Lysosome and Phagosome Stability in Lethal Cell Injury

Morphologic Tracer Studies in Cell Injury Due to Inhibition of Energy Metabolism, Immune Cytolysis and Photosensitization

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In two types of cell injury in a tissue culture system, the possibility was tested that lysosome rupture may be a lethal cellular reaction to injury, and thus an important general cause of irreversibility of damage in injured tissue. Prior labeling of secondary lysosomes with the fluorochrome acridine orange, or with ferritin, was used to trace changes in lysosomes after applying an injury. The metabolic inhibitors iodoacetate and cyanide were used together to block the cell's energy supply, or attachment of antiserum and subsequent complement attack were used to damage the surface membrane, producing rapid loss of cell volume control. Living cells were studied by time-lapse phase-contrast cinemicrography and fluorescence microscopy, and samples were fixed at intervals for electron microscopy. The cytolytic action of complement was lethal to sensitized cells within 2 hours, but results showed that lysosomes did not rupture for approximately 4 hours and in fact did not release the fluorescent dye until after reaching the postmortem necrotic phase of injury. Cells treated with metabolic inhibitors also showed irreversible alterations, while lysosomes remained intact and retained the ferritin marker. The fluorochrome marker, acridine orange, escaped from lysosomes early after metabolic injury, but the significance of this observation is not clear. The results are interpreted as evidence against the concept that lysosome rupture threatens the survival of injured cells. The original *suicide bag* mechanism of cell damage thus is apparently not operative in the systems studied. Lysosomes appear to be relatively stable organelles which, following injury of the types studied, burst only after cell death, acting then as scavengers which help to clear cellular debris (Am J Pathol 68:255–288, 1972).

DURING THE PAST TEN YEARS, the concept has frequently been advanced that lysosomes may burst open within injured but still living cells, and that the subsequent release of hydrolytic enzymes into the cell sap and inappropriate self-digestion may be significant

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factors in causing the death of the cell.[1-3] This hypothesis has two parts: first, that release of enzymes from lysosomes should occur, following a specific injury, prior to the time of cell death; and second, that this extent of antemortem enzyme release should be sufficient to cause the death of injured cells in some cases. Cell death is considered to occur when cell injury becomes irreversible—*ie,* the cell is dead at the time when it loses its ability to recover normal functions if the injury were removed. Events occurring after cell death comprise the phase of cellular necrosis.[4]

A great deal of biochemical evidence, taken from many different models of cell injury, has been presented in support of the concept of lysosomes as threats to the survival of injured cells. In most cases the data are insufficient to firmly establish this concept, however, since it is very difficult, if not impossible, to distinguish the enzyme release from lysosomes which might occur in intact injured cells from the enzyme solubilization which occurs during the processes of homogenizing the cells and preparing subcellular fractions. This induced solubilization may well be greater when injured cells are homogenized. Some histochemical studies have been interpreted as suggesting rupture of lysosomes before cell death,[5,6] and some have yielded evidence inconsistent with this interpretation.[7-9] Electron microscopic studies of well-controlled models of cell injury have generally failed to provide evidence for antemortem lysosome rupture,[8-10] but this negative evidence is inconclusive, because of the vagaries of fixation in injured cells and the uncertainty as to the expected appearance of cells whose lysosomes have discharged their enzymes. Previous studies have failed to disclose evidence of perilysosomal deterioration; however, even if lysosomal enzymes were released, it has been suggested that they would diffuse rapidly throughout the cell.[11]

The present study is an effort to estimate the time of bursting of lysosomal membranes relative to other changes of cell injury and necrosis in tissue culture cells derived from human liver (Chang, 1956). Before injury, the cells were induced to form many secondary lysosomes containing both enzymes and extraneous tracer substances which could be observed at will by morphologic technics. Cells were observed by fluorescence microscopy following labeling of lysosomes with acridine orange dye and during their subsequent reaction to injury. In parallel experiments, cells loaded with ferritin before injury were fixed at intervals and examined by electron microscopy. Two modes of rapidly lethal cell injury were applied. The first mode consisted of incubating cells with relatively high doses of sodium iodo-

acetate, potassium cyanide, or both, to inhibit the Embden-Meyerhof and oxidative routes of cellular energy metabolism. The other injury was applied by incubating cells with cell-directed antiserum without complement, then washing and exposing the cells to complement in order to produce rapid immunologic damage to the cell surface membrane. The results indicated release of acridine orange dye from lysosomes before irreversible changes were seen after metabolic injury. Release of dye occurred, however, only after apparent cell death following immune cytolysis. It is uncertain exactly what changes in the lysosomal membrane were indicated by this loss of acridine orange labeling. Following both modes of injury, ferritin was not released from within secondary lysosomes until the postmortem phase of cell necrosis, when irreversible structural alterations had already occurred.

Materials and Methods

Cells

Chang tissue culture cells, originally derived from human liver,[12] were maintained as monolayers in Parker's or Eagle's media supplemented with 10% fetal calf or horse serum, penicillin, streptomycin and glutamine. Chang cell suspension cultures were kindly provided by Dr. Eva Eliasson, Wenner-Gren Institute, Stockholm, Sweden; in later work a derivative of the Chang cell line of the American Type Culture Collection was used.

Observation Chambers

Cells were grown in sterile Sykes-Moore double-coverslip chambers,[13] or in a simple enclosed chamber fabricated from a microscope slide and cover slip, dental wax and Parafilm. For fluorescence studies in which a larger surface area of cells was needed, cells were grown on oversized coverslips (Carolina Biological Supply Co) in Falcon plastic petri dishes, and were inverted onto a shallow (1 mm) well in a Lucite sheet. Coverslips were held in place either by a spring clip or by another sheet of Lucite attached around the shallow well. All chambers were made to fit the mechanical stage of the microscope and included ports fitted with catheters so that the media could be changed at will. Although the first light-microscopic observations were made at room temperature, all results were confirmed using Sykes-Moore chambers maintained at 37 C by a homemade, thermistor-controlled, air-curtain incubator.[13]

Markers

Euchrysine 3R from E. Gurr Ltd, London (Michrome 1197; Color Index 46005), was stored as a 10^{-3} g/ml aqueous solution. Euchrysine is a commercial name for the dye usually called acridine orange. Cell monolayers were exposed to a 1:100 dilution of this dye solution in the standard medium (10^{-5} g/ml) for 15 minutes; they were then incubated in fresh medium without dye for at least 60 minutes before any further handling. This resulted in bright red fluorescent granular staining in the cytoplasm, with faint green nuclear fluorescence. Lower concentrations of dye were found to produce this pattern if larger volumes were used.

By light and electron microscopy, cells appeared unchanged after this treatment, and mitotic cells with granular fluorescence could be observed on the following day. Furthermore, specific activities of the four lysosomal enzymes assayed were normal in cells exposed to acridine orange.

Ferritin, 2X crystallized cadmium-free (Pentex), was passed through a column of Sephadex G-25 (coarse), centrifuged for 2 hours at 100,000g and redissolved in the standard medium at 15 to 25 mg/ml. Monolayers were exposed to this medium overnight (12 to 18 hours) and the medium was changed 1 hour before the start of the experiment.

Phase-Fluorescence Microscopy

Cells on coverslips were observed using either Reichert *Anoptral* or Leitz *Heine* phase-contrast optics, with a green filter. Fluorescence microscopy was done using an HBO-200 mercury arc source, Jena UG-1 source filter, Leitz 510 nm barrier filter, and a Leitz long-working-distance dark-field condenser. Photographs were taken on Kodak Ektachrome HS 35 mm film, daylight type, ASA 160. Time-lapse phase-contrast movies were taken of the cells' reactions to each type of injury, using a Sage time-lapse unit with shutter and a Bolex 16 mm camera, on Kodak Tri-X or Plus-X reversal film.

Electron Microscopy

Cells grown in Falcon plastic dishes were fixed in ice-cold 3% glutaraldehyde for 30 minutes, washed and postfixed overnight in sucrose-containing 1% osmium tetroxide. Alternatively, monolayers were fixed for 30 minutes on ice in a freshly prepared cacodylate-buffered mixture containing glutaraldehyde (4%) and osmium tetroxide (1%),[14,15] then stained in Veronal-buffered uranium acetate for 30 minutes.[16] After dehydration in graded alcohol solutions, 2 to 4 ml of propylene oxide were added to each dish. A few seconds after partial dissolution of the plastic substrate, the sheets of cells floated free; the released monolayer fragments were poured into vials of propylene oxide and washed several times to remove dissolved plastic.[17] The monolayers were then infiltrated with epoxy resin and embedded in Epon 812 according to Luft,[18] or in an Epon-Araldite mixture. Thin sections were stained with uranium acetate and alkaline lead hydroxide, or with uranium acetate alone, and examined in Siemens Elmiskop 1 or Hitachi HS-8 electron microscopes. Unless otherwise noted, all electron micrographs shown are of cells fixed in the glutaraldehyde-osmium mixture and stained only with uranium acetate.

Photosensitization

In order to demonstrate that lysosome rupture would cause release of the ferritin marker to the cell sap space, and that ferritin would remain in that space throughout processing for electron microscopy, a positive control experiment was carried out. Cells which had phagocytized ferritin overnight as described were also treated with acridine orange as above, and fluorescence observation revealed bright red granular fluorescence as usual. These cells were then placed on the microscope base and irradiated for 30 minutes with filtered (BG-12) blue-violet light from the HBO-200 mercury arc light source. The monolayer was fixed in the glutaraldehyde-osmium mixture and processed for electron microscopy as described. According to Allison,[19] such intense irradiation with light absorbed by the dye in lysosomes would be expected to cause photooxidative damage to the lysosomal membrane.

Enzyme Assays

Total activities of 4 lysosomal enzymes (acid phosphatase, aryl sulfatase, β-glucuronidase and cathepsin) were measured in homogenates of acridine orange-treated cells and compared with those in control cells. Two Falcon petri plates were inoculated with the same number of cells, and when both were confluent, one was treated with acridine orange as described. Cells were removed from each plate by scraping with a rubber policeman, frozen, suspended in deionized water, homogenized with 10 strokes of a Potter-type homogenizer and then frozen and thawed 10 times in deionized water containing 0.1% Triton X-100. Cathepsin D was assayed at pH 3.6 with acid denatured bovine hemoglobin (1.75 w/v) (Sigma) as the substrate.[20] Acid phosphatase was measured at pH 5.4 with β-glycerophosphate (63 mM) as the substrate.[21] Aryl sulphatase was determined at pH 5.7 according to the method of Roy [22] as modified by Bowers *et al.*[21] The activity of β-glucuronidase was estimated at pH 5.7 with phenolphthalein-glucuronic acid (1.25 mM) (Sigma) as the substrate.[21] Protein was determined by the method of Lowry *et al.*[23]

Histochemistry

In order to validate the use of ferritin as a tracer for secondary lysosomes in this system, acid phosphatase electron microscopic histochemical staining was performed on untreated and ferritin-loaded cells. Monolayers in 60 mm plastic Petri dishes were fixed for 10 minutes at room temperature in 4% glutaraldehyde buffered with 0.1 M sodium cacodylate. The monolayers were then washed for 1 hour in 0.1 M cacodylate buffer and incubated for acid phosphatase activity for 30 minutes, according to Gomori.[24] After incubation the monolayers were post-fixed for 1 hour in cacodylate-buffered 2% osmium tetroxide and processed for electron microscopy.

Cell Injury Protocols

Metabolic

Cells in the logarithmic phase of growth were washed twice in serum-free medium, and 30 minutes later were exposed to serum-free medium which was 10 mM in iodoacetic acid (IAA) and 5 mM in potassium cyanide (KCN). As controls, cells were incubated in serum-free medium or in medium containing only KCN or IAA. In other experiments IAA and/or KCN were present in complete medium (containing serum). The chemicals used were reagent grade, and all stock solutions were titrated to pH 7.0. The cells' responses were recorded continuously by time-lapse, phase-contrast microscopy, and in other experiments samples were fixed for electron microscopy 30, 60, 90, 120, 360 and 240 minutes after poisoning.

Cytolytic

Cells were exposed for 10 minutes to standard medium containing 5 or 10% immune rabbit serum. This antiserum was prepared by repeated injections of whole Chang cells in Freund's adjuvant, and was heat inactivated before use (56 C, 30 minutes). The titer of the antiserum was 6400 when tested by the mixed hemadsorption test.[25] The monolayer was then washed twice in standard medium, and 20 minutes later was exposed to medium containing 10% guinea pig serum as a source of complement. Controls consisted of treatment with immune serum or guinea pig serum alone.

Results

Definitions

The terminology used in this paper for the various parts of the lysosomal system was proposed by DeDuve and Wattiaux.[26] *Primary lysosomes* contain only enzymes and other components characteristic of all lysosomes of a tissue and have never participated in a digestive event. *Secondary lysosomes* are or have been involved in a digestive process and, in addition to the components of primary lysosomes, may contain additional material from within the cell (autolysosomes) or from outside the cell (heterolysosomes), in any stage of digestion. *Phagosomes* are considered to be either membranes with enclosed intracellular material (autophagosomes) or plasma membrane derivatives with enclosed extracellular material (heterophagosomes), but are devoid of lysosomal hydrolases. After fusion with primary or secondary lysosomes, phagosomes would become incorporated into new secondary lysosomes.

The terms *rupture, bursting,* and *breaking* of lysosomes are used synonymously in this paper and refer to the appearance, for any length of time, of a defect in the lysosomal membrane large enough to be easily demonstrable by electron microscopy if preservation and orientation of the tissue were ideal—ie, 100 Å or larger in its smallest dimension.

Increases in the permeability of the lysosomal membrane, or in the number of compounds which could cross the membrane freely, could conceivably occur without rupture.

Characteristics of Markers

Acridine orange was accumulated by living cells to give bright red lysosomal fluorescence under appropriate conditions, as previously reported[27,28] (Figure 5). The minimum concentration of dye which gave useful fluorescence was about 10^{-6} g/ml if adequate volumes were used. Concentrations above 10^{-4} g/ml at first gave extensive orange cytoplasmic staining which later disappeared in most cells as the fluorescent granules became larger and brighter. Acridine orange dye exhibits concentration-dependent metachromasia, so that concentrations of 10^{-3} g/ml or more in water show maximum fluorescence above a wavelength of 580 nm (red), and concentrations of 10^{-4} g/ml or less fluoresce maximally around 530 nm (green). Similarly, small amounts of dye bound to macromolecules such as DNA fluoresce green, whereas if more dye is bound, the fluorescence color

changes to red. A detailed theory developed by Bradley[29] accounts for these fluorescence color changes on the basis of the self-association of single dye molecules (which fluoresce green) into dimers with a characteristic red fluorescence emission. Whenever a given amount of acridine orange is concentrated into a small enough volume or a small enough number of binding sites, the proportion of dimers increases and red fluorescence is observed. If this amount of dye gains access to a larger volume or many more binding sites, the dimers dissociate and the fluorescence color changes to green. When dye diffuses away from the lysosomes, therefore, the resultant cytoplasmic and nuclear staining should be bright green, and this was consistently observed. Since red and green are primary colors, this color shift is very striking to the eye and readily recorded on color film.

Assay of 4 lysosomal enzymes showed approximately the same activities in homogenates of cells treated with Euchrysine 3R as in homogenates of identical untreated cultures. Thus the fluorescent marker did not inhibit lysosomal enzymes in the doses used.

Overnight incubation of monolayers with ferritin resulted in accumulation of many well-filled heterophagosomes and heterolysosomes in all cells; several were visible in nearly all random sections (Figure 1). The ferritin-containing structures were predominantly multivesicular bodies. In cells loaded with ferritin and incubated for the ultrastructural demonstration of acid phosphatase, reaction product was regularly found within ferritin-containing multivesicular bodies (Figure 4). Reaction product was present in similar structures in cells which were not exposed to ferritin. With the procedure described ferritin does appear to mark sites of acid phosphatase activity, which must be (heterophagic) secondary lysosomes. Single ferritin molecules could easily be recognized in electron micrographs by the high electron density, characteristic size (60 Å) and irregular shape of the iron core. The cells continued to grow and divide quite well in the ferritin-containing medium.

The observed uptake of ferritin was in agreement with previous findings in this laboratory on phagocytic pathways in HeLa cells.[30] According to those studies and the present work, particulate tracers appear to be taken in by cells in small vesicles which rapidly fuse into larger phagocytic vesicles and then internalize part of the vesicle membrane to form multivesicular bodies. Concurrently, within about an hour, fusion occurs with small primary lysosomes or secondary lysosomes, forming new heterophagic secondary lysosomes, which

are also multivesicular bodies morphologically. The membranes of these secondary lysosomes presumably include both material derived from the plasma membrane and other membrane derived from primary lysosomes. With extensive, rapid loading, it may be possible to incorporate most if not all of the cell's primary lysosomes into new secondary lysosomes. In rat liver loaded with Triton WR-1339, for example, the bulk of all acid hydrolases are found in vesicles (secondary lysosomes) which contain phagocytized Triton and thus have a lower than normal density.[1,31]

Phase-Contrast Cinemicrography and Fluorescence Studies

Continuous phase-contrast observation of cells spread on coverslips in a 37 C chamber revealed a characteristic, quite distinct pattern of reaction to each type of injury. The same sequence of changes was observed in each case whether or not the cells had been prelabeled with acridine orange.

Metabolic Injury

Cells treated with KCN and IAA in serum-free medium showed no changes at first, but after approximately 10 minutes, nuclear chromatin began to clump and marginate slowly. Fifteen to twenty minutes after adding the inhibitors, the normal surface ruffling and orderly spreading was lost, and long tubular extensions formed at the cell surface membranes; some of these formed vesicles at their tips, which later seemed to pinch off and float away. These tubular membrane extensions showed continuous disorderly, asynchronous writhing motions. Between 30 and 90 minutes of incubation the fibrillar network of organelles in the Golgi region changed to a pattern of many small round densities (Figure 8). By 1 hour after beginning the injury a few phase-lucent vacuoles appeared near the cell center and slowly enlarged in most cells. By 90 minutes, more and larger vesicles were present and many cells showed swollen, phase-lucent nuclear envelopes. Only about 2 hours after injury did cells appear to swell throughout, but after 3 hours of incubation most cells had swollen and become roughly spherical; nuclear chromatin was in the form of multiple dense clumps, and Brownian motion was extensive in the cytoplasm (Figure 9). No further changes were visible by light microscopy for at least 2 more hours.

In control experiments, cells treated with KCN and IAA in standard medium containing 10% serum showed no visible alterations by

24 hours. Nonspecific binding of IAA by serum proteins presumably accounts for this unexpected finding. Cells exposed to serum-free medium containing either KCN or IAA showed no changes within 4 hours, except for margination of nuclear chromatin in a few cells. In vital dye viability tests, cells treated for 4 hours with serum-free medium containing either KCN or IAA showed less than 5% staining with 0.1% Nigrosin. After being treated with both inhibitors in serum-free medium for 3 hours, most cells stained with Nigrosin and thus were considered nonviable.

By fluorescence microscopy, many cells began to show increasingly bright green nuclear fluorescence beginning about 30 minutes after adding KCN and IAA in serum-free medium, although they retained their red lysosomal fluorescence. By 1 hour this red granular lysosomal fluorescence had faded in most cells and become orange or yellow, and the green nuclear fluorescence appeared brighter than that of the lysosomal granules. After 2 hours of incubation the nucleus and cytoplasm of most cells were stained diffusely bright green, with no red cytoplasmic granules remaining visible (Figure 10). Control cells treated with either inhibitor alone continued to show red granular fluorescence for at least 4 hours.

Cytolytic Injury

Cells pretreated with rabbit antiserum showed no changes for 10 to 15 minutes after adding guinea pig complement, but then chromatin clumping occurred very rapidly; the cytoplasm became more coarsely granular in appearance and large portions of the cell membrane bulged and then ballooned out in most cells. The typical appearance 20 minutes after adding complement was that of a grossly swollen cell with one or two large round, phase-lucent outpouchings of its surface membrane (Figure 15). Within 30 minutes after addition of complement, one or several large phase-lucent cytoplasmic vacuoles appeared and slowly enlarged in most cells, and frequently a very large clear space developed around one side of the nucleus. As the cells became distended, many organelles were seen to enlarge and subsequently become invisible. By 60 to 90 minutes after addition of complement, all cells were extremely swollen, with very pale cytoplasm and densely clumped nuclear remains; the only activities observed in the next several hours were further organelle swelling and Brownian motion (Figure 16). Cells incubated for 4 hours in antiserum alone or complement alone were indistinguishable from untreated controls.

By fluorescence microscopy the red granular staining of lysosomes persisted during cytolytic injury, however, and even 2½ hours after adding complement nearly all cells showed bright red granular fluorescence of lysosomes and only faint green nuclear fluorescence, closely resembling the fluorescence pattern of control cells (Figure 17). Complement-damaged cells were very fragile, and occasional cells, whose surface membranes ruptured, released red-fluorescent granules into the medium. By 4 hours after injury, bright green nuclear staining had developed in most cells, but red-orange granules remained in most, indicating partial release of lysosomal dye.

Electron Microscopic Observations

Controls

Electron micrographs of untreated cells showed numerous elongated mitochondrial profiles, one or more Golgi complexes often containing 5 to 10 dictyosomes each, several multivesicular bodies, many small circular profiles of smooth membranes and long narrow profiles of rough endoplasmic reticulum (Figure 1). In cells fixed with the glutaraldehyde-osmium mixture, nuclear chromatin was finely granular and evenly dispersed, and prominent nucloli were visible (Figure 2). Cells which were incubated for 4 hours in medium containing antibody only, complement only, acridine orange, or in medium lacking serum, all appeared the same as untreated cells. Since cells loaded with ferritin showed the same changes following injury as unlabeled cells, they will be described together. In uninjured cells ferritin molecules could never be found in the cell sap (Figure 1).

Ferritin-loaded cells which were photosensitized by uptake of acridine orange dye (Figures 3, 5) and exposed for 30 minutes to intense blue-violet irradiation showed a number of interesting changes, including enlargement of endoplasmic reticulum profiles, mitochondrial swelling, and the presence of coarsely granular flocculent material in the cell sap (Figures 6, 7). Ferritin was present throughout the nuclear and cell sap spaces and frequently seemed to be bound to the exterior surfaces of organelles. It is not known, of course, whether the cellular damage observed was a consequence of the release of lysosomal hydrolases, of propagated lipid peroxidation, or of direct photodynamic damage by the dye to the cell membrane or other structures after its release from lysosomes. The results indicate, however, that photosensitization damaged the lysosomal membrane sufficiently to allow dispersion of the ferritin marker, and that this

marker, once released, could easily be seen throughout the cell sap in electron micrographs.

Metabolic Injury

Thirty minutes after addition of KCN and IAA in serum-free medium, the only consistent difference from controls was the appearance of long, irregular extensions of the surface membrane resembling long microvilli or large folds. Slight dilatation of rough and smooth endoplasmic reticulum (ER) was commonly seen, and there was slight margination of nuclear chromatin.

By *1 hour* the surface membrane extensions were still present, and somewhat more extensive ER dilatation was seen. Many mitochondria showed matrix condensation with enlarged intracristal spaces. Most mitochondrial profiles were ovoid; elongated profiles were rare. Ferritin-laden multivesicular bodies (secondary lysosomes) resembled those in control cells.

By *90 minutes*, many mitochondrial profiles were large and circular, but no mitochondrial densities were seen. Swelling of ER was more pronounced than at the previous interval, but ribosomes were still attached and the Golgi apparatus still contained flat cisternae.

Cells *2 hours* after exposure to KCN and IAA still showed moderate swelling of ER and mitochondria. The surface extensions remained in most cells, but some appeared swollen, with rounded surface contours, pale cytoplasm and much more extensive swelling of organelles. Pronounced chromatin clumping was observed in many cells (Figure 11). Occasional ferritin-filled lysosomes appeared enlarged and less densely packed than those of controls. However, their membranes appeared intact, and except for a few cells clearly damaged in processing, no ferritin molecules could be found in the cell sap or on the exterior surfaces of organelles.

By *3 hours* all organelles were grossly swollen, and the cytoplasm was often very pale; all mitochondria showed pronounced swelling, disorganization and flocculent matrix densities, and no narrow profiles of ER cisternae nor any rough ER could be found; nuclear chromatin was densely clumped. Many lysosomes were apparently unchanged, but many appeared swollen; ferritin molecules could not be found in the cell sap nor adherent to the outer surfaces of mitochondria or smooth membrane profiles (Figure 12).

Four hours after adding KCN and IAA the ultrastructural appearance was much the same as at 3 hours; cells and organelles were grossly swollen and disorganized. Although all lysosomes of most

cells appeared intact and retained their ferritin, a small amount of ferritin was sometimes present in the cell sap, indicating release from ruptured secondary lysosomes (Figures 13, 14).

Immune Cytolysis

Some cells fixed *15 minutes* after addition of complement showed no change from controls, but most showed indications of cell swelling, with moderate dilatation of most of the cell's smooth-surfaced vesicles or cisternae, and part of the Golgi apparatus. Mitochondria were mostly of normal shape, but showed matrix condensation, which was most pronounced in glutaraldehyde-fixed tissue. Mitochondria in altered cells also showed dark, granular circular densities in their matrices, and in some cells this peculiar mitochondrial change was the only visible alteration.

By *30 minutes* most cells showed dilatation of ER and margination of nuclear chromatin. Occasional mitochondria showed matrix condensation, forming very long, narrow profiles. Most mitochondria appeared circular and enlarged in section, however, and nearly all showed strikingly electron-dense, small circular densities (Figure 18). These were within the matrix space but very closely apposed to the mitochondrial inner membrane at the bases of the cristae; they were composed of very fine, dark granules and contained clear centers (Figure 21). These mitochondrial changes closely resemble those which have been reported to appear in a variety of circumstances all of which involve active accumulation of calcium by mitochondria. These circumstances include the *in vitro* experiments of Vasington and Greenawalt,[32] studies of toxic liver injury by Reynolds,[33] the model injuries in flounder tubules reported from this laboratory,[34–36] and the studies of heart muscle ischemia reported by Jennings *et al.*[37,38]

One hour after adding complement, various degrees of cellular swelling were observed. Most cells showed very extensive enlargement of all organelles, including the nuclear envelope, and pronounced chromatin clumping (Figure 19). Nearly all mitochondria showed the typical appearance of severe swelling, and fluffy, gray mitochondrial matrix densities were often seen. The great majority of mitochondria contained even more numerous (3 to 5 per organelle section) dark circular densities. Ferritin-containing secondary lysosomes frequently appeared markedly enlarged, with diluted contents, but intact surrounding membranes could be seen, and no ferritin molecules could be found in the cell sap.

After *two hours* the appearance was similar but with nearly all cells showing even more extensive organelle swelling: ferritin was still retained within multivesicular secondary lysosomes (Figure 20).

By *four hours* after complement treatment essentially all cells showed very pronounced swelling, irregularity and disorganization of all organelles: ferritin-containing organelles were still generally intact, but clear evidence of ferritin release could be obtained in some cells (Figures 22–27).

Nine hours after complement injury, swollen mitochondria with both flocculent and round granular densities and irregular smooth and rough-surfaced ER membranes were still present. Many intact ferritin-loaded lysosomes remained but some clearly had disintegrated, releasing their content of ferritin.

Discussion

The present findings of retention of macromolecular lysosomal contents until late in the course of cell injury are contrary to what one would expect on the basis of considering lysosome rupture as a universal mechanism leading to irreversibility of cell injury. Thus it may be important to reevaluate the evidence which does suggest a destructive role for lysosomes in injured cells.

Biochemical studies of injured cells have an inherent, well-recognized problem of interpretation since changes in the subcellular distribution of lysosomal enzymes can be evaluated only after grinding the cells in a homogenizer. As DeDuve has frequently emphasized, it is important not to misinterpret increases in unsedimentable or free enzyme activity which occur during homogenization as evidence for enzyme release in the intact injured cells. Lysosomes of injured cells should not be expected to behave exactly like those of control cells during drastic isolation procedures. Changes in lysosomes such as increased size, following heterophagy or autophagy, cause them to rupture more easily during homogenization but do not cause rupture in intact cells.[39–41] The most likely artifact in this situation would thus lead to overestimation of intracellular rupture of lysosomes, which actually might only be less stable. It would be possible and quite interesting, in this regard, to obtain estimates of the mean volume and osmotic behavior of intact lysosomes from injured tissue by means of centrifugation analyses.

Enzyme cytochemical studies must be interpreted cautiously for several reasons, of which structural and enzymatic artifacts of fixation are probably the most serious.[24] Lysosomal membranes clearly be-

come more permeable to small molecules following fixation, and fixative damage might conceivably cause release of lysosomal enzymes in itself. However, the most likely artifact would seem to be more rapid inactivation by the fixative of any enzyme that was released to the cell sap than of lysosomally bound enzyme.[42] This would lead to an underestimate of lysosomal enzyme release. Lysosome histochemistry in unfixed tissue culture cells, although yielding interesting information, is again a type of stability test, since the incubation mixture is sufficiently toxic to give lysosomal staining (interpreted as partial activation of lysosomes or moderate damage) within a few minutes even in normal cells.[43] Indeed, the interpretations of lysosomal changes based on centrifugation of homogenates have often conflicted with interpretations based on histochemical studies of the same or similar systems.[8,44,45]

Ultrastructural observations of lysosomes in ischemic cells are also difficult to interpret. Artifacts of fixation, for example, may account for false images of breakage or fusion of membranes. In a previous study of mouse liver, it was noted that lysosomes in injured cells, though mostly intact, often showed points of membrane interruption in osmium-fixed tissues.[8] Such breaks were only rarely observed after fixation in glutaraldehyde, perhaps because of the better protein-stabilizing properties of the latter fixative. These findings are probably best interpreted as evidence of increased fragility of lysosomal membranes in injured cells—*ie*, increased likelihood of artifactual rupture during processing.

On the other hand, sizable gaps in lysosomal membranes could be missed by electron microscopy due to sectioning in planes where the membranes might appear normal. Furthermore, lysosomal rupture might involve rapid disintegration or collapse of the lysosomal membrane with rapid diffusion of lysosomal contents throughout the cell, leaving little or no morphologic evidence.[11]

It seemed that an independent method of estimation of lysosome stability would be helpful in approaching this elusive problem. Our efforts, therefore, have been directed toward developing technics by which the lysosomal space could be specifically labeled before a controlled cell injury was applied, and the fate of lysosomal contents could be followed after injury, with a minimum of experimental manipulation of the injured cells.

The original *suicide-bag* hypothesis in its broadest form, that lysosome rupture may be a final common pathway leading to death in most kinds of cell injury, is not now accepted widely or without

reservation. However, for the reasons outlined above, it has not been possible to show unequivocally that it is not a common cause of cell death. In several specific types of cell injury, including photosensitization by dyes and prophyrins,[19] and silica and urate toxicity to phagocytes and polymorphs,[46–49] substantial cases have recently been made that lysosome rupture does kill cells. Lysosomes have also been implicated in viral cytotoxicity[3] and in the programmed cell death of insect metamorphosis.[50]

The purpose of the present study was to test, in a relatively direct and well-controlled way, the simple question of whether lysosomes of cultured cells burst open prior to cell death, either following inhibition of energy metabolism or following immunologic cell membrane damage. Another possibility, that lysosomal permeability to substrate or even to enzymes may increase during cell injury, was approached with the use of a low molecular weight fluorescent vital dye, but without yielding definitive results. The general question of interest was whether lysosomes are sensitive to the reversible changes of cellular reaction to injury, and thus threaten the survival of injured cells, or alternatively are stable organelles which are not altered until after cell death. This is significant not only in gaining an accurate understanding of cell injury and death, but also in rationally planning therapy to affect lysosomes.[47,51,52] The present work represents only a first approach to the problem, in which the presence or absence of secondary lysosome rupture was tested after 2 rapidly lethal cell injuries.

Types of Injury Applied

Metabolic

The two inhibitors, cyanide and iodoacetate, were used together in an effort to curtail the synthesis of ATP by the cells. Iodoacetate at low doses specifically inhibits 3-phosophoglyceraldehyde dehydrogenase,[53] thus effectively blocking the glycolytic pathway, which is well developed in most tissue culture cells. If, in the present study, the concentration of IAA in the cell interior reached that of the IAA applied, other effects would be expected; however, the fact that application of IAA alone caused no cytopathic effects by 4 hours suggests that any nonspecific effects must not severely compromise the cells' homeostatic ability. In the presence of a block in the Embden-Meyerhof glycolytic pathway, substrates such as acetate, pyruvate and succinate may be oxidized to provide ATP via the Krebs citric acid cycle in the mitochondria. When oxygen utilization is blocked by

cyanide, this pathway becomes inoperative. It seems that Chang cells are able to survive, at least for several hours, with ATP synthesis proceeding either via substrate-level phosphorylation in the anaerobic glycolysis pathway (in the presence of cyanide), or via oxidative metabolism alone (in the presence of iodoacetate). The synergistic effect of these two inhibitors in causing cellular degeneration is interpreted as being due to inhibition of both the glycolytic and oxidative pathways for ATP synthesis. The use of both inhibitors simulates the effects of total ischemia, since in the absence of a blood supply both glucose and oxygen are lacking, whereas IAA and KCN together block the utilization of both glucose and oxygen.

Cytolytic

In the method used, exposure to antiserum produced against whole Chang cells should lead to binding of antibody at the cell surface. Antibodies against cell organelles, though possibly present, would not be expected to penetrate the cells' plasma membranes, and so could not bind internal cellular antigens.[54] A small amount of antiserum may, however, be taken up by pinocytosis during the 10 minutes of its exposure to cells or the 20 minutes of incubation of cells in fresh medium. During washing with two changes of medium, essentially all antibody not bound or enclosed by the cell membrane should be removed. Thus when complement is added, one would expect it to be activated by antigen-antibody complexes and to begin to cause damage only at the cell surface membrane. (The small amount of complement which could be taken up by pinocytosis before cessation of this process might also damage a few new phagocytic vacuoles which might contain membrane-bound antibody.) Surface membrane damage caused by activated complement has been shown in other systems to lead to an immediate efflux of potassium from cells, together with cell swelling, followed by loss of cytoplasmic enzymes and even ribosomal RNA.[55] Similar cellular responses are to be expected in the present system.

Interpretation of Fluorescence Changes

Acridine orange, an aminoacridine dye of molecular weight near 300, is apparently incorporated by living cells in the same way as many other basic vital dyes.[56] The distribution of acridine orange within living cells, under appropriate conditions, has been shown to be indistinguishable from that of acid phosphatase in HeLa cells by histochemistry and in rat muscle cells by rate-zonal centrifugation

studies.[28,57] Acridine dyes can be shown to bind to isolated lysosomes and even to a purified lysosomal glycoprotein,[58] where, under appropriate conditions, red fluorescence is observed. Dye bound by intact isolated lysosomes can be displaced by adding protamine, and the same quantity of dye is bound whether the lysosomes are intact or disrupted.[58] Both findings suggest that the component which passively binds acridine dye is associated with the lysosomal membrane. Surprisingly, extensive studies of acridine orange staining in nonliving tissue culture cells have failed to show a significant amount of dye binding to lysosomes; in freeze-dried or acetone-ethanol-fixed cells the bulk of the acridine orange is bound to RNA and DNA, which can be quantitatively assayed by this technic.[59] In living cells with pronounced red-fluorescent acridine orange labeling of lysosomes, it was observed that exposure to buffered glutaraldehyde causes fading of lysosomal fluorescence within seconds and the simultaneous appearance of bright green nuclear and cytoplasmic staining.

Acridine orange labeling of lysosomes in living tissue culture cells has been studied carefully by Robbins, Marcus and Gonatas,[27,28] and this process is apparently much more complex than dye binding *in vitro*. These authors showed that even in cells deprived of glucose or incubated at 0 C lysosomes did accumulate enough acridine orange to fluoresce red; however, addition of more than a critical amount of dye resulted in additional diffuse red-orange cytoplasmic staining under these conditions. If the cells were warmed or provided with glucose, this cytoplasmic reddening disappeared as the lysosomes became brighter red and larger; if deprived of substrate, however, the cells degenerated.

By electron microscopy, lysosomes of acridine orange-labeled cells appeared to be enlarged; after several days of exposure to dye myelin figures appeared in lysosomes. Thus it seems that acridine orange staining of lysosomes in live cells occurs by at least two processes: passive binding by a lysosomal component, and a distinct energy-requiring process which can accumulate much more dye in lysosomes, perhaps by a membrane transport process. One attractive hypothesis is that only the nonionized form of the dye freely equilibrates across the lysosomal membrane, the concentration of the predominant cationic form depending upon the pH, and thus being much higher in the acid interior of the lysosome.[60] In this case, actively maintaining a low pH in lysosomes would allow protonation and trapping of larger amounts of dye, and the loss of a pH gradient across the membrane would lead to leakage of dye from lysosomes.

The observation that acridine diffuses out of lysosomes to the nucleus and cytoplasm early in the cell's reaction to metabolic inhibition could have several reasonable interpretations; therefore, its significance is not clear. Changes in the concentration of hydrogen or other ions or metabolites in the cell sap could interfere with passive binding of dye to the lysosomal glycoprotein or trapping of dye within lysosomes. Alternatively, the lack of metabolic energy could prevent the operation of whatever mechanism is normally capable of transferring dye from the cell sap to lysosomes. This would make apparent any slow diffusion of dye from lysosomes, even if it occurs continuously in normal cells. A third possibility is that the lysosomal membrane would normally retain most of the acridine orange within labeled lysosomes and that release of dye would reflect an increase in permeability of this membrane.

It is interesting to note that lysosomal staining persisted throughout immune cytolysis, by contrast, long after the composition of the cell sap was drastically altered by damage to the cell membrane and after subsequent equilibration with the external medium.

It was important to rule out the possibility that the fluorescent marker used might inhibit lysosomal enzymes. The finding that all four enzymes assayed did retain full activity in Euchrysine-treated cells indicates that the possibility of cell damage by lysosomal hydrolases was not affected by the use of the fluorescent dye.

Ultrastructural Findings

The progression of ultrastructural changes seen in each of these types of injury was quite similar to previously described patterns of many other forms of lethal injury in this and other cell types. Cytolytic complement attack led to a more rapid progression of cellular responses, in which early, extensive cell swelling and the appearance of small, circular, fine-grained densities in mitochondria were characteristic. Similar responses have been reported in other forms of primary plasma membrane injury in other systems.[35,36]

The presence of ultrastructural changes in mitochondria suggestive of calcification seems to indicate that the conditions necessary for mitochondrial calcification were present in complement-damaged cells. Isolated mitochondria require both calcium and a source of energy (either as ATP or as oxidizable substrate) to accumulate calcium from the medium. In complement-damaged cells, one would expect that the increased membrane permeability would allow extracellular calcium to enter the cells, and the supply of substrates, oxygen and even ATP

to the cells need not be greatly decreased. In cells with inhibited energy metabolism, on the other hand, calcification could not proceed in the absence of ATP or usable oxygen, even if calcium were present in increased amounts in the cytoplasm.

The use of ferritin as a marker for secondary lysosomes is convenient, since individual molecules can be identified in thin sections and routine fixation and staining may be used in preparation of the tissue. The use of a tracer such as ferritin overcomes some of the problems of assessing the integrity of lysosomal membranes by electron microscopy. The presence of ferritin in the cell sap should clearly indicate the existence of any lysosomal membrane breaks 100 Å or more in diameter, even if these are transient, obscured by fixation artifacts or missed by the plane of section. A single ferritin-loaded lysosome, if it burst open, should spread evidence of its breakdown throughout the cell, and thus should be detected efficiently. The results of the photosensitization experiments lend support to this concept.

The finding that no ferritin could be observed in the cytoplasm outside single-membrane profiles for 3 hours after a rapidly lethal cell injury (immune cytolysis) clearly indicates that rupture of lysosomes was not an early, sublethal reaction to injury in this model system.

Ferritin could not be shown to escape from lysosomes for at least 3 hours following inhibition of energy metabolism in Chang cells. It is difficult to pinpoint the time of cell death in this system, however. Many cells by 2 hours, and most cells by 3 hours, showed very dense chromatin clumping and mitochondrial swelling with flocculent matrix densities, which are ultrastructural lesions typical of the irreversible or necrotic phase of cell injury.[10] As late as 4 hours after KCN–IAA poisoning, when irreversible structural changes were uniformly seen, it was clear that the great majority of lysosomes continued to retain the ferritin marker within a morphologically intact membrane although some release of ferritin to the cell sap was demonstrated.

In conclusion, the findings do not indicate that the suicide bag mechanism of lysosomal rupture prior to cell death was operative in the two systems studied. On the contrary, the lysosomes appeared to be relatively stable organelles which only burst in the postmortem phase of cellular necrosis. However, it is important to note that only the resistance of secondary lysosomes to membrane rupture was well evaluated. It will be of interest in the future to study the fate of lysosomal tracers smaller than ferritin to test for lysosomal permea-

bility changes without rupture, and to study other more gradual modes of cell injury.

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Legends For Figures

Fig 1—A ferritin-loaded, otherwise untreated control Chang liver cell, fixed in the glutaraldehyde and osmium tetroxide solution, stained only with uranium acetate. *M* = mitochondria, *SL* = secondary lysosomes, *N* = nucleus (× 23,000).

Fig 2—An untreated Chang cell without ferritin. RER = long cisternae of rough endoplasmic reticulum, *N* = nucleus with dispersed chromatin (× 18,500).

Fig 3—Part of a cell exposed to ferritin overnight, washed, and treated for 15 minutes with acridine orange 1 hour prior to fixation (× 41,500).

Fig 4—A secondary lysosome in a cell exposed to ferritin overnight, then fixed in 4% glutaralydehyde, incubated for acid phosphatase, postfixed in 2% osmium tetroxide and stained only with uranium acetate. *Arrows* indicate histochemical reaction product (× 95,000).

Fig 5—The appearance by fluorescence microscopy of living Chang cells after labeling with acridine orange as described. The cytoplasmic granules (lysosomes) fluoresce brightly red, and nucleoli fluoresce faintly green (× 700).

Figs 6 and 7—A cell exposed to ferritin overnight, treated with acridine orange as in Figure 5, then irradiated with blue-violet light as described. Ferritin molecules, identifiable in Figure 6, are present throughout cell sap and nucleoplasm but have not penetrated other organelles. *FD* = fat droplet, *MvB* = multivesicular body (× 50,000 and × 20,000).

Fig 8—Phase-contrast photomicrography of a typical cell after 90 minutes of incubation with both KCN and IAA in serum-free medium. Filamentous processes (*F*), some resembling strings of beads, protrude from the surface of a poorly-spread cell (× 1100).

Fig 9—Cells after 2 hours of incubation with KCN and IAA in serum-free medium, illustrating rounding up and vacuolation of cells, and the ballooning out of local regions of the cell surface (*arrows*) (× 600).

Fig 10—The same cells as in Figure 9, by fluorescence microscopy.

Fig 11—Electron micrograph of a cell 2 hours after exposure to KCN and IAA in serum-free medium. Note enlarged, round mitochondrial profiles with flocculent matrix densities (*M*) and swollen endoplasmic reticulum profiles (*ER*) (× 23,000).

Fig 12—A cell pre-loaded with ferritin. Three hours in KCN and IAA, no serum. Note the clumping of nuclear chromatin (*N*), the narrow extension of cell surface membrane (*arrow*) and the intact second lysosomes (SL). G = Golgi region (× 16,000).

Figs 13 and 14—Four hours in KCN and IAA, no serum. Figure 13 shows the typical lysosomal retention of ferritin, Figure 14 documents its apparent release to the cell sap, observed occasionally ×25,000 and ×30,000.

Fig 15—Phase photomicrograph of a cell pretreated with heat-inactivated antiserum, washed and exposed to guinea-pig complement for 30 minutes. Cytoplasmic vacuolation and multiple outward bulges or "blebs" (B) of the surface membrane are apparent (× 1140).

Fig 16—Sensitized cells, prelabeled with acridine orange, two hours thirty minutes after addition of complement (× 800).

Fig 17—Same cells as in Figure 16, photographed by fluorescence immediately after Figure 16 was taken. The lysosomal granules fluoresce brightly red (× 800).

Fig 18—Mitochondria of a sensitized cell 30 minutes after adding complement (× 35,000).

Fig 19—Electron micrograph of a sensitized cell after one hour of exposure to complement. Extensive chromatin clumping is apparent in a pale nucleus (*N*); endoplasmic reticulum profiles are grossly dilated (*RER*), and mitochondria (*M*) contain multiple finely granular, peripheral densities (× 19,000).

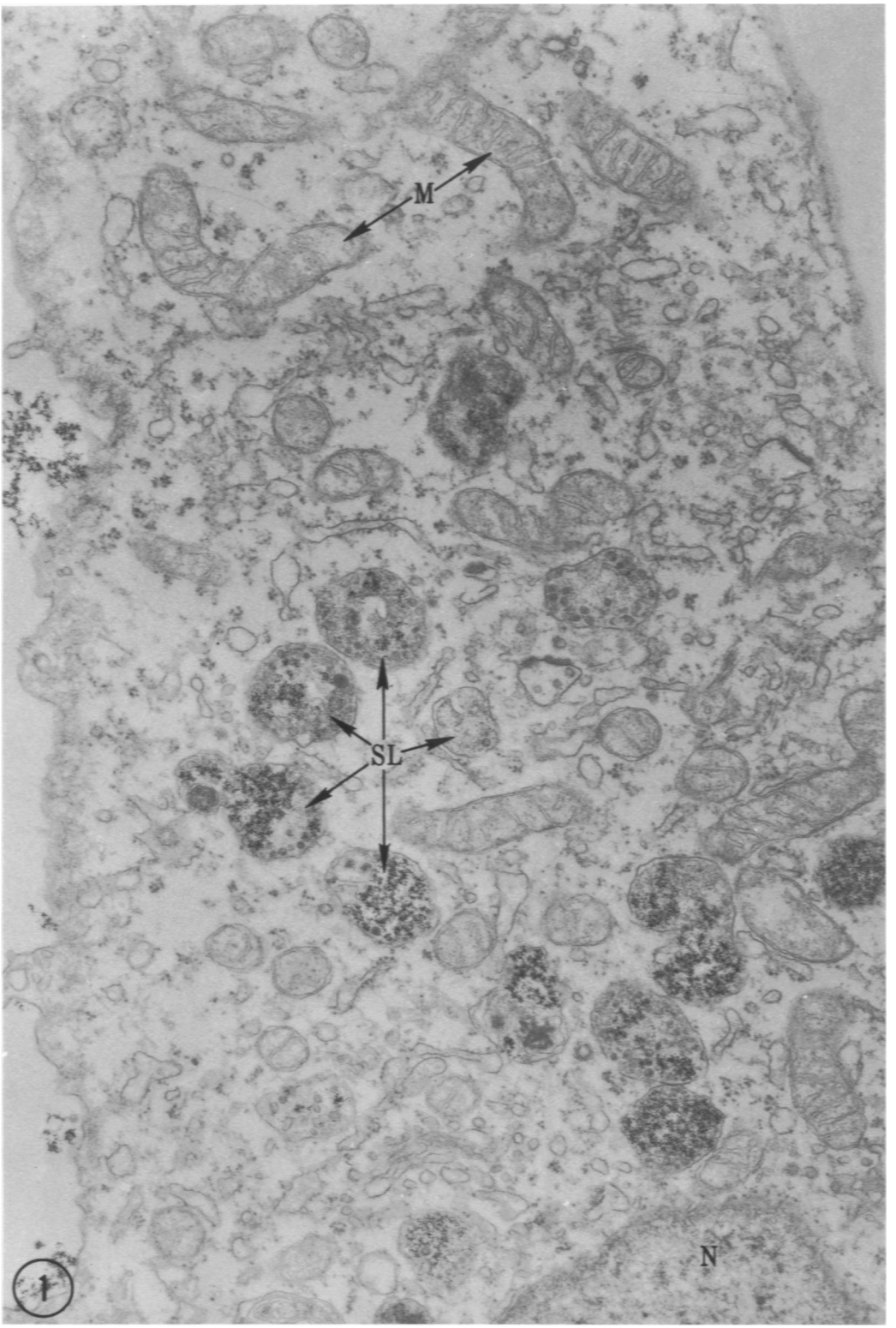
Fig 20—Two hours after antibody-complement treatment. Mitochondrial changes (*M*), and dilatation of endoplasmic reticulum (*RER*) are apparent. Ferritin-filled secondary lysosomes seem intact, since ferritin cannot be found in the cell sap (× 24,000).

Fig 21—A mitochondrion (*M*) from a sensitized cell one hour after complement treatment, without ferritin preloading. The mitochondrial densities are finely granular, circular in profile and have clear centers (*GD*). They appear nearly surrounded by evaginations of the inner mitochondrial membrane into cristae (*CM*) (× 34,000).

Figs 22 and 23—Four hours after antibody-complement treatment. Nuclear chromatin is present (*N*) but is no longer surrounded by a nuclear envelope. Secondary lysosomes (*SL*) appear completely intact in this typical cell (× 40,000 and × 18,000).

Figs 24, 25, 26—A less common appearance four hours after antibody-complement treatment. Nuclear chromatin clumping (*N*), and mitochondrial changes (*M*) including the appearance of flocculent matrix densities, may be seen. Most ferritin-filled secondary lysosomes appear intact, but some ferritin may be found free in the cell sap × 28,000, × 75,000 and 200,000.

Fig 27—Enlargement of a mitochondrion four hours after antibody-complement treatment. Two types of densities may be seen: a finely granular membrane-associated density (*GD*) and a flocculent, coarsely granular matrix density (*FD*) (× 50,000).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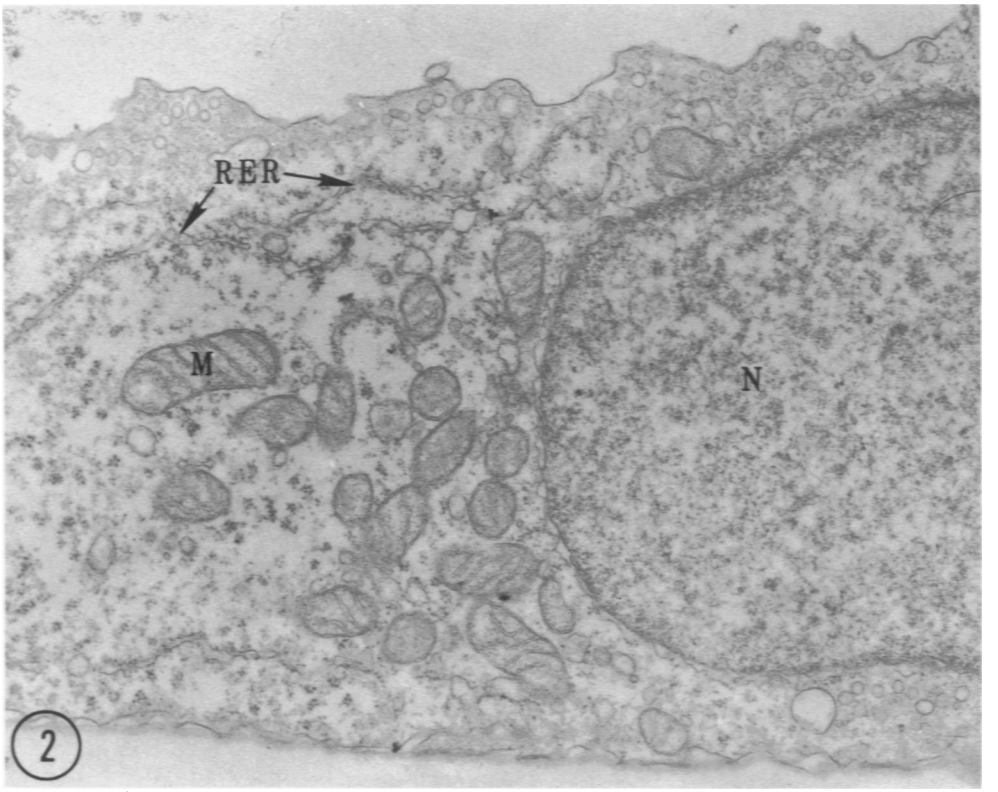
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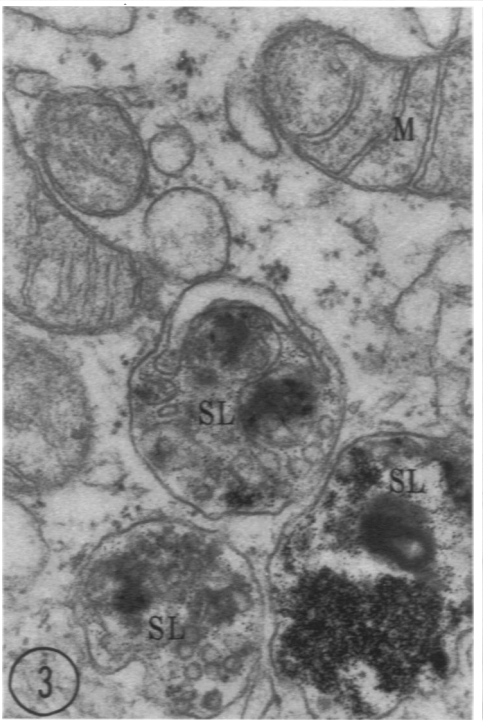
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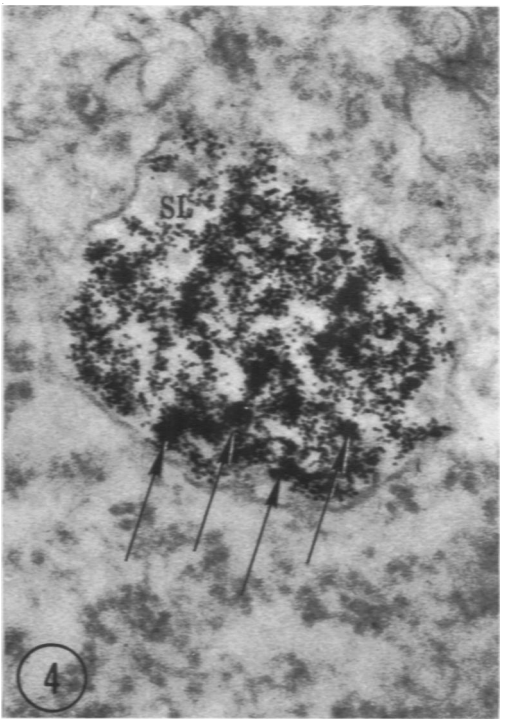
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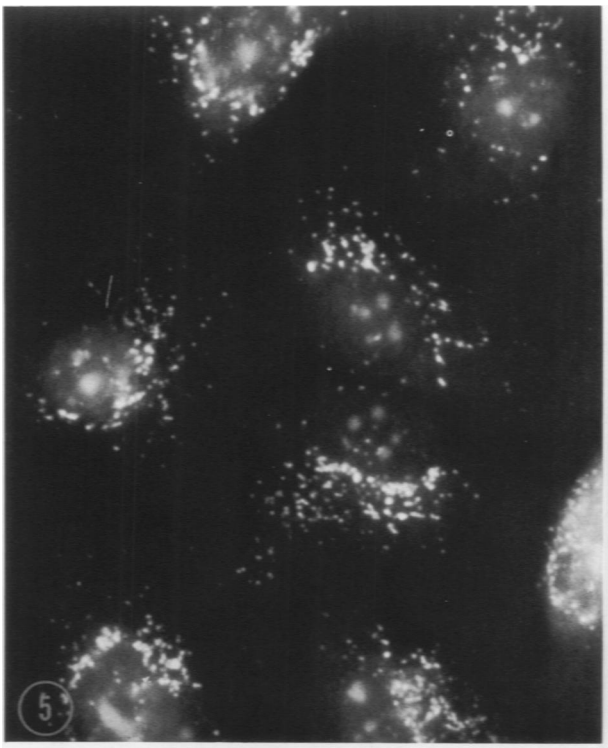

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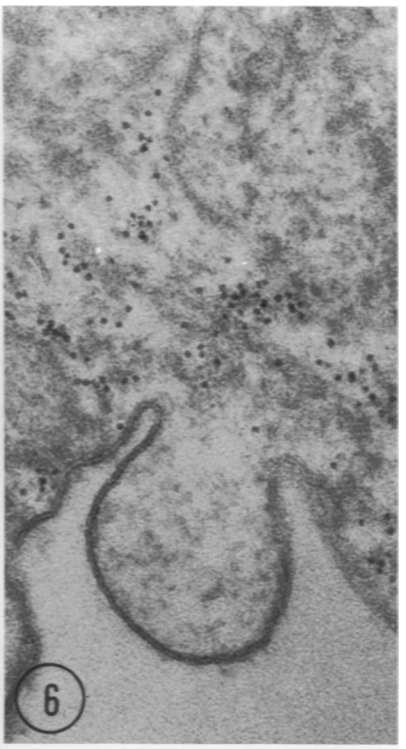

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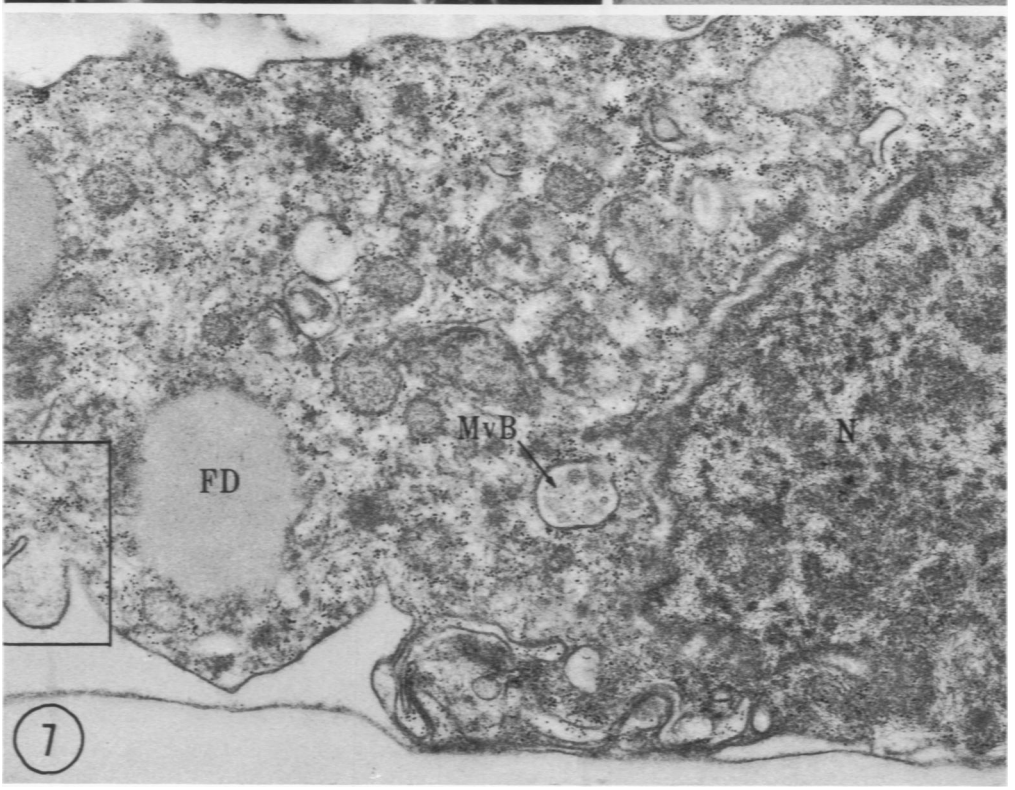

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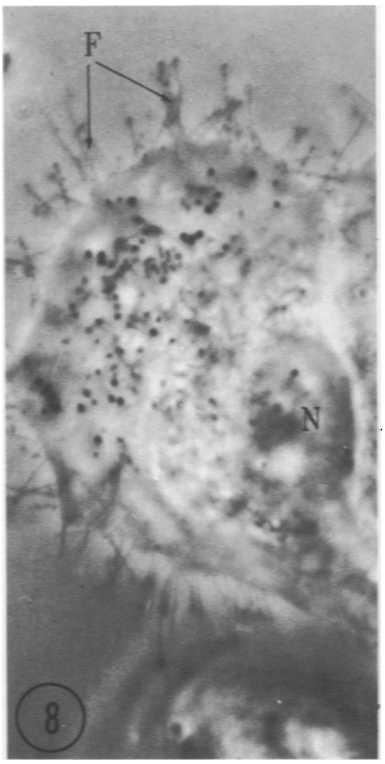
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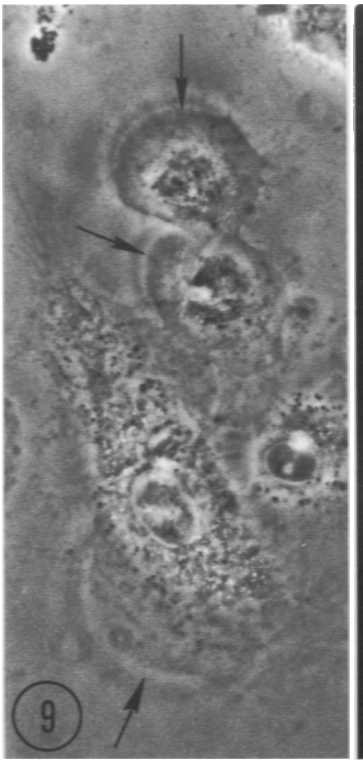
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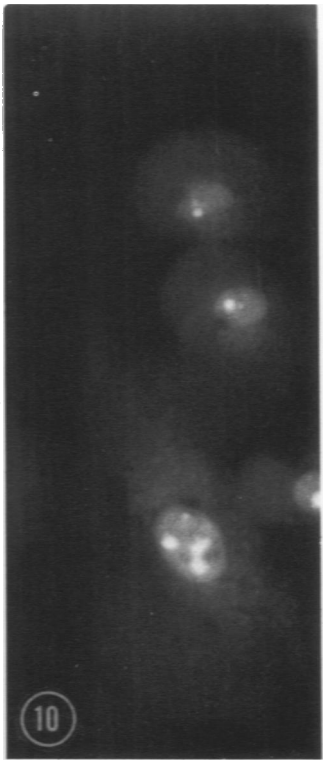
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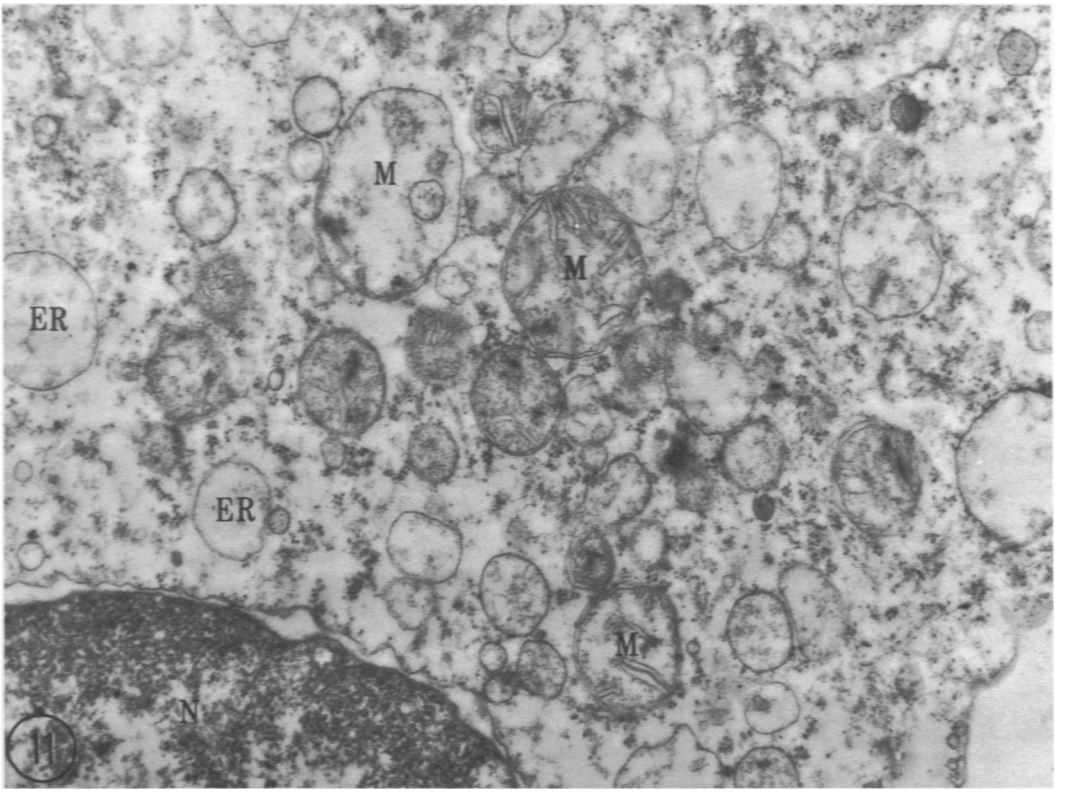
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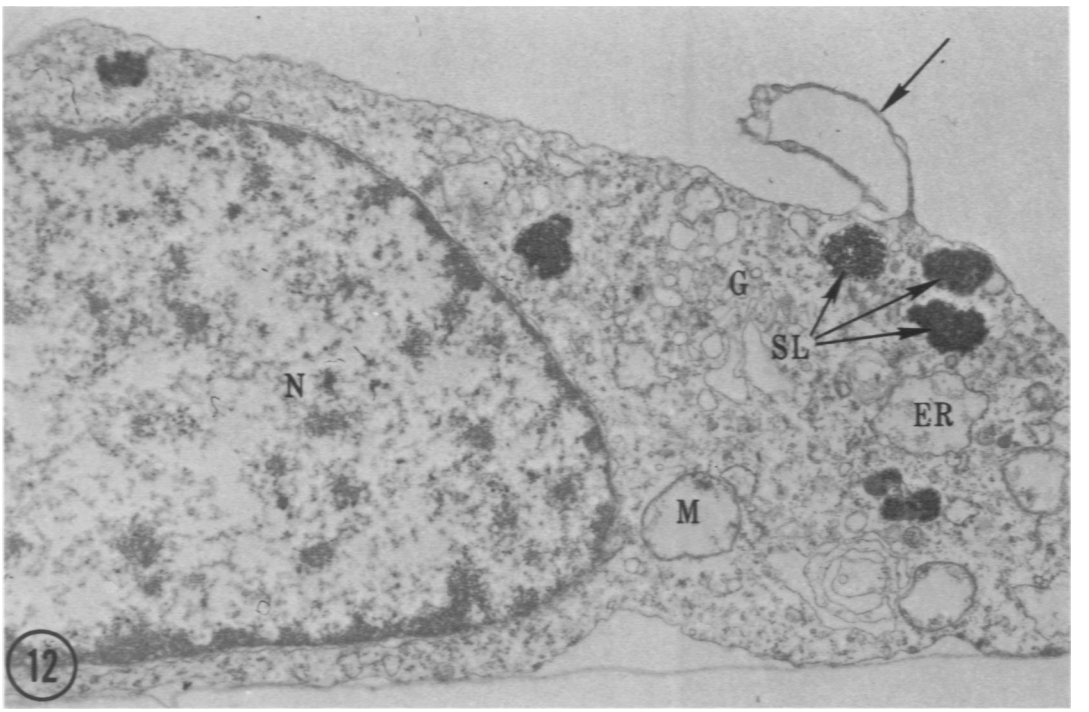

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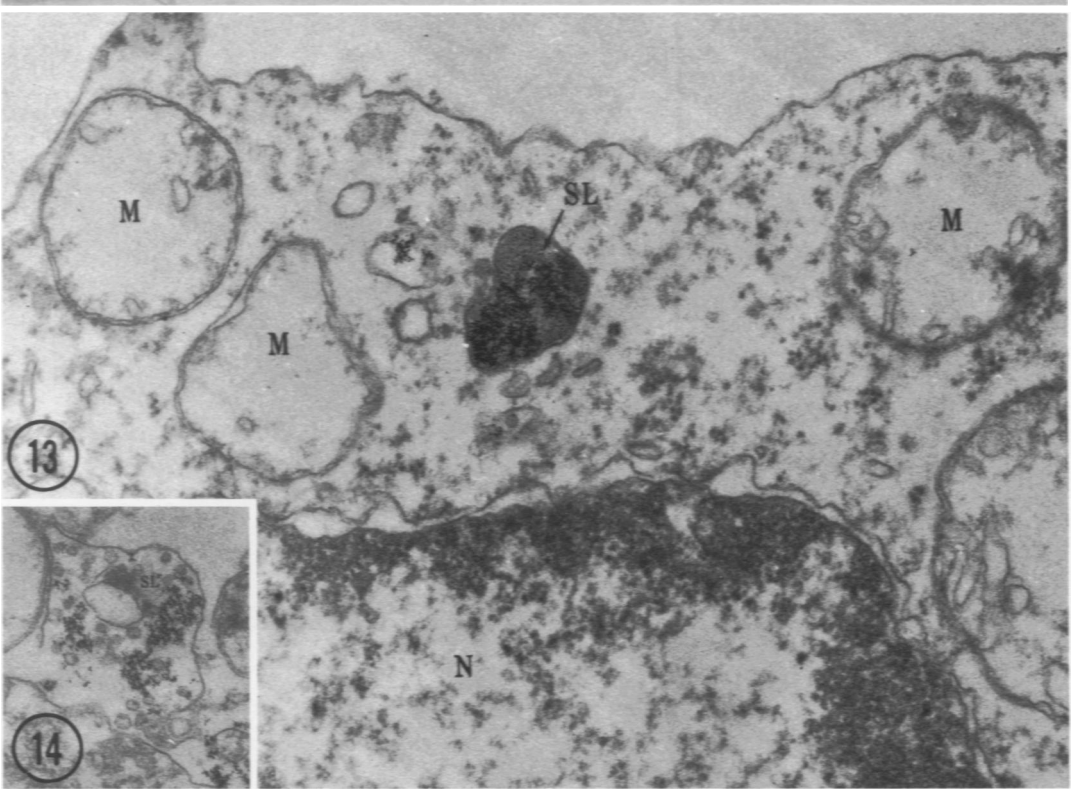

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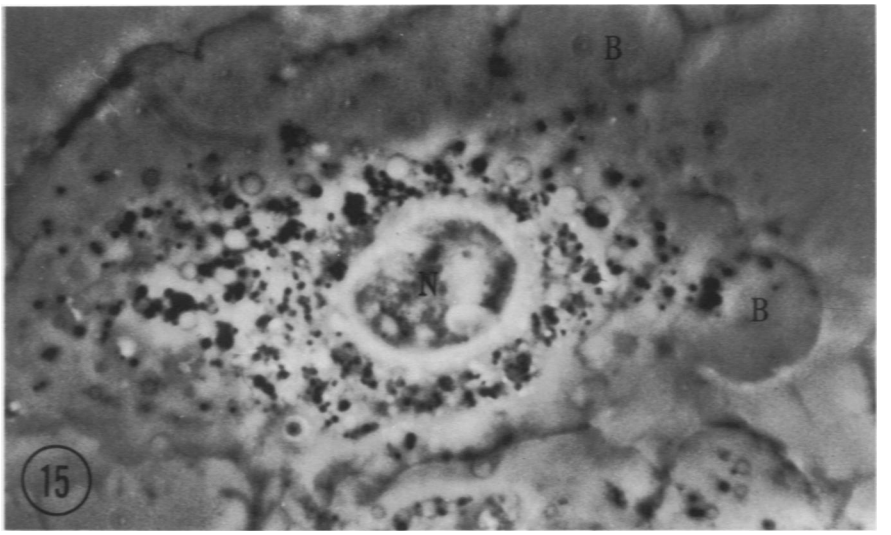
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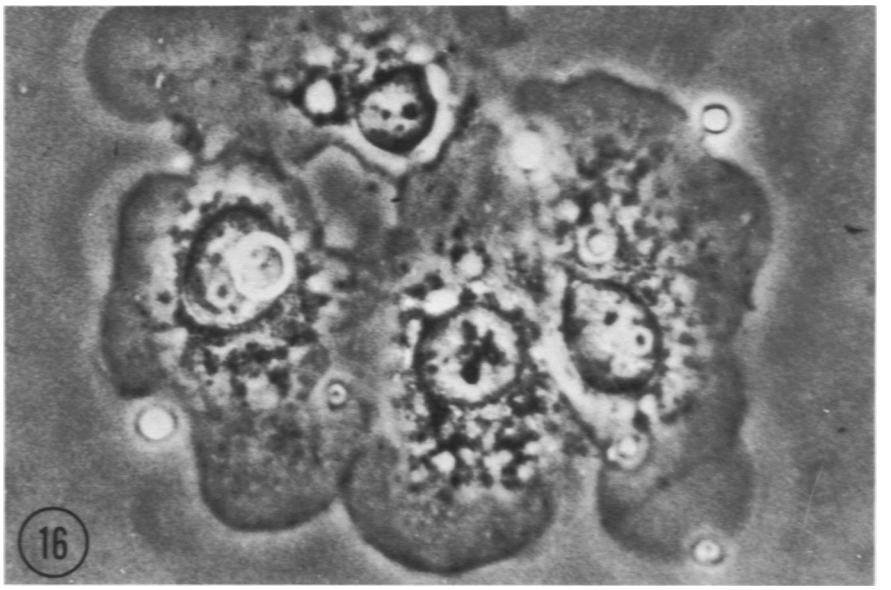
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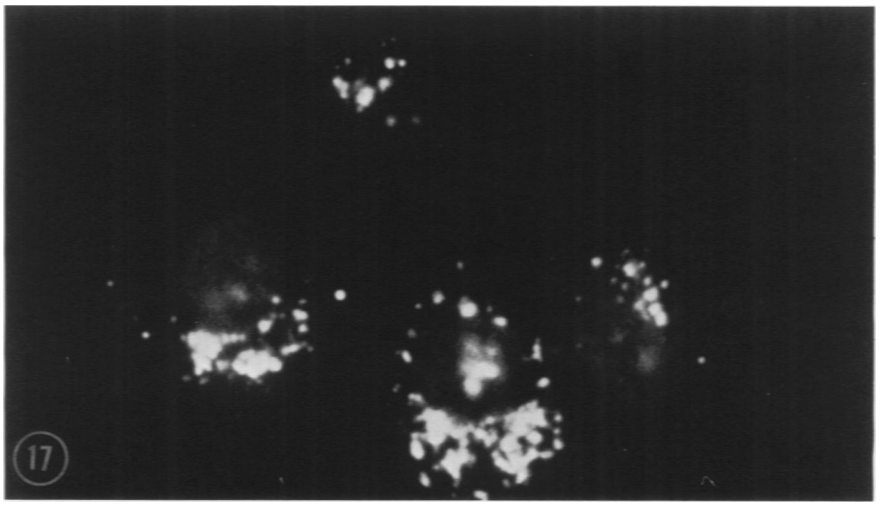
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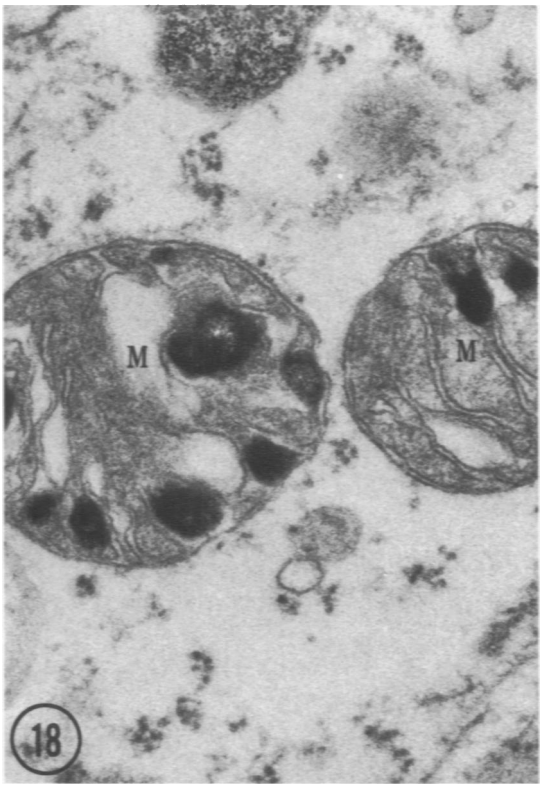

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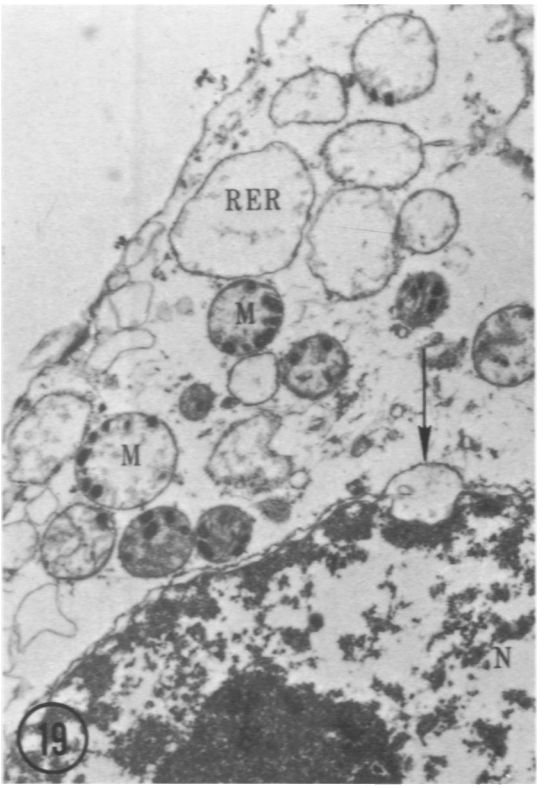

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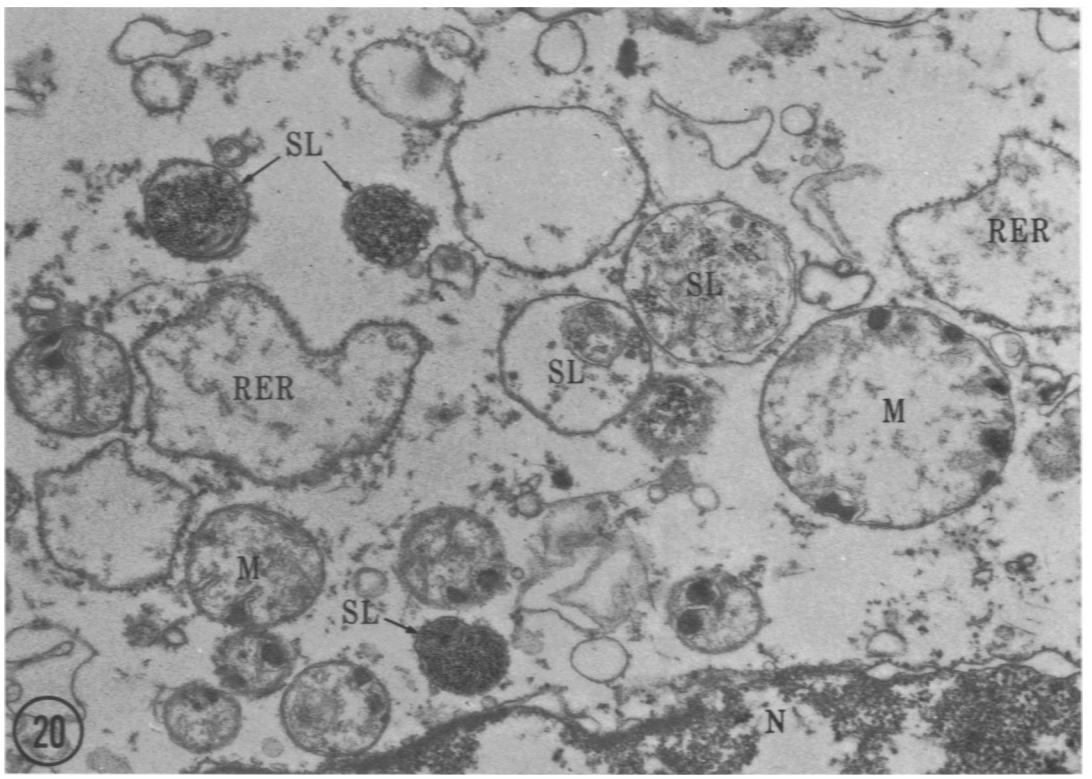

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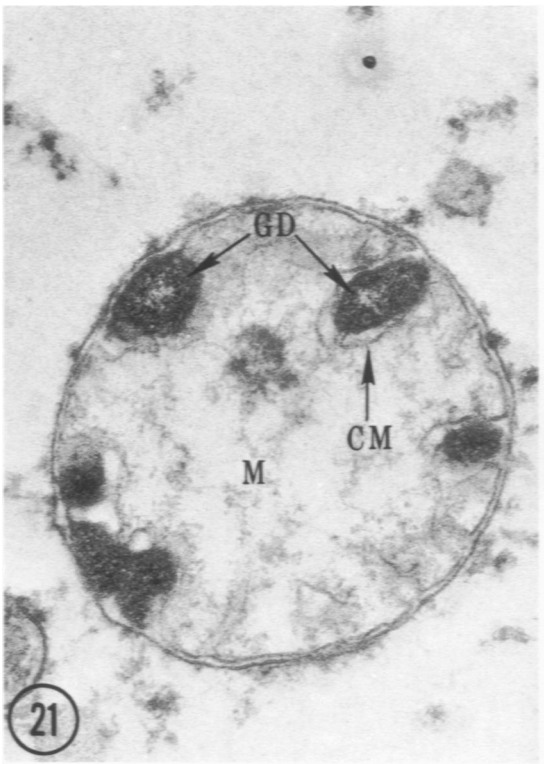
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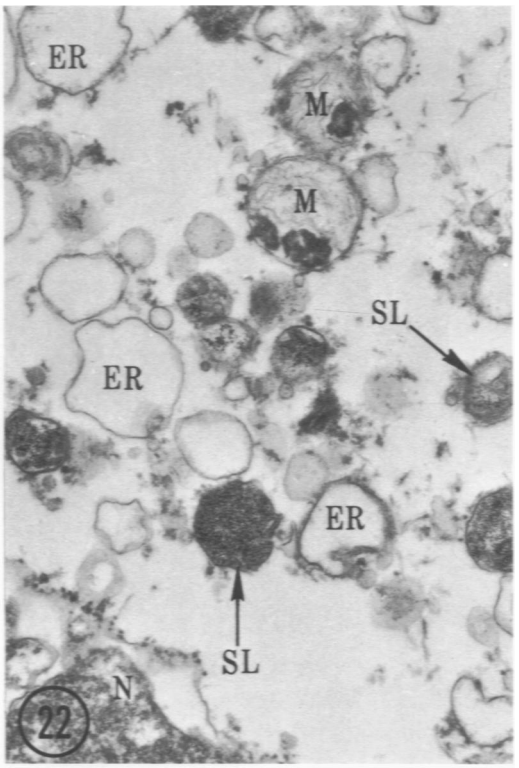
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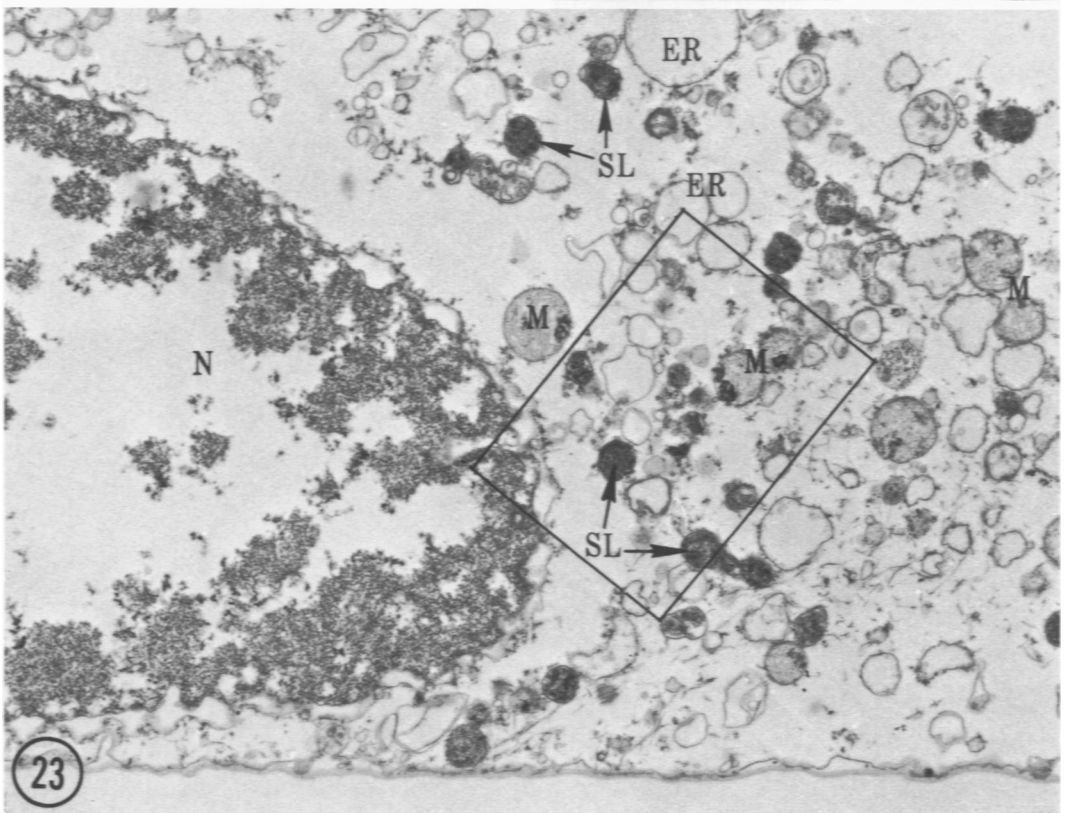
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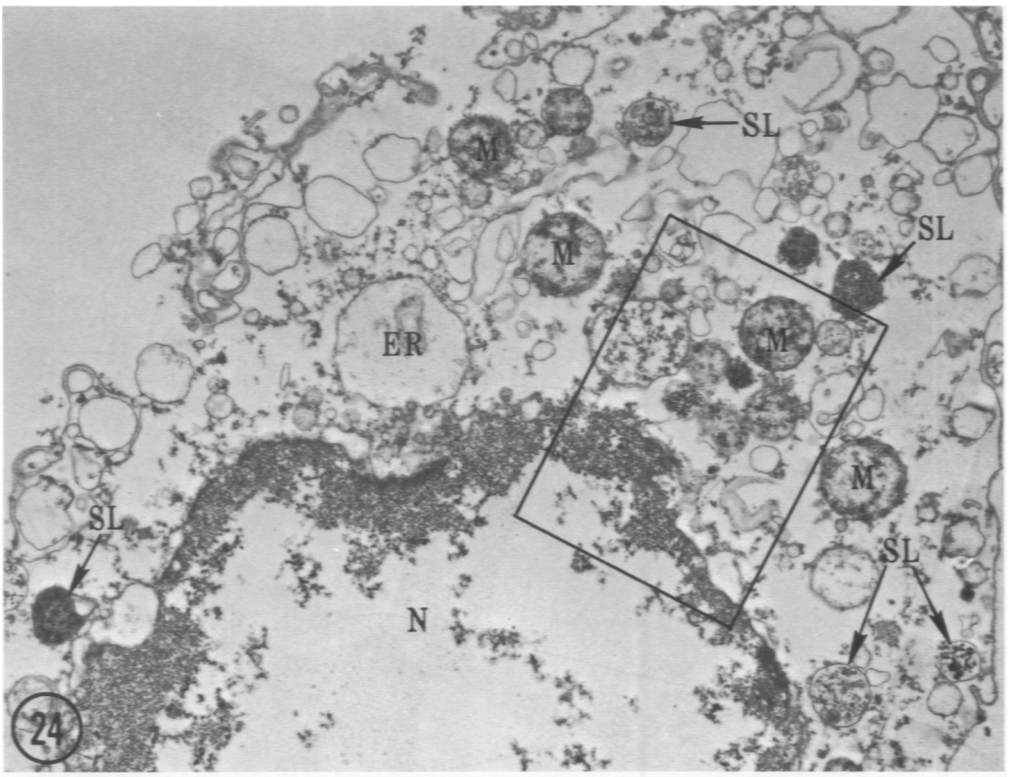
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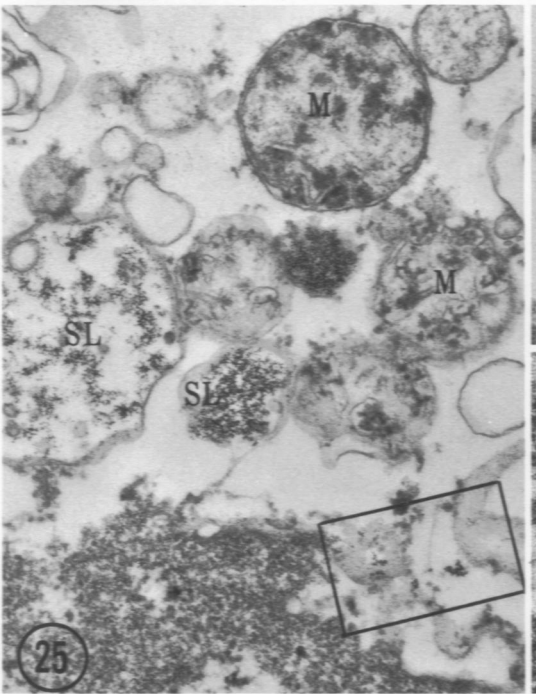
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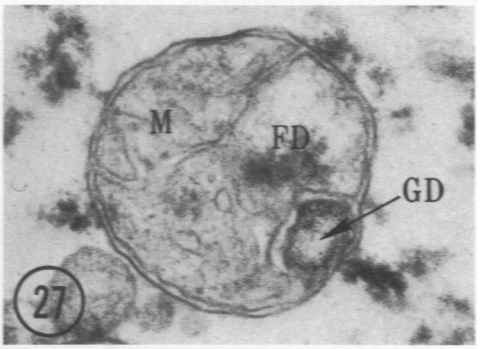
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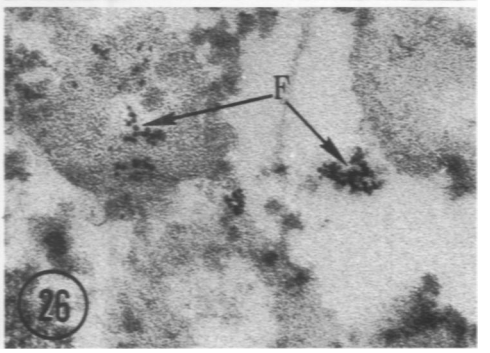
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