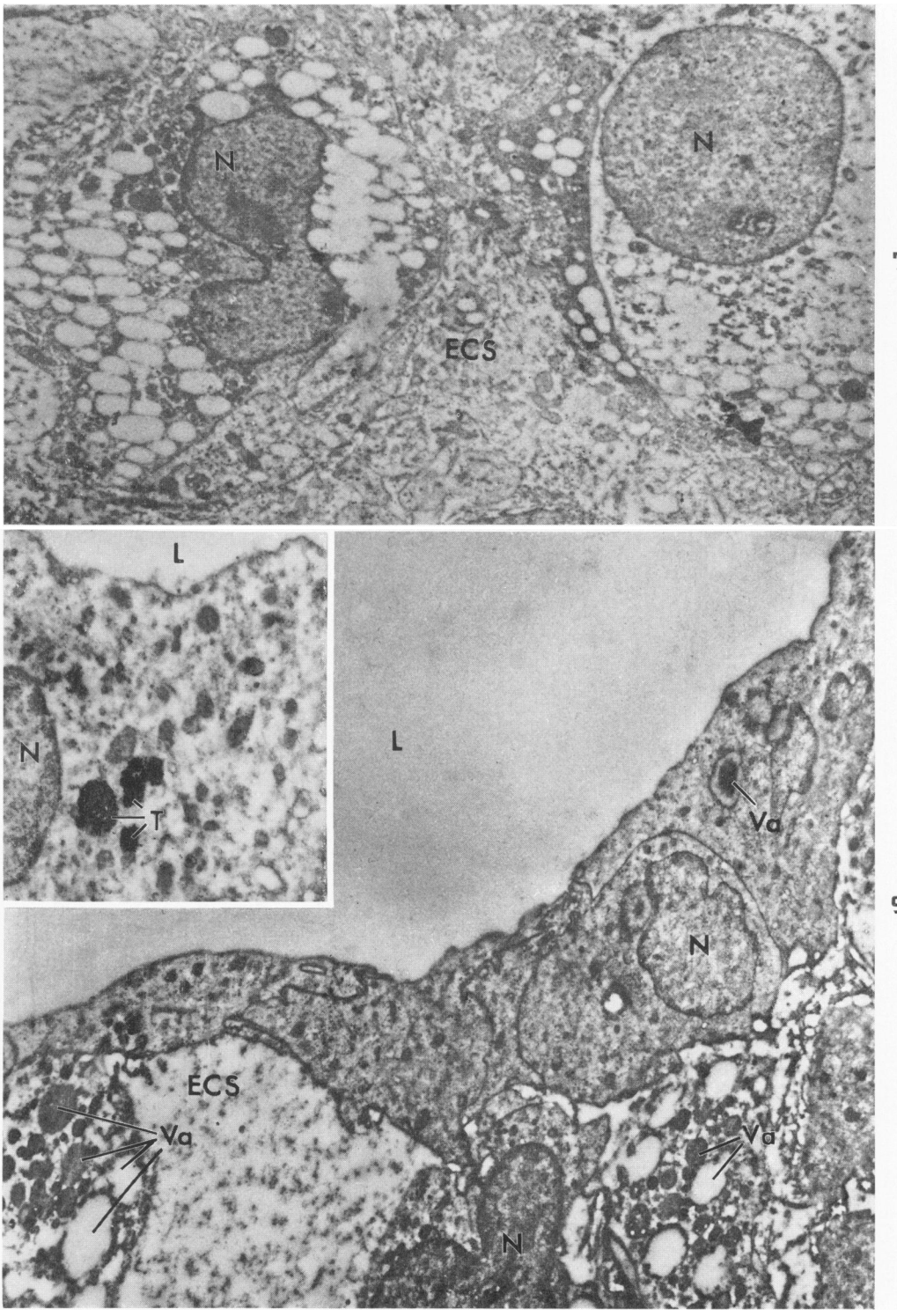
FIG. 7. Rabbit on high cholesterol diet for 5 months. Interior of a large plaque, showing two of the large, highly vacuolated cells interpreted as "foam cells." The extracellular space contains granular material and, in places, fibrils of collagen. × 6,000.

FIG. 8. Surface cell of lesion from a rabbit on high cholesterol diet for 5 months. An intravenous injection of 5 ml. of Thorotrast was given 24 hours before sacrifice. Particles of thorium dioxide are packed into cytoplasmic inclusions. × 22,000.

FIG. 9. Aorta of a rabbit fed 1.5 per cent cholesterol and 5 per cent corn oil for 6 months. In the surface layer of cells are vacuoles containing dense material, interpreted as osmiophilic lipid. Vacuoles of two types are found in other cells of the lesion; i.e., dense vacuoles, considered to be osmiophilic lipid droplets, are scattered among some light vacuoles in the cytoplasm of two cells below the surface layer (lower left and lower right). × 8,000.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