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Ultrastructure of Lymphocystis Virus

LUTZ O. ZWILLENBERG AND KEN WOLF

Department of Hygiene and Medical Microbiology, University of Berne, CH-3008 Berne, Switzerland; and Bureau of Sport Fisheries and Wildlife, Eastern Fish Disease Laboratory, Kearneysville, West Virginia 25430

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Lymphocystis virus obtained from bluegills (*Lepomis macrochirus*) was cultured in the permanent bluegill cell line BF-2 and examined by electron microscopy in ultrathin sections of cell cultures and in negative-contrast preparations from cells and from centrifuged culture medium. According to negative-contrast preparations, the icosahedral virions have an overall diameter close to but not exceeding 300 mμ. Delicate filaments seem to issue from the vertices. In collapsed virions, an ordered array of morphological units was seen. Positively contrasted virions in ultrathin sections show a shell with three dark (heavy metal-stained) layers alternating with and separated by two clear layers. The acquisition of an additional outer membrane during release from the cell, as found in African swine fever virus, was never seen. Morphologically, lymphocystis virus is considered to be closely related to Tipula iridescent virus.

Lymphocystis is a virus disease of various teleost fish species, a disease known for half a century (19, 20). The ultrastructure of the lymphocystis virus has been investigated by Walker (16), Walker and Weissenberg (17), and Walker and Wolf (18), but the technique did not allow high resolution. Moreover, an examination by means of negative staining seemed necessary in order to supplement the information from ultrathin sections.

MATERIALS AND METHODS

Lymphocystis virus from bluegills (*Lepomis macrochirus*) was inoculated onto monolayers of BF-2 cells, a permanent fibroblast-like cell line derived from bluegill fry and propagated in the Eastern Fish Disease Laboratory. The infected cultures were sent to Berne for electron microscopic processing. They were kept at room temperature in the dark. After 21 days, when the cytopathic effect typical for lymphocystis virus (21) had become well developed, some of the cultures were washed with Millonig's phosphate buffer and fixed in situ with 4% reagent grade Formalin dissolved in the same buffer. The cell sheet was then scraped off with a rubber wiper and centrifuged. Pieces of the pellet were postfixed in 2% osmium tetroxide, dehydrated in ethyl alcohol, treated overnight with 1% uranyl acetate in ethyl alcohol, washed again in ethyl alcohol, and embedded in Durcupan ACM (FLUKA Aktiengesellschaft, Buchs SG, Switzerland), with propylene oxide as an intermediate solvent. Ultrathin sections were cut with glass knives on an LKB Ultrotome III, collected on grids coated with collodium and carbon, poststained with lead citrate, and examined in a Philips EM 200 electron microscope.

Other cultures were examined 10 to 25 days after inoculation, either with the unmodified negative-staining technique described by Zwillenberg and Bürki (24), or by using distilled water containing about 0.01% bovine serum albumin as a suspension medium instead of the 5% ammonium acetate solution. In other cases, the supernatant medium was withdrawn from the culture and centrifuged at 2,500 × *g* for 10 min at room temperature. The sediment was washed with Millonig's phosphate buffer (same centrifugation speed and time) and suspended either in 5% ammonium acetate solution or in distilled water, both containing about 0.01% bovine serum albumin, prior to mixing with 2% sodium phosphotungstate solution (*p*H 7.0).

RESULTS

In ultrathin sections of infected BF-2 cells, virus particles with an overall diameter averaging 250 mμ were found in the cytoplasm, either in more or less coherent groups or surrounding clear areas of viroplasm (Fig. 1). The particles had a shell with a hexagonal profile (Fig. 2 and 3). In this profile, a central dark (electron opaque) line, about 4.8 mμ in thickness, was separated by a clear (electron-translucent) space from an outer and inner dark line. These lines were about 2 mμ thick and about 12 mμ apart from each other. The shells were either filled with a granular or fibrillar mass, or they were empty. Some shells had a more or less crumpled appearance (Fig. 2). A few shells were not closed but showed a more or less wide gap. In many instances a fuzzy halo, about 40 mμ in thickness, was surrounding the shells (Fig. 3).

Special attention was paid to any images suggesting the passage of particles through the cell membrane and indicating an acquisition of an additional outer membrane during the passage. Such images were never seen.

When negatively stained specimens were prepared, with 5% ammonium acetate solution as the suspension medium, icosahedral virus particles of an overall diameter of about 300 mμ were observed in an apparently intact state, the ridges of the icosahedra being clearly set off by an outer electron-translucent band (width about 6 mμ) and an inner electron-opaque line (Fig. 4). When distilled water was used, a large proportion of the particles had collapsed and showed an ordered array of morphological units along the facets of the icosahedra. These units were resolved with difficulty and their images deteriorated rapidly under electron bombardment. They gave the impression of rings with a diameter of about 5 mμ. They were arranged in rows, some of them nearly radial and some of them seemingly crossing each other under an oblique angle (Fig. 5 and 6). The electron-translucent margin of the collapsed particles was separated from the above rows by a dark line. The margin was often fragmented. The fragments showed a close relation to the rows of morphological units. They seemed to be their extension.

Sediment from centrifuged culture medium contained not only single virions, but more often clusters of them. In one instance, these clusters were huge. The clustered virions were sometimes not in close contact, but seemed attached to each other by means of filaments, which had become entangled. Such filaments could be better studied in occasional free-lying virions or in small, loose clusters (Fig. 4). The filaments had a width of the order of 4 mμ and a length of 200 to 300 mμ. Although various cell debris were present in the preparations, comparable filaments were not found outside virion clusters or outside the immediate vicinity of virions. In those cases, in which crowding of virions and filaments was not serious, so that the course of single filaments could be followed to the contours of presumptive virions of origin, such filaments seemed to issue preferentially from angles in the virion contours or from identifiable vertices of apparently intact virions (Fig. 4 and 5). Comparable filaments were not seen in specimens prepared from whole cells.

DISCUSSION

The findings in ultrathin sections of infected BF-2 cells accord well with those of previous investigators (16–18). In addition, they show more detail of the virus shell and possibly a morphological substrate for the osmiophilia (19) and the ether-sensitivity (21) of the virus. In fact, the hexagonal profiles are reminiscent of those quintuple-layered membranelike structures (with three electron-opaque and two electron-translucent layers after a comparable fixation and staining procedure), which are the result of lipoprotein aggregation in degenerating cells. For fish cells, such structures have been pictured in remnants of erythrocytes ingested by trout spleen reticulum cells (23). This morphological resemblance does not prove a structural similarity but lends support to the supposition that the lymphocystis virus shell contains a lipidic component. However, both the very regular, icosahedral shape of the shell and the images produced by negative staining show that the shell is certainly different from an ordinary lipoprotein membrane and that an ordered array of capsomeres is integrated in the shell. The precise nature of this integration is not clear.

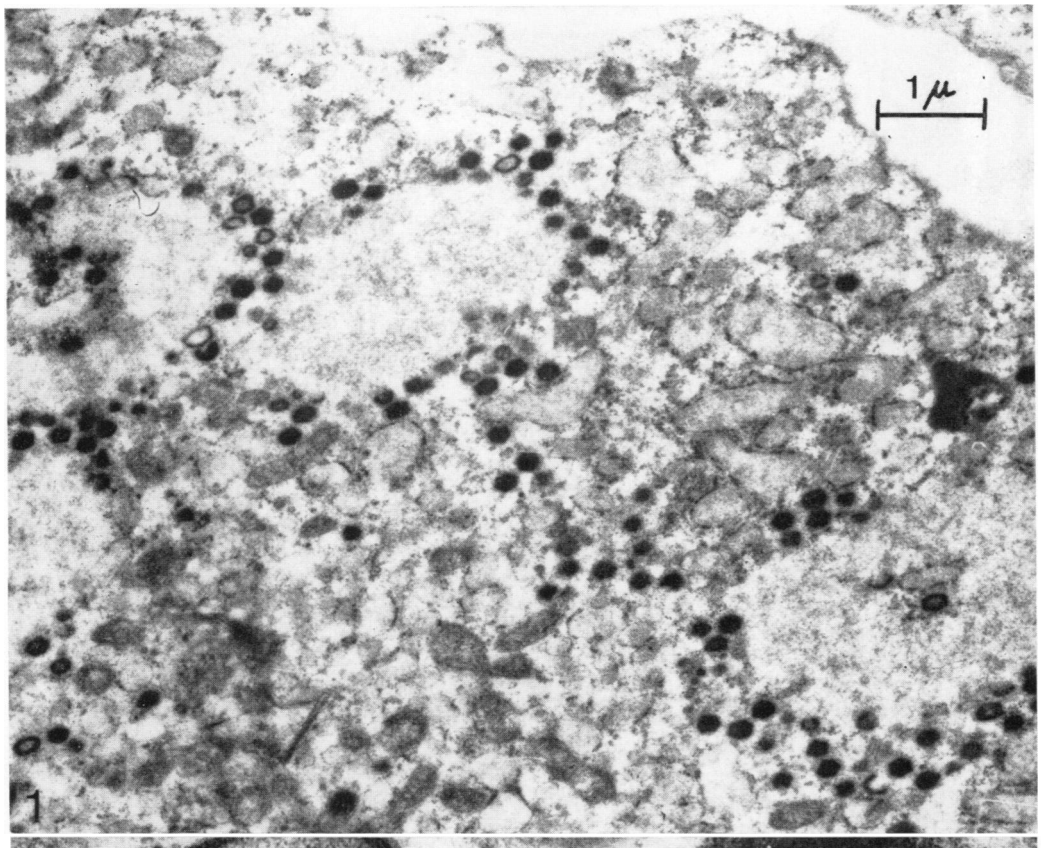
While the granular image of any dark line in the sections must be interpreted with caution on account of possible phase-contrast effects of the carbon substrate, the fragmentation of the margin of collapsed, negatively stained virions is certainly real. As a purely tentative interpretation, the heavy central line in the sections and the electron-translucent marginal band of negatively contrasted virions may be correlated with the capsomeres, and these may be backed, both to the outside and the inside, by lipoprotein layers.

The greater virion diameters, as compared to former ultrastructural studies, are explained, at least in part, by the stronger shrinkage of the methacrylate used in those studies. Even so, there was evidently some shrinkage and knife compression in our material also. The diameter in collapsed negatively stained virions is certainly exaggerated by flattening. However, the greater diameter of noncollapsed negatively stained virions, as compared to sectioned material, cannot be ascribed to flattening only. The actual virion diameter should therefore be quite close to but not in excess of 300 mμ.

Though there is no absolute guarantee that

FIG 1. *Ultrathin section of a BF-2 cell infected with lymphocystis virus. Virus particles surrounding clear spaces.* × *14,000.*

FIG. 2. *Lymphocystis virus in the cytoplasm of a BF-2 cell. Note particle content and structure of the shell.* × *174,000.*



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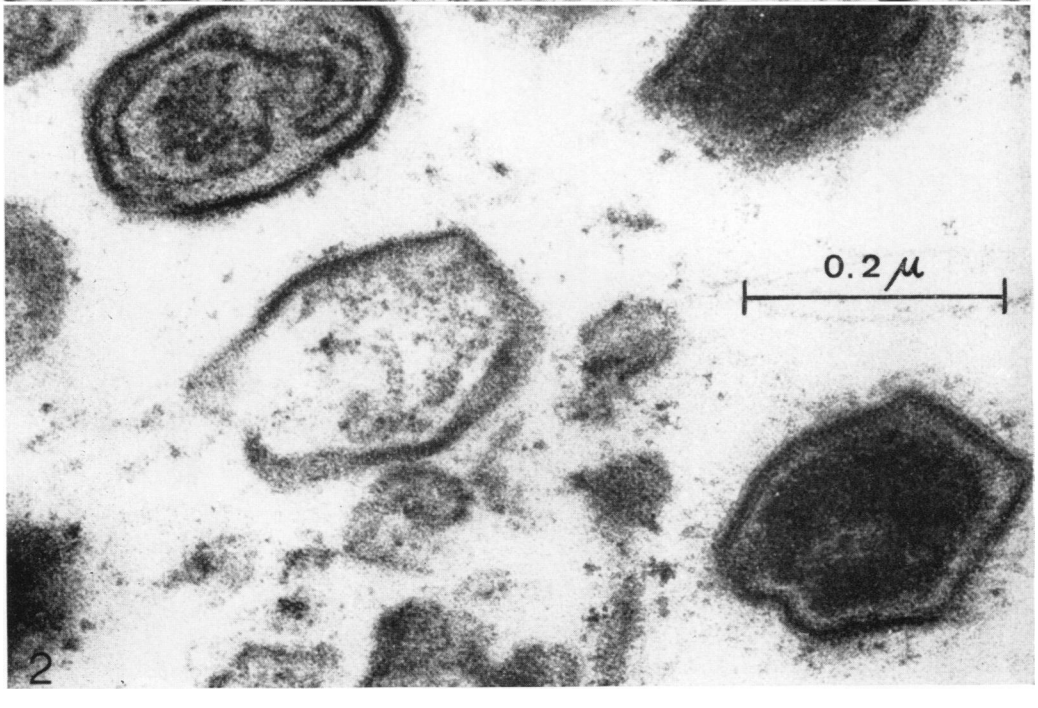

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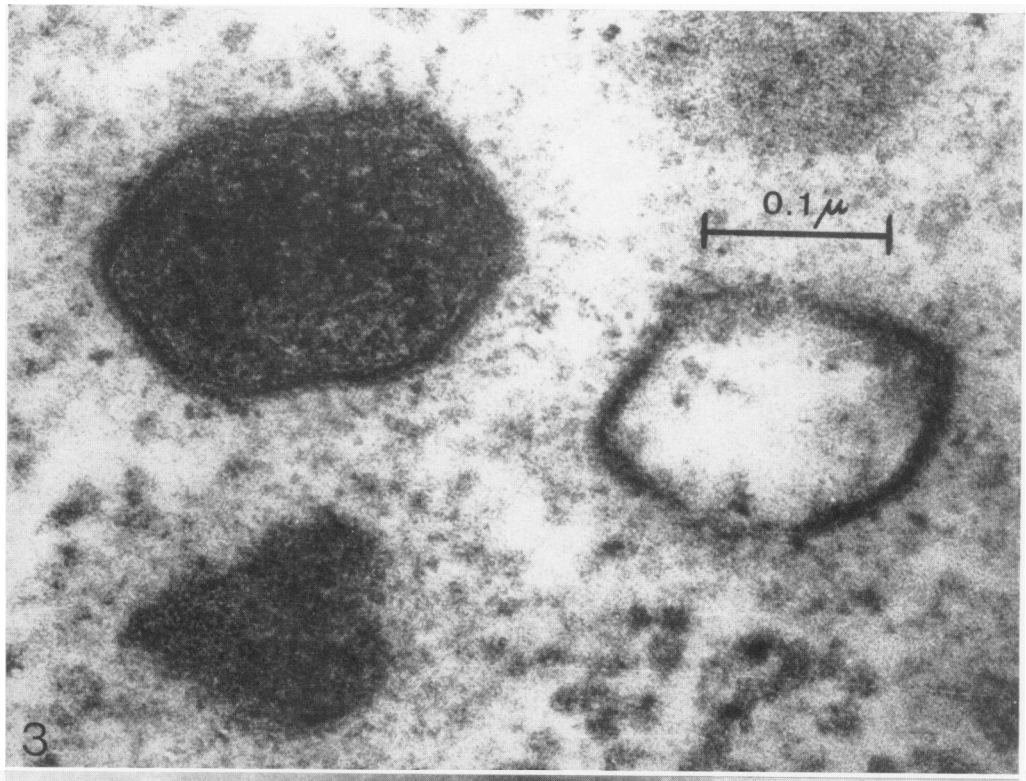

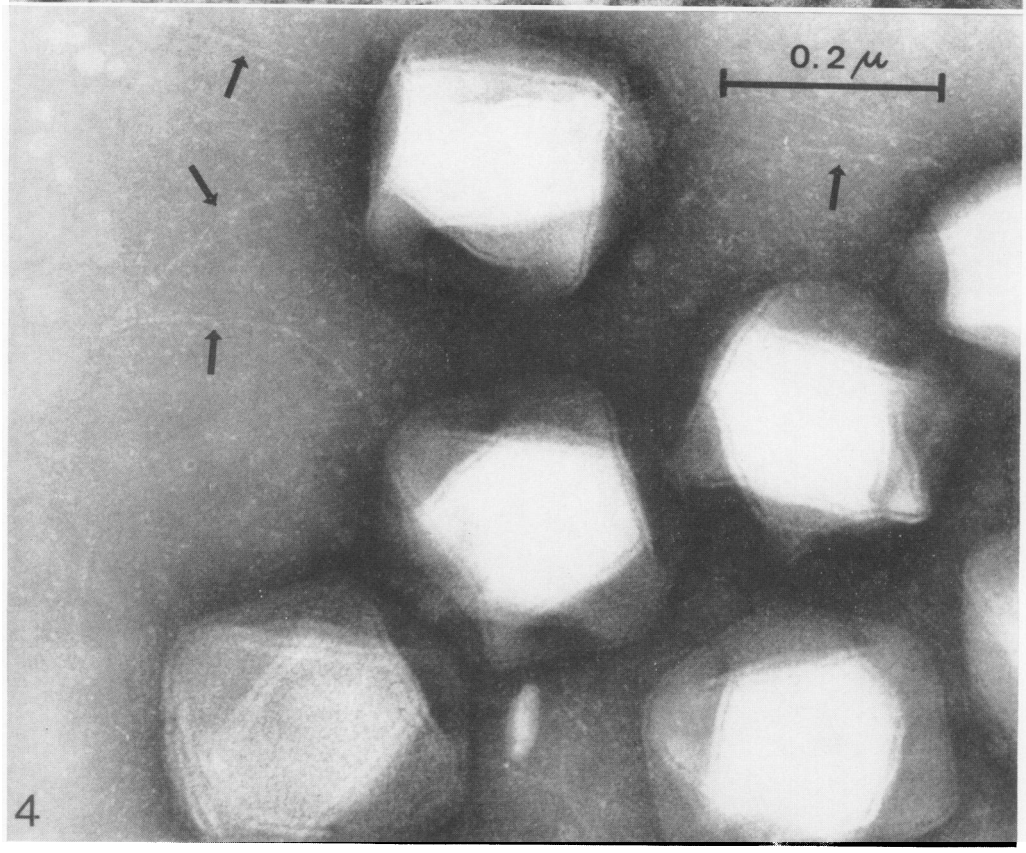

FIG. 3. *Lymphocystis virus in the cytoplasm of a BF-2 cell. Note structure of the shell and fuzzy halo around the particles.* × *250,000.*

FIG. 4. *Negatively stained lymphocystis virus from culture medium; suspended in 5% ammonium acetate. Arrows point to filaments.* × *150,000.*

the observed filaments are viral appendages, both the visual observations on the microscope screen and the recorded images created this impression, which was strengthened by the lack of comparable filaments outside the immediate vicinity of virions. If the filaments are actually appendages issuing from virion vertices, this would constitute a certain parallel to the fibers of adenoviruses (9, 12, 15). Up to the present time, there is no evidence that lymphocystis virus has hemagglutinating or hemadsorbing properties (21). African swine fever virus, which is morphologically similar, shows the phenomenon of hemadsorption (5).

Although African swine fever virus and Tipula iridescent virus are morphologically related (1), there is at least one property, which separates them: the porcine virus acquires an additional membrane during its passage through the cell membrane (4, 6). This has not been observed either in Tipula iridescent virus (2, 3, 22) or in lymphocystis virus. It has been observed in morphologically similar frog viruses (7, 8, 10, 11).

Our results suggest that lymphocystis virus should be placed taxonomically close to Tipula iridescent virus. Other comparable viruses are Sericesthis iridescent virus (14) and the gecko virus formerly named *Pirhemocyton tarentolae* (13).

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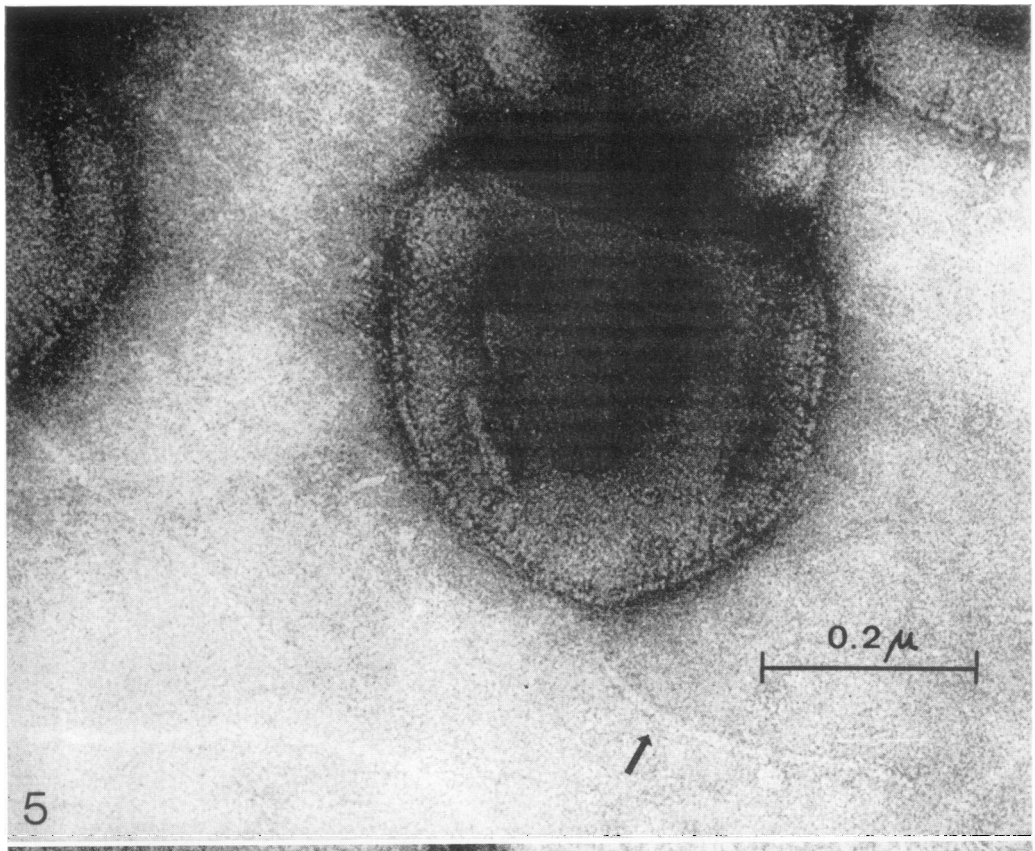

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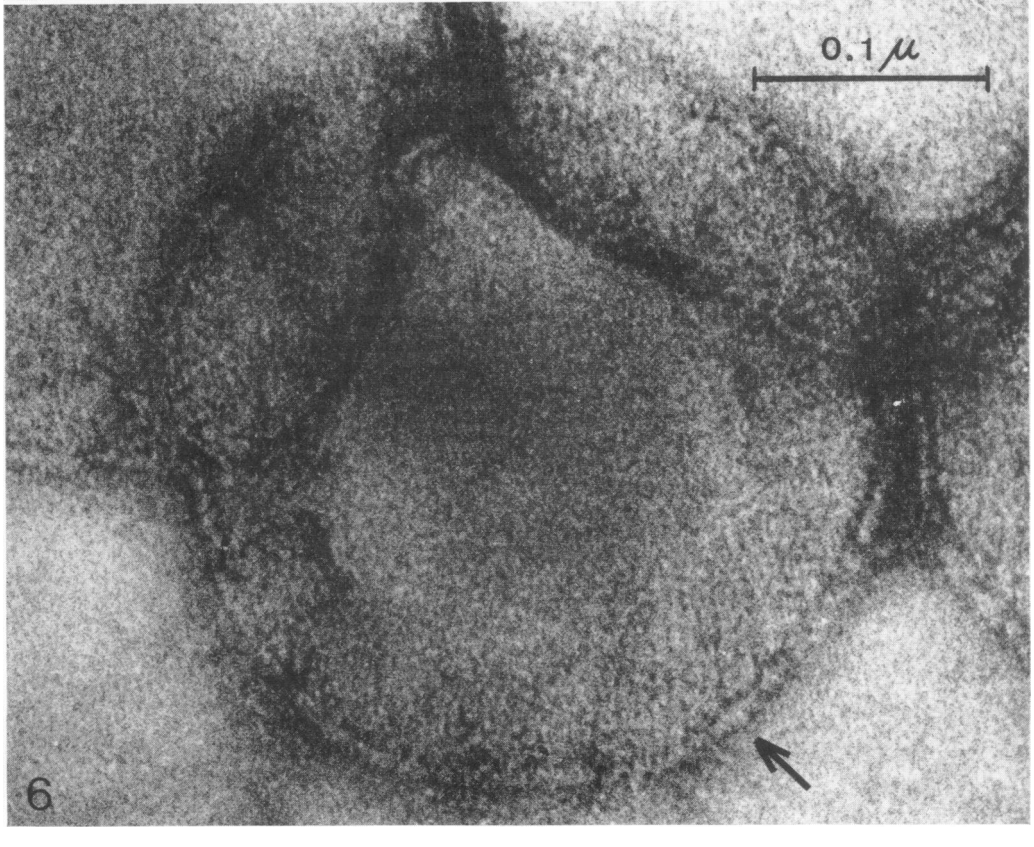

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FIG. 5. *Collapsed, negatively stained lymphocystis virus; suspended in distilled water. Arrow points to filament.* × *145,000.*

FIG. 6. *Collapsed, negatively stained lymphocystis virus; suspended in distilled water. Arrow denotes area where rows of subunits can be discerned.* × *311,000.*