ELECTRON MICROSCOPIC STUDIES ON THE DEVELOPMENT OF VESICULAR STOMATITIS VIRUS IN KB CELLS

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ABSTRACT

The development of vesicular stomatitis virus in KB cells was studied by electron microscopy. Sections of infected cells were made 1, 4, 7, 10, and 20 hours after inoculation of the cell cultures, and at the same intervals the supernatant fluid was assayed for virus titer by the plaque test in chick embryo cells. At 10, 14, and 20 hours after inoculation, virus rods were observed attached to cytoplasmic membranes, inside cytoplasmic vacuoles, and attached to the membranes delimiting these vacuoles; they were also found on the surface membrane of the cells. Besides the rods, spherical particles of different sizes and shapes were seen. The possibility that these structures are related to the development of virus rods is discussed. A similarity was noted between the site of maturation of vesicular stomatitis virus rods and that of some other arbor viruses.

Vesicular stomatitis (VS) virus particles are rod-shaped. Their dimensions have been reported to be 210 × 60 (1) or 175 × 69 mμ (2). Furthermore, Bradish *et al.* (2) found non-infectious spherical particles, 65 mμ in diameter, possessing a virus-specific complement-fixing activity. By negative staining with phosphotungstic acid, it was shown (3, 4) that each rod had a round and a square end, and there was evidence of an internal component, probably helical (4). In many virus particles, a central channel of variable depth was seen (4), and in some rods an "inclusion" was visible (3). On the basis of x-ray inactivation studies of extracellular virus particles, a radiation-sensitive sphere with a diameter between 36 and 47 mμ was assumed (5).

Rods and 52-mμ spherical particles were observed in ultrathin sections of the mouth mucosa taken from calves infected with VS virus (6). Both components were seen only in the intercellular space. In chorioallantoic membranes of eggs infected with VS virus, cells of the chorionic and allantoic layers bordering the mesoderm revealed granular areas on their surface. Only in these areas, which were separated from the cytoplasm by a membrane, were virus rods and 52-mμ particles visible (3). From studies of VS virus–infected L cells (4), it was concluded that the virus particles were assembled at the cell membrane.

In studies on the growth of VS virus in chick embryo cells and monkey kidney cells, Franklin (5) demonstrated that virus was released by a continuous process; the eclipse phase was between 1 and 2 hours and the release time approximately 2 to 3 minutes.

Our main interest was to study the development of VS virus in KB cells and to check whether at an early stage mature rods can be found inside the cells or only at or near the surface membrane.

MATERIALS AND METHODS

Virus

The Indiana strain of VS virus, kindly supplied by Dr. C. Palacios, Head of the División de Investiga-

ciones Veterinarias in Maracay, Venezuela, was used. Material of the first passage through the suckling mouse was then subjected to 12 passages through KB cell cultures. Infectious culture medium of the twelfth passage through KB cells served as the inocula for cell cultures used in the electron microscope study.

Plaque Assay and Tissue Cultures

The method employed in our laboratory for the plaque assay in chick embryo cells and for the preparation of KB cell cultures has been described previously (7). In the present studies, the KB cells were grown in disposable plastic flasks (TCF-25 ml culture flasks No. 3004, from Falcon Plastics, Los Angeles, California).

Virus Multiplication in KB Cells

Virus inoculum (10 plaque-forming units (PFU) per cell) was added to the growth medium of 2-day-old KB cell cultures containing $5 \cdot 10^5$ cells per flask. After an incubation period of 1 hour at 37°C, the culture medium containing non-adsorbed virus was removed. Then the cell layers were washed three times with phosphate-buffered saline, pH 7.2, and 8 ml of fresh growth medium were added.

Three culture flasks each were harvested 1, 4, 7, 10, 14, and 20 hours after inoculation. Pools were made, and after centrifugation for 5 minutes at 2500 RPM, the supernatant was assayed by the plaque test. The cells remaining in the flasks were examined by electron microscopy.

Electron Microscopy

The cells of the three flasks harvested at the intervals mentioned above were removed with trypsin, pooled, centrifuged, and washed once with isotonic NaCl-M/90 phosphate buffer, pH 7.2. The cell pellets were fixed in buffered osmium tetroxide (1 per cent). After dehydration in graded dilutions of ethyl alcohol, the cells were embedded in methacrylate. A detailed description of the method used in our laboratory for embedding is given elsewhere (7). A Fernández-Morán-type microtome equipped with a diamond knife (8) was used for making sections 400 A thick. Observations were performed with a Siemens Elmiskop I electron microscope.

RESULTS

Table I shows the increase of extracellular virus during multiplication of VS virus in KB cell cultures. Rods could only be detected in sections of cells taken 10, 14, and 20 hours post inoculation (p.i.) of the cell cultures. At these intervals, the titers of extracellular virus were higher than 10^7 PFU per ml of the culture medium.

Rods were observed at the cell surface membrane, inside the cell, and in the intercellular space. This is illustrated in Fig. 1 (14 hours p.i.),

TABLE I

Increase of Extracellular Virus during Multiplication of VS Virus in KB Cell Cultures

After inoculation	PFU/ml in growth medium
Hrs.	
1	$2.5 \cdot 10^3$
4	$1.8 \cdot 10^5$
7	$1.6 \cdot 10^6$
10	$1.2 \cdot 10^7$
14	$6.2 \cdot 10^7$
20	$6.0 \cdot 10^7$

in which numerous dense rods are seen in the space between cells and attached by one of their ends to membranes bordering cells or attached to homogeneous, structureless material. At the lower left of this figure, a cytoplasmic vacuole filled with rods is visible and an aggregate consisting of oval forms is observed in the upper part of the same cell. Two of several serial sections through this aggregate are shown in Figs. 2 and 3 at higher magnification. One can see that the oval structures, which are believed to be cross-sectioned rods for reasons which will be discussed later, display an orderly arrangement only in the upper part of the pictures. The presence of rods within cytoplasmic vacuoles is also demonstrated by Fig. 4 (20 hours p.i.). The relatively large cytoplasmic vacuole seen in this

FIGURE 1

Numerous virus rods are attached to the surface membrane of two cells, at the lower right, and to membranes bordering homogeneous masses. Note the core (*co*) and channel (*ca*) seen in some rods. A vacuole in the cytoplasm of the cell at the lower left is filled with rods; an accumulation of oval forms is seen in the upper part of this cell. (14 hours p.i.) × 25,000.

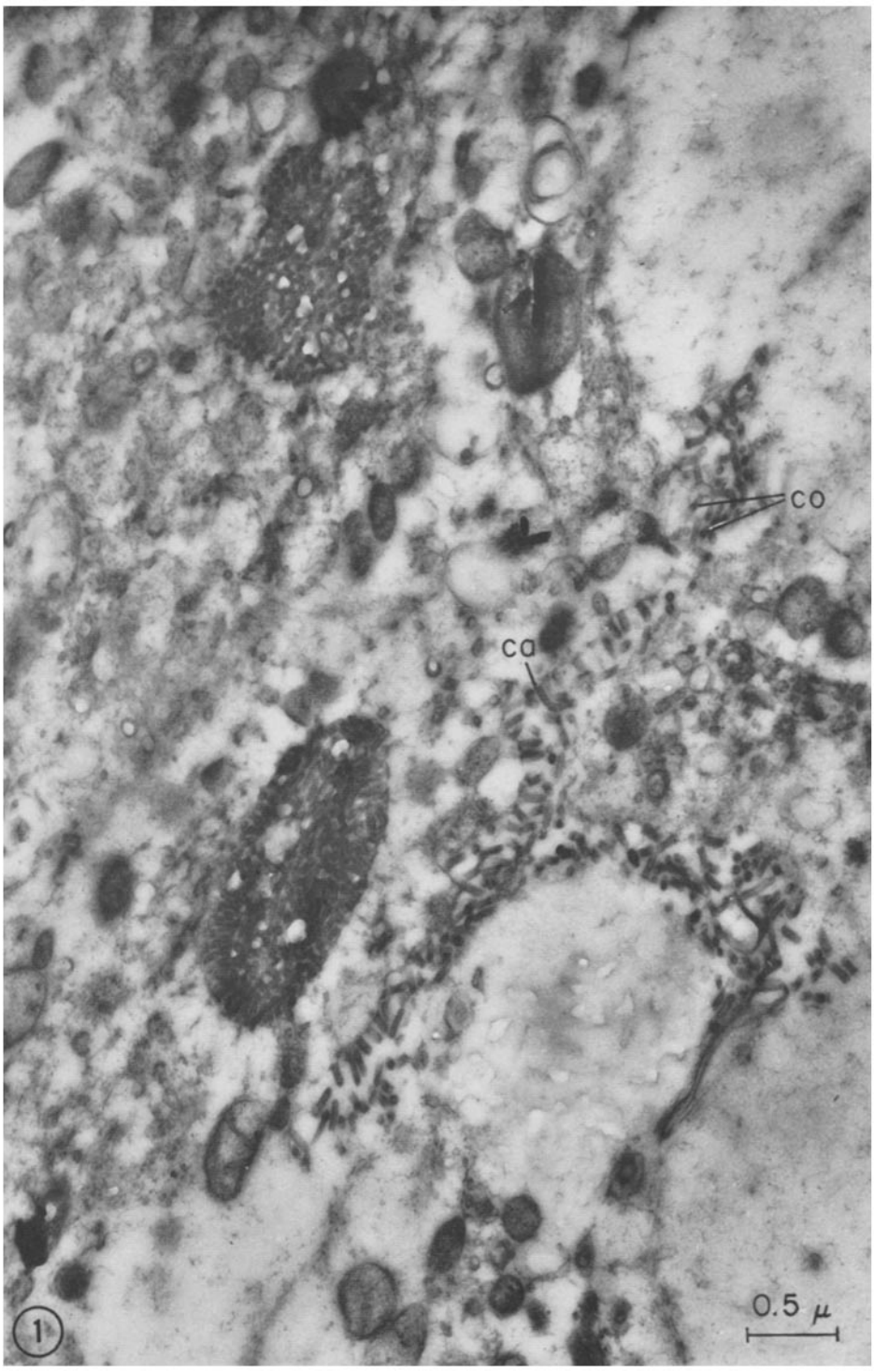

co
co
0.5 μ
1

figure contains numerous rods. Some of these rods seem to be attached to the inner side of the vacuole membrane. At two sites in the vacuole, three rods are connected to each other by one of their ends forming three 120° angles. In the cytoplasm, rods could be observed not only within cytoplasmic vacuoles but also attached by one end to cytoplasmic membranes, as seen in Fig. 5 (14 hours p.i.). The upper rod seems to lie alongside the membrane. The presence of rods in the extracellular space is observed in Fig. 6 (20 hours p.i.).

The observed rods have an average length of 180 mμ and an average diameter of 50 mμ. In some rods a dense oval core with a 65-mμ major axis is seen (Figs. 1 and 6). Some longitudinally sectioned rods exhibit in the center a channel with a diameter of about 25 mμ; this channel is less dense than the rod material surrounding it (Fig. 1). The observation of this channel leads us to believe that the oval structures in Figs. 2 and 3, with average dimensions of 60 mμ and 40 mμ for their major and minor axes, respectively, represent cross-sectioned rods, because these structures display a center of low density with nearly the same diameter as that of the channel. This assumption is supported by the appearance of the structures in the lower part of Figs. 2 and 3, which gives one the impression that most of them are obliquely sectioned rods. Virus rods that are not attached to a membrane display two round ends (Figs. 4 and 6).

Besides the rods, spherical particles of different sizes and shapes were observed within the cytoplasm. Fig. 7 (10 hours p.i.) and Fig. 8 (20 hours p.i.) show such structures inside cytoplasmic vacuoles. It should be noted that the vacuole in Fig. 7 also contains a rod in the lower part.

At least three types of spherical structures can be distinguished: (*a*) spherical particles of different densities and with a diameter of about 65 mμ (Fig. 7); (*b*) spherical particles with a diameter of 90 to 100 mμ consisting of a dense membrane separated from a dense 60- to 65-mμ core by a zone of lesser density (Fig. 8); and (*c*) particles without a dense core exhibiting two membranes (Fig. 8).

DISCUSSION

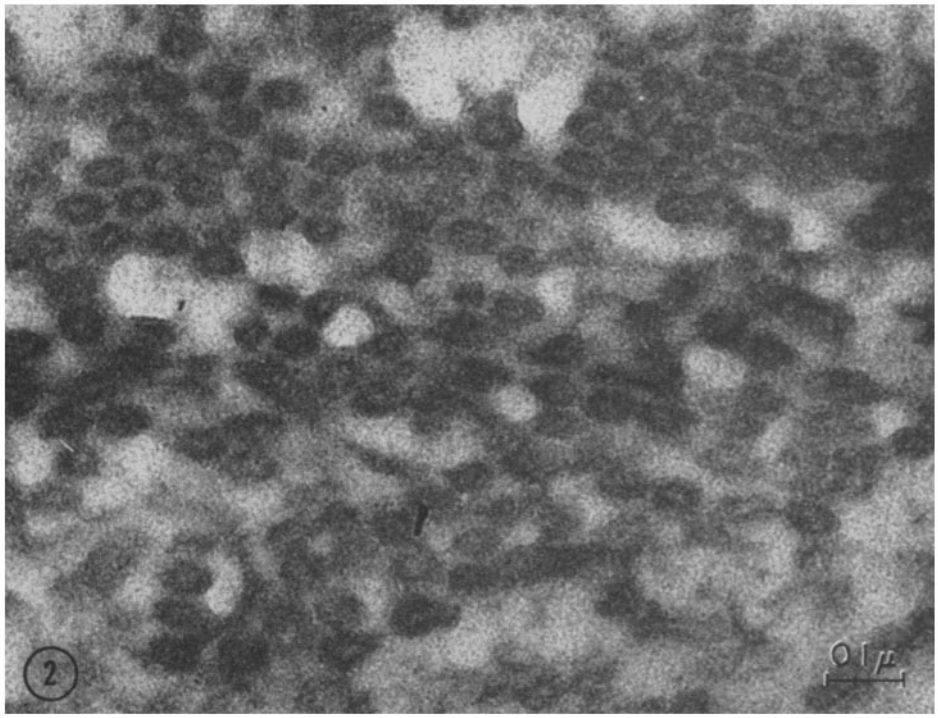
In the present study VS virus rods were seen inside cytoplasmic vacuoles, attached to cytoplasmic membranes and to membranes bordering vacuoles. No explanation can be offered for the origin of the cytoplasmic membranes to which the rods were seen attached. Virus maturation apparently occurs at those sites, which is contrary to the assumption of Howatson and Whitmore (4), who believe that the principal site of VS virus formation is at the cell membrane. The opinion of Reczko (3) that VS virus matures in granular areas separated from the cytoplasm by a membrane could also not be confirmed. The discrepancy between Reczko's findings, Howatson's and Whitmore's assumption, and our results may be due to different mechanisms of VS virus development in different hosts (chorioallantoic membrane, L cells, and KB cells), or to the rapid release of VS virus (5) which makes it difficult to detect rods in the cytoplasm.

No evidence was obtained that the cytoplasmic vacuoles play a role in the release process of VS virus. According to our observations, the rods are transported through the cytoplasm to the surface membrane of the cell from where they are extruded. The extrusion process apparently occurs without severe damage to the cell membrane (Figs. 1 and 6), an observation which may explain why the osmotic barrier of the cell membrane of VS virus–infected cells is not disturbed (9).

The fact that it was impossible to find virus-like structures before 10 hours p.i. makes it difficult to correlate the observed different forms (rods and spherical particles) with a sequence of developing structures. One can speculate, however, about the development of the virus rods from the spherical particles for the following reasons: (*a*) the 60- to 65-mμ particles (which possibly represent the noninfectious complement-fixing particles described by Bradish *et al.* (2)) and the virus rods were visible inside the same vacuole (Fig. 7); and (*b*) the diameter of 60 to 65 mμ was also found for the core of the 90- to 100-mμ particles (Fig. 8) and for the

FIGURES 2 AND 3

Two of several serial sections through the particle aggregate seen in the upper part of Fig. 1 at higher magnification. The oval particles in the upper left and right groups of both pictures exhibit a regular pattern suggesting a three-dimensional crystalline-like arrangement. (14 hours p.i.) × 100,000.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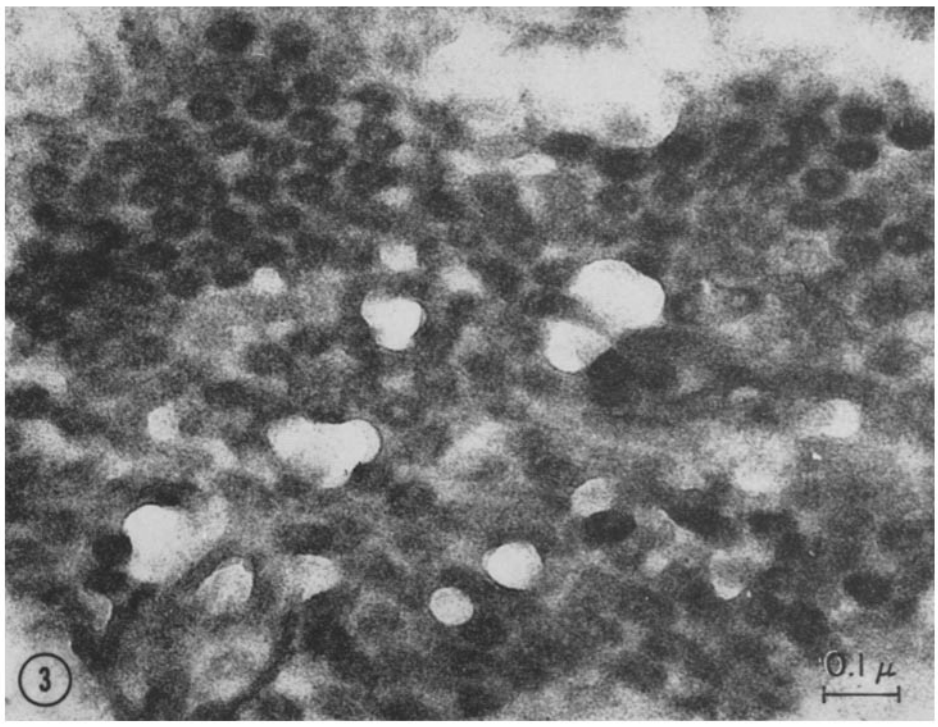
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core which was sometimes seen in the rods (Figs. 1 and 6). However, it cannot be ruled out that the spherical particles are formed by the breaking up of the rods into subunits.

The average length of 180 mμ measured for the rods which were observed in our thin sections is in close agreement with the mean length value determined for the infectious rods by Bradish *et al.* (2) and Howatson and Whitmore (4). The measured mean value of 50 mμ for the diameter of the rods is smaller than those given by other authors, namely 60 (1), 69 (2), and 68 mμ (4), but it is in good agreement with the 52-mμ value reported by Reczko (3). In some horizontal- and cross-sectioned rods, a 20- to 25-mμ channel was visible (Figs. 1 to 3), and some rods exhibited a dense core (Figs. 1 and 6). This core may be identical to the "inclusion" seen by Reczko (3) after staining the rods with phosphotungstic acid. No dense core was visible in rods which showed a channel. The presence of either a core or a channel, or the absence of both, possibly reflects different stages of maturation of the rods. Rods not attached to a membrane displayed two round ends (Figs. 1 and 6); this observation does not agree with that of Reczko (3) or Howatson and Whitmore (4), who demonstrated purified rods with a round and a square end after phosphotungstic acid treatment.

Virus maturation at membranes of cytoplasmic vacuoles as was observed in the present study was also described for Western equine encephalitis (10) and Venezuelan equine encephalitis (7), both arbor viruses. The similarity between these two arbor viruses and VS virus in respect to the sites of maturation is of some interest because it supports the classification of VS virus as an arbor virus, proposed when we observed that VS virus multiplies in *Aedes aegypti* (L.) mosquitoes and is transmitted by it under laboratory conditions (11).

The demonstration of virus rods in cells infected with VS virus bears some similarity to observations made in studies of influenza virus. Besides spherical influenza virus particles, filamentous forms of this virus are often detected in preparations of recently isolated strains (12–15), and evidence was reported that these filaments are infective (14–16). However, the filaments seem to be formed by extrusion from the virus-infected cell (17–22). Therefore, the mechanisms of the formation of influenza virus filaments appear to be quite different from the development of VS virus rods.

The authors wish to thank Dr. G. H. Bergold for the support of this work and for many helpful discussions. The excellent technical assistance of Mrs. Mussgay, wife of one of the authors, and of Mrs. Higinia de Volpe is gratefully acknowledged. Our thanks are also due to the Photographic Department of I.V.I.C.

Received for publication, January 10, 1962.

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FIGURE 4

Numerous rods inside a cytoplasmic vacuole. Some of the rods are obliquely sectioned. Note the formation of three 120° angles by three rods (*indicated by arrows*). Altered mitochondria are visible in the lower left corner and at the upper left margin. (20 hours p.i.) × 100,000.

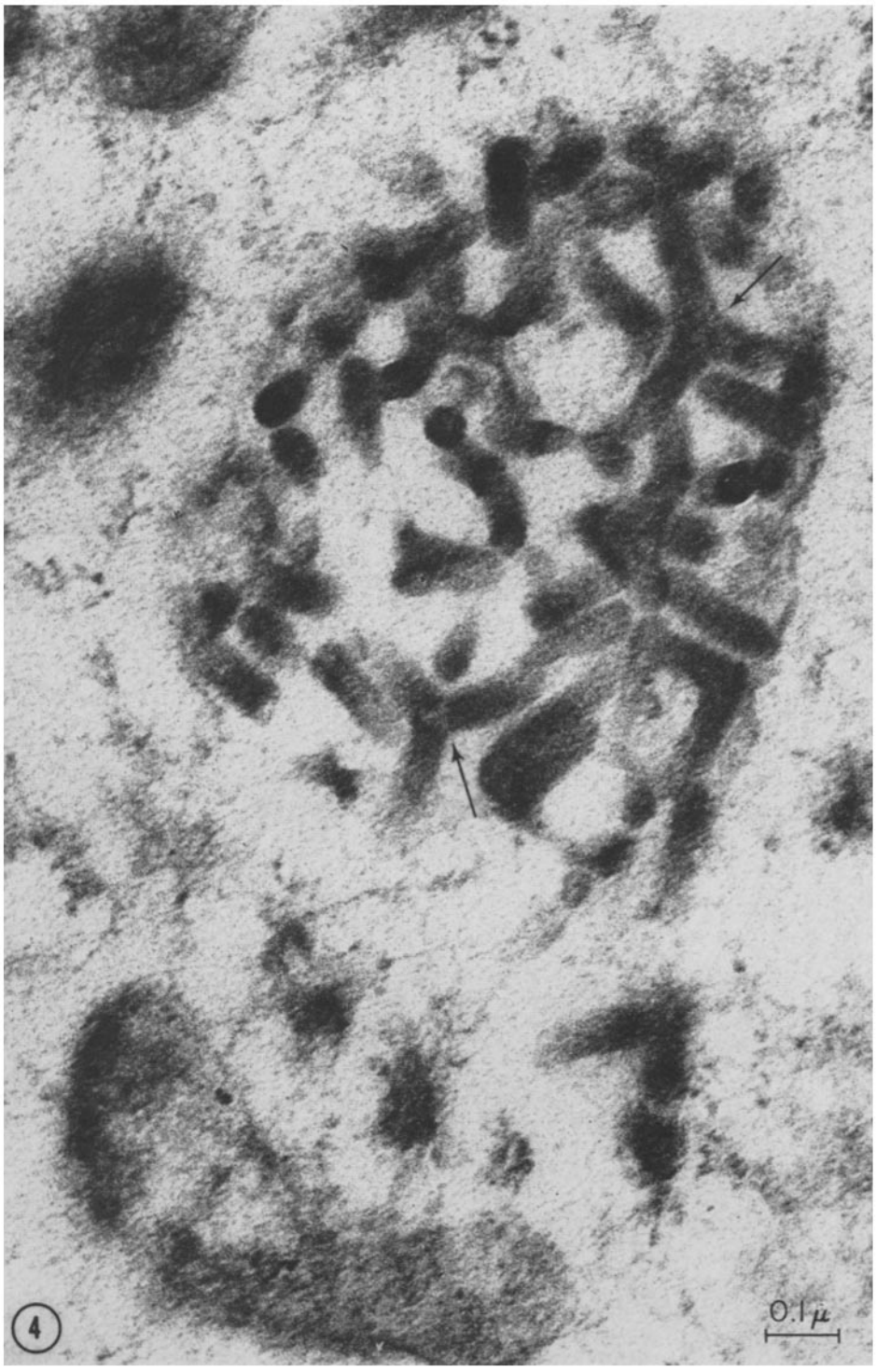
4
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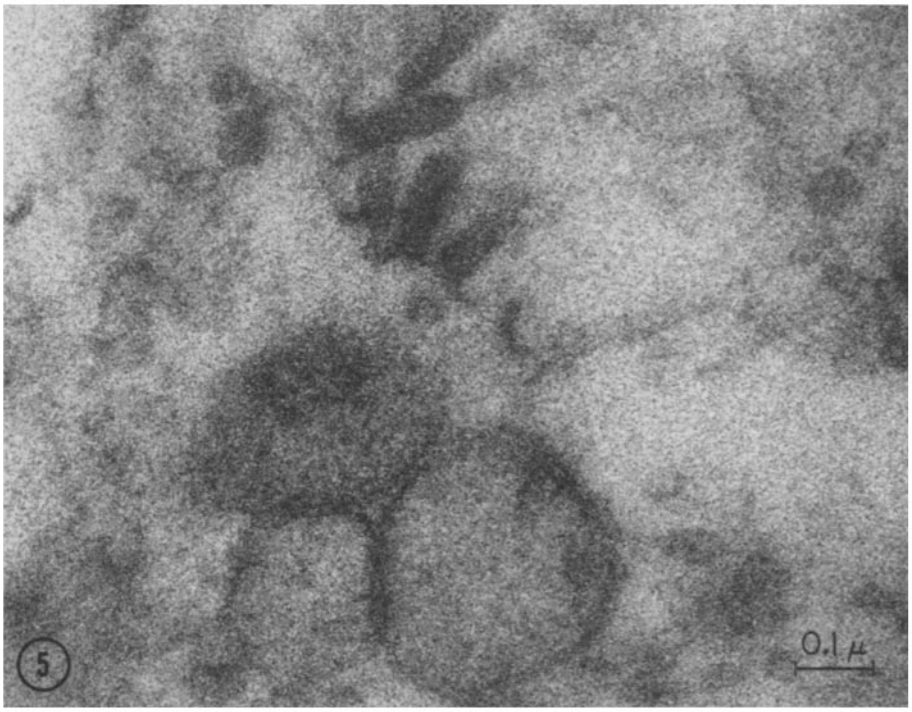
FIGURE 5

Rods attached to a cytoplasmic membrane. It is uncertain whether the membrane to which the rods are attached surrounds a not clearly visible vacuole, or whether this membrane is part of the endoplasmic reticulum. (14 hours p. i.) × 100,000.

FIGURE 6

The upper part of the picture is occupied by cytoplasm of a cell. The particle in the cytoplasm in the upper left corner is believed to be a VS virus particle. Several rods, some of them obliquely sectioned, are seen in the extracellular space. A core (*co*) is visible in one of the rods. (20 hours p.i.) × 150,000.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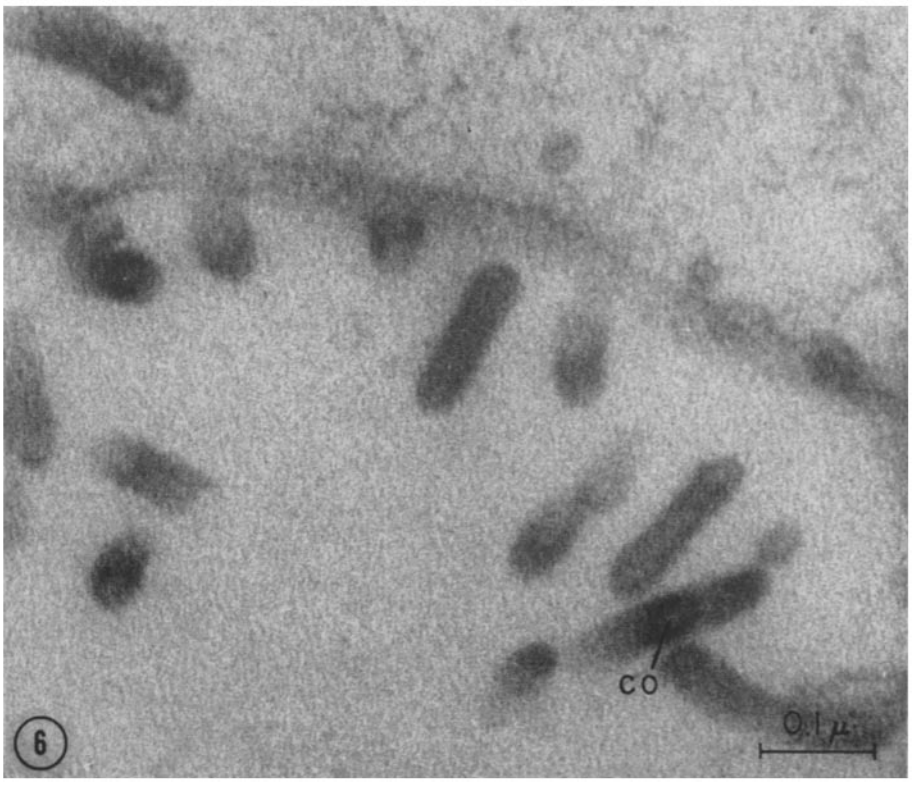
CO
6
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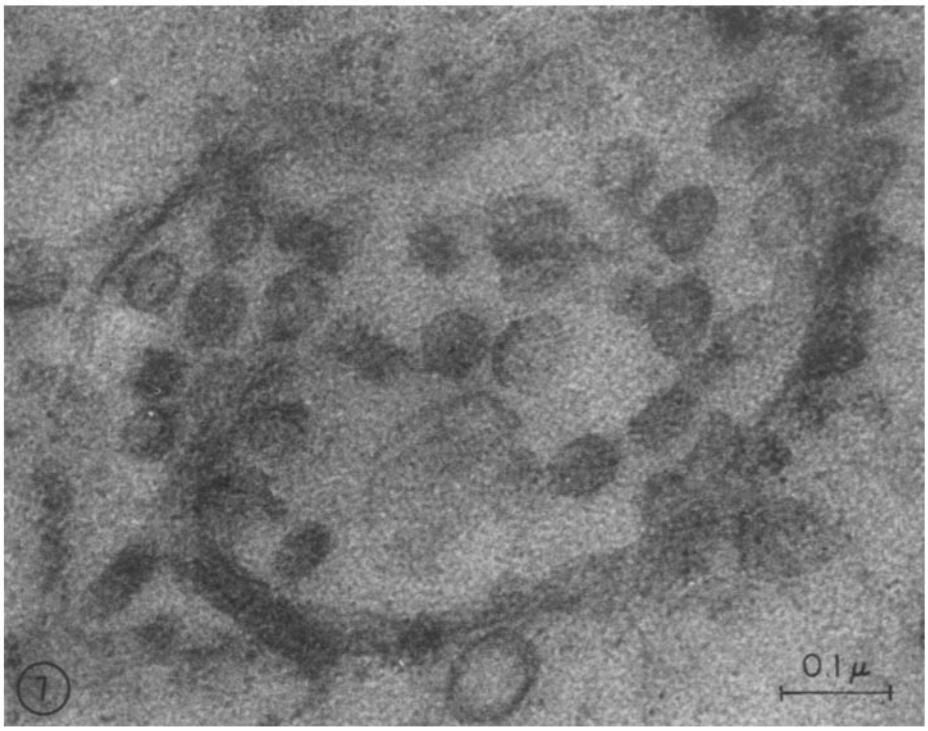
FIGURE 7

A cytoplasmic vacuole limited by an ill defined membrane. Inside the vacuole, several 60- to 65-mμ particles and one rod are visible. Some ill defined particles are lining the outer side of the vacuole membrane (right margin). (10 hours p. i.) × 150,000.

FIGURE 8

A vacuole near the nucleus (*N*) encloses several particles of different sizes and shapes. (20 hours p.i.) × 100,000.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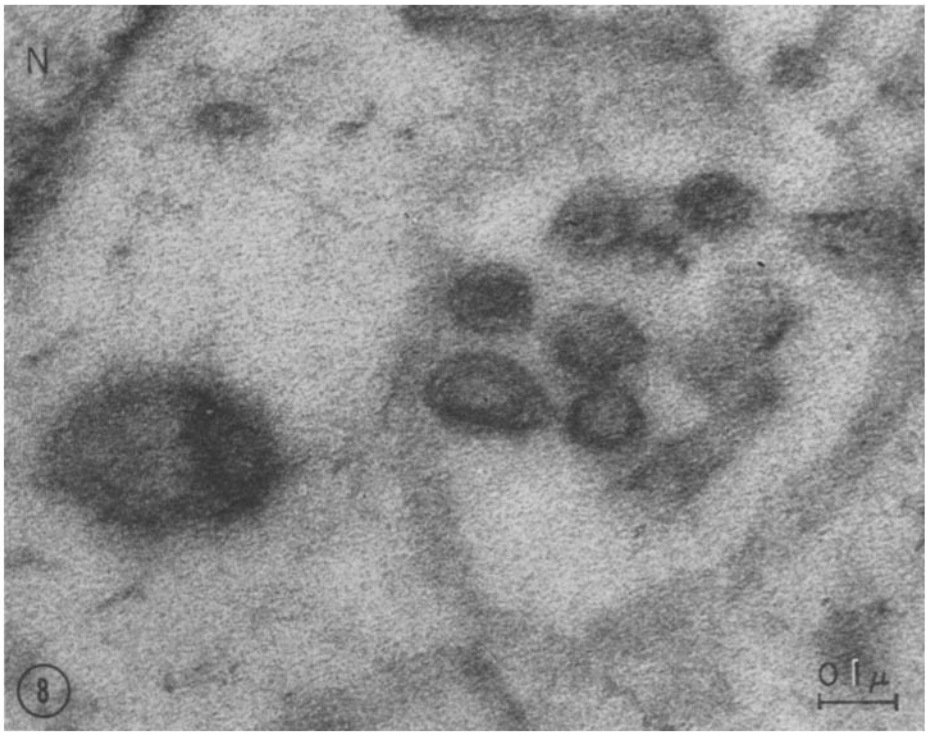
N
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8