INTRACELLULAR FORMS OF MENINGOPNEUMONITIS VIRUS

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Plates 50 to 53

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Viruses belonging to the psittacosis-lymphogranuloma group have morphological peculiarities which have made them the subject of controversy for many years. They are so large that the desirability of including the group with the viruses has often been questioned. Bedson and his colleages (1–4) described a life cycle for the virus of psittacosis based on the observation of intracellular forms even larger than the elementary bodies. These structures appeared as a graded series of spheres in the cytoplasm, suggesting a high degree of organization and, possibly, binary fission at some stage of development. Dense cytoplasmic inclusions (plaques, morulae) were often associated with the spherical forms.

A number of investigations of the morphology of psittacosis and other members of the group have been reported, and the problem was reviewed recently by Meyer (13). It was felt that a study by means of electron microscopy of virus-infected tissues should provide new information. Consequently, chorioallantois infected with meningopneumonitis virus, a representative member of the psittacosis-lymphogranuloma group, was selected for examination at the electron microscopic level.

Methods

Inoculation[1].—An allantoic fluid suspension of the "Cal 10" strain (18) of meningopneumonitis virus was inoculated on the chorioallantoic membrane of 12 day eggs. The virus was from the 11th egg passage (allantoic cavity) and a heavy dose (0.5 ml. of undiluted fluid having a titer in mice greater than 10^{-7}) was used in order to have a large number in initially infected cells. Electron microscopic observations of normal membranes were made and described in an earlier study (8).

Harvest and Fixation.—Sigel, Girardi, and Allen (18) showed that new virus appeared sometime between 24 and 48 hours after the inoculation of this strain of virus into the al-

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lantoic cavity. For that reason, membranes were harvested at 48, 72, and 96 hours after inoculation. Whole membranes were removed as rapidly as possible. Small pieces, 3 to 4 mm. square, were immediately placed in buffered osmium tetroxide (15) and fixed for 4 hours.

Imbedding and Sectioning.—Fixed tissue was imbedded in a 1 to 8 mixture of methyl to butyl methacrylate (14). The blocks were sectioned with a commercial microtome (16) using glass knives (11). Sections were usually flattened by flotation on water at 45–50°C. for several hours before mounting on collodion-covered 80-mesh Cu-Ni grids.

An RCA EMU microscope equipped with an intermediate lens and a 1 mil objective aperture was used.

Suspensions.—Suspensions of elementary bodies were prepared by centrifuging the inoculum first at low speed in a clinical centrifuge for 5 minutes and then at 20,000 *g* for 30 minutes. After several washings with distilled water the pellet was resuspended in distilled water to $\frac{1}{10}$ the original volume. Final preparations were shadowed with palladium.

RESULTS

In the cytoplasm of affected cells there were round to oval structures of varying size which were assumed to be forms of the virus of meningopneumonitis. Circular structures similar to those described by Bedson and Bland (2) for the virus of psittacosis could be identified. In any one cell nearly all the forms described below were present, but not always in the same ratios to each other. The structures identified here with virus were catagorized as follows[2]:—

Forms of the Virus

(*a*) *Elementary Bodies.*—Observation of shadowed suspensions prepared by centrifuging ground membranes revealed typical elementary bodies as reported by earlier workers (7, 9, 10, 17). They were 375 to 435 mμ in diameter and resembled empty membranes flattened down over a central mass (Fig. 3). Particles identified as elementary bodies in tissue sections averaged only 250 to 300 mμ in diameter (Fig. 5).

The elementary bodies were easily recognized in sections because they were dense to electrons. An even more dense central granule could often be detected within them. These particles were numerous in dead cells at the surface of an infected membrane (Fig. 5), but apparently absent from some infected cells. Neither elementary bodies nor any other recognizable form of the virus were ever seen extracellularly in these preparations.

(*b*) *Circles 300 to 400 Mμ in Diameter.*—There were many forms slightly larger than elementary bodies and less dense to electrons in all infected cells. These structures usually contained a dense central mass occupying about a third of the area of the entire particle. Occasionally, two or three limiting zones of differing density were visible around the particle (Fig. 6). In the top layers of tissue these particles were located in spaces formed by strands or membranes which might have been remnants of larger structures (Fig. 7), but the spaces could have been cytoplasmic vacuoles. The cells having this appearance were found in the same areas where cells packed with elementary bodies were located (Fig. 5).

(*c*) *Circles 400 to 500 Mμ in Diameter.*—Particles of the 400 to 500 mμ range were dominant in fibroblasts (Figs. 10, 11). They were found (with the other types of particles) in small or medium sized vesicles in the cytoplasm. Each fibroblast usually contained more than one vesicle, unlike the ectodermal cells in which the virus was in a large single space.

A repeatedly found and characteristic feature of the 400 to 500 mμ particles were double circles joined by a continuous membrane. The membrane was constricted and gave a lem-

[2] Descriptions of the various forms are given here in terms of plane figures as observed. The character of the third dimension has not been established.

niscoid appearance reminiscent of budding yeast cells. The segments so separated were either of equal or unequal size (Fig. 1, 10, 11). Double or triple membranes or dense granules were not observed in these particles.

(*d*) *Circles 500 to 600 Mμ in Diameter.*—Circles with single membranes, low electron density, and slightly larger than the segmented type of structure were observed usually in ectodermal cells and macrophages. These forms often contained two or three dense granules similar in size to the single granules observed in the 300 to 400 mμ structures, (Fig. 1, 4, 6). Mitochondria (Fig. 6) contained many smaller granules of a similar density but their smaller size made it possible to differentiate between the two types of granules. In addition cristae mitochondriales, or remnants thereof, could be identified.

(*e*) *"Large" Circles.*—Forms circular to elliptical in shape with axes of a micron or greater were seen in ectodermal cells and macrophages. These structures were less uniform than those described above and their identity as virus forms was doubtful. Sometimes they seemed to be septate (Fig. 4), but mitochondrial christae often appear as septa. At other times the large structures were outlined by a discontinuous zone (Fig. 6, 9). The ruptures or discontinuous membrane occasionally contained small protrusions of slightly increased density (Fig. 9). The protrusions were rounded and resembled some of the smaller circles described above.

(*f*) *Forms Observed Infrequently.*—Certain structures were observed more or less rarely which were not normal to the cells and which were in close association with structures assumed to be virus. One such observation was a circle of the 500 to 600 mμ size surrounding a cluster of smaller circles (Fig. 11). The small circles varied in size and some contained dense central masses. Another infrequent observation was the presence of small granules (similar in size to the central granules of the virus) free in the cytoplasm (Figs. 1, 11). Similar particles could be found in normal tissue, especially fibroblasts.

Abnormal Cell Inclusions Not Assumed to Be Virus.—In the cytoplasm of many cells a dense, amorphous, intensely osmiophilic material could be seen. It occurred in all types of cells under study and was present in infected tissue in amounts roughly inversely proportional to the amount of virus present. In ectodermal cells and macrophages it tended to assume a round shape 1 to 2 μ in diameter (Fig. 2). Single or multiple patches of the material might be present in one cell. In fibroblasts (Fig. 8) more irregular shapes were common and they tended to be smaller. The amorphous material could be present in the absence of recognizable virus (Fig. 8) and was usually absent from cells filled with virus (Fig. 1). In cases in which both were present in one cell, (Fig. 11) the dense material was situated at random in small amounts throughout the cell.

Tissue Response

Macroscopically, membranes appeared somewhat thickened with a uniform diffuse gray or hazy appearance. No focal lesions were present at any time, and the only change during the 4 day observation period was the degree of opacity and thickness.

The tissue response as observed in stained sections was similar to that described by Burnet and Rountree (5) for psittacosis, the most striking change being the occurrence of a stratum of dense, compressed cells overlying the ectoderm, which bore a superficial resemblance to normal cornified epithelium. At 48 hours the layer consisted of cells filled with the circular form of the virus to such an extent that the cytoplasm appeared to be a lacy network. At 72 hours the cells, apparently dead, were shrunken and the virus particles were closely packed. Cell nuclei were seldom visible at this stage. By 96 hours

most of the ectoderm was entirely sloughed off and it was replaced by an exudate of leucocytes, macrophages, and fibroblasts. Virus free in the intercellular spaces was never observed.

The thickened entoderm consisted of several layers of cells, and signs of infection were present in the form of the dense osmiophilic material, especially in 72 and 96 hour membrane. Non-specific vacuolization of the outermost layer of cells (8) was present but at no time could the entoderm be said to be heavily infected. The mesoderm was thickened and moderately infiltrated by leucocytes and erythrocytes. Many immature blood cells were present near the ectoderm, and erythrophagocytosis by macrophages was common, but no virus-like particles were ever seen in or on erythrocytes. Forms believed to be virus or closely related to it were seen in mesoderm in the fibroblasts as early as 48 hours. Infected fibroblasts often assumed a rounded shape, and although the fibroblast nuclei resembled those of ectodermal cells, identification was usually not difficult because the fibroblasts characteristically displayed a prominent "endoplasmic reticulum" different from that seen in any other cell in the chorioallantoic membrane (Fig. 8).

DISCUSSION

Examination of the cells infected with meningopneumonitis virus showed a high incidence of the 500 mμ circles occurring in pairs with continuous membranes. A line of increased density traversing the constriction could be seen in some of these structures. If this configuration is part of a life cycle of the virus, these observations suggest that the particles are capable of dividing by fission at this stage.

There is a form intermediate in size between the above mentioned segmented forms and the elementary bodies. Like the elementary bodies, it has a dense central mass, and in addition, it is surrounded by two or three membranes. Elementary bodies might then represent a form derived from the 500 mμ circles through a series of condensations around a "nucleus." This reasoning is suggested by the finding that the dead cells at the top of a membrane contain elementary bodies and/or the intermediate forms. If elementary bodies are the terminal stage of virus development, then the forms associated with them in such cells most likely are in the next to the last stage.

A cycle, as outlined, would be similar to that of a spore-forming bacterium but it does not account for virus forms larger than 500 mμ. Large circles often with multiple dense granules within them, as well as suggestions of septa, were observed frequently in the ectoderm. Other structures bounded by discontinuous membranes, appeared to be ruptured. It may be that several smaller particles were formed within the large bodies. Such a process would be analogous to the multiple sporulation seen in some fungi and, accordingly, it would mean that the virus of meningopneumonitis might have two separate cycles of development. This would explain the difference in viral forms in

different cell types of the same tissue. Forms larger than 500 mμ are seldom seen in fibroblasts and the joined pairs are common. In the ectoderm, and associated cells, larger circles are the rule and pairing is rare.

Earlier workers (9, 12, 19) have already suggested that the virus of psittacosis may multiply by fission, but this concept has not been universally accepted. It would seem from the present study that division is occurring at the stage when the circles are about 500 mμ in diameter.

Elementary bodies when seen in tissue sections are only about one-half as large as they seem when prepared from an air-dried suspension of free particles (7, 9, 10, 17). This can readily be accounted for by the flattening of the membrane of the free virus on the collodion membrane during dessication. In fact, Crocker (6) has found that the diameter of elementary bodies is about 260 mμ when they are prepared by the freeze-dry method. This is the same as that found for the intracellular elementary bodies in the present study. That the smaller structures are actually elementary bodies is suggested by their shape, density, and location, in large numbers, in the top layer of dead membrane cells (Fig. 5).

The frequent observation of a dense, amorphous, osmiophilic substance in the cytoplasm of cells in an infected membrane could lead to the conclusion that this material is related to the virus. However, the dense material seems to be present at times in cells infected with other viruses (8) and even in cells traumatized by other means. In addition, its random distribution in a cell is different from that of the virus particles which are confined in vesicles or large aggregated masses. The osmiophilic material is assumed to be a nonspecific substance produced by cells under stress and, possibly, by normal macrophages.

SUMMARY

Electron micrographs of intracellular meningopneumonitis virus have shown several types of particles which are presumably representative of different stages of a life cycle. They are: (*a*) Elementary bodies—dense particles 250 to 300 mμ in diameter with very dense central granules. (*b*) Intermediate forms—less dense than elementary bodies and larger. They are 300 to 400 mμ in diameter, contain a very dense central granule, and often have two or three limiting zones. (*c*) Circles 400 to 500 mμ in diameter—homogeneous structures with single membranes and no internal granules. They are often elongated and constricted at the center in the manner of budding yeast cells. (*d*) Circles 500 to 600 mμ in diameter with single membranes.—One, two, and three dense granules may be present in some of these structures. (*e*) "Larger" structures, circular to elliptical, often with discontinuities or ruptures in their membranes. They sometimes seem to have internal septa.

It has been inferred from these observations that the virus can multiply by binary fission or by multiple endosporulation and that elementary bodies are a spore-like stage.

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EXPLANATION OF PLATES

PLATE 50

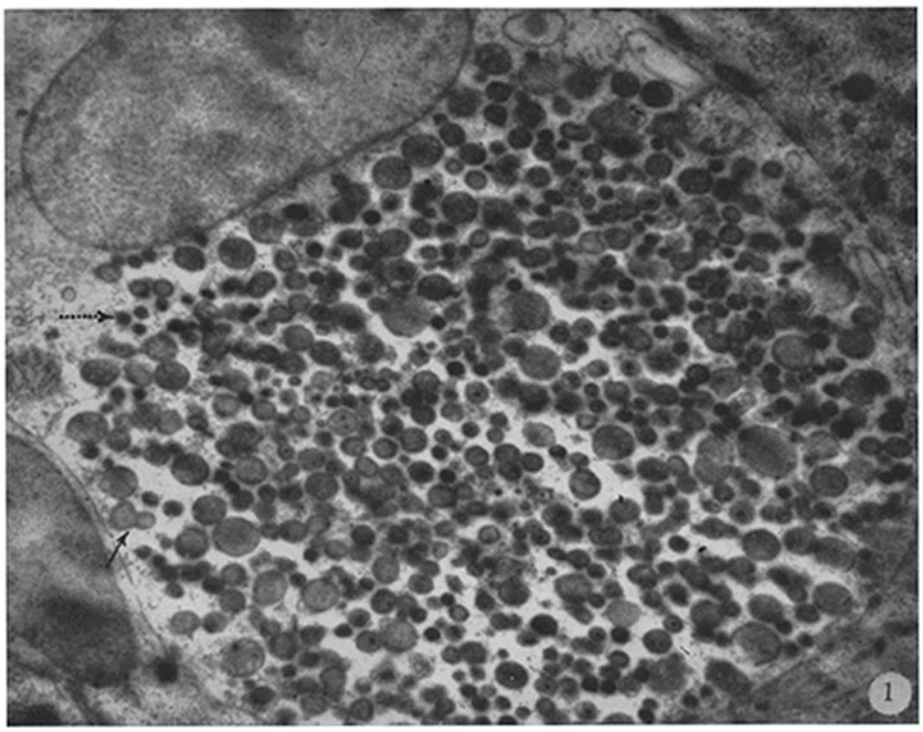
FIG. 1. Chorioallantoic ectodermal cell infected with the virus of meningopneumonitis. Large and small circles and segmented forms (arrow) can be seen. The most dense particles are elementary bodies (dotted arrow). Single dense granules are present in many cases and one medium-sized circle contains two granules. × 7,300.

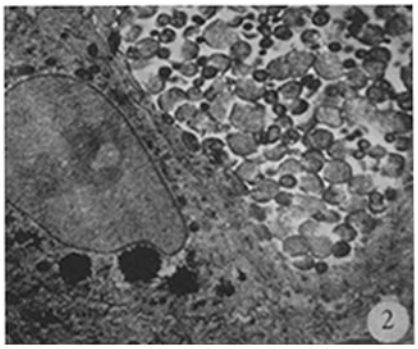
FIG. 2. Ectodermal cell containing three large masses of dense, osmiophilic material. No elementary bodies are visible but the presence of this non-specific material suggests early infection. Part of an infected cell is visible showing many large forms which appear to have ruptures. × 3,500.

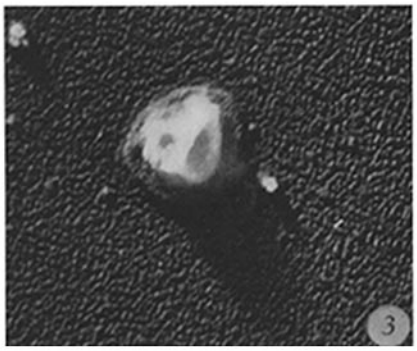
FIG. 3. Elementary body as seen after drying down from a washed suspension of macerated tissue. The outer membrane has flattened and presumably spread out causing these particles to appear larger than elementary bodies seen in tissue sections. Negative print, palladium-shadowed. × 38,000.

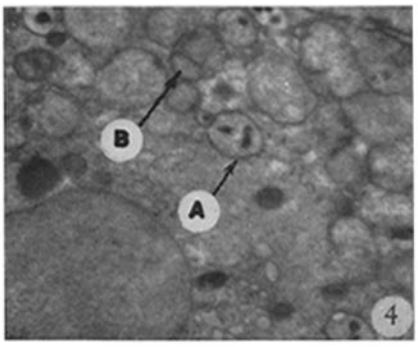
FIG. 4. Cytoplasm of an ectodermal cell, containing large forms. One, two, and three granules as well as an elongate granule (*A*) can be seen within single circles. An internal septum appears at *B*. × 9,300.

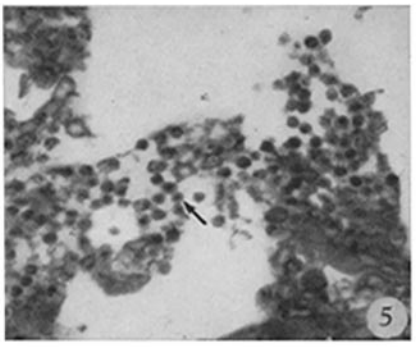
FIG. 5. Remnants of a cell at the outermost layer of compressed ectodermal cells which was torn open revealing many elementary bodies. Most of them have dense central granules (arrow). × 7,100.









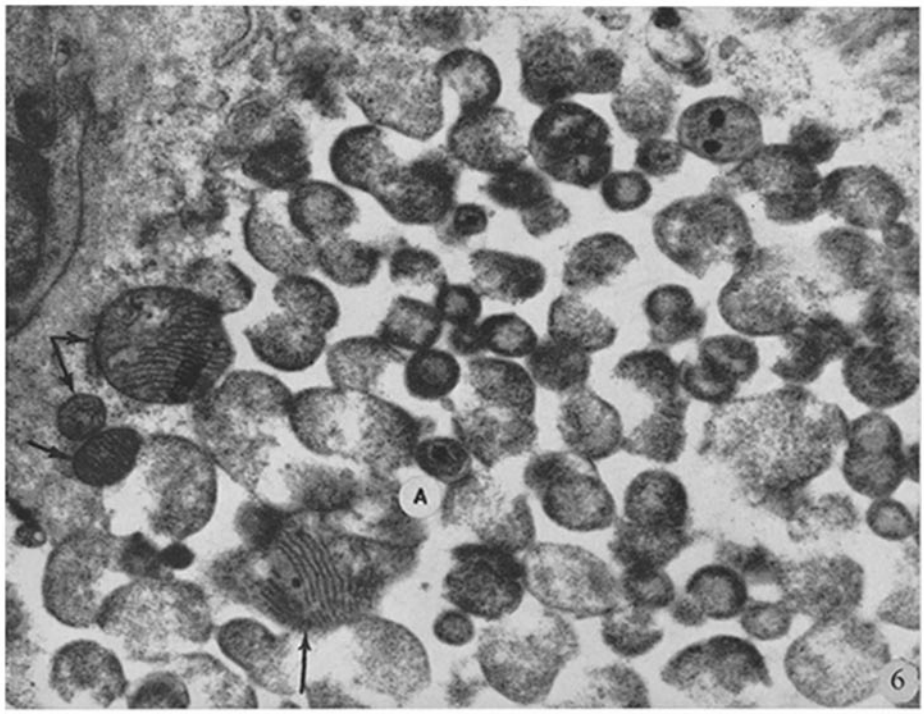


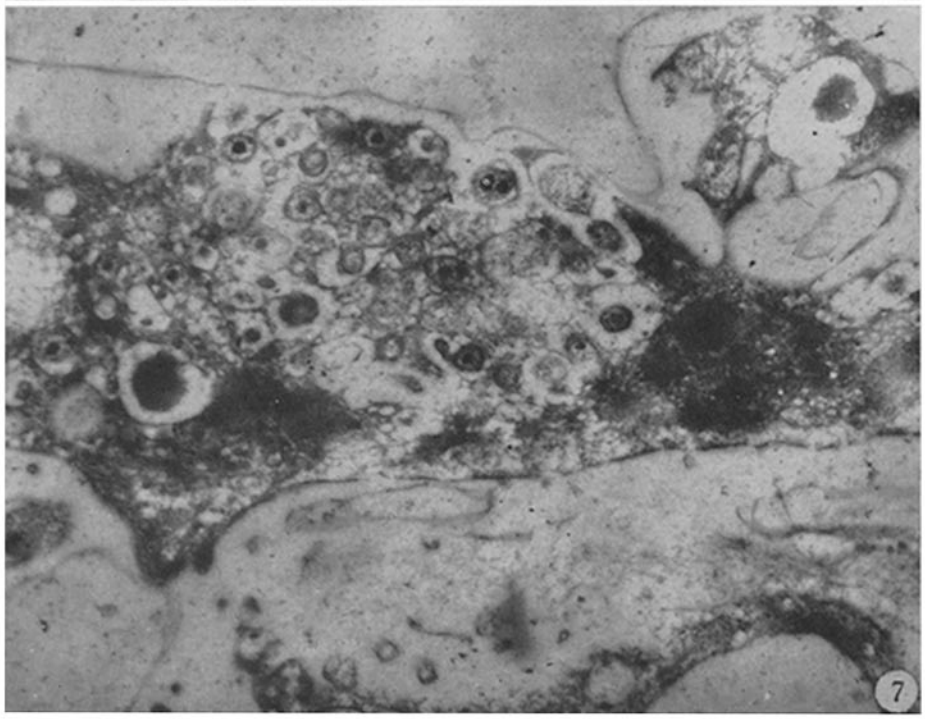
(Gaylord: Intracellular forms of meningopneumonitis virus)

PLATE 51

FIG. 6. Portion of infected cell showing various forms associated with the growth of the virus compared to mitochondria (arrows). Most of the larger forms are ruptured. This is thought to be a real phenomenon rather than artifact because all other structures are well preserved, especially the mitochondria with their delicate cristae. The small, dense granules in the mitochondria are not related to the virus. The intermediate form at *A* is bounded by two membranes. Zones of differing density with a part of a third outside them. × 16,200.

FIG. 7. Compressed, dead cell from the outer layer of ectoderm with intermediate forms enmeshed in the cytoplasm. These forms are slightly larger than elementary bodies and usually contain a dense granule. Their presence in this environment suggests that they are at a terminal stage of development. × 13,600.



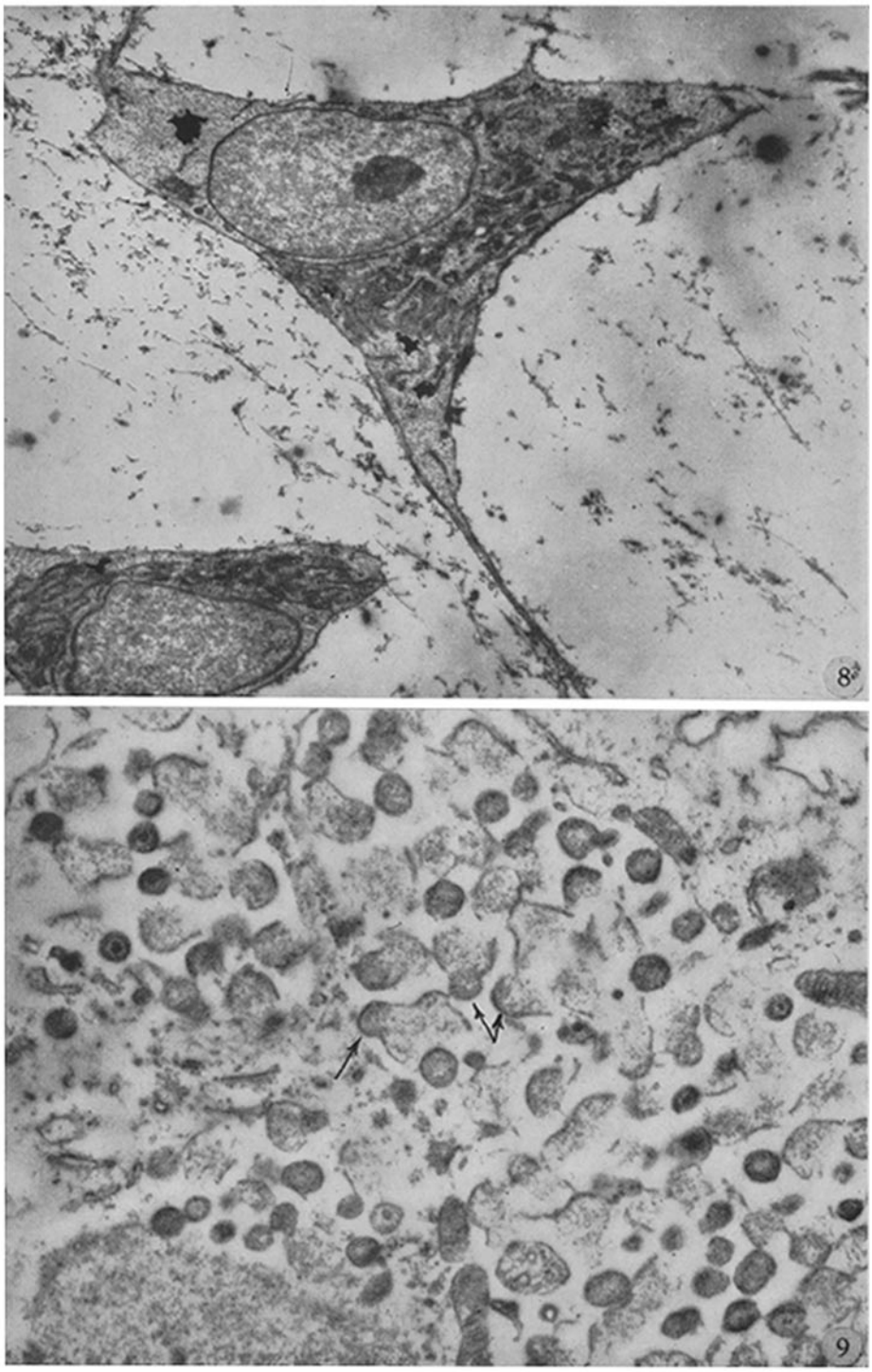


(Gaylord: Intracellular forms of meningopneumonitis virus)

PLATE 52

FIG. 8. Fibroblast containing small isolated areas of dense osmiophilic material similar to the large masses seen in Fig. 2 but no virus forms are present. Cell appears normal in all other respects. × 5,600.

FIG. 9. Portion of cytoplasm in which only remnants of large forms appear as discontinuous lines. In some places "buds" (arrows) seem to be formed and several intermediate forms can be seen. The diameters of the "buds" and intermediate forms are of the same order of magnitude (approximately 300 mμ). × 15,500.

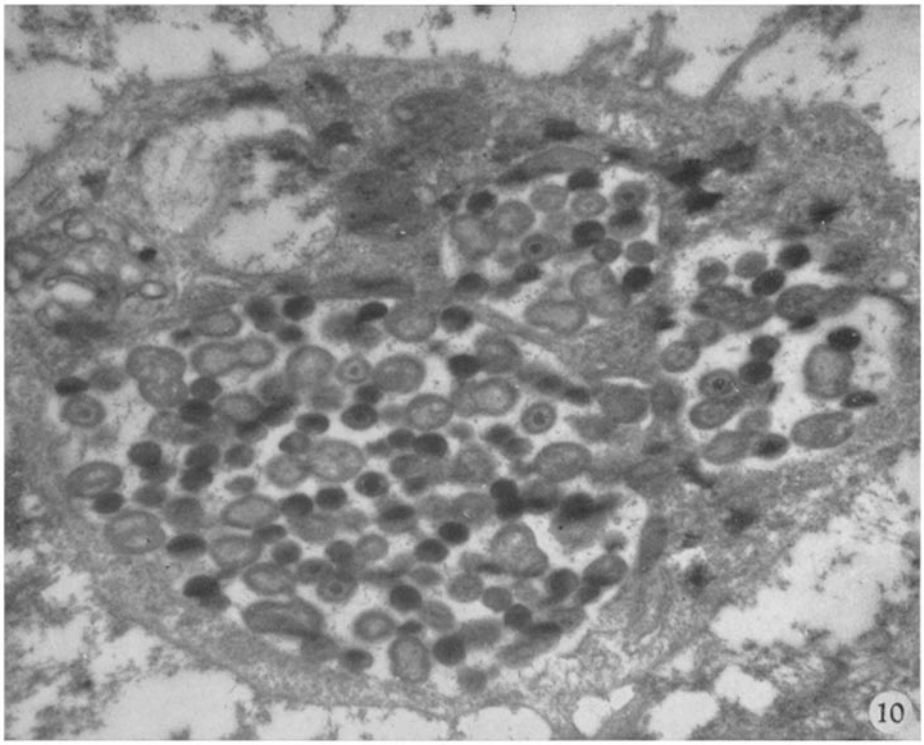


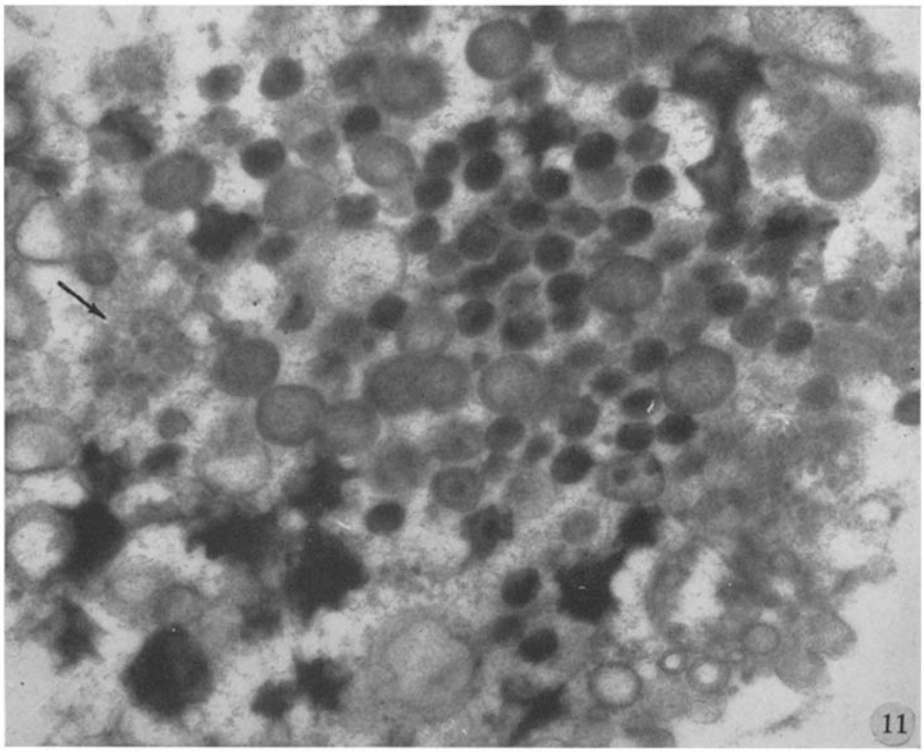
(Gaylord: Intracellular forms of meningopneumonitis virus)

PLATE 53

FIG. 10. Three vesicles in a cell similar to that of Fig. 8. Only circles of the size from 500 mμ (segmented forms) down are present. The frequency of elongated forms apparently segmenting would suggest that particles of this size divide by fission. The dense elementary bodies resemble spores and the form with a single granule is thought to be a stage in the cycle between the vegetative form and the spore (see Fig. 7). The structure upper left to the large vesicle may represent a segmenting large form. If so, each vesicle may start as a large sphere which breaks down. × 14,900.

FIG. 11. Vesicle of a fibroblast containing many elementary bodies and circles of the type which apparently show budding. Patches of the amorphous osmiophilic material shown in Figs. 2 and 8 are also present. The structure marked with an arrow is rare. × 22,200.





(Gaylord: Intracellular forms of meningopneumonitis virus)