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Ultrastructural Study of Long-Term Measles Infection in Cultures of Hamster Dorsal-Root Ganglion

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The morphogenesis of the Edmonston strain of measles is described in cultures of hamster dorsal-root ganglion maintained for as long as 63 days postinoculation. The patterns observed confirmed those previously reported in both neural and non-neural tissue. However, in the present tissue, the development of viral material could be followed chronologically within different cell types such as neurons and Schwann cells. Active replication was visualized up to 63 days postinoculation. The appearance of cytoplasmic nucleocapsid preceded that of intranuclear nucleocapsid, the latter occurring after 14 days. These intranuclear inclusions were formed after the transformation of the nucleoli into bizarre pleomorphic bodies which eventually segregated into clumps of nucleocapsid. These intranuclear inclusions mimic those seen in subacute sclerosing panencephalitis, now known to be etiologically related to a measles-like virus.

Although spontaneous paramyxovirus infections of the mammalian nervous system are common phenomena, little is known of the viral morphogenesis in such instances. Experimental inoculation of animals with nervous system-adapted or known neurotropic strains (in particular, measles) generally leads to rapidly developing, necrotic conditions only superficially resembling the natural diseases. In these experimental situations, viral morphogenesis is difficult to evaluate (4, 8, 29). In an earlier study (25), we approached this problem by using organized cultures of hamster cerebellum in which the patterns of measles virus development were found to parallel those previously described in similarly infected, short-term replicate cell systems of non-neural origin (3, 4, 17, 18, 20). In our nervous tissue culture system, measles particles evolved as detached buds from areas of cell membrane beneath the surface of which viral nucleocapsid had become aligned. The longer life-span of measles-infected nervous tissue explants permitted expanded chronological study, which revealed that cytoplasmic viral material appeared before intranuclear inclusions, the latter being a feature of chronic infection. Furthermore, these intranuclear viral conglomerates encountered morphologically mimic those seen in brain biopsies obtained from patients with subacute sclerosing panencephalitis (SSPE; references 6, 12, 24, 27, 30), a disease suspected for a number of years as having a measles-like etiology. The association between measles and SSPE has already been demonstrated by a number of investigators (10, 16), and, more recently, co-cultivation techniques have shown that a measles-like virus can be recovered from SSPE tissue (9, 13, 15, 19, 22, 23). This SSPE virus has also been compared with known strains of measles virus in short-term cultures of monkey kidney cells (21). These latter results somewhat conflict with ours in that intranuclear inclusions were not seen in the Edmonston measles-infected cells, and smooth filamentous viral material, described by us as being restricted to the nucleus, was also claimed to be present in the perikarya of infected cells. Moreover, SSPE virus showed some divergence from the usual pattern of measles replication since profiles interpreted as virus particles were devoid of precisely arranged nucleocapsid beneath the plasma membrane.

On the basis of immunological evidence, several other diseases, for example, multiple sclerosis, are suspected of having a measles-like etiology (1); consequently a broad understanding of the pathogenesis of lesions and viral development in measles-infected central nervous system tissue is important. The common in vitro systems

employed in the study of measles infection have been performed on suspension or monolayer cultures of single cell lines, namely HeLa, BSC-1, or CV-1 cells (3, 14, 17, 18, 20). These, of course, are of non-neural origin and survive only a few days after measles infection. As a consequence of the short life-span, viral events are condensed and it is frequently impossible to distinguish acute from chronic appearances which usually occur simultaneously.

Measles-infected hamster dorsal-root ganglia (DRG) cultures have been found to retain structural integrity much longer than did the cerebellar explants reported previously (25). With this DRG system, we have been able to define once more sequelae leading up to chronic infection. These substantiate our earlier findings.

MATERIALS AND METHODS

Culture techniques. Cultures of 15-day-old embryonic hamster DRG were prepared by the methods of Bunge et al. (7) on collagen-coated cover slips (6). Three DRG were explanted per cover slip, and the cultures were maintained in vitro for 8 or 11 days before inoculation with the Edmonston strain of measles virus.

Virus and infection. Techniques of virus propagation, infection of nerve tissue, and assay for infectious virus were performed as previously described (11, 25). The Edmonston strain of measles virus, obtained from Fred Rapp (The Milton Hershey School of Medicine of the Pennsylvania State University), was propagated and assayed in BSC-1 cells (26). Hamster DRG cultures were infected with 3×10^5 plaque-forming units (PFU) of measles virus which had never previously been in contact with nervous tissue. The virus was allowed to adsorb for 1 hr at room temperature, and the cultures were then incubated at 34 C in growth medium. The nutrient consisted of 48% Eagle's minimum essential medium with L-glutamine, 10% rat embryo extract, 33% horse serum, and 9% balanced salt solution supplemented with 600 mg of glucose per 100 ml, 100 μg of cortisone per ml, and 1.7 μg of tetracycline per ml. Samples of virus were collected as previously described (25). Multiplicity of infection could not be determined since significant amounts of virus also adsorbed to the collagen-coated cover slips.

Light and electron microscopy. Cultures were examined in the living state throughout the period of infection. Measles-infected DRG cultures were sampled for electron microscopy (EM) at 3, 5, 10, 14, 18, 49, and 63 days postinfection. Normal sister cultures were taken for controls. Fixation was by immersion in 2.5% glutaraldehyde followed by 1% osmic acid, both buffered to *p*H 7.3 with PO_4. Dehydration was carried out in ethyl alcohol and the cultures, removed during dehydration from the collagen-coated cover slips, were embedded flat in Epon 812. They were later reoriented end-on in gelatin capsules containing fresh resin, polymerized once more, and sectioned at right angles to the plane of growth. Complete details of the procedure are given in the preceding paper (25). Sections were cut on LKB, Porter-Blum, or Reichert ultramicrotomes. After fixation and embedding for EM, 1-μm sections were made from each explant and stained with Toluidine Blue for light microscopic examination. Sections for EM were double-stained with uranyl acetate and lead citrate. Grids were examined in Siemens Elmiskop 1A and 101 electron microscopes.

RESULTS

Viral assay. The replication of measles virus in hamster DRG cultures is shown in Fig. 1. Small amounts of virus were detected as early as 3 days postinfection, and significant amounts of virus were recovered from the nutrient media of these cultures for periods as long as 25 days postinfection. Other experiments have revealed that a 10- to 100-fold greater concentration of virus remains associated with the tissue mass or explant. Several cultures, although surviving for longer periods than 25 days postinfection and although undergoing obvious cytopathic effects (CPE), failed to produce infectious virus which could be recovered in the nutrient media. These same cultures were examined by EM and the results are presented below.

Light microscopy: living cultures. Irrespective of the age at which the DRG cultures were

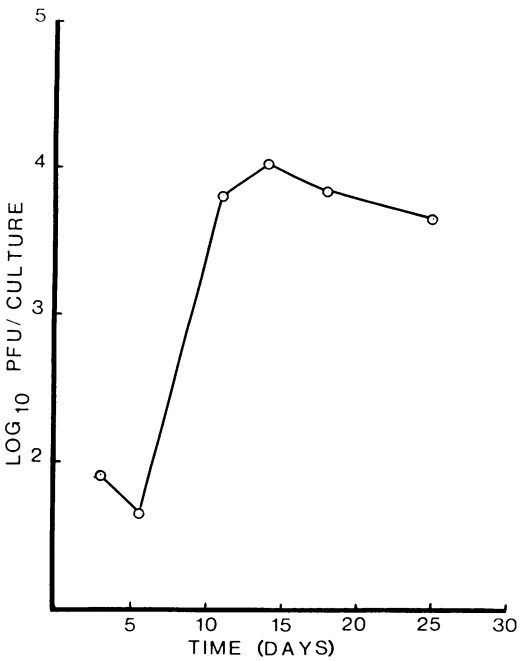


FIG. 1. *Growth of measles virus in hamster DRG cultures. Cultures were inoculated with 3×10^5 PFU of virus, and supernatant fluids were assayed as described.*

inoculated with measles virus (i.e., 8 or 11 days in vitro), all developed CPE and eventually completely degenerated. The pattern of degeneration was essentially identical between the two groups, although the 11-day group developed CPE earlier.

Neuronal abnormalities were primary manifestations of CPE. This initially involved the displacement of the nucleus towards the periphery of the cell, occurring at 5 to 10 days after inoculation. The nucleolus appeared misshapen and the cytoplasm more granular than normal. With increased severity of CPE, definition of cellular detail diminished. Nuclei appeared to increase in size and resembled fluid-filled sacs occupying one-quarter to one-third of the neuronal soma. Nucleoli gradually underwent changes, becoming nonrefractile, pleomorphic, and usually enlarged. In more severely affected neurons, the clear, liquified nuclei increased in size and finally occupied one-half to three-quarters of the entire cell. Satellite cells appeared to dissociate from neurons. Giant cells were observed throughout the course of infection. By 63 days, it appeared that changes had reached a terminal stage and the tissue was finally succumbing to measles virus infection.

Light microscopy: 1-μm sections. Normal cultures revealed the usual pattern of organization for DRG cultures with the large neurons centrally located in the explants, each surrounded by a layer of capsule cells (7). Spindle-shaped Schwann cells were distinguishable by virtue of their size, location, and densely staining, elongate nuclei. About 90% of the cell population was made up of these two cell types.

In the infected cultures, no change was seen before 10 days, after which the close packing of elements was disrupted and the neurons became separated from one another. Examination of cultures 14 days postinfection and later revealed massive intraneuronal inclusions, which in serial section could be shown to be of cytoplasmic origin, the nucleus being uninvolved at this stage (Fig. 2). After 49 days, similar intracytoplasmic masses were still common, particularly within neurons. Even at this time, neurons could still be identified from surrounding Schwann and interstitial cells. On occasion, discrete "empty" circular areas were visible within the nuclei, whereas in other cases floccular material could be seen within the liquified nucleochromatin (Fig. 3). The reduced thickness of the explant indicated that some tissue loss had occurred. By 63 days, the entire explant was reduced to a sheet of tissue two to three cells thick.

EM. The ultrastructural features of long-term cultures of DRG are well documented (7). The neurons are large with centrally located nuclei, each with a prominent nucleolus and an extensive, well-defined perikaryon, rich in organelles. Each neuron is intimately surrounded by capsule cells. In the interstices, Schwann cells envelop myelinated and nonmyelinated axons. The latter cell type possesses a characteristic basement membrane.

Cytoplasmic changes. Within measles-infected DRG cultures, viral material was first encountered after 5 days in the form of regularly aligned, granular, filamentous rods beneath the outer cell membranes of both neurons and Schwann cells. These are identical to the nucleocapsids described by others (2, 28). Each nucleocapsid was invested by an amorphous "fuzzy" matrix. Additional amorphous electron-dense material occupied the spaces between the regularly aligned nucleocapsids. This contributed to the dense inner lining of the altered region of plasma membrane (Fig. 4 and 5). A blurred coating, morphologically analogous to basement membrane, also appeared on the cell surface overlying each affected area of plasma membrane. Outward protuberances of these regions resulted in the formation of viral buds which eventually became detached and rounded up to form typical measles particles (Fig. 4 and 5). Each tubular nucleocapsid, known from previous studies to be a tightly wound helix of nucleoprotein (2, 28), was situated beneath the thickened cell membrane and always possessed a coating of amorphous material. The diameter of the central nucleocapsid was 20 nm and, together with the coating material, varied between 40 and 50 nm (Fig. 4 and 7). The mode of replication described here is identical to that described both in measles-infected hamster cerebellar explants and non-neural systems (3, 14, 17, 18, 20). These phenomena were observed in DRG cultures from 5 to 63 days.

The subsequent development of cytoplasmic nucleocapsid occurred either in haphazard distributions or in paracrystalline arrays (Fig. 6). These arrays corresponded to the inclusions seen with the light microscope (Fig. 2 and 3). Whenever nucleocapsid was located within the cell perikaryon, it always bore the amorphous coating mentioned above (Fig. 6, inset). Neurons were the sites of most prominent viral inclusions which in many cases occupied more than half of the perikaryon. In neurons infected for 5 to 18 days, the nucleus invariably became eccentrically located, but, apart from a slight irregularity in the nuclear contour, both the nucleochromatin and nucleolus remained normal (Fig. 6). When cytoplasmic paracrystalline arrays were formed, normal cellular organelles became displaced and there was an apparent increase in neurofilaments.

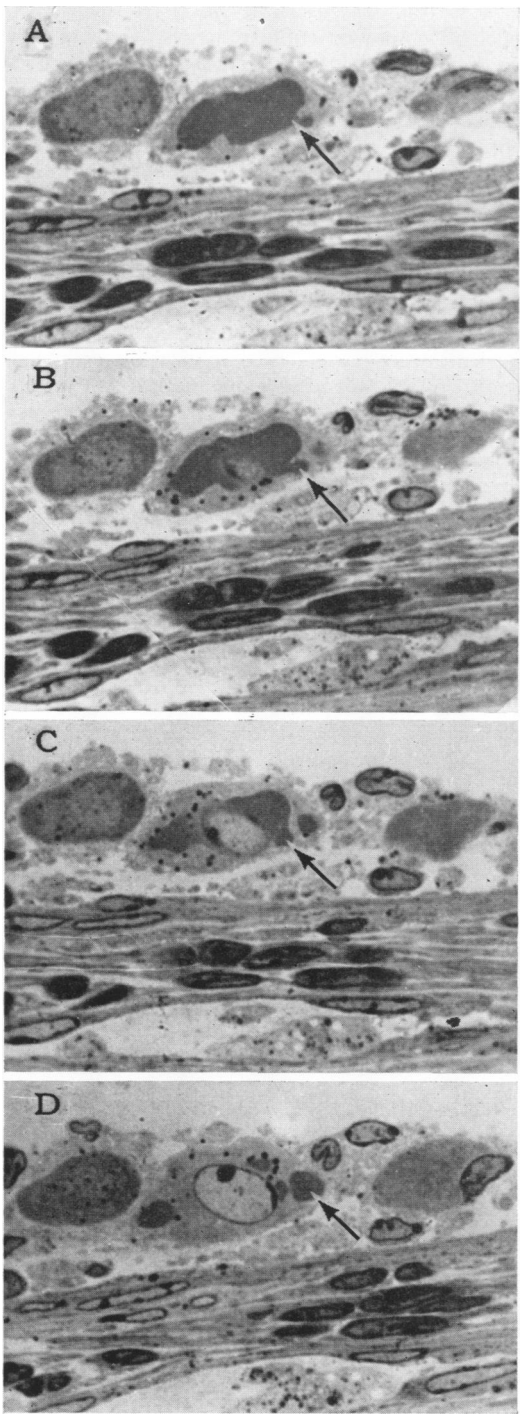


FIG. 2. *DRG culture 14 days postinfection. Serial sections A to D are through a large cytoplasmic measles inclusion (arrow) within a neuron. The nucleus begins to appear in (B) and is cut almost equatorially in (D). The inclusion body is represented in (D) by several dispersed masses. Elongate Schwann cell nuclei are seen below the three neurons in each figure. Toluidine Blue stained 1-μm Epon section. × 480.*

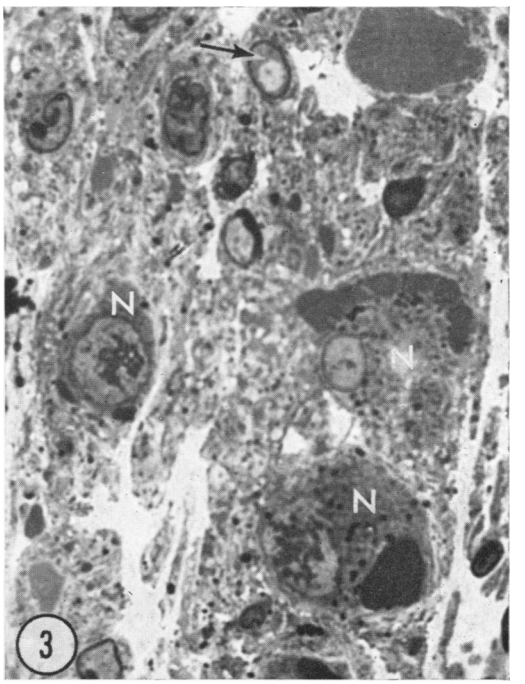
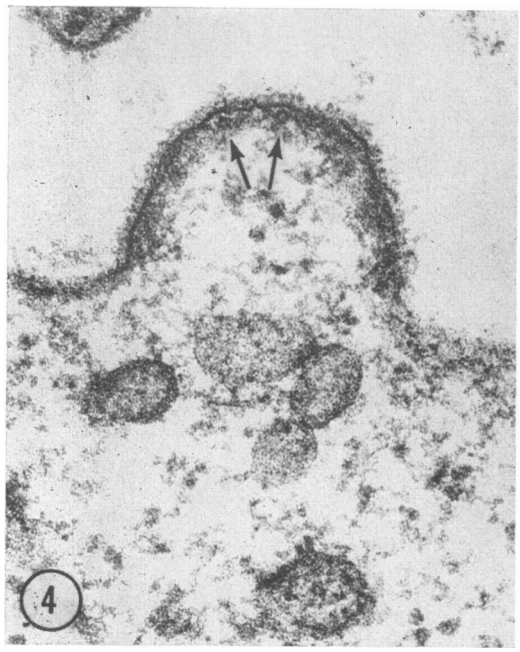


FIG. 3. *DRG culture 49 days postinfection. Many DRG neurons (N) contain large cytoplasmic inclusion bodies, one of which (upper right) shows a striated pattern. Nuclei, mostly belonging to Schwann cells, show emargination of nucleochromatin, and one (arrow) contains a clear, round inclusion. Toluidine Blue stained 1-μm Epon section. × 560.*

The capsule-cell layer encompassing each neuron remained intact for 14 days, but later it was frequently disrupted. In time, some cytoplasmic arrays lost their ordered structure and became amorphous granular masses (Fig. 7 and 8). Autophagic vacuoles, or sequestration of altered viral inclusions by endoplasmic reticulum, a common appearance within cerebellar explants (25), was a rare phenomenon.

Nuclear changes. In chronically affected explants, cytoplasmic viral material was frequently present within neurons (Fig. 9). However, by this time (18 days and later), distinctive nuclear changes became apparent. These changes began with the transformation of nucleoli into granular reticulated masses (Fig. 9) or several small clumps of material (Fig. 10) which at higher magnification contained suggestions of tubular



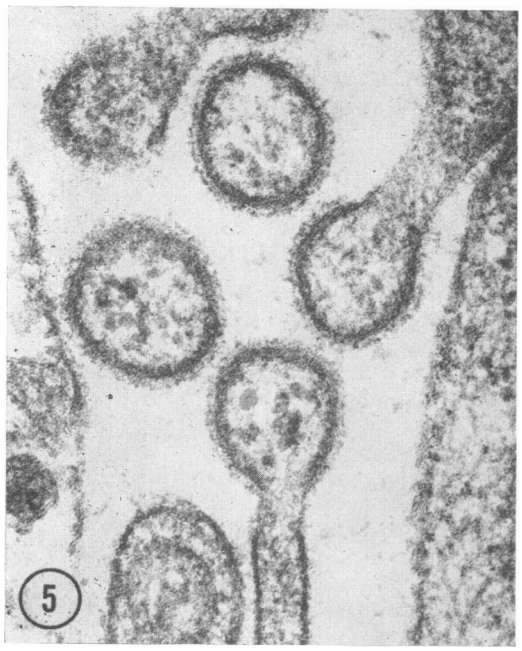


FIG. 4. *DRG culture 18 days postinfection. A viral bud is seen at the surface of an infected cell. Tubular nucleocapsid material (arrows), together with associated amorphous material, lies beneath the plasma membrane. An additional blurred membrane surrounds the budding particle.* × *100,000.*

FIG. 5. *DRG culture 18 days postinfection. Viral buds lie within the extracellular space, some not yet detached from the processes of parent cells.* × *75,000.*

arrays (Fig. 10, inset). By 49 days, nearly all nucleoli contained tubular arrays. The nucleoli gradually increased in size and became floccular conglomerates containing nucleocapsids, visible under higher magnification (Fig. 11). Such appearances complemented those seen in the light microscope (Fig. 3). These transformed nucleoli then appeared to segregate into smaller collections of tubular arrays between which haphazardly oriented nucleocapsid later appeared (Fig. 14 to 16). These inclusion bodies were made up of 20-nm helical rods, each having a vermiform appearance. By this time, normal nucleoli were rare. The haphazardly arranged masses of helical rods became more frequent and eventually occupied large areas of the nucleus, displacing the denser nucleochromatin towards the periphery. These intranculear rods differed from their cytoplasmic counterparts in their lacking an outer amorphous coat. This facilitated resolution of detail. This type of "smooth" nucleocapsid was never encountered beyond the confines of a nuclear envelope unless, as occasionally happened in longer infected explants, the cell had begun to degenerate. Then the nuclear membrane usually became disrupted, thereby exposing the cytoplasm to the contents of the nuclei.

Some neurons possessed highly lobate nuclei, which in thin sections gave the impression of multinucleated giant cells. On serial section, however, these lobes could be shown to be connected by narrow bridges (Fig. 12). Although the majority of intraneuronal cytoplasmic viral masses tended to lose their regular organization with time, many well-preserved paracrystalline arrays could still be found after 49 days. In addition to this, cytoplasmic budding of measles particles containing precisely aligned nucleocapsid was still a common phenomenon (Fig. 13).

Similar, but somewhat delayed changes also occurred within Schwann cells except that these cells rarely developed the massive intracytoplasmic inclusions seen in neurons. However, by 49 days intranuclear tubular arrays were also encountered. Denser nucleochromatin was displaced towards the periphery by these inclusions. Giant cell formation was seen on rare occasions at the edges of cultures after 49 days. Whether these polykaryocytes were the result of cell fusion or repeated nuclear division could not be ascertained (Fig. 14 to 16).

The longest surviving cultures remained for 63 days. Even in these explants, active cytoplasmic budding and prolific, intranuclear nucleocapsid masses were evident.

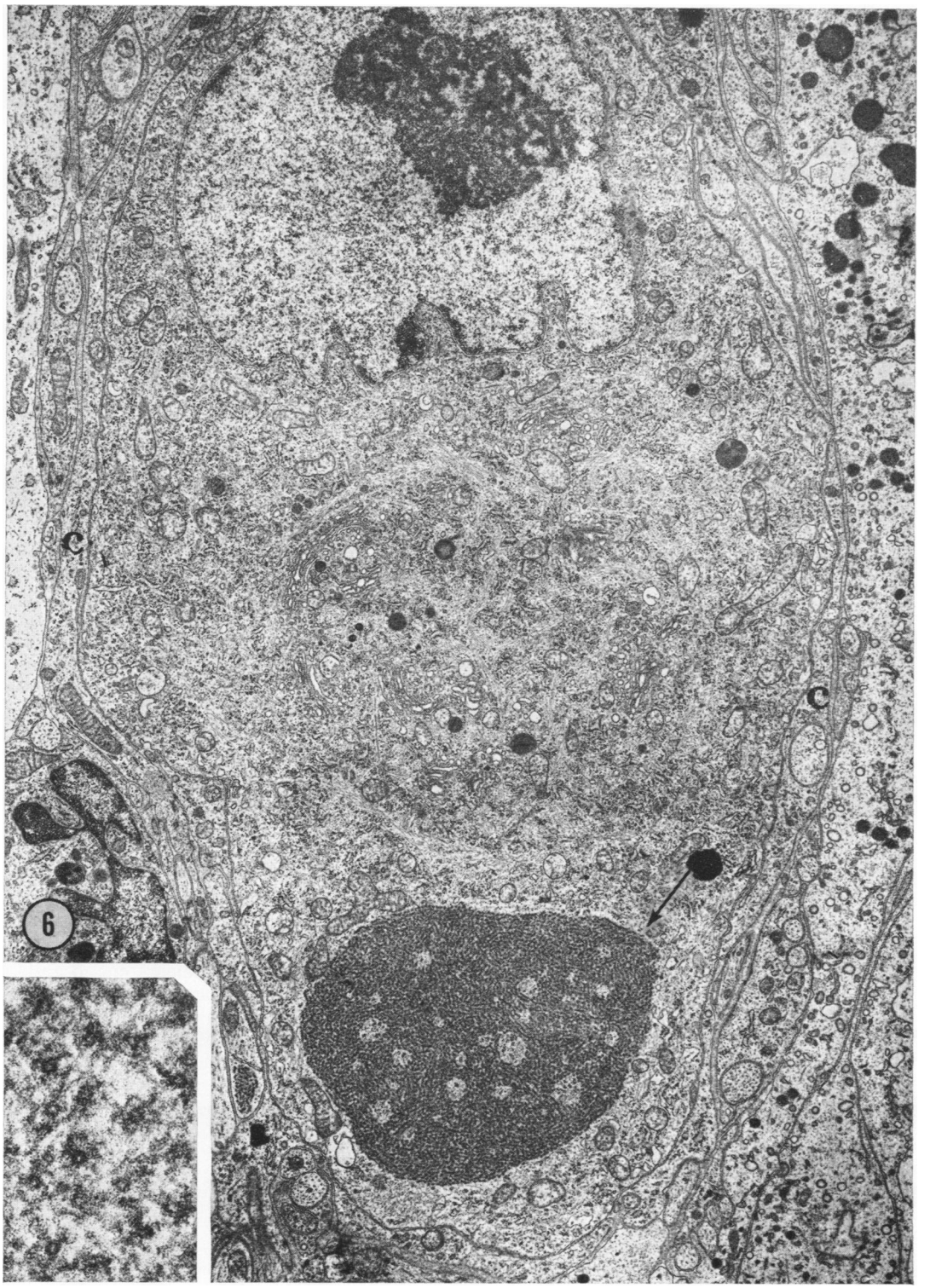


FIG. 6. *DRG culture 14 days postinfection. A large neuron, still surrounded by its capsule layer (C), contains a cytoplasmic measles inclusion (arrow). The nucleus has been displaced towards the upper pole of the cell but, apart from a slight irregularity in contour, shows no abnormalities. × 5,400. Inset: detail of cytoplasmic viral material. Note amorphous coating around each tubular nucleocapsid. × 114,000.*

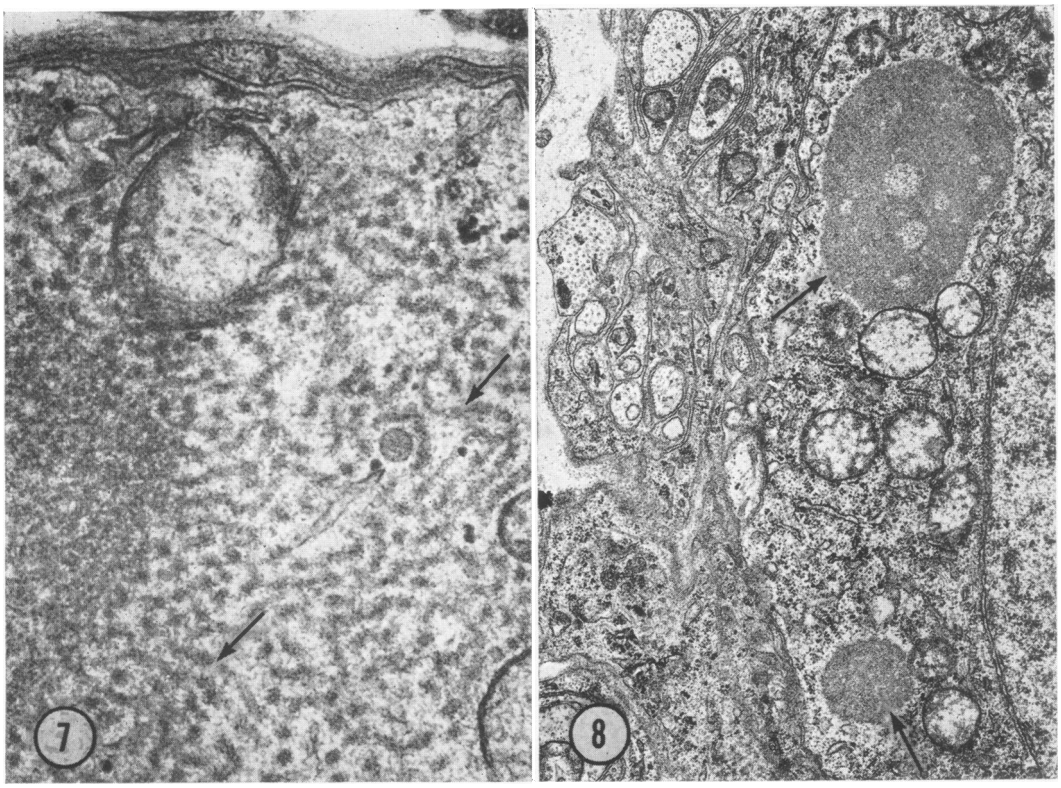


FIG. 7. *Portion of a neuron displays a transitional stage in the degradation of viral material. Intact cytoplasmic nucleocapsids (arrows) are apparently to become incorporated into the amorphous mass on the left. Capsule cell layer (including basement membrane) of infected neuron is seen at the top.* × *35,000.*

FIG. 8. *DRG culture 18 days postinfection. Degraded cytoplasmic measles inclusion bodies (arrows) appear as amorphous masses within the perikaryon of a neuron. Note normal axons and surrounding Schwann cell elements.* × *14,000.*

DISCUSSION

These data demonstrate that measles virus replicates in organotypic cultures of hamster DRG for as long as 63 days. Synthesis of infectious measles virus continues for periods as long as 25 days. Ultrastructurally, there was neither an alteration of the structure of the budding particles nor of their number during the later stages of infection (26 to 63 days). The failure in some instances to detect infectious virus in the supernatant fluid after the 26th day postinfection is, therefore, not explained in these studies. It may be due to "trapping" of the particles within the interstices of the tissue. It should be noted that, with increasing time post-inoculation, viral buds in a previous study on cerebellar explants tended to lose the uniform arrangement of nucleocapsid which was characteristic of the early stages of infection (25). This transformation was not observed in this study.

The morphogenesis of measles virus in cultures of hamster DRG resembles patterns reported in a variety of non-neural systems (3, 14, 17, 18, 20). Massive aggregates of rodlike nucleocapsid material, surrounded by amorphous material, were present in the cytoplasm of infected DRG cells. These aggregates persisted throughout the entire period of infection. Their appearance preceded that of the intranuclear collections of smooth rods. The two types of filamentous rods were never intermixed within the same part of the cell unless the cell had undergone necrotic changes. Oyanagi et al. (21) clearly showed a necrotic nucleus, filled with nucleocapsid, bringing its contents into close apposition to cytoplasmic nucleocapsid (Fig. 7 and 8). This was interpreted as simultaneous formation of the smooth and granular rods within the cytoplasm of infected cells.

Unlike measles-infected cerebellar explants

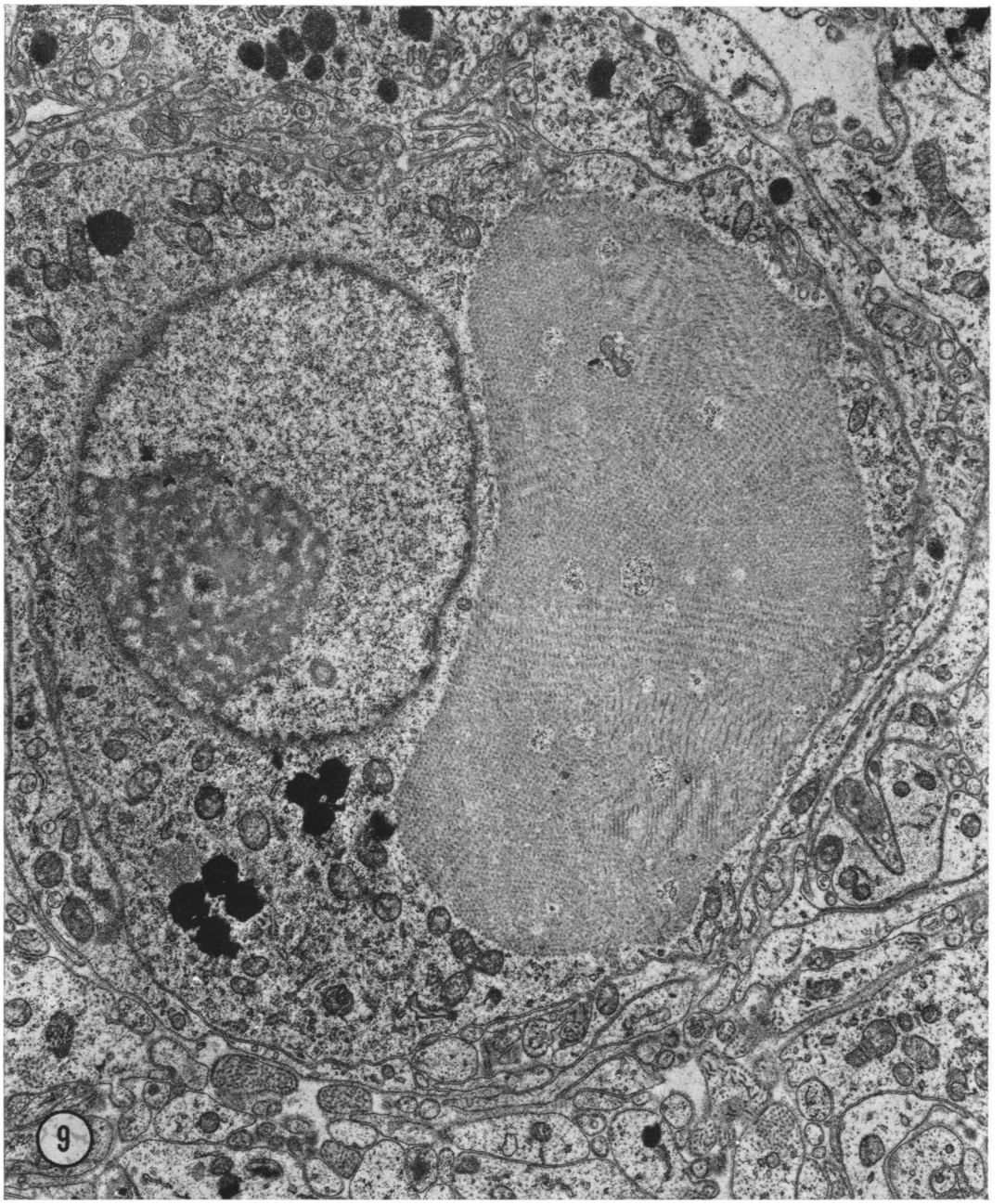


FIG. 9. *DRG culture 49 days postinfection. A DRG neuron with a slightly disrupted capsule layer contains a massive cytoplasmic paracrystalline array of nucleocapsids cut in various planes. The nucleolus appears enlarged and reticulated. Measles particles are evident within the extracellular space.* × *9,500.*

(25), cellular identification within DRG cultures was less of a problem. Schwann cells always retained their basement membranes. Neurons remained large and surrounded by a capsule layer. These features usually persisted throughout the course of infection. The present study shows for the first time unequivocal involvement of neurons during measles infection. Cerebellar

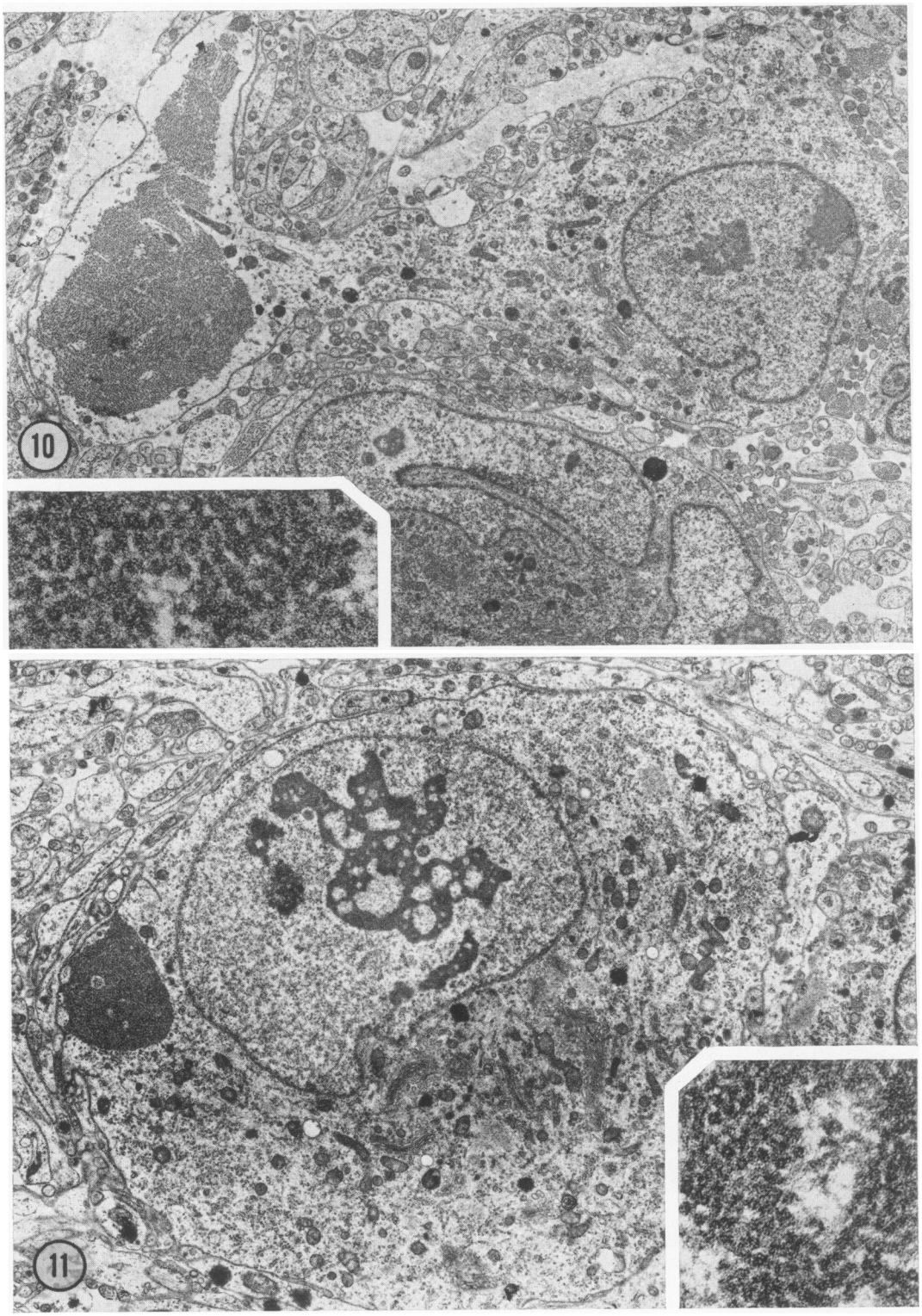
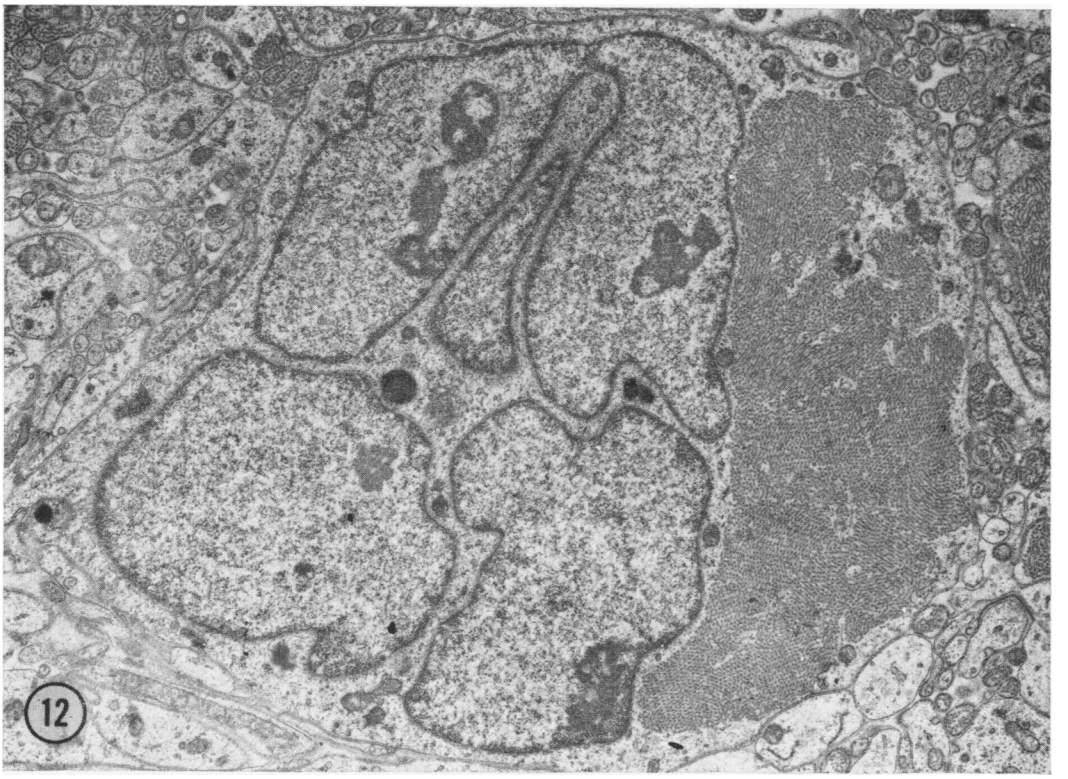


FIG. 10. *DRG culture 49 days postinfection. A DRG neuron possesses a massive collection of nucleocapsids within its swollen axon hillock. The nucleolus consists of small dense clumps. The capsule cell layer is highly disrupted. Note measles particles in interstices.* × *5,000. Inset: detail of altered nucleolus from above figure. Note the suggestion of tubular profiles.* × *114,000.*

FIG. 11. *DRG culture 49 days postinfection. A bizarre pleomorphic nucleolus occupies a large area of the nucleus within an infected neuron. A cytoplasmic mass of viral material is also present (arrow).* × *7,000. Inset: detail of transformed nucleolus showing tubular arrays in longitudinal and transverse section.* × *114,000.*



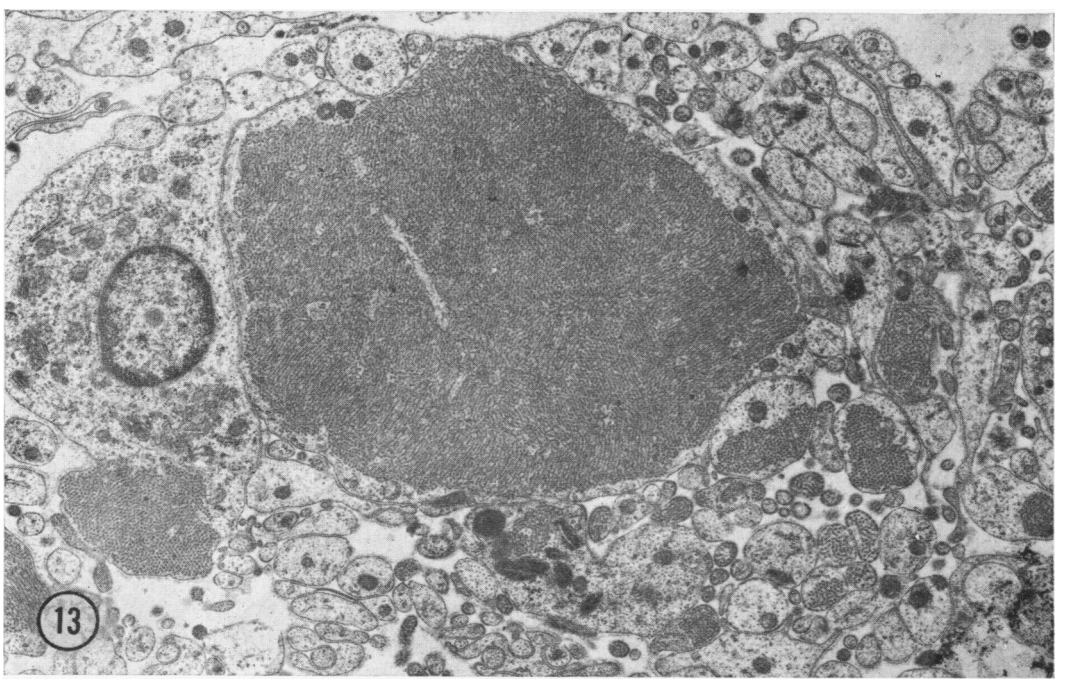
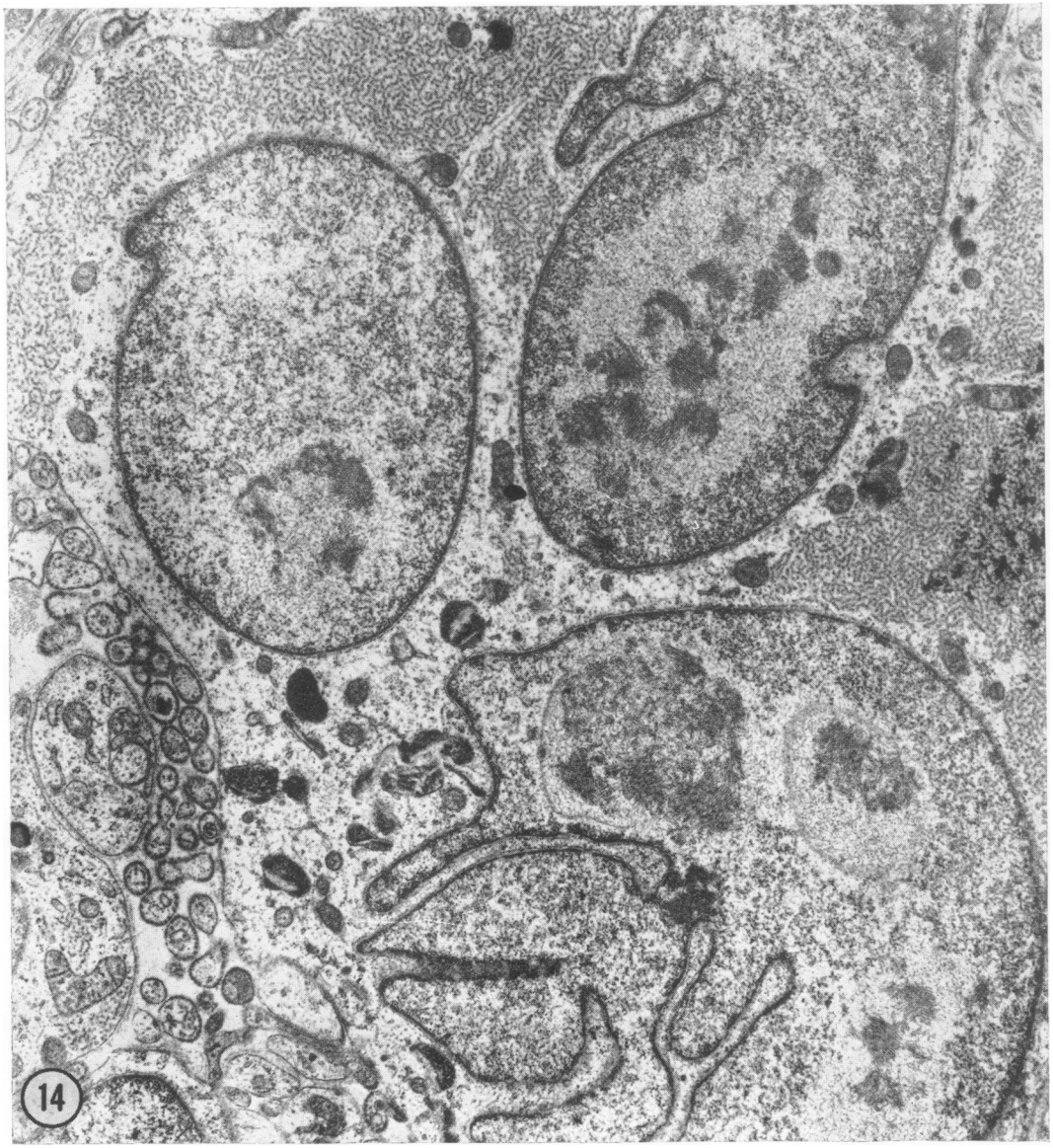
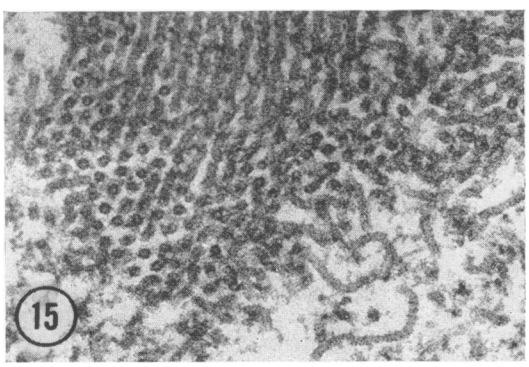


FIG. 12. *DRG culture 49 days postinfection. A measles-infected neuron shows a multilobate nucleus containing several fragments of nucleoli. A large collection of cytoplasmic viral material is also evident. The capsule cell layer is disrupted. Measles particles can be seen in the interstices.* × *6,000.*

FIG. 13. *DRG culture 49 days postinfection. A large paracrystalline array of cytoplasmic viral material occurs within a neuronal cell process. It is surrounded by a Schwann cell (left) and many Schwann cell processes, some of which show budding viral particles.* × *6,000.*





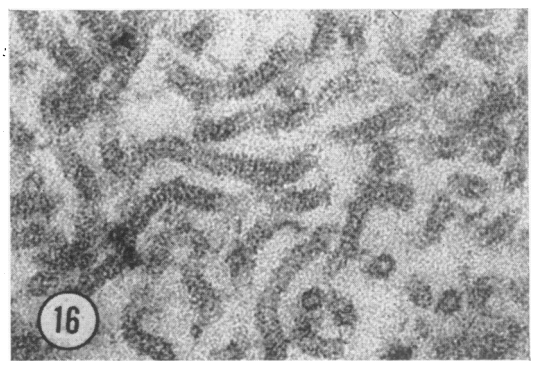


FIG. 14. *DRG cultures 49 days postinfection. A giant cell of Schwann cell origin sectioned near the periphery of an infected explant. Note both haphazard and paracrystalline arrays of intranuclear nucleocapsid. Scattered cytoplasmic viral material is also present. Many measles particles can also be seen within the interstices.* × *12,000.*

FIG. 15. *DRG culture 49 days postinfection. Detail of intranuclear nucleocapsid array. Note absence of outer amorphous coating around each tubular profile.* × *66,000.*

FIG. 16. *DRG culture 49 days postinfection. Haphazardly arranged vermiform nucleocapsids from an affected nucleus. Suggestions of a helix are evident within several of the tubular structures.* × *154,000.*

neurons generally lost all criteria of identification and possibly died the moment CPE became evident.

The morphological similarity between the intranuclear inclusions witnessed here and those described as characteristic of SSPE (6, 12, 24, 27, 30) support the contention that in SSPE tissue we are visualizing a chronic or latent measles infection in which measles-like genome is in a nonpermissive state. Co-cultivation techniques, which lead to the recovery of measles-like virus from SSPE tissue (9, 13, 15, 19, 23), would therefore suggest that the viral genome is once more exposed to permissive conditions. With the exception of active replication, the overall pathological picture obtained from the present study resembles closely that associated with SSPE. We are presently investigating the possibility that these long-term infections in vitro are transitional states in the process of establishing nonpermissive conditions for measles virus in nervous tissue.

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