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Electron Microscopy of a Plant-Pathogenic Virus in the Nervous System of Its Insect Vector

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The central nervous system of wound tumor virus (WTV)-infected *Agallia constricta* was studied by electron microscopy to obtain information concerning the virus distribution in the nervous system. Wound tumor virions were mostly found in the cytoplasm of the ganglion cells and less frequently in the glial cells. WTV was occasionally observed in the perineurium cells, nerve axons, tracheoblasts, and lateral nerves. In the ganglion cells, virions appeared as individual isolated particles (V_1), in tubular formation (V_2), and occasionally in aggregates (V_3). In the glial cells, the virions were mostly seen in the V_3 formation, and very seldom in the V_1 and V_2 formations. In the perineurium cells and tracheoblasts, only small V_3 formations were observed. The isolated virions were usually surrounded with polyribosomes, and often appeared around the foci of the viroplasm. Sometimes degenerating ganglion cells infected with the WTV were encountered. These damaged cells strongly indicated that WTV exerted a cytopathogenic effect on the nerve cells.

The detection in 1964 (22) of wound tumor virions in thin sections of infected plant and insect tissues led to a systematic study of virus distribution in host cells by electron microscopy. The virus was demonstrated in the cytoplasm of systemically infected plants (20) and in various organs and tissues of systemically infected *Agallia constricta* (19) and *Agalliopsis novella* (Granados et al., J. Invertebrate Pathol., *in press*). In the last-mentioned study, the presence of mature virus particles was demonstrated for the first time in the nervous system of insect vectors. This was not surprising, since serological tests (23) had earlier indicated the presence of viral protein in the brain of infected *A. constricta*. However, the serological method employed could not distinguish between virions, incomplete particles, soluble viral antigen, or viral coats. There was no information about the possible multiplication of the plant-pathogenic virus in the nervous system, as compared to accumulation and storage, nor were there any data as to whether the virus is present solely in the cytoplasm or also in the nucleus of a nerve cell. Therefore, a detailed study was undertaken to obtain accurate information concerning the distribution of wound tumor virus (WTV) and the kind of viral entities present in different parts of this system. The preliminary information obtained with *A. novella* revealed the occurrence of virions inside of ventral ganglia, which indicated that the whole nervous system might serve as an important site for virus multiplication in the insect body. The present study was also aimed at obtaining data on the possible effects of the plant-pathogenic virus on nerve cells.

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MATERIALS AND METHODS

To obtain viruliferous insects, virus-free *A. constricta* nymphs in the second and third instar stage were confined on wound tumor-diseased crimson clover plants, *Trifolium incarnatum*, for 44 days. The central nervous system of 10 infected insects, including parts of the lateral nerves emerging from the ventral ganglia, was excised and fixed in cold 2% glutaraldehyde in 0.14 M Veronal-acetate buffered solution (3). Excised organs were transferred to fresh fixative at 4 C for 30 min. The organs were postfixed in 1% osmium tetroxide in 0.14 M Veronal-acetate buffered solution at 4 C for 2 hr. Fixation was followed by dehydration in graded ethyl alcohol, and by embedding in epoxy resin (13). Ultrathin sections were cut with diamond knives on a Porter-Blum MT-1 microtome, and were stained with uranyl acetate, followed by lead citrate (24). Thick sections, cut with glass knives, were stained with 0.05% methylene blue solution in order to identify specific regions of the central nervous system. Observations were made with a Siemens Elmiskop I electron microscope. Insects reared on healthy plants, and of the same age as insects on infected plants, served as controls. To study the general histology of the nervous system, whole bodies of healthy insects were also fixed in Carnoy's fixative for 3 hr. Serial sections were made following

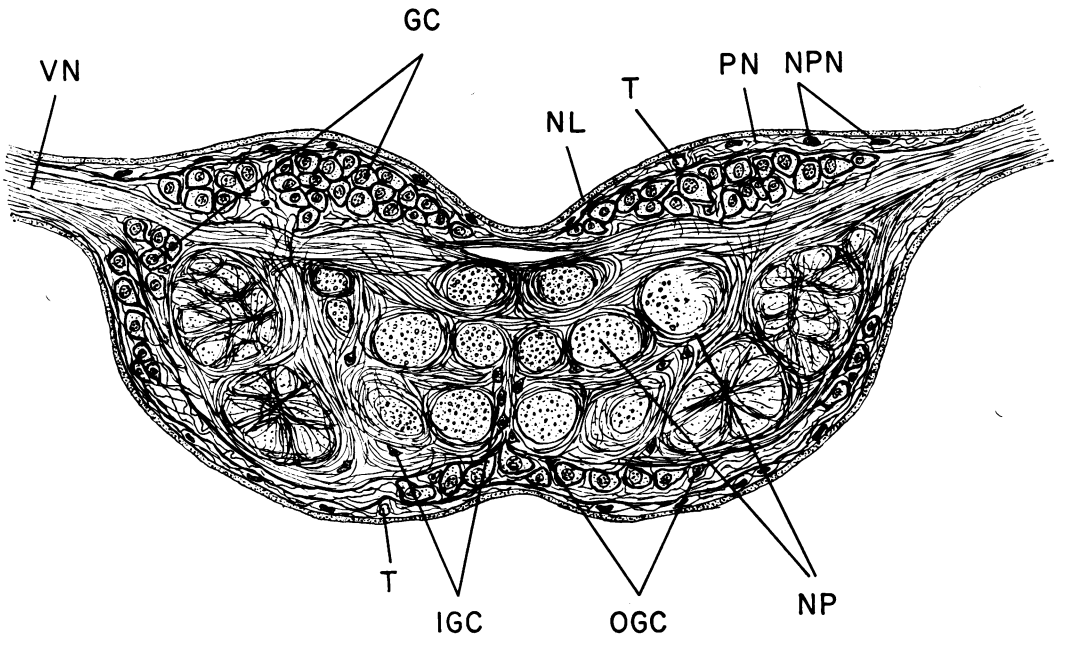


FIG. 1. *Schematic drawing of a transverse section of the prothoracic ganglion. The entire ganglion is enclosed with the neural lamella (NL) and perineurium (PN). The ganglion cell bodies (GC) are situated at the periphery. The neuropile (NP) occupies the central core of the ganglion. The outer glial cell (OGC) and the inner glial cell (IGC) are situated around the ganglion cell bodies and nerve fibers, respectively. The tracheae (T) penetrate into the perineurium and neuropile. NPN = nuclei of perineurium cells, VN = ventral nerve.*

general histological procedures and stained by one-step trichrome stain (9). Other details of the methods have been described elsewhere (Granados et al., *in press*). Terminology for the general anatomy of the nervous system of *A. constricta*, as used by Gil-Fernandez and Black (8), was followed in this paper.

RESULTS

General anatomy and histology. The findings concerning the general anatomy of the central nervous system in *A. constricta* agreed with those of Gil-Fernandez and Black (8). The brain (supraesophageal ganglion) is composed of the proto-, deuto-, and tritocerebrum. The protocerebrum consists of median protocerebrum and optic lobes. Three pairs of ventral ganglia are fused to form a single complex mass. These ganglia consist of the subesophageal ganglion, the thoracic ganglion, including the pro-, meso-, and metathoracic ganglia, and the abdominal ganglion. The brain is connected to the ventral ganglia with the circumesophageal ring, which is formed by the posterior region of the tritocerebrum, the circumesophageal connectives, and the anterior region of the subesophageal ganglion surrounding the pharynx. The central nervous system is concentrated in the head and the thorax.

The entire central nervous system is enclosed in the perilemma, which is formed by an outer noncellular layer (neural lamella or basement membrane, Fig. 1, NL), and an inner cellular layer (perineurium, Fig. 1, PN). In the ganglia, most of the ganglion cell bodies are situated in the periphery (Fig. 1, GC). Outer glial cells (Fig. 1, OGC) situated beneath the perineurium surround the ganglion cell bodies. The cytoplasm of some glial cells extends into the neuropile (Fig. 1, NP), which occupies the central core of the ganglion. The neuropile consists of large and small fibers forming complex fiber networks, often collected into nerve-tracts. The fibers are usually surrounded by the cytoplasm of inner glial cells. Some of the fibers are directly adjacent to others. Synapses were observed between plasma membranes of the fibers and inner glial cells (Fig. 16, S). Sometimes they were also observed between ganglion cells and outer glial cells. Synaptic vesicles (SV) were seen in the synaptic region of the axoplasm (Fig. 16). The axoplasm contained a few mitochondria and numerous neurofilaments (Fig. 16, NF). The cytoplasm of the glial cells was composed of loosely scattered cytoplasmic organelles (Fig. 13). The lateral nerves were also enclosed in the perilemma. Some nerve axons in the lateral nerve were invested with a primitive type of "myelin sheath" (26), in a spiral course,

formed by the glial cells (Fig. 19, SH). The tracheae penetrated into the perineurium of either the ganglia or the lateral nerves (Fig. 2 and 19, T). Sometimes they also penetrated into the periphery of the neuropile (Fig. 16, T).

WTV in the nervous system: Perilemma. Wound tumor virions were not observed in the neural lamella. In the cytoplasm of the perineurium, only small dense inclusion bodies containing a few loosely aggregated virions were occasionally found.

Ganglion cells. Many ganglion cells, including neurosecretory cells, either in the brain or ventral ganglion complex, were infected with WTV. There were various gradations of WTV infection among these cells. Some were infected with only a few wound tumor virions, and others were infected with many (Fig. 2). Cells in which the entire cytoplasm was infected with a considerable number of virions were often observed (Fig. 3). The virions were found only in the cytoplasm but not within the nucleus.

There were three different formations of virus in the cytoplasm of the ganglion cells. The first type was represented by individual *isolated virions* (V_1). These were loosely scattered in the cytoplasm. No membrane structure was observed surrounding the capsid of the virions (Fig. 4, V_1). Sometimes these isolated virions were accumulated in cytoplasmic vacuoles. The second type consisted of *a tubular formation* (V_2). The virions were in a single file within tubular structures which had a single membrane (envelope). Figure 4 shows longitudinal sections (LV_2) and transversal section (TV_2) of the tubular formations. In the tubular structures, a substance of low electron density was observed between virions. At the terminal of the tubules, virions were occasionally observed directly adjacent to cytoplasmic organelles, such as ribosomes. Tubules with closed terminals were never observed. In some regions of the cytoplasm, the membrane of the tubules appeared to be irregular and filamentous. In these regions, the core and capsid of individual virions were not distinguishable (Fig. 5). Virions of both types often surrounded a dense matrix area (Fig. 6, DM), which consisted of electron-dense fibrous structures. In these areas, many free ribosomes were often observed. Many fine filamentous structures, approximately 20 mμ in diameter, were occasionally observed among virions of both types (Fig. 7, F). In some infected ganglion cells, very long tubular formations of the second type were observed (Fig. 8). Some of them extended through the entire length of the cell. The tubular structures were straight. A few of the tubular structures were sheathed with an outer membrane, which had almost the same thickness and electron density as the plasma membrane (Fig. 9, arrow). These outer sheaths sometimes did not completely surround the tubular structures, and occasionally the same outer sheath held more than two tubules.

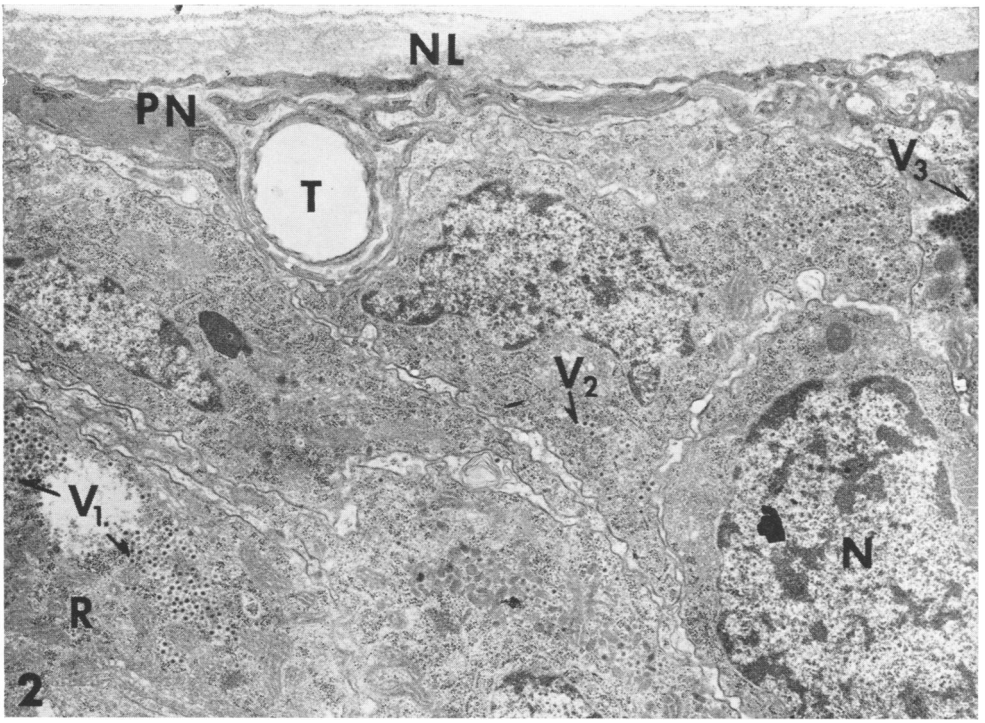
The third type of virus distribution was *an aggregated formation* (V_3). The virions of this type were aggregated in electron-dense inclusion bodies (Fig. 8, V_3). The inclusion bodies usually consisted of a homogeneous, highly electron-dense matrix, with a single limiting membrane. In some inclusion bodies, only a few virions were scattered, but in most others numerous virions were tightly aggregated. Clusters of virions tightly packed in parallel rows were often observed in the aggregated formation (Fig. 8).

In the cytoplasm of the ganglion cells, the virions were distributed mostly in the V_1 and V_2 formations and less often in the V_3 formation.

Occasionally, many irregular inclusion bodies, without virions, were seen in the cytoplasm of the infected cells. However, in most infected cells no significant changes in the fine structure of the cytoplasmic organelles were found, except in degenerating cells which are described below.

Occasionally, degenerating ganglion cells were encountered. The cytoplasm of these cells had a high electron density in comparison with non-infected ganglion cells (Fig. 10 and 11). Figures 11 and 12 show the changes in the ultrastructure of a degenerating cell. The cytoplasmic matrix of the cell was transformed into homogeneous fine granules. The membrane structures of cytoplasmic organelles, such as the endoplasmic reticulum, the nuclear membrane, and the Golgi apparatus, were indistinct. No normal mitochondria were found in the cytoplasm. The ribosomes of the cell were loosely coagulated. The nuclear substance was also changed to a homogeneous fine granular substance. A number of virions, either in the isolated virions or in the tubular formation, were clearly observed in the cytoplasm of these degenerating ganglion cells.

Glial cells. In glial cells, most of the wound tumor virions were observed in the aggregated formation (V_3) of the virus distribution. Figure 13 shows that the virions were arranged tightly within an electron-dense inclusion body in the cytoplasm of an outer glial cell that surrounded the ganglion cells. The same type of virus aggregation was often found in inner glial cells which surrounded the nerve fibers of the neuropile. Several different types of virus inclusion bodies were found in the glial cells. Large irregular bodies consisting of a substance of high electron density were occasionally observed. In these inclusion bodies, numerous virions were tightly aggregated. The core and capsid of each virion



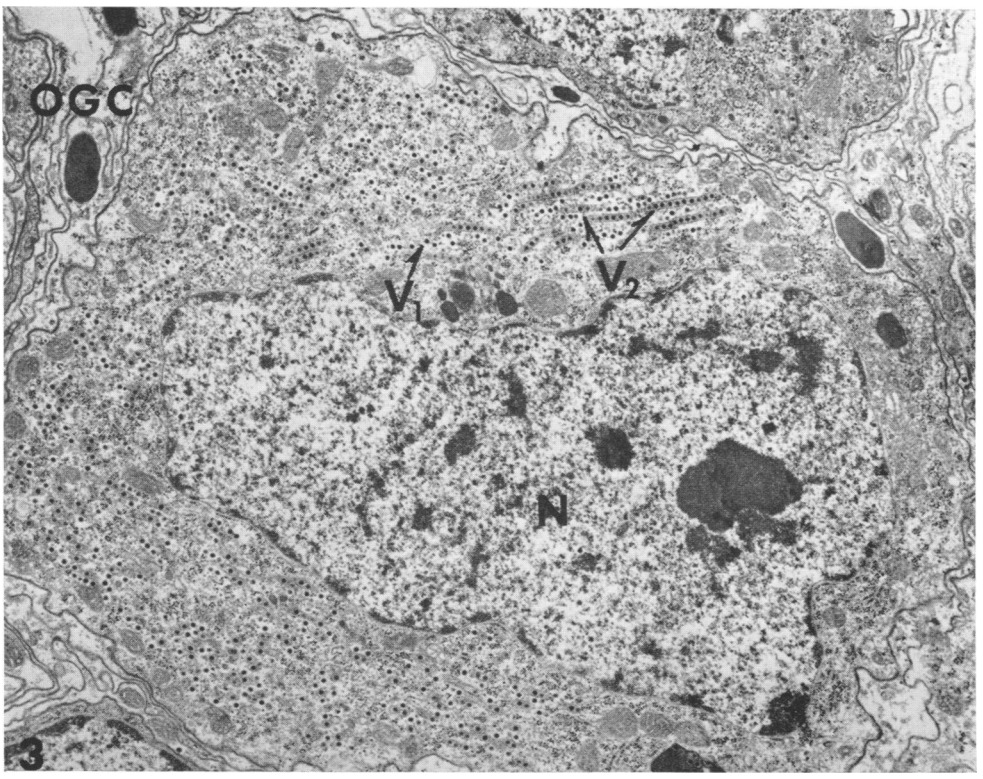


FIG. 2. *Peripheral region of the prothoracic ganglion. Various gradations of wound tumor virus infection (V_1 = isolated, free virion; V_2 = tubular formation; V_3 = aggregated formation) were seen among the ganglion cells. N = nucleus of the ganglion cell, NL = neural lamella, PN = perineurium, R = ribosome, T = trachea.* × *11,000.*

FIG. 3. *Subesophageal ganglion cell, in which the entire cytoplasm was infected with wound tumor virus, represented by isolated, free virions (V_1) and by virions in tubular formation (V_2). N = nucleus of the ganglion cell, OGC = outer glial cell.* × *12,000.*

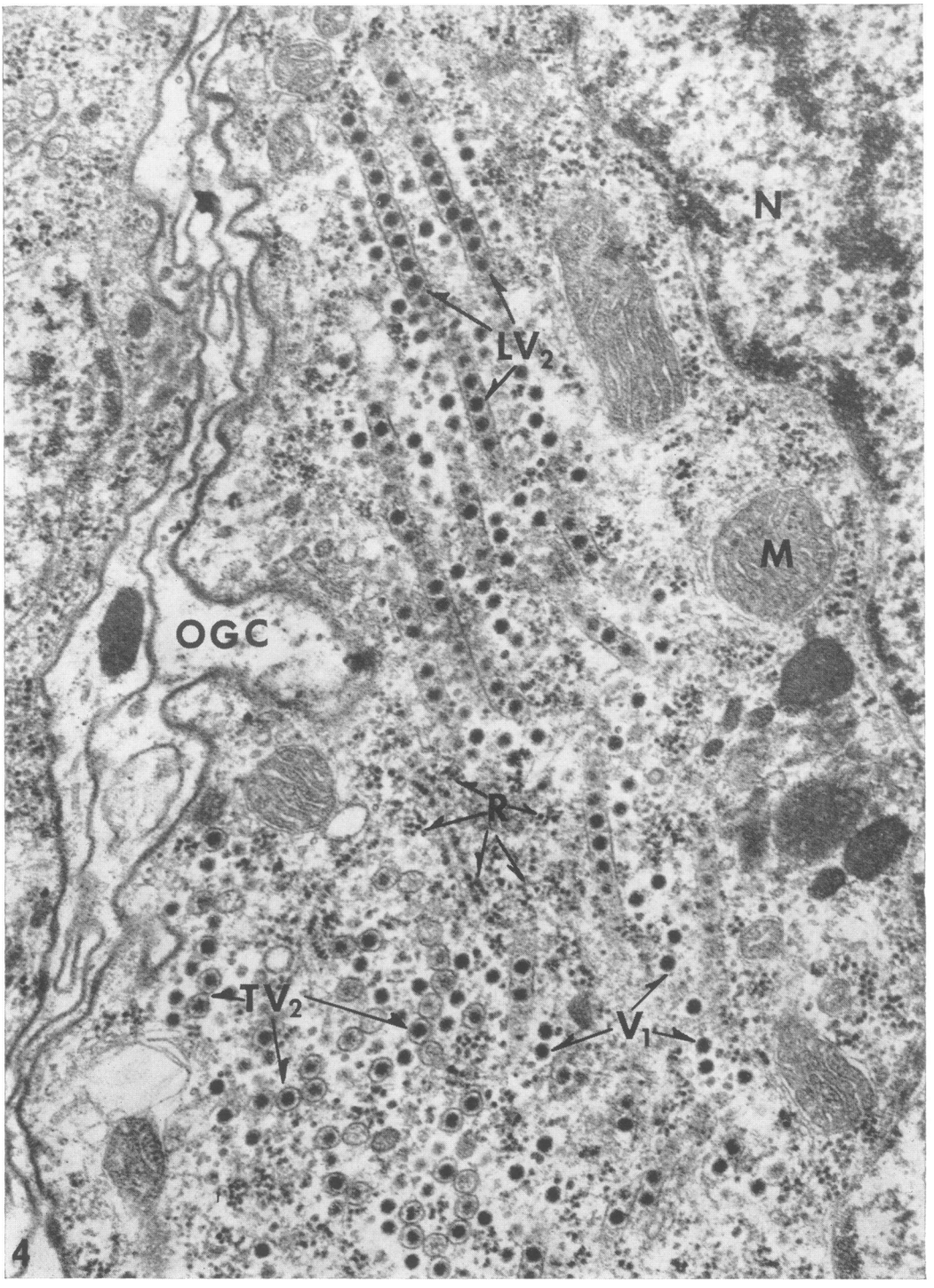
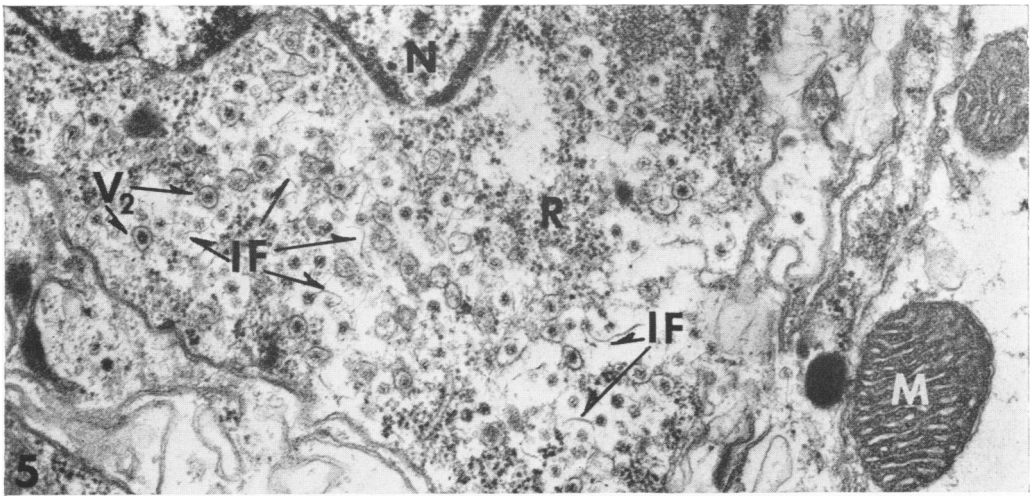
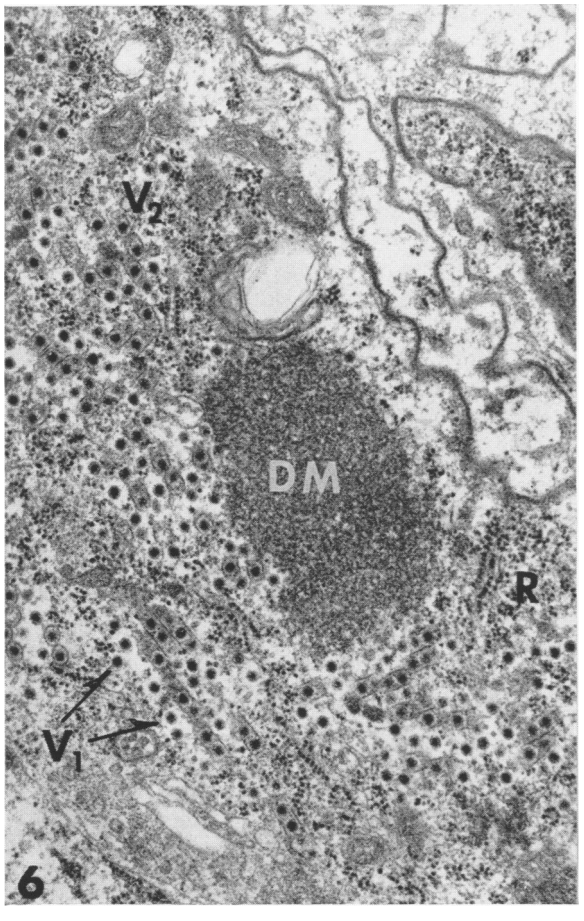


FIG. 4. *Portion of an infected subesophageal ganglion cell. Isolated, free virions (V_1) and virions in tubular formation were surrounded by numerous ribosomes (R). M = mitochondria, N = nucleus of the ganglion cell, OGC = outer glial cell, LV_2 = longitudinal sections, TV_2 = transversal sections.* × *49,000.*





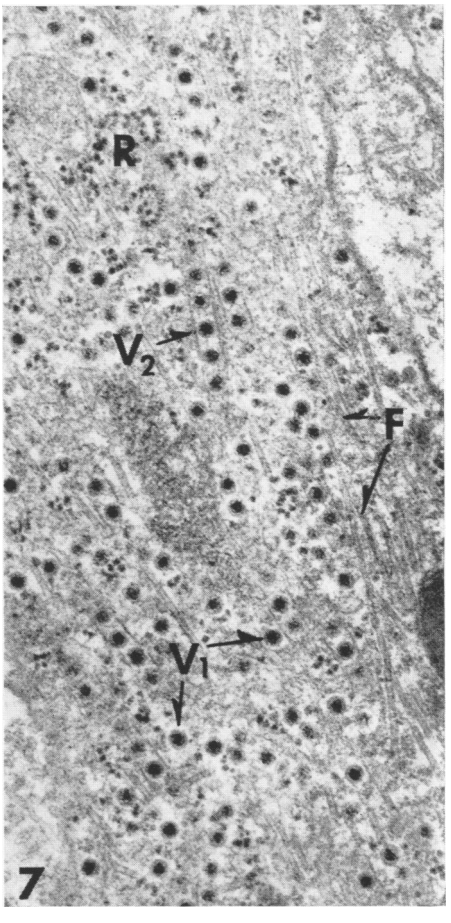


FIG. 5. *Wound tumor virions in a protocerebrum cell. The core and capsid of some virions surrounded with irregular filamentous membranes (IF) of the tubular form (V_2) were not clearly distinguishable. M = mitochondrion, N = nucleus of the ganglion cell, R = ribosomes.* × *35,000.*

FIG. 6. *Electron-dense matrix area (DM) surrounded by wound tumor virions, either isolated (V_1) or in tubular formations (V_2), in the cytoplasm of a subesophageal ganglion cell. Numerous ribosomes (R) were seen in this area.* × *30,000.*

FIG. 7. *Prothoracic ganglion cell. Note fine filamentous structures (F) among the wound tumor virions in the isolated (V_1) and tubular (V_2) formations. R = ribosomes.* × *42,000.*

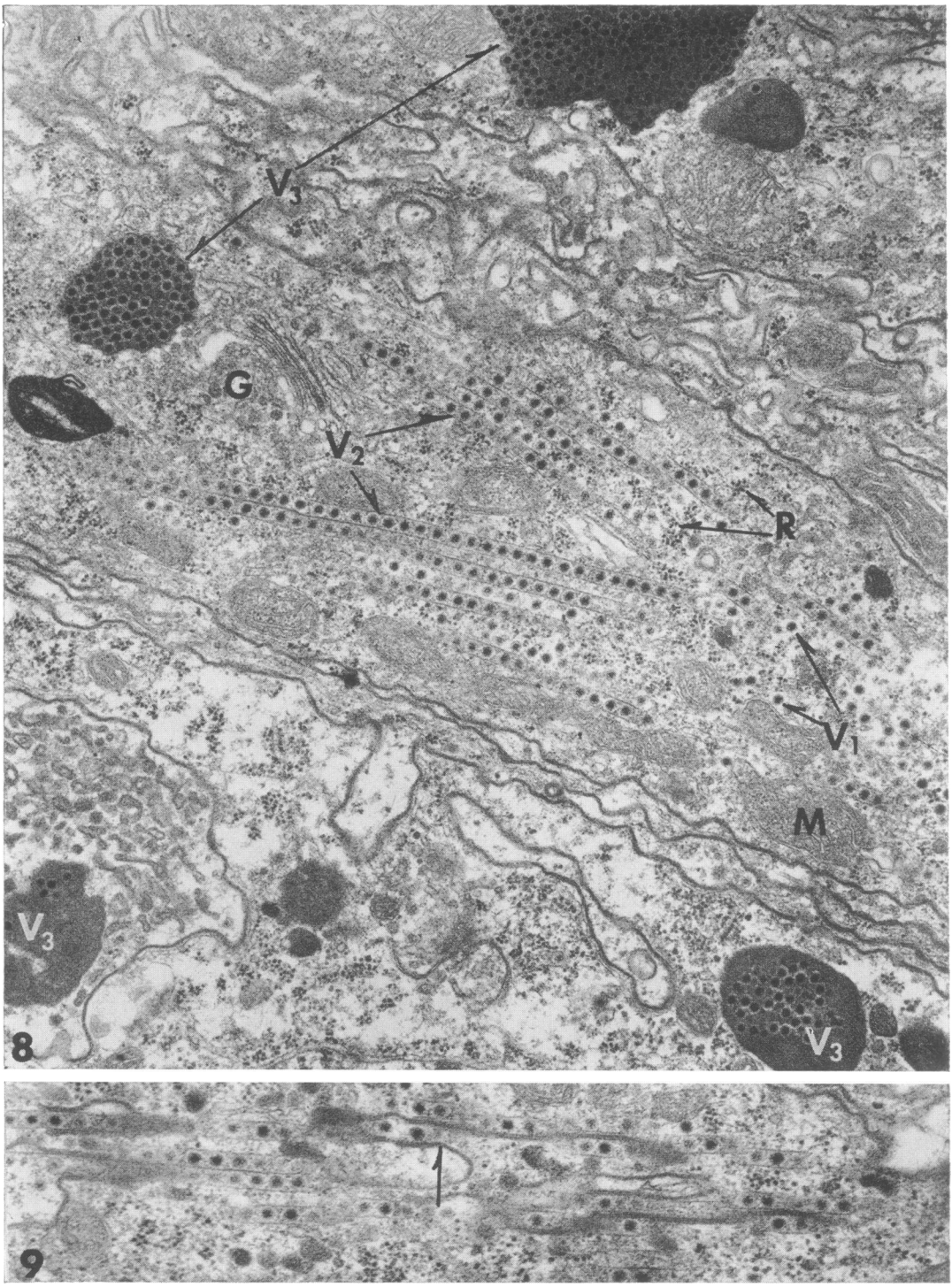
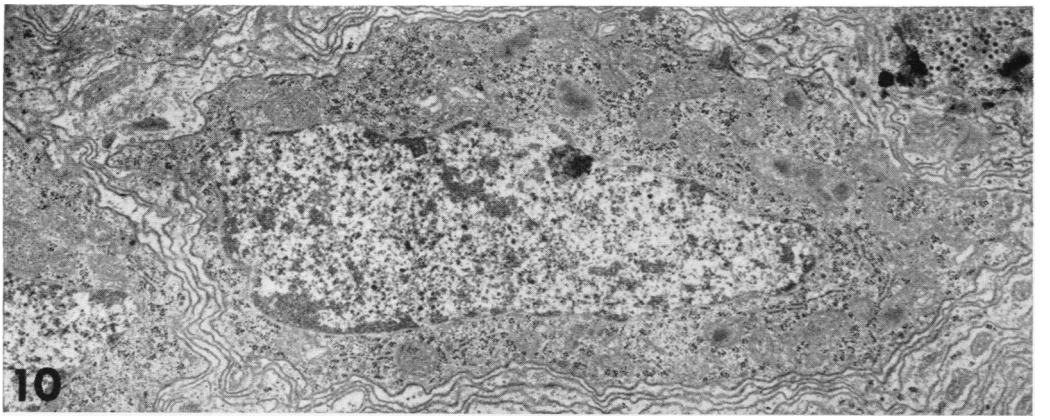
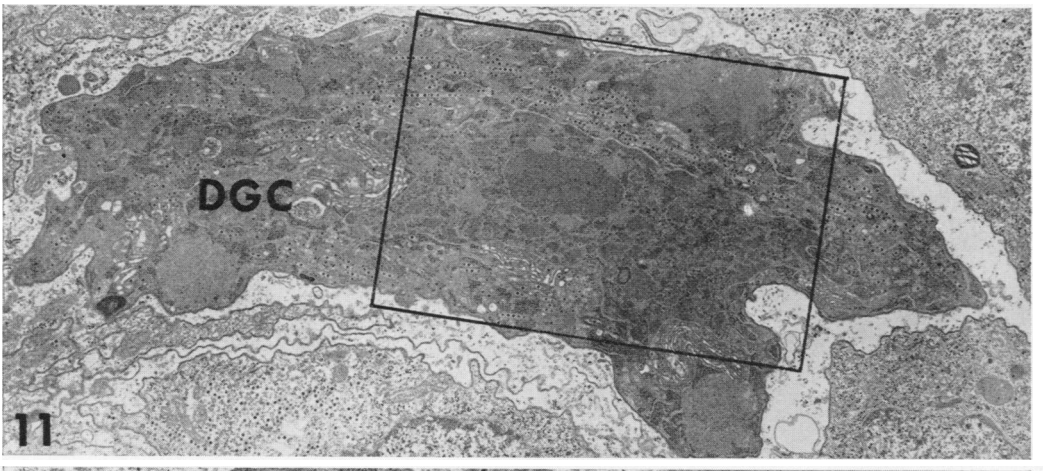


FIG. 8. *Cytoplasm of prothoracic ganglion cells with isolated, free virions (V_1), the long tubular form (V_2), and the aggregated form (V_3). G = Golgi apparatus, M = mitochondrion, R = ribosomes.* × *30,000.*

FIG. 9. *Outer membranes (arrow) enclosing the tubular structures with wound tumor virions in a tritocerebrum, cell.* × *35,000.*





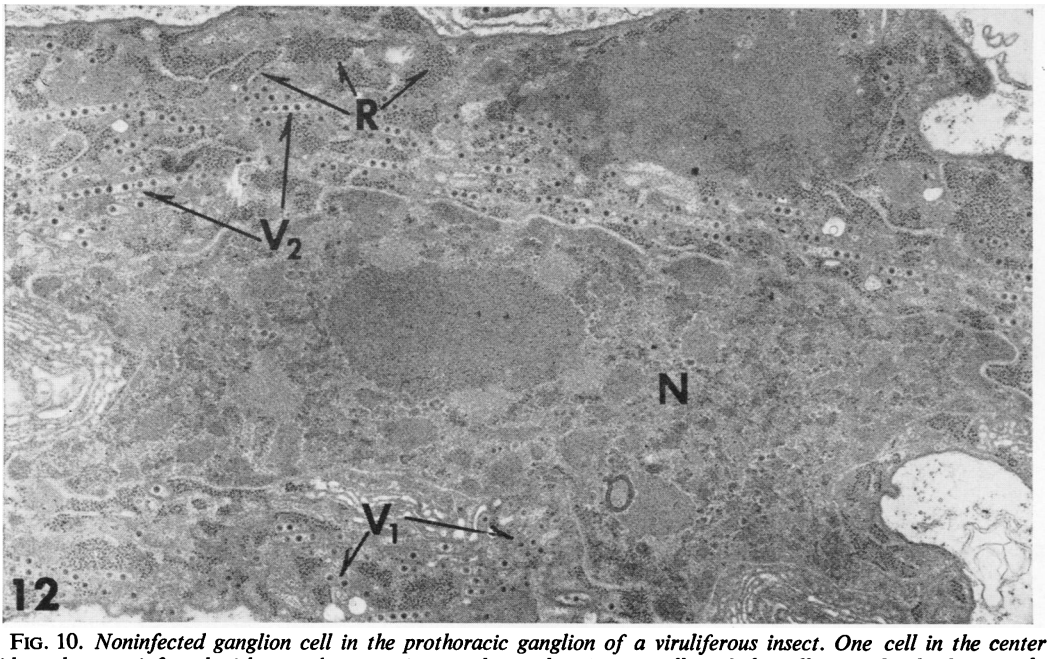


FIG. 10. *Noninfected ganglion cell in the prothoracic ganglion of a viruliferous insect. One cell in the center did not become infected with wound tumor virus, and cytoplasmic organelles of the cell were clearly distinguishable.* × *14,400.*

FIG. 11. *Degenerating ganglion cell (DGC) surrounded by an outer glial cell in the prothoracic ganglion.* × *8,400.*

FIG. 12. *Portion of the degenerating ganglion cell shown in Fig. 11. Wound tumor virus in the isolated, free virions* (V_1) *and the tubular formation* (V_2) *were seen in the cytoplasm. The cytoplasmic matrix was transformed into highly electron-dense fine granules. Membrane structures were indistinct. No mitochondria were seen. Ribosomes (R) were loosely coagulated. N = degenerated nucleus.* × *18,400.*

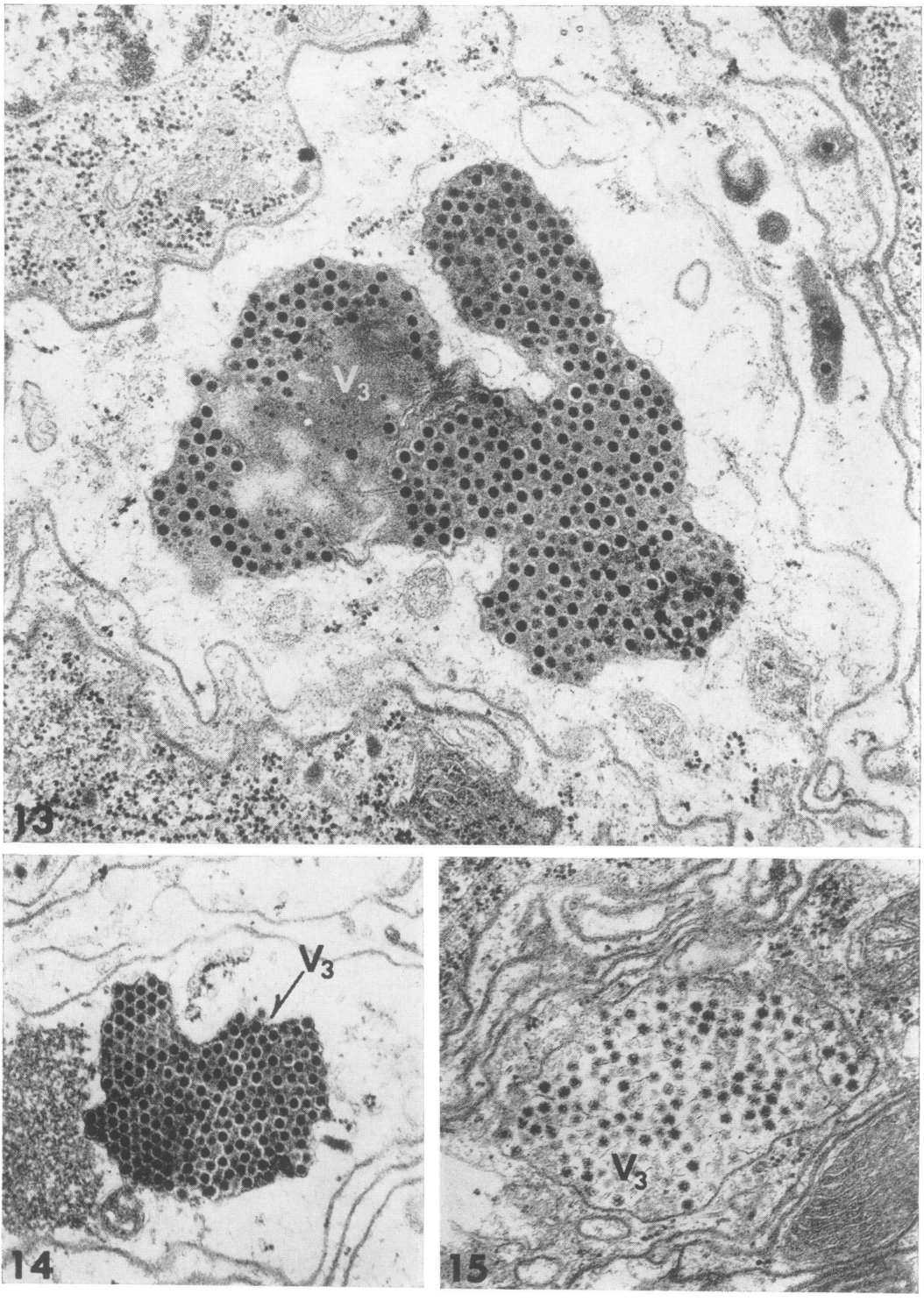


FIG. 13. *Large aggregated formation (V_3) of wound tumor virus in the cytoplasm of an outer glial cell in the prothoracic ganglion. Note sparse cytoplasm in cytoplasmic organelles.* × *42,000.*

FIG. 14. *Crystalline arrangement of wound tumor virions (V_3) in a tritocerebrum cell.* × *32,500.*

FIG. 15. *Wound tumor virions of the aggregated form (V_3) in a slightly electron-dense inclusion body in a prothoracic ganglion.* × *35,800.*

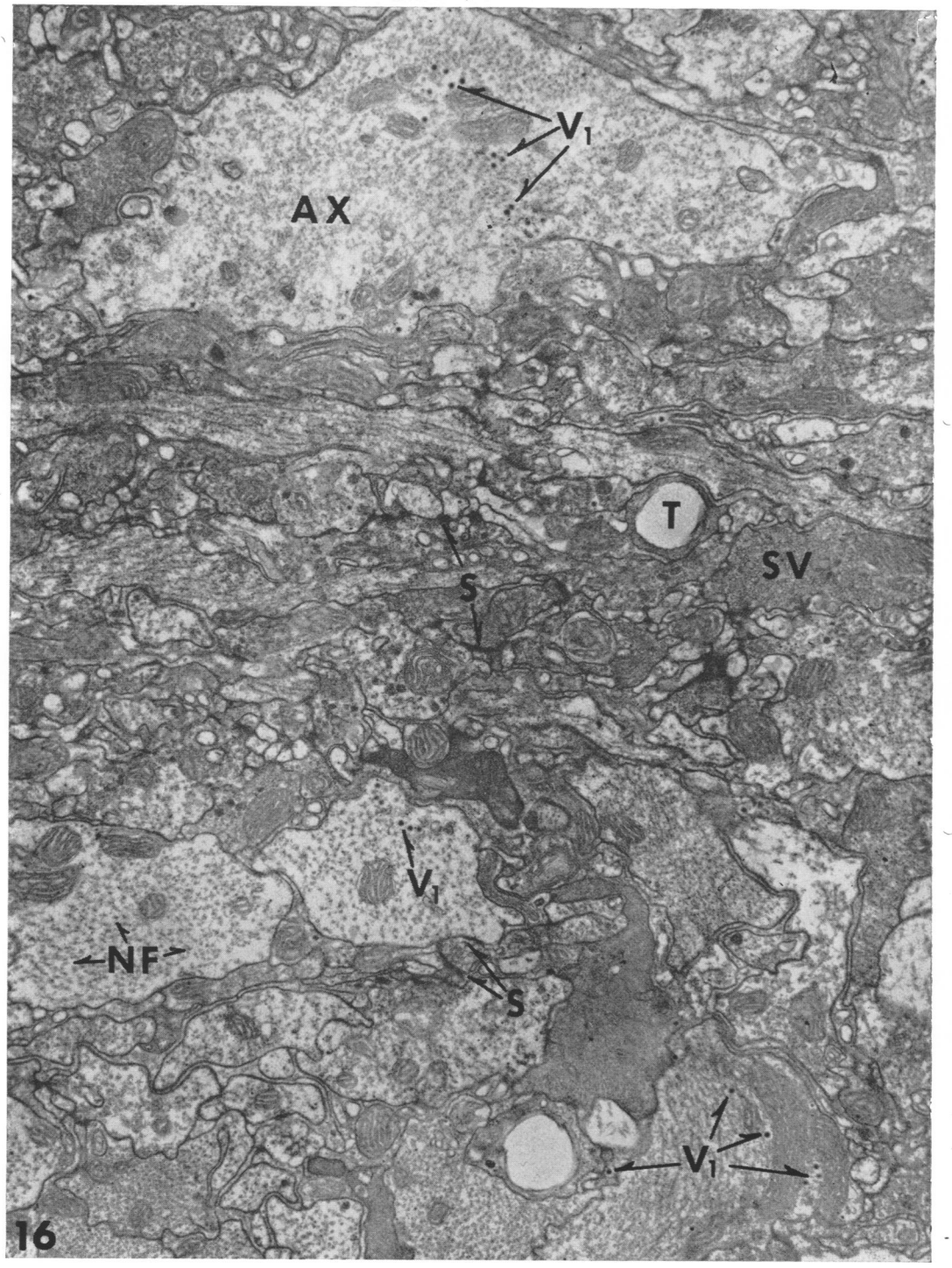
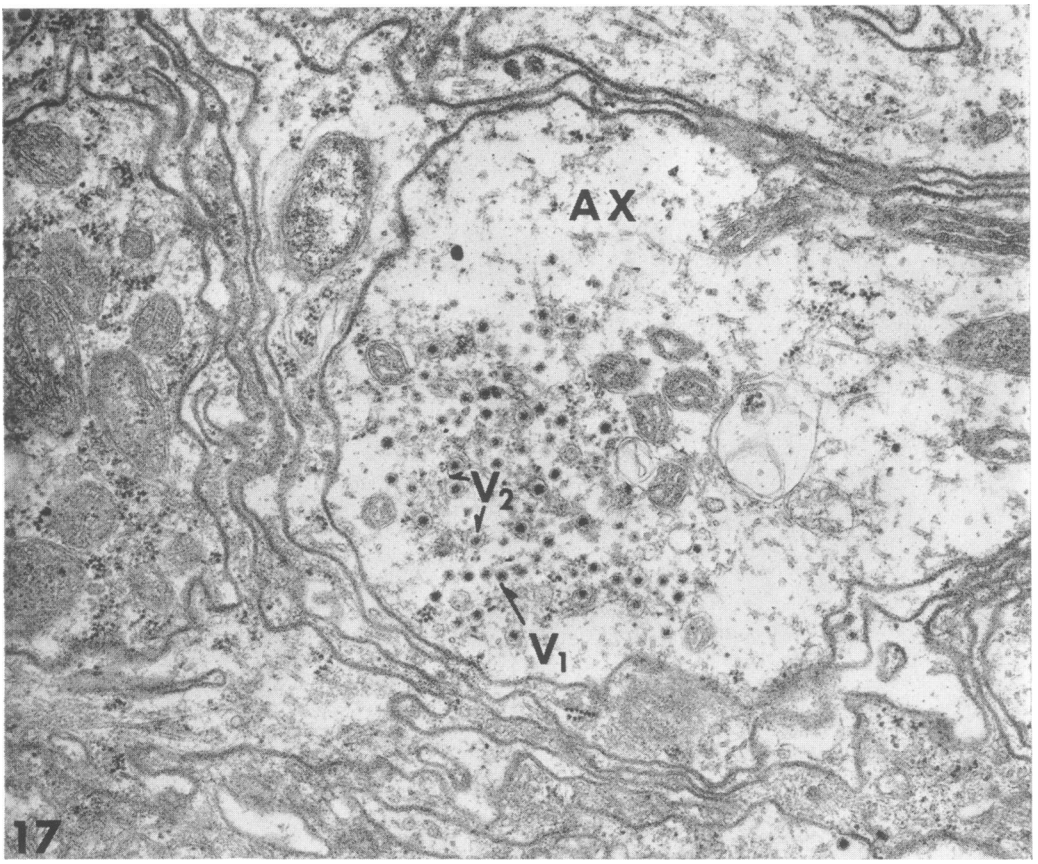


FIG. 16. *Portion of neuropile in the prothoracic ganglion. Isolated, free virions (V_1) were distributed in the axoplasms of either large or small axons. AX = axon, S = synapse, SV = synaptic vesicle, T = trachea, NF = neurofilament.* × *16,500.*



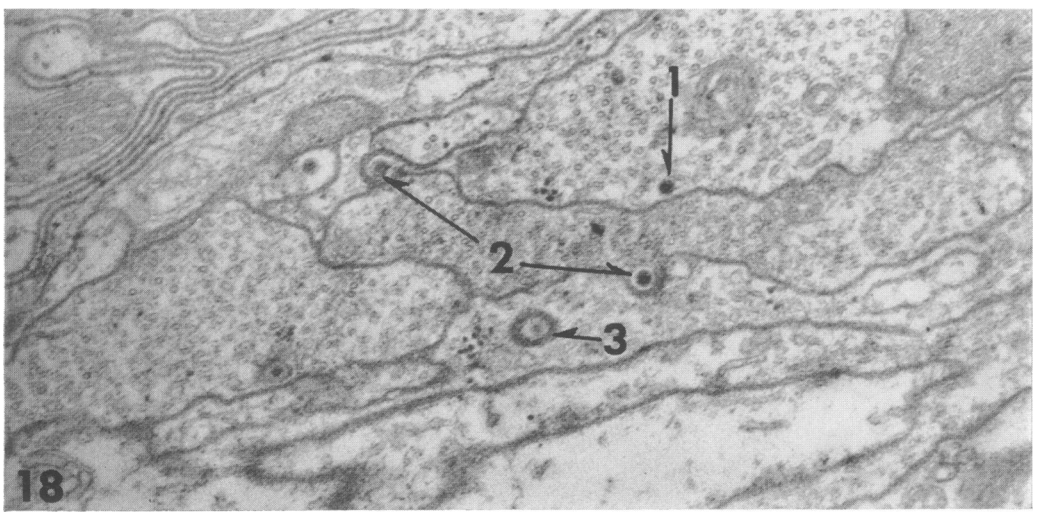


FIG. 17. *Wound tumor virus as isolated, free virions (V_1) and in the tubular formation (V_2) in a large axon (AX) in the subesophageal ganglion.* × *30,000.*

FIG. 18. *Isolated, free virions in small axons in the prothoracic ganglion; 1 = a single virion attached to the plasma membrane, 2 = virions in the invaginations of the plasma membranes, 3 = a virion enclosed with plasma membrane-like envelope.* × *42,000.*

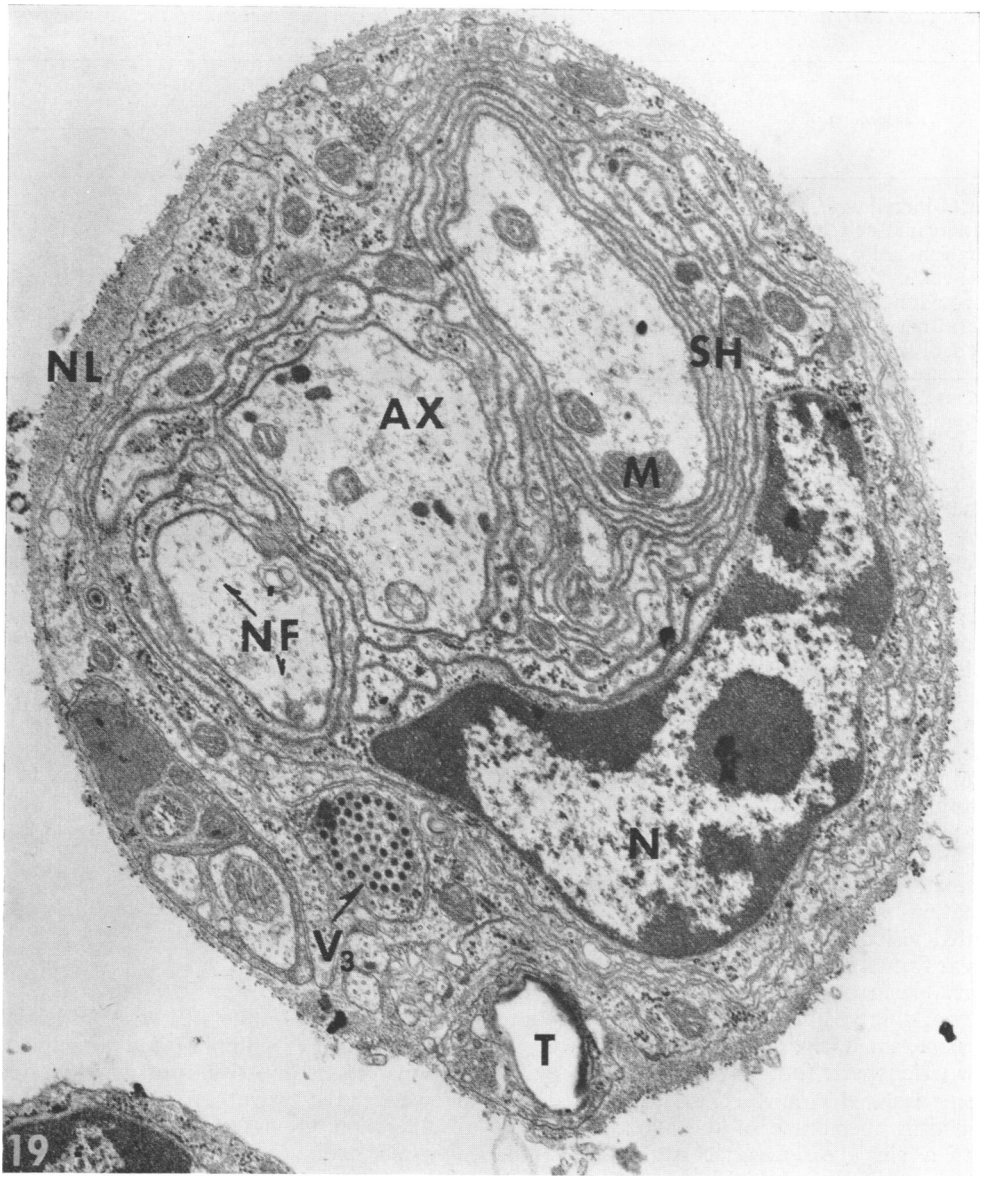


FIG. 19. *Transverse section of a lateral nerve. Aggregated formation (V_3) was seen in the perineurium cell. AX = axon, M = mitochondrion, N = nucleus of a sheath cell, NF = neurofilament, NL = neural lamella SH = spiral sheath, T = trachea.* × *30,000.*

were clearly distinguishable, and no membrane structure of the tubular sheath was observed. In some inclusion bodies, the virions were tightly packed in crystalline arrangement (Fig. 14). On rare occasions, very highly electron-dense inclusion bodies were seen. Some other types of loosely constructed inclusion bodies were composed of a substance of comparatively low electron density, and some virions were enveloped with the membrane of the tubular sheath. Figure 15 shows irregular filamentous structures among the virions in an inclusion body of low electron density. Usually, the core and the capsid of each virion that appeared with filamentous structures were not clearly distinguished. These inclusion bodies sometimes had more loosely scattered virions with

TABLE 1. *Occurrence of three patterns of WTV distribution in various regions of the nervous system of Agallia constricta and Agalliopsis novella, 6 weeks after virus acquisition*

Virus location	*A. constricta* distribution patterns[b]			*A. novella*[a] distribution patterns		
	V_1	V_2	V_3	V_1	V_2	V_3
Neural lamella	−	−	−	−	−	−
Perineurium cell	−	−	+	−	−	+++
Ganglion cell	+++	+++	+	−	−	−
Glial cell	+	+	++	−	+	+
Nerve axon	+	+	+	−	−	−
Lateral nerve	+	−	+	N	N	N
Tracheoblast	−	−	+	−	−	+

[a] Data from Granados, Hirumi, and Maramorosch (*in press*).

[b] Symbols: −, not observed; +, occasionally observed; ++, often observed; +++, very often observed; N, not yet established. V_1, isolated, free virus; V_2, tubular formation; V_3, aggregate formation.

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filamentous structures, and they were enclosed by an incomplete limiting membrane.

Very few of the isolated virions (V_1) and tubular formations (V_2) were found surrounding the electron-dense fibrous matrix areas in the cytoplasm of the glial cells. Although the cytoplasm of the glial cells was sparse in ribosomes (Fig. 13), slightly higher accumulations of ribosomes were observed near the matrix area.

Nerve axons. Wound tumor virions were seldom found in the nerve axons in the neuropile of both the brain and the ventral ganglia. Most of the virions observed in the axons were isolated and were distributed in the axoplasms of either large or small axons (Fig. 16). Virions in tubular formation were sometimes found with some isolated virions in large axons (Fig. 17). Short tubular formations were occasionally observed in the axoplasm of small axons. Large aggregates were not observed in the axons; however, on rare occasions, small dense inclusion bodies enclosing a few virions were found in large axons.

Some isolated virions seemed to pass through the plasma membrane of the axon. Figure 18 shows a single virion attached lightly to the plasma membrane, and virions protruding into invaginations of plasma membranes. Occasionally, the virions enclosed with thick membrane structures, which had almost the same thickness and electron density as the plasma membrane, were also seen in the axoplasm. Virions in the short tubular structures sheathed with the double sheaths, one an inner thin tubular structure and the other an outer thick sheath, were observed between the infected inner glial cell and the adjacent axon. The plasma membrane structures of both cells were often ruptured in those areas.

Tracheoblasts. Small inclusion bodies containing wound tumor virions were occasionally found in the cytoplasm of the tracheoblasts which had penetrated into the central nervous system.

Lateral nerves. In the lateral nerves emerging from the ventral ganglia, wound tumor virions in aggregated formations were often located in the cytoplasm of the perineurium and the sheath cells which enclosed the nerve axons in a spiral manner (Fig. 19). The isolated virions were occasionally seen in the axoplasm of the lateral nerves.

No particle similar in size and shape to WTV was found in the nervous system of noninfected *A. constricta*. The various types of virus distribution in different regions of the nervous system of *A. constricta* are summarized in Table 1 and compared with previous results obtained with *A. novella*.

DISCUSSION

It was known from earlier work in this laboratory that WTV multiplies and accumulates in various organs and tissues of its insect vectors. However, the extent of the apparent virus proliferation, the different types of virion formations in nerve cells, and the degeneration of infected ganglion cells, were unexpected findings. The cytoplasmic matrix of degenerating ganglion cells was changed to a homogeneous, electron-dense granular substance (Fig. 11 and 12). Similar cytoplasmic substances were also seen in plant cells in necrotic areas of WTV-induced tumors (20). No direct deleterious effects or behavioral disorders were observed in virus-transmitting *A. constricta*, but it has to be emphasized that no precise methods were employed to detect such changes. Although it has not been established whether the virus causes a disease *sensu strictu* of the insect vector, these degenerating ganglion cells strongly indicate that

WTV exerts an intensive cytopathogenic effect on the nerve cells of its invertebrate host and vector.

Anatomical and histological features of the nervous system of *A. constricta* were basically similar to the previous reports concerning the nervous system of the same (8) and other species (11).

The size and shape of WTV observed in the nervous system corresponded to previous findings in purified materials of WTV (1, 2), in various organs, such as fat-body tissues, mycetomes, guts, muscles, salivary glands, Malpighian tubules, and tracheoblast, of WTV-infected *A. constricta* (19, 22), of *A. novella* (Granados et al., *in press*), and in WTV-infected plants (20).

The matrix areas of dense fibrous material surrounded by isolated wound tumor virions observed in this study were similar to areas which were considered as foci of the viroplasm of some animal viruses, such as poxvirus (5, 10, 15), reovirus (17), and poliovirus (4). This finding strongly suggests that WTV may also multiply within or at the periphery of the dense matrix area, and that the isolated virions surrounded with polyribosomes, mostly observed in the ganglion cells, would be an early active stage of virus multiplication. If this assumption is correct, it further implies that the cytoplasm of ganglion cells may contain sites of WTV multiplication in the leafhopper vector, *A. constricta*. It should be emphasized that the central nervous system is not the only region supporting WTV multiplication inside the insect body, since similar dense fibrous matrix areas were also found in the cytoplasm of other organs of the same vector and in plants infected with WTV (Shikata and Maramorosch, Virology, *in press*).

The aggregated formation (V_3), usually found in glial cells of *A. constricta* and in the perineurium cells of *A. novella* (Granados et al., *in press*), was usually found without specific connection with the matrix area or polyribosomes. It is therefore assumed that the aggregated formation may not be an active stage of virus multiplication, but one of virus storage.

The WTV present in the brain resembled that found in ventral ganglia of *A. constricta*. However, there were conspicuous differences in the distribution of virus among the ganglion cells, glial cells, perineurium cells, and nerve axons (Table 1). Wound tumor virions were most frequently found in the cytoplasm of the ganglion cells, secondarily in the glial cells, and occasionally in the perineurium cells and the nerve axons. In *A. novella*, on the other hand, the virions were mostly found in perineurium cells and very seldom in other regions of the nervous system. Three different types of WTV distribution described in this study were found with varying frequency in different cell types. In the cytoplasm of ganglion cells, the wound tumor virions were observed most frequently as isolated, free virions (V_1), or in the tubular formation (V_2), and occasionally in an aggregated formation (V_3). In the glial and perineurium cells, the virions generally occurred in aggregates and very seldom as isolated virions or in tubular formation. These results strengthen previous conclusions (Granados et al., *in press*) that *A. novella* does not support WTV multiplication to the same extent as does *A. constricta*.

The tubular structure observed in this study has also been reported in other organs of *A. constricta* (19), *A. novella* (Granados et al., *in press*), and in plants (20) infected by WTV. Similar tubular structures were also found in both insect and plant tissues infected with several other plant-pathogenic viruses, such as rice dwarf virus (6, 14, 16, 18), Brazilian tomato spotted wilt virus (12), maize rough drawf virus (7, 25), and pea enation mosaic virus (21). The tubular structures of these plant-pathogenic viruses seem to be different from the tubelike structure observed in L cells infected with vaccinia virus (5). The tubular structures enclosing plant-pathogenic viruses consist of a single limiting membrane (Fig. 4 and 8), whereas the tubelike structures enclosing vaccinia virus consist of lamellae and very flat vesicles. The tubular structures seem to be common in the host cells infected by arthropod-borne plant-pathogenic viruses, but the significance and origin of these structures, as well as of the fine filamentous structures (Fig. 7, F), remain unknown. Sometimes, the tubular structures were enclosed within outer thick membranes (Fig. 9). These outer sheaths most likely originated from the plasma membrane.

Information concerning the penetration of arthropod-borne plant-pathogenic virus into host cells, and from infected cells to other cells, is very limited (16). It is suggested that one of the possible mechanisms of WTV penetration into insect host cells may be by means of phagocytosis. Virions were occasionally found within invaginations of the plasma membrane (Fig. 18). Virions were sometimes enclosed with plasma membrane-like structures around an area where plasma membranes were ruptured. Leafhopper tissue culture cells might provide a useful tool for obtaining more detailed information on WTV multiplication. Therefore, attempts are now under way to study the fate of WTV in leafhopper vector cells in vitro.

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