RIFT VALLEY FEVER VIRUS HEPATITIS

Light and Electron Microscopic Studies in the Mouse

Malcolm H. McGavran, M.D.,* and Bernard C. Easterday, D.V.M., Ph.D.†

From the Department of Anatomy, Washington University School of Medicine, St. Louis, Mo., and the United States Army Chemical Corps Biological Laboratories, Fort Detrick, Md.

Rift Valley fever virus (RVFV) infects a wide range of species including man.[1] Total hepatic necrosis is seen in the highly susceptible species, e.g., the lamb, mouse and hamster.[2] Under natural conditions or when these animals are inoculated with minimal doses, the lesions are initially focal, gradually enlarge and increase in number, and finally, between 60 and 90 hours, all of the hepatic parenchymal cells become necrotic in a precipitous manner. Mims showed that by intravenous inoculation with very large doses of RVFV in mice a synchronous cycle of infection and necrosis occurred within 10 hours.[3] We chose to study RVFV in a similar system in order to minimize sampling errors and to determine whether the cells actually were infected, where the virus was produced and the significance of the nuclear inclusion.

Material and Methods

The source, production and assay of RVFV were as previously described.[4] Thirty-six 10- to 12-gm. Swiss-Webster mice were given intravenous inoculations of 0.5 ml. of lamb serum containing $1 \times 10^{9.7}$ mouse intraperitoneal median lethal doses ($MIPLD_{50}$) of RVFV. Fourteen mice were inoculated with 0.5 ml. of normal lamb serum. Two of the infected and one of the control mice were killed at time zero and then at hourly intervals for 12 hours. The remaining infected mice were observed until they died. Samples of blood, liver and lung from each mouse were assayed for virus.

Portions of the liver were frozen, and the remainder was fixed in cold buffered formalin, Carnoy's fixative (6:3:1), absolute methanol, acetone or 1 per cent osmium tetroxide in White's saline at pH 7.2. The frozen and acetone-fixed tissues were used in fluorescent antibody studies. The osmium-fixed tissues were dehydrated in ethanol and propylene oxide, and embedded in Epon 812. They were then cut with glass knives on a Porter-Blum microtome, stained with uranyl acetate and examined with an RCA EMU-3F microscope at 50 KV. The remaining tissues were processed through paraffin, cut at 4 μ and stained with hematoxylin and eosin, methyl green pyronine, and with the periodic acid-Schiff method with and without prior diastase digestion, the Feulgen method, and the method for demonstrating the Millon re-

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* Trainee in Anatomy. Present address: Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

† Present address: Department of Veterinary Science, University of Wisconsin, Madison, Wis.

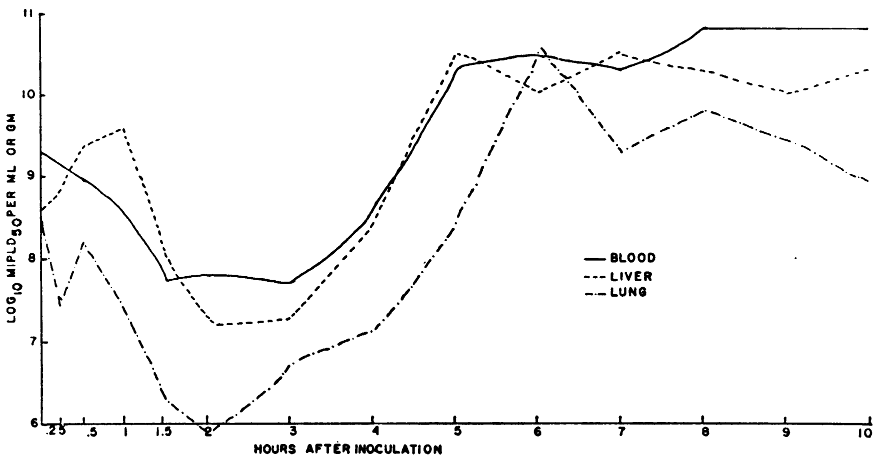
action. Frozen sections were stained with 0.1 per cent acridine orange at pH 3.6 for 10 minutes and with a globulin fraction, derived from the serum of sheep that had survived RVFV infection, conjugated with fluorescein isothiocyanate.

Results

The mice died between 14 and 18 hours after inoculation and manifested total hepatic necrosis associated with mild hemorrhagic manifestations. No morphologic evidence of infection or necrosis was found in other organs. The control mice did not die and showed no changes at necropsy.

Virus Assay

The mean values of the virus titers in the blood, liver and lung from the two mice killed at each point are shown in Text-figure 1. The concentration of virus in the liver exceeded that of the blood from the second through the fourth hour and was essentially the same thereafter.



Text-figure 1. Levels of Rift Valley fever virus in mice following intravenous inoculation of 1×10^{10} $MIPLD_{50}$ of RVFV.

Light Microscopy

The hepatic parenchymal cells underwent a synchronous sequence of alterations. Glycogen depletion was rapid and complete by the sixth hour even though the mice had not been fasted and were left on food and water. As the glycogen content decreased, the clumping of the ergastoplasm diminished as indicated by basophilia, pyroninophilia, or of red fluorescence in acridine orange stained sections. From the seventh to the tenth hours the ribonucleoprotein content of the cytoplasm decreased, and by the twelfth hour only faint staining was seen in a few cells. No increase in lipid was found. Between the eighth and twelfth hours the cells separated from each other, and erythrocytes became enmeshed, first singly, then in groups of 2 or more, and then in clusters in the interstices. Some cells became hyalinized, but the majority disintegrated, leaving a mixture of necrotic but recognizable cells, cellular debris and erythrocytes. There was significant absence of inflammatory cells (Fig. 1). Microthrombi containing cellular debris, presumably hepatic in origin, were found in the small pulmonary arteries.

The nuclear chromatin was distinctly marginated by the eighth hour, and thereafter a pale eosinophilic inclusion appeared in the nuclear sap. The inclusions failed to stain for deoxyribose nucleic acid (DNA), ribonucleic acid (RNA), virus antigen, basic protein, mucopolysaccharide or glycogen (Figs. 2 and 3). We did not find any change in nucleolar staining at any time.

Excessive nonspecific staining by the fluorescein-conjugated globulin was encountered, in spite of repeated extractions and changes in procedure. On the few occasions when the controls indicated satisfactory specificity, distinct perinuclear cytoplasmic staining was found. At no time was staining in the nuclei observed, nor in the Kupffer cells. In experiments reported elsewhere [5] specific immunofluorescence was not found in the nuclei of lamb liver cells and in tissue cultures of hamster kidney and Chang human liver cells infected with RVFV.

Electron Microscopy

The mode of attachment or ingress of the virus was not observed. After the first hour small aggregates of oval and round particles appeared in dilated endoplasmic sacs and lacunas (Figs. 4 to 8). This was not preceded by the condensation of precursor particles on the outer surface of the membranes lining these spaces, nor were particles seen crossing these membranes. Rather, the initially relatively electron-lucid bodies appeared to condense and develop a distinct limiting membrane and a dense nucleoid. The initial amorphous particles varied from 70 to 120 mμ in diameter. When fully formed, the virus measured approximately 90 mμ in diameter. A thin section of RVFV, centrifuged from lamb serum at 100,000 *g* for 1 hour, is shown in Figure 12. It has the same structure and size. Vesicles containing virus appeared to migrate toward the plasma membrane. The walls of these vesicles then fused with the plasma membrane and the mature virus was released into the space of Disse. The virus did not acquire another membrane, nor did it lose one during the process of release.

This cycle occurred rapidly so that by 6 to 8 hours it was difficult to find virus within the cells (Figs. 9 to 12). At no time were virus or aggregates of amorphous matrix found within the nucleus, nor did the nucleoli enlarge.

Depletion of glycogen and ribosomes was recognizable by 3 hours and continued. Aggregates of smooth-surfaced endoplasmic reticulum appeared, as well as an unusual form of the ergastoplasm. Normally the ergastoplasmic membranes are studded with ribosomes on their outer surface. The distance between adjacent membranes is variable and within the space unattached ribosomes are seen. The alteration found in the RVFV-infected liver cells is illustrated in Figures 13 to 15. Adjacent ergastoplasmic membranes were uniformly approximated, the distance between them averaging 100 Å. In this space a relatively regular banding appeared. These cross bars were spaced at 100 to 200 Å intervals. In some areas the banded or ladder-like pattern merged into a fine lamellar fraying or stacking of less well defined membranes. These variations were probably the result of differing planes of section. It was concluded that the composite structure that would account for them was composed of shelf-like cross bands, rather than isolated granules or particles, at right angles to the approximated ergastoplasmic membranes. The ergastoplasmic sacs were dilated in these areas and electron-lucid. Although the density of their matrix increased progressively, the mitochondria remained intact until the cells disintegrated. As the cells shrank they separated from each other, except at the parabiliary desmosomes (Fig. 15). Finally, distinct myelin figures appeared and the plasma membranes ruptured. No increase in dense bodies preceding the dissolution of these cells was observed (Fig. 16).

Discussion

The mechanisms whereby viruses kill cells are poorly understood. In some instances, particularly when large doses are used, toxic destruction without apparent infection or viral multiplication occurs. The results of the assay (Text-fig. 1) and the electron microscopic findings of multiplication indicated that the hepatic necrosis produced by RVFV was not merely a toxic phenomenon. Another hypothesis suggests that the virus-infected cell is forced to transfer its anabolic functions into virus production so completely that it becomes "exhausted" and dies. The condition in RVFV hepatitis might fit this latter hypothesis although estimates of the amount of virus synthesized by the liver are hardly sufficient to exhaust these active cells. It is more probable that this type of virus infection irrevocably alters vital enzymatic systems as indicated by Ginsberg [6] and Jones and Cohen.[7] The aberration of the ergastoplasm described may be a structural manifestation of some of the metabolic changes associated with virus production or its aftermath.

RVFV is formed in a manner which differs from that described for certain other animal viruses.[8,9] It forms within a membrane-lined system which is generally agranular and thus resembles the Golgi apparatus.

We may assume that the virus nucleic acid as well as the coat proteins and lipids are formed in the ergastoplasm and transported to the Golgi apparatus wherein they are assembled. Thereafter, the egress of the virus is similar to that of secretory products of exocrine cells. It does not bud from the cell surface as does the virus of influenza and many of the tumor viruses.

An increasing number of reports on experimental virus hepatitis are available. These include studies on yellow fever,[10–12] ectromelia (mouse pox),[13] and a series of papers on the mouse hepatitis viruses (MHV).[7,14,15] The pitfalls of sampling, particularly in asynchronous systems, and the difficulties of differentiating changes that are antecedent and causally related to virus multiplication from those that are subsequent and probably degenerative are well illustrated. Even within an apparently synchronous system such as used in this study the limitations of sampling and perhaps the rapidity of multiplication prevented the finding of virus in each cell. It may only be surmised that all the cells were infected by the comparable secondary changes.

Tigertt and associates,[10] Ruebner and Miyai,[14] and Jones and Cohen[7] have noted changes in the Kupffer cells that precede the appearance of histologic damage in liver cells. They have suggested that Kupffer cell damage may be the primary event and that hepatocellular necrosis is secondary. Similar changes in the Kupffer cells were found at 2 to 3 hours after infection in this study. However, this did not precede the appearance of virus infection of the parenchymal cells as seen electron microscopically. Infected Kupffer cells were not found. Thus, it is difficult to adopt the hypothesis that hepatic necrosis is secondary to primary injury to the Kupffer cells.

Summary

River Valley fever virus produces a necrotizing hepatitis in mice that is similar to certain other forms of virus hepatitis. The virus infects the hepatic parenchymal cells and new virus is formed in the cytoplasm within a membrane-limited system resembling the Golgi apparatus. Unique structural alterations of the ergastoplasm are associated with this process and may be manifestations of metabolic dysfunction. The acidophilic nuclear inclusion is not formed of virus matrix or virus particles and may represent a degenerative phenomenon. Changes in the Kupffer cells follow infection of the parenchymal cells.

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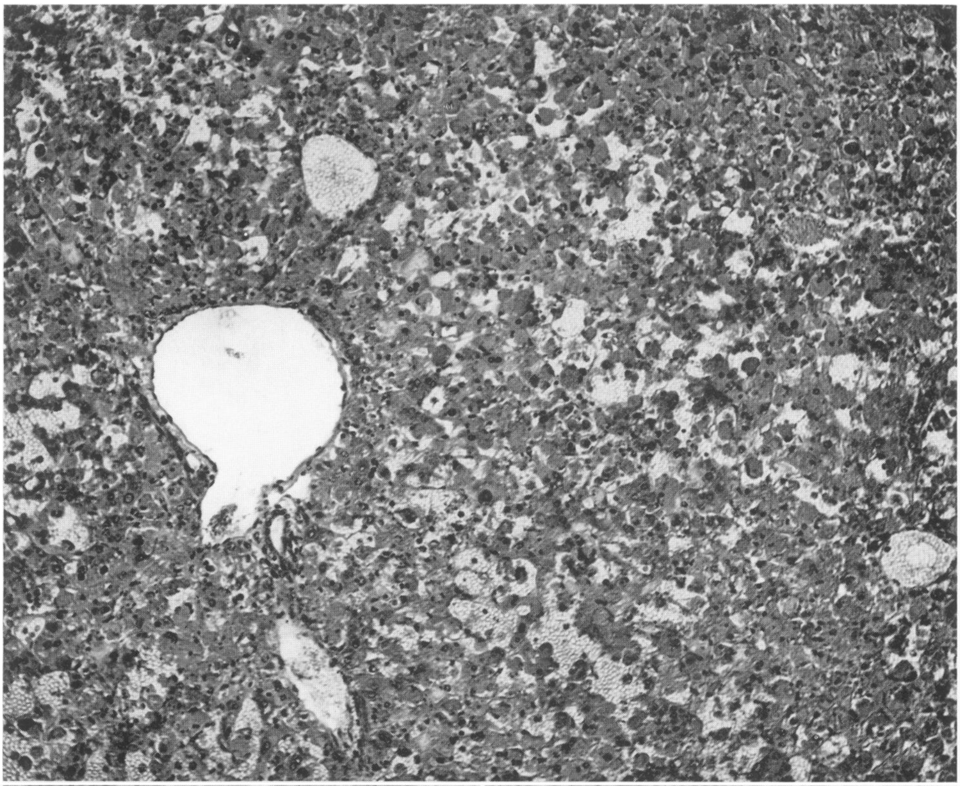
LEGENDS FOR FIGURES

FIG. 1. The necrotic mouse liver 12 hours after infection with a massive dose of Rift Valley fever virus. Hematoxylin and eosin stain. × 150.

FIG. 2. The nuclear alteration found 8 to 10 hours after infection. The chromatin is distinctly marginated, leaving a pale central sphere in which an irregularly shaped, faintly eosinophilic inclusion is found. Hematoxylin and eosin stain. × 1,000.

FIG. 3. An adjacent section to that shown in Figure 2 stained by the Feulgen reaction. The nuclear inclusion does not react for DNA. It is stained by the light green. × 1,250.



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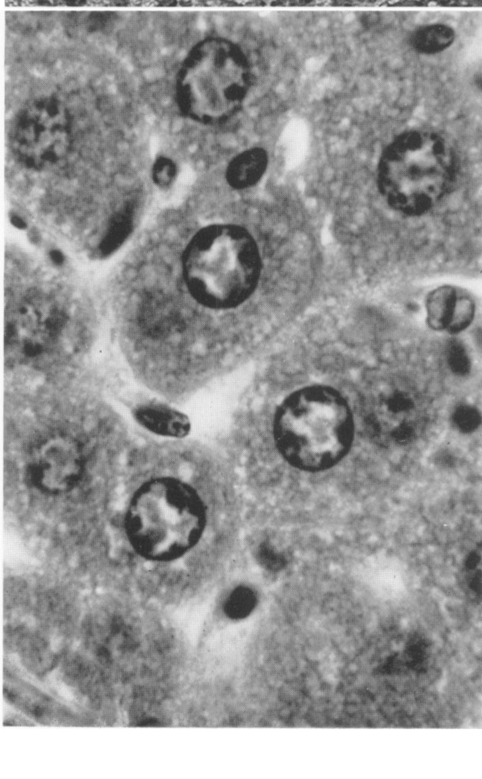

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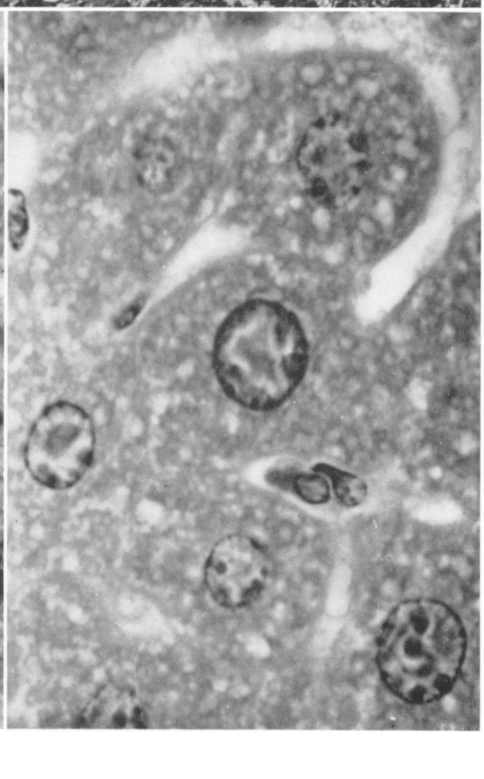

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FIG. 4. Many incompletely formed viruses (arrows) are present within a membrane-limited complex 2 hours after infection. The pale areas (gl) toward the left and bottom are deposits of glycogen that have been leached during fixation. A portion of the liver cell nucleus (n) is seen at the upper left and to the right is a mitochondrion (m). Approximately × 31,000.

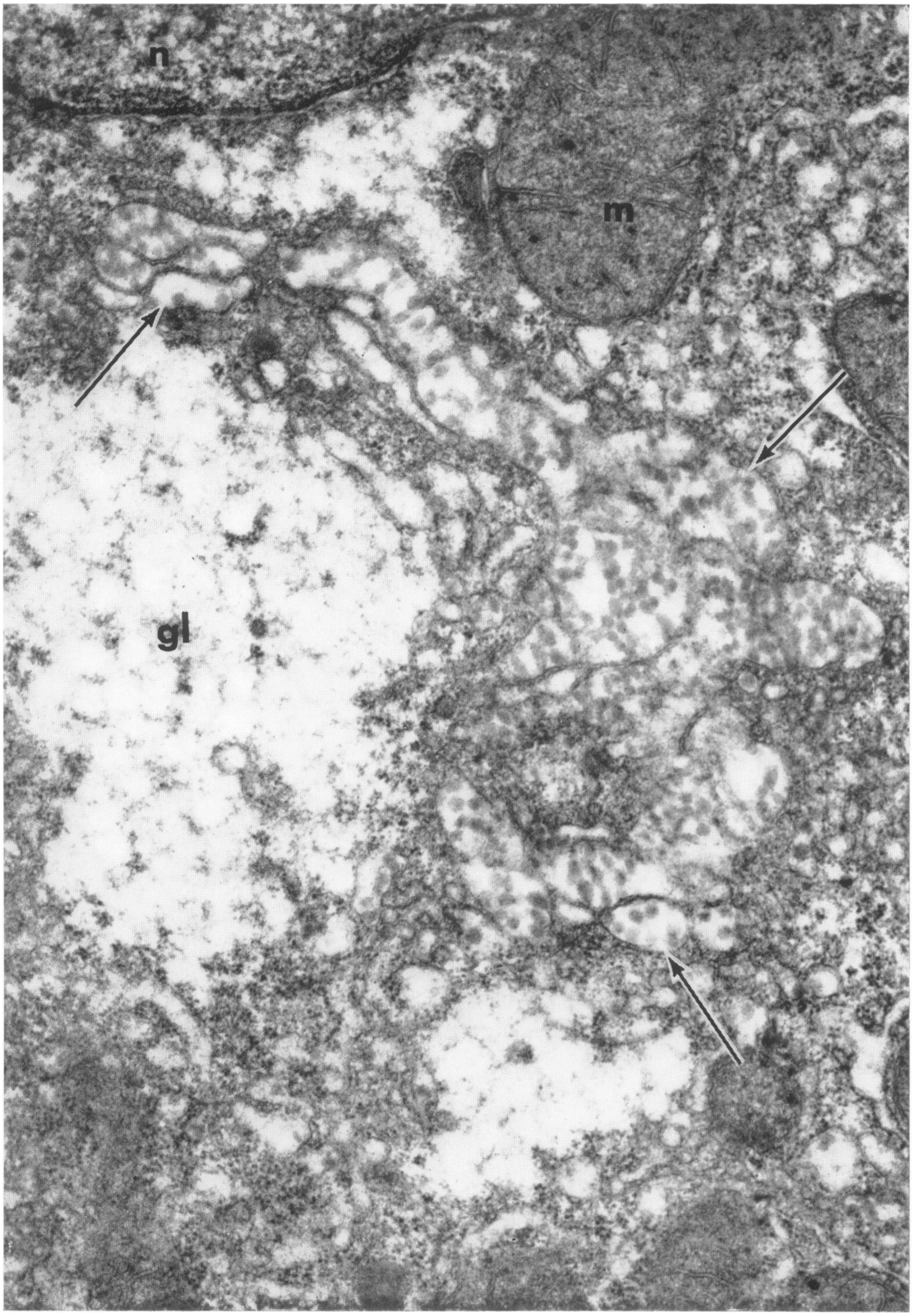

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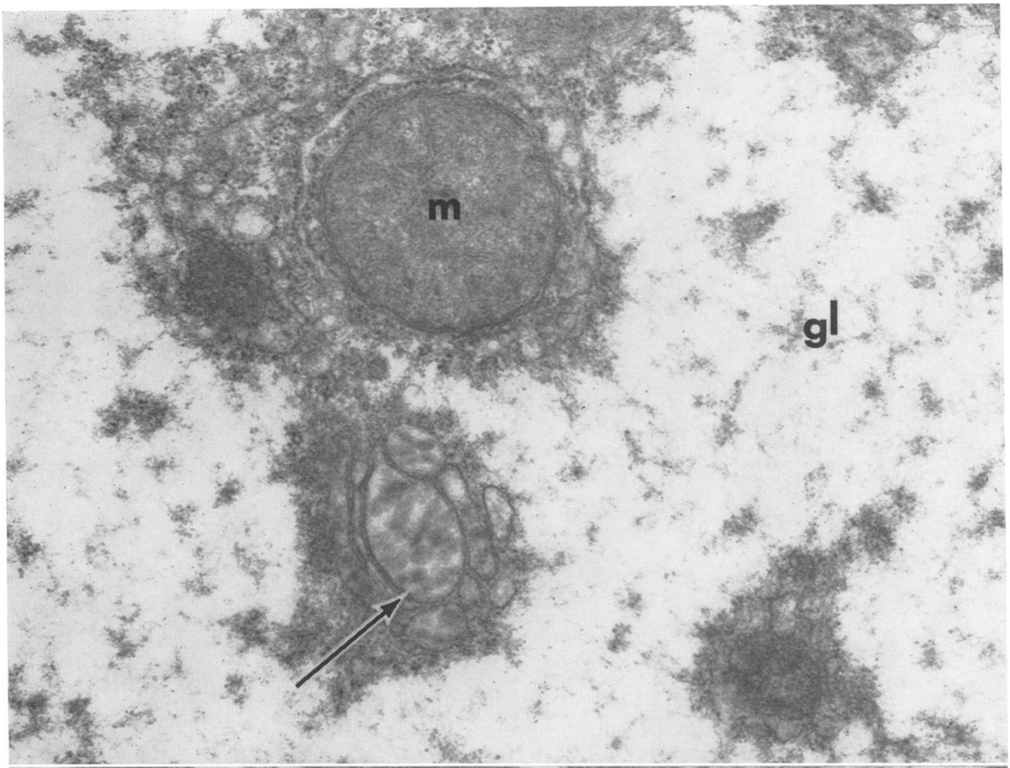
FIG. 5. The oval and round shapes of the forming viruses are seen in the vesicles (arrow) beneath the mitochondrion (m). Approximately × 31,000.

FIG. 6. The varying sizes of the viruses and the agranularity of the limiting membrane (arrow) are shown. Approximately × 35,000.



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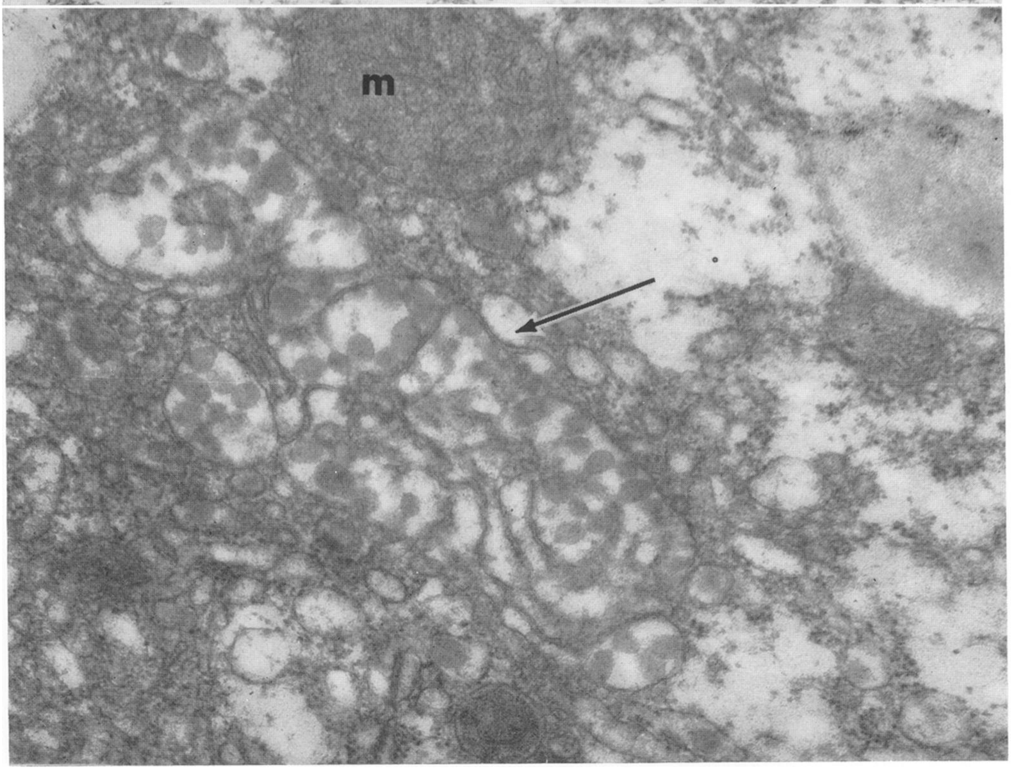

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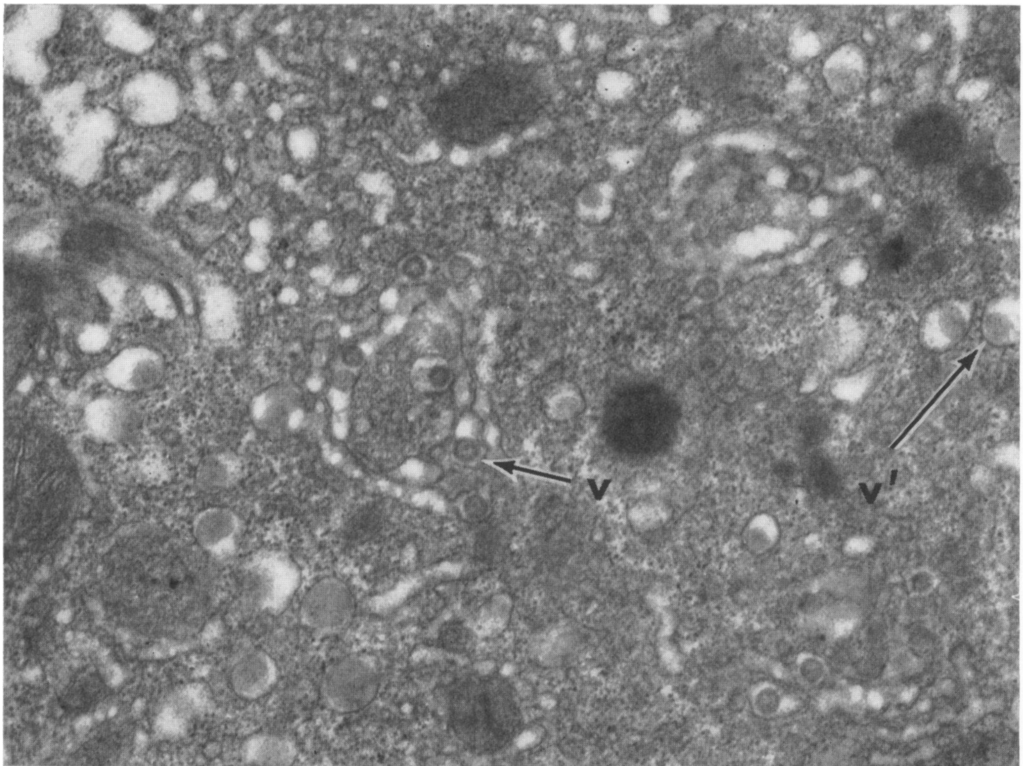
FIG. 7. Four hours after infection some viruses have a distinct membrane and nucleoid (v) while others remain amorphous (v^1). Approximately × 30,000.

FIG. 8. Six hours after infection some viruses are still present. The ribosomes are fewer, the mitochondrial matrix (m) flocculated and a fine lamellar alteration of the endoplasmic reticulum is noted near the nucleus (arrows). Approximately × 30,000.



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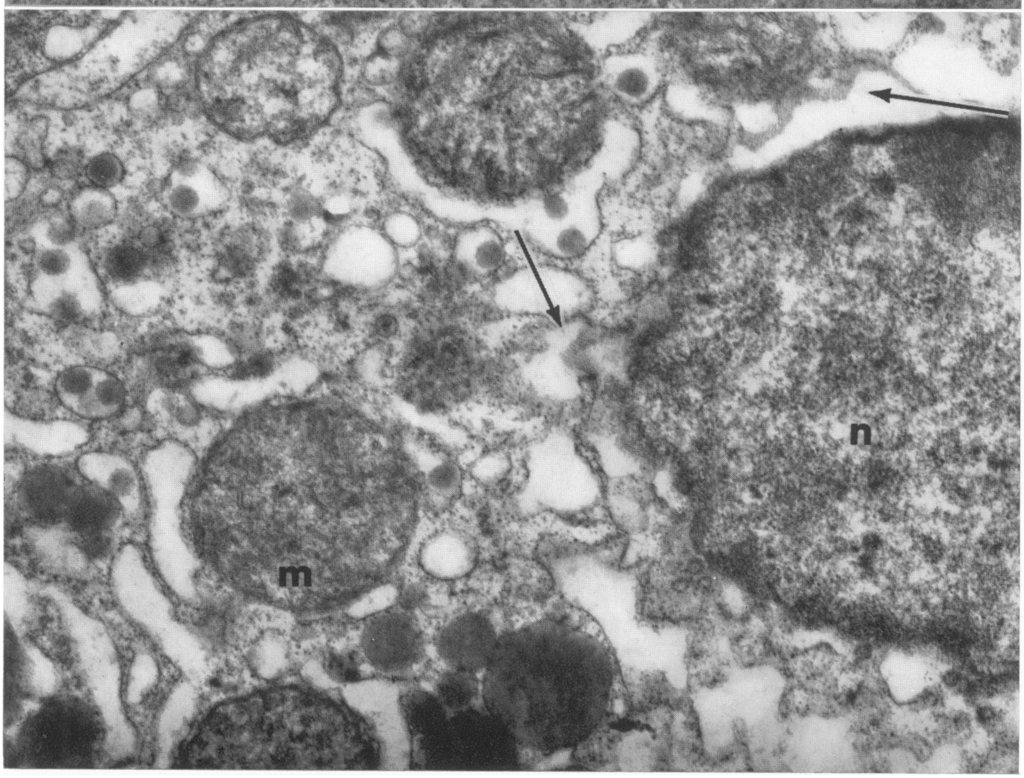

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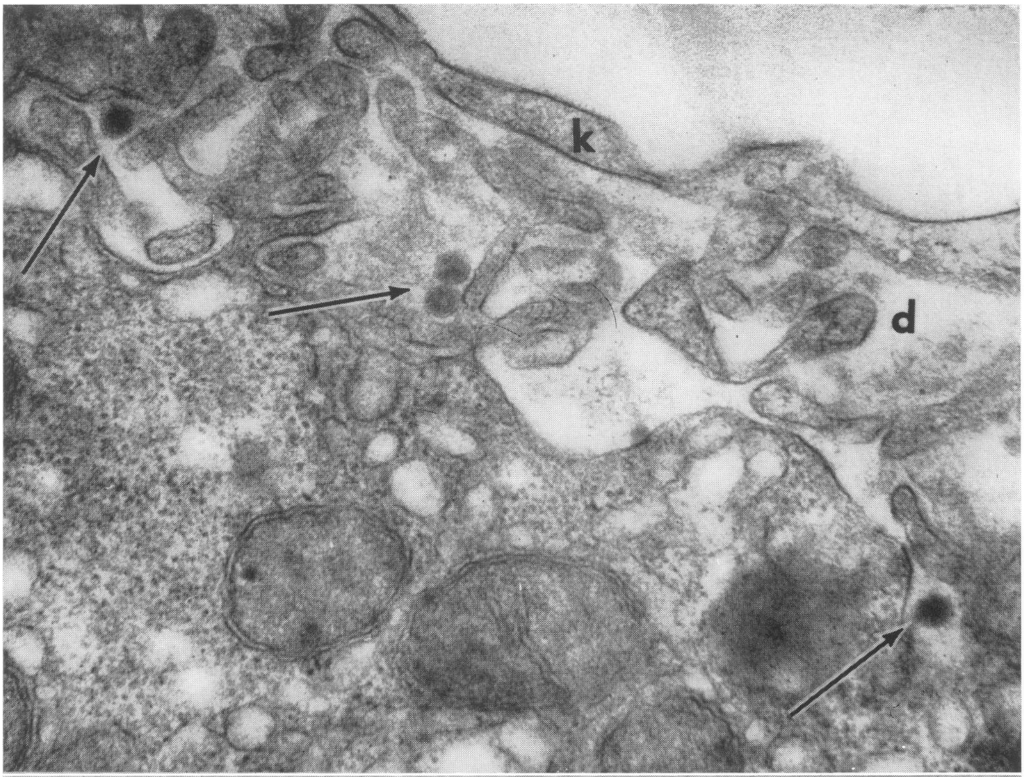
FIG. 9. Four viruses (arrows) are present in the space of Disse (d) between the liver cell microvilli and intact Kupffer cell cytoplasm (K). Approximately × 57,000.

FIG. 10. A collection of virus (v) in the extracellular space 7 hours after infection. Approximately × 14,000.



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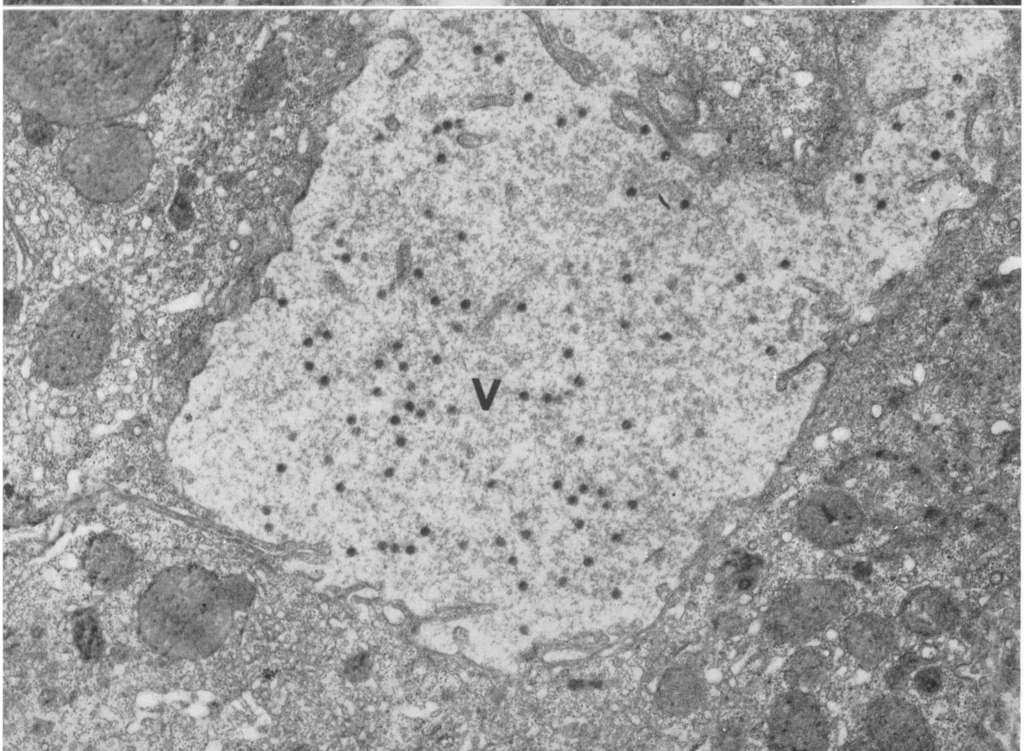

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FIG. 11. Three viruses in the space of Disse. The one on the left is superimposed on the tip of a microvillus and has a distinct hexagonal nucleoid. The plasma membrane is indistinct beneath the middle one and the one on the right overlies the base of a microvillus (see text). Approximately × 80,000.

FIG. 12. A thin section of a Rift Valley fever virus centrifuged from lamb serum. It has the same characteristics as those shown in Figure 10. Approximately × 88,000.

FIG. 13. The aberration of the endoplasmic reticulum found 6 to 9 hours after infection. In some planes it is finely lamellar (l) while in others it has a regular cross banding (b) between adjacent membranes. Approximately × 44,000.

FIG. 14. An example of the concentric form of the ergastoplasmic change which encircles a mitochondrion (m). At the tip of the arrow (b) 8 pairs of banded membranes are seen. Approximately × 60,000.

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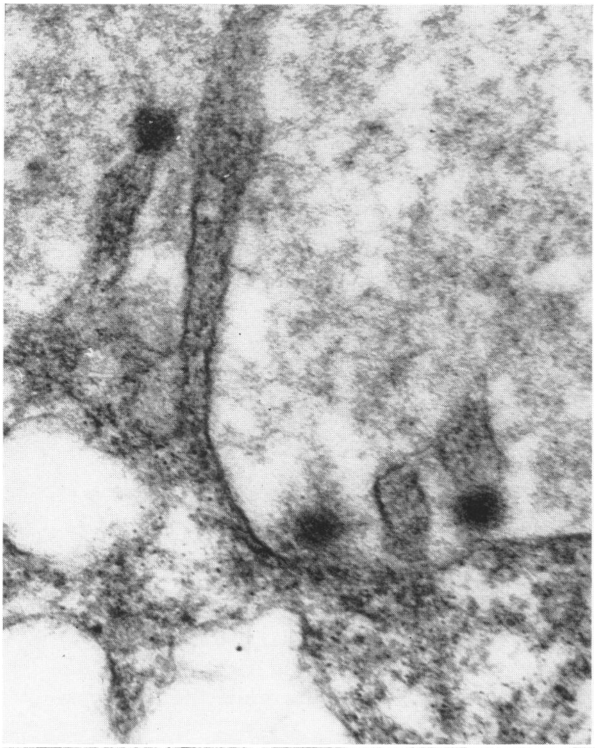

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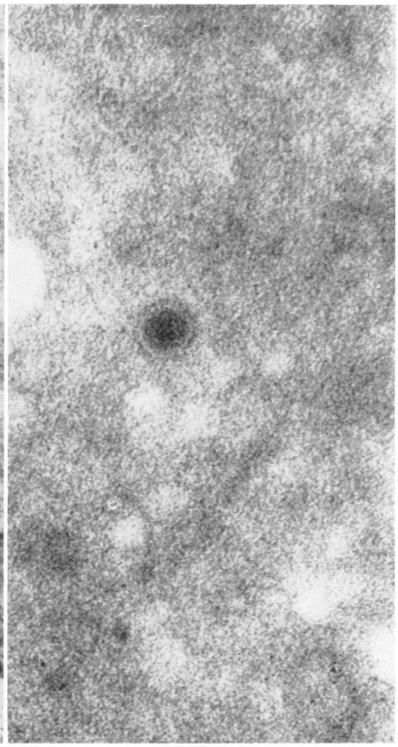

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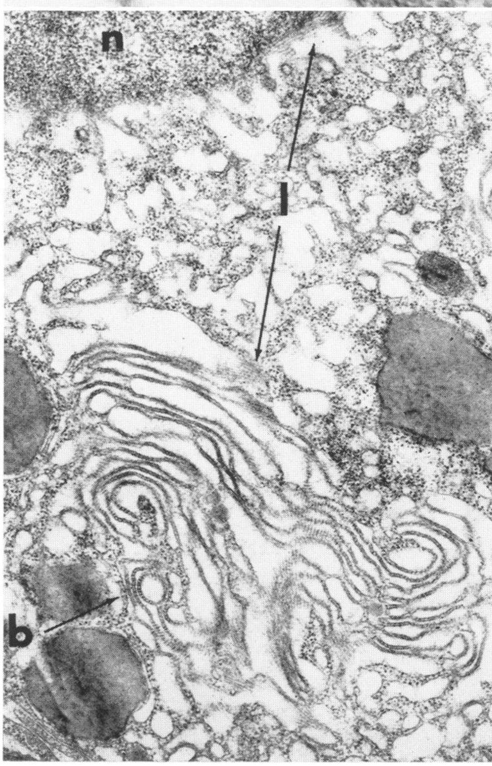

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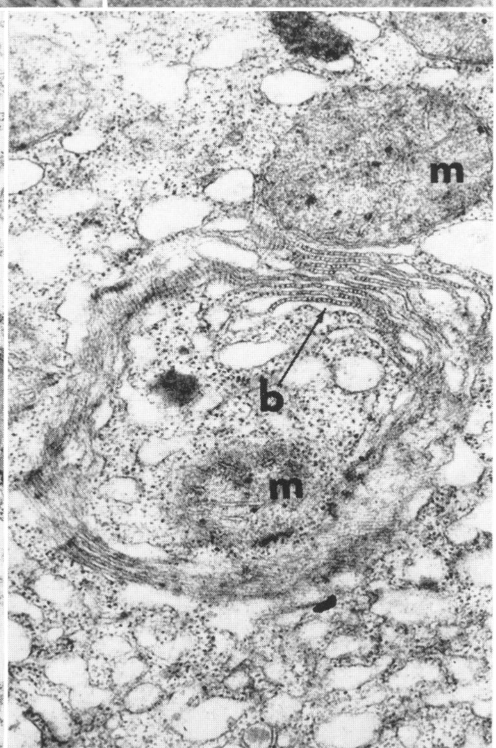

FIG. 15. A detailed view of the alteration in the ergastoplasm in which at the tip of the arrow marked (b) 3 pairs of cross-banded membranes are seen. Followed toward the bottom of the plate they merge into the lamellar pattern (arrow, 1). The cross bars are smaller than the ribosomes. The right wall of the ergastoplasmic sac (s) is formed of banded membranes while the left is unaltered. Approximately × 80,000.

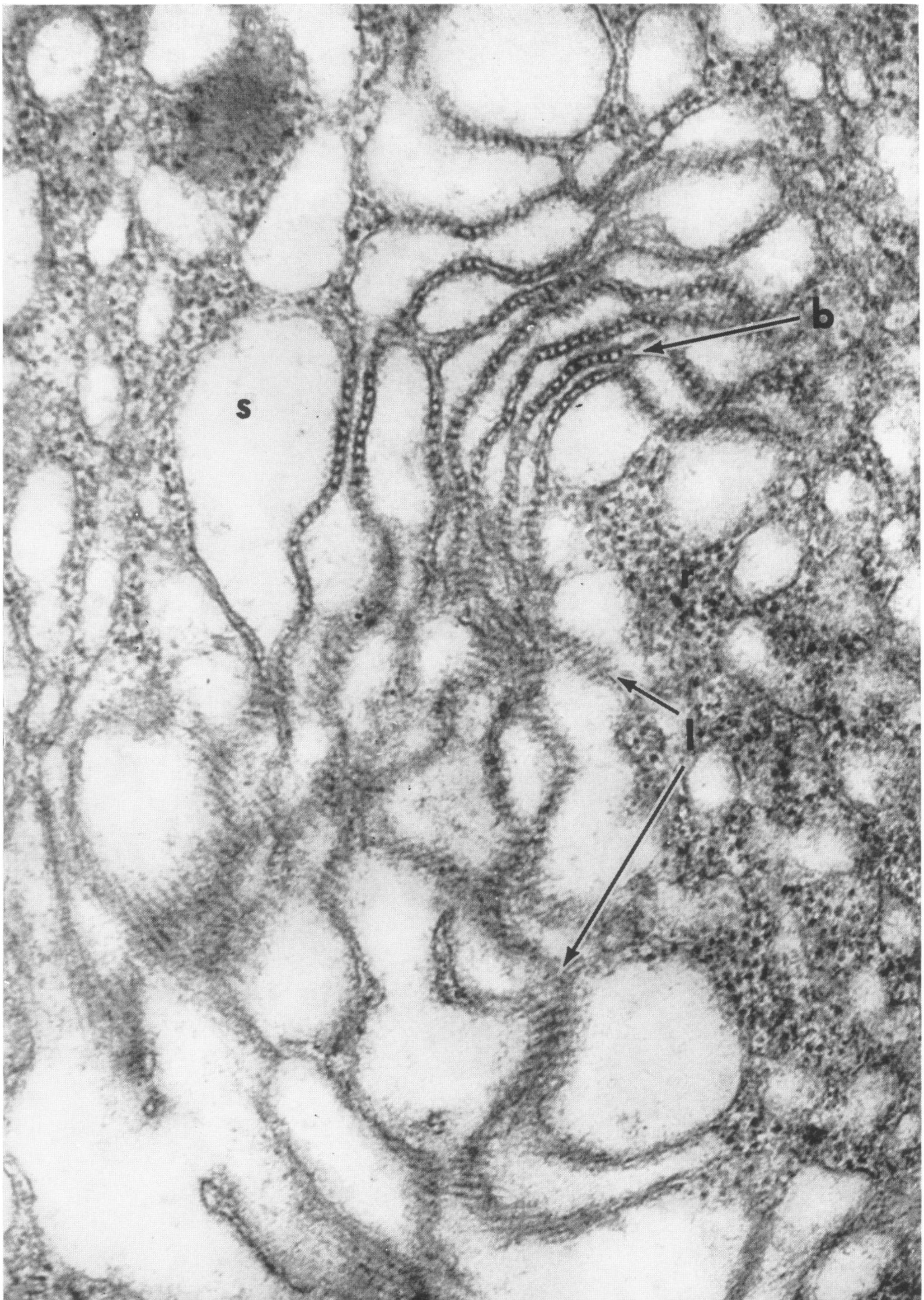

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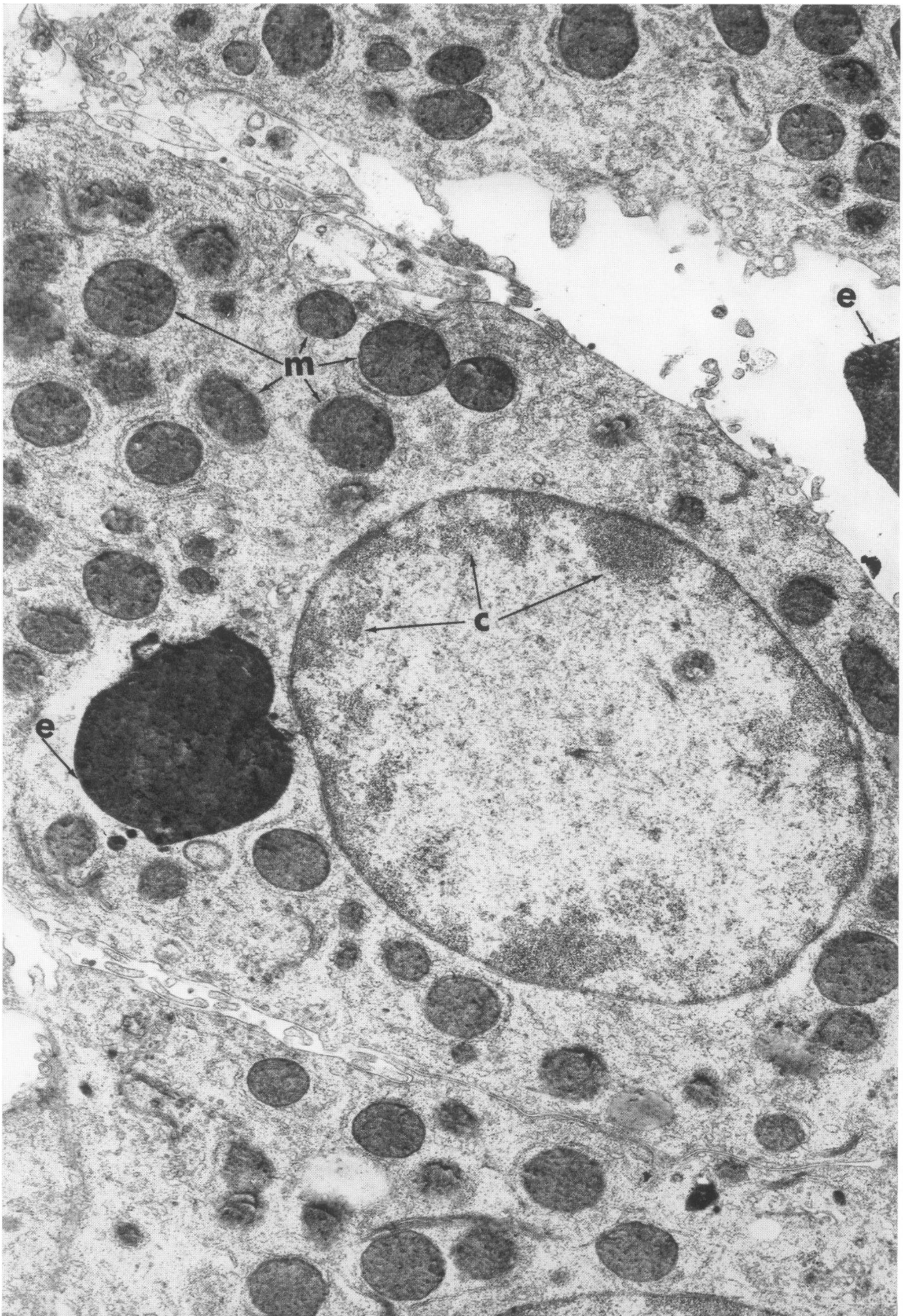
FIG. 16. The dying liver cell, 8 hours after infection, is devoid of virus and depleted of glycogen and ergastoplasm. A few ribosomes are scattered between the remaining membranous components. The mitochondria (m) are intact. Their matrix is exceptionally dense. The chromatin (c) is marginated and the nuclear sap faintly granular. A portion of an erythrocyte (e) is present to the left of the nucleus. Approximately $\times$ 11,000.



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