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Degeneration of Arterial Smooth Muscle Cells: Ultrastructural Study of Smooth Muscle Cell Death in Control and Cholesterol-Fed Animals*

H. Imai, S. K. Lee, S. J. Pastori, and W. A. Thomas
Department of Pathology, Albany Medical College, Albany, New York 12208

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Summary. Bifurcations of common carotid arteries of rhesus monkeys and trifurcations of terminal abdominal aortas of Yorkshire swine were examined by electron microscopy for dead and dying smooth muscle cells (SMC). Ultrastructural criteria are proposed for classifying SMC in arterial walls as dead or dying based on *in vitro* data reported by others. We were not able to make clear distinction between the dead and dying SMC and the term "degenerative forms" is used to include both. Degenerative forms were classified into two categories: rarefied and mummified. The frequency of apparent dead and dying SMC averaged 1.9% in carotid bifurcations of control monkeys. Four months of cholesterol feeding elevated the frequency to 4.2%. The frequency of degenerative forms in aortic trifurcations of control swine averaged 1.2%, and 3 days' cholesterol feeding elevated the value to 3.2%. Thus one of the early effects of oral administration of cholesterol appears to be injury to arterial SMC resulting in aggravation of cell death. Functional damage to the cell membranes is viewed as the most likely primary event that induces the sequence of morphologic changes associated with cell death.

Introduction

Advanced atherosclerotic lesions in man and in experimental animals are characterized by accumulations of pleomorphic smooth muscle cells (SMC), lipids, collagen, elastica and other extracellular materials leading to localized architectural distortion of the arterial wall. The predominant cell type in normal and diseased arteries has been identified by numerous investigators as SMC and only a relatively small subpopulation is represented by macrophages laden with lipids and debris, granular and agranular leukocytes and cells lacking identifying features (Langhans, 1866; Pease and Molinari, 1959—60; Rhodin, 1962; Buck, 1963; French *et al.*, 1965; Geer, 1965; Ghidoni and O'Neal, 1967; Lee *et al.*, 1970).

In a previous study, we found that some of the apparently dead and disintegrating cells in the atheromata of middle cerebral arteries of swine had ultrastructural features suggesting their origin from SMC (Imai and Thomas, 1968). Even in control animals, individual, randomly scattered apparently dead arterial SMC were found, and these were more common in animals on high fat diets. No evidence of sublethal injury, however, was recognized in either the diseased or control arteries. Our initial attempts at hypothetically linking viable SMC with dead cells by identifying images of dying cells were futile when we were using grossly normal, non-branching segments of middle cerebral arteries from swine

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fed high cholesterol or control diets. In retrospect it seems that convincing data were not gathered because of the scarcity of dying cells in these straight portions. Known predilection of the branching sites for the occurrence of early lesions prompted us to search for the missing link at such sites. Two such sites were chosen on the basis of ease of obtaining ample specimens for ultrastructural study: the aortic trifurcation, as an example of large elastic arteries, and bifurcation of the common carotid arteries as an example of muscular arteries.

The aim of the present study was to ascertain the ultrastructural features consistent with degeneration and death of arterial SMC in control and cholesterol-fed animals. Endothelial cells were excluded from this study because they are not directly involved in the architectural distortion which ,in our working hypothesis, is one of the salient features in the development of atherosclerosis. Special attention was focused on identifying the earliest change recognizable by electron microscopy, prior to the onset of unequivocal signs of death of SMC with an expectation of disclosing possible clues in regard to the target organelles of cell injury.

Materials and Methods

Eight male rhesus monkeys with an average body weight of 2.5 kg were used to obtain the bifurcations of common carotid arteries. Three monkeys (cholesterol group) were given 2 g cholesterol mixed in a tablespoon of banana-flavored commercial food for primates (Agway) daily for 4 months, in addition to 360 g semisynthetic diet containing butter, 9.7%; peanut oil, 9.7%; casein, 25%; sucrose, 38.9%; salt mix, 5.6%; vitamin mix, 2.8%; alphacel, 8.0% and choline chloride, 0.3%. Another 3 monkeys (non-cholesterol control group) were given the identical diet without cholesterol. In addition, 2 monkeys (pellet control group) were maintained on commercial chow for primates (Purina). A daily tablespoonful of banana-flavored food without cholesterol was given to all control monkeys.

Aortic trifurcations were taken from 8 young male Yorkshire swine weighing approximately 10 kg. They were fed 8 g cholesterol mixed with 530 g of dry milk powder (Borden's Parlac; 2,400 calories, 22% protein, 49% fat and 29% carbohydrate) and water for 3 days. Eight control swine were fed the same amount of milk only.

All animals were given enough water for adequate hydration and caged individually in air-conditioned animal facilities. At the termination of the experiment, the animals were deeply anesthetized by an overdose of pentobarbital sodium and promptly autopsied. Aortic trifurcations including short segments of abdominal aorta and common iliac arteries were resected. They were fixed *in toto* for 2 hours in 2% acrylic aldehyde and 4% glutaraldehyde in Sorensen's 0.1 M phosphate buffer, pH 7.4. For *in situ* fixation of carotid arteries of monkeys, 0.5 ml of tetracaine was injected into each of the proximal common carotid arteries; primary fixation by perfusion with the combined acrylic aldehyde and glutaraldehyde was started within 10 minutes with 100 to 110 mmHg pressure gradient. After one half hour the carotid arteries were resected, and further fixed in an expanded state for a total of 2 hours. Both branching sites were rinsed in phosphate buffer, sliced into small blocks and post-fixed in Dalton's osmic acid fixative for 2 hours. Specimens were dehydrated in ascending alcohol series and embedded in epoxy resins. Orientation and selection of suitable areas for ultrathin sectioning were done by light microscopy using relatively thick sections of the entire tissue blocks. Thin sections were mounted on bare grids, stained with uranyl acetate and lead citrate, and examined in an electron microscope with accelerating voltage of 100 KV.

For quantitative comparisons concerning the frequencies and morphologic types of degenerative SMC in the arterial walls, 3 to 6 plastic-embedded tissue blocks were used on each animal. These tissue blocks were chosen on the basis of good orientation so that all three layers could be seen on light microscopy sections. Blocks were trimmed so that thin sections of the entire intima and media could be cut and mounted on specimen grids. The total cell counts were made at 2,400 or 5,000× magnification in the open areas of 75 × 300 mesh.

Evaluation of the degenerative cells was done at 10,000× to 40,000×, and visual impressions were confirmed or corrected using photographic prints when necessary. The counts of all specimens in Tables 1 and 2 were made by a single observer.

Results

No gross lesions were observed in any of the animals, but at the branching sites a ridge was regularly encountered in both cholesterol and control groups. Microscopically these ridges were characterized by a mound-like protrusion containing collections of cells and extracellular materials. In these areas the internal elastica was often irregular and demarcation between the intima and media was not definite.

The cells that appeared degenerative were infrequent in occurrence and seen singly, scattered at random, throughout the arterial wall. Qualitatively, the ultrastructural features that we chose as characteristic of degeneration or death were shared by both species and all dietary groups in both vascular segments.

I. Ultrastructural Features of Degenerating and Dead Cells (Degenerative Forms) in the Arteries

Ultrastructural manifestations of cells that we considered as destined to undergo eventual disintegration (impending cell death or irreversible cellular injury) were divided into two major categories, a) rarefaction, and b) mummification (condensation).

The cells that appeared damaged could usually be readily identified in regard to the cell type by the ultrastructural features indistinguishable from those in the adjacent medial SMC, such as arrays of thin cytoplasmic filaments, fusiform densities and basement membrane (Figs. 1, 2 and 6). When there had been moderate derangement of such features (Figs. 4, 5, 7 and 8), general configuration was also taken into account. Lysosomal densities were rarely seen in these relatively young animals. Lysosomes did not appear to be involved in the initial phase of cell death, but were found in the "post-mortem" phase as phagocytized bodies which we interpreted as one of the phenomena resulting from cell death, as will be described later.

a) Cell Death by Rarefaction. The slightest, possibly the earliest, manifestations of this type of degeneration appeared to be confined to the nuclear substance (Fig. 1). The cytoplasmic features in this category were indistinguishable from those of the majority of SMC in the arterial wall (Figs. 5 and 9) and conformed to the reported norm (Pease and Molinari, 1959—60; Rhodin, 1962). Normal SMC were characterized by abundant longitudinal myofilaments and fusiform densities throughout except for the perinuclear cytoplasm where profiles of granular endoplasmic reticulum, free ribosomes and mitochondria were concentrated. Ultrastructural features included mainly marginal micropinocytotic vesicles and sarcolemma with undisrupted plasmalemma and adjacent basement membrane. In slightly rarefied SMC the perinuclear cistern of endoplasmic reticulum, nuclear membrane and nuclear dense zone appeared intact but the nuclear contents did not have the usual density. The interior of the nucleus was occupied by clear

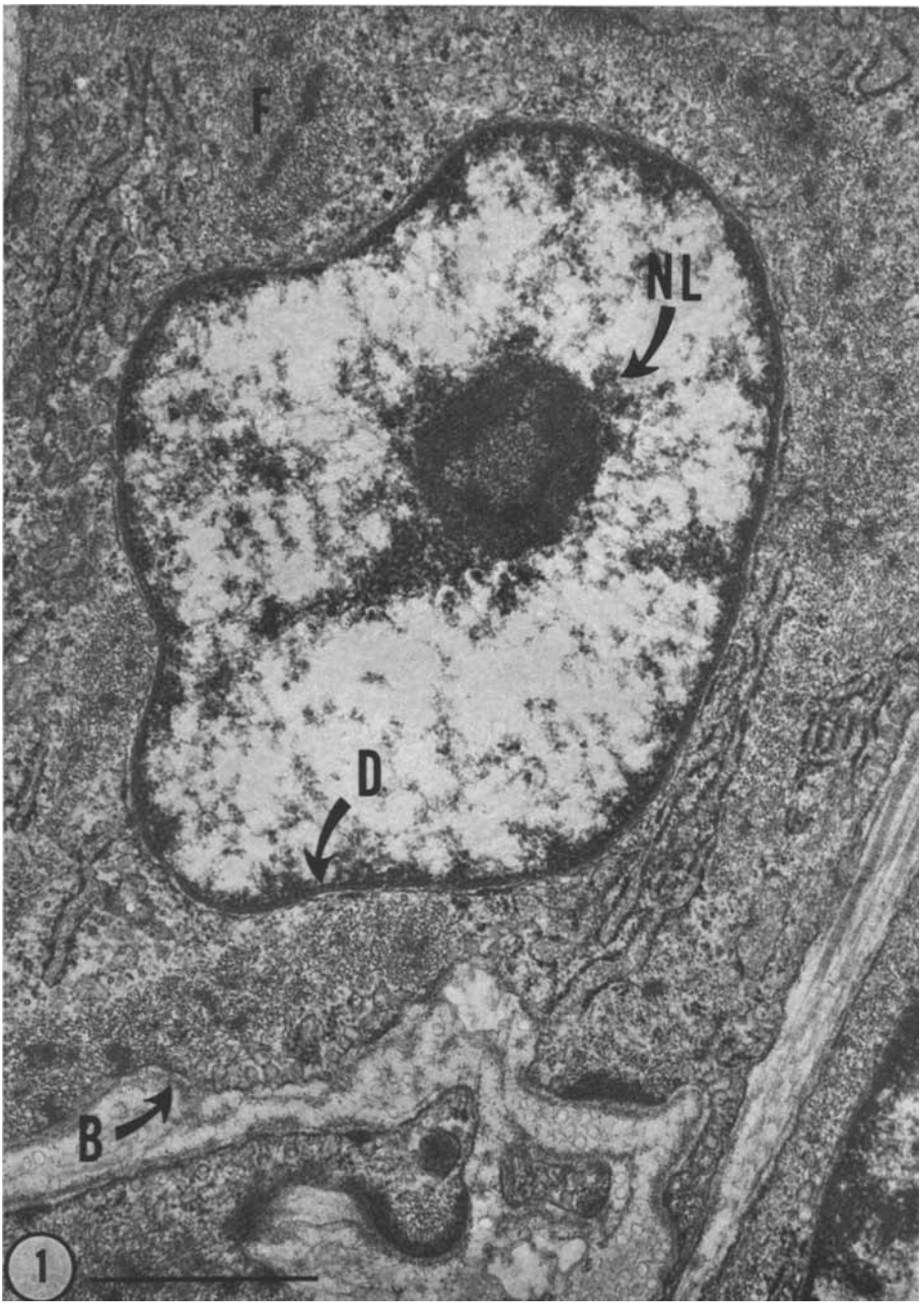

Fig. 1. A cross-sectioned smooth muscle cell. A large portion of the nucleus is electron-lucent, making the nucleolus and associated chromatin (*NL*) appear prominent. Other ultrastructural features including endoplasmic reticulum, fusiform densities (*F*), myofilaments and basement membrane (*B*) are indistinguishable from those of the adjacent SMC. *D* nuclear dense zone. See also Figs. 5 and 9 for normal nuclei of SMC. All illustrations were taken from tissues fixed in the combined aldehyde and osmium fixatives for 2 hours each. Black horizontal bar near the figure number indicates 1 micron, and white and black bar 0.5 micron

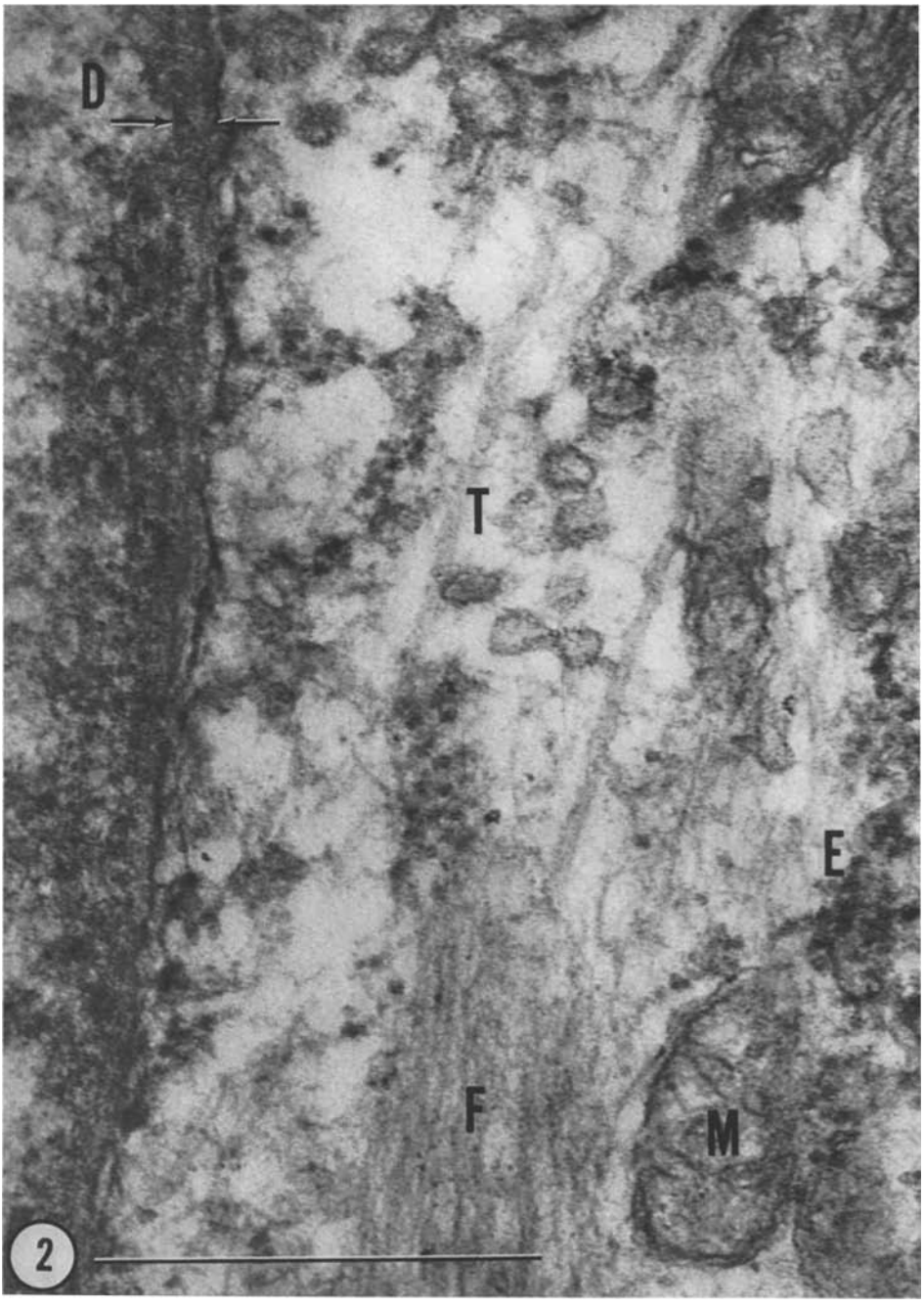

Fig. 2. A small portion of rarefied nucleus and intact nuclear dense zone (*D*) are on the left. The cytoplasm is focally rarefied making the filamentous and particulate organelles more distinct than usual. *T* microtubules; *M* mitochondria; *E* endoplasmic reticulum; *F* myofilaments

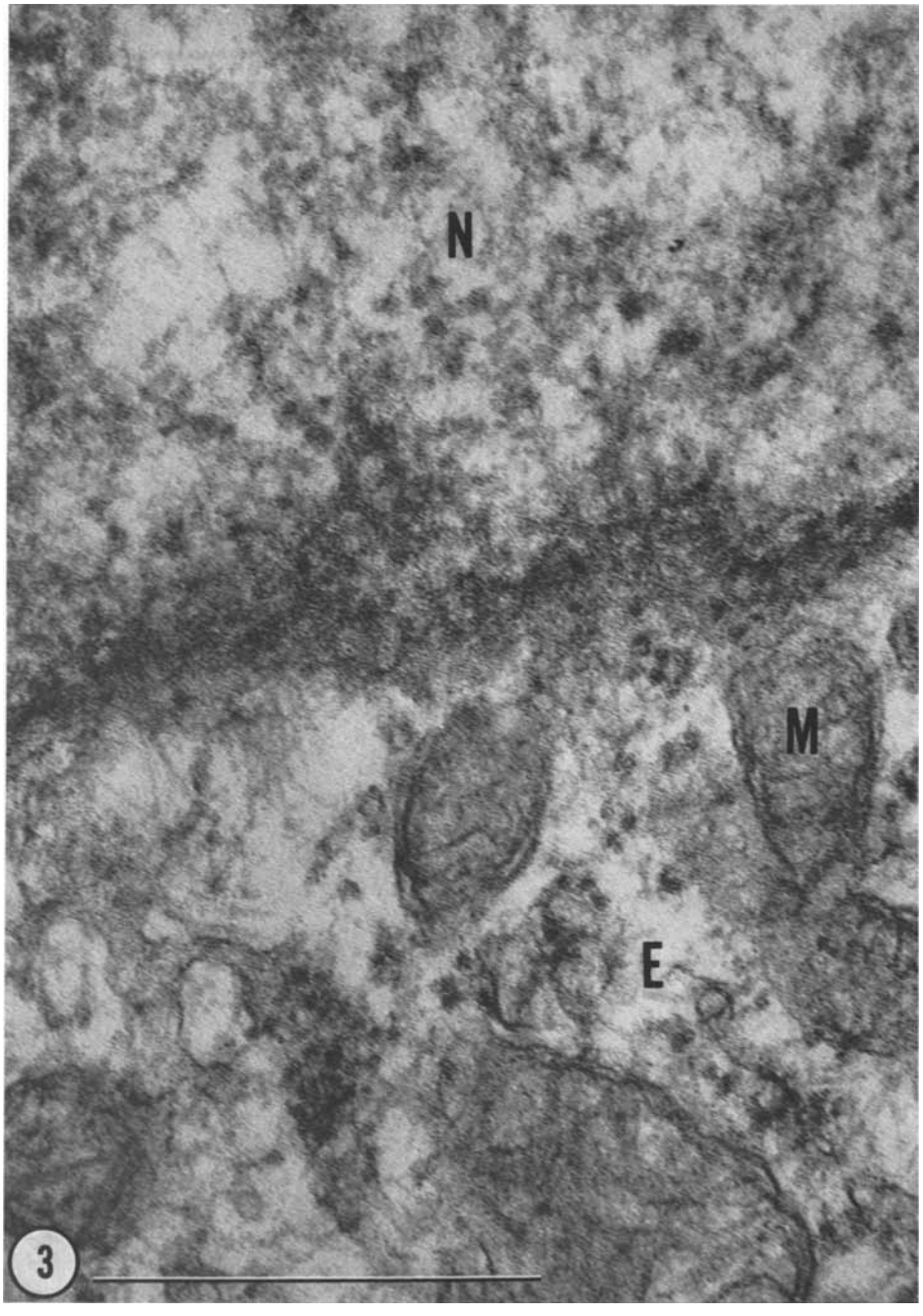

Fig. 3. The cell is severely rarefied throughout, yet most of the mitochondria (*M*) appear intact. Arrangement of the ribosomes associated with endoplasmic reticulum (*E*) appear random and less orderly than usual. *N* nucleus

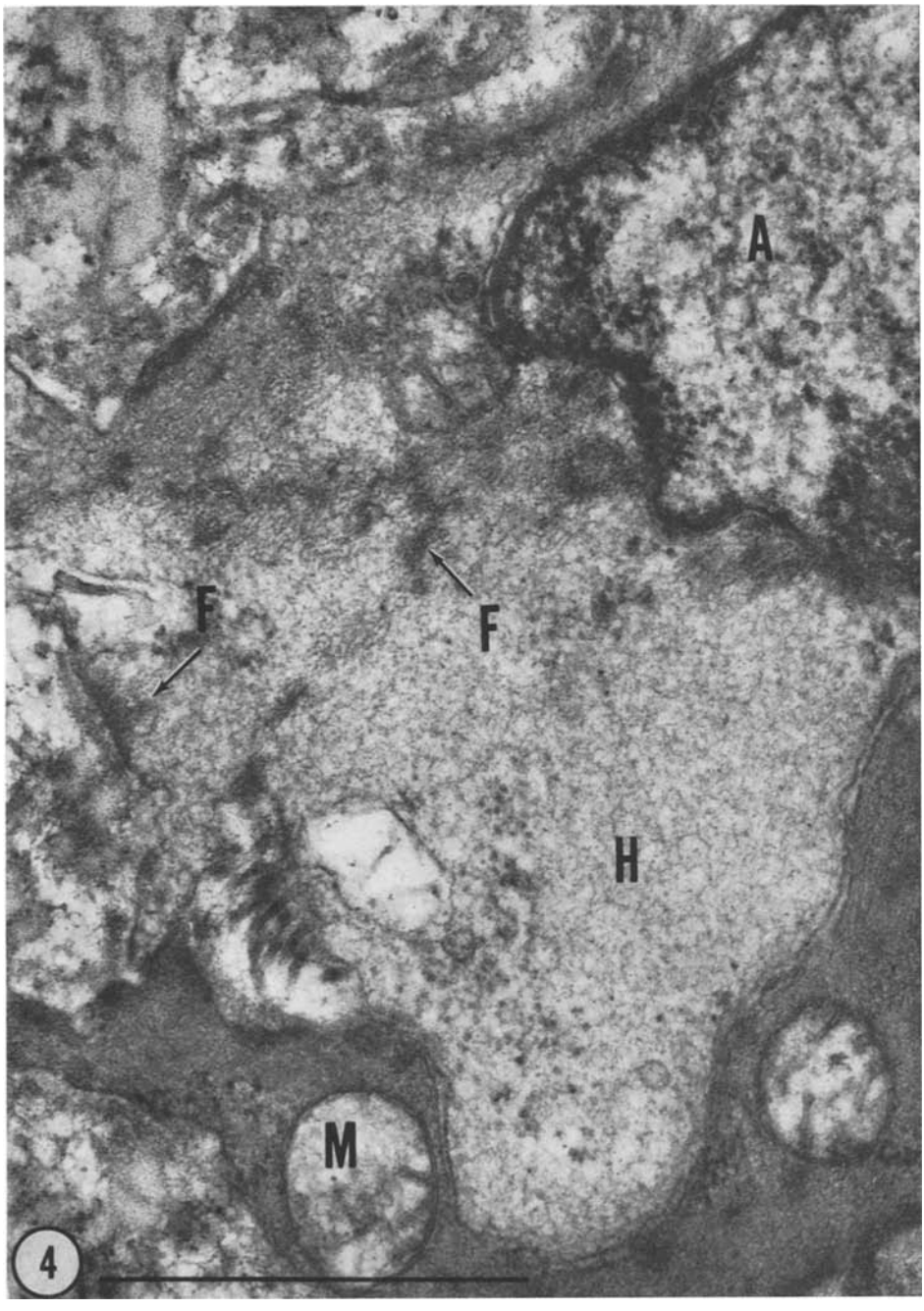

Fig. 4. Both nucleus (*A*) and cytoplasm are moderately rarefied. Cytoplasmic alterations are uneven. One area is nearly structureless (*H*), and closely abutted by an adjacent SMC that is darker than usual and has swollen mitochondria (*M*). The rest of the cytoplasm retains filamentous appearance with coarse fusiform densities (*F*)

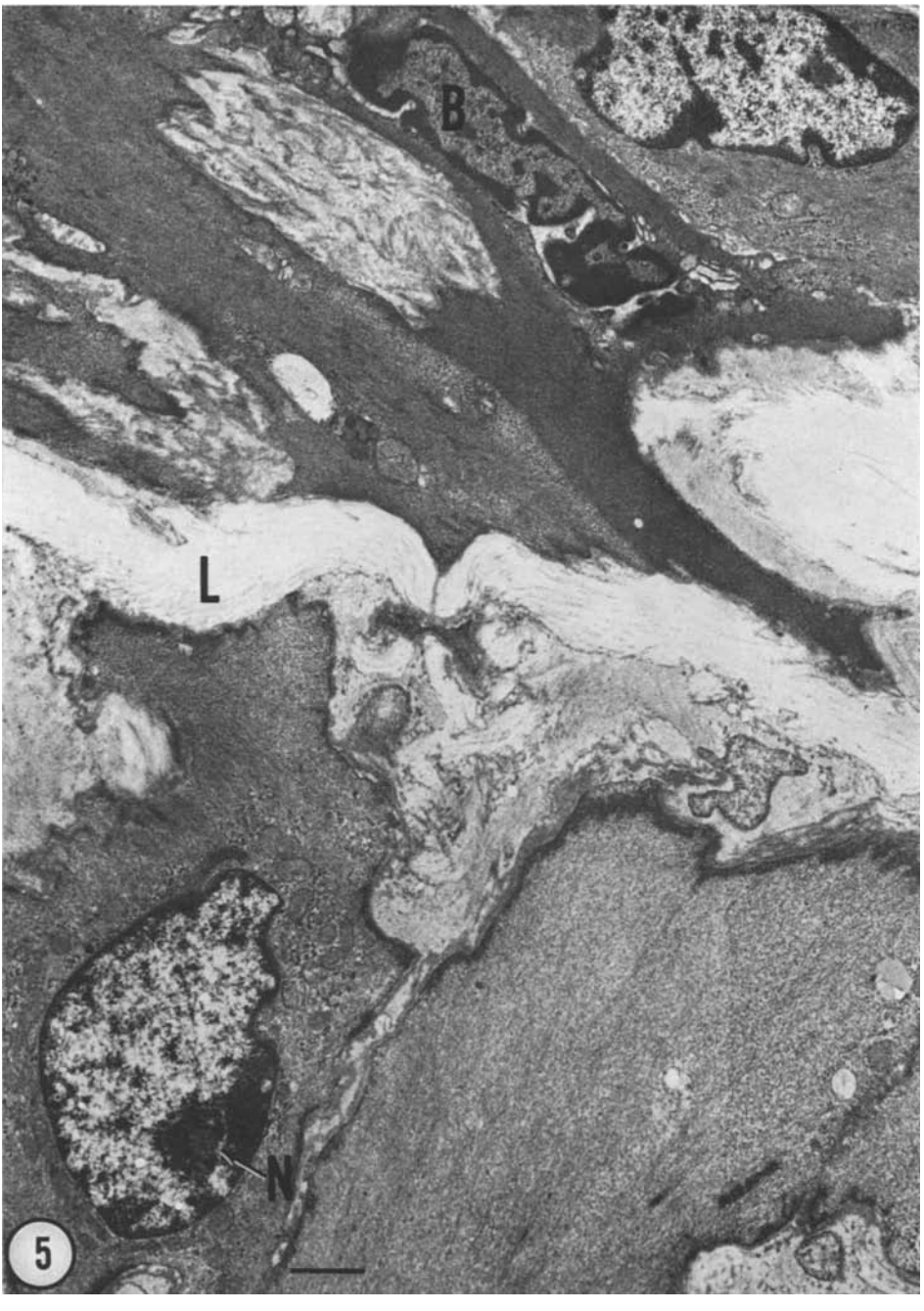

Fig. 5. There are two rows of SMC separated by a layer of elastica (L). The cell B is moderately condensed, although it appears to be a native constituent of the row of SMC. The nuclei at the right upper and left lower corners appear normal being composed of diffuse fine granular densities, occasional clumps of chromatin and homogeneous gray background material throughout. N nucleolus and its associated chromatin

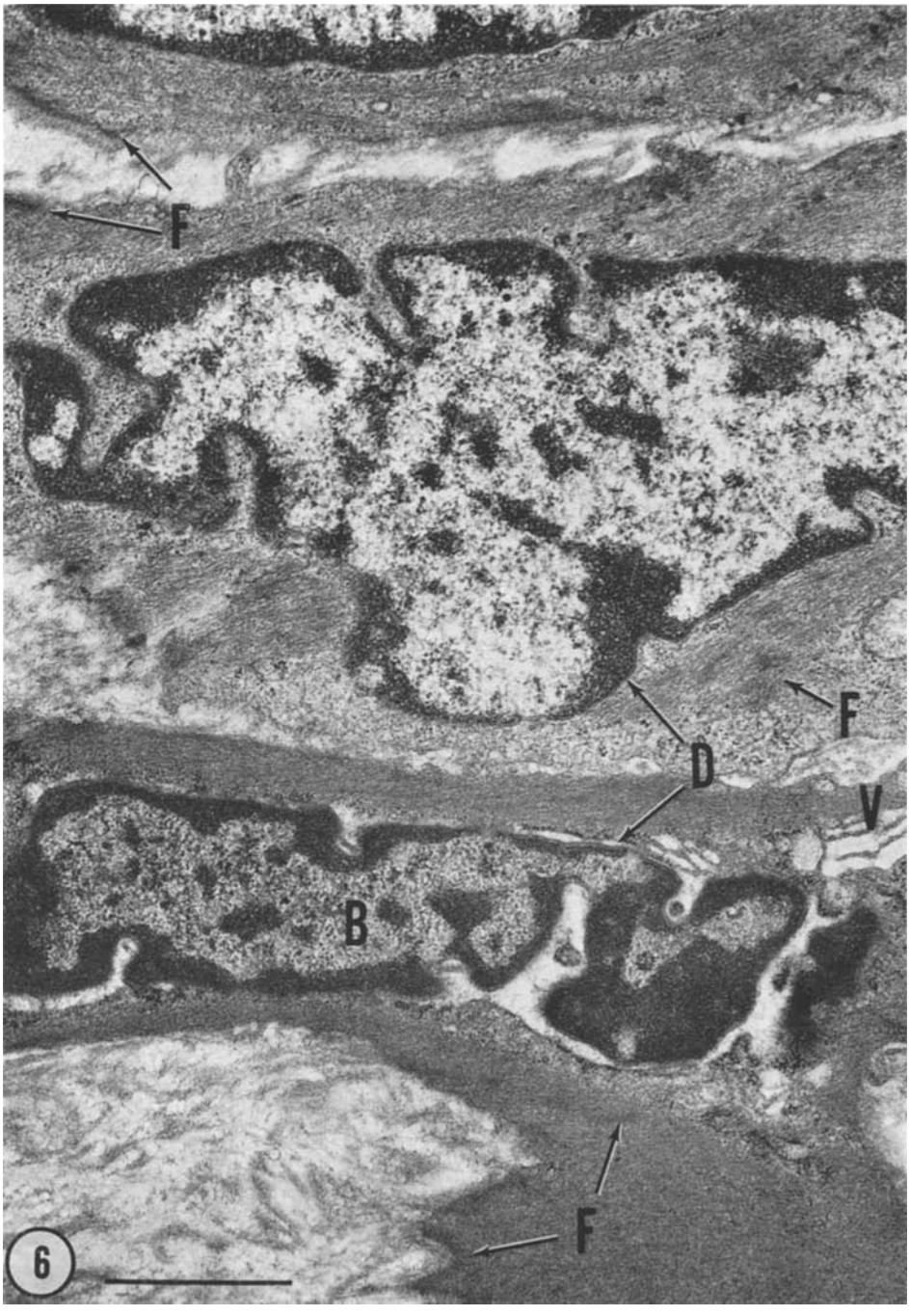

Fig. 6. The same condensed cell (*B* in Fig. 5). As if to compensate for the general condensation and probable shrinkage, some of the vesicles (*V*), probably Golgi, and the perinuclear cistern appear overdistended. The cytoplasmic arrays of filaments are compact, and fusiform densities (*F*) can be seen as in the usual SMC above. *D* nuclear dense zone

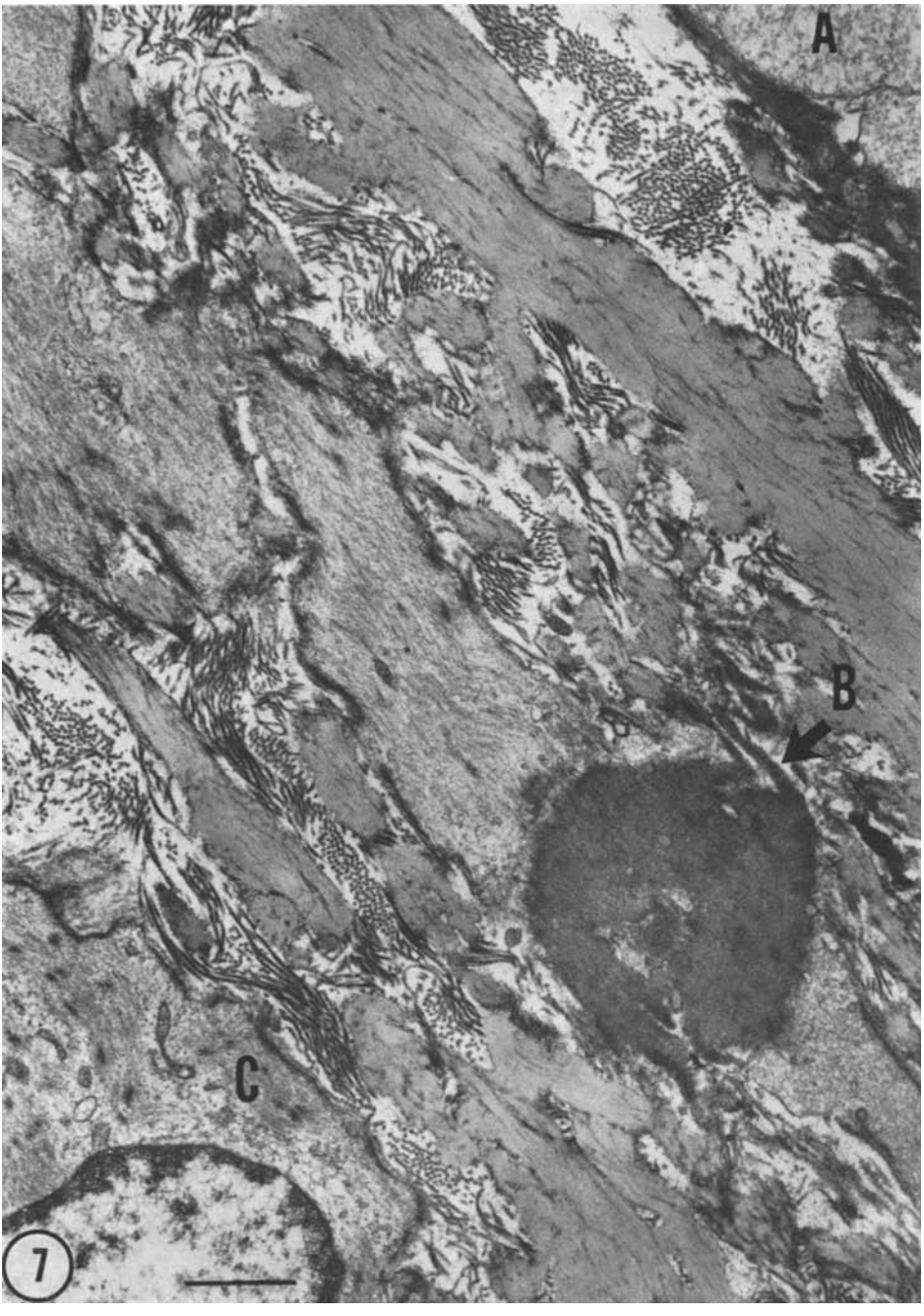

Fig. 7. Within a small area, rarefied cytoplasm (*A*), moderately condensed cytoplasm (*B*) and a SMC (*C*) with a slightly rarefied nucleus are seen. Note that the portion *B* appears to be surrounded by a SMC with focal concentration of vesicles

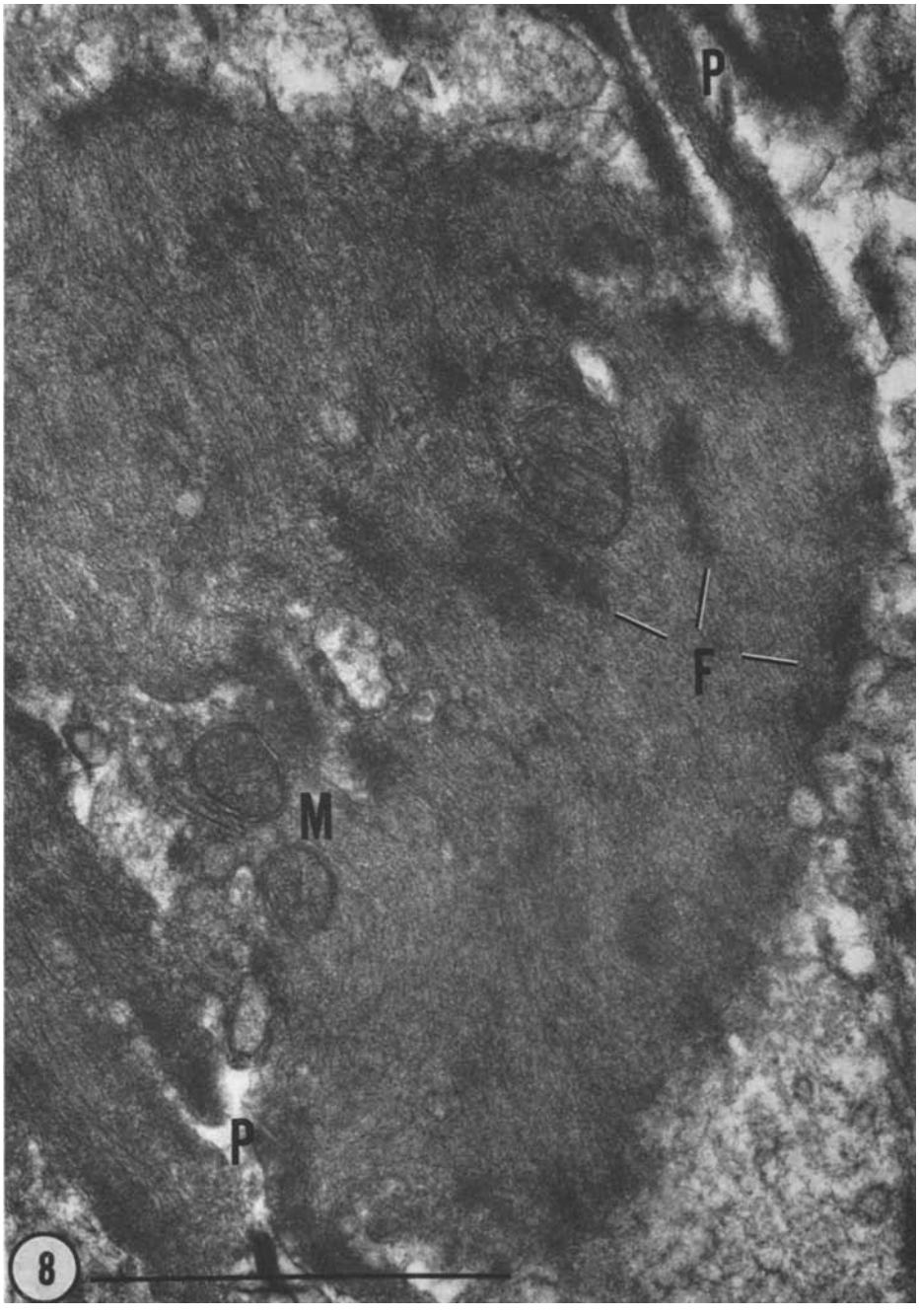

Fig. 8. Higher magnification of the area with the condensed cytoplasm (*B* in Fig. 7). It illustrates relatively well preserved, yet distorted fine structures. Altered, but recognizable, mitochondria (*M*), fusiform densities (*F*), thick filaments and vesicular organelles can be seen. Cytoplasmic processes (*P*) appear folded

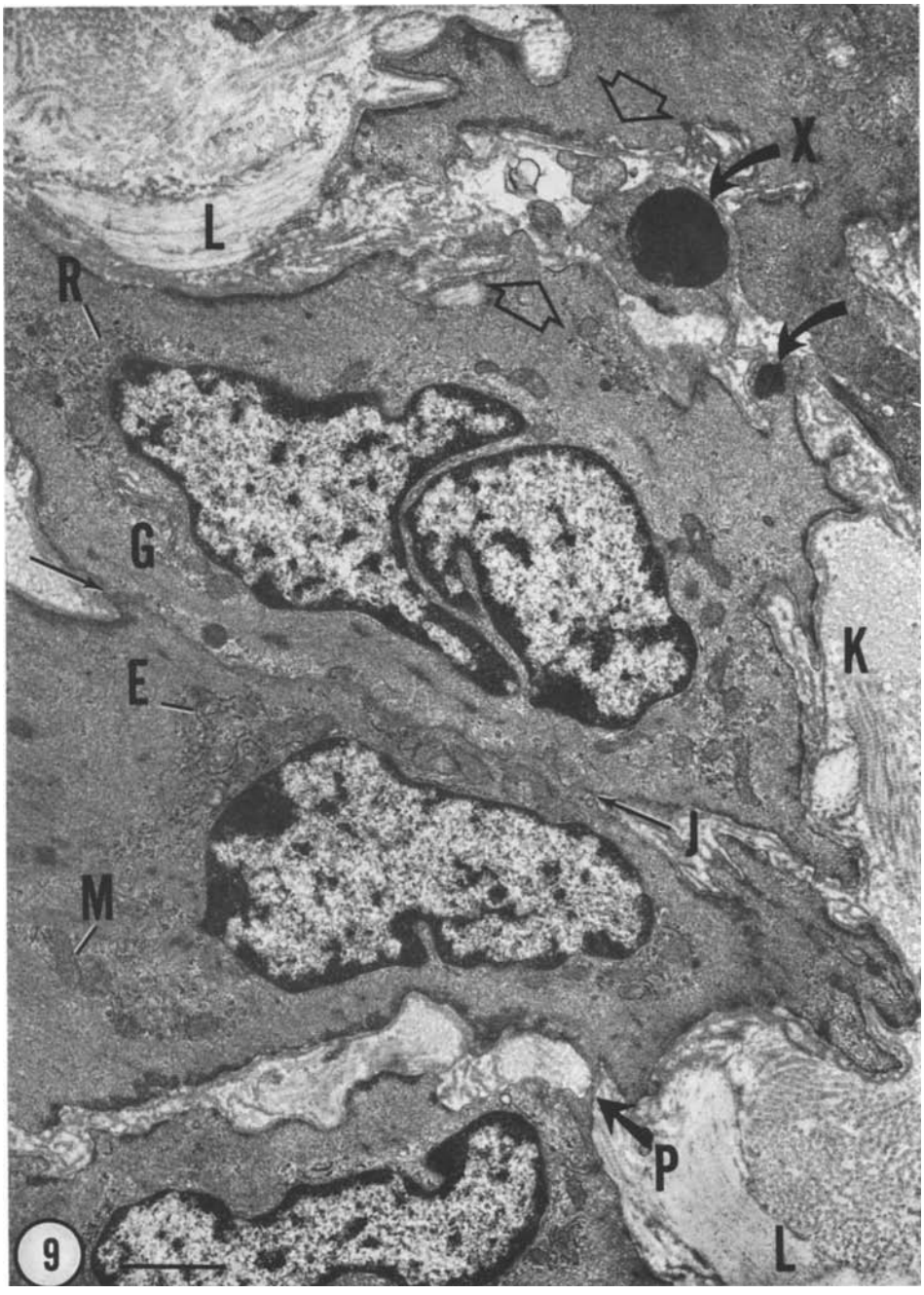

Fig. 9. A regular row of SMC between the elastic laminae (L) and bundles of collagen (K). The usual junction of SMC is either a nexus with a short process (P) or with two relatively long and straight abutting segments of cell membranes (J). Two opposing clear arrows indicate the area with slight departure from the usual cell junction. A relatively large dense body (X) and small densities (curved arrow) are also noted. Two SMC in the middle illustrate the

blebs that appeared to coalesce progressively leading to relative prominence of the intact nucleolus and its associated chromatin (Fig. 1). This phase of degeneration will be referred to in Section II on quantitative comparisons, as A-1 degeneration. No cell with intact nucleus and damaged cytoplasm was found.

According to the degree of derangement of cellular features manifested the next phase (A-2 degeneration) was characterized by more severe rarefaction of the nuclear contents accompanied by focal reduction in electron density along the nuclear membrane and formation of cytoplasmic blebs making individually intact organelles appear prominent by contrast (Fig. 2). Endoplasmic reticulum, including the perinuclear cistern, did not appear dilated.

In the extremely rarefied and swollen SMC (A-3 degeneration), comparable to cell death by cytolysis in light microscopy, the distinction between the nucleus and cytoplasm was no longer obvious due to diffuse loss of electron density. The entire nucleus was occupied by low density material and dispersed fine granules (Fig. 3). The nucleolus and nuclear dense zone were not demonstrable, and the perinuclear cistern was obscure or appeared to have detached as vesicles. The rest of the endoplasmic reticulum was either within normal limits or slightly dilated with relatively few ribosomes. Arrangement of particulate densities seemed random; for instance, no distinct whorly pattern of the ribosomes was noted. Most of the individual organelles, however, appeared intact. Co-existence of intact mitochondria and a few grossly swollen mitochondria within individual SMC was encountered in degenerative and non-degenerative forms (Imai *et al.*, 1966; Imai and Pastori, 1968).

Unlike the necrotic cerebral arterial lesions described in a previous report where products of cell death seemed to represent multiple cells more often than single cells (Imai and Thomas, 1968), degenerative SMC at the branching sites in this study were seen singly. Nonetheless, some of the adjacent SMC appeared to have responded to the presence of damaged cells as scavengers, as was found in the necrotic lesions (Figs. 4 and 7). With little architectural distortion of the area, severely damaged SMC were intimately surrounded by neighboring SMC as if being sequestered. In addition to such images that were consistent with breakdown of SMC *in toto*, examination of multiple neighboring sections revealed focal extracellular collections of dispersed, altered organelles that probably represented rupture of the plasmalemma with release of its contents into the stroma (Fig. 12).

b) Cell Death by Mummification. As in cell degeneration and death by rarefaction, the cells that appeared to be undergoing mummification occurred singly. Within the relatively orderly rows of SMC the entire cell body of individual SMC appeared to have been condensed, except for some of the mitochondria, vesicles

usual features of obliquely sectioned medial SMC. Elongated cell bodies are surrounded by adjacent basement membrane. The cell membranes appear thickened in places because of fusiform densities. They are also seen along the myofilaments which can be recognized only as fine granularity or streaks at this low magnification. Small areas around the nucleus are non-filamentous and are occupied by Golgi (*G*), rough endoplasmic reticulum (*E*), free ribosomes (*R*) and mitochondria (*M*). Indentations of varying depths are often seen in the central nuclei of SMC fixed in a contracted state

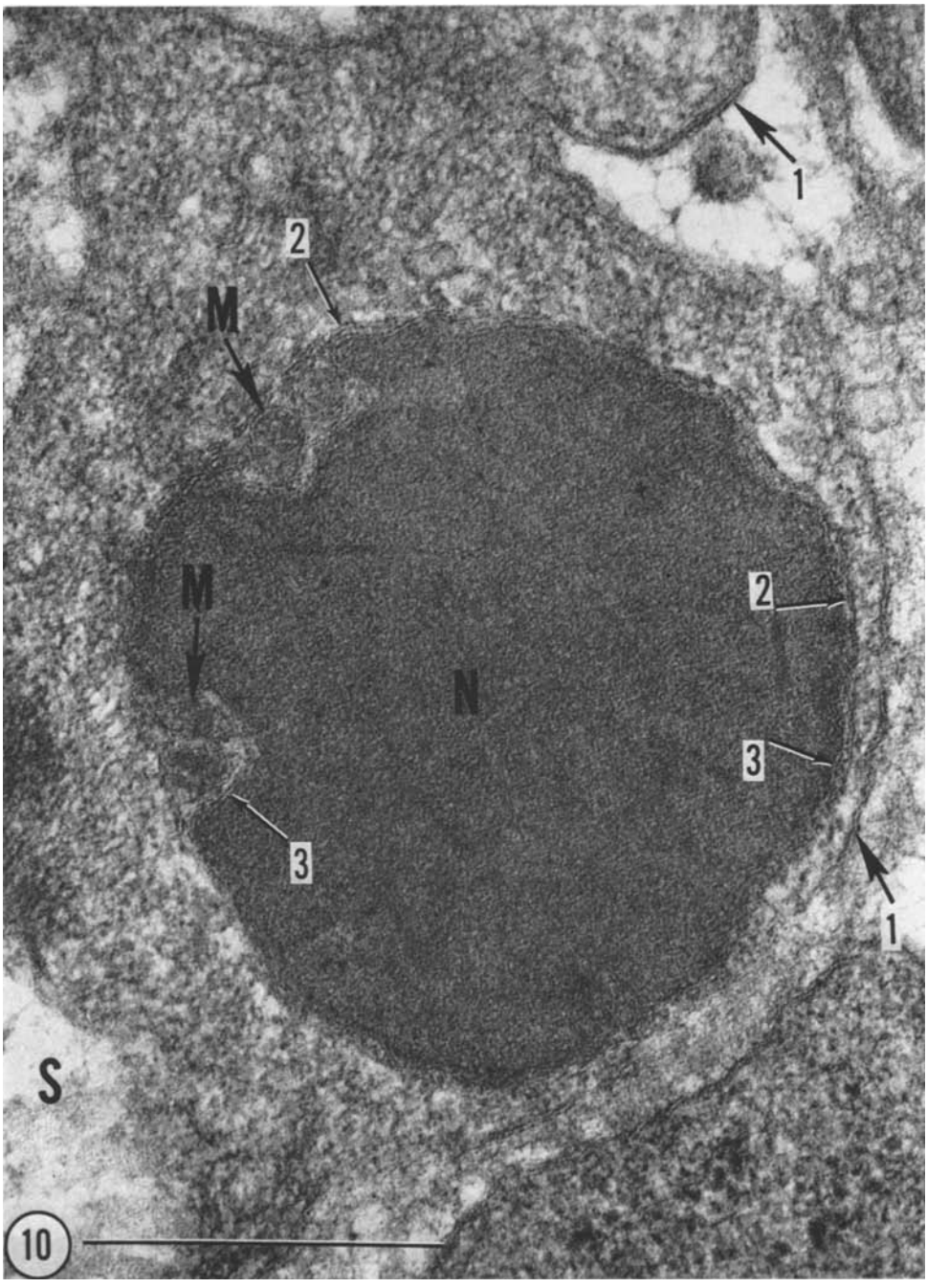

Fig. 10. The large dense body (*X* in Fig. 9) is shown at a higher magnification revealing that there are two compartments in this structure. The inner, relatively homogeneous part (*N*) is probably the nuclear debris, and the narrow outer, slightly lighter area is probably the cytoplasmic debris with dense particles, some of which are reminiscent of mitochondria (*M*). The plasmalemma of SMC is indicated by the arrows (*1*). A slightly thinner membrane limiting the debris (possibly the remains of plasmalemma or the wall of a phagocytic vacuole) is labeled (*2*). The arrows (*3*) point the limiting membrane of the assumed nuclear debris. *S* fibrous stroma

and perinuclear cistern, that appeared in contrast clear and distended (Figs. 5 and 6). The cytoplasm appeared to have shrunken slightly and condensed diffusely and uniformly without gross distortion of the arrangement of organelles. Cytoplasmic thin filaments and fusiform densities could be demonstrated as coarse streaks. The nuclear membranes appeared to have been pulled away from the perinuclear cistern, which contained a small amount of fine granular material. Condensation of chromatin was diffuse and uniform (comparable to pyknosis in light microscopy), but the nuclear dense zone could be seen in places. Such an image was considered as slight mummification and will be referred to as B-1 degeneration.

B-2 degeneration was characterized by the addition of structural distortion and progression of condensation (Figs. 7 and 8). Condensed cell bodies could be differentiated revealing ultrastructural details, but the distortion of structures and changes in their arrangement were evident. As in cell death by rarefaction, some of the surrounding SMC appeared to respond as scavengers (Figs. 7, 9—11).

Mummified cells that had undergone further deterioration and fragmentation were seen either in the stroma or apparently phagocytized by SMC (Figs. 10, 11 and 12). Dense and homogeneous pyknotic nuclei or nuclear fragments could be distinguished from slightly lighter cytoplasm containing a few of the persistent, although greatly altered particulate or filamentous organelles (Fig. 10). Both the nuclear and plasma membranes appeared to consist of relatively simple trilaminar (unit) membranes.

II. Quantitative Comparisons of Numbers of Degenerative Forms in Arterial Walls in Various Groups

a) Bifurcation of Common Carotid Artery in Rhesus Monkey. Counts and computations of SMC death by rarefaction and mummification at the bifurcation of common carotid arteries after 4 months of cholesterol feeding are presented in Table 1. At this segment of muscular arteries the rarefaction type appeared to predominate in all dietary groups. Frequency of the mummification type was low in all groups, but was significantly higher in the cholesterol group than in controls. Individually or combined, both A and B types occurred more frequently in the cholesterol group than in the non-cholesterol (high fat) or pellet control group. No significant difference was noted between the non-cholesterol (high fat) and pellet control groups in the frequency of degenerative forms.

Within the cholesterol group, the highest frequency of the rarefaction type was 5.7% and the lowest 3.4%. The highest frequencies in the non-cholesterol and pellet control groups did not reach the lowest value in the cholesterol group. Severe degenerative changes were infrequent, especially in the rarefaction type in all groups. No consistent pattern was noted in the occurrence of cell fragments, either free in the stroma or apparently phagocytized by SMC.

b) Aortic Trifurcation in Swine. Table 2 is a summation of the results obtained from 4 swine fed the milk-cholesterol and 4 fed the control milk diet for 3 days. The results are similar to those obtained with the carotid bifurcation of rhesus monkeys. Cholesterol feeding appeared to double the incidence of degenerative forms at the aortic trifurcation in swine. In regard to the types of degeneration, however, mummification was consistently more frequent in both the cholesterol and non-cholesterol groups.

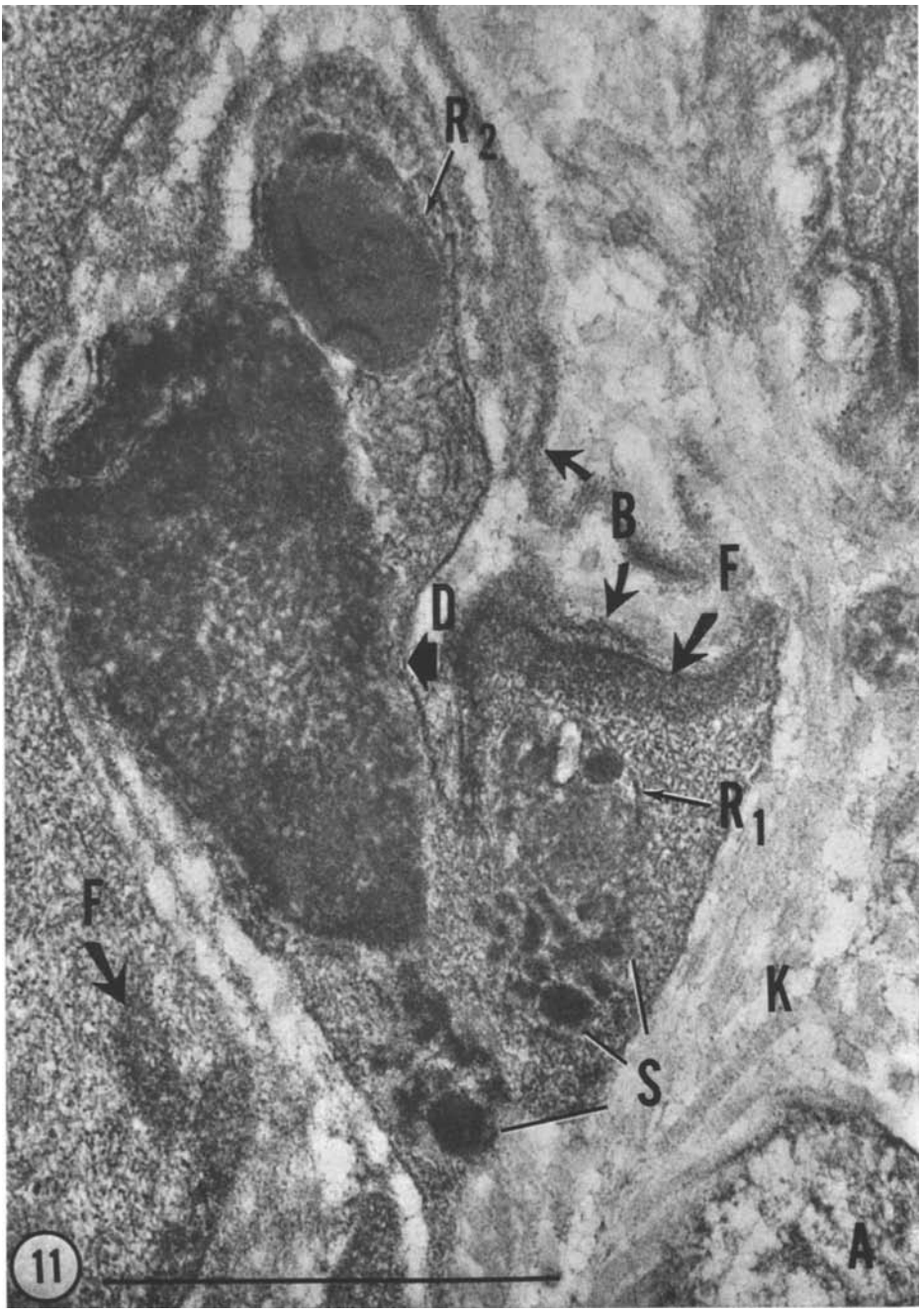

Fig. 11. Condensed cell debris (*D*, *R*, *S*) in a cell that can be identified as SMC by fusiform densities (*F*), arrays of cross-sectioned thin filaments and basement membrane (*B*). While small inclusions (*S*) appear to have no membranous enclosure, R_1 has at least a partial one. R_2 appears to be contained in a vacuole. *A* rarefied cell debris. *K* collagen

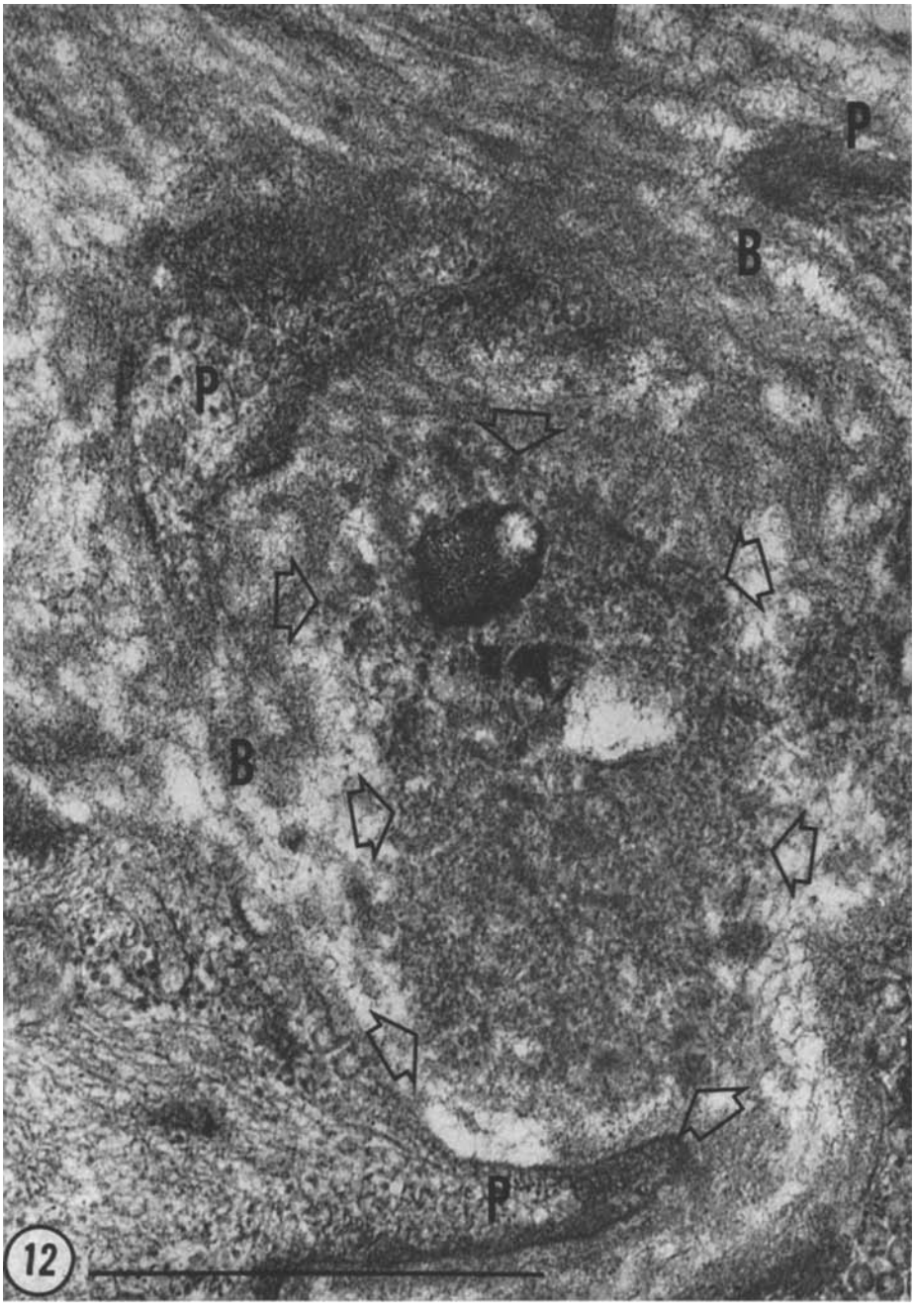

Fig. 12. Processes (*P*) of SMC and multilayered basement membrane (*B*) appear to sequester a stromal aggregate of vesicular and particulate debris (clear arrows)

Table 1. *Degenerative SMC in common carotid bifurcation in 3 dietary groups of rhesus monkeys (terms of the classification are defined in the text)*

Dietary group	Rarefaction				Mummification				Total [a] degenerative SMC/ SMC counted
	A1	A2	A3	Sub-total A	B1	B2	B3	Sub-total B	
Cholesterol	81	18	3	105	15	4	2	21	126/2,865
(%)	2.8	0.6	0.1	3.5	0.3	0.1	0.05	0.7	4.2% (3.4—5.7%)
Non-cholesterol	43	4	0	47	6	1	0	7	52/2,644
(%)	1.6	0.2	0	1.8	0.2	0.03	0	0.3	2.1% (1.2—2.8%)
Pellet control	30	2	0	32	1	0	1	2	34/2,024
(%)	1.5	0.1	0	1.6	0.05	0	0.05	0.1	1.8% 1.2—2.3%)

[a] Total SMC counted: the number of smooth muscle cells with nuclei in the plane of section.

Significance levels by Chi square test

1. A vs. B in Cholesterol	$P = 0.02$
Non-cholesterol	less than 0.001
Pellet control	less than 0.001
2. Comparison of A:	
Cholesterol vs. Non-cholesterol	less than 0.001
Cholesterol vs. Pellet control	less than 0.001
Non-cholesterol vs. Pellet control	not significant
3. Comparison of B:	
Cholesterol vs. Non-cholesterol	less than 0.001
Cholesterol vs. Pellet control	$= 0.025$
Non-cholesterol vs. Pellet control	not significant

Discussion

Search for images of dead and dying SMC in the arterial wall yielded two hypothetical series of degenerative forms linking the usual SMC and products of cell death. This is in accord with and supports the hypothesis formulated previously (Imai and Thomas, 1968) that most of the necrotic debris in the atheromatous lesions of cerebral arteries originated from degenerated SMC. Conversely we have described mitotic division in aortic cells and identified most of them as mature SMC (Imai *et al.*, 1970). Since both proliferative and degenerative processes have been demonstrated in controls, we may assume that the cell renewal cycle in the arterial wall is operative as in many other tissues of adult multicellular organisms (Biggers, 1964). Orderly death occurs concurrent to rapid cell divisions in embryos of many metazoan animals (Glücksmann, 1951) including the aortic wall of 5- to 7-day chick embryos (Hughes, 1943). The proliferative process in arteries of adult animals has been demonstrated to be accelerated by dietary cholesterol (McMillan and Stary, 1968; Florentin *et al.*, 1969). Results of the present study indicate that there is also an accelerating effect of dietary cholesterol on degeneration of arterial SMC at the dosages given to these animals.

Table 2. *Degenerative SMC of aortic trifurcation in swine on diets for 3 days (terms of classification are defined in the text)*

Dietary group	Rarefaction				Mummification				Total [a] degenerative SMC/ SMC counted
	A1	A2	A3	Subtotal A	B1	B2	B3	Subtotal B	
Cholesterol	33	1	2	36	95	14	16	125	161/5,054
(%)	0.6	0.02	0.04	0.7	1.9	0.3	0.3	2.5	3.2% (2.5—4.4%)
Milk control	10	0	0	10	42	4	3	49	59/5,050
(%)	0.2	0	0	0.2	0.8	0.1	0.1	1.0	1.2% (0.5—1.8%

[a] Total SMC counted: the number of smooth muscle cells with nuclei in the plane of section

Significance levels by Chi square test

1. A vs. B in Cholesterol	*P* less than 0.001
Milk control	*P* less than 0.001
2. Comparison of A	*P* less than 0.001
Cholesterol vs. Milk control	*P* less than 0.001
3. Comparison of B	*P* less than 0.001
Cholesterol vs. Milk control	*P* less than 0.001
4. Comparison of A + B	*P* less than 0.001
Cholesterol vs. Milk control	*P* less than 0.001

Based on *in vitro* observations of human leukocytes and various cultured mammallian cells, Bessis (1964) categorized phenomena of cell death into three phases, namely *death agony*, *death proper* and *necrosis* (or disintegration of dead cells). *Death agony* was characterized by irreversible damage. Manifestations of death agony were described by Bessis as more varied than those at the moment of death and included (1) changes in the movement of pseudopods, (2) changes (gelatification or liquefaction) in the cytoplasm with increased permeability leading to plasmolysis and loss of cyclosis, and (3) swelling of mitochondria, Golgi vacuoles and endoplasmic reticulum. Nuclear damage was manifested by nuclear edema with accentuation of nucleoli, which either preceded or followed cytoplasmic edema. *Death proper* (moment of cell death) was characterized by the loss of integrity of intracellular milieu and manifested by edematous balooning of cells allowing energetic Brownian movement of cytoplasmic particles, spherical deformation of nuclei with homogenization of nuclear contents except for the accentuated nucleoli which later disintegrated. Death proper could also be characterized by sudden fragmentation into uneven spheres or rapid onset of rigidity of cells, which might last for several minutes to hours. Furthermore, he indicated that the majority of dead cells underwent *necrosis* with eventual disintegration of organelles, probably by enzymatic digestion, while a small population underwent mummification. The nuclei showed liquefaction followed by rearrangement into small spheres; progressive shrinkage or pyknosis; and fragmentation or karyorrhexis.

The morphologic criteria for cell death as established *in vitro* by Bessis were used in the present study, with modifications as necessary for translation to

electron microscopic terms. Arterial SMC in the hypothetical series of degeneration presented in this study can be placed into appropriate categories in the classification of Bessis. Images consistent with necrotaxis and phagocytosis of injured and dead cells were observed to be in accord with the classification of cell death proposed by Bessis (1964). The images were also similar to those we have previously described in necrotic atheromatous lesions (Imai and Thomas, 1968) but were on a lesser scale. Factors determining the occurrence of single cell death versus *en masse* necrosis are obscure.

In regard to the major underlying mechanisms for the varied manifestations of cell injury noted above, Bessis defined the moment of death as that time at which the cell can no longer maintain the integrity of its internal milieu with respect to the external environment. Thus, free entry and binding of various vital dyes in the cell can be used as a criterion for cell death. Holmberg (1961) reported the *in vitro* use of Lissamine green and other vital dyes for differentiation of viable and irreversibly damaged cells that had been exposed to graded insult of rapid freezing and thawing. He noted that irreversibly damaged cells became promptly permeable to Lissamine green, and that such a change in permeability was associated with cessation of active movement of the plasma membrane, reduction in oxygen consumption and enzyme activity. Because of the lack of a suitable marker for use in this ultrastructural study, the point of no return in cell injury and death was not defined for the arterial SMC.

If dietary cholesterol accelerates cell death in arterial walls, the mechanisms or series of events leading to this ultimate effect is unknown. It is not even known whether cholesterol *per se* is the injurious agent. However, since cholesterol is one of the normal components of cell membranes, such mechanisms might be related to changes in the cholesterol content and hence chemical structure of cell membranes. Ultrastructural changes of the degenerative cells appear to be consistent with changes in functional properties of the cell membrane. Little is known about the functional properties of the nuclear membrane, but its capacity as a powerful metabolic barrier has been demonstrated in several kinds of cells by means of electrical conductance and potential (Kanno and Loewenstein, 1963; Loewenstein and Kanno, 1963; Mitchison, 1966). It may be postulated that in cell death by rarefaction the first manifestation of irreversible cell injury is the loss of barrier function of the nuclear membrane.

Identification of the target organelle (the structure that by changing primarily is responsible for initiating the sequence of events) in mummification of SMC was made difficult by the fact that the entire cell body including the nucleus appeared to become condensed simultaneously, i.e. we did not see intermediate forms between the usual "viable" SMC and B-1 degeneration. The fact that these condensed forms were surrounded by normal appearing SMC and the usual fibrous stroma suggests that the mechanisms for such a change (rapid in progress and confined within the cell membrane) may be associated with an internal shift of ions, such as calcium, rather than with abnormal entry from the exterior. The role of calcium ion in the maintenance of cell permeability has been documented by Shanes (1958). In a series of studies on liver cell injury, Judah and associates (1963 and 1964) postulated that damage to cell membranes resulted in the mobilization of membrane calcium, which in turn caused an increased entry of sodium and a reduction

in the level of ATP because of the increased demands upon the sodium pump, and a critical depletion of the energy supply ensued.

Differences between the morphologic characteristics of cell death by rarefaction and mummification might be explained by the severity (and rapidity) of damage to cell membranes. Based on observations made following various physico-chemical manipulations of cells, Dixon (1967) postulated that catabolic enzymes were contained inside membranous compartments and were thus inaccessible to their substrates in the cell, and that such a dynamic equilibrium could be maintained by the energy of the respiratory metabolism of the cell. Ischemia removes this limitation on the accessibility of catabolic enzymes to their substrates, leading to proteolysis and destruction of the cell. If the cell injury is only moderate, the function of cell membranes as an osmotic barrier is partly retained, leading to osmotic swelling, intracellular proteolysis and subsequent loss of protein. On the other hand, if the injury was severe the cell membranes lose their property of functioning as an osmotic barrier and no swelling or loss of protein occurs. In the present study the predominance of the mummification type in the grossly normal aortic trifurcation and the predominance of the rarefaction type of death in the carotid bifurcation were in sharp contrast, and could not be ascribed to the differences in fixation techniques. The possibilities for such a difference would include variations or modifications of metabolic requirements of SMC depending upon the thickness of arterial wall and the amount and nature of the extracellular materials, which would in turn determine the severity (and rapidity) of the damage to cell membranes.

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Hideshige Imai, M.D., Ph.D.
Department of Pathology
Albany Medical College
Albany, New York 12208