AN ELECTRON MICROSCOPE STUDY OF THE DEVELOPMENT OF A MOUSE HEPATITIS VIRUS IN TISSUE CULTURE CELLS

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ABSTRACT

Samples taken at different intervals of time from suspension cultures of the NCTC 1469 line of mouse liver—derived (ML) cells infected with a mouse hepatitis virus have been studied with the electron microscope. The experiments revealed that the viruses are incorporated into the cells by viropexis within 1 hour after being added to the culture. An increasing number of particles are found later inside dense cytoplasmic corpuscles similar to lysosomes. In the cytoplasm of the cells from the samples taken 7 hours after inoculation, two organized structures generally associated and never seen in the controls are observed: one consists of dense material arranged in a reticular disposition (reticular inclusion); the other is formed by small tubules organized in a complex pattern (tubular body). No evidence has been found concerning their origin. Their significance is discussed. With the progression of the infection a system of membrane-bounded tubules and cisternae is differentiated in the cytoplasm of the ML cells. In the lumen of these tubules or cisternae, which are occupied by a dense material, numerous virus particles are observed. The virus particles which originate in association with the limiting membranes of tubules and cisternae are released into their lumen by a "budding" process. The virus particles are 75 mμ in diameter and possess a nucleoid constituted of dense particles or rods limiting an electron transparent core. The virus limiting membrane is sometimes covered by an outer layer of a dense material. In the cells from the samples taken 14 to 20 hours after inoculation, larger zones of the cell cytoplasm are occupied by inclusion bodies formed by channels or cisternae with their lumens containing numerous virus particles. In the samples taken 20 hours or more after the inoculation numerous cells show evident signs of degeneration.

INTRODUCTION

Acute hepatic disease of mice resulting from virus infection was described in 1951 by Gledhill and Andrews (36). Shortly thereafter, Nelson (58), in the course of serial transmission of a spontaneous leukemia in Princeton mice by means of splenic implants, detected a virus responsible for the induction of hepatic lesions in his experimental mice and for the concomitant loss of the transplanted leukemic cells. These and subsequent reports of virus-induced acute hepatitis (6, 45, 55, 56) in experimental mice permit the conclusion that the specific agents responsible for the disease are widely disseminated in mouse populations in a latent state, and that activation follows the systemic stress imposed during experimental procedures. Since some strains of virus produce pathological lesions in the mouse liver similar to those found in human hepatitis (44), and because trans-

mission and study of the human disease is beset with difficulties, the study of hepatitis in mice has received increased attention. During recent years, the biology and pathology of the disease has been under investigation, and some electron microscopic observations have been published (51, 69, 72). These studies demonstrated the presence of virus in mouse liver and described the ultrastructural alterations occurring in affected hepatic cells. Neither the site nor the mode of virus replication has been reported. In an effort to clarify some of the obscure points of the cell-virus relationship, we undertook an electron microscope study of virus-cell interaction employing cultured cells. The morphological changes observed in infected cells during the first 24 hours postinfection are described.

MATERIALS AND METHODS

CELLS: The NCTC 1469 line of mouse liver-derived cells was obtained from Dr. Virginia Evans (30, 43) and maintained in serial subculture in our laboratory. The cells are herein designated ML cells.

NUTRIENT MEDIUM: The ML cells were adapted in our laboratory to growth on Eagle's minimal essential medium (28) supplemented with 10 per cent unfiltered horse serum previously heated 30 minutes at 56°C, and containing 50 μg of kanamycin per ml of complete medium. The same medium proved satisfactory for suspended ML cell cultures in spinner flasks.

VIRUS: The A59 mouse hepatitis virus isolated by Manaker *et al.* (50) was available for this study. The virus pool used had undergone 25 passages in ML cells, 7 passages in L 929 cells, and 3 further passages in ML cells. Fluids from infected cultures induce acute hepatitis in mice.

In the course of this investigation, another morphologically different particle, herein termed the VL particle, was observed budding from cell membranes. No evidence of overt disease which might be attributed to this agent was detected in mice inoculated with control ML cell culture fluids, nor did this agent induce intracellular change in the control cultures comparable with those observed in cells infected with the hepatitis virus.

Hepatitis virus titration was made in ML cells, which are destroyed by this agent. Twenty-four hours after tube cultures containing 375,000 cells were infected with 5.6×10^2 tissue culture (T.C.) ID_{50} of virus, large syncytia involving more than half of the cell sheet were evident. A cytopathic response was evident within 3 days in those tubes that received virus following inoculation of the limiting dilution. On this basis a titer of 5.6×10^6 T.C. ID_{50} was determined by the method of Reed and Muench for the virus pool used in this study.

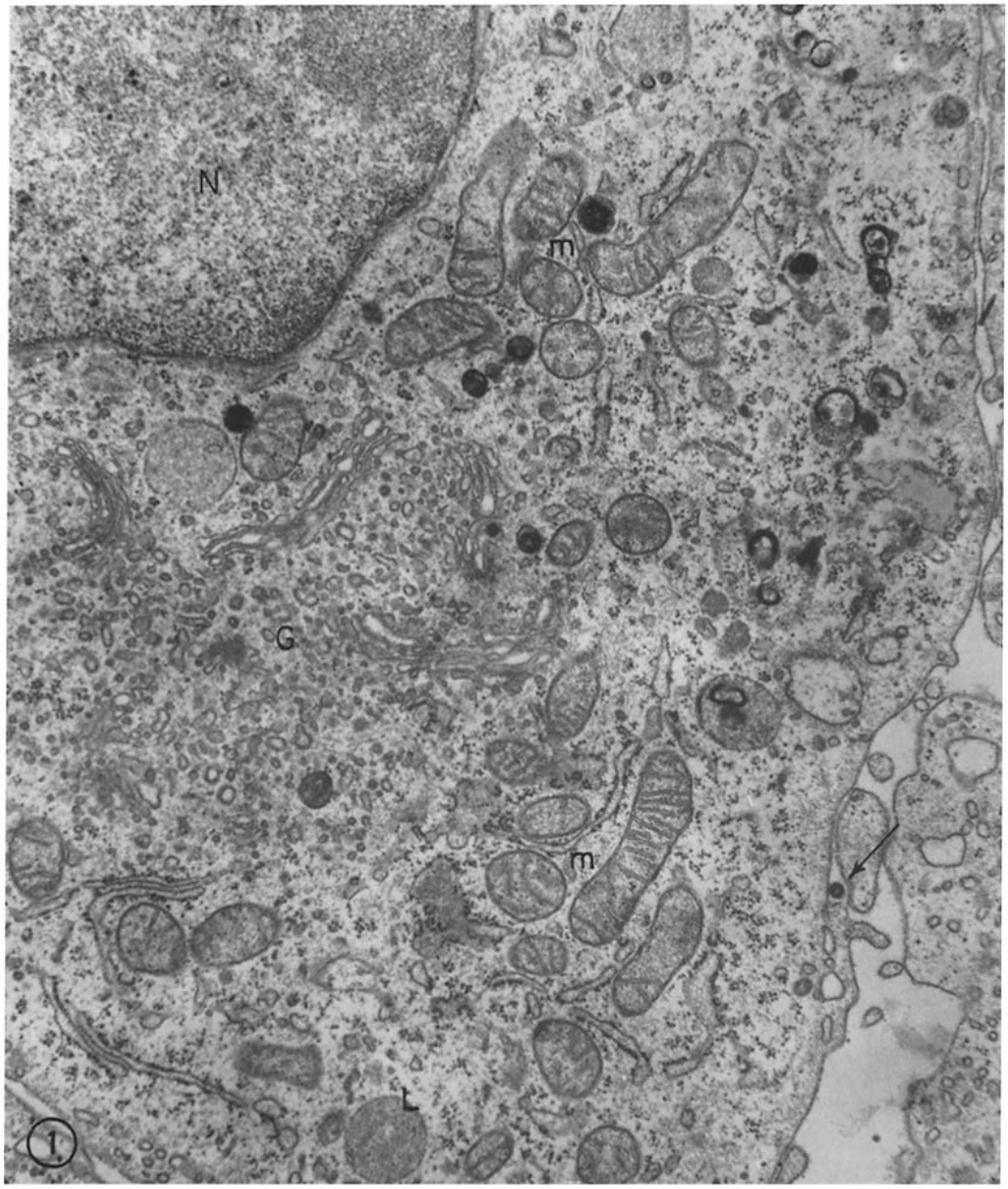
INFECTION AND SAMPLING OF CELLS: ML cells were grown in T-60 flasks at 36.5 to 37°C. Heavy cultures were shaken lightly to dislodge the cells. 100 ml of growth medium in which was suspended 3.3×10^7 cells was introduced into a spinner flask and incubated at 36.5 to 37°C. The culture was infected with 8×10^6 T.C. ID_{50} of virus. A similar uninfected suspension culture provided a control. At intervals over a period of 24 hours, beginning 2 hours after addition of virus, cells were withdrawn from the cultures for electron microscopic examination. These samples were compared with cells removed from the control cultures at the same times.

ELECTRON MICROSCOPY: Samples taken from the controls or from the infected cultures were centrifuged at 1000 RPM for 10 minutes and the pellets obtained fixed for 1 hour in chrome-osmium tetroxide fixative (15), in 1 per cent osmium tetroxide phosphate-buffered at pH 7.4, or in 2 per cent potassium permanganate (48, 52). After fixation the blocks were dehydrated in increasing concentrations of ethanol and embedded in Epon 812 mixtures (49). The thin sections were cut on an LKB ultratome with a diamond knife and picked up on Formvar-coated grids. In order to improve the contrast we double-

FIGURE 1. Portion of the nucleus (*N*) and cytoplasm of an ML cell from a non-infected culture. In the cytoplasm are seen mitochondria (*m*) dense corpuscles (*L*) and the Golgi apparatus (*G*). The Golgi apparatus is surrounded by numerous small vesicles. The arrow on the cell surface indicates a VL-particle of the type habitually seen in these cells. × 24,000.

FIGURE 2. Detail of the surface of an ML cell showing an infolding of the plasma membrane. Its cytoplasmic side is covered by fibrous material. × 60,000.

FIGURE 3. Virus-like particle budding from the cell membrane of a non-infected ML cell. This particle, which is similar to the "immature" C particles observed in the murine leukemias, has its outer membrane covered by small spicules. × 120,000.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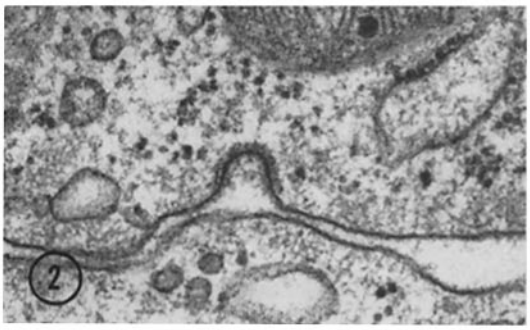
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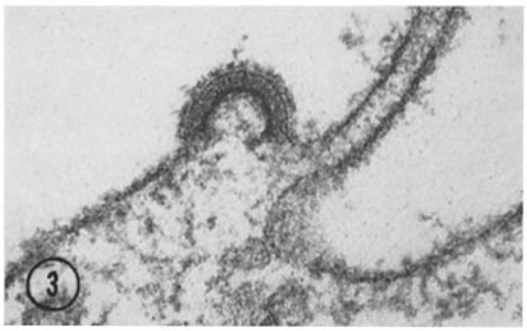
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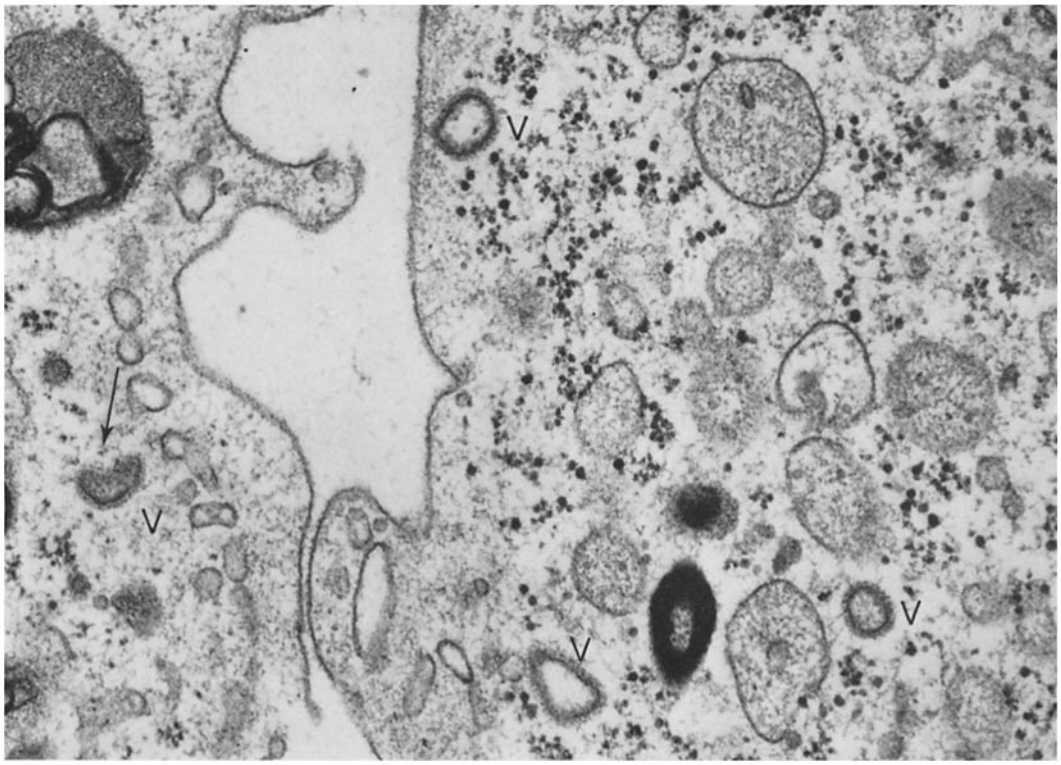


FIGURE 4. Portions of two non-infected ML cells presenting different aspects of the special pinocytotic vesicles (*V*). Some contain an amorphous substance. The vesicle designated by an arrow has its limiting membrane partially disrupted. × 45,000.

stained the sections for 60 minutes with a saturated solution of uranyl acetate in 50 per cent alcohol and for 30 minutes with lead citrate (66). This method has given very good and constant results. The observations and the electron micrographs were made in a Siemens Elmiskop I working at 60 kv.

OBSERVATIONS

Fine Structure of the Uninfected ML Cells

The ML cells (Fig. 1) are round or oval and approximately 80 to 120 μ in diameter. They possess one or two nuclei centrally located which present some variation in size and shape from cell to cell. The nuclear envelope as usual is formed by two membranes with pores, and the nucleoprotein consists of a granular or filamentous component randomly distributed. In each nucleus one or two nucleoli are habitually seen.

In the cytoplasm, the Golgi complex is apparent generally in a juxtanuclear position (Fig. 1). Mitochondria, ribosomes, rough membranes of the endoplasmic reticulum, and occasionally small lipid inclusions are diffusely scattered through the cytoplasm.

Another cytoplasmic component observed in variable number in almost all the cells is round or oval dense corpuscles of different dimensions (Fig. 1). They are limited by a membrane and have a dense, generally homogeneous matrix in which myelin-like structures are sometimes observed. In some cells fine fibrils 70 to 75 A in width have been observed in the cytoplasmic matrix. Their amount and distribution is variable. They can be irregularly distributed among the cell organelles, but sometimes they are concentrated into oriented bundles near the nuclear membrane.

In a small percentage of the cells, dense granules 200 A in diameter with the staining characteristics of glycogen granules are observed scattered among the cytoplasmic organelles.

At some points of the plasma membrane a

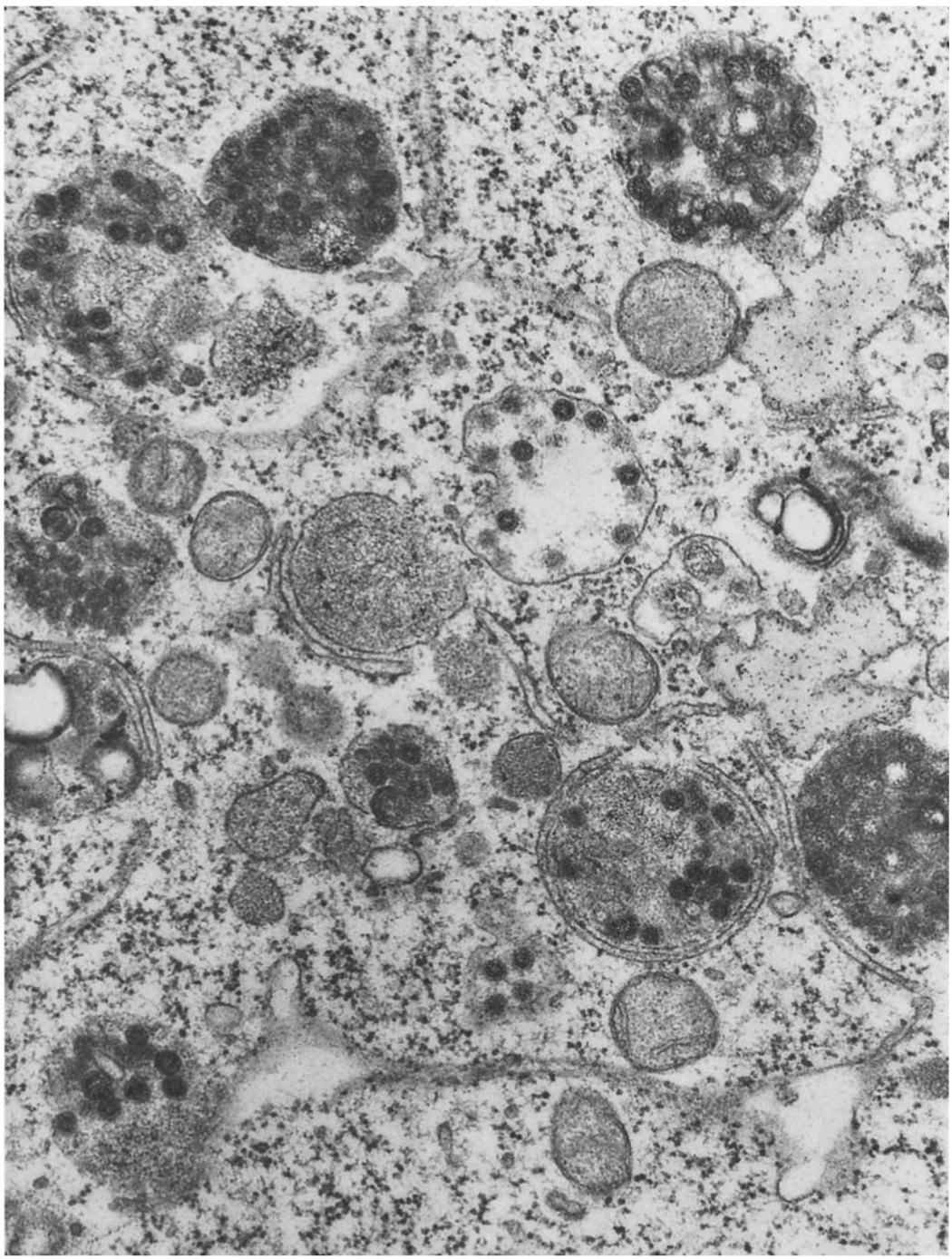


FIGURE 5. Part of the cytoplasm of an ML cell infected with hepatitis virus, showing different aspects of the dense bodies containing virus. The appearance of the particles inside the corpuscles is variable. × 45,000.

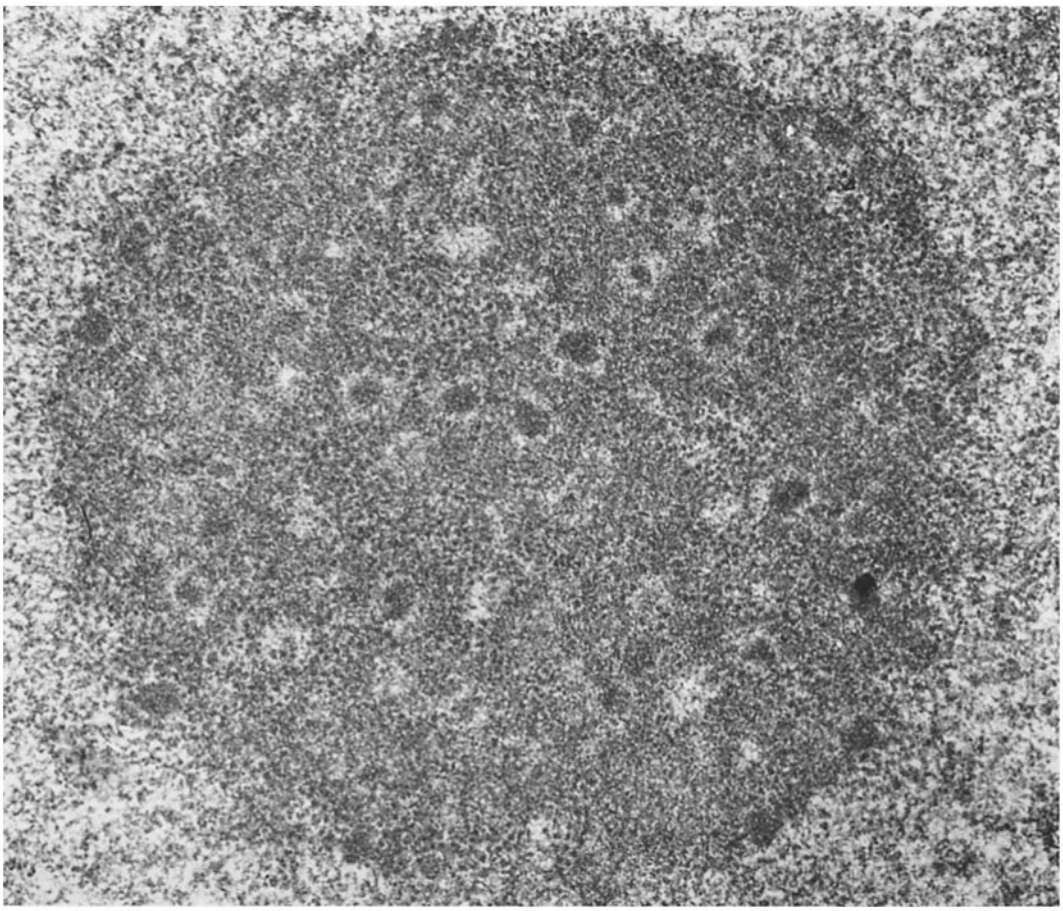


FIGURE 6. Large nucleolus of an ML cell from an infected culture, showing numerous dense spots. × 40,000.

peculiar differentiation 1200 to 1600 A long is frequently observed, characterized by a denser aspect of the cell membrane which is covered on the cytoplasmic side by a dense fibrous material. Some of these formations are observed invaginated into the cytoplasmic matrix (Fig. 2). Special cytoplasmic vesicles presenting a limiting membrane with the same constitution as these infoldings have been observed near the cell surface and in other regions of the cell. All the transitions between the infoldings and the cytoplasmic vesicles have been found (Fig. 4). They are similar to the "cell pits" described in liver cells (68) and in the thymus of chickens with myeloblastosis (25).

Although we have seen these special vesicles in various regions of the cytoplasm in our material, they are observed in significant amounts near the Golgi zone. Frequently we have noted vesicles of this type with their membrane partially disrupted and their contents lying free in the cytoplasm (Fig. 4).

Another peculiarity seen in the controls as well as in the infected cells is the presence of virus-like particles attached to or in the process of budding from the plasma membrane (Figs. 1 and 3). They are spherical in shape with an average diameter of 100 mμ. Their nucleoid, 60 mμ in diameter, is electron-transparent, and it is limited by an electron-opaque membrane. The space between this internal membrane and the outer membrane is occupied by a dense material, and sometimes in this region an intermediate membrane is identifiable. The outer surface of the particle is covered

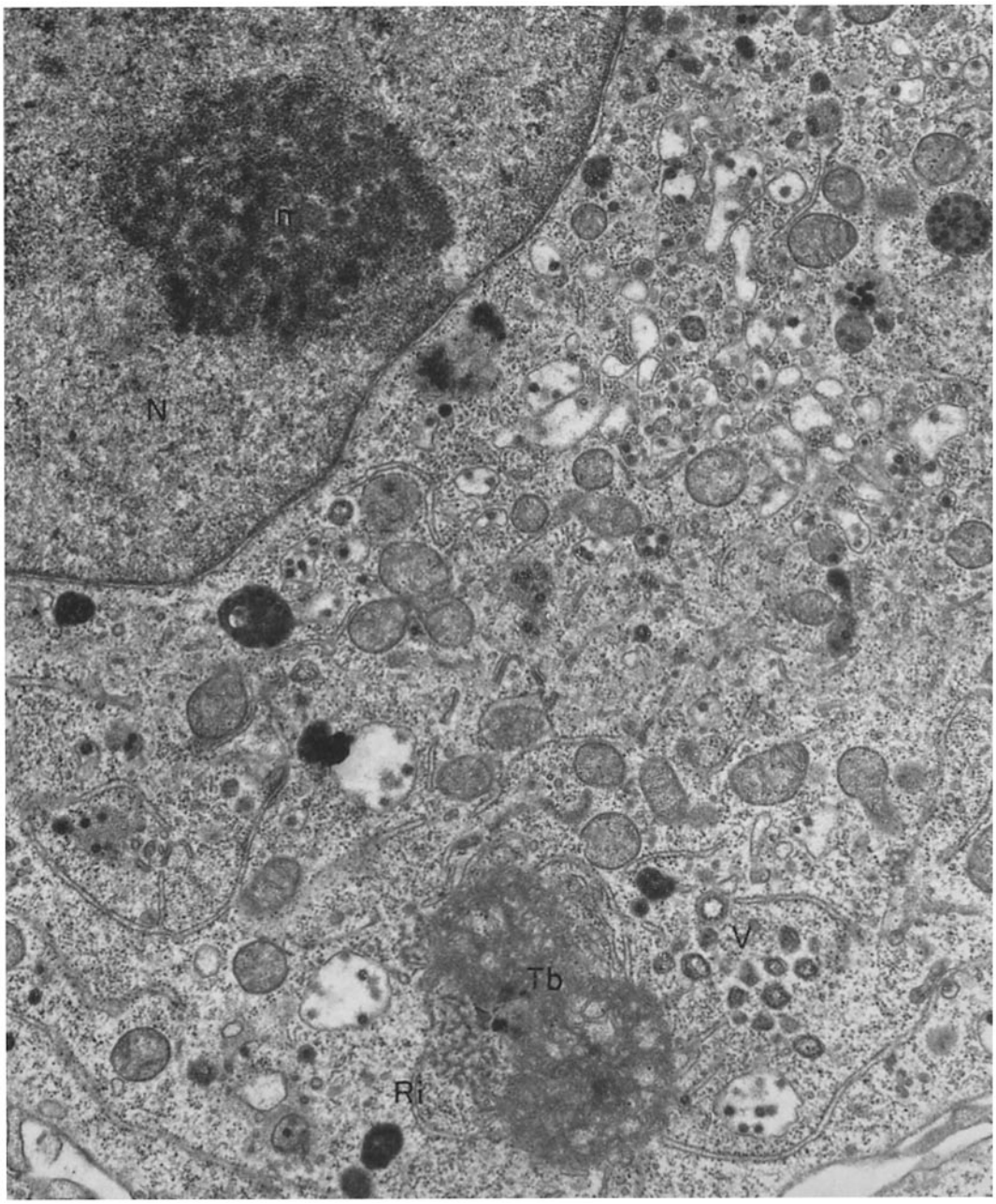


FIGURE 7. Portion of the nucleus (N) and cytoplasm of an ML cell from an infected culture. Numerous vacuoles and dense bodies containing virus are seen in the cytoplasm. In the lower part of the micrograph are seen a tubular body (Tb), a small reticular inclusion (Ri), and associated vacuoles (V). × 20,000.

by "spicules" similar to those referred to by Zeigel (76) in the particles of the chicken pancreas agent.

These virus-like particles are seen in almost all the cells observed, one to four particles per cell section, generally budding from the plasma membrane. Occasionally similar particles are also seen inside cytoplasmic vesicles.

Cells Infected by the Mouse Hepatitis Virus

The early association between the hepatitis virus and the ML cells was observed in the samples taken within 1 hour after the culture inoculation. In these cells the virus is present in the outer surface of the plasma membrane sometimes partly enclosed by a cell process or inside cytoplasmic vesicles near the cell surface.

In the samples taken 2 to 3 hours after the culture inoculation virus particles are found in increasing numbers inside dense cytoplasmic corpuscles (Fig. 5). These dense bodies are spherical and range in size from 400 to 700 mμ. They are bounded by a membrane in which, when cut normally to its surface, the components of the unit membrane are recognizable. In the preparations fixed with potassium permanganate the matrix of the dense bodies is less dense and their content of virus particles more evident (Fig. 14).

The number of virus particles per dense body varies greatly; some have just one or two particles, but others are completely packed with them. The appearance of the particles inside these corpuscles is also variable (Fig. 5). In some only intact particles are seen, but in others the particles are partially disintegrated (Fig. 5). Dense bodies containing structures similar to virus membranes are also observed.

During the first 5 hours after infection the changes observed in the ML cells, besides those already described, are slight increases in the number and size of the pseudopodia, in the number of intracytoplasmic vesicles and vacuoles, and in the number of dense bodies with virus. The nucleoli of the infected cells are frequently hypertrophied and present dense spots on the nucleolonema (Fig. 6).

In the cells from the samples taken 7 hours and more after the inoculation two organized structures never noticed in the controls or, to our knowledge, in any type of normal cell are observed in the cytoplasm (Fig. 7). These structures, almost always associated, are of two type: one, that we have called the reticular inclusion, is composed of threads of a dense filamentous material disposed in a reticular pattern and was the first to be noticed; the other, named tubular body, consists of membrane-limited tubules arranged in a very complex pattern.

The reticular inclusion (Fig. 8), although it varies in size and shape from cell to cell, is usually round or oval and is between 1 to 2 μ in diameter. Its threads are 250 to 400 A in width and are composed of a dense matrix wherein are dispersed dense granules approximately 35 A in diameter (Fig. 9). We have observed one to three of these structures per cell section.

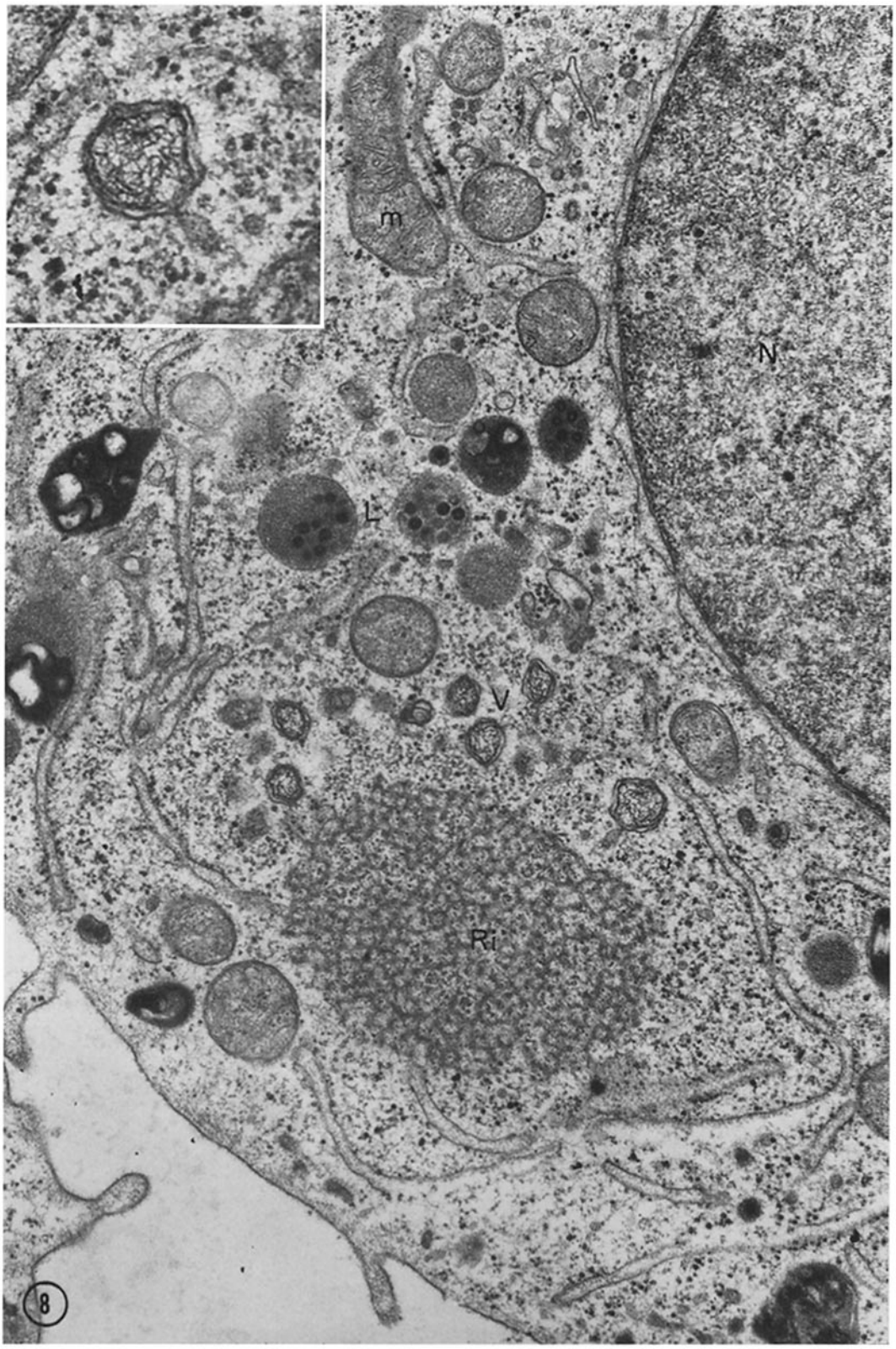
Always present in the vicinity of the reticular inclusion and generally disposed around it are several vacuoles with a diameter of about 200 mμ (Fig. 8). They have a dense limiting membrane 50 A thick in which at some points the three layers of the unit membrane are recognized.

These vacuoles are generally empty but sometimes there is seen in their interior a coiled filament 30 A in width (Fig. 8 *a*).

The tubular body (Figs. 10 and 11), which is composed of tubules with a medium diameter of approximately 160 to 250 A, has a round or oval outline in the thin sections and is between 1 to 1.7 μ in diameter. The form of the whole inclusion is spherical. The elements of the tubular body are frequently in continuity with a system of membrane-limited tubules and cisternae that develop in the cytoplasm of the infected cells (Fig. 10). With the progression of the infection the system of tubules develops and occupies progressively larger zones of the cytoplasm.

FIGURE 8. Portion of the nucleus (*N*) and cytoplasm of an ML cell infected with mouse hepatitis virus. In the cytoplasm is seen an inclusion constituted of threads of dense material disposed in a reticular pattern (*Ri*). Around this reticular inclusion are observed vesicles containing a coiled filament (*V*). Note several dense corpuscles containing virus or myelin-like structures (*L*). × 32,000.

In the insert one of the vesicles associated with the reticular inclusion is shown at higher magnification. × 64,000.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The tubules or cisternae are 310 to 370 A wide (Figs. 10 and 12), are limited by smooth membranes, and their lumens are occupied by a dense substance. Free in the cytoplasmic matrix separating the cisternae are numerous dense granules 150 to 200 A in size interpreted as ribosomes. Virus particles are seen inside the cisternae or in contain numerous virus particles are observed in the cytoplasm. In these stages the reticular inclusion and the tubular body are seen less frequently. Also the Golgi apparatus, which is so evident in the uninfected cells, is not apparent. The possibility exists that it has been transformed during the virus infection.

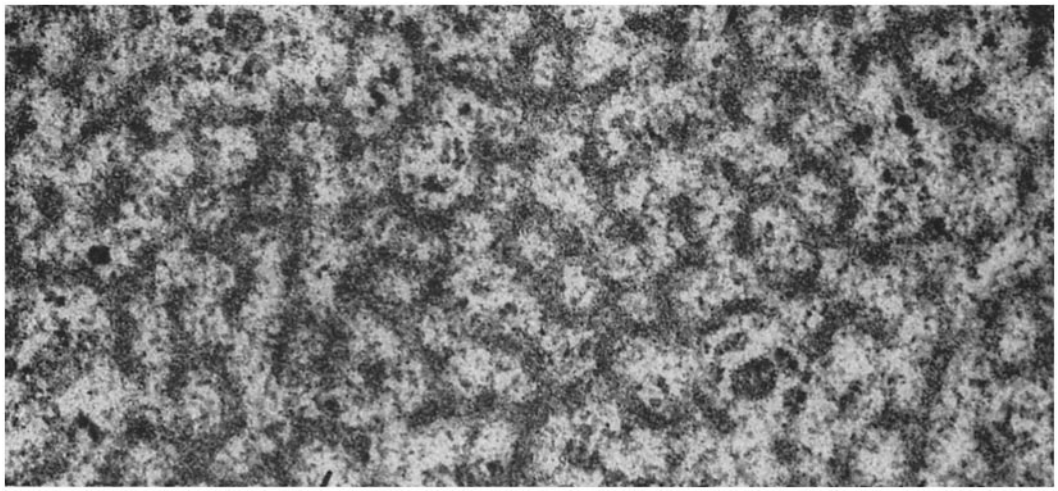


FIGURE 9. High magnification of a reticular inclusion observed in the cytoplasm of an infected cell. × 110.000.

the process of budding from the limiting membranes into the cisternae (Fig. 12).

The virus particles have a circular outline and a median diameter of 75 mμ. Their nucleoid, 55 mμ in diameter, is formed by dense granules and rods composing a ring which limits a central electron-transparent space. The limiting membrane of the virus, 30 A thick, is separated from the nucleoid by a clear space 80 A in width. The outer surface of the virus is covered by a layer of moderately dense material.

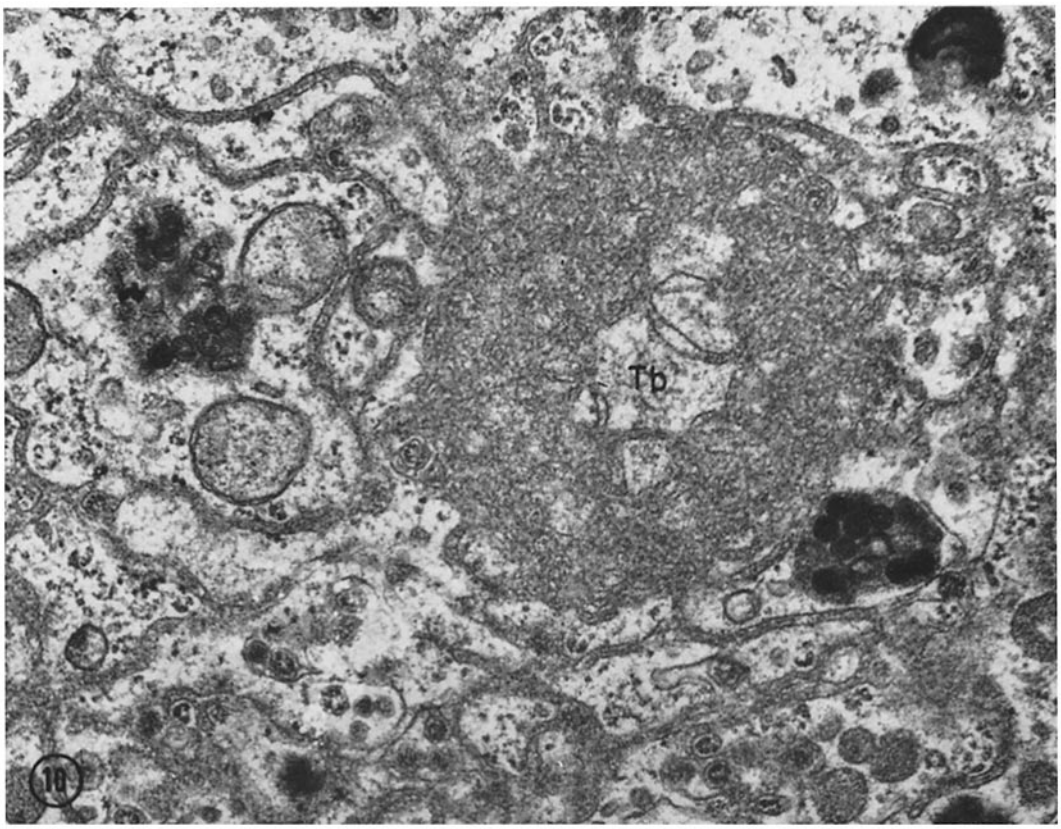
In the cells taken from the samples 14 to 20 hours after the virus inoculation, large inclusions consisting of cisternae (Fig. 13) whose lumens

In preparations fixed with potassium permanganate the reticular inclusion, tubular body, and the system of tubules and cisternae are clearly seen and their relations are evident (Figs. 14 and 15). Inside the cisternae the virus particles are observed but their nucleoid is not seen (Fig. 15). In these preparations we sometimes noted between the two components of the nuclear membrane round formations which might correspond to virus particles. This point is not yet clear and needs further investigation.

In the samples taken 20 hours or more after the inoculation numerous cells present evident signs of degeneration (Fig. 16). Their nuclei have very

FIGURE 10. Part of the cytoplasm of an infected ML cell showing an inclusion (*Tb*) formed by closely interwoven, membrane-limited tubules (tubular body). Note its relation with the double-membraned system developed in the cytoplasm of these cells after inoculation of the hepatitis virus. × 60,000.

FIGURE 11. Higher magnification of a tubular body showing its complex organization. In its periphery are seen several incompletely formed viruses (arrows). × 60,000.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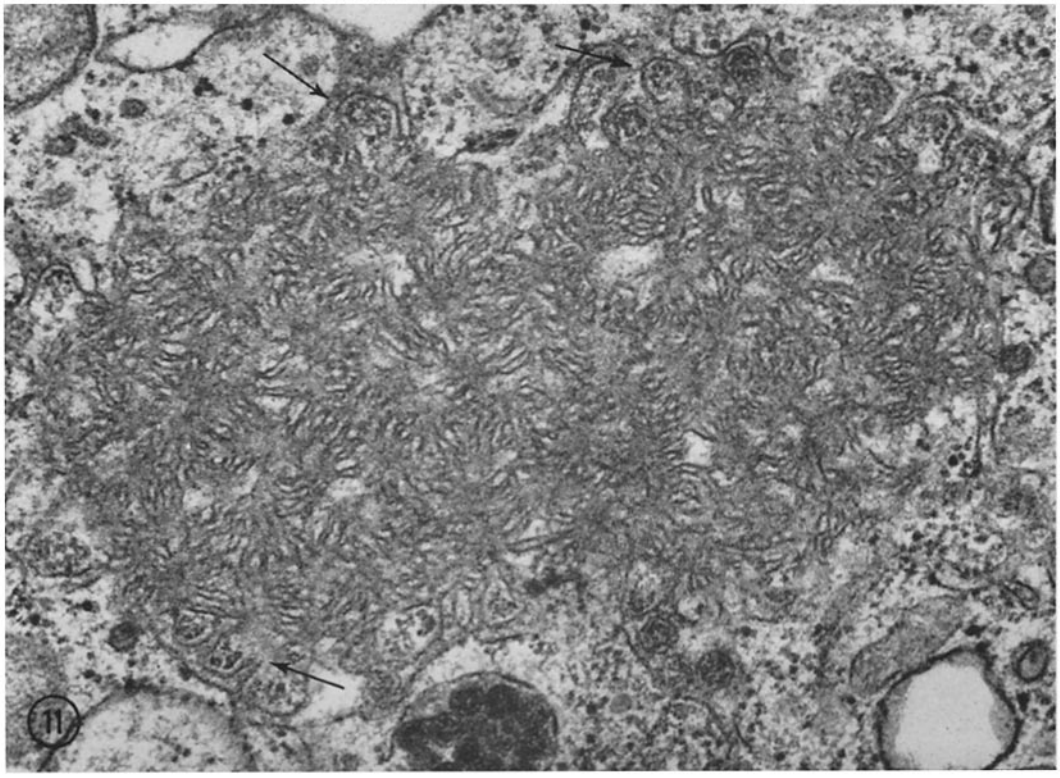
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dense marginated chromatin, and their cytoplasm shows a complete lack of organization. In the cytoplasm of the degenerated elements (Fig. 17) accumulations of dense granules 150 A in diameter (ribosomes?) and inclusions having the appearance of lipids are observed. Virus particles are seen inside vacuoles or cisternae or lying free. Around these dying cells are frequently seen masses of cytoplasm originating from their disruption (Fig. 16). They contain abundant dense granules.

DISCUSSION

Before discussing the data concerning the infected cells, we need to comment on three points from the observations made on the controls: (*a*) the presence of virus-like particles in these cells; (*b*) the localized plasma membrane specializations, their infoldings, and related cytoplasmic vesicles; (*c*) the bundles of 70 to 75 A fibrils existing in the cytoplasm of some of the cells.

(*a*) The virus particles observed attached to or budding from the plasma membrane of the ML cells are, by their morphology and process of formation, similar to the "immature" C particles described in the murine leukemias (16).

Not long ago, Dales and Howatson (13) identified morphologically similar particles in their cultures of Earle's strain L cells. Specific biological activity could not be associated with these entities. In our experiments the particles observed apparently did not interfere with multiplication of the mouse hepatitis virus in the ML cells. However, their presence in this cell line provides an example of the caution which must be exercised in drawing conclusions from observations of virus-like particles in cultured cells employed in the search for new viruses. As Bernhard (3) points out, "the morphologist is ahead of the biologist and has many unlabeled virus particles to sell."

(*b*) The localized differentiations observed in the plasma membrane of the ML cells as well as their related invaginations and special cytoplasmic vesicles are morphologically identical with the formation reported by Roth and Porter (68) in hepatic cells and by de-Thé *et al.* (25) in blast-like elements in the thymus of chickens with myeloblastosis. We have recently observed similar structures in several other types of cells. These observations all suggest that such formations have a general significance in cell physiology. A dynamic interpretation provides two possibilities: they may represent different steps of a process of incorporation into the cell, or they may be related to a phenomenon of cellular excretion. Roth and Porter (68), on the basis of their observations on the mosquito oocyte, concluded that the membrane differentiations and their infoldings are special sites for protein uptake. However, additional investigation is required to permit precise conclusions concerning the functional significance of these structures. For this purpose tissue cultured cells provide a good study system.

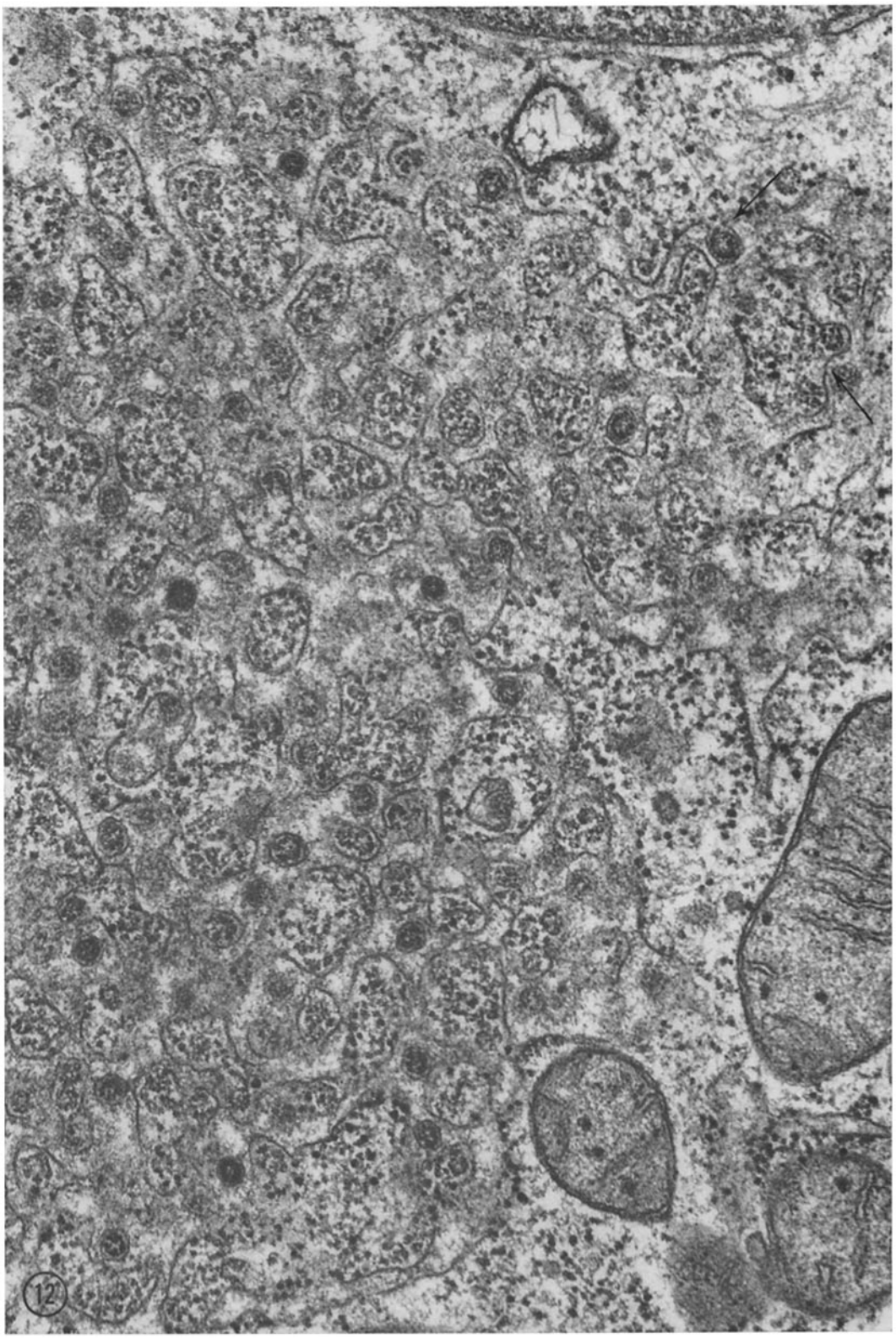
A point of interest that emerges from the present observations is the localization of these *special vesicles* in the Golgi zone. Although the significance of this observation can at the moment be only a matter of speculation, a possible relationship is indicated between these vesicles and the function of the Golgi apparatus.

(*c*) The fibrils 75 A in diameter observed in bundles in the cytoplasm of some of the ML cells (controls and infected) are identical to the fibrils described in normal or virus-infected cells by several authors (14, 24, 54). Dales and Siminovitch (14) have suggested that similar fibrils which they described in L cells could be a response to injurious stimuli.

Another plausible hypothesis about their significance is that of De Petris *et al.* (24) who suggest that they are contractile in nature and probably related to cell movements.

The data reported herein show that the hepatitis virus enters the cell by phagocytosis. This mechanism of cell penetration by virus was first suggested by Fazekas (32) who named the process viropexis. More recently, utilizing the electron microscope, Dales and his colleagues have

FIGURE 12. Cytoplasm of an ML cell infected with mouse hepatitis virus, showing the double-membraned tubules or cisternae which differentiate in the cytoplasm of these cells 15 to 18 hours after virus inoculation. In their lumen an amorphous substance and numerous virus particles are observed. Between the cisternae small dense granules are dispersed. In the upper right corner of the micrograph the arrows indicate virus particles in the process of budding into the cisternae. × 60,000.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demonstrated that it is by viropexis that the vaccinia virus (10, 14), adenovirus (9), and influenza virus (11) are incorporated into cells infected *in vitro*. The same mechanism has been reported by Heine *et al.* (42) in the virus of avian erythroblastosis, by Zamboni and Biberfeld (75) in the polyoma virus, and by Mussgay and Weibel (57) in the Newcastle disease virus. Tanaka *et al.* (73) who studied a mouse hepatitis virus *in vitro* also concluded that it is by engulfment that this virus is incorporated by the cells.

The data available at present suggest that viropexis is a general mechanism of cell penetration by a virus and probably one of the first steps in cell infection. However, from the morphological evidence, we cannot eliminate the possibility that, simultaneously with the entry of intact particles, the cell is also penetrated by disassembled "infectious material" more directly related to the initiation of the cellular infection.

According to the observations presented, after the mouse hepatitis virus enters the cell by viropexis, a process which generally does not involve more than two particles, it is observed inside dense cytoplasmic corpuscles. The number of virus particles in each corpuscle is variable, but most corpuscles contain large numbers. This indicates that after incorporation the virus is concentrated inside these bodies.

The localization of virus particles in dense cytoplasmic corpuscles is an observation reported after cellular infection with different types of virus. Bonar *et al.* (5) and de-Thé *et al.* (26) described in avian myeloblasts, cultivated *in vitro*, the presence of virus in dense corpuscles. Haguenau *et al.* (40) noted dense bodies with virus in Rous sarcoma cells cultivated *in vitro*. Dales (9) reported the presence of adenovirus inside dense cytoplasmic corpuscles in HeLa cells infected *in vitro*.

The process of accumulation into cytoplasmic bodies is not a special reaction of the cell to the entry of a virus, and has been repeatedly reported after cellular phagocytosis of different types of extraneous material (2, 31, 41, 60, 61, 64). Evidence has been accumulated that the dense cytoplasmic bodies, generally described as being associated with cell phagocytosis, are identical to Straus's phagosomes (70, 71) or de Duve's lysosomes (20, 21, 59).

The concentration of virus in the dense cytoplasmic corpuscles raises the question of how they enter and are accumulated in these corpuscles. One can speculate that vesicles, containing virus, are incorporated in preformed dense corpuscles by fusion of their limiting membranes, but another possibility is that several phagocytotic vesicles containing particles fuse together and a dense corpuscle is subsequently formed by progressive concentration of the dense matrix in the vesicle. A similar mechanism has been postulated by Farquhar and Palade (31) for the dense bodies containing ferritin observed in the visceral epithelium of rats previously inoculated with a solution of ferritin.

The most interesting observations on hepatitis virus-infected ML cells were made 7 hours after their exposure to the agent. At this time, two formations, the reticular inclusion and the tubular body, were noticed. The reticular inclusion, the first of the two to appear, resembles in some characteristics the cytoplasmic inclusions observed in other virus infections. For example, inclusions of a filamentous substance were reported to develop in L cells after their infection with mengovirus (Dales and Franklin, 12) or with vaccinia virus (Dales, 10). It is in these areas that the immature vaccinia virus particles were later observed.

Dales calls the dense material viroplasm and interprets these inclusions as "factories" where the viral nucleoprotein is synthesized. Dense inclusions of the same type were described long ago with different names (matrix area, ground substance, viroplasm) in cells infected with viruses, especially those from the pox group (4, 27, 33, 35, 47, 53, 62, 67, 74). In all these observations it is evident that the final virus formation is related to the dense cytoplasmic inclusions. In our experiments the relation between the inclusions observed and the virus is difficult to establish because the virus formation is never observed in these areas.

Recently Kuhn and Harford (46) found cytoplasmic inclusions in HeLa cells infected with parainfluenza virus and observed that these inclusions consist of dense areas of filaments and granules which they claim to be viral ribonucleoprotein. They never observed virus particles in these areas and they suggest that the absence of complete virus can be explained by the hypothesis that the "viral capsid is only acquired when the viral nucleoprotein passes through the cell membrane."

In our material we can also hypothesize that the reticular inclusion is the site where the viral nucleoprotein replicates, although the final formation of the virus takes place in the tubules and cisternae by budding from their membranes.

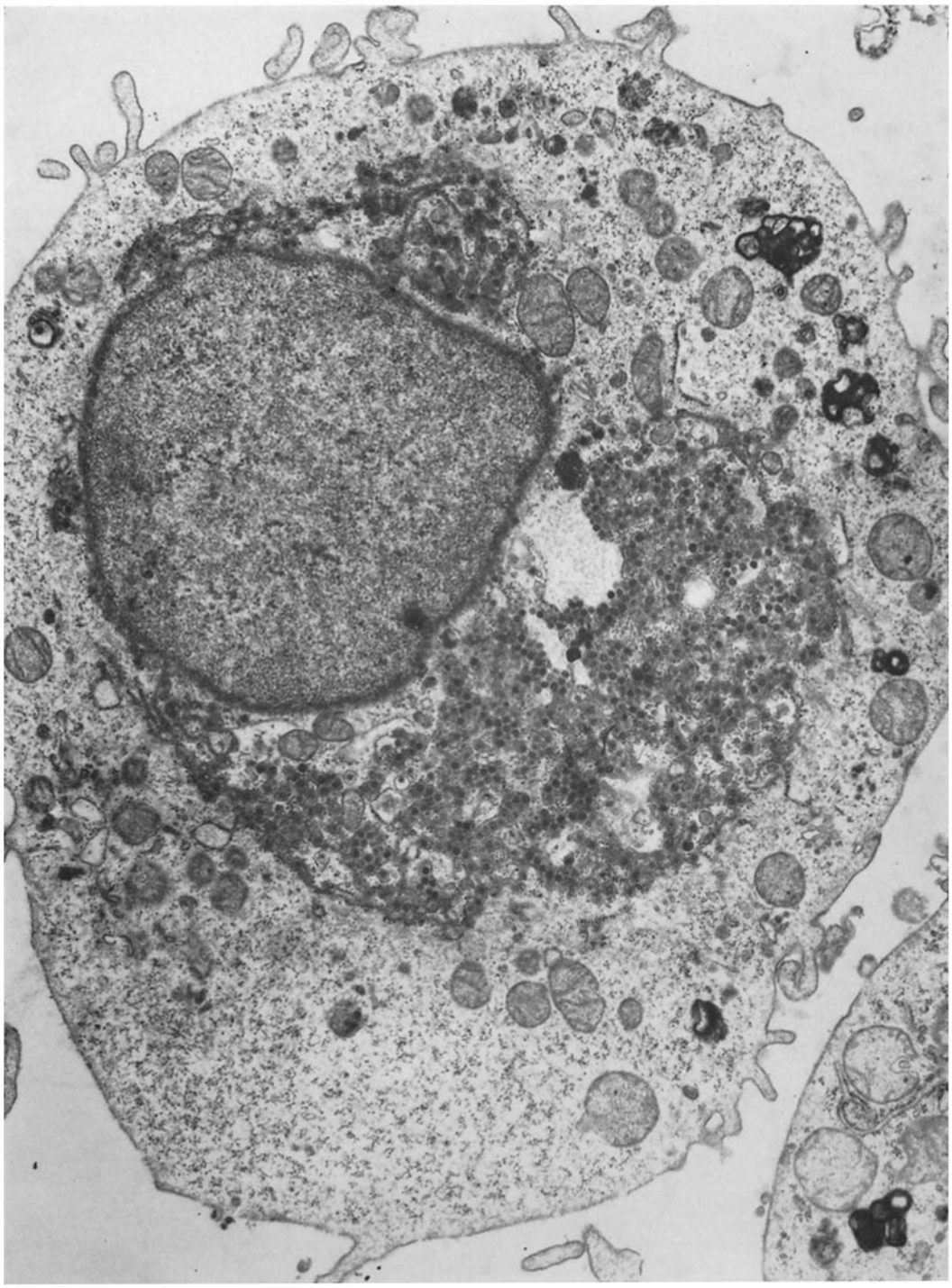


FIGURE 13 ML cell sampled 20 hours after inoculation with mouse hepatitis virus. A big cytoplasmic inclusion formed by tubules and cisternae containing virus is observed in the cytoplasm. × 15,000.

Another possibility is that the reticular inclusion is a precursor material from which the tubular body originates.

The significance of the vesicles observed in close relationship with the reticular inclusion is not clear. Similar vesicles have been observed in the vicinity of the cytoplasmic inclusions in vaccinia virus-infected cells (10) as well as near the dense inclusions of parainfluenza virus-infected cells (46). Dales interpreted the vesicles noted in the vaccinia virus-infected cells as virus "shells" remaining in these areas after the release of the DNA core. In our material this interpretation, although not excluded, is unlikely because of the great differences in size between the vesicles and the hepatitis virus.

The tubular body, which is usually found in the vicinity of the reticular inclusion, is a peculiar structure which, to our knowledge, has not been previously detected in normal or infected cells. Its origin is obscure, but it is evident that it is always connected with the system of tubules and cisternae differentiated in the infected cells. It can be interpreted as the center from which the development of this membranous system is initiated.

As a tentative interpretation of the significance of the reticular inclusion, the tubular body, and their relationship to each other, we can speculate that the reticular inclusion represents the fabric where the synthesis of the viral nucleoprotein takes place and that the tubular body is the center from which is developed the membrane-limiting tubules and cisternae where the final formation of the virus is realized.

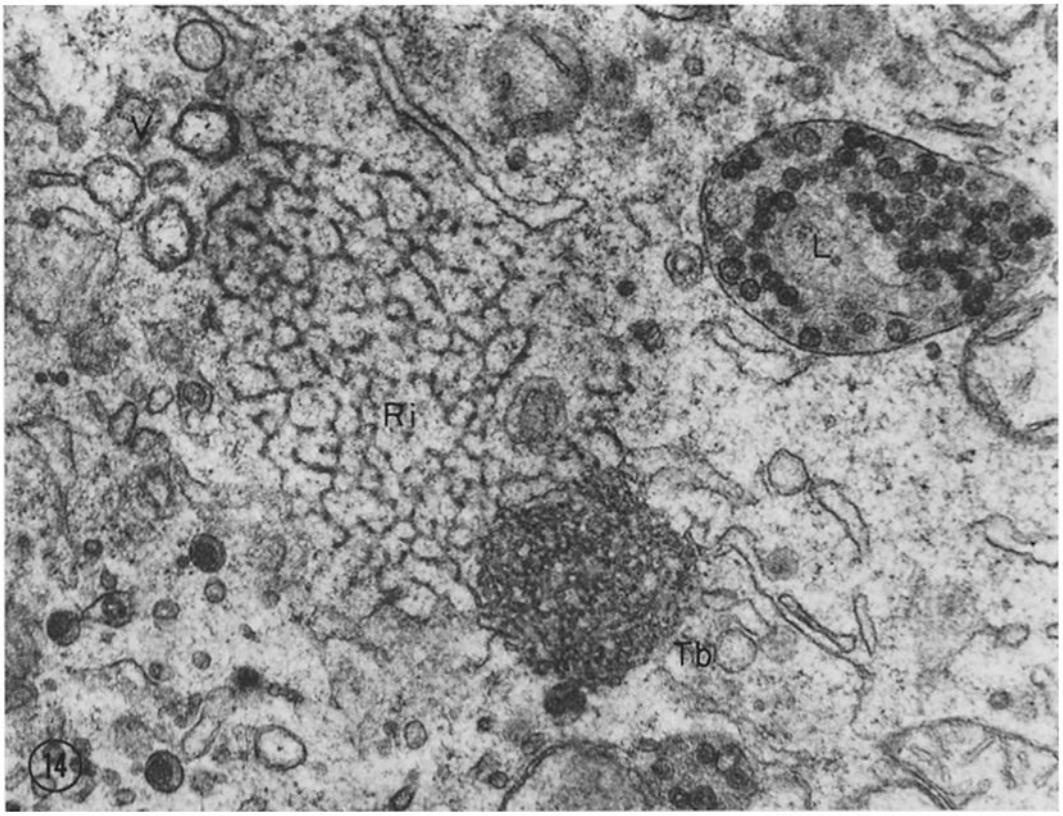
When we compare the ML cells from the controls with cells sampled 14 to 20 hours after virus inoculation, the most impressive element is the existence of the developed system of membrane-limited tubules and cisternae in the cytoplasm of the infected cells. The appearance of membranous structures, vesicular or tubular, in the cytoplasm of virus-infected cells is a phenomenon that has been reported in the literature by several authors (4, 12, 39, 54). Dales and Siminovitch (14) interpreted these formations as "an example of cellular response in a variety of animal cells which is elicited not only by virus, but also by toxic agents," and compared them with the membranous structures appearing after different experimental conditions. The membrane-limited tubules and cisternae described in our experiments are much more regular formations and have been interpreted from their morphology as belonging to the agranular reticulum (7, 63). Unfortunately, the physiological significance of the agranular reticulum, which has been lately discussed in several papers (8, 29, 65), is not yet clear.

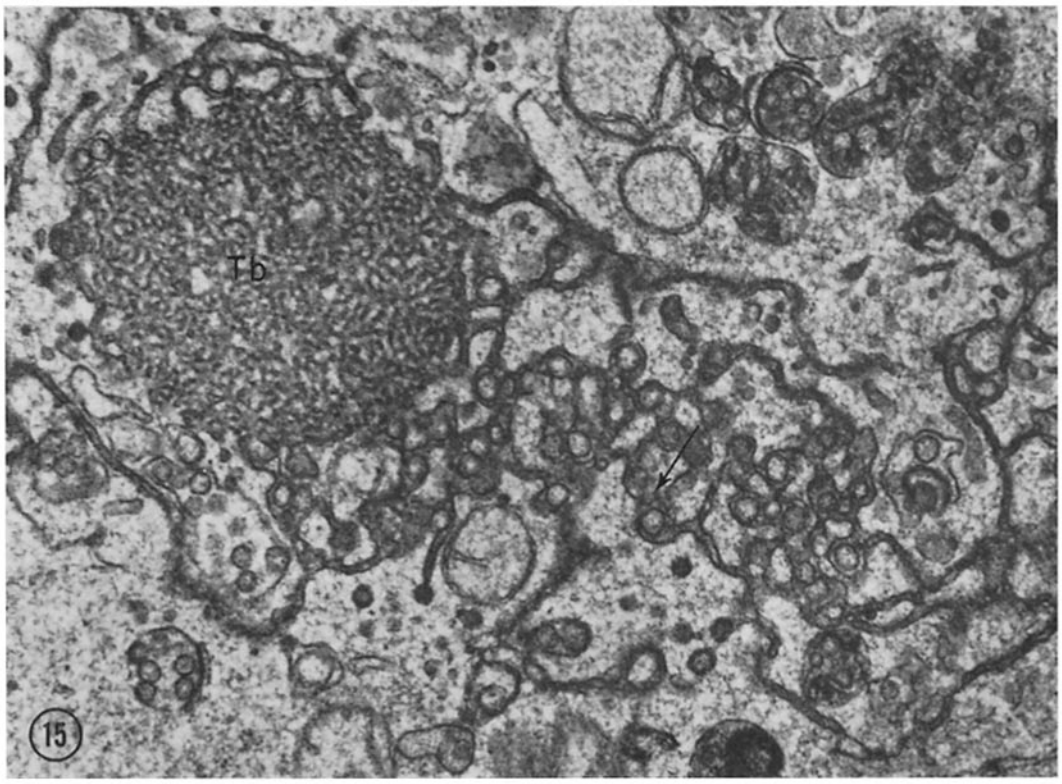
In our experiments the tubules and cisternae which contain a dense substance elaborated or accumulated therein are intimately related with the virus formation. As the virus particles bud from the cisternae limiting membranes and are released into the lumens, their outer membranes become covered by the dense substance present in the cisternae.

The formation of virus or virus-like particles in vesicles or cisternae of the endoplasmic reticulum is a process reported several times in the last few years. Friedlaender and Moore (34), and Adams and Prince (1) observed such formation in the endoplasmic reticulum of the Ehrlich ascites tumor cells. Dalton and Felix (17) described virus-particles inside intracytoplasmic vesicles in S 37 tumor cells. Type A particles were reported inside or budding from the limiting membranes of the endoplasmic reticulum in plasma cell tumors (19) and in a fibroblastic sarcoma (38). In murine leukemias it was established that the virus forma-

FIGURE 14. Portion of the cytoplasm of an ML cell infected with mouse hepatitis virus. Fixation with potassium permanganate. The relationship between the tubular body (*Tb*), reticular inclusion (*Ri*), and associated vacuoles (*V*) is clearly seen. A dense body containing virus particles is also evident (*L*). × 35,000.

FIGURE 15. Portion of the cytoplasm of an ML cell infected with mouse hepatitis virus. Fixation with potassium permanganate. In the upper part of the micrograph a tubular body (*Tb*) and its relation to the system of the cytoplasmic tubules are clearly seen. Inside the tubules small vesicles corresponding to virus particles are indicated by an arrow. × 32,000.


V
L
Ri
Tb
14


Tb
15

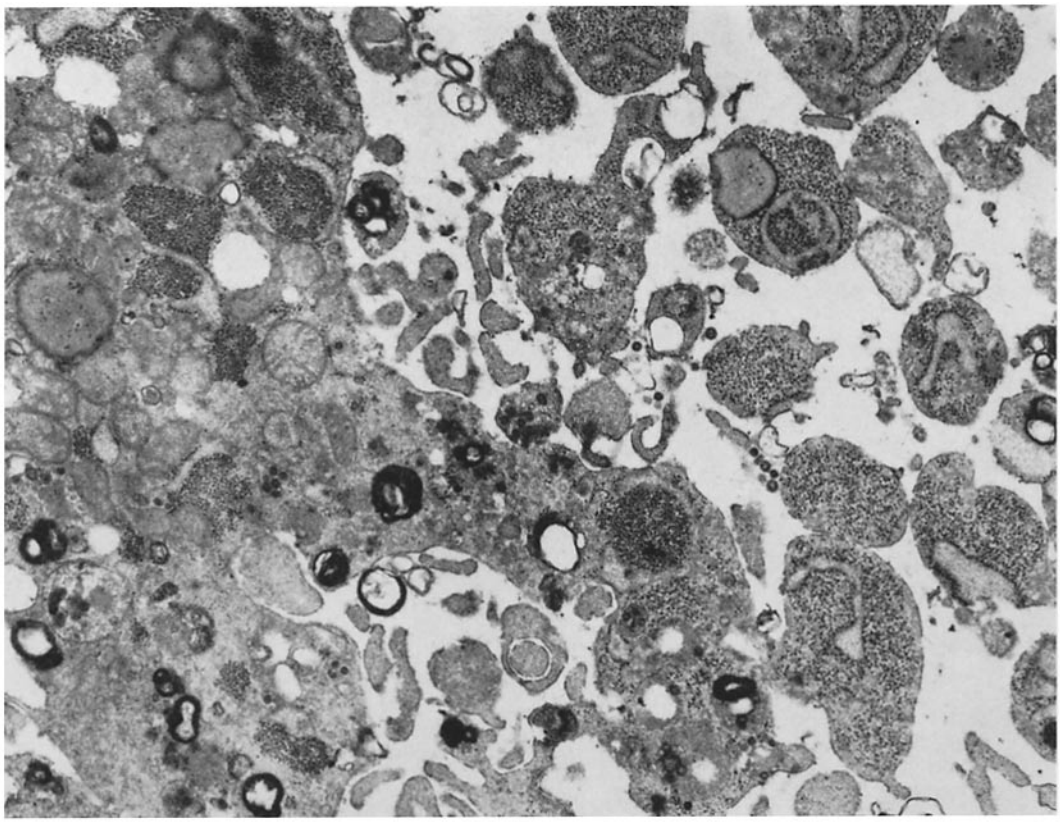


FIGURE 16. An infected ML cell presenting evident signs of degeneration. In the cytoplasmic fragments are observed accumulations of dense granules, and inclusions with the appearance of lipids. × 20,000.

tion takes place in the limiting membranes of the intracytoplasmic channels of the megakaryocytes (18, 22, 23).

In all these cases it was not established that there was any difference in the organization and amount of the endoplasmic reticulum (granular or agranular) before and after the presence of the virus. Only in the murine leukemias is it known that the system of intracytoplasmic channels from which the virus buds is a normal component of the megakaryocytes. More recently Granboulan and Wicker (37), reporting the formation of a simian virus in hamster fibroblasts cultivated *in vitro*, inferred that the appearance of the virus nucleoid "est accompagnée par la formation de systemes membraneux trés developpés, plus au moins associés avec les grains RNP cytoplasmiques."

In our experiments it is evident that the tubules and cisternae of the endoplasmic reticulum are developed after the entry of the virus. They grow with time following infection and are intimately associated with the virus formation.

In the light of this observation it seems appropriate to conclude that the hepatitis virus, once integrated into the cell, is able to originate the differentiation of a cellular system never observed in the controls. Speculating about the differentiation of this system, we can surmise either that it is a result of information transferred to the cell by the virus material or that it is the development of a cell potentially which is activated after the virus infection.

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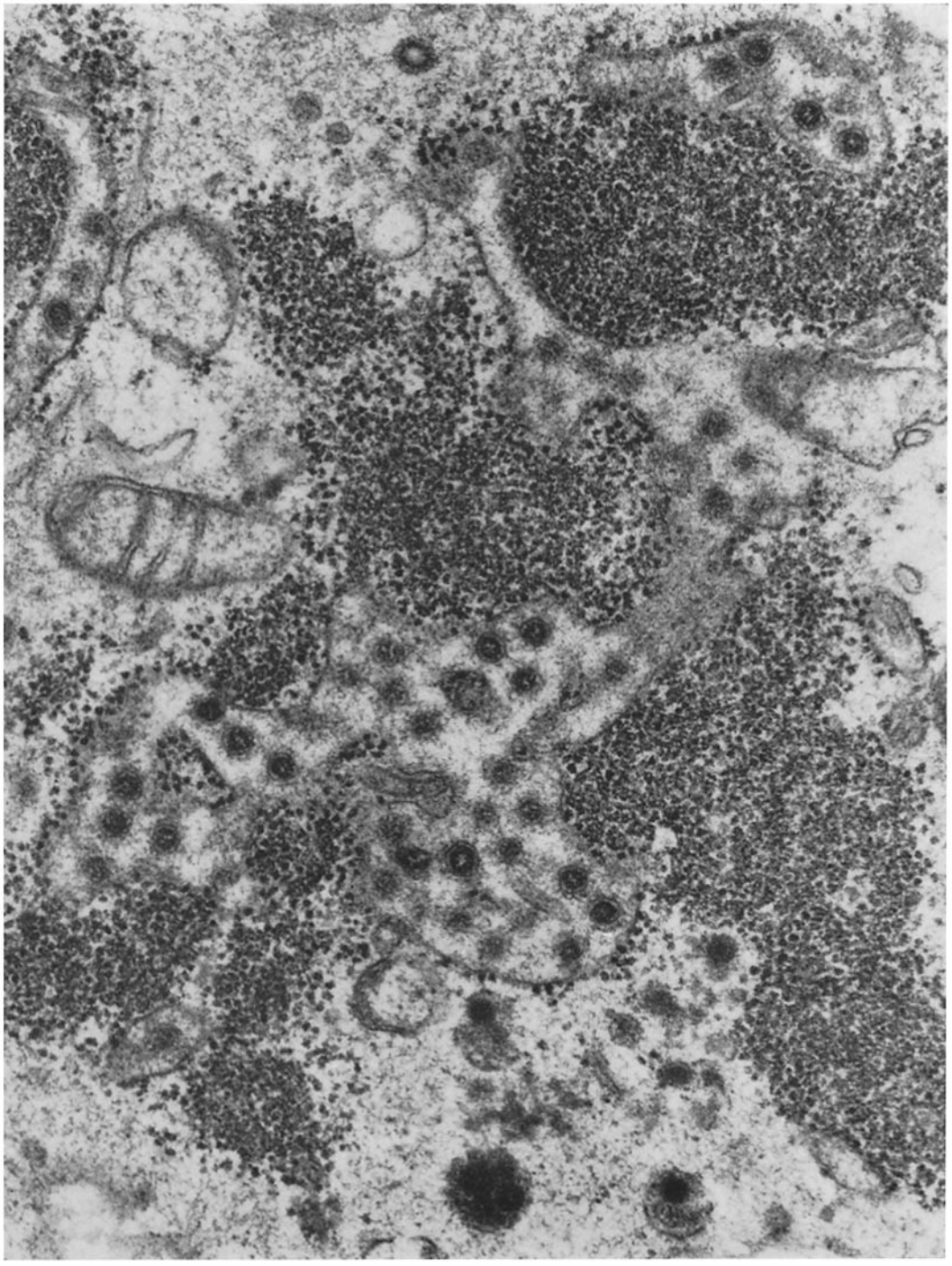

FIGURE 17. Portion of the cytoplasm of a partially disintegrated, infected ML cell. Numerous virus particles are observed inside dilated cisternae. Accumulations of dense granules are seen. × 60,000.

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