Cellular Autophagocytosis Induced by Deprivation of Serum and Amino Acids in HeLa Cells

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Morphologic alterations in HeLa cells deprived of serum and amino acids were studied by light and electron microscopy. After complete deprivation for 1, 3, or 6 hours, many cells contained autophagic vacuoles enclosing clearly recognizable cell organelles. The proportion of cells showing autophagy rose from 4 to 37% in the first 3 hours of deprivation. Acid phosphatase reaction product was localized within many of the autophagic vacuoles by a modified Gomori method. After 12 or 24 hours of deprivation, many membranous residual bodies were noted within the starved HeLa cells. These electron microscopic studies indicate that complete deprivation of serum and amino acids provides a useful model for the further study of cellular autophagocytosis. (Am J Pathol 83:485–498, 1976)

AUTOPHAGOCYTOSIS occurs to some extent in normal cells, and appears to increase in pathologic cellular conditions as a sublethal reaction to injury.[1-3] Some evidence of this phenomenon has been observed in cultured cells under such conditions as iron toxicity,[4] increased pH of the culture medium,[5] chloroquine exposure,[6] and arginine deprivation and irradiation by ultraviolet light and x-rays.[7] A reproducible and physiologic tissue culture model of autophagy would be highly desirable. Histochemical demonstration of lysosomal enzymes within autophagic vacuoles or other evidence that their contents are completely sequestered from the remaining cell sap and objective evidence that the number of autophagic vacuoles is increased as compared to controls have often been lacking in previous reports. A tissue culture model of autophagy would provide advantages for study of its mechanism, including control of the chemical and hormonal environment of the cell and the presence of a single cell type. *In vivo* studies have shown that starvation can cause autophagy,[8] and this observation encouraged us to study deprivation of serum and amino acids in HeLa cells.

Materials and Methods

Monolayer cultures of HeLa cells were cultivated in plastic Falcon flasks (25 sq cm and 75 sq cm) at 37 C with air as the gas phase and Eagle's minimal essential medium as the liquid phase. The medium contained 10% fetal calf serum, 0.005 M sodium bicarbonate and 0.015 M TRICINE buffer,[9] 100 units ml penicillin and 100 μg ml streptomycin. The

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cell cultures were maintained in the logarithmic phase of growth during at least five subcultivations before they were used in the starvation experiments. Cells were subcultivated before reaching confluence using crystallized trypsin and EDTA, and medium was replaced shortly after attachment of cells. Possible contamination was monitored with bacteriologic [10] and ultrastructural methods,[11] and no evidence of bacterial, viral, or mycoplasmal contamination was found.

The deficient medium for the starvation experiments was prepared by simply omitting all amino acids and fetal calf serum from minimal essential medium, made from powdered components (Microbiological Associates, Bethesda, Md.); the medium contained Earle's salts, vitamins, glucose, buffers, and penicillin and streptomycin in the same concentrations as the standard medium. The final osmolality was 285 to 300 mOsmoles. For the starvation experiments, flasks were washed twice with the deficient medium, and then, every 60 minutes, the deficient medium was aspirated and immediately replaced with fresh deficient medium. After 1, 3, 6, 12, and 24 hours in the deficient medium, duplicate monolayer cultures were fixed for 30 minutes at room temperature, in the plastic flasks, by replacing the medium with a mixture composed of 4% glutaraldehyde and 1% osmium tetroxide in 0.05 M sodium cacodylate and 0.05 M *s*-collidine buffers.[12] Replicate flasks of control cultures, incubated in complete medium prepared from separate components to include 10% fetal calf serum and all essential amino acids, and also replicate cultures, which were not washed and fed with fresh medium after the final three subcultivations, were fixed in the same manner. The fixed monolayers of cells were stained *in situ* with 0.5% uranyl acetate in veronal acetate buffer at pH 4.7 prior to dehydration in graded ethanol solutions. Propylene oxide (Polysciences) was poured into each flask, and the fixed cells were floated free of the substratum.[13] After three washes in propylene oxide the cells were infiltrated with Epon 812, centrifuged, and placed onto BEEM capsules filled with Epon (DuPont). The capsules were centrifuged for 2 hours at 1900*g* to form a pellet of cells at the tip of the block. Thin sections were stained with uranyl acetate and lead citrate. Acid phosphatase reaction product was demonstrated according to the method of Brunk and Ericsson[14] following fixation for 4 hours in cold 2% glutaraldehyde (Ladd Industries) in 0.1 M sodium cacodylate buffer.

In order to estimate the proportion of cells which contained autophagic vacuoles at each time period, several low magnification (90×) electron micrographs were used to form montages of each entire grid. These were prepared using a Jeolco JEM-100B electron microscope in the scanning mode at 60 kV, with objective lens off and objective aperture removed. Using these montages as a guide, every cell on each grid was examined using a Hitachi HS-8 electron microscope operating at 50 kV, at 12,000× screen magnification with a 50-μ objective aperture and beam current of approximately 25 μA. Electron micrographs were made of representative cells. Each cell was recorded as containing or not containing autophagic vacuoles, which were defined as single membrane-bounded or double membrane-bounded vacuoles enclosing recognizable cytoplasmic organelles.

Results

Controls

The plasma membranes of control HeLa cells (Figure 1) were smooth, with occasional microvilli; rough endoplasmic reticulum was present in small amounts, and polyribosomes were quite numerous throughout the cytoplasm. Profiles of smooth endoplasmic reticulum, both vesicular and compact, were numerous especially in the Golgi region. There were commonly several Golgi complexes, usually located near the nucleus. A

few multivesicular bodies were seen in some sections. These features have been described previously in HeLa cells.[15,16] Single membrane-bounded bodies, approximately 0.2 to 0.5 μ in diameter, were located near the Golgi region. A few cytosomes contained extremely dense, tightly packed membranous residues and resembled the peribiliary dense bodies of hepatocytes. Autophagic vacuoles were rarely seen in sections of control cells. In the acid phosphatase preparations, lead phosphate reaction product was observed in Golgi lamellae, Golgi vesicles, and primary lysosomes.

Electron microscopy of HeLa cells which were not maintained in the log phase of cell growth and not regularly refed with complete medium demonstrated the importance of these conditions in maintaining normal cellular morphology.

Cells which were subcultivated without replacing the medium present during cell attachment, which contains debris of cells killed during trypsinization, often had in their cytoplasm large, irregular, single membrane-bounded vacuoles which contained several types of single- and double-membrane-limited bodies and also condensed nuclear chromatin material (Figure 2). In some instances the double membrane-limited structures were similar in appearance to swollen mitochondria with flocculent densities and microcrystalline densities, probably due to calcification.[17] Similar clusters of vacuoles and chromatin were often present adjacent to viable cells.

Cells which were never refed with fresh medium and were examined 4 and 13 days after forming confluent monolayers contained many osmiophilic bodies filled with tightly packed, concentric membranous arrays (Figure 3).

Starved Cells

In HeLa cells deprived of serum and amino acids, many autophagic vacuoles were noted at 1, 3, and 6 hours. These vacuoles were small and usually round, with a diameter of about 0.5 μ, and contained clearly recognizable cytoplasmic components, such as mitochondria, rough and smooth endoplasmic reticulum, Golgi vesicles, ribosomes, and lipid droplets (Figures 4–7). They were located throughout the cytoplasm but were most common near the Golgi zone. The vacuoles were usually limited by two trilaminar membranes. From the appearance of the organelles within them, it was not possible to determine morphologically whether degradation had occurred. Acid phosphatase reaction product was localized in many of the autophagic vacuoles, and sections of the starved cells showed more acid phosphatase-positive lysosomes than sections of control cells. At 12 and 24 hours many cells contained large vacuoles with material similar

to myelin figures or residual bodies, which usually gave a positive acid phosphatase reaction.

A striking increase was found in the number of cells which contained autophagic vacuoles after 1, 3, and 6 hours of starvation (Table 1). Of 115 control HeLa cells examined, only 4% contained autophagic vacuoles. After 1, 3, and 6 hours of starvation, respectively 26, 37, and 35% of all cells examined contained recognizable autophagic vacuoles. Using the Chi squared test, significant differences were found between the number of autophagic vacuoles in control cells and cells which were in the deficient medium for 1, 3, and 6 hours, with $P < .01$ in each case.

Discussion

A striking increase in the number of autophagic vacuoles has been observed in HeLa cells deprived of serum and amino acids, as compared to optimally nourished controls. This model of cellular autophagy could well be used for further study of the mechanisms involved in the control of autophagy. HeLa cells are apparently able to respond to nutritional deprivation by digesting their own cytoplasm. Although neural, hormonal, and other environmental influences are excluded in this system, the specific intracellular change which triggers autophagy remains obscure.

Autophagy has been documented after starvation in the livers of rats deprived of essential amino acids.[5] Also, biochemical studies by Eagle[19] and Righetti *et al.*[20] have shown that extensive protein and amino acid turnover occurs in HeLa cells. A few investigators have reported morphologic observations of autophagy in tissue culture,[2-7,21] but only one of these—arginine deprivation in cultured KB cells[7]—involved a physiologic or perhaps adaptive mechanism. In the iron toxicity and chloroquine studies, autophagy was interpreted as a result of abnormal vacuole formation with accidental entrapment of cytoplasmic elements.[4,6]

Table 1—Counts of Random Thin Sections of HeLa Cells Which Contained Recognizable Autophagic Vacuoles

Time in deficient medium (hrs)	Percent of cells containing autophagic vacuoles
0 (control)	4% (115)
1	26% (53)*
3	37% (87)*
6	35% (108)*

The total number of cells examined is shown in parentheses.
* These are statistically significant differences from the control ($P < .01$ in each case).

An alternative explanation for the increased number of autophagic vacuoles observed in starved HeLa cells in these experiments is that these findings represent a normal process which is drastically slowed. Thus autophagic vacuoles might be formed at the same rate in starved cells as in control cells, but degradation of the contents by hydrolytic enzymes or extrusion of secondary lysosomes into the surrounding medium could be inhibited. Autophagic vacuoles would then persist longer, giving a false impression of increased autophagic vacuole formation. This is a reasonable interpretation of the autophagy seen in fibroblasts with increased pH of the culture medium[5] or in chloroquine-treated macrophages,[6] since increased intralysosomal pH would inhibit the acid hydrolases.[22] However, there is no apparent basis for postulating that a decreased rate of degradation would cause the large increase in the number of autophagic vacuoles which was observed within 1 hour in starved HeLa cells. An attractive hypothesis is that the HeLa cells undergo autophagy to provide amino acids for necessary enzyme synthesis or substrates for ATP production. Thus this autophagy would represent a survival mechanism for the cell.[1]

HeLa cells are highly phagocytic, and it is important to differentiate heterophagocytosis of cellular debris in the culture medium from autophagocytosis.[23] In the HeLa cells cultured without refeeding and removing cell debris after subcultivation, heterophagic vacuoles were more frequently observed than autophagic vacuoles. These heterophagic vacuoles were easily distinguished since they were larger, were devoid of the granular ribosome-like material commonly seen in autophagic vacuoles, and often contained chromatin material and mitochondria with microcrystalline densities (Figure 2).

Kerr [24] has introduced a new term, *apoptosis*, to represent a phenomenon of physiologic cell deletion in which cellular condensation occurs, followed by fragmentation into ultrastructurally well-preserved fragments, which are then taken up into other cells by phagocytosis. According to Kerr, apoptosis is common in many tissues undergoing atrophy or involution, and phagocytosed apoptotic bodies have probably been mistaken for autophagic vacuoles in such tissues. The presence of nuclear chromatin is the most helpful criterion in differentiating apoptosis and autophagocytosis; it was not observed within the vacuoles of the starved cells but was commonly noted in the larger heterophagic vacuoles.

It cannot be stated whether deprivation of serum only or deprivation of amino acids only would produce increased numbers of autophagic vacuoles, since only the combined deprivation was studied in these experiments. It would be of interest to identify precisely the factor responsible

for the observed increase, since this might lead toward identification of the factors controling physiologic autophagocytosis.

These observations indicate that withdrawal of all amino acids and serum from well-buffered tissue culture medium causes HeLa cells to respond with a large, rapid increase in their rate of autophagocytosis. Initially double membrane limited and acid phosphatase negative, these vacuoles acquire acid phosphatase activity and accumulate as lysosomes of the multilamellar residual body type. Intralysosomal protein degradation would be expected to provide a source of amino acids for cellular metabolism, thus perhaps enhancing survival under conditions of starvation. This method of producing autophagy in a well-controlled *in vitro* system offers significant advantages for studies of the cellular consequences, metabolic controls, and pharmacologic alteration of the autophagic process.

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[*Illustrations follow*]

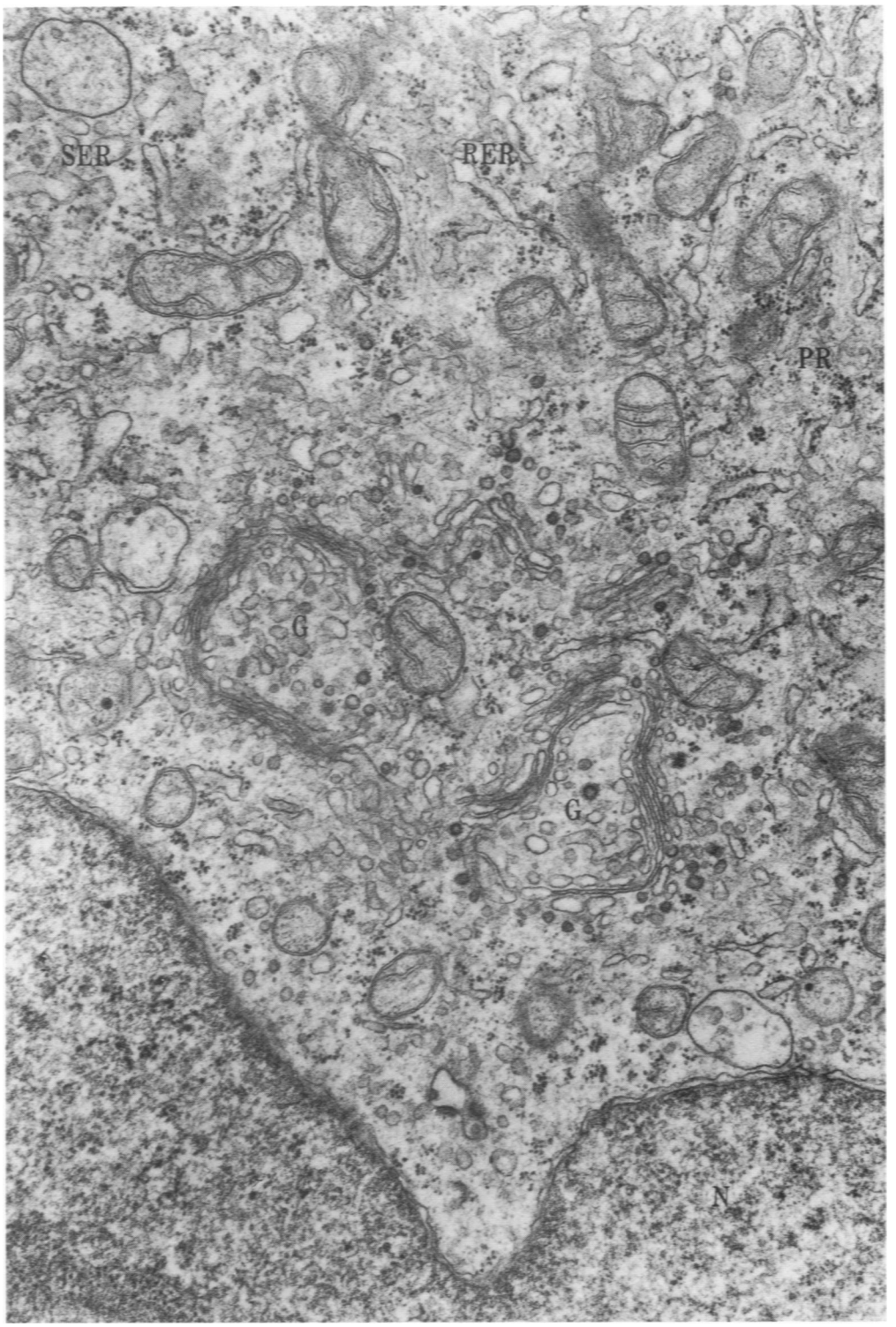


Figure 1—Electron micrograph of a control HeLa cell fixed in a mixture of glutaraldehyde and osmium tetroxide. Note the nucleus (*N*), and characteristic organelles. The Golgi region (*G*) is extensive, with numerous dictyosomes. Polyribosomes (*PR*), smooth endoplasmic reticulum (*SER*) and rough endoplasmic reticulum (*RER*) are prominent. Note the absence of autophagic vacuoles. (× 27,300)

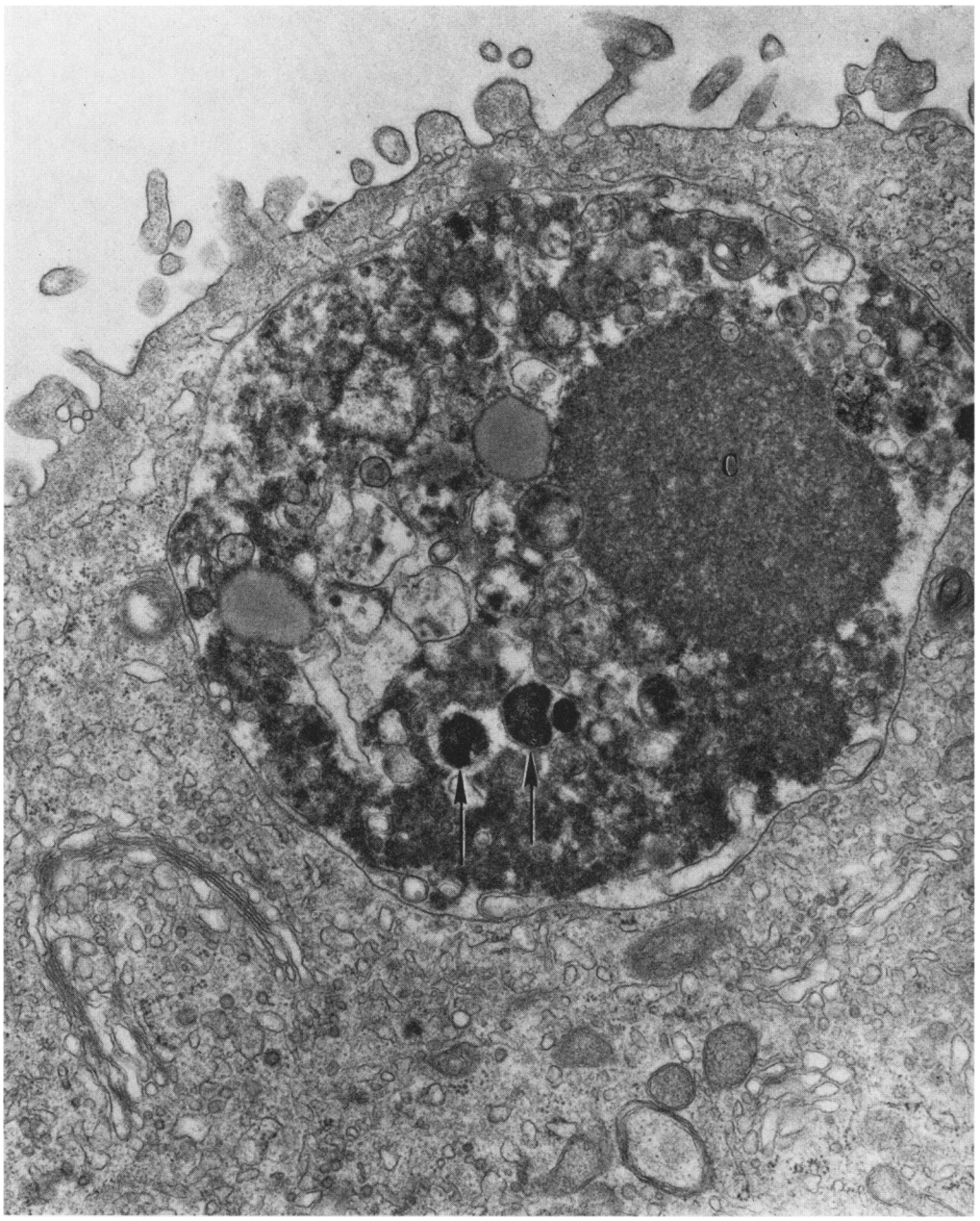
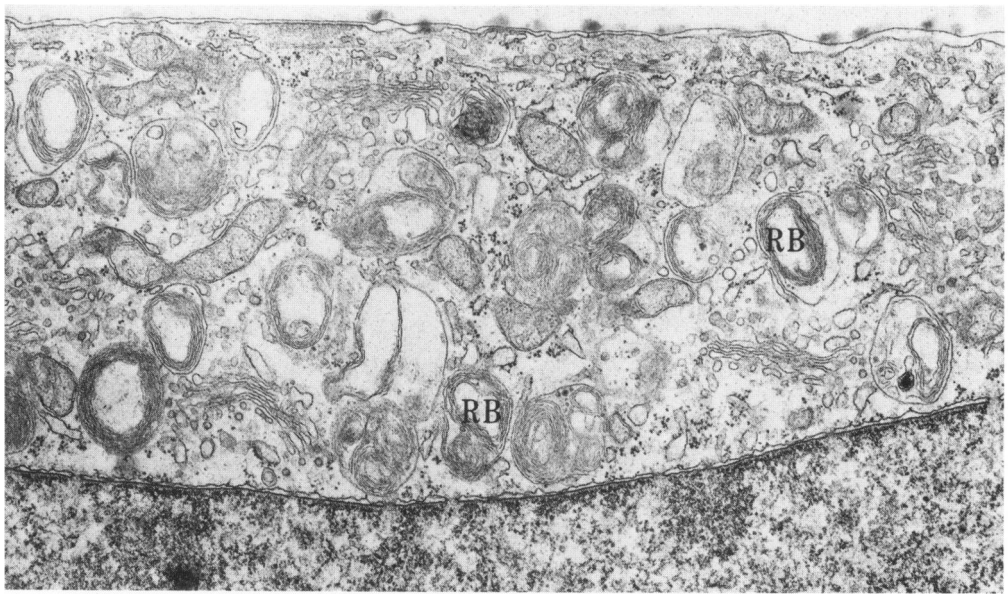


Figure 2—Electron micrograph of a large phagocytic vacuole within one cell of a HeLa cell culture which was subcultivated without replacing the medium after cell attachment. This single membrane-bounded vacuole contains chromatin (*C*) and smaller membrane-bounded bodies, some of which are extremely electron opaque and could represent calcifications within mitochondria (*arrows*. × 22,000)



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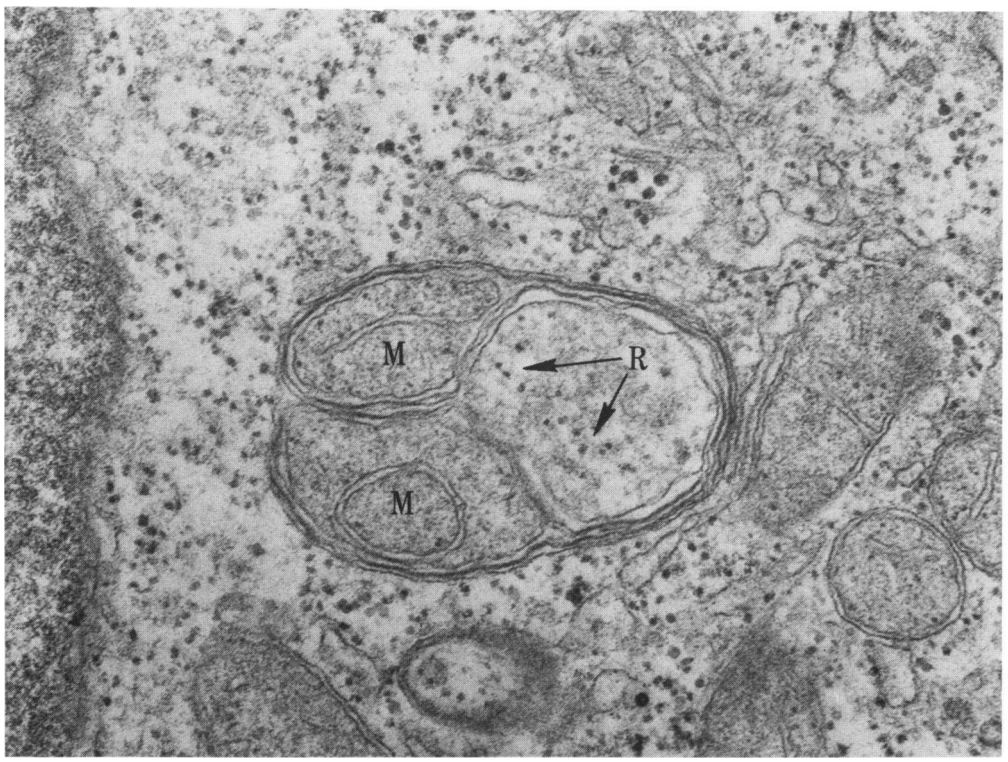

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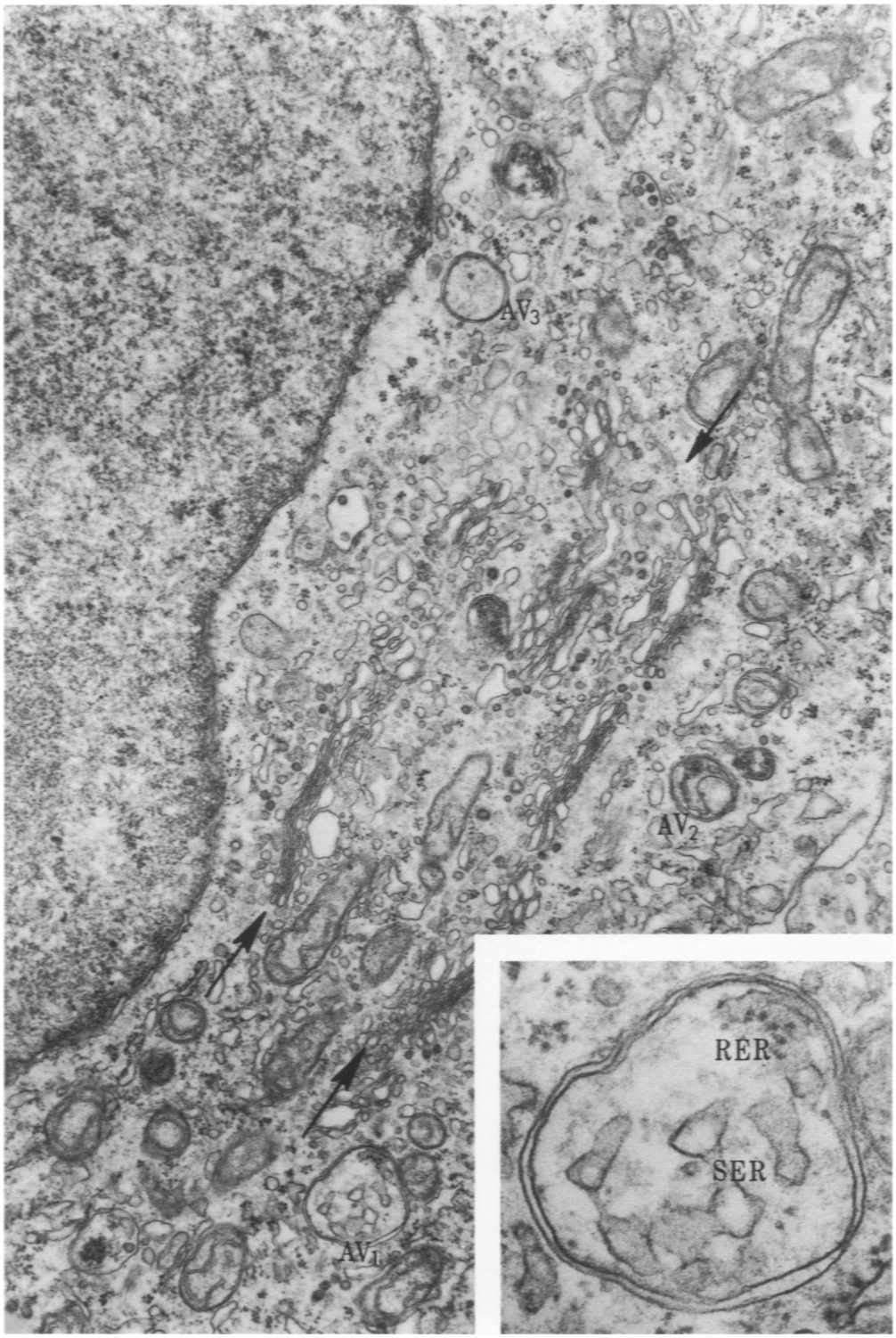
Figure 3—HeLa cell 13 days after forming a confluent monolayer, without refeeding. The cytoplasm is filled with residual bodies which contain lamellar debris (*RB*). (× 22,00) **Figure 4**—HeLa cell after 3 hours in the medium deficient in serum and amino acids. This double membrane-bounded autophagic vacuole contains two mitochondria (*M*) and ribosomes (*R*). (× 58,500)

Figure 5—HeLa cell after 1 hour in the medium deficient in serum and amino acids. Numerous autophagic vacuoles are present (*AV_1*, *AV_2*, *AV_3*). The **inset** is a higher magnification of *AV_1* demonstrating smooth endoplasmic reticulum (*SER*) and rough endoplasmic reticulum (*RER*). Note that these autophagic vacuoles are in the region of a large Golgi apparatus (*arrows*). (× 21,200; **inset,** × 76,500)


AV3
AV2
AV1
RER
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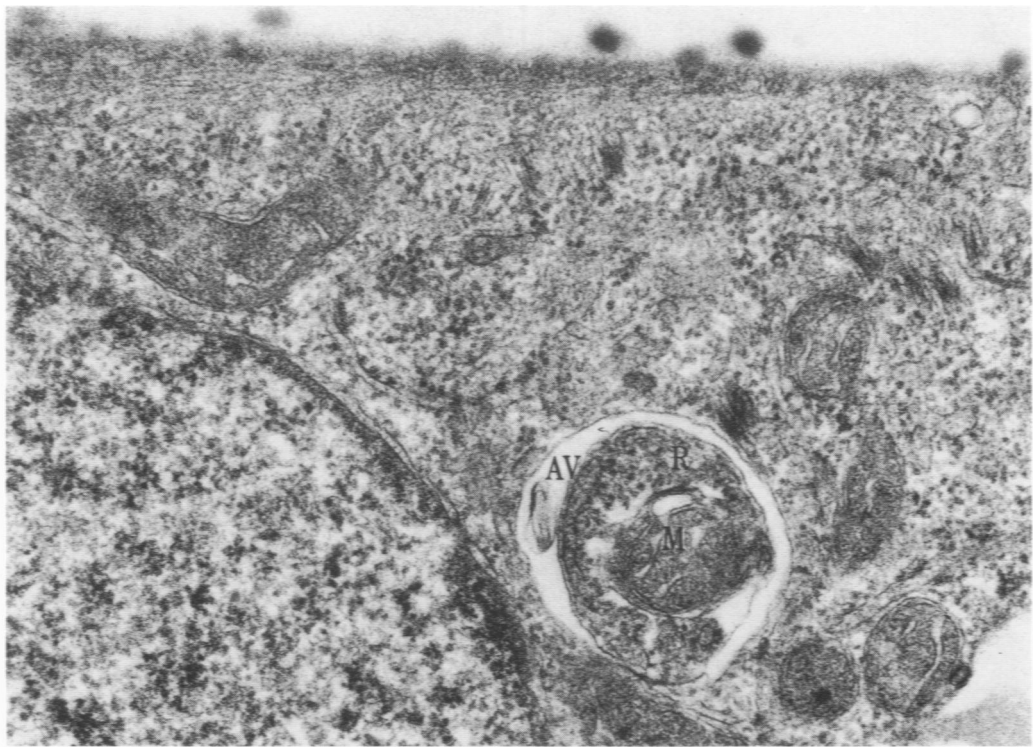

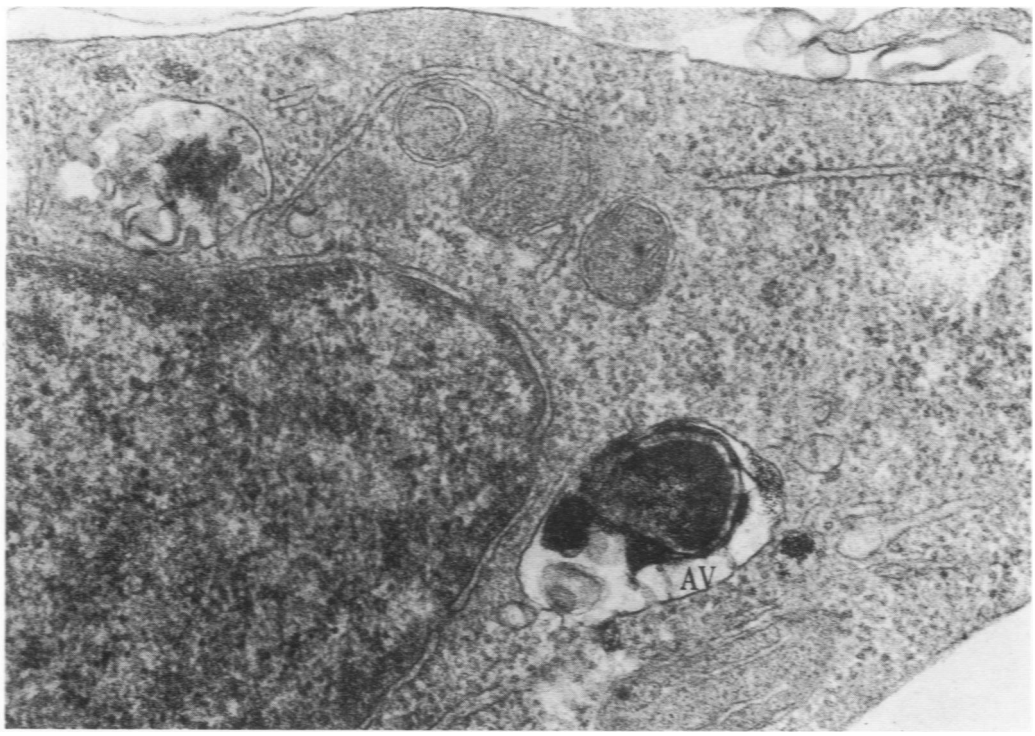

Figure 6—HeLa cell after 1 hour in the deficient medium, acid phosphatase preparation. Ribosomes (*R*) and a mitochondrion (*M*) are present within this autophagic vacuole (*AV*); acid phosphatase reaction product is absent. (× 47,000) **Figure 7**—HeLa cell after 1 hour in the deficient medium, acid phosphatase preparation. This autophagic vacuole (*AV*) contains acid phosphatase reaction product. The sequestered organelle could possibly have been a mitochondrion. (× 70,400)