STRUCTURE AND DEVELOPMENT OF VIRUSES OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP OBSERVED IN THE ELECTRON MICROSCOPE[1]

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The morphology of viruses of the psittacosis-lymphogranuloma group, including their developmental cycle, has been studied by many investigators. Bedson (1933), Bedson and Bland (1932, 1934), and Bland and Canti (1935) described, from their observations on different developmental forms, that psittacosis virus undergoes a complex developmental cycle and that the virus may multiply by division at some stage of development. Other workers who investigated the developmental cycle of viruses of the psittacosis-lymphogranuloma group obtained results similar in the essential facts to those described by Bedson and his colleagues, and among these investigators Levinthal (1935), Yanamura and Meyer (1941), Rake and Jones (1942), Heinmets and Golub (1948), Bedson and Gostling (1954), and Swain (1955) have attached importance to the fission as a mode of multiplication of viruses of this group. Observing thin sections of the chorioallantoic membrane infected with meningo-pneumonitis virus, a member of this group, Gaylord (1954) inferred that the virus could multiply by binary fission or by multiple endosporulation and that elementary bodies represented a sporelike stage.

It should be noted, however, that after elementary bodies had been introduced into the susceptible tissue and until initial bodies appeared in the cytoplasm of infected cells, there was a period of 5 to 18 hr when no viral elements were observed at all (Bedson and Bland, 1932; Bland and Canti, 1935; Findlay *et al.*, 1938; Rake and Jones, 1942; Weiss, 1949; Bedson and Gostling, 1954; Swain, 1955). Accordingly, the mechanism leading to the formation of the apparently homogeneous virus masses, or the plaques, described by the earlier workers, has never been demonstrated. Sigel *et al.*, (1951) and Girardi *et al.*, (1952) suggested that an eclipse phase was

present in the developmental cycle of meningopneumonitis virus. From the review of these papers, it is considered that the simple hypothesis of binary fission does not completely explain the mode of multiplication of viruses of this group.

In order to obtain further information concerning the structure and development of viruses of this group, thin sections of infected tissues harvested at different time intervals were studied with the electron microscope. The results of the study are described.

MATERIALS AND METHODS

Viruses. Two strains of the viruses of the psittacosis-lymphogranuloma group were used. One is the Maeda strain isolated by Kiuchi and Inaba (1952) from a cow and identified by Omori *et al.* (1953) as a member of the psittacosis-lymphogranuloma strain. After isolation, this strain was subjected to 91 passages in mouse lungs and then 116 passages in the yolk sac of embryonated eggs. The other is the Cal 10 strain of meningopneumonitis virus, which had been transferred many times in the yolk sac of embryonated eggs before being received by our laboratory. We passed the strain in embryonated eggs by the allantoic route and used the 6th passage of it.

Inoculation. The Maeda strain was inoculated intranasally in mice under light ether anesthesia. The inoculum was a 10 per cent broth suspension of infected yolk which had an LD_{50} titer of $10^{9.5}$ in 7-day-old chick embryos by yolk sac inoculation. A dose of 0.05 ml was inoculated.

The inoculum of the Cal 10 strain was undiluted infected allantoic fluid which gave an LD_{50} titer of $10^{7.5}$ in 7-day-old chick embryos by yolk sac inoculation. A dose of 0.2 ml was inoculated on the chorioallantoic membrane of 10- to 12-day-old eggs and the same dose into 9-day-old ones by the allantoic method.

Harvest and fixation. Mice inoculated with the Maeda strain were sacrificed by threes at 2, 3, 4, 5, and 7 days after inoculation. Small pieces

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were excised from pneumonic foci immediately after death and fixed for 4 hr in 1 per cent osmium tetroxide buffered at pH 7.4 as recommended by Palade (1952).

From embryonated eggs inoculated with the Cal 10 strain by the chorioallantoic method, small pieces were excised from the inoculated area of chorioallantoic membrane of every 3 eggs at 2, 4, 8, 15, 20, 24, 30, and 40 hr and 2, 3, and 4 days, respectively, after inoculation. From those inoculated with the same strain by the allantoic route, small pieces were excised from the chorio-allantoic membrane and amnion-allantois of every 3 eggs at 10, 20, 24, and 30 hr and 2, 3, 4, and 5 days, respectively, after inoculation. All the excised pieces were fixed in 1 per cent osmium tetroxide for 2 to 4 hr.

When materials for electron microscopy were harvested, those for stained preparations for light microscopy were also collected from the same tissues so that the interpretation of electron micrographs might be facilitated.

Imbedding and sectioning. Fixed tissues were dehydrated in graded dilutions of ethyl alcohol and imbedded in methacrylate. The blocks were sectioned with a thermal expansion microtome of type JUM using glass knives. Sections were flattened by flotation on a mixture of 30 per cent acetone and 70 per cent water and picked up directly on formvar-coated grids. Sections were examined without removal of the imbedding medium. A JEM type 4 C electron microscope was used.

RESULTS

Maeda strain in mouse lung. Viral forms were easily identified in those sections prepared from inflamed areas of the lung, because they had formed such viral colonies in the cytoplasm of alveolar and bronchiolar epithelial cells as resembled the structure of inclusion bodies observed in light microscopy. Those forms which we identified as virus were classified into four types, as mentioned below, by size and structure. Between these types, however, there were various transitional forms, which sometimes made it difficult to establish a clear-cut classification. The size of a viral form is expressed by the length of the minor axis.

(1) Elementary bodies:—They were of round

to oval shape and approximately 240 to 300 mμ in diameter (figures 1 and 4). Their appearance was uniform. They were more dense to electrons than other viral forms. A central granule which was more dense to electron beam than the other part of the body was often recognized.

(2) Intermediate forms:—These forms were mostly round to oval in shape and 300 to 500 mμ in diameter (figures 2 and 8). They had a dense central granule. The viroplasm which was so termed by Morgan *et al.* (1954) consisted of fine granular material of moderate density or was almost perfectly electron-transparent. Under this classification were also included many which were considered as transitional forms to elementary bodies because of their decreasing size and increasing density of viroplasm. Limiting membranes, often with 2 or 3 discernible layers, were clearly observed.

(3) Large forms:—These forms were mostly round to oval in shape and 500 to 1,000 mμ in diameter (figures 2, 5, and 8). There were some which often presented irregular shapes (figure 3). Most forms had a single limiting membrane and some a partly doubled membrane. The viroplasm consisted of fine granular material or presented reticular structure. No central granule was recognized, but sometimes fine granular material was condensed in the central area, showing such a stage as was considered to precede the central-granule formation (figure 8). Of the large forms, some were dumbbell shaped with a constricted limiting membrane in the middle part of the body (figures 2 and 4); some resembled chains due to membranes constricted in several portions; from some budlike protrusions sprouted; and still others had an internal septum (figure 6). Furthermore, two forms were sometimes connected with a fine fiber, resembling those which were at a stage immediately before the completion of segmentation (figures 4 and 7). It was not unusual to observe one form of such a pair in the process of segmentation showing characteristics of the intermediate forms or one whose morphology resembled that of elementary bodies with an increased density to electrons (figures 3, 4, and 7).

(4) Incomplete forms:—The viroplasm of these forms looked similar to that of the large forms, but its limiting membrane was discontinuous and did not cover its whole perimeter (figures

Figures 1–10. Electron micrographs taken from thin sections of lungs of mice inoculated intranasally with the Maeda strain. Scale length at bottom of each figure represents 1 μ.
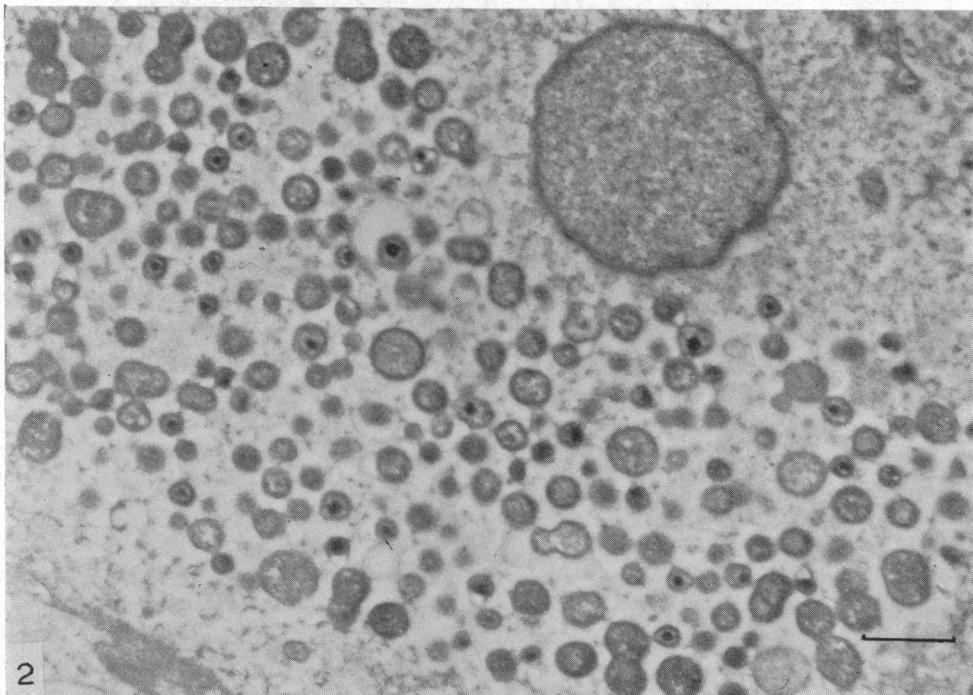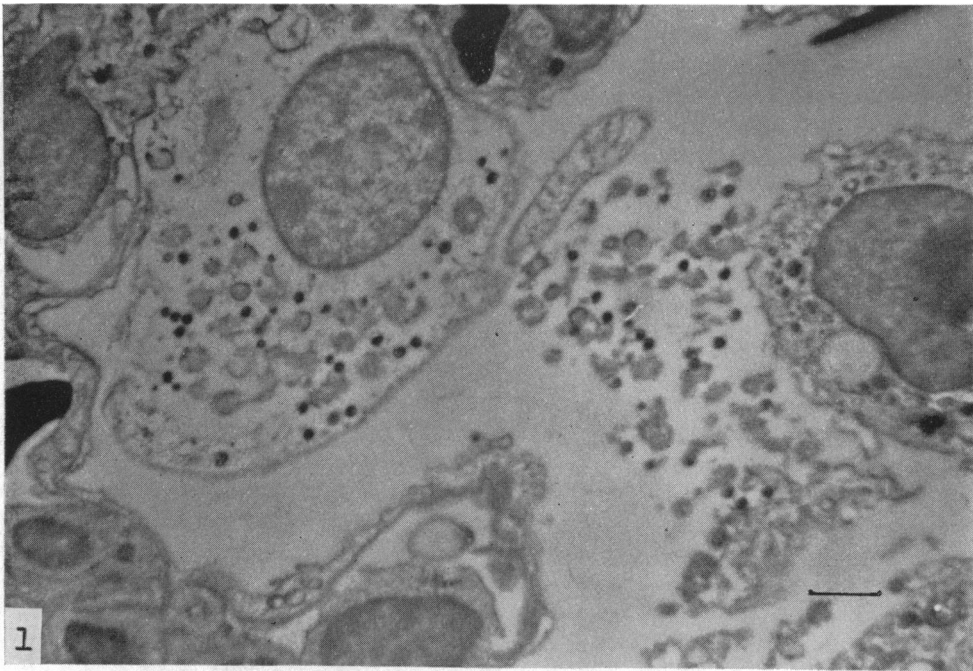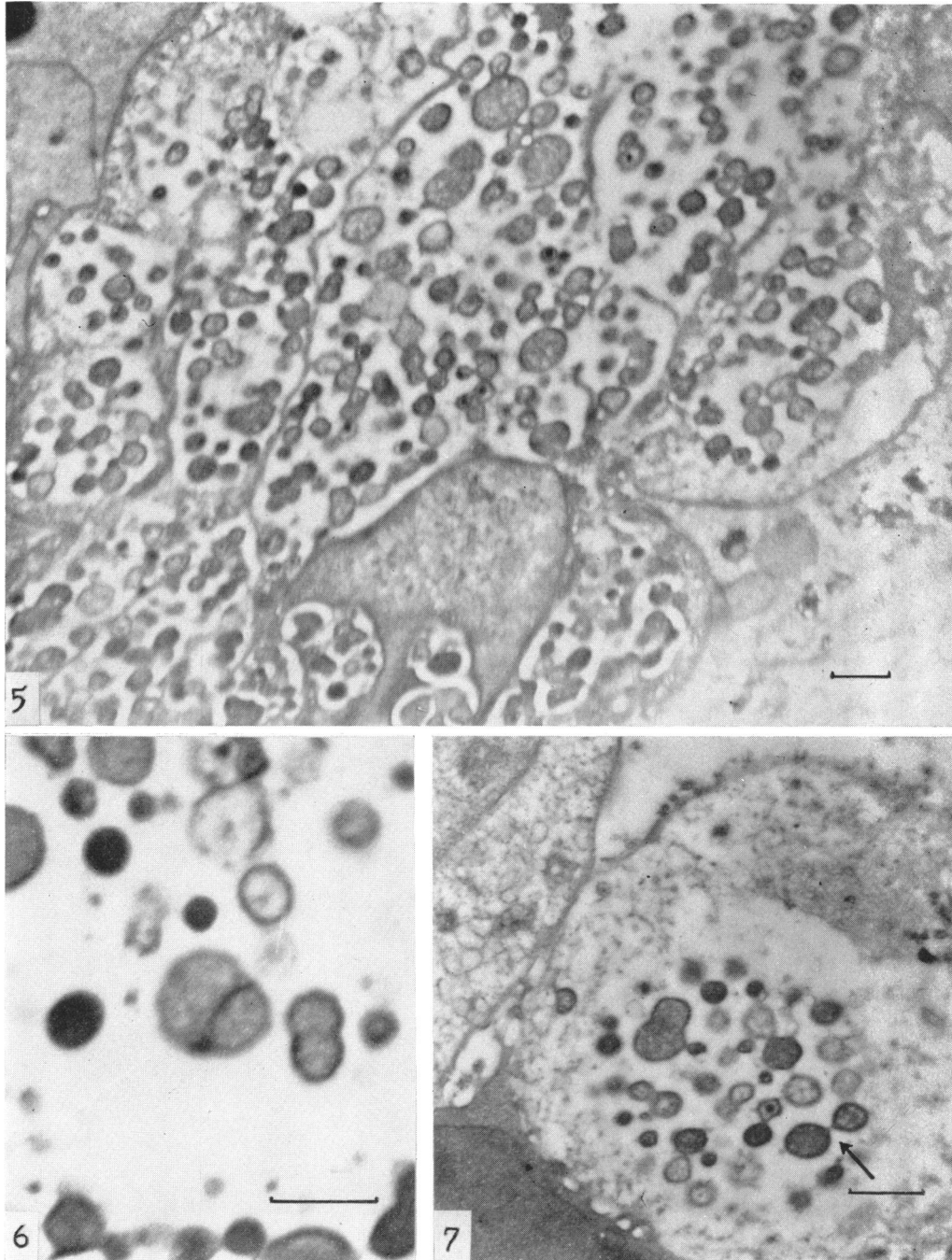
Figure 1. 2 days after inoculation. One alveolar epithelial cell in the upper left corner of the picture contains an inclusion body composed of various viral forms and matrices. Aggregates of viral structures are sparse, but elementary bodies are already intermingled. The cytoplasm of the same cell reveals diminution of submicroscopic cytoplasmic organelles in the region of the inclusion body. In the alveolar lumen, released viral forms and cellular debris are seen.

Figure 2. 5 days after inoculation. An inclusion body consisting of many large forms and intermediate forms. A number of elongated forms suggesting segmentation can be seen. In some intermediate forms two limiting zones of differing density are discernible. A nucleus is present at the upper right corner of the picture.
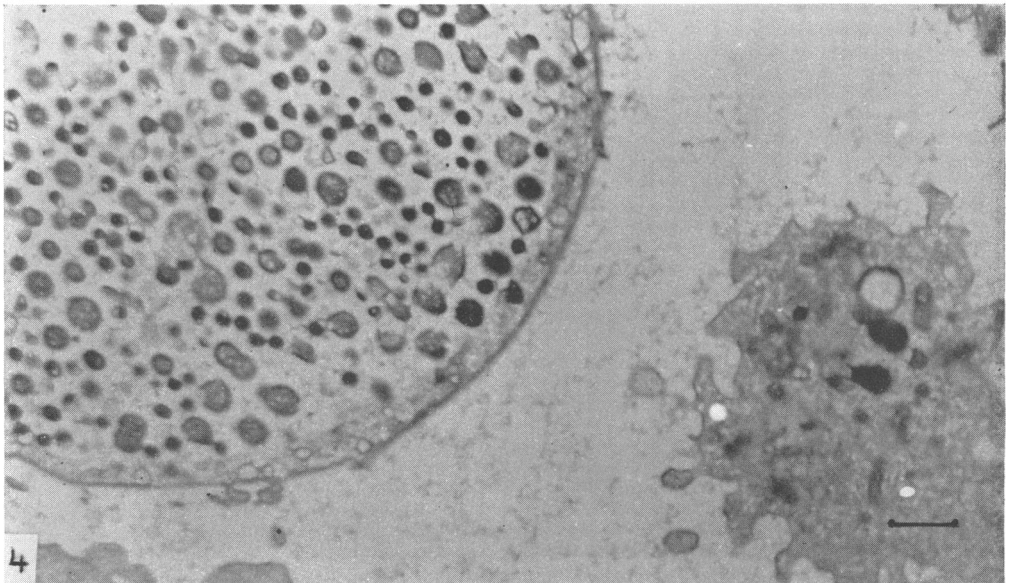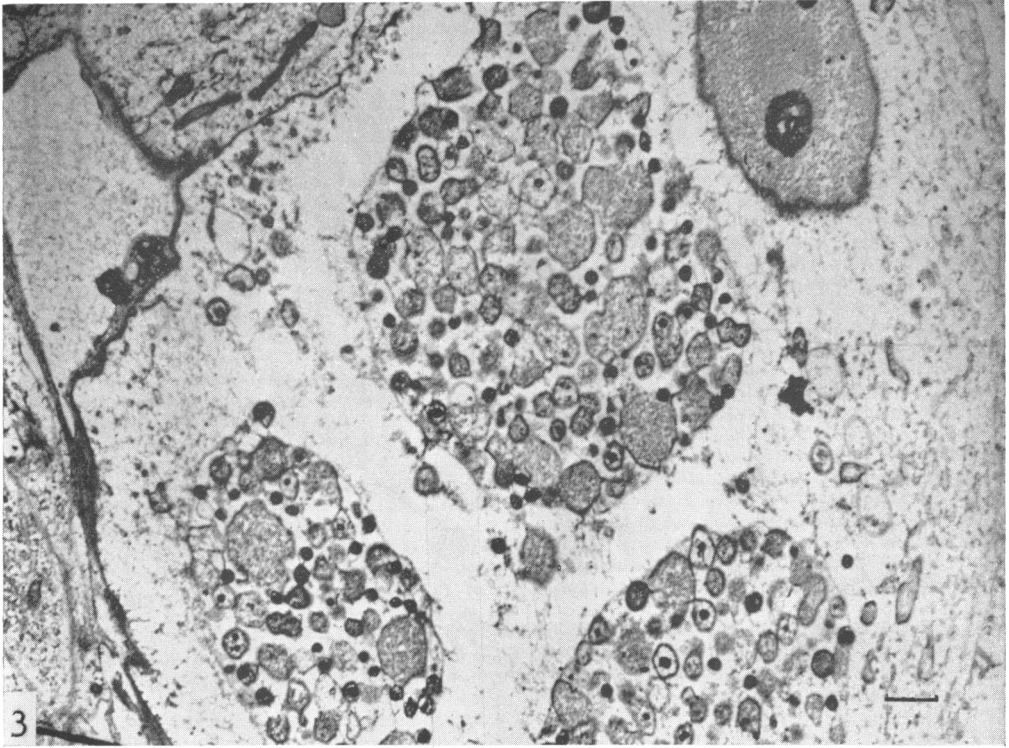
Figure 3. 4 days after inoculation. Three discrete inclusion bodies composed mainly of large forms are seen in the cytoplasm of an epithelial cell.
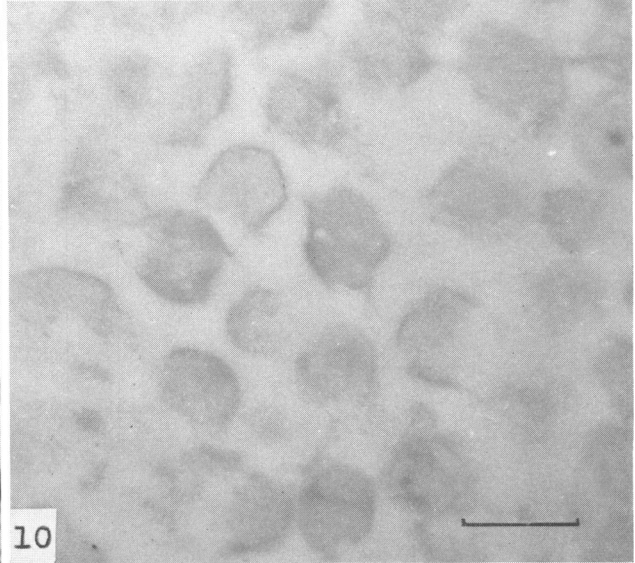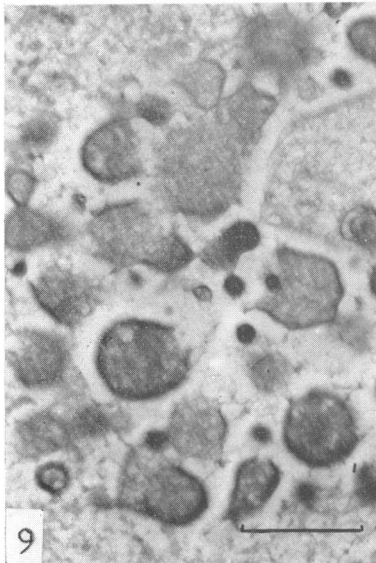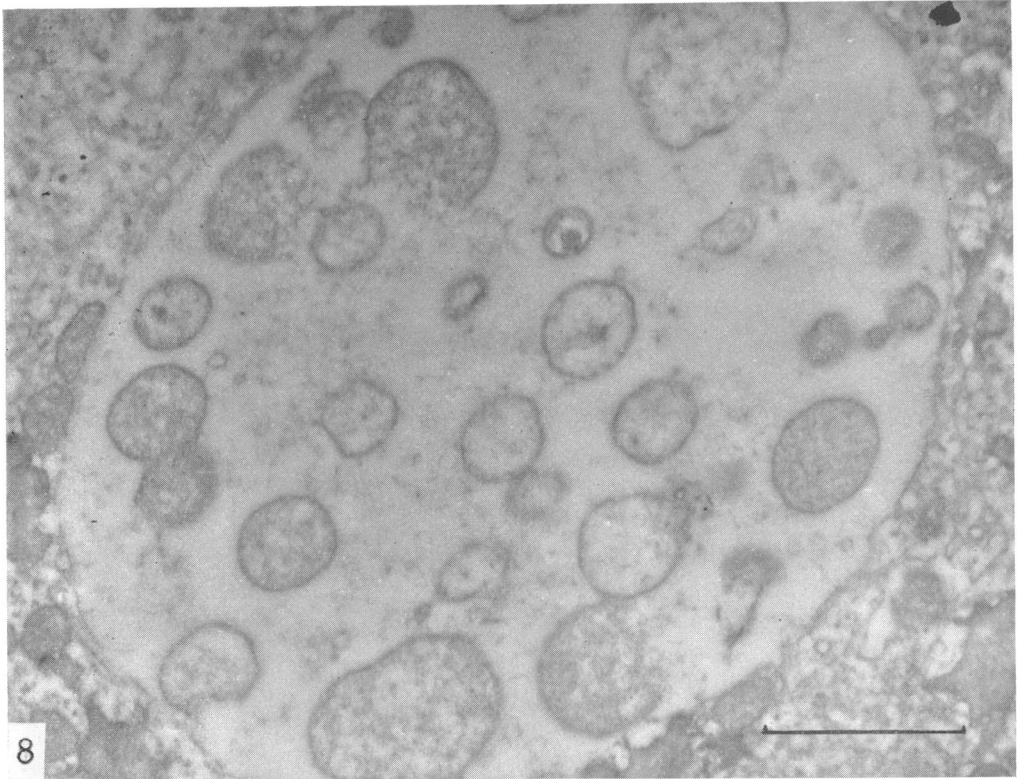
Figure 4. 4 days after inoculation. Portion of a large inclusion body in the cytoplasm of an alveolar epithelial cell. Elementary bodies and intermediate forms predominate but a fair number of large forms are also present. In the large forms, various stages of segmentation are seen. Some viral forms are in pairs connected with a find thread. The elements of the endoplasmic reticulum are pressed against the cell membrane.

Figure 5. 7 days after inoculation. Many large forms and intermediate forms scattered throughout the cytoplasm of an alveolar epithelial cell. Such an extensive aggregation of viral forms appears to have resulted from the coalescence of several inclusion bodies. The nucleus is severely indented.

Figure 6. 5 days after inoculation. Portion of an inclusion body containing mostly large forms. A large form near the center of the picture has an internal septum.

Figure 7. 5 days after inoculation. Inclusion body in the cytoplasm of an epithelial cell. Large forms connected with a fine thread are shown by the arrow.

Figure 8. 5 days after inoculation. Portion of the cytoplasm of an epithelial cell showing a sparse aggregation of large, incomplete, and intermediate forms. Near the left border of the inclusion body lies an incomplete form constricted in the middle. In some of the large forms, the fine granular material is condensed in the central area, suggesting the development of a central granule observed in the intermediate form. The submicroscopic organelles are crowded out of the inclusion body.

Figure 9. 5 days after inoculation. Portion of an inclusion body showing matrices and incomplete forms. Various transitional viral forms can be seen between the incomplete forms and matrices.

Figure 10. 4 days after inoculation. Portion of an inclusion body composed almost exclusively of incomplete forms.

8–10). Some of these forms were considered to be at a stage of segmentation because of their formation of budlike protrusions or constrictions of the limiting membrane (figures 8 and 9). The incomplete forms usually had a minor axis of 700 to 1,200 mµ in length and were less uniform than other viral forms, which made it often difficult to measure their size.

Structure assumed to be a pre-existing component of incomplete forms. In addition to the viral forms mentioned above, there was observed an amorphous material which existed in close association with them. This material presented a fine granular appearance modestly dense to electron beam, resembling the viroplasm of the large and the incomplete forms (figures 1 and 9). As these structures could not be classified in viral forms because of their ill-defined and irregular shape, we termed them the matrix. It seemed that the matrix was partly provided with an incomplete limiting membrane due to condensation of the substance in its periphery. There were also cases where some incomplete limiting membranes were formed separately in the periphery of a larger matrix, giving such an appearance that some incomplete forms were developing from a matrix (figure 9). Such being the case, the matrix was interpreted as a structure at a stage immediately before the development of the incomplete forms.

Those types of viral forms and matrices which were described above formed, by aggregating, intracellular colonies clearly set off from the cytoplasm of the host cells. Such viral colonies corresponded to inclusion bodies observed with the light microscope. In some cases more than two discrete inclusion bodies were present in the cytoplasm of a single cell (figure 3). Inclusion bodies had no limiting membrane and developed always intracellularly. The cytoplasm of host cells was usually remarkably swollen, and reduction in quantity of the submicroscopic cytoplasmic organelles was observed in the area occupied by the inclusion body (figure 1). Well developed inclusion bodies were often found in cytoplasmic vacuoles. At the advanced stages of infection host cells eventually ruptured, and released the contents of their inclusions.

In individual inclusion bodies, different types of viral structures were usually contained at various ratios, but, for sake of convenience, we classified the inclusion bodies in the following four

types chiefly according to the kind of the viral structure predominantly contained.

Type I inclusions:—The inclusion bodies of this type were mainly composed of matrices and were generally small in size, making their appearance in the cytoplasm of cells at the early stage of infection (figure 9).

Type II inclusions:—Inclusion bodies of this type were those which had the incomplete forms as principal constituent. These inclusions were small in size and contained a small number of viral forms (figure 10).

Type III inclusions:—These were inclusion bodies chiefly consisting of the large forms. They were relatively small in size and the total number of viral forms contained in them was small (figures 2, 7, and 8).

Type IV inclusions:—Inclusion bodies of this type contained only elementary bodies or elementary bodies and intermediate forms as principal constituent. They were large in size and included a large number of viral forms (figure 4). They tended to appear in those cells which were at the advanced stages of infection.

Although these types of viral forms and inclusion bodies were presumed to be the representatives of different stages of a developmental cycle, it was difficult to correlate the morphological sequence of changes of viral structures in initially infected cells to the lapse of time. The examination of materials at early stages, however, was difficult, since it was at 48 hr post-inoculation or later that pneumonic foci became discernible macroscopically in mouse lung. For this reason the following experiment was carried out.

The Cal 10 strain in chorioallantois of embryonated egg. In a preliminary experiment by means of light microscopy, it was confirmed that the Cal 10 strain inoculated on the chorioallantoic membrane produced, after 24 hr, inclusion bodies in ectodermal cells in such density that they might be easily detected in electron microscopy The main experiment was not undertaken, in which inoculated chorioallantoic membranes were harvested at various periods of incubation and examined by electron microscopy. This experiment had two principal purposes, one to compare the results with the findings in mouse lungs infected with the Maeda strain and the other to establish the time relationship of the various types of viral forms and inclusion bodies.
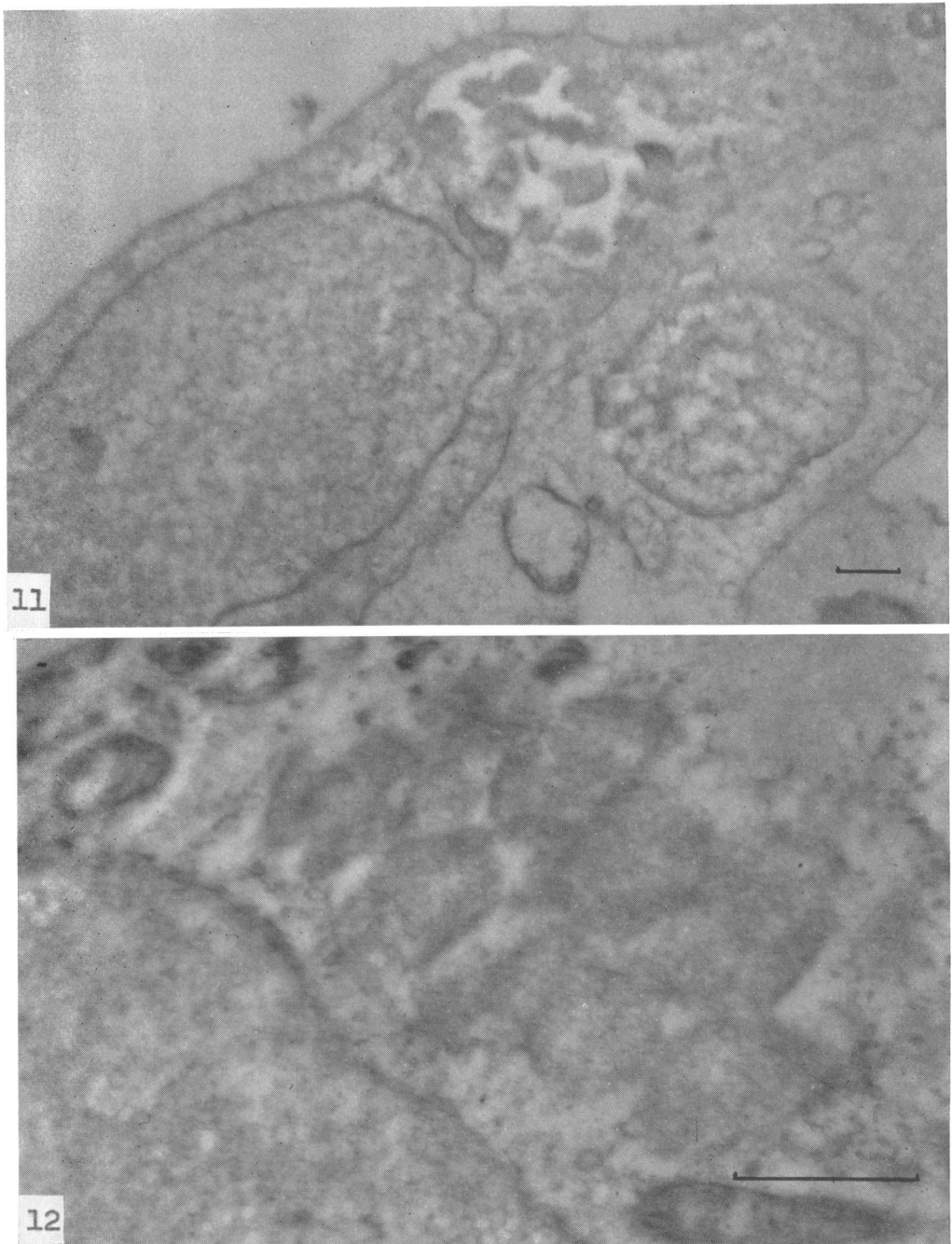
Figures 11–22. Electron micrographs of the Cal 10 strain. Figures 11, 16, 17, 19, 21, and 22 are from chorioallantoic membranes of embryonated eggs inoculated by the chorioallantoic method, and the re- mainders from allantoic membranes of embryonated eggs inoculated by the allantoic route. Scale length is 1 μ in each electron micrograph.
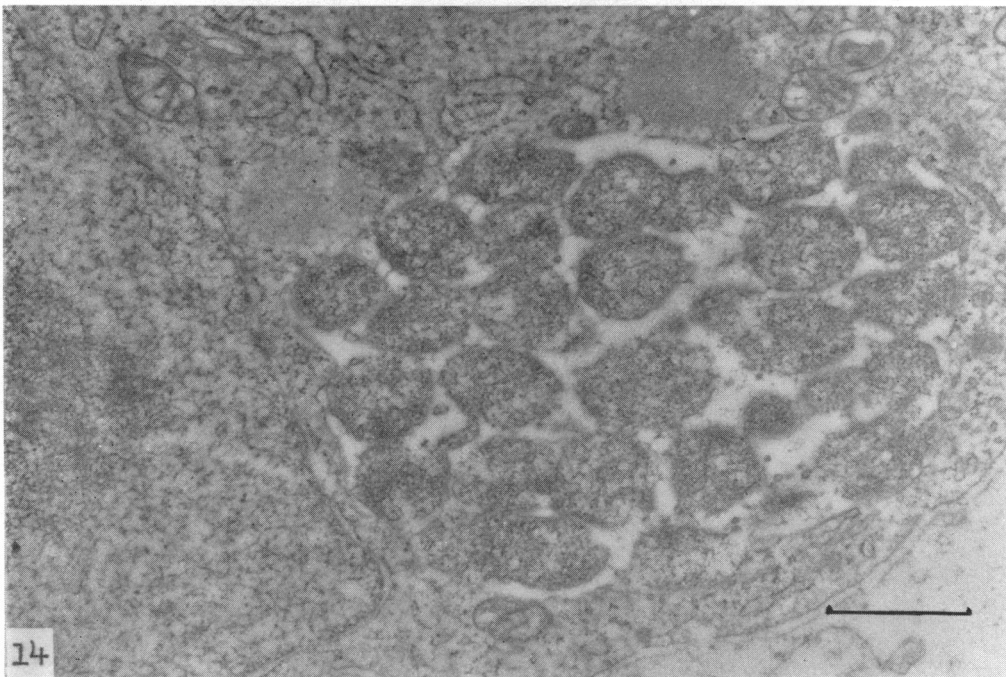
Figure 11. 24 hr after inoculation. Type I inclusion lying in a cytoplasmic vacuole of an ectodermal cell.

Figure 12. 24 hr after inoculation. Type I inclusion in the cytoplasm of an entodermal cell. A few mitochondria lie near the inclusion. Part of a nucleus occupies the lower left corner of the illustration.
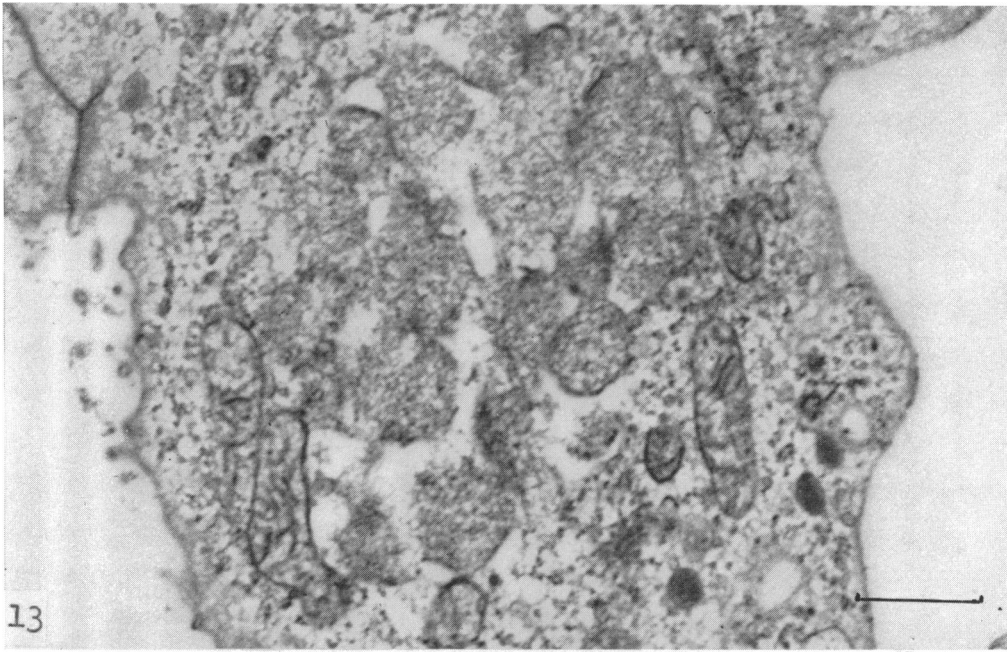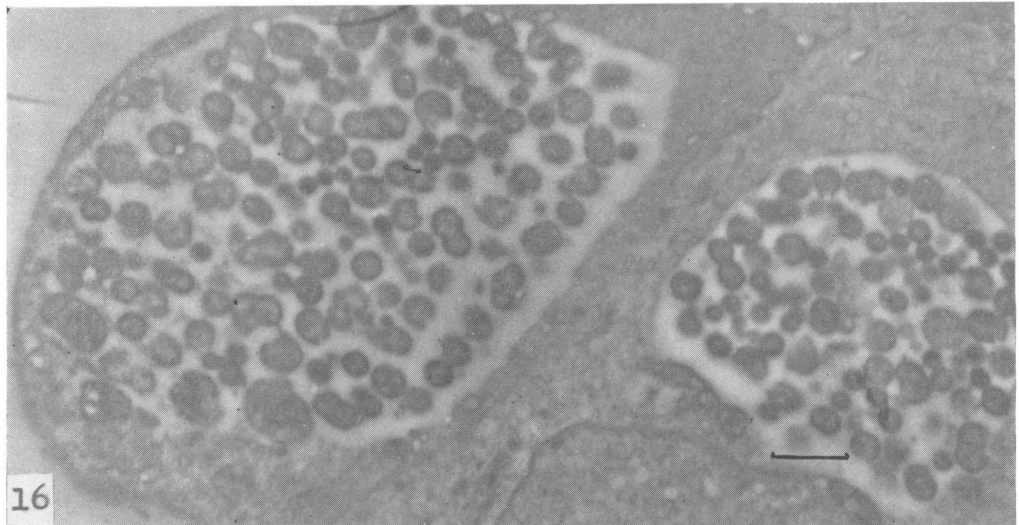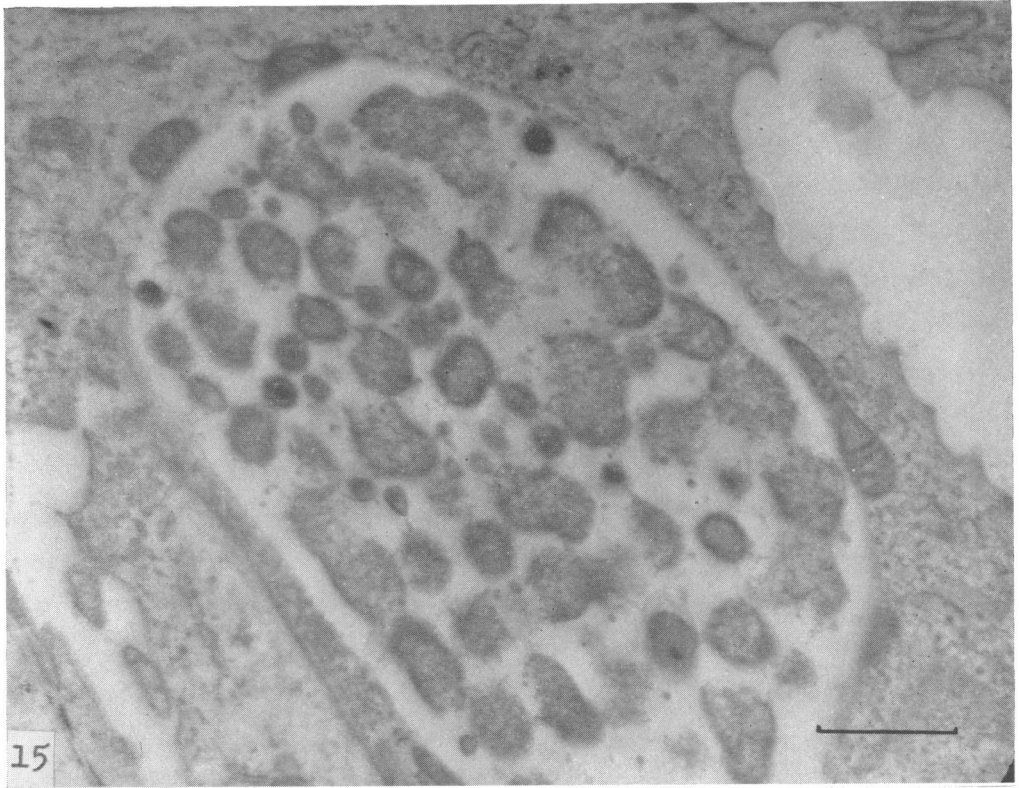
Figure 13. 24 hr after inoculation. Type I inclusion lying in the cytoplasm of an entodermal cell. Some matrices are provided with an incomplete limiting membrane. Several swollen mitochondria are seen around the inclusion. The nucleus is not shown.

Figure 14. 30 hr after inoculation. Type II inclusion in the cytoplasm of an entodermal cell. Around the inclusion several mitochondria and the elements of the endoplasmic reticulum are clearly visible. Portion of a nucleus occupies the left one-third of the picture.

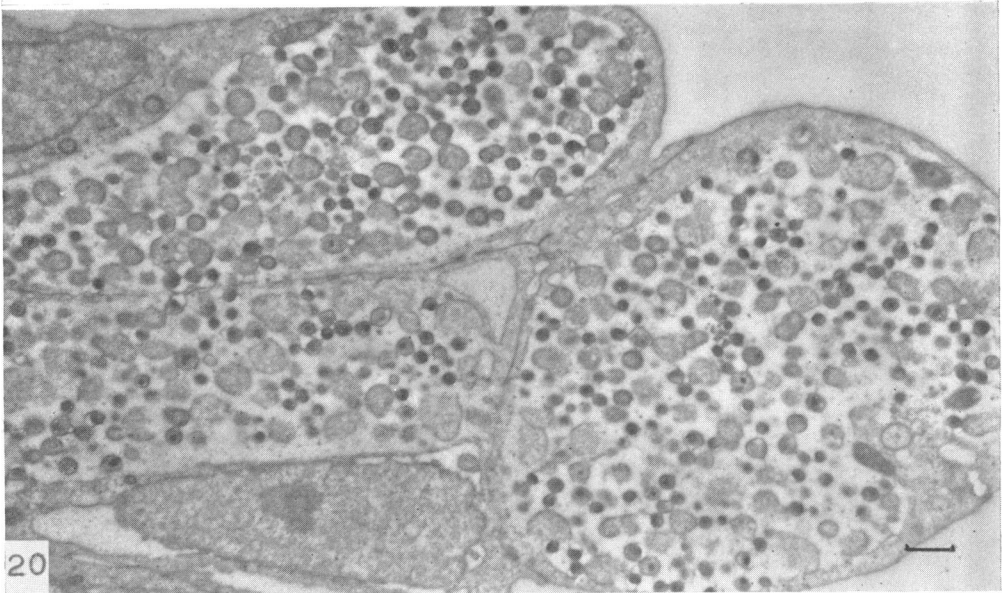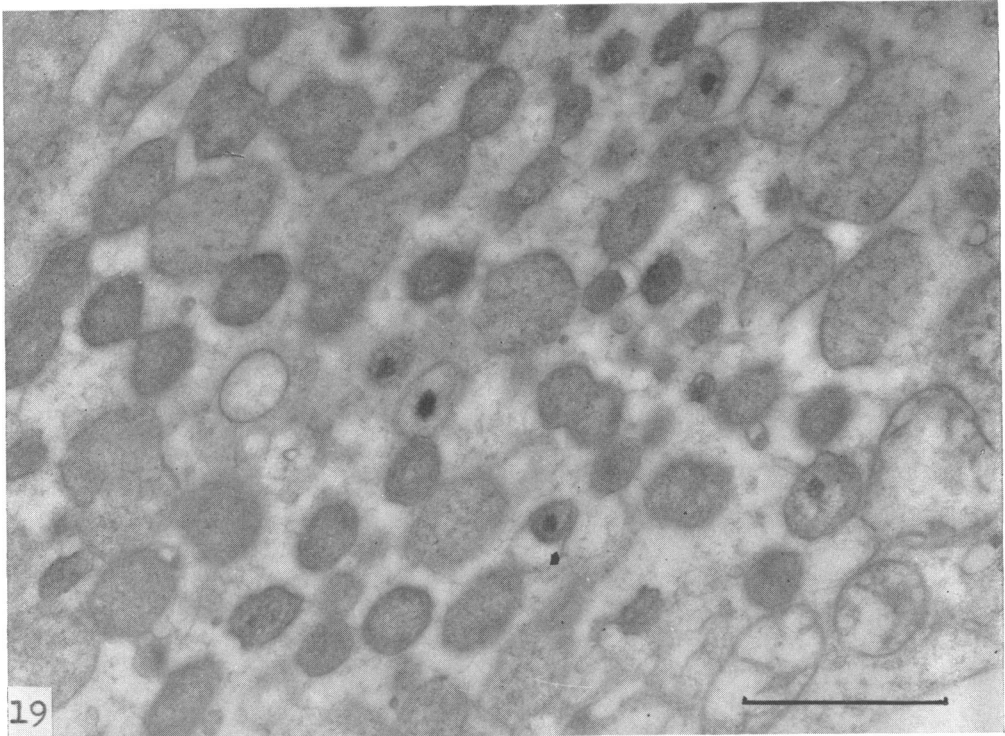*Figure 15.* 30 hr after inoculation. Type II inclusion lying in a cytoplasmic vacuole of an entodermal cell. In addition to incomplete forms, several large and intermediate forms are present.

Figure 16. 40 hr after inoculation. Type III inclusions in the ectodermal layer. A few intermediate forms and elementary bodies are intermingled. Near the lower border of the left inclusion lie 4 mitochondria.
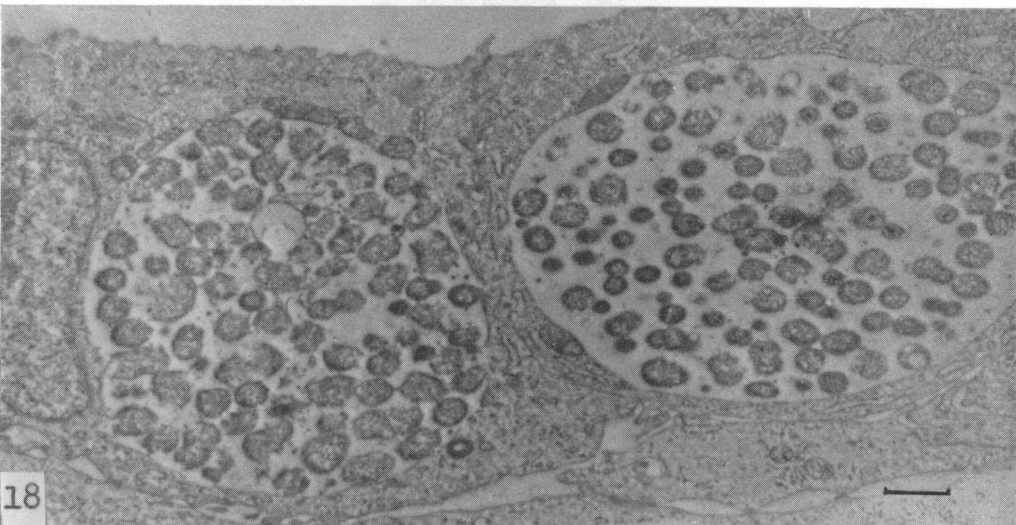
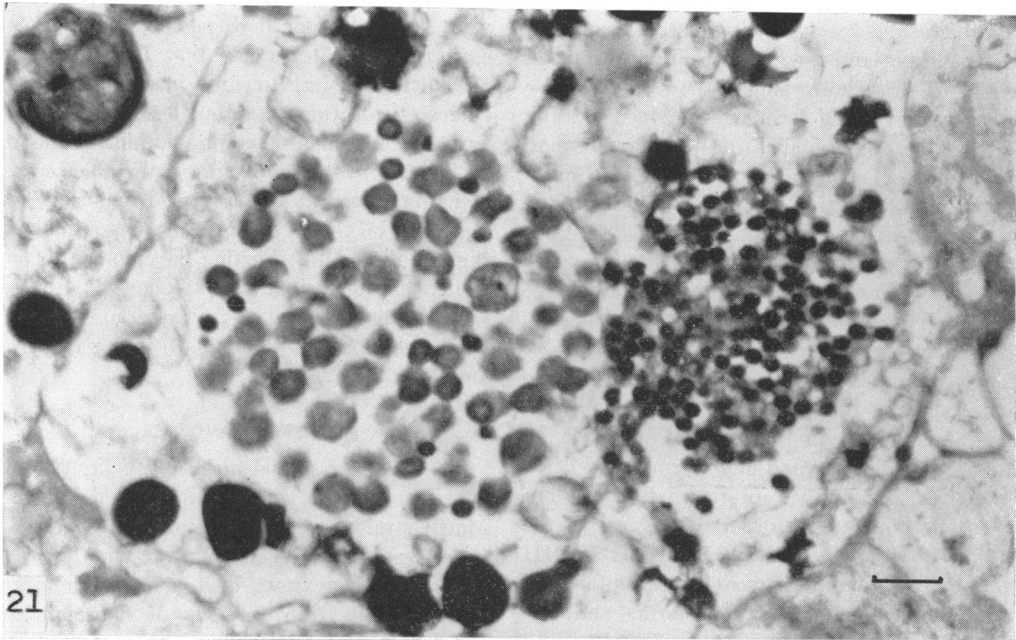*Figure 17*. 2 days after inoculation. An inclusion body containing various viral forms in cytoplasm of an ectodermal cell. A few large matrices are also present. The dense, osmiophilic material forming amorphous masses is believed to be a nonspecific material produced by the cell.

Figure 18. 4 days after inoculation. In the entodermal layer are shown a type II inclusion on the left and a type III inclusion on the right.

Figure 19. 4 days after inoculation. Type III inclusion contained in an ectodermal cell. One large form at the upper left corner is constricted at several places and gives a chainlike appearance. Near the border partly shown of the inclusion lie several swollen mitochondria which are of almost the same size as the large form but which can be identified by their internal structure.

Figure 20. 4 days after inoculation. Three large inclusions in the entodermal layer of allantoic membrane. Elementary bodies and intermediate forms hold a large majority, but a fair number of large forms are also present.
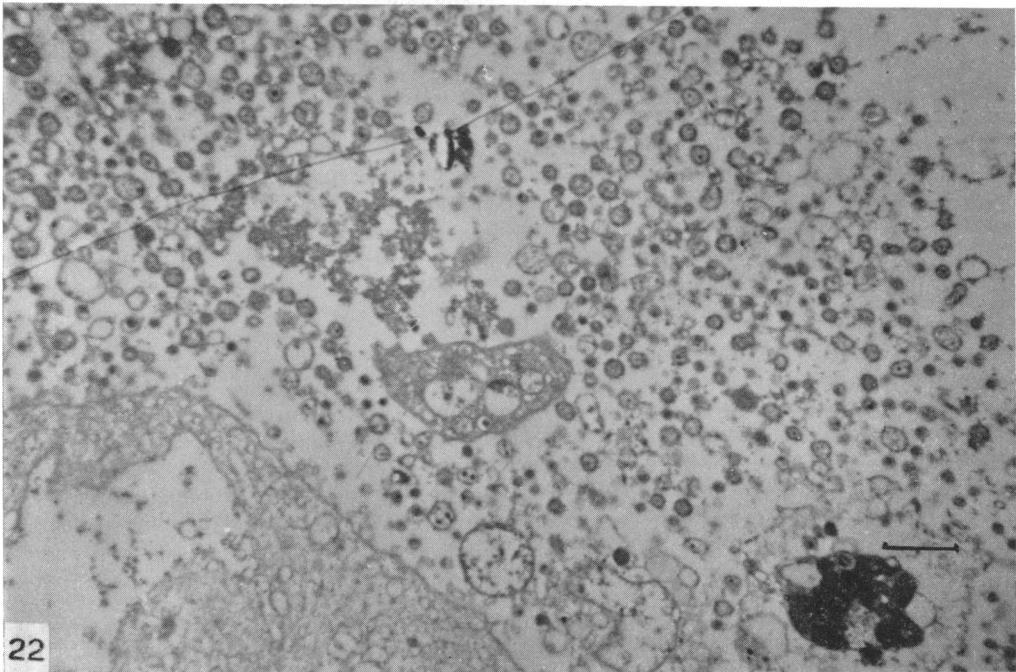
Figure 21. 4 days after inoculation. Of two adjoining ectodermal cells, the left one contains a type III inclusion and the right one a type IV inclusion. Several irregularly shaped masses of osmiophilic material described in figure 17 are seen around the inclusions.

Figure 22. 4 days after inoculation. The outermost layer of ectoderm showing numerous viral forms released from ruptured cells. This figure is believed to represent the final stage of infection.

Through this experiment it was confirmed that the appearance and the size of each viral form of the Cal 10 strain were almost the same as those of the corresponding form of the Maeda strain, and that inclusion bodies of both strains had almost identical structure. In sections from specimens harvested within 20 hr after inoculation, however, no general changes could be detected in the host cells, and neither viral forms nor inclusion bodies recognized with certainty. In the "24 hr" preparations, type I inclusions generally appeared in the cytoplasm of outermost cells of the ectodermal layer (figure 11). Matrices were situated in the cytoplasm or cytoplasmic vacuoles. Some inclusion bodies contained a small number of incomplete and large forms in addition to matrices, but neither intermediate forms nor elementary bodies were observed as yet. In the "30 hr" specimens, inclusion of types II and III were mostly observed and sometimes contained a small number of intermediate forms and elementary bodies in addition to incomplete and large forms. In the "40 hr" specimens type III inclusions were predominant (figure 16), but there were also many transitional forms to type IV inclusions. In the "48 hr" sections type IV inclusions appeared in the outer layer of the ectoderm and some of them were released from ruptured cells (figure 17). In the inner layer of the ectoderm type III inclusions were observed very often. In specimens collected at 3 days after inoculation or later, inclusion bodies increased in number and appeared not only in ectodermal cells but also in fibroblasts and macrophages in the mesoderm. Consequently, it was quite easy to find inclusion bodies in such specimens by electron microscopy. All types of inclusion bodies were seen in these specimens, especially type IV inclusions in the ectoderm, and very frequently inclusions of the younger types in the mesoderm (figures 19, 21, and 22).

In the chorioallantoic method of inoculation, the chorioallantoic membrane was detached from the shell and undiluted virulent allantoic fluid dropped directly on the membrane. So there was a possibility of causing additional nonspecific changes in the membrane. An additional experiment was then undertaken by employing the allantoic route for inoculation. In this experiment, allantoic membrane was harvested at different time intervals after inoculation and submitted to both light and electron microscopy. In light microscopy, a large number of inclusion bodies

were seen in entodermal cells of the allantois as early as 24 hr after inoculation. It was relatively difficult, however, to find inclusions at early stages of infection with the electron microscope. Viral forms or inclusions were not detectable until 20 hr after inoculation. First detectable in specimens harvested at 24 hr, type I inclusions were observed in the cytoplasm of entodermal cells (figures 12 and 13). Further development of viral forms and inclusion bodies followed a pattern such as was observed in the materials inoculated by the chorioallantoic method (figures 14, 15, 18, and 20).

DISCUSSION

The viral forms and inclusion bodies observed in thin sections of lungs of mice infected with the Maeda strain were classified into four overlapping types, respectively. These types of viral forms and inclusion bodies were also, and more clearly, recognized in thin sections of chorioallantoic membranes of embryonated eggs infected with the Cal 10 strain. In the experiments performed with the Cal 10 strain, the developmental cycle was studied in the viral forms and inclusion bodies by periodic observations of infected chorioallantoic membranes. The first viral structure which was observed in the cytoplasm of host cells at 24 hr after inoculation was an ill-defined, amorphous, fine granular material. This structure was called matrix by us.

Regarding the development from the matrix to the final viral forms, i.e., elementary bodies, evidences obtained up to the present have led us to the following hypothetic conception. In the periphery of the matrix, incomplete limiting membranes partly develop by the condensation of fine granular material to form the incomplete forms. The limiting membranes enclose portions of the matrix progressively, and then the large forms develop. The incomplete and the large forms are divided each into two or more viral forms of equal or unequal size by the constriction of the limiting membrane. The viral forms which are constricted at several places present a chain-like appearance. The large forms gradually reduce their size, and the fine granular material in the viroplasm is condensed in the central area to form a dense central granule. At this stage of development, two or three limiting zones of differing density occasionally appear around the viral form. Intermediate forms develop in this way. The intermediate forms gradually reduce

their size, increase in density, and transform into elementary bodies. The elementary body is considered to be at the terminal stage of development.

If one developmental cycle is defined as the period of time between the inoculation of the original virus and the first appearance of newly formed elementary body in the cytoplasm of infected cells, it is assumed to be less than 48 hr for the Maeda strain in mouse lung and about 30 hr for the Cal 10 strain in the chorioallantois. In both cases, inclusion bodies of infected tissues increased in number and manifested remarkably varying phases of development 3 days or more after inoculation. These observations are probably due to a new infection caused by such elementary bodies as were released after the completion of the first developmental cycle.

The present study has given no evidence with regard to the mode and mechanism of the invasion of elementary bodies into cells and of the formation of the matrix. Nevertheless, it is quite definite that the matrix provides the fundamental element from which the virus of various stages develops. If this is true, the claim made by many other authors that the elementary body grows into an initial body by increasing its size and that new elementary bodies develop from the initial body by repeated simple fission, must be amended. Bedson and Bland (1932) put forward two alternative hypotheses to explain the developmental cycle of psittacosis virus. According to one hypothesis, the apparently homogeneous masses, or the plaques, seen in the early stages of virus multiplication were considered to be truly homogeneous, in fact, plasmodial and at a given state of maturity they divided into a number of more or less equal parts which in turn divided and subdivided with the final production of elementary bodies. They (Bedson and Bland, 1934) revoked this hypothesis in their subsequent paper because the particulate nature of the apparently homogeneous masses was demonstrated, but, if the terms "plasmodium" and "subdivision" used in their hypothesis are replaced by "matrix" and "budding," respectively, a life story develops which seems to be close to the results of the present study. However, we have at no time seen any evidence to lead us to believe that the plaques are formed by coalescence of elementary bodies. The mode of formation of the limiting membrane is analogous to that of viruses of the pox group (Morgan et al., 1954; Tajima and Kubota, 1956).

In the case of viruses of the pox group, however, no configuration was observed where the incomplete and the large forms are segmented by the constriction of their limiting membranes. Thus in viruses of the psittacosis-lymphogranuloma group, it seemed that two different modes existed in the apparent development in their life cycle. The segmentation of larger forms caused by the constriction of limiting membranes, however, may also be interpreted as reflecting a process in which incomplete limiting membranes begin to enclose the matrix. Experiments are now under way to elucidate this point.

In thin sections of the chorioallantoic membrane infected with the Cal 10 strain, Gaylord (1954) has observed the structures considered to be identical with the incomplete forms described in the present paper and stated that "their identity as virus forms was doubtful." From our observations described above, however, this structure is believed to be a viral form existing between the matrix and the large form.

SUMMARY

The intracellular development of two virus strains of the psittacosis-lymphogranuloma group has been studied with the electron microscope using thin sections of infected tissues.

In lungs of mice infected with the Maeda strain isolated from a cow, viral forms were classified into (1) elementary bodies, (2) intermediate forms, (3) large forms, and (4) incomplete forms. In addition to these viral forms, the matrix which was considered to be a pre-existing component of viral forms was described. Inclusion bodies, which were intracellular colonies of these viral structures, could be divided into four types according to the prevailing viral structures contained.

In chorioallantoic membranes of embryonated eggs inoculated with the Cal 10 strain of meningopneumonitis virus, various types of viral forms and inclusion bodies similar to those described for the Maeda strain in mouse lung were observed quite clearly. In the same kind of materials, the development of viral forms was followed according to the lapse of time after

inoculation. The matrix was a viral structure which appeared first in the cytoplasm of infected cells. In the next stage, incomplete limiting membranes developed in the periphery of the matrix and enclosed portions of the matrix progressively, thus giving rise to the incomplete forms. Then large forms developed by the completion of limiting membranes. Viral forms in these stages of development were segmented by the constriction of limiting membranes. The large forms reduced in size gradually to transform into intermediate forms, from which elementary bodies, the virus at its terminal stage of development, were produced.

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