EFFECTS OF ARGININE DEPRIVATION, ULTRAVIOLET RADIATION, AND X-RADIATION ON CULTURED KB CELLS

A Cytochemical and Ultrastructural Study

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ABSTRACT

Cultured KB cells (derived from a human oral carcinoma) grown in monolayers were injured by one of three agents: starvation by arginine deprivation or treatment with high doses of either ultraviolet radiation or x-radiation. The different agents produced changes in nucleolar structure and varying accumulations of triglyceride and glycogen. All three agents produced an increase in number and size of lysosomes. These were studied in acid phosphatase preparations, viewed by both light and electron microscopy, and, occasionally, in vital dye, esterase, and aryl sulfatase preparations. Ultrastructurally, alterations in lysosomes suggested that "residual bodies" developed in a variety of ways, *i.e.*, from the endoplasmic reticulum, multivesicular bodies, or autophagic vacuoles. Following all three agents the endoplasmic reticulum assumed the form of "rough" or "smooth" whorls, and, after two of the agents, arginine deprivation or ultraviolet radiation, it acquired cytochemically demonstrable acid phosphatase activity. Near connections between the endoplasmic reticulum and lysosomes raise the possibility that in KB cells, at least when injured, the endoplasmic reticulum is involved in the formation of lysosomes and the transport of acid phosphatase to them.

INTRODUCTION

Strains of cultured cells grown for short periods in nutrient media deficient in a single amino acid remain viable, for restoration to complete medium results in survival of a proportion of the cells (8, 13). Similarly, cells treated with high-level (lethal) doses of radiation remain viable for a certain length of time before the onset of death (33, 57). Such injured cells grown in controlled fashion lend themselves to a study of the events leading to cell death.

This report describes the profound changes in the structural organization and cytochemical properties of KB cells following injury; in two instances (ultraviolet and x-irradiation) the changes are irreversible while in the other (arginine deprivation) they are still reversible. The most dramatic alterations occur in the endoplasmic reticulum and the lysosomes.

MATERIAL AND METHODS

The heteroploid KB line, derived from a human oral carcinoma (12), was used in these investigations. The cells were grown at 37° as monlayers on glass coverslips in Petri dishes containing Eagle's minimum essential medium (14) with added 10 per cent calf serum, non-essential amino acids, glutamine, and antibiotics. Cultures were refed every other day.

Amino acid–deprived cells were grown in a deficient

medium from which arginine, an essential amino acid for KB cells (15), had been omitted. They were placed in this medium after attaining the log phase of growth (1 to 3 days) in complete nutrient medium. Coverslips were removed and the cells studied after intervals of deprivation of 16, 40, 48, and 64 hours, and 3, 4, and 5 days; others, after deprivation for similar time intervals, were restored to complete medium and left in it for periods of 1, 2, 3, 4, 5, or 7 days.

Ultraviolet radiation was generated by a 30-watt germicidal lamp (Sola), backed by a polished reflector and mounted 88 cm from a designated target plane. The cells were subjected to ultraviolet irradiation for

FIGURE 1 Living KB cells, deprived of arginine for 40 hours; phase contrast. Large, multiple nucleoli (*N*) are evident within nuclei. Arrows indicate the spike-like cytoplasmic extensions characteristic of injured cells. × 310.

FIGURE 2 Unfixed cells, deprived of arginine for 16 hours, vitally stained with neutral red. The colored granules are concentrated in the nuclear hof or Golgi area, but some, mostly small, are evident elsewhere in the cytoplasm. × 780.

FIGURE 3 Cells deprived of arginine for 64 hours; acid phosphatase preparation. Fixed in formaldehyde-calcium for 2 hours and incubated with β-glycerophosphate as substrate for 60 minutes. Note concentration of acid phosphatase–positive granules (lysosomes) in nuclear hof or Golgi area; compare with Figs. 2 and 4. Individual lysosomes are evident where they are not too numerous. × 1,000.

FIGURE 4 Unfixed cells, deprived of arginine for 40 hours, vitally colored by acridine orange and viewed in the fluorescence microscope. Note general correspondence in size, distribution, and number between fluorescent cytoplasmic granules and lysosomes (Fig. 3). The large, multiple nucleoli are evident within the nuclei. × 950.

FIGURE 5 Untreated cell; acid phosphatase preparation. Fixed in glutaraldehyde, and incubated with 5′-cytidylic acid as substrate for 20 minutes. Note concentration of lysosomes in nuclear hof or Golgi area. Unstained nucleoli are evident in the nucleus. × 1,400.

FIGURE 6 Cell deprived of arginine for 64 hours; acid phosphatase preparation. Fixation and incubation as in Fig. 5. The increase in lysosome number with deprivation is illustrated by comparing this cell with the untreated cell in Fig. 5. × 1,230.

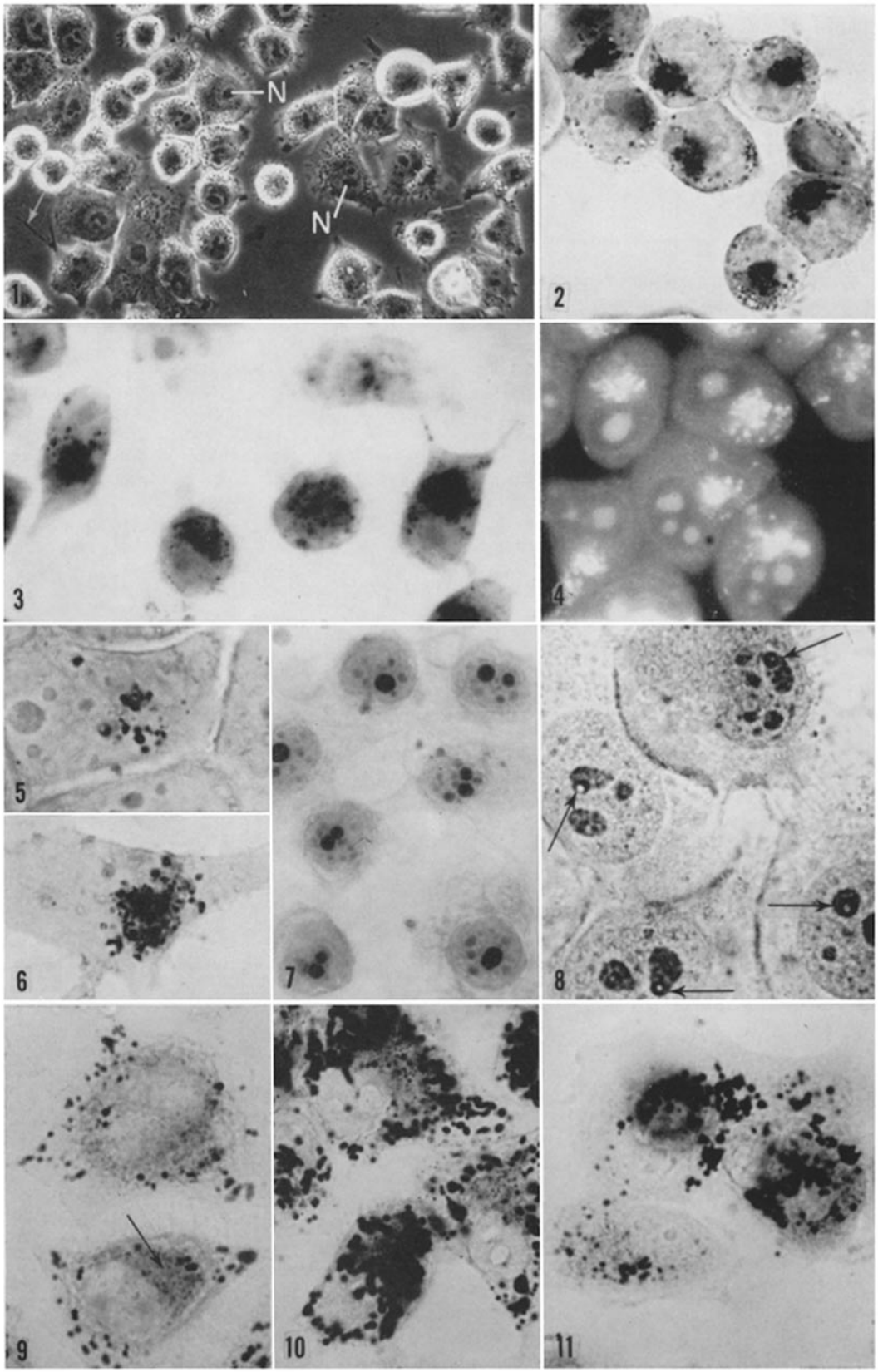
FIGURE 7 Cells 5 hours after ultraviolet irradiation. Fixed in glutaraldehyde; incubated with thiamine pyrophosphate for 2 hours. No enzyme activity is evident. However, as in substrate-free controls, the nucleoli are darkened. × 800.

FIGURE 8 Cells 22 hours after x-irradiation. Fixed in formaldehyde-calcium for 5 minutes; incubated with adenosine triphosphate for 60 minutes. As in Fig. 7, the color in the nucleoli is non-enzymatic and is also present in no-substrate controls. Arrows indicate ring-like structures. × 1,000.

FIGURE 9 Untreated cells; Sudan black B preparation. The dark spheres, mostly peripheral, are presumed to be triglyceride-rich lipid droplets. Note smaller, lighter stainng granules; some are probably mitochondria and some in the nuclear hof area (arrow) are probably lysosomes. × 1,300.

FIGURE 10 Cell deprived of arginine for 40 hours; Sudan black B preparation. Lipid droplets presumably rich in triglyceride have increased greatly (*cf.* Fig. 9). × 760.

FIGURE 11 Cell 23 hours after ultraviolet irradiation; Sudan black B preparation. Their increase in lipid droplets presumed to be rich in triglyceride is less marked than in x-irradiated or arginine-deprived cells (Fig. 10). × 870.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56 seconds, without attenuation by mesh filters. This gave a dose of 790 erg/mm^2 or 7.3 hits per cell, a lethal dosage for 99.9 per cent of a population of HeLa cells (33). During exposure to the ultraviolet radiation the cells were covered by 10 ml of phosphate-buffered saline; after treatment this was replaced by 20 ml of fresh complete nutrient medium. Cells were studied at intervals of 4, 5, 16, 17, and 24 hours after irradiation.

X-radiation was delivered by a Picker instrument operated at 280 kv and 20 mA, at a distance of 55 cm (or 70 cm) with a half value layer of 2.0 mm Cu, at an exposure rate of approximately 97 r (or 47.5 r) per minute. The cells were subjected to an exposure of 1300 r as determined by a Victoreen chamber and electrometer; Tolmach and Marcus (57) have shown that this dosage results in the death of HeLa cells in 2 days (60 to 80 per cent) or 5 to 15 days (20 to 40 per cent). During irradiation the cells were in complete nutrient medium; it was replaced by fresh medium following treatment. Cells were studied at 24 hours, 2-, and 5-day intervals after irradiation.

Normal, uninjured cells, grown in complete medium for 2 to 5 days, were studied as controls in each separate experiment. The experiments on amino acid deprivation were repeated on five separate occasions; the ultraviolet and x-irradiation experiments, on three.

Light Microscopy

Live cell preparations were examined by the phase microscope or colored with the vital dyes neutral red (2 drops of 0.5 per cent aqueous solution in 2 ml medium) and acridine orange (0.2 ml of 1/2000 aqueous solution in 20 ml medium). After staining for 10 minutes, the cells were examined by light microscopy, the acridine orange preparations in a Leitz-Wetzlar ultraviolet fluorescence microscope.

Fixed cell preparations were used for cytochemical studies. Fixation was carried out at room temperature in formaldehyde-calcium (1) for 5 minutes, or in 1.5 to 3 per cent glutaraldehyde (55), in basal nutrient medium or Earle's salt solution, for 1½ to 3 minutes. The coverslips bearing the cells were washed in Earle's balanced salt solution before fixation, in 7.5 per cent sucrose after fixation, and in 7.5 per cent sucrose after incubation. The fixed cells were incubated for the following enzyme activities: (1) acid phosphatase, by a modified Gomori technique (26) with cytidine 5′-monophosphate (40) or thymidine 5′-monophosphate (48) or β-glycerophosphate (26) as substrate, and by the Barka-Anderson procedure (4) with naphthol AS-TR phosphate as substrate; (2) thiolacetic esterase (37); (3) indoxyl esterase (29); (4) DPNH-tetranitro blue tetrazolium reductase (DPNH-TNBT) (See reference 47, substituting

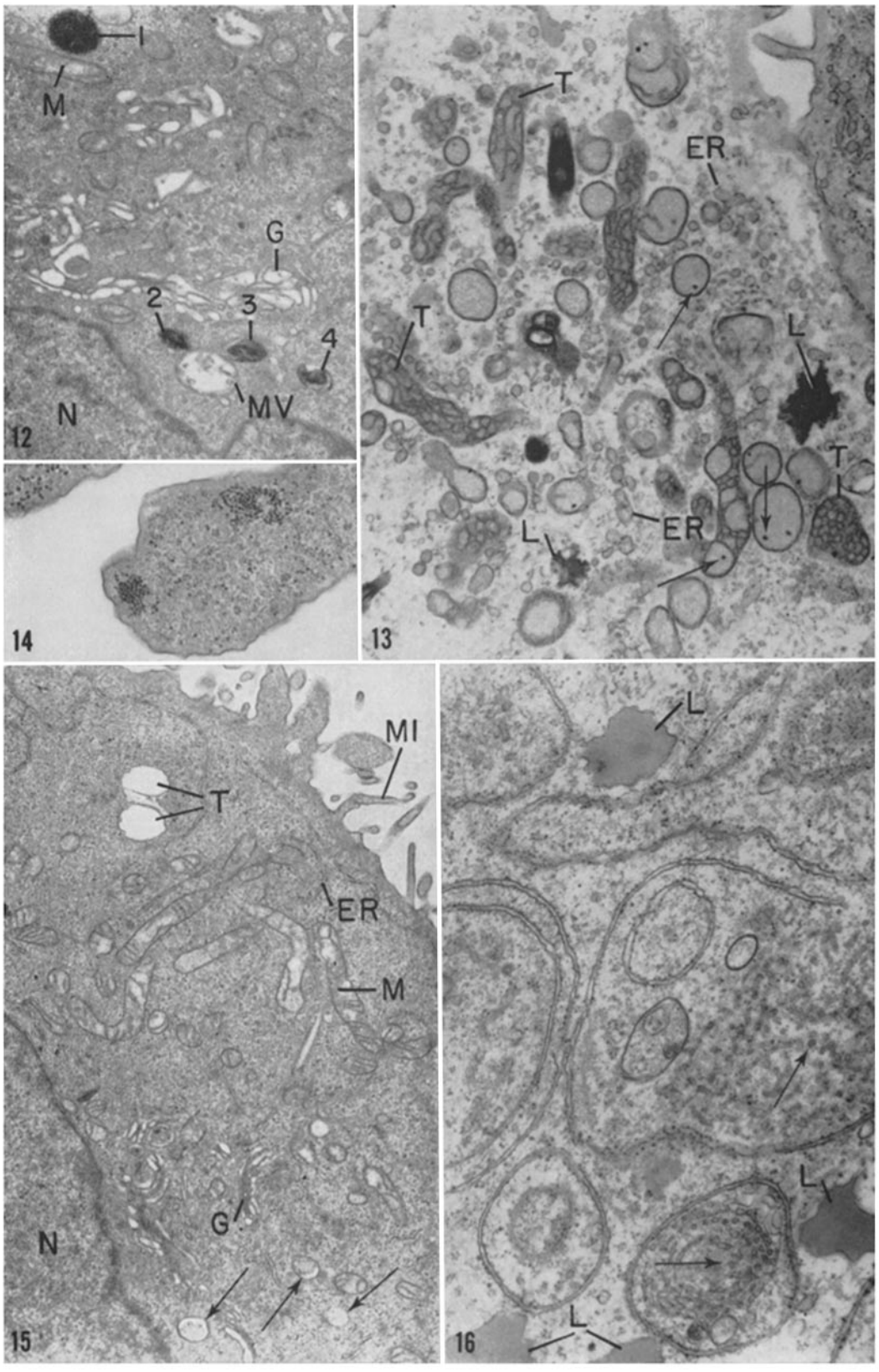
FIGURE 12 Portion of uninjured cell. This region shows Golgi apparatus (*G*); dense bodies, numbered *1* through *4*; and multivesicular bodies (*MV*). Also labeled are nucleus (*N*) and mitochondria (*M*). × 13,000.

FIGURE 13 Portion of cell deprived of arginine for 64 hours. The mitochondria show: (1) tubule-like structures (*T*), seen in both transverse and longitudinal section; and (2) homogeneous, relatively structureless areas in which are seen granules (arrows) that may be the original intramitochondrial granules. The vesiculated endoplasmic reticulum, *ER*; lipid, probably rich in triglyceride, *L*, are seen. × 18,000.

FIGURE 14 Peripheral cytoplasm (upper left) and bleb from cell deprived of arginine for 5 days and restored to complete medium for 7 days. The glycogen is present as individual granules and clumps; two clumps are visible. × 15,000.

FIGURE 15 Portion of uninjured cell. The Golgi apparatus (*G*) is extensive; elongate mitochondria (*M*) are numerous; and multivesicular bodies (arrows) are concentrated in the vicinity of the Golgi apparatus. The endoplasmic reticulum (*ER*) is sparse. The clear areas (*T*) have the size and form of Sudan black B–positive spheres (*cf.* Fig. 9), and it is presumed that they were occupied *in vivo* by triglyceride-rich lipid that was extracted during processing for electron microscopy. The edge of the nucleus is seen at *N* and some microvilli at *MI*. × 9,000.

FIGURE 16 Cell deprived of arginine for 16 hours and restored to complete medium for 72 hours. The endoplasmic reticulum (note ribosomes on its surface) is in the form of whorls and vesicles (arrows). *L* indicates material presumed to be triglyceride-rich material that has crenated during the processing for electron microscopy. × 14,000.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TNBT for NBT); (5) thiamine pyrophosphatase (TPPase) or nucleoside diphosphatase with inosine diphosphate as substrate (42); (6) nucleoside triphosphatase with adenosine triphosphate as substrate (58); and (7) aryl sulphatase at pH 5.7 (24).

Sudan black B (3) was used to color lipids, especially those rich in triglycerides, and the acid hematein test with pyridine extraction control, to visualize phospholipids (2). These tests were made on cells fixed in formaldehyde-calcium for 5 minutes at 4° or room temperature.

Electron Microscopy

Cells were grown on coverslips previously coated with a thin layer of carbon (50). They were fixed by one of the solutions described by Robbins and Gonatas (51) for cultured HeLa cells. Best results were obtained with 2 per cent aqueous osmium tetroxide diluted with an equal volume of Earle's balanced salt solution, (1:1), for 20 minutes at room temperature, followed by 60 minutes' fixation in a solution composed of 10.6 ml 2 per cent osmium tetroxide, 0.6 ml 40 per cent formaldehyde, and 10 ml 0.15 M $NaHCO_3$, 0.15 M NaCl (1:3). Dehydration with Cellosolve (ethylene glycol mono-ethyl ether) was followed by embedding in Epon 812, Araldite, or Maraglas. For embedding, the coverslip, cells upward, was placed on a glass slide and covered with enough embedding medium to keep it in place. Gelatin capsules filled with embedding medium were then placed, open end down, over the cell monolayers (31, 50). When hardened, the capsules were snapped off at the carbon layer–coverslip interface; the cells remained on the flat block surface, ready for sectioning.

For demonstrating acid phosphatase activity, coverslips were treated as described for light microscopy, with 5′-cytidylic acid as substrate and with 5 per cent sucrose present in the medium. The incubation time varied from 15 to 120 minutes, depending on the staining intensity as monitored by the light microscope. Following incubation, the cells were washed in 7.5 per cent sucrose and postfixed and embedded as described above for unincubated cells. As controls, cells were incubated either in the acid phosphatase medium without substrate or in complete medium containing 0.01 M sodium fluoride.

Thin sections were cut on a Porter-Blum MT-2 ultramicrotome and were studied unstained or following staining with ethanolic uranyl acetate (60), lead citrate (49), or both. They were examined in a Siemens Elmiskop I using the double condenser and operated at 80 kv.

OBSERVATIONS

Uninjured (Control) KB Cells

In normal KB cells spread in monolayers, cell inclusions are easily seen by phase contrast microscopy (Fig. 1). These include nuclei, nucleoli, mitochondria, granules that are probably lysosomes, and triglyceride-rich lipid droplets.

Following addition of the vital dyes, neutral red or acridine orange, color appears in granules; these are concentrated in the nuclear hof or Golgi

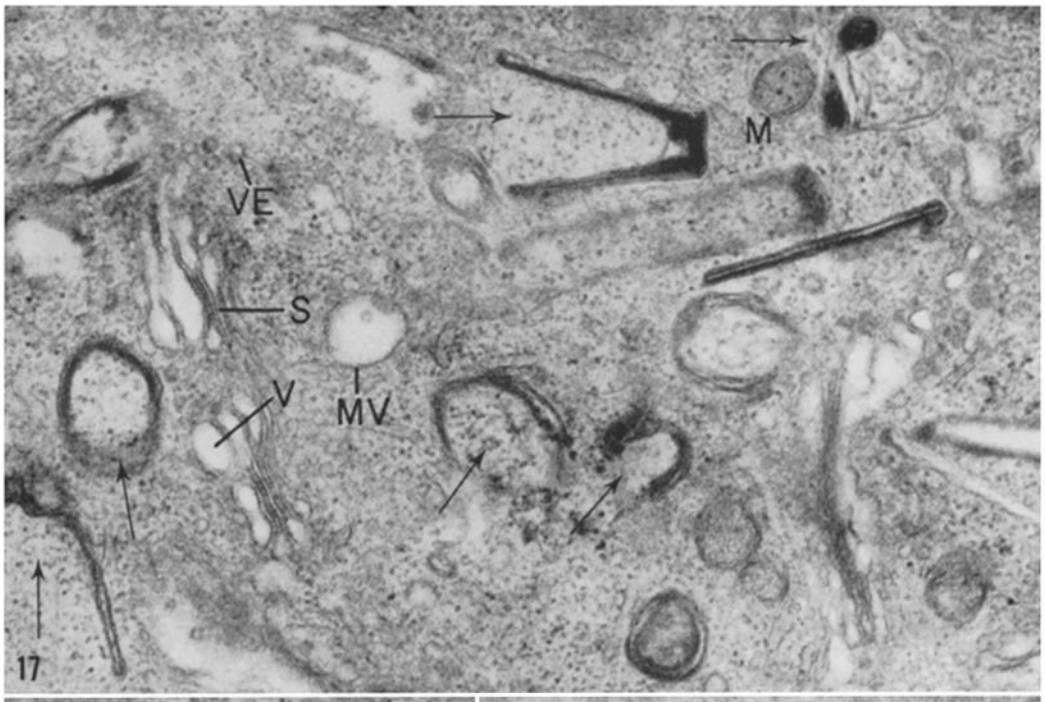
FIGURE 17 Portion of cell deprived of arginine for 16 hours. Arrows indicate accumulations of electron-opaque material in smooth membranous structures, possibly the endoplasmic reticulum. The shapes suggest that these bodies may be forming lamellated dense bodies (Figs. 18, 19). Also seen are components of the Golgi apparatus: saccules (*S*), vacuoles (*V*) and small vesicles (*VE*); a multivesicular body (*MV*); and a mitochondrion (*M*). × 33,000.

FIGURE 18 Portion of cell deprived of arginine for 5 days. The increased electron opacity and their shapes suggest a later stage in the development of lamellated dense bodies (*cf.* Fig. 17). Note mitochondria (*M*) and helical aggregates of ribosomes (arrow). × 24,000.

FIGURE 19 Portion of cell deprived of arginine for 5 days. Note numerous lamellated dense bodies, Golgi apparatus (*G*), and multivesicular body (*MV*). × 19,000.

FIGURE 20 Small portion of cell deprived of arginine for 5 days. The lamellated dense body shows parallel membrane arrays. × 45,000.

FIGURE 21 Portion of cell deprived of arginine for 5 days. The cytoplasm is more electron-opaque than normal and is highly vacuolated. Two of the vacuoles (arrows) contain structures that appear to be altered lamellated dense bodies. × 15,000.



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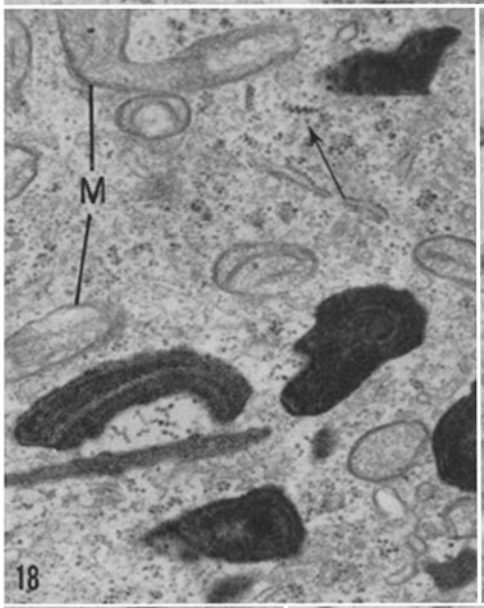

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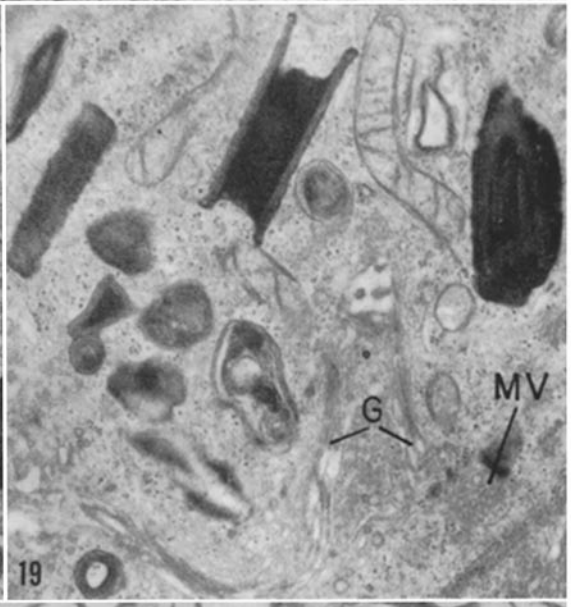

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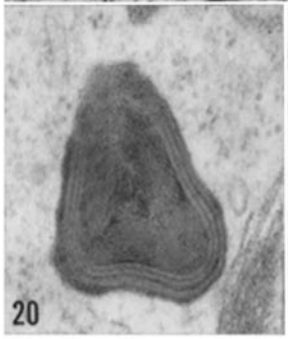

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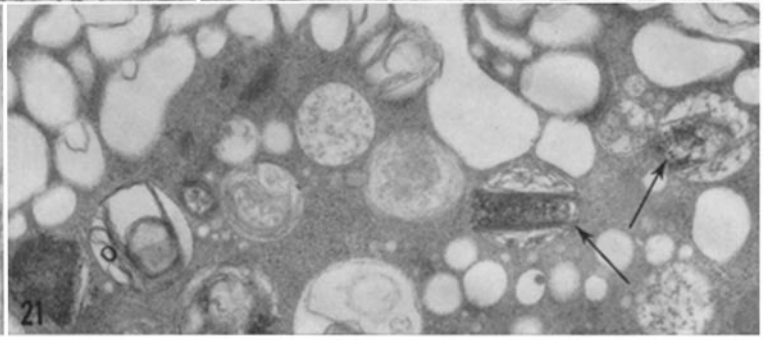

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region (Figs. 2 and 4). They are probably lysosomes since granules of similar size and distribution are seen in fixed sections incubated for acid phosphatase activity (Figs. 3, 5, and 6), the "marker" used for lysosomes (*cf.* reference 41); in general, the Barka procedure gave the same results as the Gomori technique. Bodies like them are also seen in esterase and aryl sulphatase preparations, and in Sudan black B or acid hematein preparations (Fig. 9). (Sudan black B stains triglyceride droplets more intensely.) In these cells it is not possible, by light microscopy, to attack the problem of biochemical heterogeneity among lysosomes.

At the level of light microscopy no reaction product is seen when cells are incubated to demonstrate nucleoside diphosphatase or thiamine pyrophosphatase activity under our conditions. These preparations were not studied by electron microscopy.

DPNH-TNBT reductase preparations show many filamentous and granular mitochondria throughout the cytoplasm. They are sometimes visible after Sudan black B staining (Fig. 9) and, infrequently, after incubation for nucleoside triphosphatase activity, as described by Essner *et al.* (18).

Non-enzymatic staining of nucleoli occurs when KB cells are incubated for thiamine pyrophosphatase and nucleoside tri- and diphosphatase activities. This enhances the visibility of some aspects of their structure (Figs. 7 and 8).

There is relatively little endoplasmic reticulum in control cells (Fig. 15). Using the presence of acid phosphatase activity and a delimiting membrane as a tentative identification of lysosomes (39, 40), these are of two types in KB cells: multivesicular bodies and dense bodies of moderate electron opacity (Figs. 12 and 15). They are concentrated in the nuclear hof area where the Golgi apparatus is located.

Other structures seen in electron micrographs include: (1) mitochondria, spherical or elongated in thin sections, with cristae sometimes extending across their width (Figs. 12 and 15); (2) ribosomes, singly or in rosettes, spirals, or helices[1], generally considered to be polysomes; some are attached to the endoplasmic reticulum but most seem to lie in the cytoplasmic matrix; (3) microtubules, about 23 mμ in diameter, more numerous near the cell periphery, and preserved rather well by osmium

[1] Helical aggregates of ribosomes have previously been reported only in differentiating plant cells (16) and embryonic animal cells (5, 59).

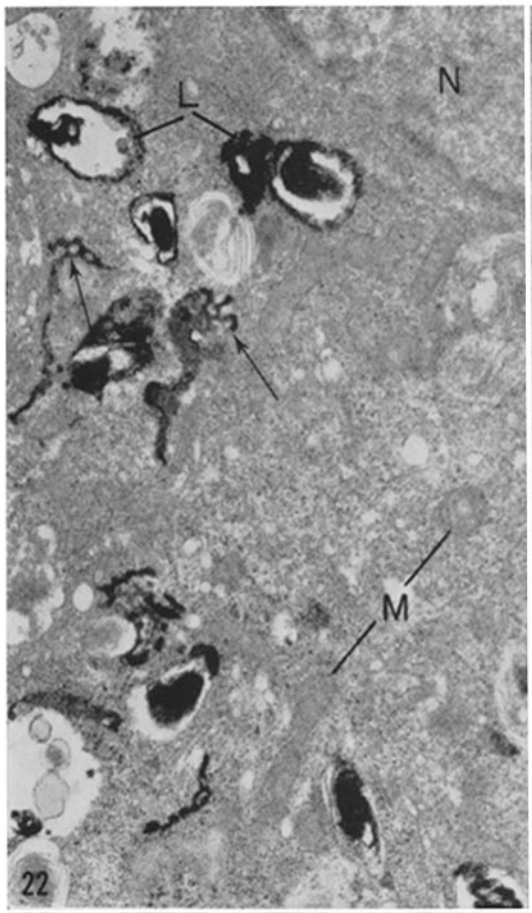
FIGURE 22 Portion of cell deprived of arginine for 64 hours; incubated for acid phosphatase activity, with 5′-cytidylic acid as substrate. In unincubated material such cells have numerous lamellated dense bodies (Figs. 17 to 19). In this incubated preparation the bodies are moderately well preserved and show abundant reaction product (*L*). Reaction product is also seen in Golgi saccules with fenestrated regions (arrows). The nucleus (*N*) and mitochondria (*M*) are free of reaction product. × 18,000.

FIGURE 23 Portion of cell deprived of arginine for 5 days. At arrow, one of the lamellated dense bodies shows a near connection to a region of smooth endoplasmic reticulum containing electron-opaque material. Note ribosomes on endoplasmic reticulum farther removed from lamellated dense body. A small portion of the nucleus is seen at *N*. × 25,000.

FIGURE 24 Portion of cell deprived of arginine for 5 days. A vacuole which resembles an autophagic vacuole, with electron-opaque material within it, is seen at *AV*; and a helical aggregate of ribosomes at *P*. × 30,000.

FIGURE 25 Portion of cell deprived of arginine for 17 hours. Fixed in glutaraldehyde and incubated for acid phosphatase activity with 5′-cytidylic acid as substrate; thin section not stained with heavy metal. Reaction product is present in the whorls of endoplasmic reticulum (*ER*) and in lamellated dense bodies (*L*). The reaction product of the latter appears continuous, at arrows, with that of the endoplasmic reticulum. × 22,000.

FIGURE 26 Portion of cell deprived of arginine for 16 hours. Fixation and incubation as in Fig. 25. Note reaction product in lysosomes containing membranous arrays. × 25,000.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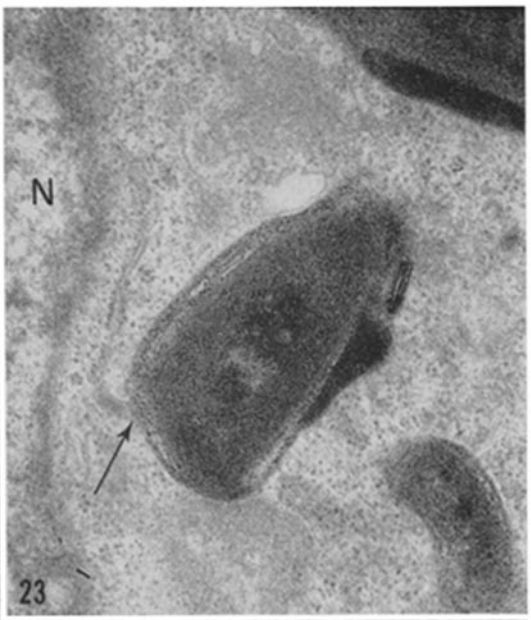
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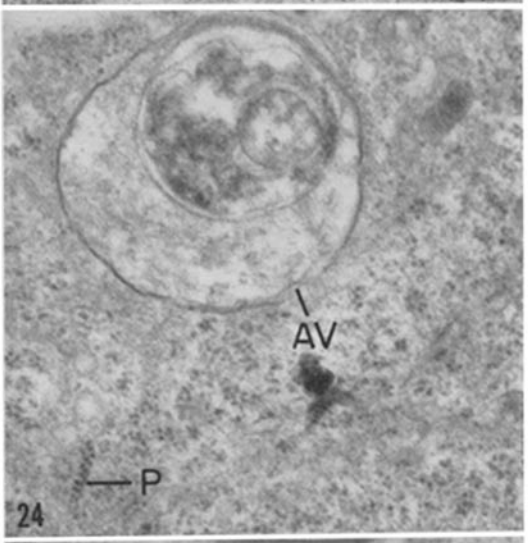
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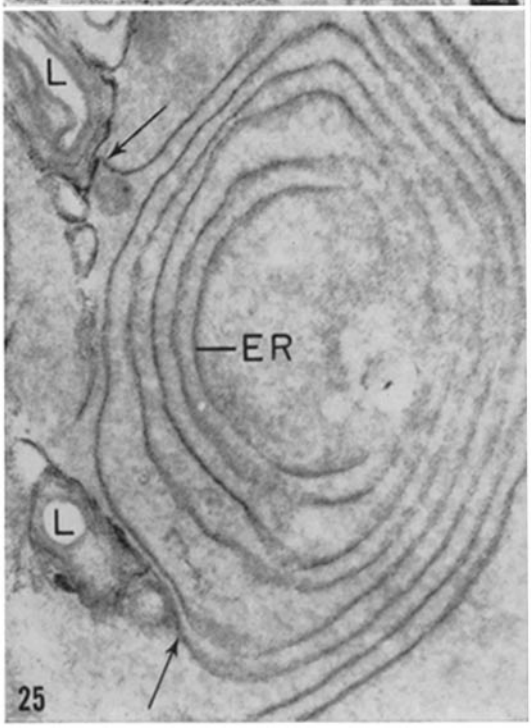
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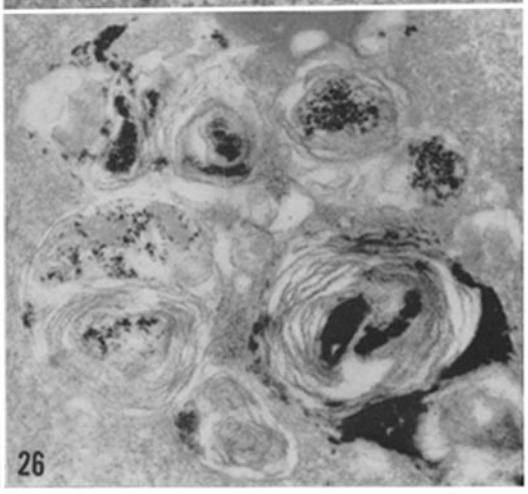
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tetroxide fixation; (4) microvilli at the surface (Fig. 15); (5) desmosomes between adjacent cells; (6) annulate lamellae, sometimes clearly seen as continuous with the endoplasmic reticulum (Fig. 27); and infrequently (7) centrioles (Fig. 38).

Injured KB Cells

After all three agents employed, variable numbers of cells round up and show blebbing; many produce cytoplasmic strands extending from the surface (Fig. 1). Considerable variety is seen among individual cells. We will attempt to describe the more typical changes.

I. Arginine Deprivation

Damage to the cells increases with increasing deprivation time. Sudanophilic spheres, presumably triglyceride-rich lipid, appear early and accumulate in considerable numbers (Fig. 10). Mitochondria may appear normal in many cells even after 5 days of deprivation, but in some cells profound alterations are apparent after 64 hours. An uncommon finding is the presence of tubule-like structures which course in roughly parallel fashion throughout the length of the mitochondria (Fig. 13).

Helical and spiral ribosomal aggregates are found after even a 5-day period of deprivation (Figs. 18, 24). Their persistence is interesting in light of reports, from electron microscopy (23, 54) and biochemical assay (34), that polysomes disappear following amino acid deprivation or vitamin deficiency.

In early stages of degeneration, the nucleoli seem little changed in morphology except that in some cells they show electron-opaque "caps" at their periphery. With more advanced degeneration, nucleoli and nuclei show radically altered morphology. Microtubules are seen except in badly damaged cells.

The endoplasmic reticulum becomes much more extensive and commonly forms whorls (Figs. 16, 25). Whether it is straight or in whorls, the endoplasmic reticulum is often vesiculated. The influence of different fixatives upon these appearances was not studied (*cf.* reference 53).

In the early stages of injury, the endoplasmic reticulum (including the nuclear envelope) shows reaction product resulting from acid phosphatase activity (Figs. 25, 29)[2], but this is rarely seen beyond 16 hours. Acid phosphatase reaction product is also seen in one or more of the Golgi saccules and in cytoplasmic bodies of characteristic shape (Fig. 22). These bodies, which we speak of as lamellated dense bodies, in unincubated cells show clear delimiting membranes and numerous membranous lamellae inside (Fig. 20). They appear in section as rings and shapes resembling wishbones (Fig. 17); their three-dimensional form is probably like that of water tumblers. They increase in size and electron opacity with increasing deprivation time, to form rectangular and triangular bodies (Figs 18 to 20). Presumably many of the numerous larger lysosomes seen by light microscopy (Fig. 6) are of this type.

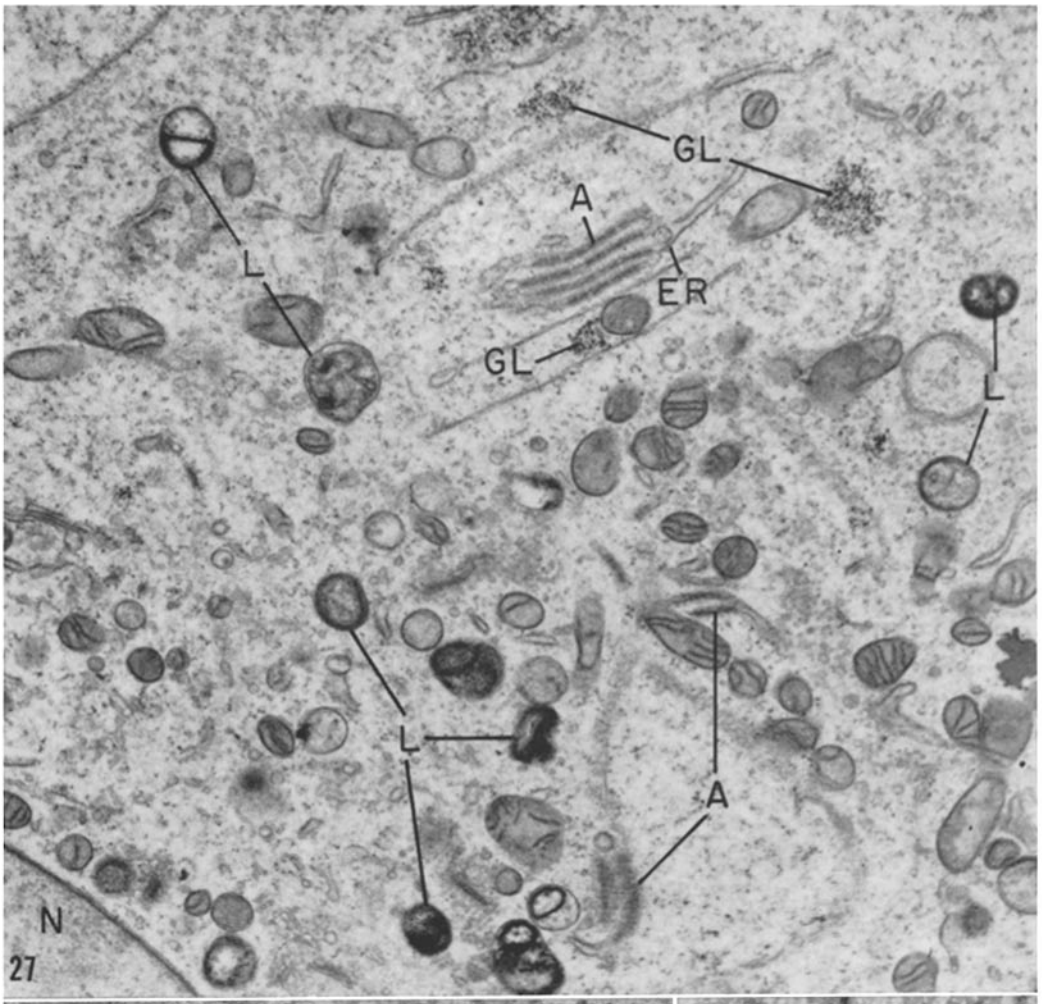
The lamellated dense bodies often lie close to the endoplasmic reticulum, and near connections between the two have been noted on several oc-

[2] Cells injured by all three agents show no reaction product in the endoplasmic reticulum, Golgi saccules, or lysosomes when incubated in substrate-free or fluoride-containing media (Fig. 37).

FIGURE 27 Portion of cell deprived of arginine for 16 hours and restored to complete medium for 48 hours. Note annulate lamellae (*A*) including one that shows continuity with the endoplasmic reticulum, at *ER*; aggregations of glycogen particles (*GL*); and a variety of lysosomes (*L*). A small portion of the nucleus is seen at *N*. × 12,000.

FIGURE 28 Portion of cell deprived of arginine for 64 hours and restored to complete medium for 24 hours. Lysosomes contain arrays of membranes which showed a "unit-membrane" structure in the original photograph, at arrows. × 20,000.

FIGURE 29 Portion of cell deprived of arginine for 17 hours. Fixation in glutaraldehyde and incubation for acid phosphatase activity with 5′-cytidylic acid as substrate. Reaction product is present in nuclear envelope (*NE*), endoplasmic reticulum (*ER*), and outer part of lamellated dense body (*L*). Arrows indicate proximity of the endoplasmic reticulum and lamellated dense bodies. × 27,500.



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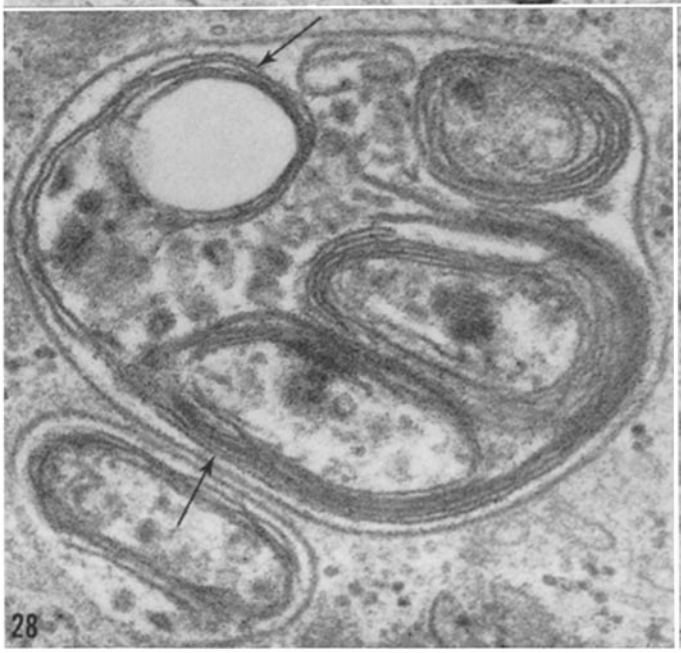
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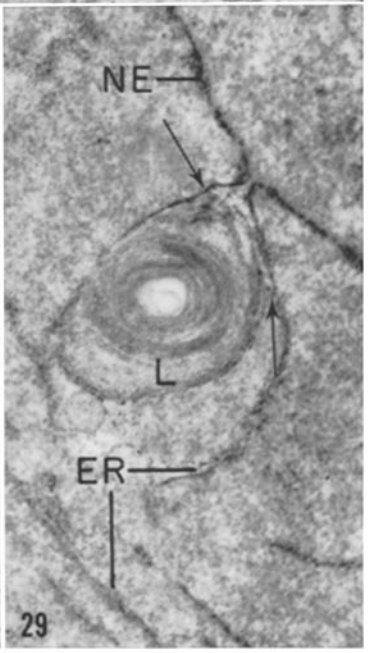

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casions (Fig. 23). In incubated sections the rcaetion product in endoplasmic reticulum strand and lamellated dense body may form a continuous layer (Figs. 25, 29).

Bodies resembling autophagic vacuoles are also seen in unincubated sections. Some seem to be early stages in which cytoplasm is sequestered within a membrane. The origin of this membrane is not established by our observations, but appearances are encountered which are consistent with its origin from the endoplasmic reticulum (Fig. 31), as suggested for rat hepatocytes (43). Membranous arrays and other electron-opaque materials characteristic of "residual bodies," to be considered in the Discussion, are encountered in these sequestered areas (Fig. 24). Such residual bodies have demonstrable acid phosphatase activity (Fig. 26); lamellated dense bodies are encountered in vacuoles in later stages of degeneration (Fig. 21), but whether these are autophagic vacuoles is unknown.

II. Restoration of Arginine-Deprived Cells

When arginine-deprived cells are restored to complete nutrient medium, many recover. After 2 days of deprivation, about 90 per cent appear injured. If at that time they are restored to complete medium, at the end of 7 days only 10 per cent of the cells on the coverslips show signs of injury. However, if cells are first deprived for 3 to 4 days, up to 40 to 50 per cent of the cells on the coverslip are still injured even after restoration for 7 days.

Even when cells are deprived for only short periods before restoration, more than the usual number of annulate lamellae and glycogen clumps are found (Figs. 14, 27). Glycogen clumps are not seen in cells that have not been deprived and then restored to complete medium. In cells deprived for extended periods (5 days) before restoration, the glycogen becomes even more prominent and is no longer confined to focal areas.

Lysosomes are present in the restored cells, apparently as numerous as in deprived cells. They generally contain membranous arrays of various forms (Figs. 27 and 28). The endoplasmic reticulum may remain in whorls, at least for 3 days after restoration.

III. Ultraviolet Irradiation

The ultraviolet dosage used produces more rapid change than arginine deprivation does. By

FIGURE 30 Portion of cell 24 hours after ultraviolet irradiation. The following structure are labeled: nucleus, *N*; Golgi apparatus, *G*; vesiculated endoplasmic reticulum (*ER*); and lysosome-like granules with inner membranes (arrows). Small electron-opaque structures may be sections through forming lysosomes. × 21,000.

FIGURE 31 Portion of cell 4 hours after ultraviolet irradiation. The endoplasmic reticulum (*ER*) is more abundant than in untreated cells; it is vesiculated in regions (*V*); and it appears (at arrows) to be isolating regions of cytoplasm. × 8,000.

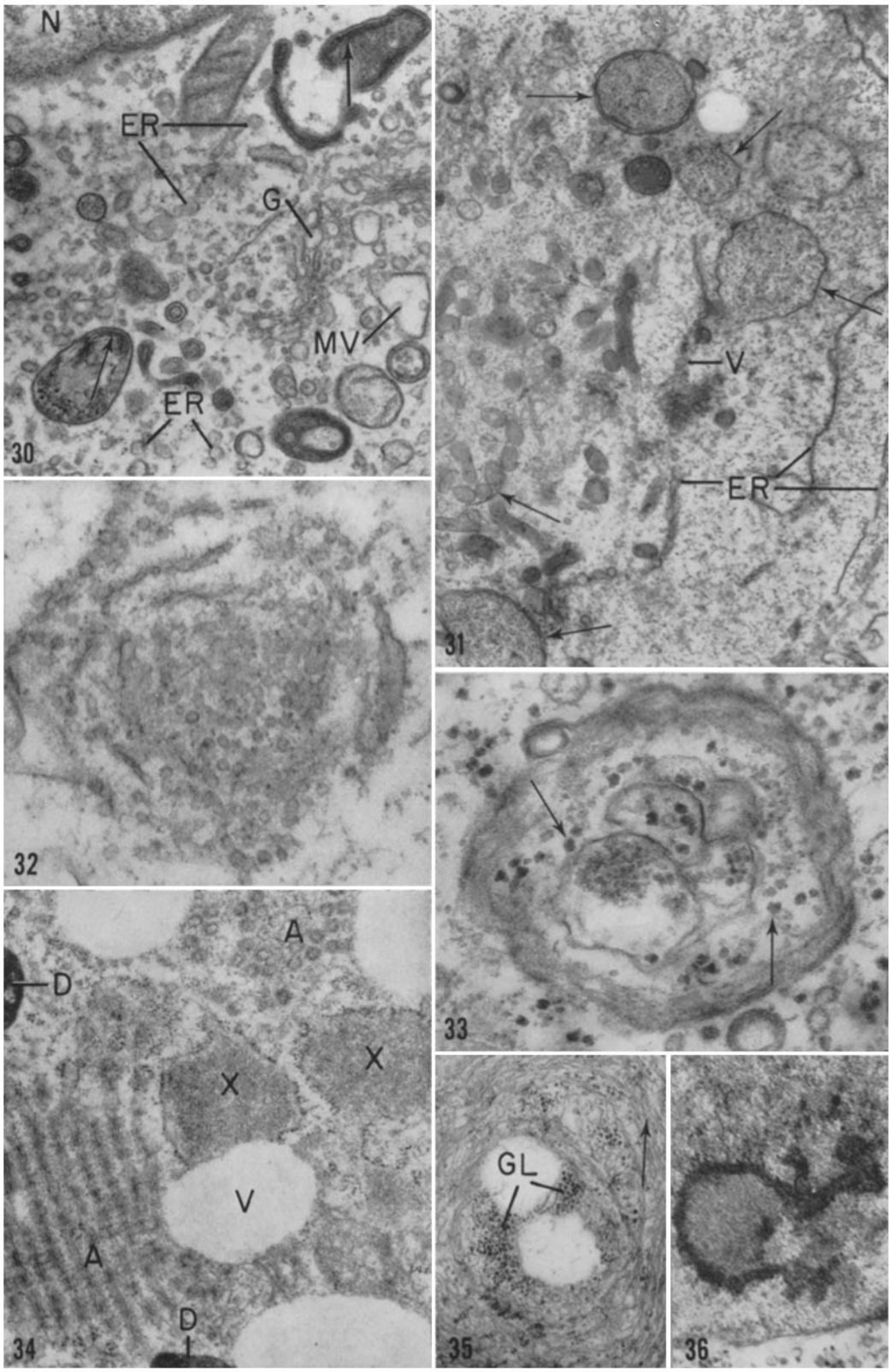
FIGURE 32 Portion of cell 24 hours after ultraviolet irradiation. A whorl of endoplasmic reticulum shows extensive vesiculation. × 21,000.

FIGURE 33 Portion of cell 24 hours after ultraviolet irradiation. Whorls of membrane, probably smooth endoplasmic reticulum, have included numerous glycogen granules (arrows). × 45,000.

FIGURE 34 Portion of cell 5 days after x-irradiation. Visible in the field are annulate lamellae (*A*), the one above sectioned transversely and the one below longitudinally; clear vacuoles (*V*), probably resulting from extraction of triglyceride-rich lipid during processing for electron microscopy; a moderately electron-opaque material (*X*) within ribosome-studded endoplasmic reticulum; and edges of two dense bodies (*D*). × 19,500.

FIGURE 35 Portion of cell 24 hours after ultraviolet irradiation. Whorl of endoplasmic reticulum (arrow) encloses a mass of glycogen granules (*GL*). × 15,000.

FIGURE 36 Portion of nucleus 24 hours after ultraviolet irradiation. The periphery of the nucleolus is of high electron opacity and extends into the nucleus in irregular fashion. × 20,000.


N
ER
G
MV
ER
30
V
ER
31
32
33
A
D
X
X
V
A
D
34
GL
35
36

24 hours after irradiation, the cells are injured about as much as when grown in deficient medium for 5 days. In some ways, the responses to the two types of injury are different.

The following differences may be noted in the irradiated cells: (1) Fewer triglyceride-rich lipid droplets are present (Fig. 11); however, they are more numerous than in untreated cells (Fig. 9). (2) Less glycogen is present. (3) The numerous nucleoli (Fig. 7) show electron-opaque material at their periphery (Fig. 36). (4) Although multivesicular bodies (of greater electron opacity than in normal cells), bodies with a ring-like appearance, and other apparent residual bodies are present (Figs. 30 and 38), few, if any, lamellated dense bodies are found (Fig. 30). (5) The endoplasmic reticulum that forms whorls is of the "smooth" variety with few or no attached ribosomes. The whorls may enclose glycogen and other cytoplasmic entities (Figs. 31 to 33 and 35). The cytochemically demonstrable acid phosphatase activity that appears in the endoplasmic reticulum remains even 16 and 24 hours after injury (Figs. 39 to 41).

IV. X-Irradiation

At 24 hours after x-irradiation, few changes are evident, but by 5 days the cells are dramatically altered. Certain of the alterations differ from those in arginine-deprived or ultraviolet-irradiated cells. In addition to the usual droplets considered to be triglyceride-rich lipid (Fig. 34), a material that may contain lipid accumulates within the rough-surfaced endoplasmic reticulum (Fig. 34) (*cf.* reference 46 which describes triglyceride accumulation within vesicles of endoplasmic reticulum, following orotic acid feeding; and reference 6 which describes the presence of dense material within rough endoplasmic reticulum after irradiation). The nucleoli commonly show ring-like structures (Fig. 8).

Membrane-delimited electron-opaque bodies are numerous (Fig. 34); however, they are not in the same shapes as the lamellated dense bodies. Near connections between them and the endoplasmic reticulum are occasionally encountered. Vacuoles with electron-opaque materials within them are also seen. Incubation for acid phosphatase activity yields reaction product in bodies of these sizes and shapes.

The endoplasmic reticulum of these cells gives no sign of acid phosphatase activity by the methods used in this study. In these cells, in contrast to ultraviolet-irradiated cells but similar to arginine-deprived cells, the whorls of endoplasmic reticulum are rough-surfaced. Spiral aggregates of ribosomes are encountered frequently.

DISCUSSION

Among the changes noted following injury of KB cells are several reported in other cultured cells

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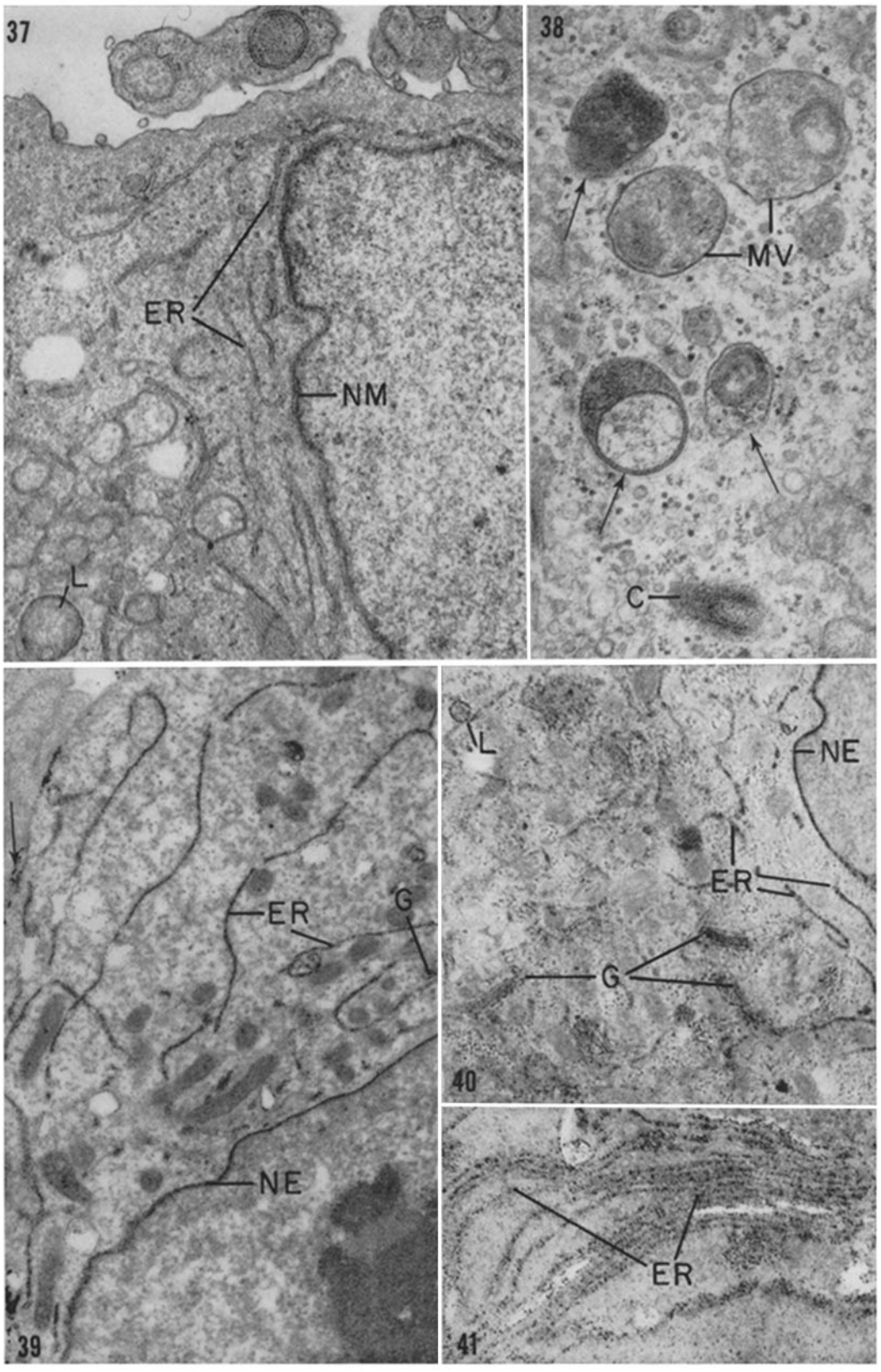
FIGURE 37 Portion of cell 24 hours after ultraviolet radiation, incubated in substrate-free control medium at pH 5.0. Neither reaction product nor lead binding is seen anywhere. Note nuclear membrane (*NM*), endoplasmic reticulum (*ER*), and lysosome-like body (*L*). × 10,500.

FIGURE 38 Portion of cell 16 hours after ultraviolet irradiation. Note multivesicular bodies (*MV*), some with electron-opaque material in areas; other lysosome-like bodies (arrows); and a centriole (*C*). × 18,500.

FIGURE 39 Portion of cell 24 hours after ultraviolet irradiation. Fixation in glutaraldehyde and incubation for acid phosphatase activity with 5′-cytidylic acid as substrate. Reaction product is seen in nuclear envelope (*NE*), endoplasmic reticulum (*ER*), and Golgi saccules (*G*). There are non-enzymatic deposits (arrow) on the plasma membrane, also found in substrate-free control cells, at interfaces between cells. × 10,500.

FIGURES 40 and 41 Portions of cells 24 hours after ultraviolet irradiation. Fixation in glutaraldehyde and incubation for acid phosphatase with 5′-cytidylic acid as substrate; thin sections not stained with heavy metal salts. Reaction product is seen in nuclear envelope (*NE*), endoplasmic reticulum (*ER*), Golgi saccules (*G*), and lysosome (*L*). Fig. 40, × 12,000; Fig. 41, × 19,500.


37
ER
NM
L
38
MV
C
39
ER
G
NE
40
L
NE
ER
G
41
ER

when damaged. These include accumulation of lipid rich in triglyceride (8, 35, 38), appearance of ring-like structures in nucleoli (38), mitochondrial change (36), structural alteration of the endoplasmic reticulum (9, 32, 38), and increased number and size of lysosomes (52). Not previously reported, to our knowledge, are increased numbers of annulate lamellae, persistence of helical ribosomal aggregates, glycogen accumulations, and the appearance of cytochemically-demonstrable acid phosphatase activity in the endoplasmic reticulum.

The common occurrence of polysomes, rough endoplasmic reticulum, and annulate lamellae suggests that synthesis of some proteins, possibly lysosomal hydrolases, continues in these injured cells.

The continuity of acid phosphatase reaction product in the endoplasmic reticulum and adjacent lysosomes has been seen on several occasions. This, together with near connections between the endoplasmic reticulum and lysosomes, seen in both arginine-deprived and x-irradiated cells, is consistent with the suggestion from this laboratory (45, 48, 30) and that of Brandes (7) that some lysosomes form from the endoplasmic reticulum. The fact that the Golgi apparatus seems not to be directly implicated in lysosomal production in these situations distinguishes them from those described in other tissues (40). However, the evidence presented here does strengthen the view (45) that acid phosphatase, and presumably other acid hydrolases, are transferred from their sites of synthesis, the polysomes, either directly to lysosomes, or *via* the Golgi apparatus, by the endoplasmic reticulum. It may be presumed that in some cells (19, 25, 44, 48) the extent of transfer is sufficiently high to be demonstrable cytochemically, but that this is not the case in KB cells until after injury. Whether injury induces increased rate of synthesis of acid hydrolases, increased concentration of enzyme proteins in the endoplasmic reticulum, or "unmasking" of enzymes is not known. Our observations are also consistent with the view (43, 56) that the endoplasmic reticulum is involved in the sequestration of cytoplasm to form autophagic vacuoles.

It is apparent that, in the absence of kinetic data, it is possible only to speculate concerning functional changes within lysosomes. It has become customary to consider the appearance of membranous arrays and other electron-opaque materials inside lysosomes as suggestive evidence of intracellular digestion and the accumulation of undigested residues (10). Admittedly, some or much of this material might, with time, have undergone further digestion. Nevertheless, we believe it warranted to consider these bodies as residual bodies (10). They appear in large numbers after all three forms of injury to KB cells. Structurally, the most striking of the residual bodies develop during arginine deprivation, and we have called these lamellated dense bodies. They sometimes resemble crystals.[3] The transformation of lysosomes into "multilaminated" bodies which can be considered residual bodies is seen when HeLa cells are exposed to acridine orange (52). Granules that are probably residual bodies are also seen in HeLa and MAC-21 cells after prolonged growth in unchanged nutrient medium with low pH (22), in HeLa cells treated with 5-bromodeoxyuridine (27) or grown in serum-free medium (32), in aging transformed FL cells (20), and in human cartilage cells after 48 hours in culture (21).

Injured KB cells may prove to be favorable material in which to study the functional significance of residual bodies, as well as their origin from the endoplasmic reticulum, multivesicular bodies, autophagic vacuoles or other lysosomes. It is not yet known which, if any, of the ultrastructural changes we have observed in such cells are reversible and which reflect irreversible damage leading to cell death.

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[3] Crystal-like structures have been reported in cultured cells (11, 17, 22, 28), but these apparently consist of exogenous materials (asbestos, etc.) engulfed from the medium and, unlike the KB material, they are enclosed within phagocytotic vacuoles.

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