STUDIES ON THE INTRACELLULAR DIGESTIVE PROCESS IN MAMMALIAN TISSUE CULTURE CELLS

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ABSTRACT

DNA-protein coacervates containing colloidal gold particles were readily phagocytized by strain L fibroblasts. During the subsequent digestion process, the gold particles served as markers which permitted the demonstration of the evolution of digestive vacuoles to multivesicular bodies and finally to dense bodies. Acid phosphatase and esterolytic activity was present in these structures. The hydrolytic enzymes were apparently brought to the phagocytotic vacuoles in small vesicles originating in the Golgi region. These vesicles entered the vacuoles prior to the digestion of the coacervates and the appearance of positive cytochemical reactions. The cytoplasmic dense bodies frequently merged with the phagocytotic vacuoles. This was demonstrated by prelabeling the dense bodies with colloidal iron prior to phagocytosis of the coacervates. In addition, evidence is presented for the interrelationship of the phagocytotic and autophagic pathways.

INTRODUCTION

In previous experiments, mammalian cells in suspension culture were shown to engulf DNA-protein coacervates and completely digest them within large vacuoles (1). These studies demonstrated the presence of several hydrolytic enzymes (acid phosphatase, 3′-AMPase, 5′-AMPase, ATPase, and E600-resistant esterase) within the digestive vacuoles. A decrease in the number of hydrolytic enzyme-containing dense bodies concomitant with the phagocytosis and digestion of the coacervates suggested that a merger of the dense bodies with the phagocytotic vacuoles occurred. The earlier experiments did not permit the determination of either the fate of the digestive vacuoles or the origin of the dense bodies.

The incorporation of a colloidal metal (gold) in the DNA-protein coacervates as a marker permitted a study of the various stages of the intracellular digestive process which will be described in this report. The role of the dense bodies in intracellular digestion is also demonstrated by labeling them with a different colloidal metal (saccharated iron) before conducting the phagocytosis experiments. This "labeling" of the dense bodies is possible because L strain fibroblasts phagocytize colloidal metals and localize them in the same manner as other cells (2–4). Hydrolytic enzyme-containing structures thought to result from the autodigestion of cell organelles (autophagic vacuoles) are known to occur in a variety of mammalian cells (5–9). These structures undergo various stages of development and eventually

may be present as dense bodies (10). The relationship of similar structures in tissue culture cells to those formed as a result of phagocytosis will be presented together with a unified concept of the structural pathways of intracellular digestion.

MATERIALS AND METHODS

Tissue Culture

Strain L (Earle) cells were grown in suspension tissue culture in 250-ml Erlenmeyer flasks agitated on a rotary shaker at 37.5°C (11). Each culture contained Eagle's basal medium (72 ml), horse serum (8 ml), penicillin (100 units/ml), streptomycin (50 μg/ml), and an initial inoculum of 200,000 to 300,000 cells/ml. Cultures in logarithmic growth with a cell concentration of 500,000 to 600,000 cells/ml were used for the phagocytosis experiments. Morphological and cytochemical studies were also performed on control cultures in the logarithmic growth phase as well as the stationary phase (aging cultures).

Phagocytosis Experiments

The DNA-protein particles used in the phagocytosis experiments were prepared by the following modification of the previously described method (12). Dilute aqueous solutions of herring sperm DNA (0.1 per cent) were added to approximately equal volumes of a 0.1 per cent solution of gelatin in colloidal gold (Colloidal Gold Reagent, Harleco). This was performed in a water bath at 50°C with the pH adjusted to 4. After suitably sized particles had formed, they were stabilized by exposure to 0.1 to 0.2 per cent cold glutaraldehyde. The particles were then dialyzed for 72 hours against normal saline. Usually 10 ml of the coacervate solution was added to each culture, an amount which was sufficient to give a particle-to-cell ratio of approximately 15:1. This ratio prompted rapid and marked phagocytosis and had no effect on cell growth. Cells were examined for phagocytosis and digestion at intervals of $\frac{1}{2}$, 1, 2, 3, 4, 8, 12, and 24 hours after the addition of the coacervates to the culture medium. The experiments were terminated by centrifugation and immediate fixation.

Experiments were also performed in which the cytoplasmic dense bodies were "labeled" with colloidal iron prior to phagocytosis of the coacervates. This was accomplished by a 2-hour incubation of the cells with 0.2 ml of saccharated iron oxide solution (Proferrin, Merck Sharp & Dohme) added to the culture medium. The cells were then gently centrifuged and resuspended in a fresh prewarmed medium and incubated for at least 2 hours before the addition of the coacervates. Cultures examined at this time showed no iron particles outside the cells, and all the intracellular iron particles were localized within cytoplasmic dense bodies.

Electron Microscopy

Fixation was carried out by gentle centrifugation of the cultures and immediate resuspension of the cells in cold 2 per cent glutaraldehyde in 0.08 M cacodylate buffer (13, 14). After 5 to 10 minutes' fixation, the cells were centrifuged and transferred in very small blocks (less than 1 mm^3) to an isosmotic wash solution (14). After a 24-hour wash, and cytochemical incubations if performed, the cells were postfixed with osmium tetroxide (15, 16). Dehydration was followed by embedding in Maraglas (17). The blocks were cut with an LKB Ultratome and thin sections were mounted on either Formvar film-supported grids or naked grids and examined in a Siemens Elmiskop 1. Sections were stained with either lead citrate (18) or uranyl acetate (19) or both. Sections of cells from cytochemical studies were also examined unstained. Serial sections for electron microscopy and 1-μ toluidine blue-stained sections (20) for light microscopy were studied when indicated.

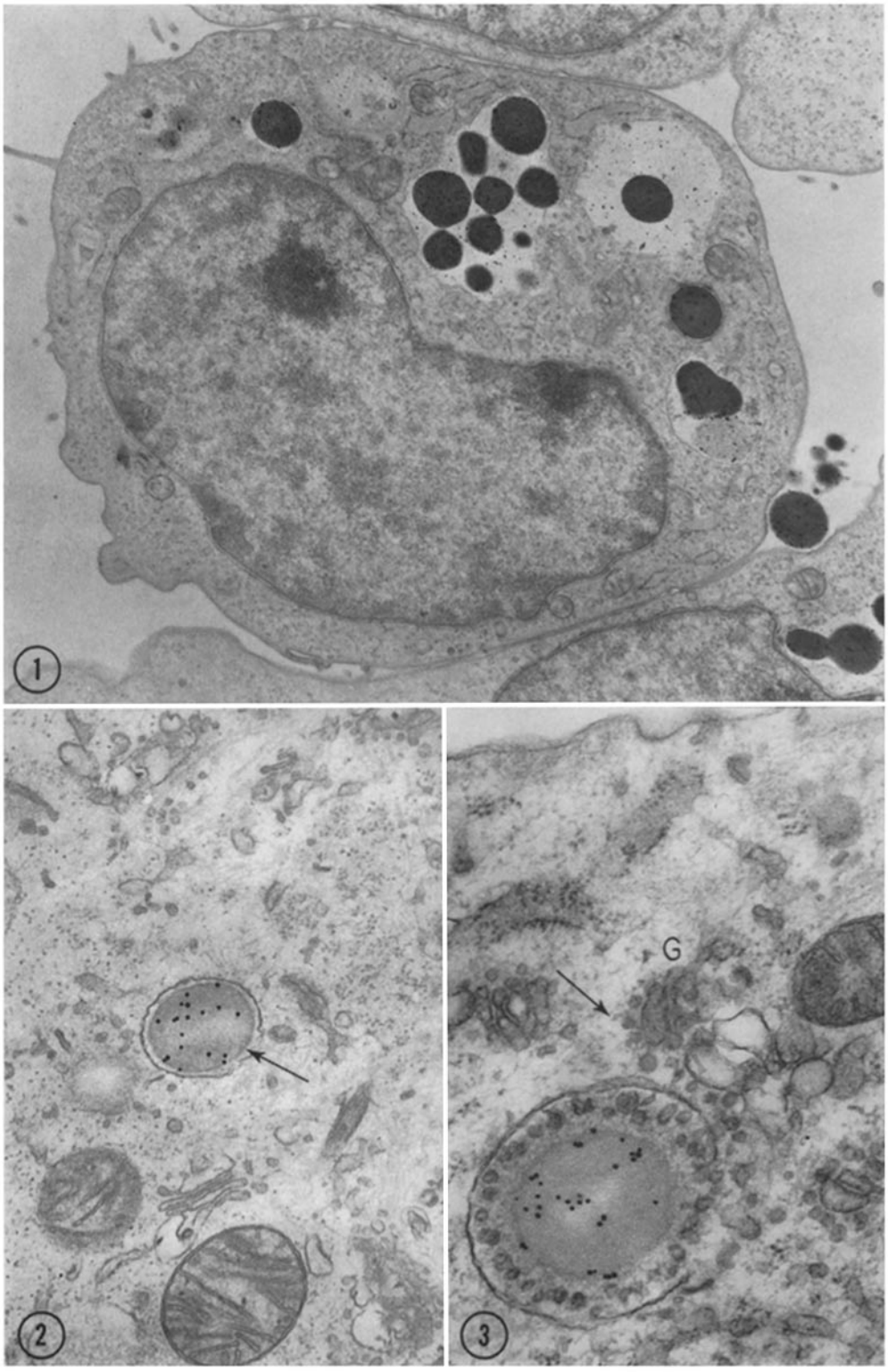
Cytochemical Studies

After fixation in glutaraldehyde, cells for cytochemical studies were thoroughly washed and incubated for either acid phosphatase or E600-resistant esterase (presumably cathepsin C (21)) activity. The esterase reactions were carried out in media containing thiolacetic acid as substrate in the presence of lead ions and 10^{-5} M E600 (diethyl-*p*-nitrophenyl phosphate) (22). Acid phosphatase activity was demonstrated by a modified Gomori technique (23, 9). Controls for acid phosphatase lacking substrate or containing 0.01 M sodium fluoride never developed

FIGURE 1 Strain L cell containing many phagocytized coacervates in various stages of digestion (see text). × 11,000.

FIGURE 2 DNA-protein coacervate (arrow) containing colloidal gold particles shortly after phagocytosis. Note that phagocytotic vacuole is in the Golgi region of the cell. × 28,000.

FIGURE 3 Coacervate within vacuole containing numerous small vesicles. Note the row of vesicles (arrow) extending between the Golgi apparatus (*G*) and the early digestive vacuole. The contents of these vesicles are more electron opaque than the hyaloplasm. × 32,000.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any reaction product. The incubation medium in each instance was adjusted to 290 milliosmols and the reactions were carried out on minute, loosely packed cell aggregates (less than 1 mm^3).

RESULTS

Phagocytosis Studies

The electron microscopic appearance of strain L fibroblasts has been previously described (1, 14, 24). Of particular importance is the appearance of the Golgi zone, which is located in the vicinity of the nuclear indentation. The Golgi apparatus consists of separate arrays of stacked cisternae and numerous small vesicles (40 to 80 mμ in diameter). Cytoplasmic dense bodies (200 to 400 mμ in diameter) limited by a single unit membrane were frequently found in the Golgi region. Cytochemical studies showed the presence of acid phosphatase and an E600-resistant esterase within these dense bodies (14). Under normal conditions, an average of 2 to 4 dense bodies were found per section (400 to 700 A thick) of an L cell. Occasionally small vacuoles (400 to 800 mμ in diameter) were seen which usually contained only homogeneous electron-translucent material. Also, small multivesicular bodies (300 to 500 mμ in diameter) were observed.

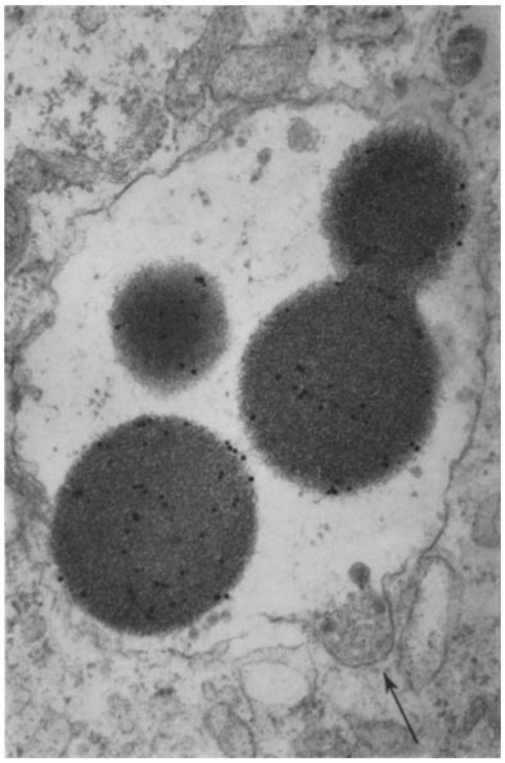


FIGURE 4 Dense body (arrow) in the process of merging with early digestive vacuole. × 40,000.

The coacervates appeared as well circumscribed spheres (300 to 1000 mμ in diameter) composed of a finely granular electron-opaque matrix, in which many very electron-opaque gold particles (100 to 200 A in diameter) were randomly embedded. In these experiments all coacervates contained gold particles, and no free gold particles were ever observed extracellularly.

Within as little as 30 minutes of incubation with the coacervates, many phagocytized particles were seen in the cells (Fig. 1). One or more coacervates were contained in each phagocytotic vacuole. At this time, several of the ingested coacervates appeared to be already undergoing hydrolysis as evidenced by a uniform loss of density. Some of the vacuoles contained particles in various stages of digestion (Fig. 1), indicating a fusion of vacuoles containing recently phagocytized particles with previously formed digestive vacuoles.

After engulfment, particles were initially found within phagocytotic vacuoles formed by the invagination of the cell membrane (1). These vacuoles were shown to migrate toward the Golgi zone (1) and were most frequently observed in this region (Figs. 1 and 2). After extensive phagocytosis, the Golgi apparatus appeared more prominent owing to a dilatation of the Golgi cisternae and an increase in the number of vesicles (Figs. 2 and 3). Many of these small vesicles were arranged in rows or clusters extending between dilated Golgi cisternae and phagocytotic vacuoles (Figs. 3 and 14). The contents of these vesicles were usually more electron opaque than the surrounding hyaloplasm (Figs. 3 and 14). Some Golgi vesicles appeared to fuse with the vacuole membrane, while many were seen intact within the vacuole (Fig. 3). Dense bodies were frequently observed in the immediate vicinity of the phagocytotic vacuoles. A marked decrease in the number of these dense bodies was evident by 1 to 2 hours after the start of phagocytosis. This suggested that many dense bodies merged with the phagocytotic vacuoles. On occasion, the membrane of a dense body was observed to be continuous with the vacuole membrane and appeared to disperse its contents throughout the vacuole (Fig. 4).

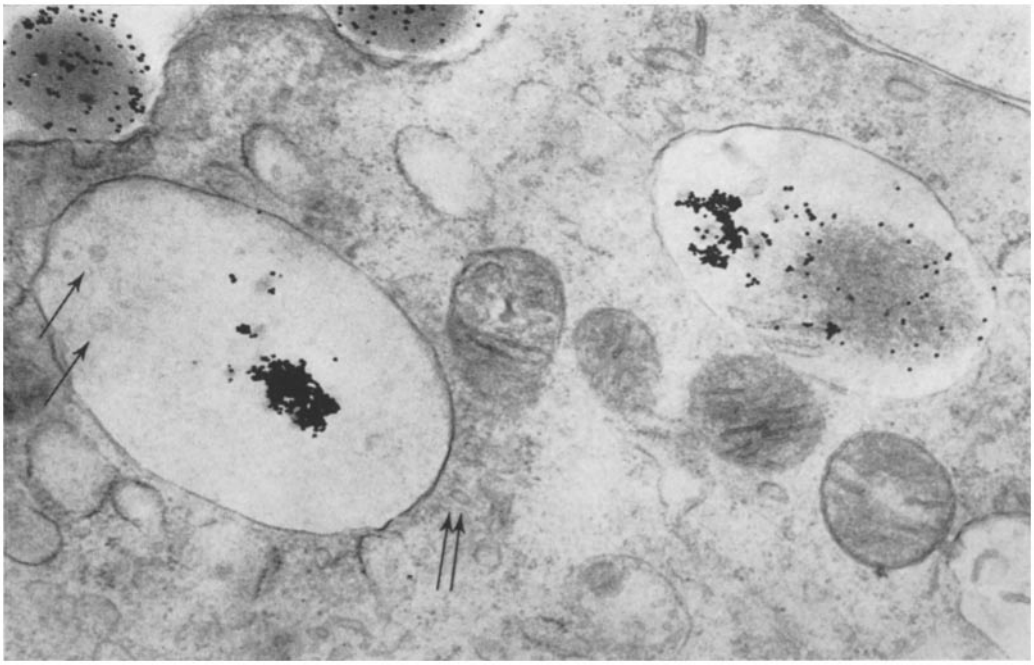


FIGURE 5 Two vacuoles containing coacervates in later stages of digestion. Clumping of the gold particles occurs as hydrolysis of the DNA-protein progresses. At this stage, a few faintly outlined vesicles (single arrows) are seen within the digestion vacuoles. Small vesicles (double arrow) are present near the vacuoles. The coacervates seen in the upper left are outside the cell. × 25,000.

After the observed association of the phagocytotic vacuoles with the Golgi vesicles or dense bodies, the protein and nucleic acid components of the coacervates were hydrolyzed, as evidenced by a diffuse loss of electron opacity (Fig. 5). Concomitant with the loss of density there was a marked increase in the size of the digestive vacuole. As the DNA and protein were digested, the gold particles formed dense aggregates (Fig. 5). Loss of coacervate density continued until the vacuoles became almost completely electron transparent (Fig. 5). At this stage the small vesicles within the vacuoles were few and had an indistinct appearance (Fig. 5). However, small vesicles still could be observed in close proximity to the digestive vacuoles. In cells examined from an experiment of 3 hours' duration, many of the digestive vacuoles began to show a decrease in size. The continuing entry of Golgi vesicles into these late digestive vacuoles resulted in the formation of multivesicular bodies containing the clumped gold residue of digested coacervates (Fig. 6). Later the vesicles within a multivesicular body became indistinct and gradually disappeared as the whole structure decreased in size and became more electron opaque. The end result was the formation of a typical cytoplasmic dense body containing an aggregate of gold particles (Figs. 7 and 8). A few small vesicles were still apparent within an occasional dense body (Fig. 8). Dense bodies containing the metallic residue of digested coacervates were commonly seen within 4 hours after the initiation of phagocytosis. Myeloid figures or double-membrane structures, resembling mitochondrial fragments, could occasionally be seen in a dense body in addition to the gold particles (Fig. 16). No gold particles were ever seen outside membrane-bounded structures, except when rarely found along one side of a digestive vacuole (Fig. 15), presumably owing to an artifactual displacement during cutting.

Cytochemical studies showed that the appearance of E600-resistant esterase and acid phosphatase activity within the digestive vacuoles coincided with the visible degradation of the phagocytized coacervates (Figs. 9 and 10). Reaction products were found in many of the small vesicles within the digestive vacuole (Fig. 9). In the later stages of digestion, enzyme activity per-

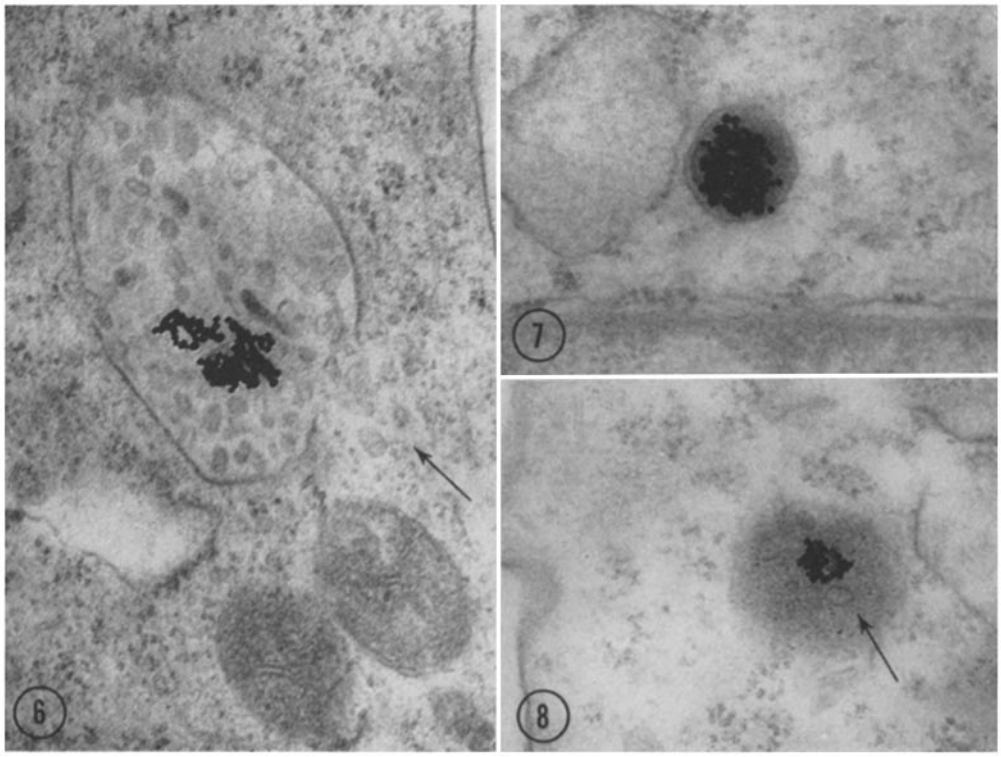


FIGURE 6 A later stage of digestion is a multivesicular body containing clumped colloidal gold. Vesicles can still be seen in the hyaloplasm near this structure (arrow). × 38,000.

FIGURES 7 AND 8 Dense bodies containing gold aggregates are the final stage in the digestion of coacervates. A few small vesicles are occasionally present (arrow, Fig. 8). Fig. 7, × 56,000; Fig. 8, × 48,000.

sisted in the vacuoles as they decreased in size (Fig. 11). The resultant dense bodies exhibited hydrolytic enzyme activity (Fig. 11).

Strain L cells incubated for 2 hours in culture medium containing saccharated iron oxide readily phagocytized the colloidal iron. After an additional 2 hours' incubation in iron-free medium, the phagocytized iron particles were localized in the cytoplasmic dense bodies (Fig. 12). Virtually all (more than 95 per cent) of the dense bodies were "labeled" with the colloidal iron in this manner, and 4 to 8 iron-containing dense bodies were found in each section of cell. These "labeled" dense bodies exhibited the same positive cytochemical reactions for acid phosphatase and E600-resistant esterase as unlabeled dense bodies (Fig. 13).

When these cells with iron-"labeled" dense bodies phagocytized gold-marked DNA-protein coacervates, the iron-"labeled" dense bodies merged with the phagocytotic vacuoles (Fig. 14). This resulted in the presence of clumps of colloidal iron within many digestive vacuoles (Fig. 15). However, when studied on serial sections, approximately one-third of the vacuoles were seen to contain no iron particles (Fig. 15). Digestion progressed as described above and resulted in the formation of dense bodies containing both gold and iron (Fig. 16). Approximately one-third of the resultant dense bodies contained no colloidal iron, a finding which correlated with the observed number of iron-free digestive vacuoles.

Autodigestion Studies

Examination of control cultures showed that cells in stationary phase ("aging cultures") have a marked increase in vacuoles, multivesicular bodies, and dense bodies as compared with cells in logarithmic growth (Figs. 17 and 18). In contrast to equivalent structures resulting from

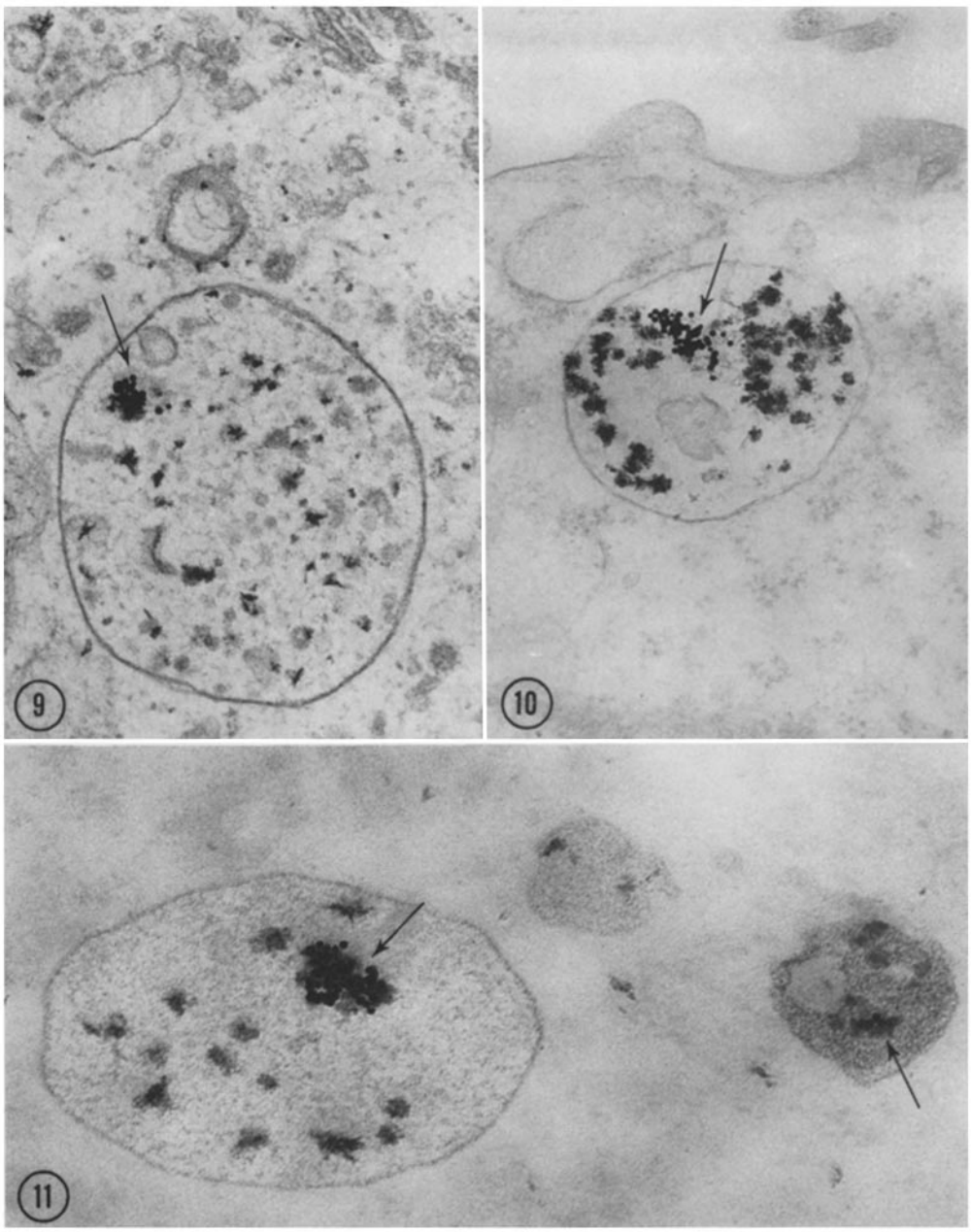


FIGURE 9 Non-specific esterase activity (E600-resistant) in a digestive vacuole containing clumped gold particles (arrow). Note the association of reaction product with some of the small vesicles. Unstained section. × 56,000.

FIGURE 10 Reaction products of acid phosphatase within digestive vacuole. Arrow points at aggregated gold particles. Unstained section. × 58,000.

FIGURE 11 Reaction products of E600-resistant esterase in digestive vacuole and dense body. Colloidal gold residue is present in both (arrows). Unstained section. × 58,000.

phagocytosis, these presented a complex appearance and contained myeloid figures and other membranous or tubular components. Acid phosphatase and E600-resistant esterase activity was present within these structures (Figs. 19 to 21). Reaction product was present over many of the small vesicles within the vacuoles and was occasionally seen over small vesicles, presumably of Golgi origin, near the vacuoles (Fig. 20). The Golgi zones in these cells resembled those in cells after extensive phagocytosis, in that the Golgi cisternae were dilated and there was an apparent increase in the number of Golgi vesicles. Typical dense bodies in which myeloid figures were seen were numerous (Fig. 21). Some of these aging cells contained residual bodies that appeared to have been formed by the fusion of several dense bodies with a less electron-opaque component having the appearance of a lipoid material.

DISCUSSION

De Duve and associates first recognized that a group of subcellular particles exist which are particularly rich in the acid hydrolases (25–27). These particles were termed lysosomes by de Duve, although admittedly the original definition was purely a biochemical one and not related to cytological structure (27). By combined cytochemical and electron microscope studies, acid hydrolase activity has since been found in a number of different morphological entities (28). These include digestive vacuoles resulting from phagocytosis or pinocytosis (23, 29, 30), multivesicular bodies (28, 31), dense bodies (14, 30), large pigment accumulations such as lipofuscin granules (30), and complex structures containing fragments of cell organelles (7, 10). The last mentioned structures have been termed autophagic vacuoles or cytolysomes (32) and are thought to occur as a result of autodigestion either during degenerative states (5–7) or in tissues undergoing physiological lysis (8–10).

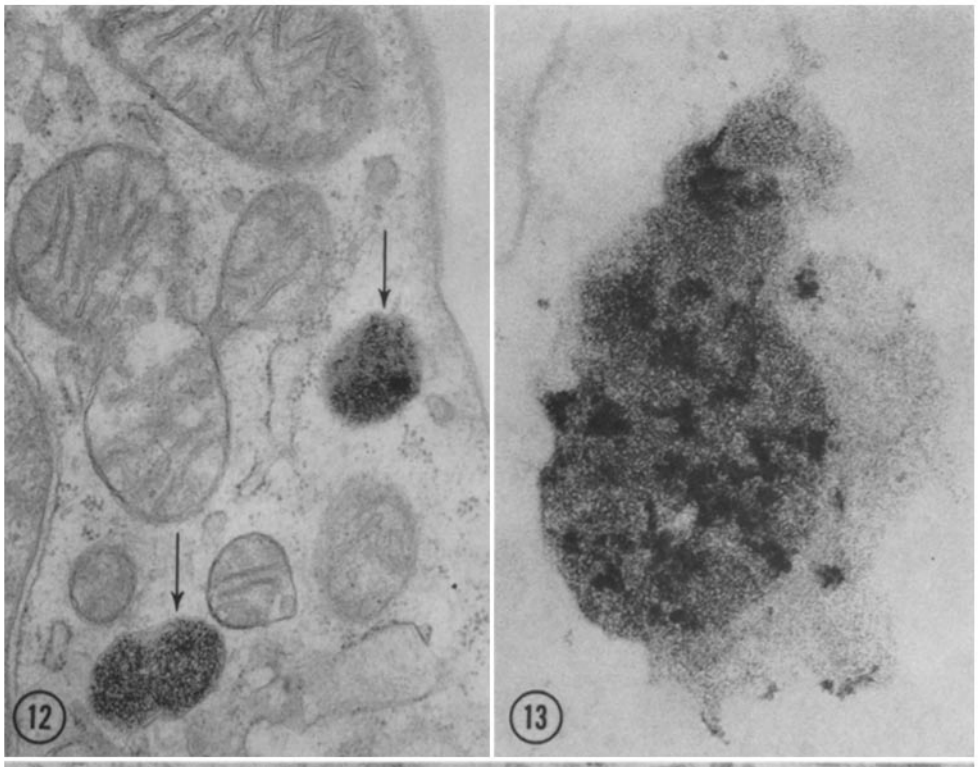
In the present study, intracellular digestion in strain L cells occurred as an ordered sequence in which various forms of lysosomes were involved. The progressive morphological alterations of the phagocytized gold-marked coacervates permitted the formulation of the structural pathway involved in their degradation. This proposed pathway is shown in chronological sequence in Figs. 2 to 7, and schematically summarized on the left of the diagram in Fig. 23. The transformation of a phagocytotic vacuole or phagosome (33) to a digestive vacuole or phagolysosome (34) occurred with the appearance of acid hydrolase activity within the vacuole. Previous studies demonstrated that the phagosomes migrate toward the Golgi region, where digestion of the coacervate occurs (1). According to Novikoff (28), phagosomes in kidney tubular cells acquire acid phosphatase activity in the region of the Golgi apparatus. The present study suggested that the Golgi apparatus plays a major role in supplying hydrolytic enzymes to the phagocytotic vacuole. Recently, Novikoff and associates (35, 36) demonstrated the presence of acid phosphatase activity within the rough endoplasmic reticulum (ER) and suggested that the hydrolytic enzymes are produced there. Structural or functional connections between the ER and the Golgi complex have been demonstrated (31, 37). Evidence has accumulated which indicates that the Golgi complex is involved in the packaging of substances produced within the ER (28, 31, 38). Acid hydrolase activity in the Golgi

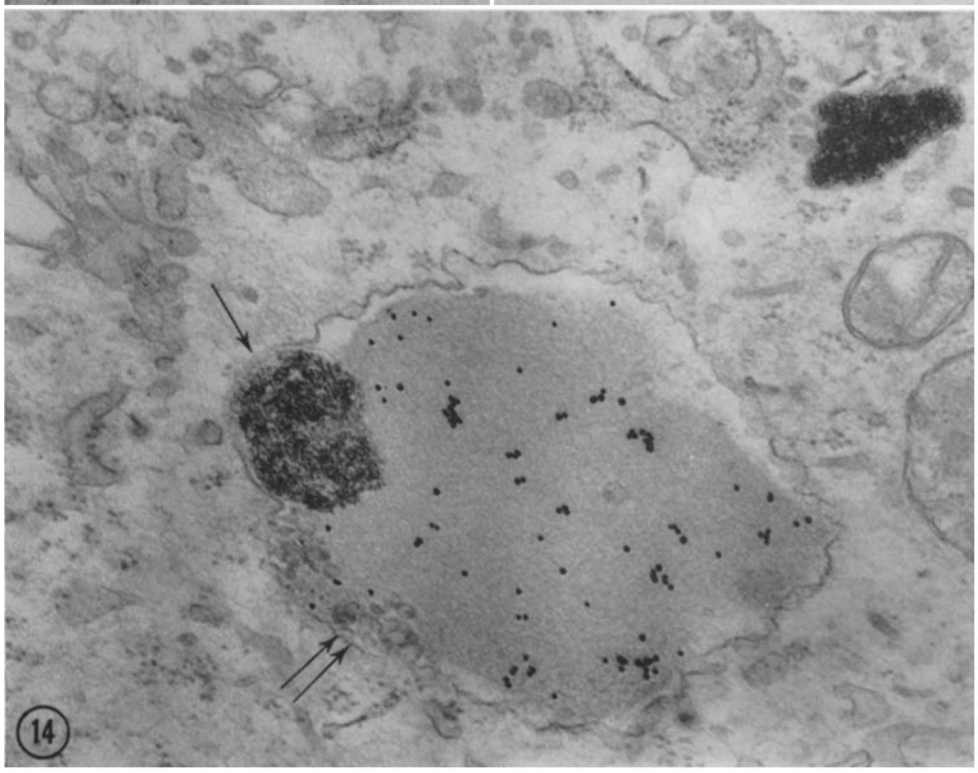
FIGURES 12 TO 16 Experiments with cells in which the colloidal iron was used to "label" the dense bodies.

Fig. 12. Before phagocytosis, the dense bodies (arrows) of the L cells uniformly contain iron particles. × 34,000.

Fig. 13. Dense body containing colloidal iron (darker area) shows coarse and fine reaction product of E600-resistant esterase. Unstained section. × 105,000.

Fig. 14. Early digestive vacuole immediately after merger with iron-marked dense body (single arrow). Another "labeled" dense body can be seen in upper right. Numerous small vesicles are present within the digestive vacuole (double arrow). Note the row of vesicles containing electron-opaque material linking Golgi cisternae (left upper corner) with the digestive vacuole. × 50,000.





complex has not been demonstrated cytochemically in most tissues (31) and was not present in the strain L cells. However, it is known that fractions of isolated Golgi membranes have a high concentration of acid phosphatase (39). The Golgi vesicles are derived from the Golgi cisternae by peripheral budding (40). The finding that many of these vesicles entered each phagosome provides a means of linking the site of probable enzyme production (the ER) to the site of utilization (the digestive vacuole). Other investigators have noted the association of small vesicles with the phagocytotic vacuole and suggested that they supply hydrolytic enzymes to the vacuole (31, 41). These were termed "microlysosomes" or "pure lysosomes" (31), but a term that better denotes their function as the original lysosomal structure in the digestive process would be "proto-lysosome." Relatively few of the Golgi vesicles may contain the enzyme studied in any single histochemical reaction. Individual Golgi vesicles may have functions other than enzyme transport, such as the formation of membranes throughout the cell including the membranes of enlarging vacuoles (10). The possibility also exists that the enzyme within newly formed Golgi vesicles is in an inactive form. In the L cells, acid hydrolase activity was observed in small vesicles in the vicinity of and within the digestive vacuoles.

As the coacervates were hydrolyzed, the digestive vacuole enlarged secondarily to the resultant osmotic alterations (1). Absorption of the hydrolysates probably accounts for the ensuing reduction in size of the vacuole (1). Golgi vesicles continued to enter the phago-lysosome during these steps. With the completion of the digestive process, the vesicles accumulated within the late stage phagolysosome and formed a multivesicular body. Eventual condensation to a dense body then occurred. An appropriate name for this final structure in the digestive process is "telo-lysosome." The presence of phagocytized particles within multivesicular bodies in glomerular epithelial cells was noted by Farquhar *et al.* (42). These authors were not able to determine the fate of the multivesicular bodies, but they did consider the end stage of phagocytosis to be the dense bodies. Also, Palade (43) believes that the dense bodies in splenic macrophages represent the terminal appearance of phagocytotic vacuoles. Others (44, 45) have been of the same opinion concerning the origin of the pericanalicular dense bodies of liver.

The residual hydrolases in the telo-lysosomes of the strain L cells can be reutilized. Light microscope observations by Straus (34) showed the confluence of phagosomes with existing acid hydrolase-containing structures in renal tubular cells. In addition, the specific granules of polymorphonuclear leukocytes have been shown to merge with phagocytotic vacuoles (46). The actual merger process in L cells must occur very rapidly, because visual evidence of the process was seldom present. However, evidence that frequent merger occurred was provided by the experiments with the iron-containing telo-lysosomes. The actual frequency of merger must depend upon factors such as the number of preexistent telo-lysosomes and the extent of phagocytosis.

Strain L cells in the stationary phase exhibit

FIGURES 12 TO 16—*continued*

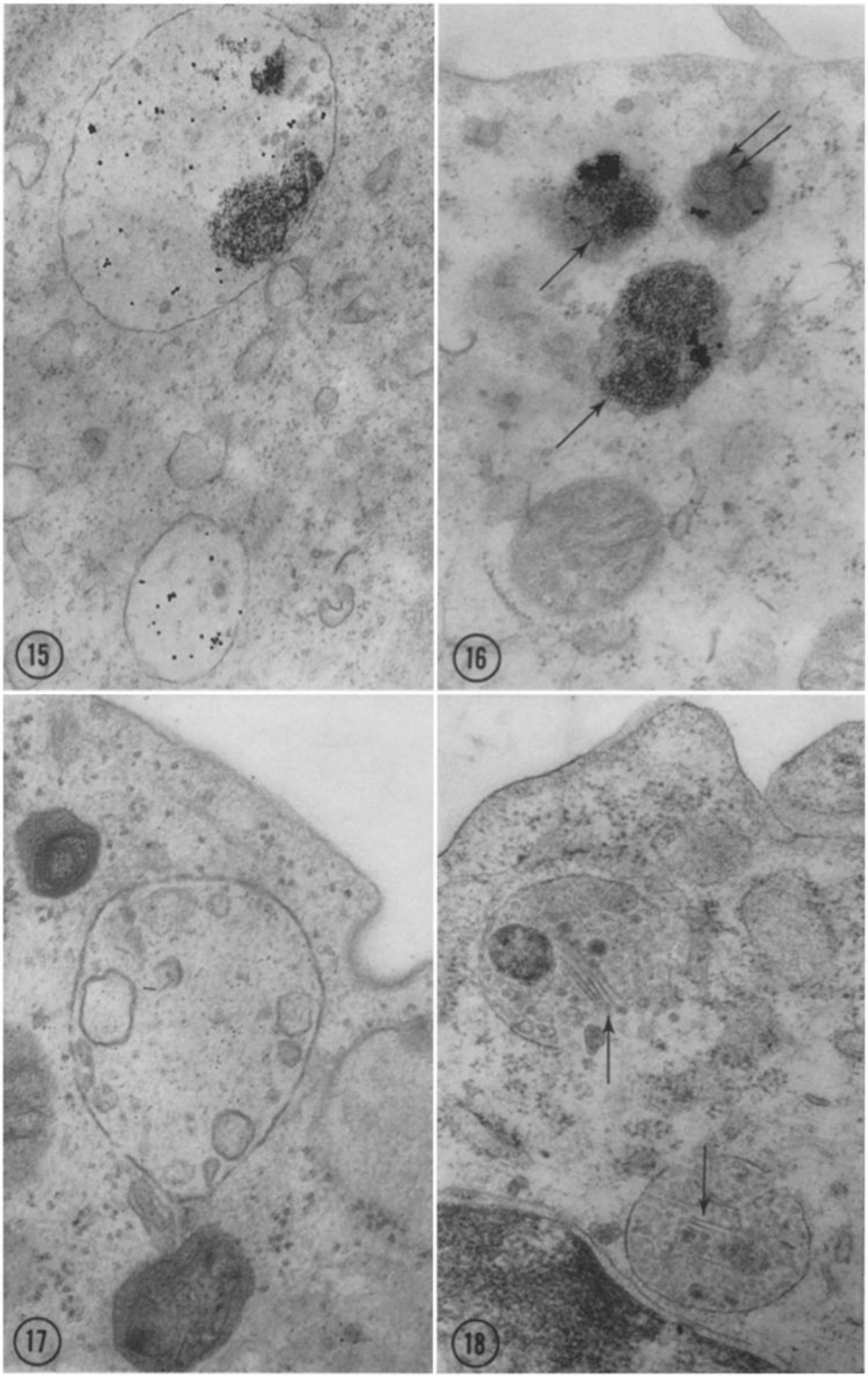
Fig. 15. Two digestive vacuoles in a cell with "labeled" dense bodies. Serial sections failed to reveal colloidal iron in the lower vacuole. × 42,000.

Fig. 16. Dense bodies containing the clumped gold residue of digested coacervates. Colloidal iron is seen in two of these (single arrows). Double arrow points at myeloid figure in dense body in which no iron marker is present. × 28,000.

FIGURES 17 TO 22 Experiments with cells in stationary growth phase.

Fig. 17. Portion of L cell with an autophagic vacuole and two dense bodies. All three contain membrane remnants. × 56,000.

Fig. 18. Multivesicular bodies containing (arrows) double-layered membrane fragments. × 45,000.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marked biochemical alterations including decreased protein synthesis and the accumulation of large amounts of lipid (47). Therefore, it is not surprising that these cells contained numerous autophagic vacuoles. The finding of structures in these cells that correspond to each stage noted in the digestion of the coacervates allowed a similar sequence of events to be postulated. This is summarized on the right half of Fig. 23. The term "auto-lysosome" is used to denote the autophagic equivalent of a phago-lysosome. The presence in old L cells of numerous telo-lysosomes containing membrane remnants indicates an autodigestive origin. In this connection, Behnke (10) traced the development of autophagic vacuoles in the epithelium of fetal rat duodenum. Among the structures noted in this development were large complex vacuoles, multivesicular bodies, and dense bodies. Behnke considered the dense bodies to be a late stage and demonstrated their involvement in the formation of new autophagic vacuoles. More recently, Robbins *et al.* demonstrated acridine orange–induced changes in HeLa cells which consisted of the conversion of multivesicular bodies to large complex vacuoles containing myeloid figures and other dense structures (48).

In the aging L cells, larger residual bodies were occasionally observed that appeared to be formed by the fusion of several telo-lysosomes with lipid droplets. Morphologically and cytochemically, these residual bodies resembled lipofuscin granules (30, 49, 50), which have been considered by some investigators to be of lysosomal origin (51). The occasional finding of telo-lysosomes containing gold particles and membrane remnants provided evidence for an interrelationship between the phagocytotic and autodigestive pathways.

Previously, it was postulated that hydrolytic enzymes for intracellular use were produced and stored in dense bodies or other structures in anticipation of a future requirement (31). This appears to be the case in the granular leukocytes. There is no increase in protein synthesis during phagocytosis in the polymorphonuclear leukocyte (52), a fact which suggests that its large complement of digestive enzymes is produced before phagocytosis. In L cells, however, the *de novo* synthesis of lysosomal structures must be of minor significance if it occurs at all. Hydrolytic enzyme–containing structures are few in cells in logarithmic growth. Pinocytosis of the culture medium—a frequently observed process—could account for the formation of these structures. In any event, a great increase in the various lysosomal forms occurs after phagocytosis or during autodigestion. This suggests that the L cell responds to either stimulus with the production of hydrolytic enzymes. Studies are now in progress to quantitate the extent of this adaptive process.

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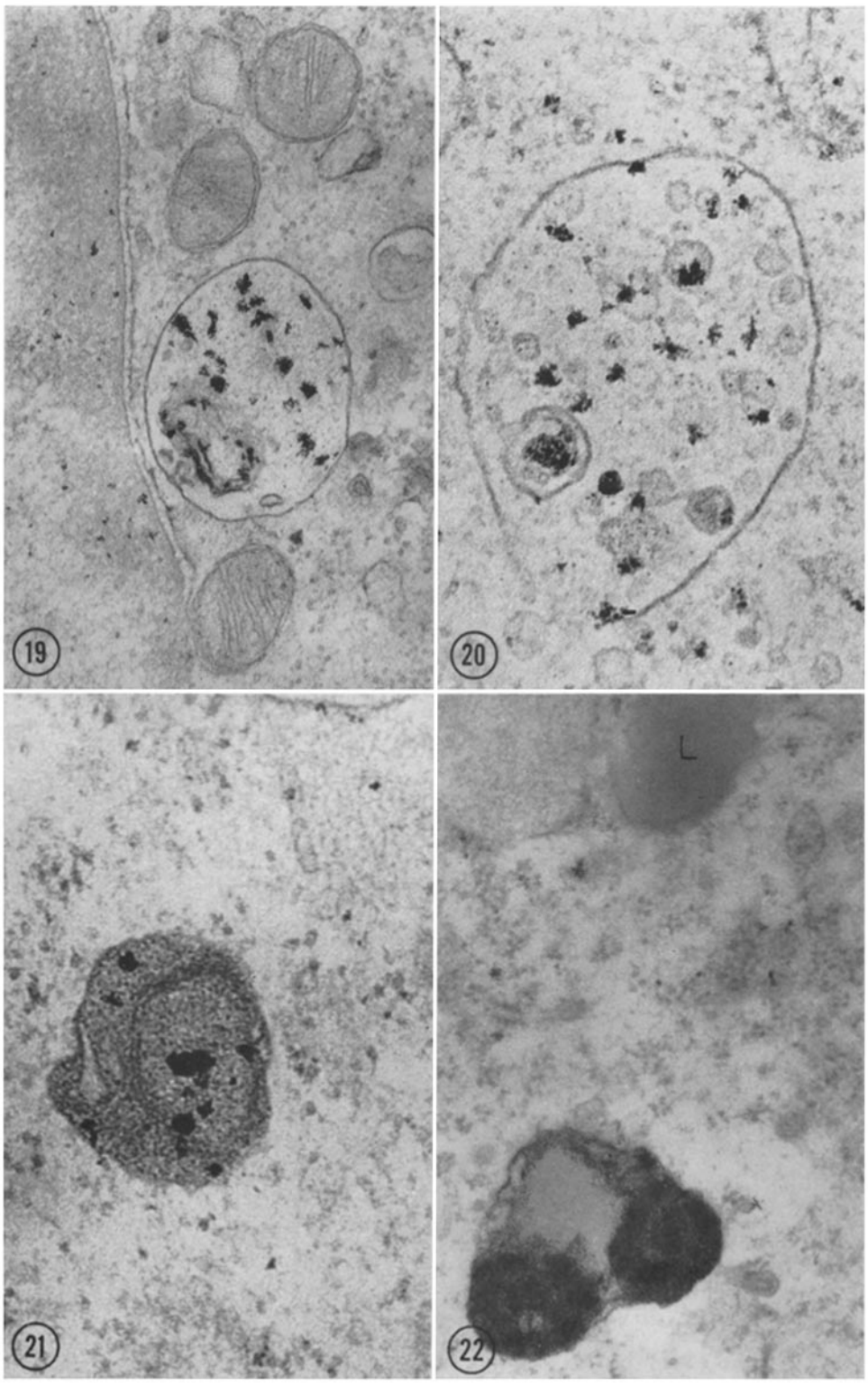
FIGURES 17 TO 22—*continued*

Fig. 19. Acid phosphatase reaction product in autophagic vacuole. Unstained section. × 56,000.

Fig. 20. Esterase activity in autophagic vacuole. Note reaction product over many small vesicles, both within and in the vicinity of the vacuole. Unstained section. × 100,000.

Fig. 21. Fine and coarse reaction products of E600-resistant esterase in dense body containing myeloid figure. × 104,000.

Fig. 22. Residual body consisting of lipoid material and two dense bodies. The reaction product of esterase is confined to the latter. Lipid droplets (*L*) are frequently seen in aging cells. × 52,000.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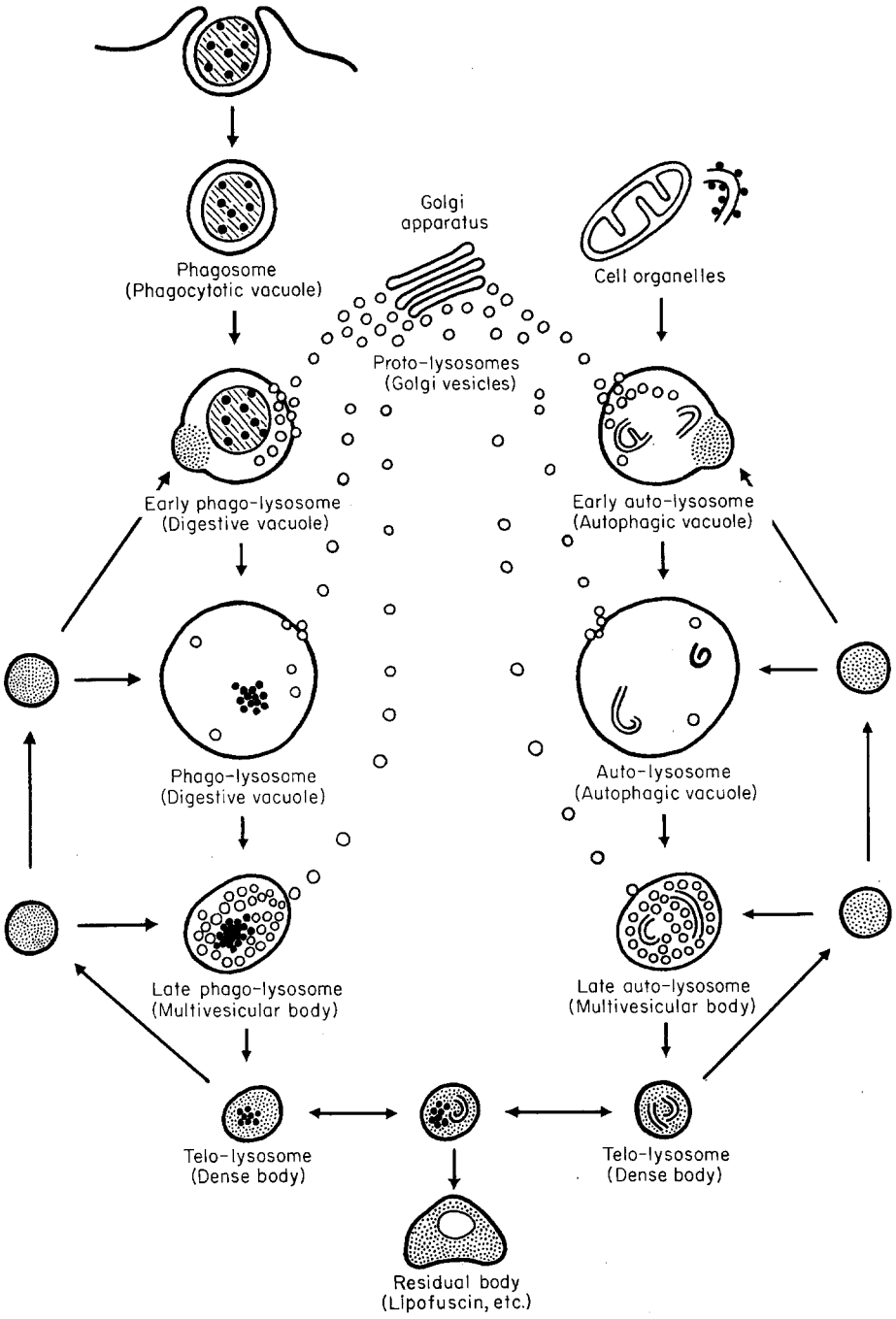

FIGURE 23 Schematic representation of proposed structural pathways of intracellular digestion in strain L cells.

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