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ELECTRON MICROSCOPIC STUDY OF THE FORMATION OF BLUETONGUE VIRUS*

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INTRODUCTION

Bluetongue is caused by an unclassified RNA virus which has a diameter of approximately 600 Å (Owen & Munz, 1966). According to these workers the particle is an icosahedron containing 92 capsomeres, with some evidence of the presence of an envelope. More recently, Studdert, Pangborn & Addison (1966) have suggested that the causal agent of bluetongue is possibly a reovirus which shows no envelope while Bowne & Jones (1966) consider it as an arbovirus with evidence of an envelope " which is derived from proliferation of cytoplasmic membranes ". The present study was undertaken to characterize the virus with respect to the site and mode of replication in the cell, the presence or absence of an envelope and the method of entry into and exit from the cell during a single infection cycle.

MATERIALS AND METHODS

Virus and cells

BHK 21/13 cells grown in Roux flasks in Eagles medium were used in the study. Cells were inoculated with an attenuated strain of bluetongue virus Type 10 at an input multiplicity of 5–10 PFU/cell.

Electron microscopy

After infection with the virus, cells were harvested at 5, 15, 30 and 60 minutes and 2, 4, 8, 12, 16 and 20 hours. Before harvesting, cells were washed with fresh Eagles medium to remove cell debris left from the virus suspension. Approximately 1×10^8 cells were freed from the surface of Roux flasks by vigorous, manual shaking. The suspensions were centrifuged at 300g for 5 minutes and pellets of cells obtained in this way were fixed for one hour in 1 per cent buffered solution of osmium tetroxide (Millonig, 1961). Pieces of these pellets were embedded in Epon mixtures (Luft, 1961) and sectioned with glass knives on a Porter Blum MT—2 ultramicrotome. Sections were double stained with 5 per cent uranyl acetate for 45 minutes followed by lead citrate for 15 minutes (Reynolds, 1963). A Siemens Elmiskop IA electron microscope was used in the investigation.

RESULTS

The uninfected cell

The BHK 21 cell maintained in our laboratory possesses large, centrally situated, lobed nuclei. The rough endoplasmic reticulum (ER) is fairly well developed. There is little smooth ER. The cytoplasmic matrix is rich in ribosomes which

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occur singly or, more commonly, in groups of five or more (polysomes). Clusters of very fine filaments occur in the cytoplasmic matrix in limited numbers. Mitochondria and the Golgi complex occur only occasionally. The surface of the cell commonly shows microvilli.

Changes resulting from infection with bluetongue virus

Penetration of the cell by virus: Cells infected with bluetongue virus at an input multiplicity of 5–10 PFU/cell showed numerous fine structural changes. Adsorption and penetration of the particle occurs within the first five to ten minutes, the particle entering the cell by means of a pinocytotic vesicle [Plate 1 (1)]. At about 30 minutes the appearance of dense bodies containing nucleoids occurs [Plate 1 (2)], and this is followed by the appearance of numerous, small dense, lysosome-like inclusion bodies in the cytoplasm at about two hours [Plate 1 (3)].

Swelling of endoplasmic reticulum: Simultaneously at two hours, the rough ER begins to show progressive swelling to form vesicles lined by ribosomes [Plate 1 (4)]. Four hour cells show further swelling of the ER with little or no flattened ER in evidence.

Bundles of tubular elements: Appearing at eight hours are bundles of parallel orientated tubular elements which appear hollow in cross section with occasional dense centres [Plate 2 (5 and 6)].

Mitochondria: At 12 hours the mitochondria show characteristic dense inclusion bodies which are absent from normal BHK cells grown under our laboratory conditions. The inclusion bodies appear to be structurally associated with the cristae of the mitochondria [Plate 2 (7)].

Fine filaments: From eight hours onwards a large increase in the amount of fine filaments present in the cytoplasmic matrix occurs. They appear to be of two types depending on whether they are dispersed or in bundles [Plates 2 (8) and 3 (9)]. The latter are often found in close proximity to mature progeny particles.

Mature progeny particles: Mature virus particles are commonly seen in clusters near the nucleus in 12, 16 and 20 hour cells and incipient particles appear in this position in 8 hour cells [Plate 3 (10)]. The latter appear to have no dense central core as do mature particles. Isolated groups of particles in various positions including some near the cell membrane are evident [Plate 3 (11)]. Some particles are enveloped by a single membrane but this is rare, most particles being liberated without an envelope. Clusters of mature particles often show extremely fine filaments attached to the core of some particles, with possible connections between filaments also evident [Plates 3 (12), 4 (13) and 4 (14)]. Such connections have also been seen in African horsesickness virus and reovirus Type 1. Mature particles are also seen lying at the centre and periphery of dense, irregular masses of granular material [Plate 4 (15)]. At 16 hours mature particles are often seen to lie in the ER vesicles and even in the perinuclear space. Particles liberated from broken cells have no envelope and breaks in the cell membrane allow passage to the cell exterior. There is no evidence of budding off from the cell membrane to liberate mature particles.

Discussion

It seems evident that once bluetongue virus has gained entrance into the cell the initial reaction of the cell is to produce dense lysosome-like inclusion bodies containing nucleoids within 30 minutes, adhesion and penetration by the particle occurring rapidly within the first 5 to 10 minutes. The diameter of these nucleoids

suggests that they are virus particles which are degraded within the lysosome-like bodies. This is followed by incipient swelling of the rough ER at about 2 hours, a process which is maximumly developed by the time mature progeny particles become evident.

Further changes occurring up to about 8 hours include an increase in size of the inclusion bodies with simultaneous loss of nucleoids and an increase in the degree of swelling of the rough ER. From 8 hours onwards an increase in the number of fine filaments occurs resulting in masses of filaments in various areas of the cytoplasmic matrix. It would appear that these filaments are similar to those reported by Dales, Gomatos & Hsu (1965) in L strain cells infected with reovirus. Both "kinky" filaments and fine filaments appear to be present in bluetongue-infected BHK cells. Their possible significance in virus replication is completely obscure. The appearance of particles in groups in areas adjacent to the nucleus is significant since mature particles are commonly found in this area from 12 hours onwards. At 8 hours, the particles are vaguely defined and this is assumed to be due to their incomplete formation at this stage. Some of these particles appear to have hollow centres, and others appear at 12, 16 and 20 hours after inoculation showing extremely fine filaments attached to the central core. This phenomenon is also noted in African horsesickness virus and reovirus Type 1. It seems evident from Plates 3 (12), 4 (13) and (14) that the particles with larger cores have no filaments attached to them, while those with smaller centres show attached filaments. This appears to suggest a possible packing in of the nucleic acid after formation of the capsid, or that the capsid has condensed around an incompletely coiled nucleic acid moiety. The contention of Kellenberger (1966) that a central scaffold is not a prerequisite for the formation of the protein capsid, appears to support this observation. An alternative but less likely explanation is that the nucleic acid core is progressively removed. To date, efforts to digest the attached filaments with ribonuclease have been unsuccessful. The connections of some of the attached filaments with each other suggest that they may contain more than one strand.

The presence of dense irregular masses of granules with mature virus particles at the centre of each mass, presumably represents a stage in maturation of the virus particles. Similar structures have been seen in BHK cells infected with African horsesickness virus. The significance of the simultaneous appearance of bundles of tubular elements in parallel array and mature progeny particles is obscure at this stage. The diameter of individual tubules roughly corresponds to the diameter of spindle tubules found to be associated with reovirus development (Dales, 1963). The position of isolated mature particles in various areas in the cytoplasmic matrix and especially at the cell membrane appears to signify movement towards the cell periphery from 12 hours onwards, i.e. soon after maturation.

Evidence for the liberation of particles through breaks in the cell membrane is substantial while no evidence exists to indicate that budding of the cell membrane liberates virus particles. Twenty hour cells are commonly found to have a disrupted cell membrane with groups of particles lying outside the cell.

The fact that a large majority of the particles leave the cell in this way and the occasional observation of particles with an envelope derived from within the cytoplasmic matrix suggests strongly that bluetongue virus has no envelope. Since no visible implication of the nucleus in virus maturation is evident it seems clear that bluetongue virus is essentially produced in the cytoplasm.

Summary

BHK 21 cells infected with bluetongue virus were sampled at various stages during a single cycle of viral replication, sectioned and studied with the electron microscope. Stages in development of the virus have been recorded including initial penetration into the cell and eventual exit from the cell of mature particles. This study indicates that bluetongue virus essentially has no envelope. Successive characteristics shown by the cells include:

(1) appearance of dense inclusion bodies;
(2) swelling of the endoplasmic reticulum;
(3) the appearance of masses of fine filaments in the cytoplasm;
(4) the presence of mitochondria containing dense inclusion bodies;
(5) the appearance of bundles of tubular elements;
(6) the presence of irregular, dense bodies containing virus particles and
(7) incipient and mature virus particles.

Agknowledgements

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PLATE 1 →

Bar equals 250 mμ

1. Virus particle (arrow) entering cell by pinocytosis. 15 min cell. Cell membrane (CM)
2. Inclusion body (arrow) containing dense, central core. 30 min cell
3. Lysosome-like bodies (arrows), one containing a dense mass of material. 30 min cell
4. Swollen, rough ER vesicles in 20 hour cell

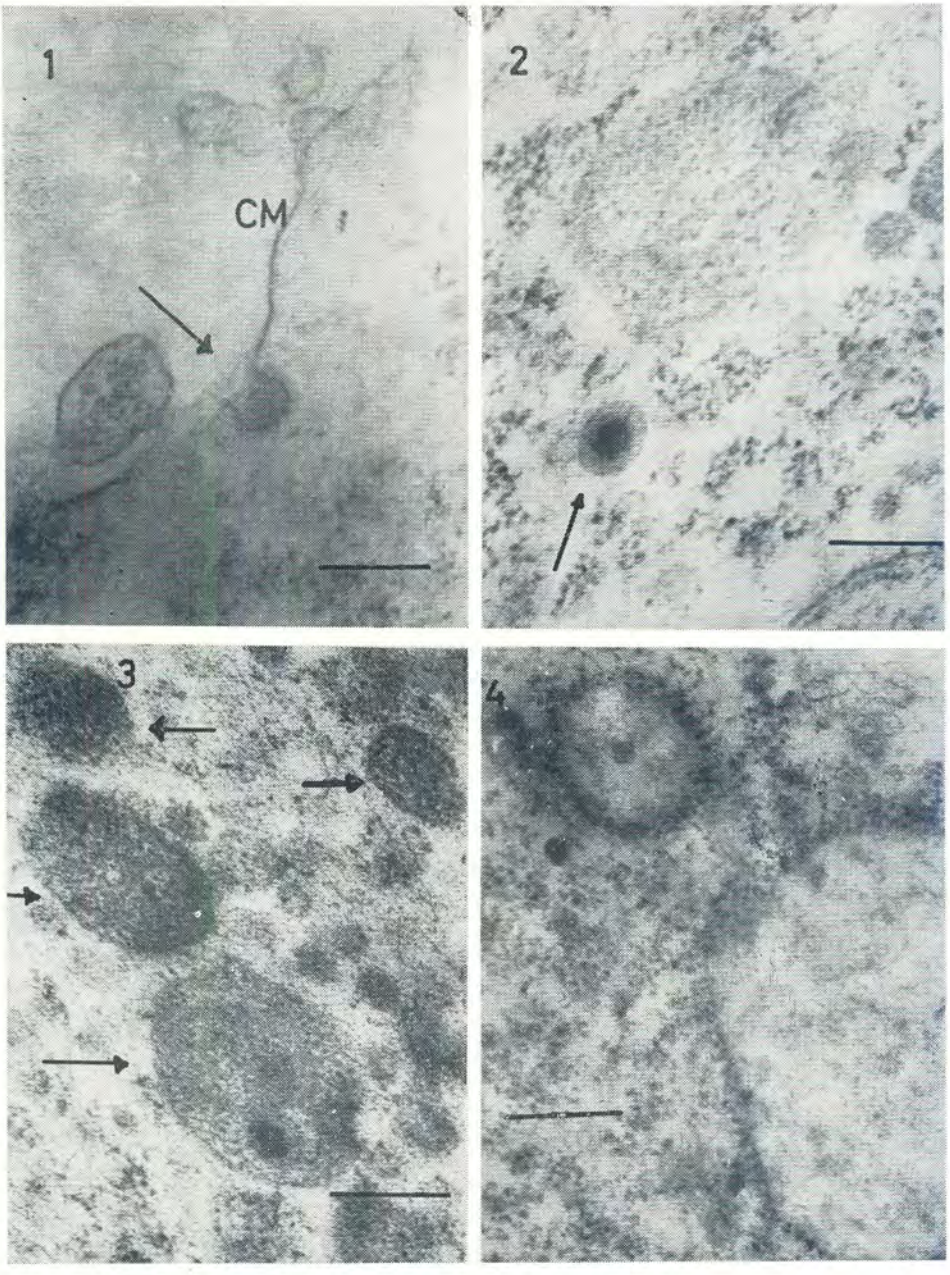
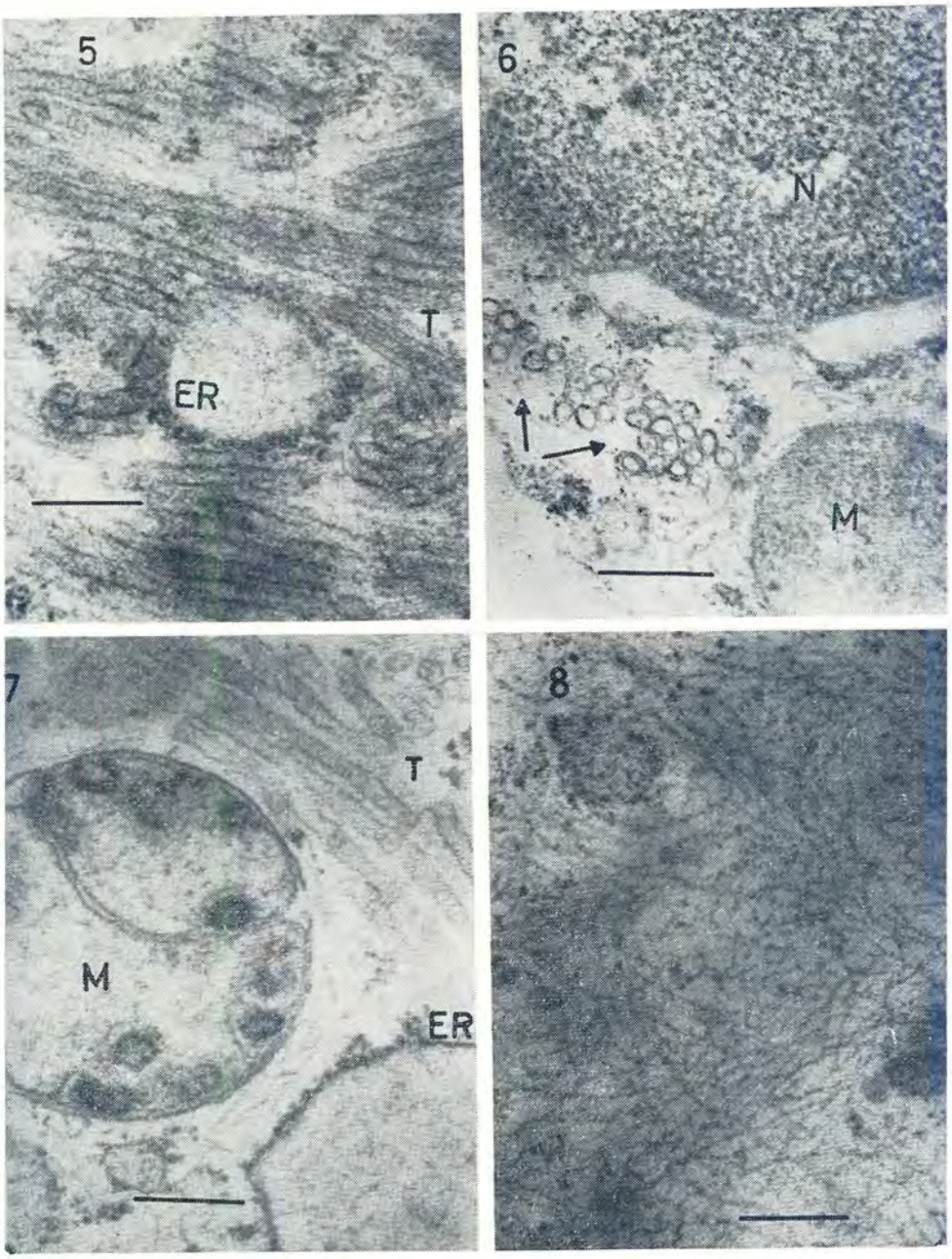

1
CM
2
3
4

PLATE 2

Bar equals 250 mμ

5. Bundles of parallel orientated tubular structures (T) adjacent to swollen, rough ER vesicle (ER). 20 hour cell
6. Tubular structures shown in cross section (arrows). 20 hour cell. Mitochondrion (M), nucleus (N)
7. Mitochondrion containing several dense inclusion bodies. Tubular elements and swollen rough ER vesicle evident. 12 hour cell. Tubular structures (T) mitochondrion (M), endoplasmic reticulum (ER)
8. Mesh of fine filaments showing granular character along their length. 8 hour cell

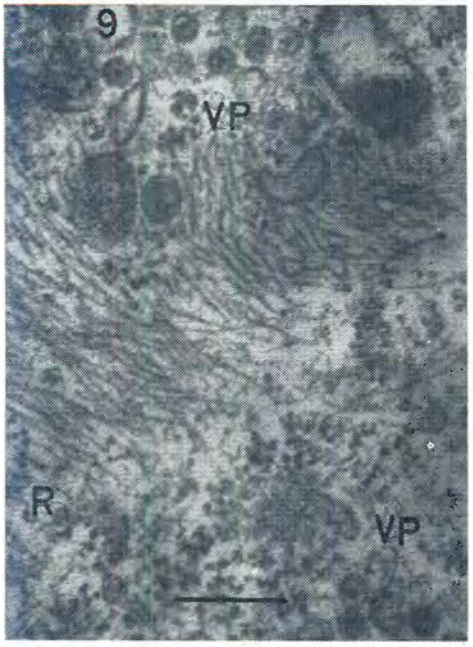


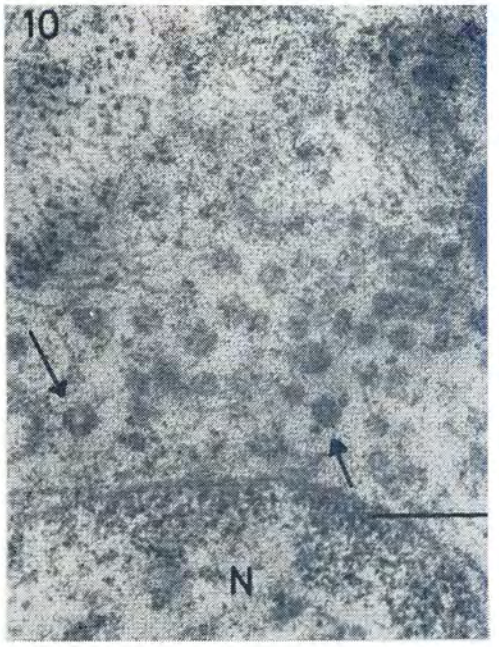
25408—10

PLATE 3

Bar equals 250 mμ

9. Bundle of fine filaments lying between two groups of mature virus particles. Polysomes are abundant in the cytoplasmic matrix. 12 hour cell. Polysomes (R), virus particles (VP)
10. Arrows indicate virus-like dense, spherical bodies, one of which shows a hollow centre. They occur in the perinuclear area and are associated with the Golgi complex. 8 hour cell. Nucleus (N)
11. Mass of mature virus particles at periphery of cell, adjacent to cell membrane. Some fine filaments are associated with the cores of the particles. 20 hour cell. Cell membrane (CM)
12. Arrows indicate mature virus particles with large central cores and no attached filaments. Other virus particles in the photograph show small cores with attached filaments. 20 hour cell. Tubular structures (T)


9
VP
R
VP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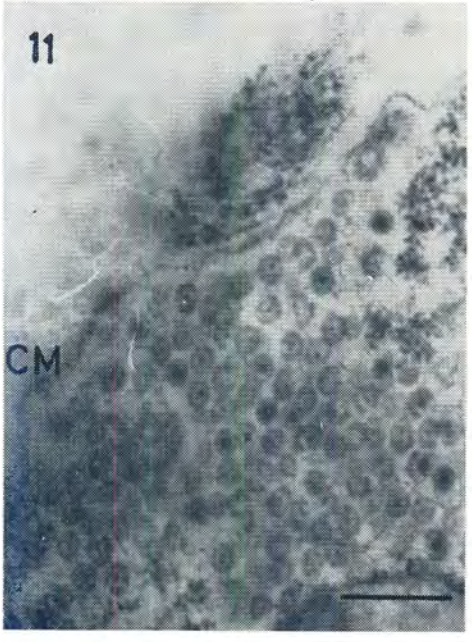
N

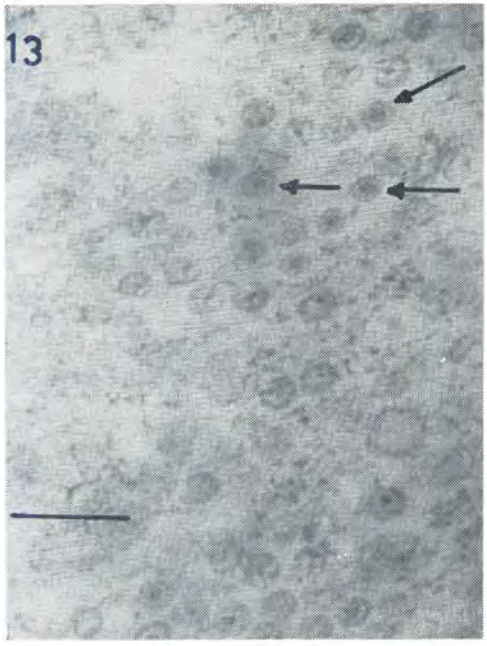

11
CM


12
T

PLATE 4

Bar equals 250 m/μ.

13. Arrows indicate mature virus particles with large central cores and no attached filaments. Other virus particles in the photograph show small cores with attached filaments. 20 hour cell
14. Mature virus particles showing connections (arrows) between filaments attached to different particles. 20 hour cell
15. Electron-dense mass of granular material showing mature virus particles at the centre and periphery (arrows). Particles appear to arise from the granular mass. 12 hour cell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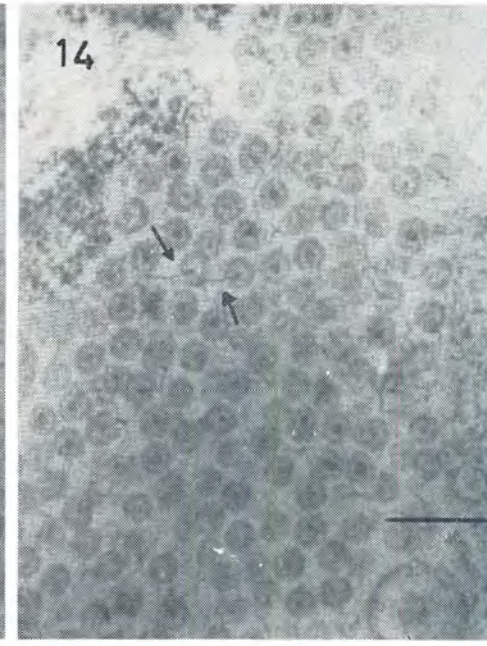
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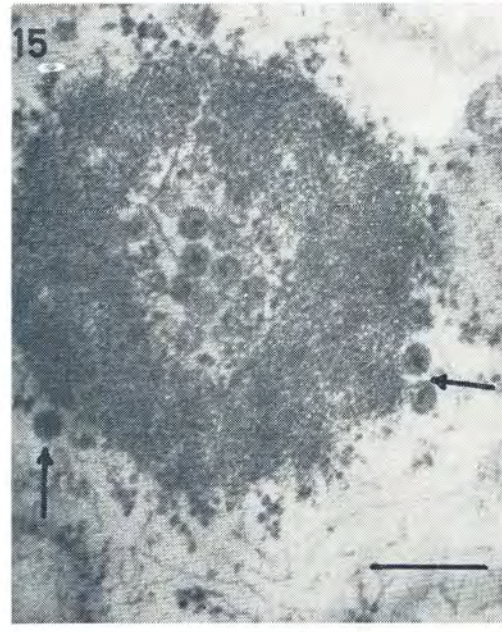
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