A NEGATIVE STAINING METHOD FOR CELL-ASSOCIATED VIRUS

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The virus host-cell relationship has been studied in the electron microscope almost entirely by the thin sectioning technique. This method has given information about the sites of virus production within the cell and the appearance of virus particles at various stages in the growth cycle (1, 2). It does not, however, permit high resolution studies on the substructure of individual particles or the manner in which this substructure may change during the development of the virus. For high resolution studies on individual particles it is more effective to use the negative staining method (3), and much has been learned about the architecture of virus particles since the introduction of this technique (4). With two exceptions (5, 6), this method has been applied to purified or semipurified preparations in which the relationship of the virus to the host cell is lost. The following method (7) has been developed in an attempt to combine the advantages of these two techniques, that is, sectioning and negative staining, and thus enable a study to be made of fine structure of virus particles and virus precursor stages as they occur in the cell.

Virus-infected cells are centrifuged for 5 minutes in a clinical centrifuge and the resulting pellet is resuspended in 0.5 ml of a 1:5 dilution of culture medium with distilled water. This hypotonic solution results in swelling and disruption of the cells without excessive disruption of the cell components and also avoids difficulties that will occur when the tonicity is too high in subsequent freezing. The cell suspension is then frozen into a block of size and shape suitable for sectioning. This can be done conveniently by making a cup-shaped mold in the following way. An approximately 1 inch square of plastic film (in this laboratory, Parafilm was used) is pressed over the end of a solid cylindrical object, such as a pencil, and the plastic secured with a small strip of adhesive

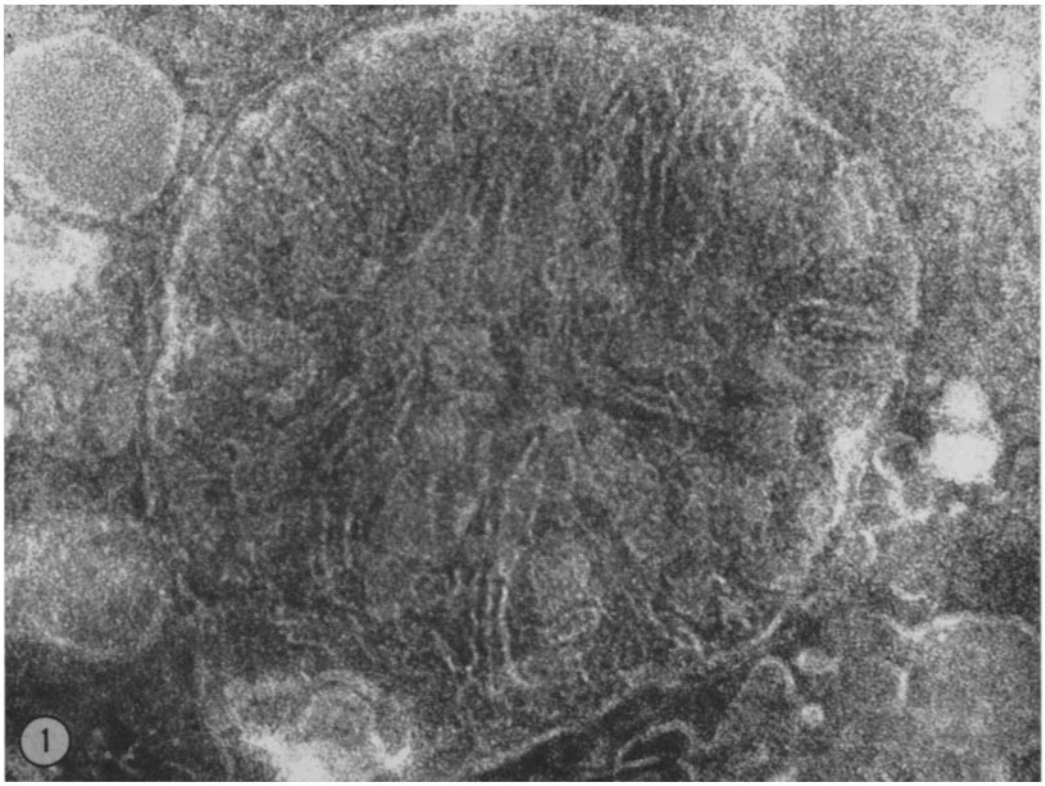
FIGURE 1

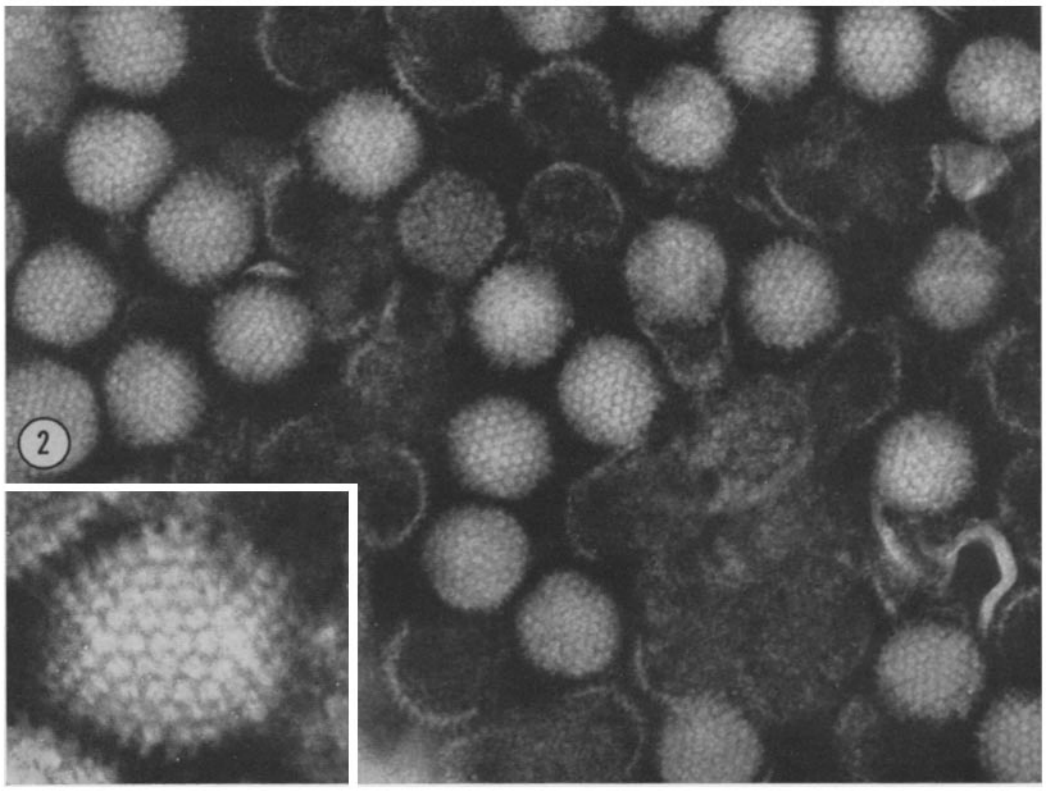
Negatively stained mitochondrion showing cristae and double enveloping membrane. The general appearance closely resembles that seen in thin sectioned material. × 140,000.

FIGURE 2

Portion of a nucleus from a cell infected with adenovirus type 12. Both complete and incomplete particles are present. × 170,000.

The insert shows one favorably oriented particle from the array. The icosahedral form of the virus is clearly seen, and virus preservation is such that the capsomeres are seen to be hollow. × 374,000.





tape. The mold is slipped off and the cell suspension pipetted into it and frozen at −20°C. The frozen pellet or block is transferred to a refrigerated cabinet or cryostat, maintained at −20°C and containing a Spencer rotary microtome. The plastic mold is stripped from the block and the latter is then attached to the microtome chuck with a drop of water. Sections are cut at a setting of 4 μ and collected in the well of a depression slide. When enough sections are collected (10 to 30, depending on the nature and concentration of the specimen), the slide is removed from the cryostat and the sections are allowed to thaw at room temperature. The resulting suspension of cell fragments is treated in the same way as a virus preparation, and negative staining is carried out as follows. A volume of 3 per cent phosphotungstic acid, buffered at pH 6, approximately equal to the volume of the sectioned cell suspension in the depression slide is added to the preparation and a large drop of this mixture is placed on a carbon-coated Formvar grid. After about a minute the excess fluid is gently withdrawn, and immediately after drying the grid is examined in the electron microscope.

Although cut nominally at a thickness of 4 μ, the cell sections provide material thin enough to be examined at high resolution. This seeming incongruity is in part due to the hypotonic suspension fluid, which ruptures and spreads the cells, and also to the spreading effect of surface tension forces that are exerted on the section during the drying process.

Although cellular architecture is not preserved nearly so well as by thin sectioning methods, sufficient cell integrity is maintained to allow recognition of the basic cell components and the study of virus particles in relation to these cell components. Endoplasmic reticulum is easily identified as sheets or tubules, and the nucleus as an area of fine granulation. Mitochondria can differ considerably in structural detail, but many have an appearance closely corresponding to that seen in thin sections (Fig. 1). The method was applied to cells infected with many different viruses, including adenovirus type 12, herpes simplex, SV_{40}, polyoma, vaccinia, rabies, and measles.

Fig. 2 shows part of a nucleus of a cell infected with adenovirus type 12. Fully formed and apparently incomplete forms of the virus are easily recognized. The insert of one favorably oriented particle present in the array shows that the icosahedral shape and capsomere arrangement are well preserved. In fact it would seem that this method of preparation subjects the particles to minimal damage and allows delicate viruses to be viewed under optimum conditions. For example, use of this technique has enabled rabies virus grown in hamster kidney cells to be visualized (8). Fig. 3 illustrates another viral component that was first detected by this method. The figure shows part of a measles virus with the internal component visible; the helix previously described as belonging to this virus is seen (9), but in several places this helix is shown to have an outer covering which gives an appearance suggestive of another helix.

When applied to vaccinia virus–infected cells, the method revealed a very wide spectrum of developmental forms. Such forms are not found in purified preparations of vaccinia virus stained with phosphotungstic acid (10). Fig. 4 shows an early form of the virus found very frequently in infected

FIGURE 3

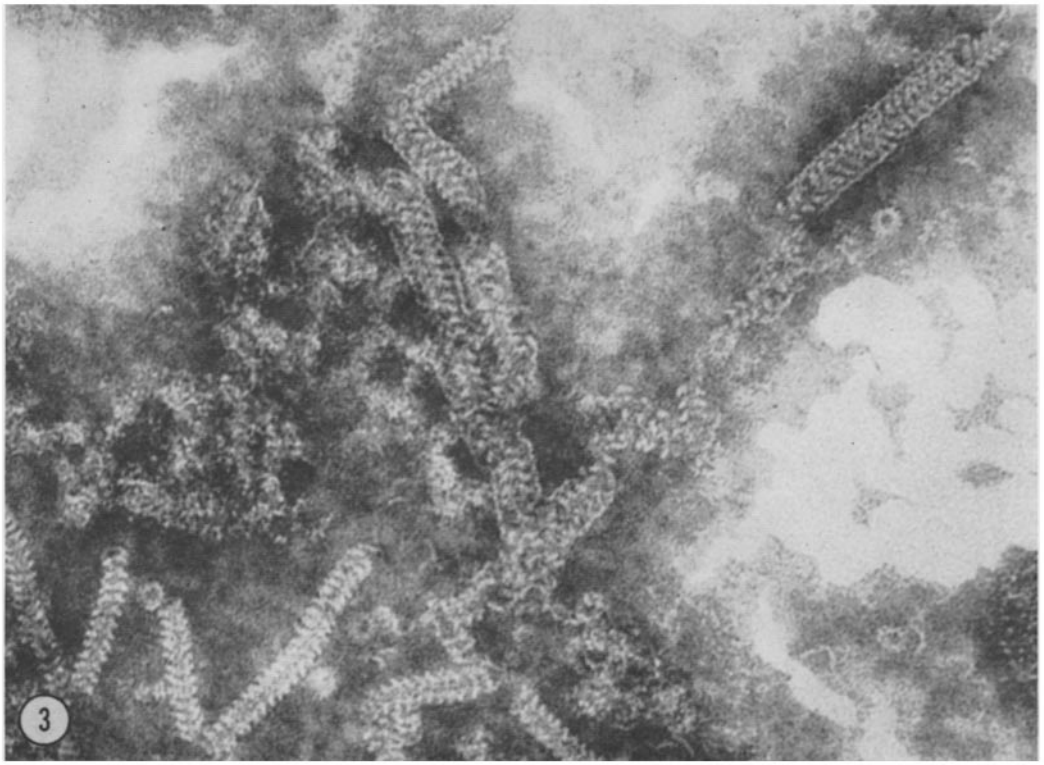
Portion of a disrupted measles virus particle showing internal component. At the bottom left of the micrograph the helical structure that has been associated with this virus is seen. In the upper part of the micrograph this helix is seen to have an outer, possibly helical, covering. × 220,000.

FIGURE 4

Vaccinia virus particles at an early stage of development. This form of the virus is composed of randomly oriented fibrils having a diameter of 25 A. × 127,000.

FIGURE 5

Three vaccinia virus particles contained within cytoplasmic membranes. In the order *a*, *b*, *c*, they seem to represent three stages in the maturation of the virus. × 127,000.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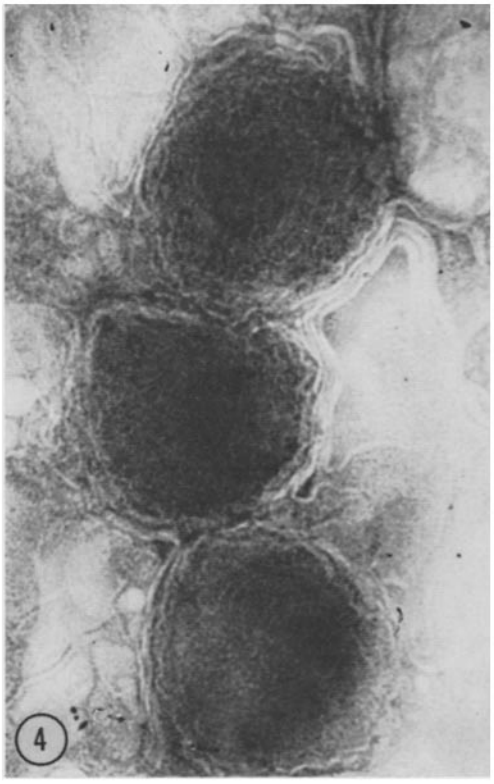
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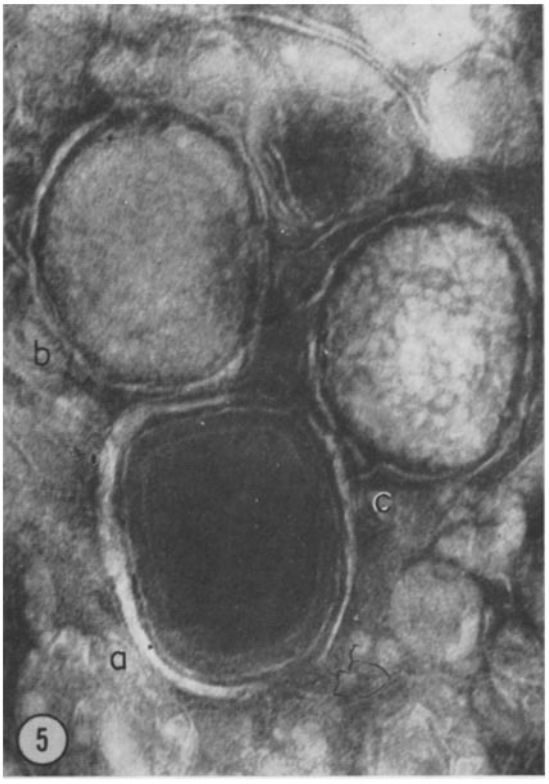
b
c
a
5

L cells during virus maturation (3 hours after infection and later). Fig. 5 shows three particles at different later stages of development. When viewed together in this way they seem to show a sequence of events in the development of the mature particle.

The technique described here, together with the similar sectioning technique of Fernández-Morán and the technique of cell spreading used by Parsons in this laboratory (11–13), offers a new approach to the study of the morphology of basic cell components in the normal cell and the virus cell relationship in infected cells.

In summary, the technique described here offers three main advantages in the examination of virus-infected cells by negative staining.

First, it is a sensitive and rapid method of establishing the presence or absence of virus in any preparation.

Second, since the material receives the minimal amount of handling, viruses retain structural details that may be lost in lengthy purification procedures.

Third, a virus can be examined in relation to other particles around it and to the constituents of the cell in which it is contained.

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