VACCINIA VIRUS MULTIPLICATION IN RABBIT-KIDNEY CELL CULTURES*

ASPECTS OF THE EVOLUTION CYCLE

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The intracellular life cycle and virus-cell interaction have been successfully studied in the electron microscope, particularly with phages, not only because of their functionally organized morphology, but also as a consequence of the use of pure virus and "cell" suspensions. Tissue culture techniques using isolated and purified cells permit to extend to a certain point the same facilities to other viruses, provided that they may be identified inside the cell and differentiated from cell components. Biochemical studies of intracellular virus and histochemistry of infected cells may be carried out and well interpreted only if preceded by the knowledge of morphology of the intracellular evolution cycle of the virus and its relation to the cell microstructures. Special interest present the viruses of the pox group, which in spite of the more complicated biochemical systems and structures will permit the observation of intracellular morphological modifications due to their larger dimensions. However, even with large viruses technical difficulties have arisen in observing all morphological transformations that occur during the evolution process from the moment of entering the cell until liberation of mature virus, with or without host cell destruction. The existence of a viral evolution phase without identifiable corpuscular structure has been responsible for difficulties in the interpretation of the morphogenesis of certain virus or pro-virus particles.

In vaccinia infected cells of chicken embryo chorioallantoic membrane, Wyckoff (6) detected virus microcolonies, in which it was not possible to distinguish the way of multiplication nor the interference of virus with cytoplasmic components. In that study, Wyckoff draws the attention to the fact that no evidence was found of an increase of volume, nor of subsequent division of the particles considered the elementary bodies of vaccinia virus.

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Ruska and Kausche (5) already made the same observation by statistical evaluation of size and shape of purified virus particles.

More recently, Morgan et al. (3) in studying the structure and development of vaccinia virus using ultrathin sections of focal areas of hyperplasia in infected chicken embryo chorioallantoic membranes, described intracytoplasmic virus particles averaging 200 x 300 mμ and containing a dense, nucleus-like body (nucleoid) separated from granular material (viroplasm) by a zone of lesser density. They are enclosed by a single membrane. Also on the cell surface and in the intercellular spaces, these authors detected the presence of another virus particle type composed of a double membrane enclosing a central body of variable shape and density.

This note gives preliminary results of an attempt made in order to detect a system of cell-vaccinia virus allowing better observation of the different evolution stages of this virus. Some phases of the evolution cycle are depicted in the micrographs.

Materials and Methods

Growth media and cells. Trypsinized epithelial kidney cells obtained from six-month-old rabbits (7) were cultured in Hanks' or Earle's balanced salt solution with 10% calf serum, 5% lactalbumin hydrolysate and antibiotics. Media were changed after 72 hours. When confluence of the cells was obtained, usually within 5 to 7 days, the same nutrient medium was used with only 2% calf serum.

Inoculum. The initial inoculation for tissue culture was obtained from cowpox virus used in routine vaccine production, maintained by passages through calves, with occasional transfers in rabbit skin.

Vaccinia infected bovine dermal pulp was homogenized, diluted 1:5 with phosphate buffered saline containing antibiotics, and centrifuged at 500 r.p.m. for 30 minutes. 0.1 ml of supernatant fluid showing pustular confluence when scarified in rabbit skin at the dilution of 1:100 was inoculated in 16 x 16 tissue culture tubes or the corresponding amount in larger flasks. Afterwards, 2 ml of nutrient medium were added.

One hundred and eight passages of the vaccinia virus were made using always cells obtained directly from rabbit kidney.

Material of each passage was tested in rabbits and some of it also in chicken embryo choriollantoic membranes. Electron microscopic controls of purified virus suspensions of tissue culture from tubes showing total cytopathogenic effect were performed with material of different passage levels; virus of the 54th passage was also tested in scarified calf skin. The results obtained in these and the cross immunization tests in rabbits and in tissue cultures demonstrate that the infective and vaccinogenic properties of the virus apparently remained unchanged.

Undiluted suspensions of virus from the 43rd tissue culture passage caused characteristical cutaneous reactions when scarified in human skin.

The cytopathogenic effect on the cultures is clearly visible before 24 hours elapsed; lysis is usually complete after 48 hours. Bacteriological controls were performed as a routine in all passages.

Electron microscopy. Micrographs illustrate vaccinia infected rabbit-kidney cells 24 hours after inoculation with virus of the 23rd passage in these artificially cultured cells, when cytopathogenic effect was already evident in some cells or groups of cells.

The fluid medium was discarded and the cultures versenized (0.02% solution) for 15 minutes at 37°C. Elimination of the Versene (di-sodium ethylene diamine tetra-acetic acid) solution was obtained by centrifugation at 500 r.p.m. for 5 minutes. The cells were fixed during one hour with 1% OsO_4 in isotonic acetate-veronal buffer (pH 7.2), dehydrated in the alcohol series, and embedded in methyl and butyl methacrylates. The changes of the alcohol series and methacrylates were performed by centrifugations and decantations. Sections made in a Porter-Blum microtome were examined in a Siemens electron microscope model ÜM 100b at 60 Kv. The original micrographs x7.200 and x15.000 were enlarged photographically.

RESULTS

Cells with intracytoplasmic inclusions and elementary bodies may be found with three hours of infection. Nevertheless, in this study we used mostly 24 hours infected cultures, at which time the beginning cytopathogenic effect is evidenced.

Further studies on cultures between 0 and 24 hours will be published elsewhere. In 24 hours infected cultures, a large number of cells presents besides the inclusion bodies, different aspects of the evolution process until the appearance of the mature virus, the structure of which is similar to that of virus of infective inoculum and which has been described by Morgan et al. (3). The intracytoplasmic parasitism of infected cells may present one of the following predominant aspects:

A) Relatively well delimited "inclusion" of variable dimensions that may achieve 3.5 μ in diameter, as the one depicted in figure 1. No virus-like particles are detected isolated or in groups in the rest of the cytoplasm. Cells with these characteristics are less frequently observed.

These inclusion bodies are composed of an agglomeration of sub-units 0.45 μ wide and 0.5 to 0.6 μ long, which we shall call "matrix", and which present poorly defined contours. Spherical or elliptical corpuscular particles are seen to originate from the whole surface of all the matrices (fig. 2). All stages, from a small protuberance to the individualized corpuscular particle measuring in average 250 mμ, may be detected on the surface of the matrices or already free between them. The space between the matrices has the same electron density and general structure as the cytoplasm outside of the inclusion. In some inclusions, the matrices are regularly disposed and the spaces measuring 370 to 770 mμ permit the corpuscular bodies to be isolated, free in the inter-matrix spaces. Corpuscular bodies, which will be referred to as "pro-viruses", apparently originate from matrices like sprouts or salien-

ces of a "morula". These sprouts present a sort of membrane only in the part protruding from the matrix towards the cytoplasm or to the spaces between the matrices. Apparently, there is no discontinuity between the inner material of the matrix and that of the sprouts. Isolated pro-viruses show circular or slightly elliptical shape, measuring 210-240 mμ x 280-320 mμ, with a membrane that appears double in some micrographs. A more electron dense, apparently homogeneous material is seen inside of those particles. Some sections show eccentric, dense bodies averaging 80 mμ, separated from the inside pro-virus material by a halo of much less electron density. A more accurate examination of matrix and pro-virus sections showed that they are not composed of simple granular or structureless material, but that at least some of these elements contain variable amounts of parallel, continuous lines or circular contours like those of vesicles (figs. 3 and 4). The diameters of the circular forms are approximately the same as the distance between the parallel lines (10 to 15 mμ), which suggests that the same structure was cut in perpendicular or longitudinal planes.

B) Most frequently, the infected cells show variable amounts of individualized corpuscles of the above described pro-virus type irregularly distributed in the cytoplasm; often they are grouped in larger numbers in some region of the cytoplasm, however, without being concentrated in a well delimited area. In some cells, hundreds of these particles may be counted in a single section. All steps of the evolution process may be followed from the inclusion body to the moment when only pro-virus particles are contained in the cytoplasm. Isolated or small groups of matrices permit better analysis of the progressive formation of pro-virus. The increase in number of these corpuscles corresponds to the progressive decrease, disintegration or consumption of the matrix. At the same time, the spreading of the pro-viruses in the cytoplasm is evidenced.

In a further step, no more matrix or similar structure is seen, and the free pro-virus particles appear embedded in a granular material which is more electron dense than the normal cytoplasm (figs. 5 and 6). When virus formation occurs in the vicinity of the Golgi apparatus, apparently a regular distance is maintained between its characteristical structures and the zone of virus particle formation and evolution. In figure 6, this fact is observed with particles in the pro-virus phase B.

Sometimes, newly formed pro-virus particles show a yet incomplete membrane or a membrane which is better defined on one side of the particle than on the other. Finally, all pro-viruses are distributed at random, individualized, show a complete membrane difficult to be distinguished whether double or

single, and contain the inner eccentric, characteristical dark mass; in the cytoplasm, no more regions are found where the spaces between the pro-viruses are filled with a dense, granular material.

C) In this study it has not been possible to follow all steps between the pro-virus phase and the appearance of a defined inner morphology of the virus particle preceding or constituting the mature virus. Before the liberation of the virus or the disintegration of the cell, virus particles fill almost all the cytoplasm in some sections (Fig. 7). Frequently, sections containing large number of this virus particle type do not show the dark inner bodies. Predominantly they tend to an elliptical shape, which does not seem to be an artifact due to pressure of the knife during sectioning, since they are irregularly orientated. Nevertheless, it is possible that this shape is partly influenced by tensions during the fixation and polymerization process, to which the viral particles in this phase may be more susceptible. In most sections, the inner structure appears dumb-bell shaped, the two enlarged extremities being orientated along the large axis of the ellipsis. Some cells present exclusively this virus phase without the presence of particles of the pro-virus type; few other particles are identical to those described in B, having the eccentric body and no defined structure. In some sections, near the cell membrane there may also be observed particles tending to be spherical, identically to those described in D.

D) Mature or free, extracellular viruses (fig. 8) tend to be spherical in shape and most frequently have dimensions near 300 mμ. No intact cells were found in which only spherical forms are contained in all the cytoplasm, but occasionally spherical, like mature particles are found in the neighbourhood of the host cell membrane. It was not possible to observe the extrusion of a virus through the cell membrane. When large amounts of mature viruses were found in the field, they were located in the intercellular space, in the vicinity or in fragments of already destroyed cells.

Typical mature viruses may be found in the periphery of the cells described in A, B or C, in 24 hours cultures, which seem to originate from cells already destroyed by the action of the virus of one or more infection cycles, as evidenced by the cytopathogenic effect observed in some cells of the culture. The intracellular location of the inclusion body A to mature virus D is progressively distant from the nucleus, approaching the cell membrane.

Sections of liberated virus particles show two circular, concentric limiting membranes, as if they were sections of two spherical bodies one inside the other, the central one containing a structureless, non electron dense substance like a vacuole; the space between the inner and the outer sphere is filled with a denser, granular material, in some sections showing one or two much denser bodies

located, if two are present, on opposite poles of the spherical virus. In some sections, these masses of dense material are larger than the inter-membranal space, protruding at the expense of the spherical shape of the outer membrane (fig. 9).

DISCUSSION AND CONCLUSIONS

The described part of the evolution cycle is based on the predominant aspects observed in hundreds of micrographs. The order of description from an inclusion to mature virus is believed to be the one which is closest to the facts, considering the impossibility to follow the viral evolution process in one culture or cell in the electron microscope.

Much more work is required to clarify the morphological intermediate stages of the main pictures we tried to fix in A, B, C and D. For example, not all the steps of the progressive modifications of the structure of what we call pro-virus to the virus described in C or from this to D were detected, even when large numbers of infected cells were observed. We believe these steps were not detected because they occur in a short period of time, so that their presence is less frequent in cells, not considering the limitations of the resolution in our micrographs.

From the point of view of electron microscopy, an inclusion body is the center of newly formed viruses or the place in the cell where pro-viruses are originated and viruses reorganized or formed from one or more matrices. This interpretation agrees with that of Prowazek (4) who described the inclusion body as being an obligatory stage of virus multiplication or a colony of virus particles. More recently, Bland and Robinow (1) confirmed this interpretation. From Wyckoff's and our own (6) observations it may be concluded that newly formed viruses are not originated by binary division or fission of one previously enlarged virus particle giving origin to new virus corpuscles. Figs. 2, 3 and 4 seem clear enough to admit that vaccinia and related viruses are not reproduced like bacteria, as one could expect, specially because of their large dimensions as compared with other viruses. The structural resemblance of fowl pox and vaccinia virus particles and the interior of pro-virus particles with small mitochondria has already been described by others. Eaves and Flewett (2) show in infected chicken embryo chorioallantoic membranes that the inner structure of elementary bodies is similar to small mitochondria, but distinguishable. The double lines inside the matrix, however, are more similar to the mitochondrial lamellae. We believe that mitochondria play some important role in the formation of the inclusion or in the process between the moment at which virus particles enter the cell and the time of rearrange-

ment of the matrices or reorganization of new virus particles in the inclusion body.

Sections of less compact inclusions or isolated matrices show too frequently the presence of mitochondria usually having the same dimensions as virus particles. Larger mitochondria are seen to be in a process of disorganization, still showing the typical trabeculae. Sometimes, matrices already giving origin to sprouts show traces of trabeculae. After the pro-virus formation, an almost complete disappearance of preserved mitochondria is observed in the place of virus formation, whilst in other areas of the cytoplasm large amounts of mitochondria are found irregularly distributed, or in areas where pro-viruses and viruses are encountered in large numbers.

From these observations it is not possible, at the present stage of the work, to draw secure conclusions regarding this relation, however, two possibilities may be considered. First, that mitochondria participate directly in the constitution of the inclusions, not only contributing with their enzymatic systems to the formation of the new pro-viruses, but also by constituting material of their structures which would be disintegrated and the disintegration products used in the viral morphogenesis. The other possibility would be that pro-viruses and the matrix have structures similar to that of mitochondria, and that the true mitochondria would influence the viral morphogenesis from distant, not being necessarily present at the inclusion or the place of development of new virus particles.

The granular material seen in the area of newly formed pro-viruses is very similar to that found in the place where groups of mitochondria are disintegrated and their inner material liberated in the cytoplasm. This material seems to be consumed between phases B and C, or somehow dispersed in the cytoplasm. We believe this material to take part in the evolution process, because it is always present in phase B (pro-virus) and always disappears when phase C is predominant.

This paper deals specially with the aspects of intracellular virus development, only mentioning the eventual relation between mitochondria and this process. It is not possible, nor do we wish to make any reference here to the modifications of the endoplasmic reticulum and the nucleus, which might, as already described by others, take part in the evolution process of the virus in the cell.

SUMMARY

In epithelial rabbit kidney cells cultured "in vitro" various phases of the vaccinia virus evolution cycle were detected and described, particularly those observed from the formation of the inclusion bodies to the appearance

and liberation of mature elementary bodies. The inclusions may be composed of one or more sub-units or matrices, on the surface of which particle formation takes place by a process of "sprouting", which in the initial phase confers the matrices the shape of a morula. Three main stages can be differentiated during the progressive modification of these particles until the stage of maturity. Indications were observed that mitochondria may participate in the formation of the inclusion matrix or even in the formation of the elementary bodies.

Studies viewing a better knowledge of the formation of the inclusions and of the structural transformations that occur during the passage from one phase to another in the evolution of the elementary body are being carried out in this laboratory.

SUMÁRIO

Vírus vacínico foi adaptado ao crescimento de células epiteliais de rim de coelho artificialmente cultivadas em meio de Hanks ou Earle, contendo sôro bovino, hidrolisado de lactalbumina e antibióticos. Cento e oito passagens iniciadas com amostra bovina utilizada para vacinação humana foram feitas ininterruptamente em células epiteliais de rim de coelho cultivadas "in vitro". Nestas células foi possível acompanhar, ao microscópio eletrônico, grande parte do ciclo evolutivo dêste vírus no citoplasma, principalmente após a formação das inclusões e até a liberação de partículas de corpúsculos elementares maturos.

As inclusões, em geral situadas na região próxima ao núcleo, são constituidas de uma ou mais subunidades ou matrizes, na superfície das quais se originam as partículas que vão sendo progressivamente liberadas no citoplasma. Nesta fase, as matrizes adquirem o aspecto de uma mórula, devido ao fato de que as partículas vão se formando por um processo semelhante ao de uma brotulação. Terminada esta fase, a matriz desintegra-se ou é consumida. No interior das partículas recém-formadas inicia-se uma série de transformações, que se evidenciam por significativas alterações morfológicas que antecedem o pleno amadurecimento dos corpúsculos elementares. Esquemàticamente, três fases principais podem ser diferenciadas no transcorrer destas transformações evolutivas. Tanto as matrizes quanto as partículas individualizadas recentemente formadas, apresentam, por vêzes, estrutura interna difícil de se diferenciar daquelas das mitocôndrias. Outros fatos analisados parecem também indicar a provável participação dêstes organelos, quer na formação das inclusões, quer na formação dos corpúsculos elementares na sua primeira fase evolutiva.

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Fig. 1 — Section of a vaccinia infected cell containing a large cytoplasmic inclusion composed of numerous sub-units (su) and also of some already individualized particles. In this phase, matrices or individualized virus particles are seen only in the inclusion area or in its vicinity. Between the centrally disposed cell and the cell at the right side of the micrograph, some mature virus particles (mv) are seen. N = nucleus; li = lipids; er = endoplasmic reticulum; mi = mitochondrion.

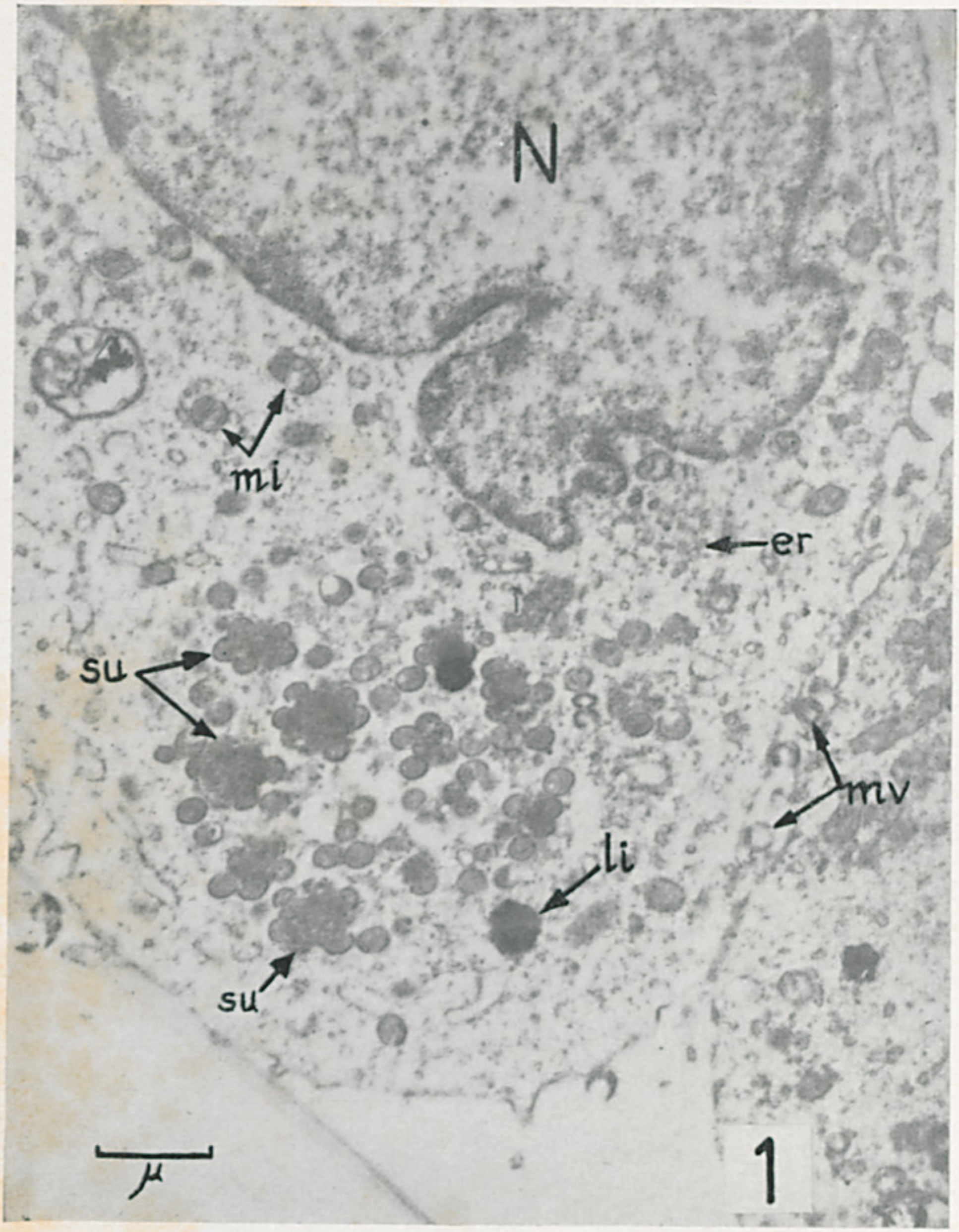
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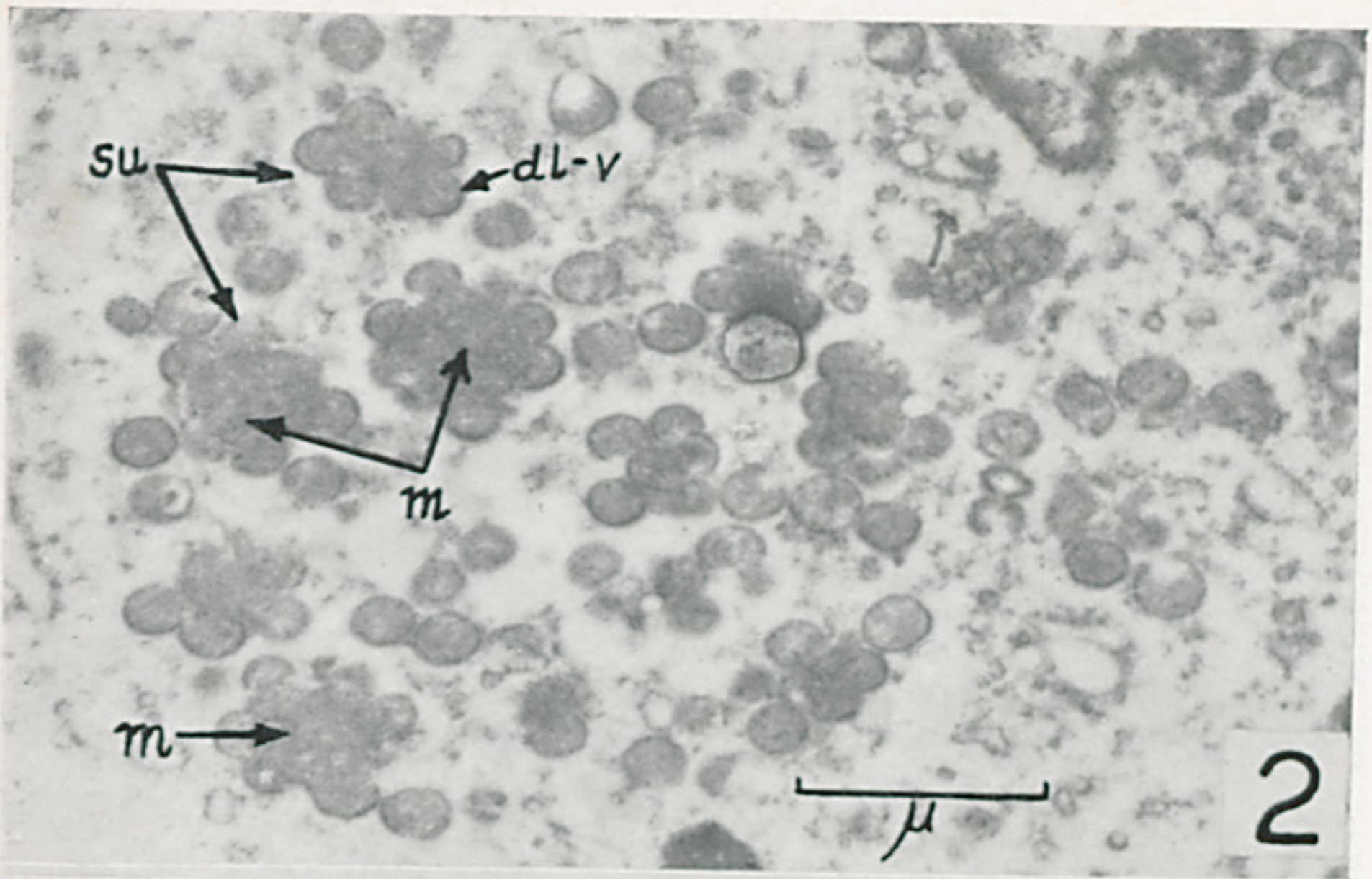
Fig. 2 — Enlargement of the inclusion depicted in fig. 1. Each sub-unit (su) is composed of a matrix (m) giving origin to new virus particles at its periphery. New spherical particles are apparently formed like sprouts from the central matrix. Generally, double lines and vesicles (dl-v) are seen inside the matrices, the sprouts or the newly formed particles.

Fig. 3 — Cytoplasmic region of granular material (g) containing particles with double lines and vesicles (dl-v) inside and an almost complete limiting membrane. These structures resemble that of mitochondria (mi). In areas of granular material where none of the completely individualized particles appear, elements (dl) are found which resemble more distinctly mitochondria, although they are not well defined because of being associated with the granular material of the cytoplasm. The aspects presented in this micrograph are held to be the final stage of activity of one or two matrices giving origin at the same time to pro-virus particles and the surrounding granular material. li = lipids.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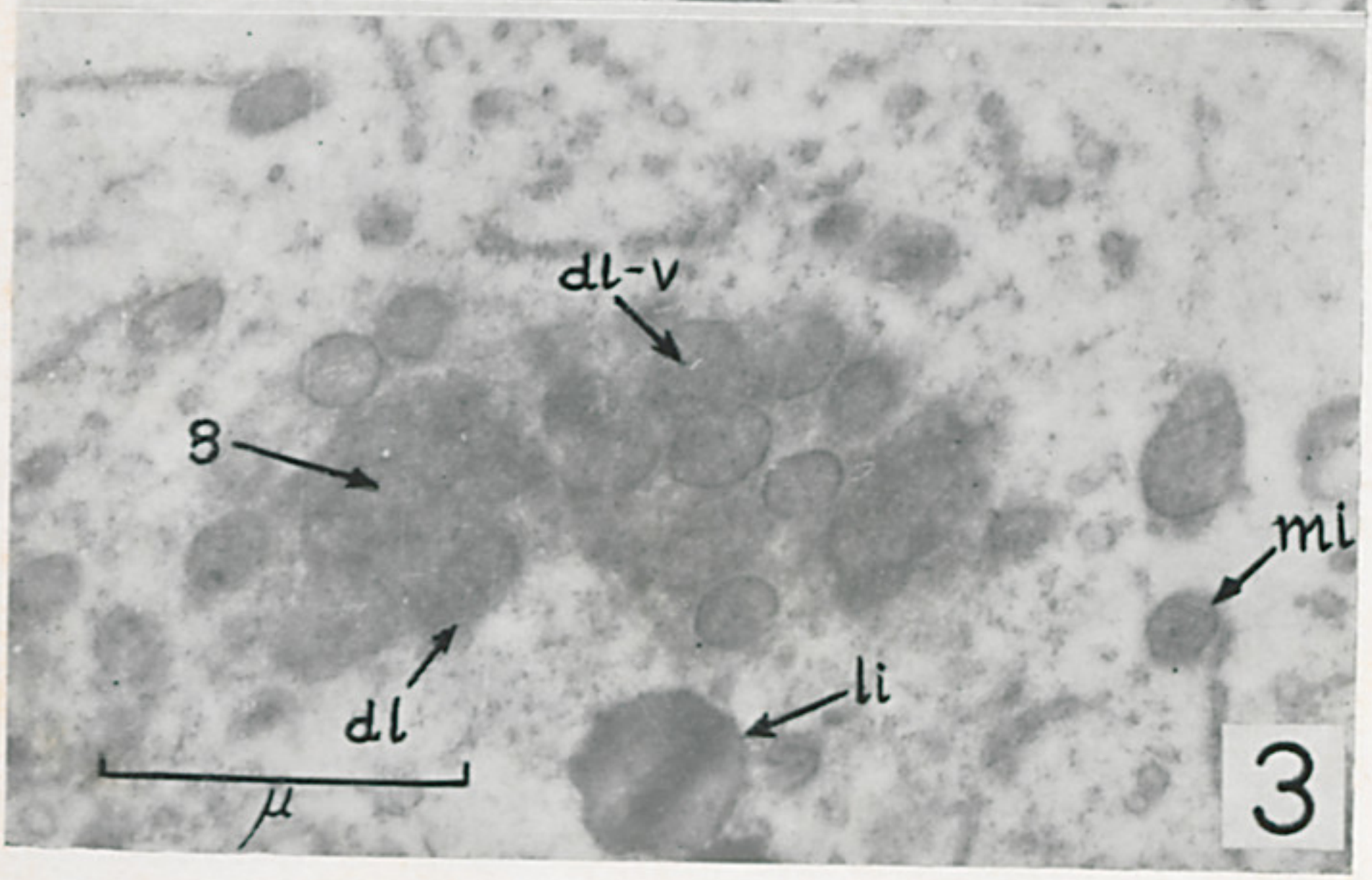
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Fig. 4 — Cytoplasm with granular, electron dense material (g) containing more or less individualized particles, some of which present a dense, eccentrically disposed body. In *mi* appears an agglomeration of mitochondria, near to which are vacuoles (v) limited by double membranes, which remind mature virus particles rarely observed in the cytoplasm. mv = mature virus particles, outside and inside the cytoplasm.

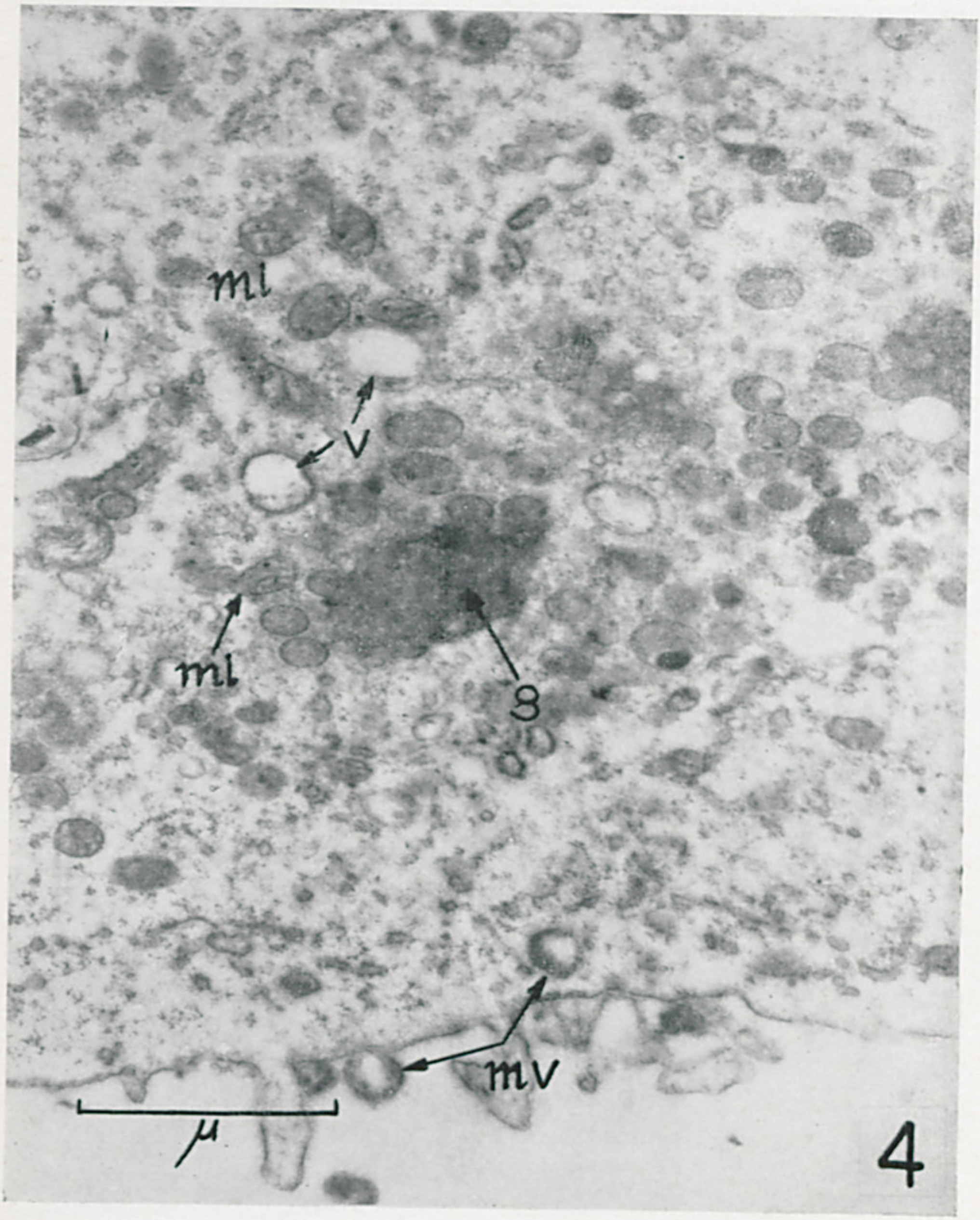
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Fig. 5 — Cell containing numerous pro-virus particles, phase B, in the cytoplasm. The majority of the pro-virus particles is already individualized and located in a more granular zone (g). Many mature virus particles (my) are adherent to the cell membrane. N = nucleus; mi = mitochondrion; li = lipids; er = endoplasmic reticulum.

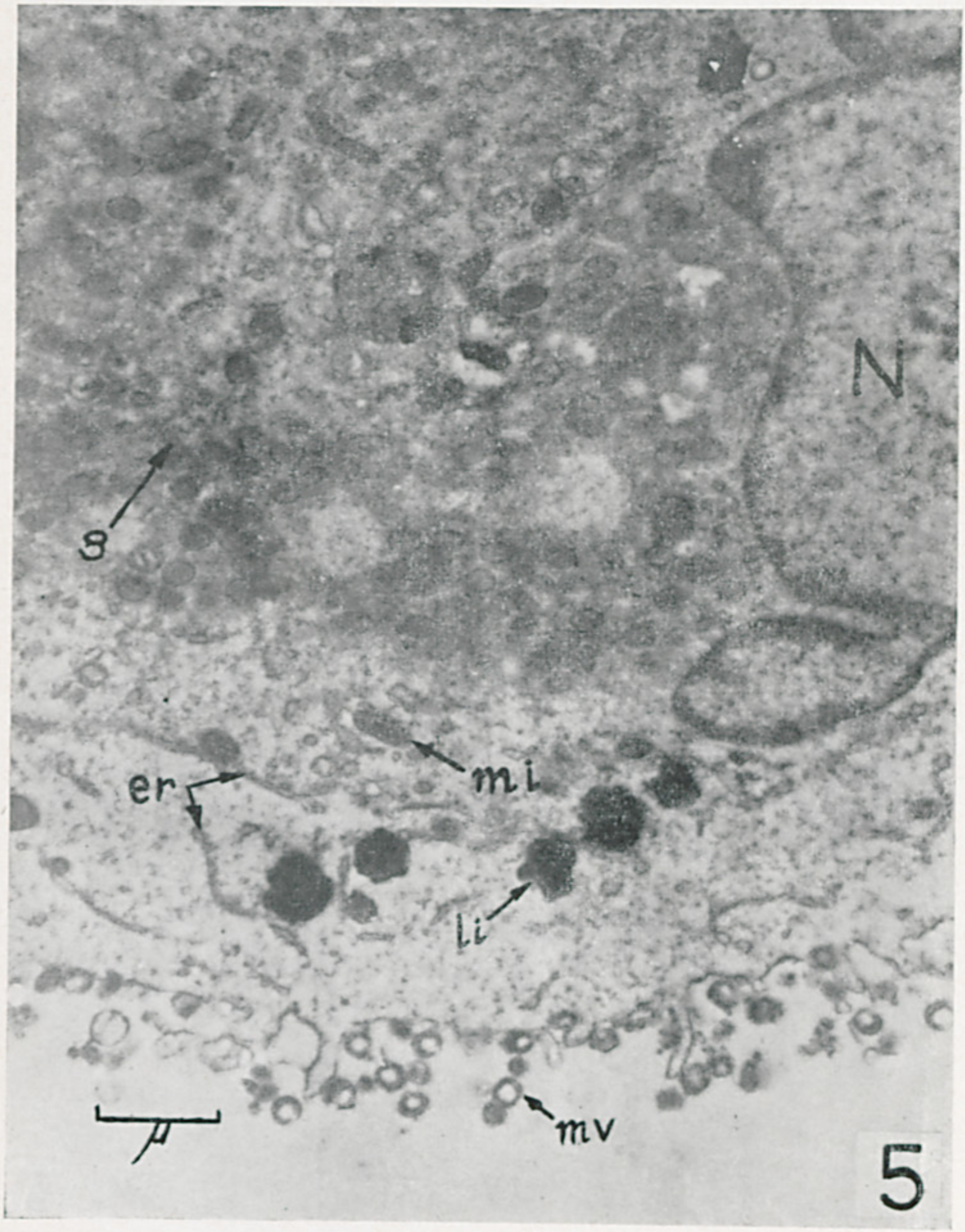
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Fig. 6 — Section of epithelial cell fixed 5 hours after infection, containing virus particles surrounded by a granular zone (g). The area of elementary body formation and evolution is apparently maintained at a constant distance from the location of the constituting elements of the Golgi apparatus (G). mi = mitochondrion; er = endoplasmic reticulum; li = lipids.

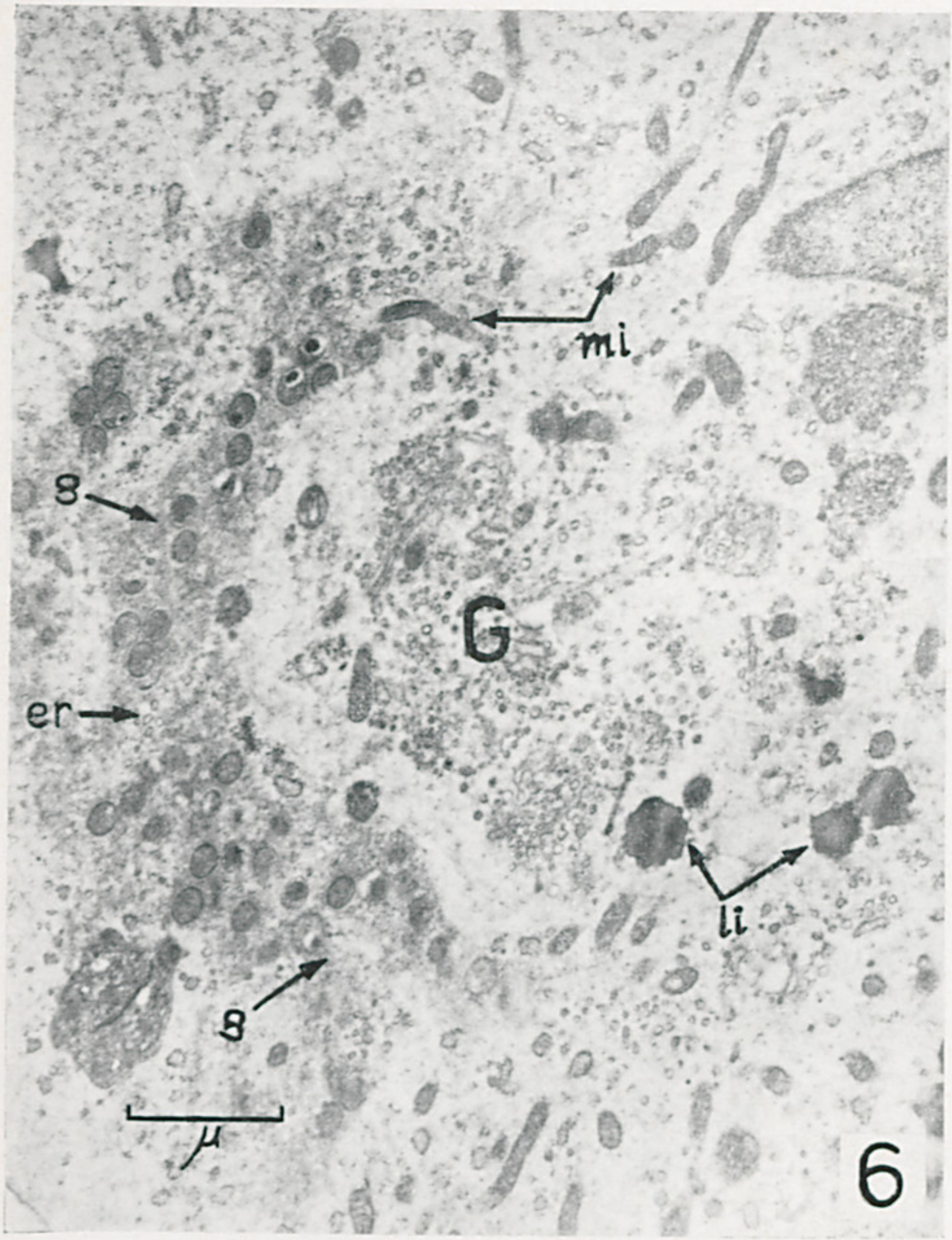
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Fig. 7 — Cell with virus particles occupying the cytoplasm to a large extent. The virus particles, which are in a more advanced stage of development than those of the preceding figures, present variable internal structures, however, generaly are characterized by the dumb-bell shape. Their elliptical sections do not seem to be due to pressure of the knife during sectioning, because there are particles orientated in different directions along the larger axis. No electron dense, granular material is seen in this phase; the cytoplasm surrounding the particles presents a similar aspect to areas without particles. N = nucleus; mi = mitochondrion; li = lipids.

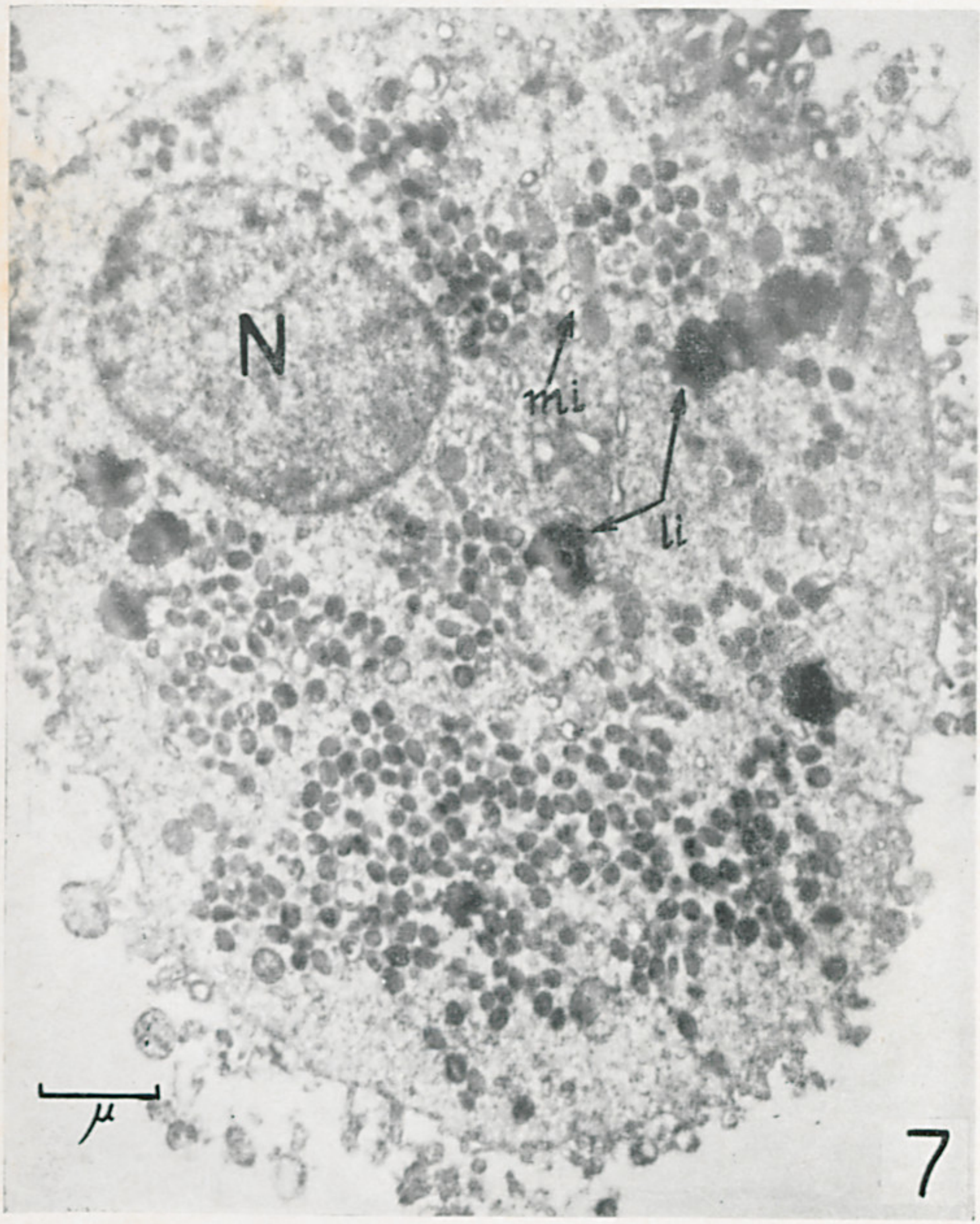
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Fig. 8 — Section of cells in different levels showing large number of mature virus particles (mv) close to the cytoplasmic membranes. The mechanism of virus entering the cytoplasm was not yet detected, however, particles similar to the external ones were already observed inside the cell in the peripherical cytoplasm. N = nucleus.

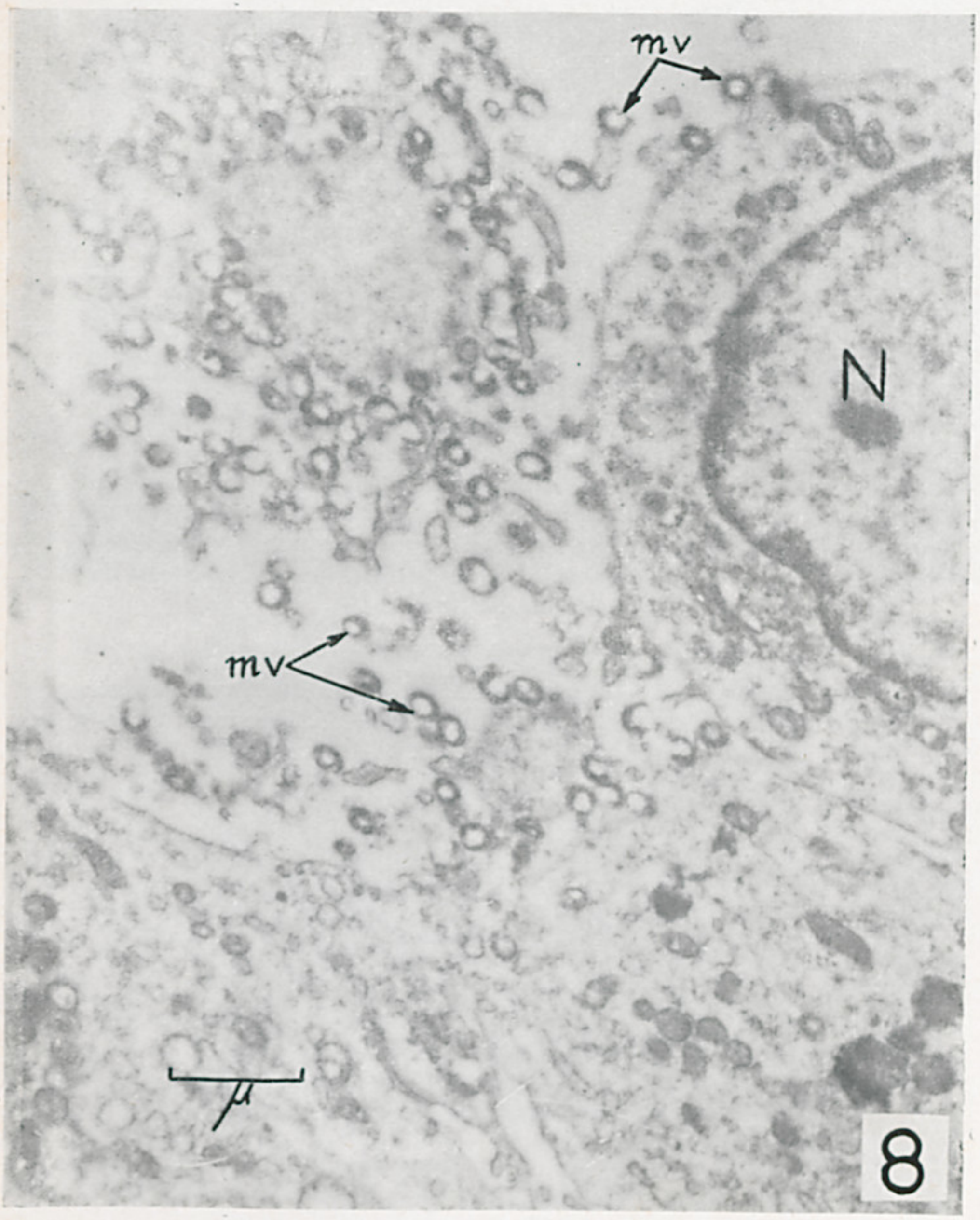
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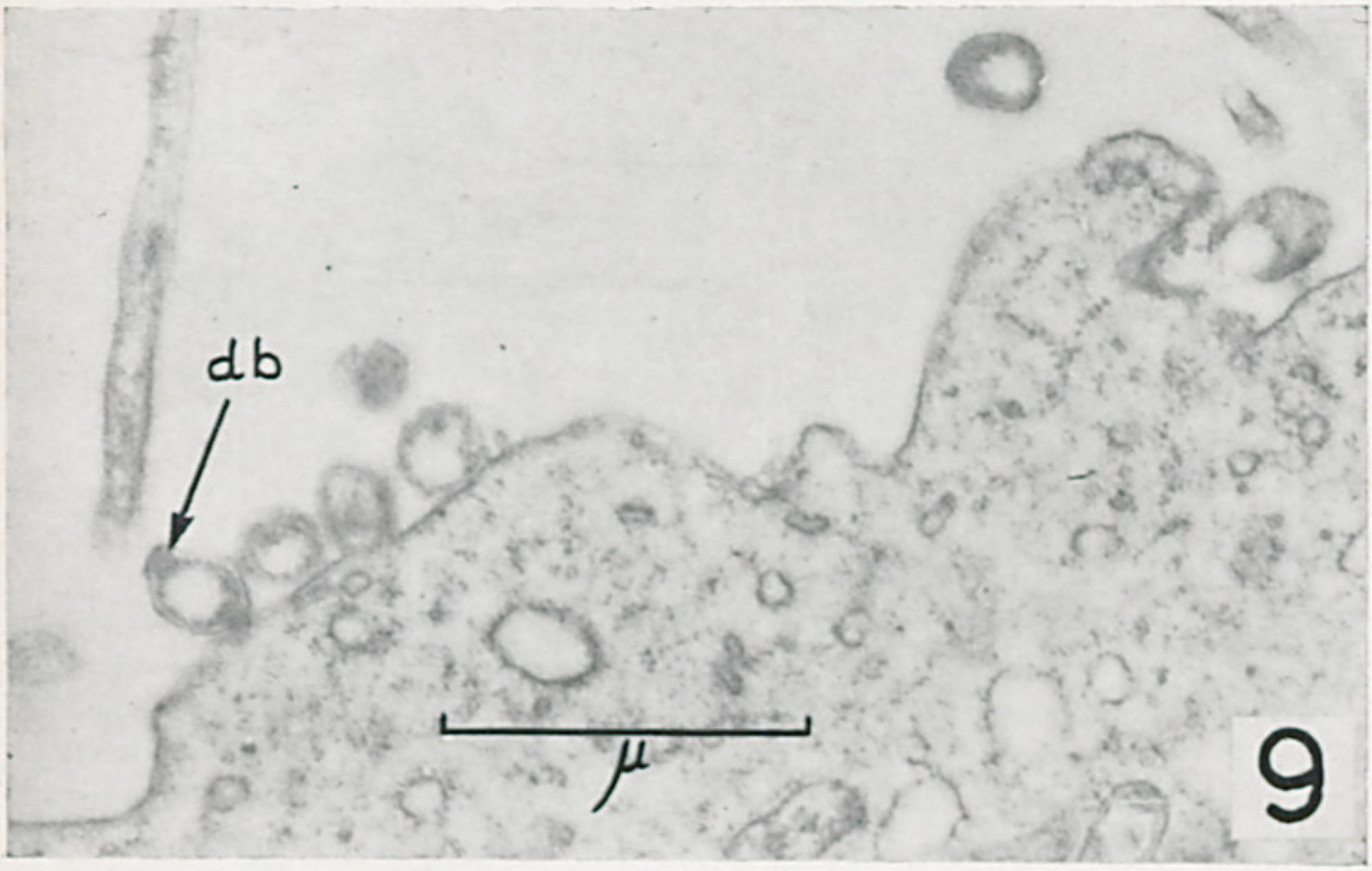
Fig. 9 — Section of cell showing peripherical cytoplasm containing some mature virus particles adhered to the cytoplasmic membrane. Generally, sections of these particles present two concentrical membranes and a transparent or slightly electron dense core. In some sections, one or two dense bodies (db) are observed between the two membranes. When two dense bodies are transected in the same virus particle, they appear diametrically opposed.

Fig. 10 — Electron micrograph of shadowed, purified vaccinia virus from a 48 hours culture of epithelial rabbit kidney cells. The infective material was constituted of virus previously submitted to 54 passages in the cells "in vitro".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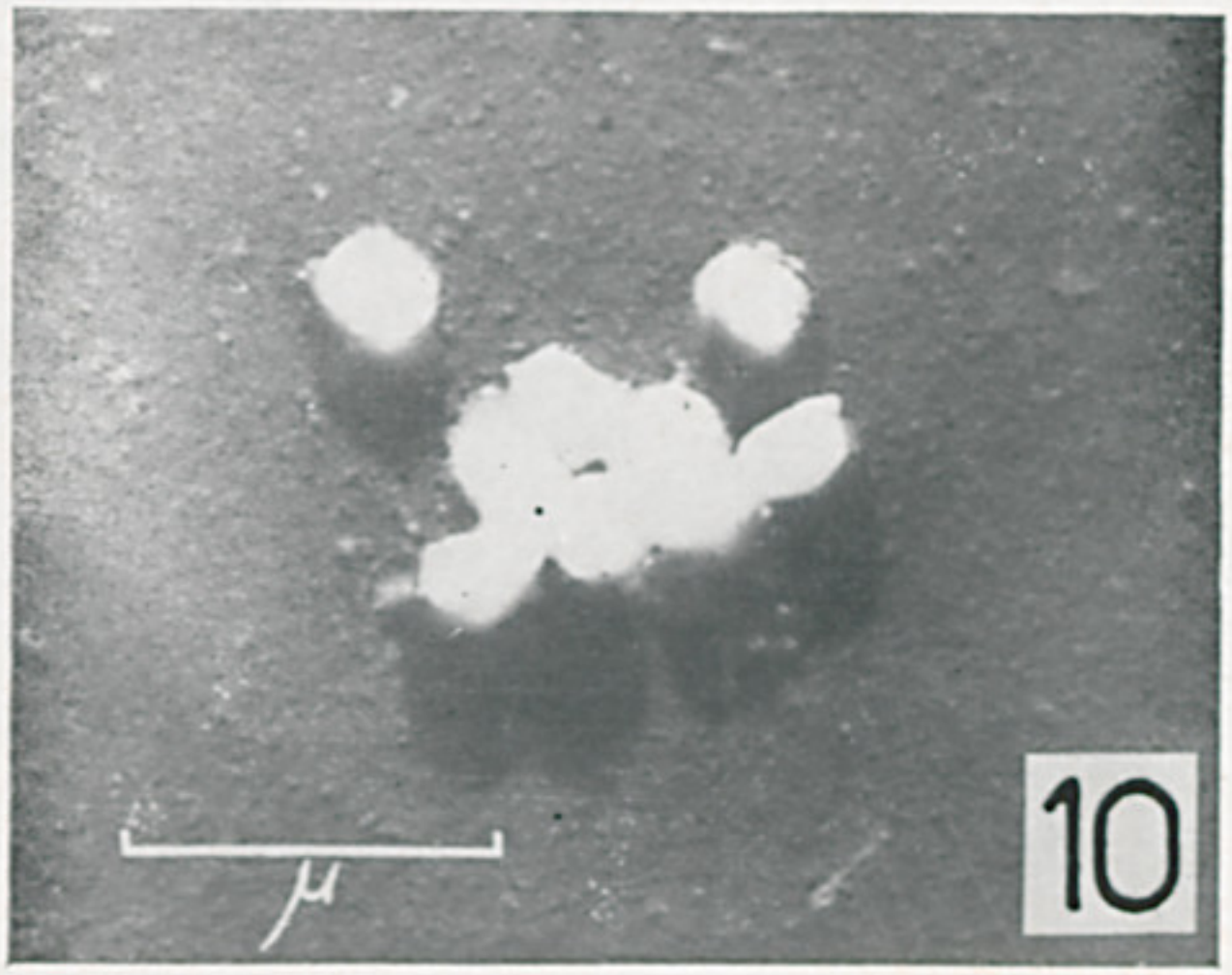
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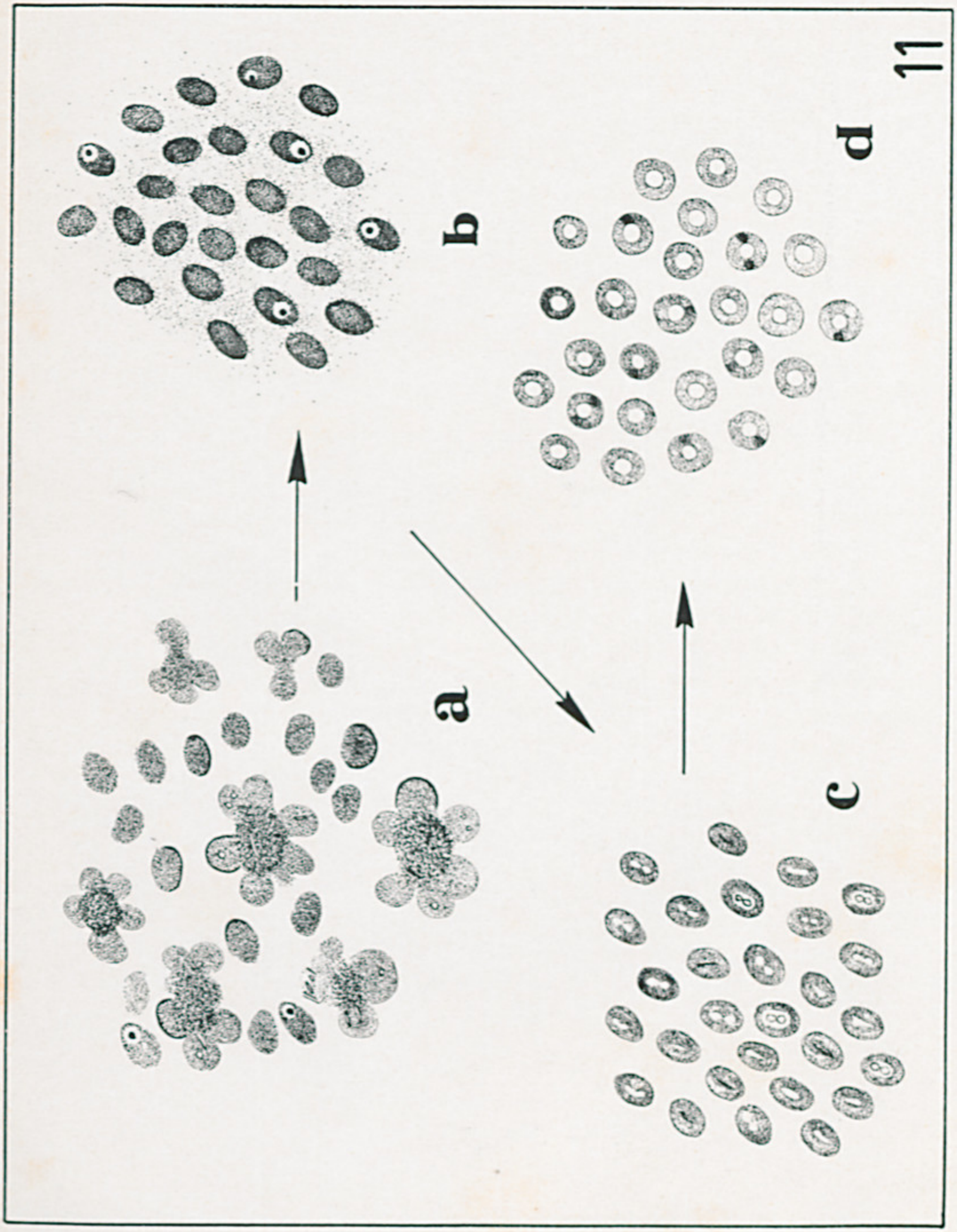
Fig. 11 — Schematic representation of predominant morphological aspects of sections of vaccinia virus during evolution from the inclusion body to the mature, free elementary body. Phases *a*, *b* and *c* are intracytoplasmic; phase *d* corresponds to free, mature virus. Rarely, type *d* particles are seen in the cytoplasm. From *a* to *b*, the characteristics are the progressive disintegration of the matrix or matrices constituting the inclusion, simultaneously giving origin to an increasing number of "pro-virus" particles surrounded by granular material; from *b* to *c*, the particles undergo internal, structural modifications, simultaneously with the disappearance of the granular material; from *c* to *d*, the virus particles are liberated from the host cell, apparently at the same time when death and disintegration of the cell occur.

The aim of this paper was not to study in detail the microstructure of the virus particles in each evolution phase, but to know their relation to the preceding and succeeding phase.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