JOURNAL OF VIROLOGY, Aug. 1971, p. 232-241

Vol. 8, No. 2
Printed in U.S.A.

Acid Phosphatase Activity in Mouse Brain Infected with Venezuelan Equine Encephalomyelitis Virus

JORGE GARCIA-TAMAYO

Electron Microscopy Laboratory, Department of Pathology, Hospital General del Sur, Maracaibo, Venezuela

Received for publication 19 March 1971

The mode of development of Venezuelan equine encephalomyelitis virus and the activity of acid phosphatase in the central nervous system of newborn mice were investigated. Precursor particles appeared to be formed in masses of viroplasm, migrating to the membrane of the Golgi cisterns and vacuoles or to the plasma membrane and being transformed into mature viral particles by budding. Mature viral particles were also found in the lumen of the blood vessels and around the myelin sheath of axons. Increased number of Golgi complexes and depletion of polysomes were the main ultrastructural alterations of the nerve cells. Acid phosphatase activity was found to be increased in the Golgi cisterns, vacuoles, and lysosomes of nerve cells. The presence of acid phosphatase activity in the rough endoplasmic reticulum and perinuclear cisterns suggests increased production of the enzyme in the nerve cells infected with Venezuelan equine encephalomyelitis virus.

Electron microscopic studies of Venezuelan equine encephalomyelitis (VEE) virus in tissue culture cells have been reported (4, 12, 14). Ultrastructural details of isolated viral particles by shadow casting and negative staining were demonstrated (7, 13); however, the development of VEE virus in animals or humans has not been previously studied with the electron microscope.

This paper deals with an investigation on the mode of formation of VEE viral particles and on the ultrastructure and histochemistry of the central nervous system of newborn mice inoculated with a suspension of VEE virus.

MATERIALS AND METHODS

Eighteen newborn Swiss white mice [Instituto Venezolano de Investigaciones Científicas (IVIC) colony, originally from NMR Bethesda strains, Navy Medical Research Institute] were used in this experiment.

Fourteen mice were intracerebrally inoculated with 0.02 ml of a suspension of VEE virus (Goajira stain), with a titer of 10^{-9} LD_{50} per 15.09 ml., and four mice were used as controls. Five mice were decapitated at 16 and 24 hr after inoculation, and the remaining nine animals were sacrificed between 30 and 48 hr, after paralysis was evident. Samples from the brain, measuring up to 0.5 cm^3, were fixed in 3% phosphate-buffered glutaraldehide (*p*H 7.2) at 4 C. Fixation time varied from 3 hr up to 1 week with similar results. Samples were cut into 0.1 cm^3 blocks and washed in cold phosphate buffer for 24 hr. Postfixation treatment was carried out in 2% phosphate-buffered osmium tetroxide. The material was dehydrated in increasing gradients of acetone, embedded in Araldite, and polymerized at 60 C for 48 hr. Thin sections were cut with a Porter Blum MT-2 ultramicrotome, equipped with a diamond knife; placed on copper or nickel grids, with or without support film; and stained with uranyl acetate for 15 to 60 min and lead citrate for 3 to 5 min. The sections were examined in a Jem 7A electron microscope, with an accelerating voltage of 80 kv and direct magnification ranging between 8,000 and 65,000 ×.

To investigate the activity of acid phosphatase in the nervous tissue, the Gomori method was used (3). For histochemical purposes, small pieces of brain were fixed in 6.25% glutaraldehyde in 0.068 M cacodylate buffer (*p*H 7.2) at 4 C. The material was washed in cold 0.1 M cacodylate solution with 7.5 g of sucrose per ml. Frozen sections 50 μm in thickness were prepared in a cryostat. The sections were floated in cold cacodylate solution and transferred into a freshly prepared Gomori medium for acid phosphatase. Incubation was carried out at 37 C for 90 min. The Gomori medium was prepared with 20% solution of sodium beta-glycerophosphate, 0.1 M acetate buffer (*p*H 5.2), and lead acetate. Control of the histochemical reaction was performed by incubation of some sections in a Gomori medium lacking the substrate, sodium beta-glycerophosphate. After incubation, the material was washed in cold 0.1 M acetate buffer (*p*H 5.3) for 1 min, briefly treated with 2% acetic acid, and placed again in the cold solution of acetate buffer. Postfixation in 2% phosphate-buffered osmium tetroxide, dehydration, embedding, and other technical details were similar to those previously described.

RESULTS

Sections from mice sacrificed 16 to 24 hr postinoculation revealed conspicuous differences when compared with those of mice sacrificed at 30 to 48 hr. The central nervous system of mice sacrificed 16 hr postinoculation showed minimal changes. In some neurons, slight dilatation of the endoplasmic reticulum and moderate hypertrophy of the Golgi complex were noted. Occasionally, small areas of granular, chromatin-like material were seen in the cytoplasm of nerve cells. The astrocytes showed moderate swelling of their foot processes.

Viral particles. In mice sacrificed 30 to 48 hr after inoculation, cytoplasmic masses of granular material were frequently seen. Round particles, measuring 28 to 30 nm, were also seen at the periphery of the granular masses (Fig. 5); these particles were osmiophilic, their contours were irregular, and occasionally their central part showed decreased electron density (Fig. 6, 13, 16). Very often, 28- to 30-nm particles appeared to be formed by clumping and grouping of the granular chromatin-like material. Round particles were frequently seen free in the cytoplasm of the nerve cells (Figs. 5, 6, 11, 16) or attached to the membranes of the Golgi apparatus (Fig. 8, 11, 13) and cytoplasmic vacuoles (Fig. 7). Penetration of the particles into the lumen of the Golgi cisterns and vesicles (Fig. 8, 11, 13, 16) or into cytoplasmic vacuoles (Fig. 7) by budding was commonly observed. While budding, 28- to 30-nm particles become enveloped by the cytoplasmic membranes and transformed into 45-nm particles (Fig. 7, 13). Budding at the plasma membrane and the release of 45-nm particles to the extracellular space were also noted (Fig. 9). Particles measuring 45 nm in diameter were slightly oval in shape, and often the central part of the core showed decreased electron density (Fig. 10). Projections measuring approximately 10 nm in length were noted on the surface of mature viral particles located in the lumen of blood vessels or in the extracellular space (Fig. 3, 14).

Glial cells and neurons. In mice sacrificed 30 to 48 hr after inoculation, alterations of the nervous tissue were more conspicuous. Glial cells were swollen, and an increased number of glycogen particles were seen in their cytoplasm. Dilatation of the endoplasmic reticulum cisternae of the neurons was noted, and, often, nerve cells showed a decreased number of ribosomes or dispersed polysomes (Fig. 11). There were some groups of ribosomes organized with a helicoidal arrangement (Fig. 18). Marked vacuolation of some nerve cells, disruption of their membranes and organelles, clumping of ribosomes, and osmiophilic masses in the cytoplasm were also noted (Fig. 20). Mitochondriae showed rupture of cristae and, occasionally, myelin figures in their matrix. Some synapses showed polyedral or elongated vesicles with dilatation of the presynaptic end (Fig. 19). In the periphery of the myelin sheath of some axons, groups of mature viral particles were occasionally seen in a paracristalline array (Fig. 4).

Golgi complex. Conspicuous alterations of the Golgi complex were noted early in VEE virus infection of the central nervous system. In mice sacrificed 16 to 24 hr after inoculation, the Golgi complexes showed an increased number of cisternae and vesicles (Fig. 1). At 30 to 48 hr after inoculation, Golgi cisternae became tortuous with elongated projections, frequently adopting a ring-shaped appearance (Fig. 11, 12). In some of these cisternae with spherical arrangement, 28- to 30-nm particles were attached on both sides of the cisternae membranes (Fig. 12). Vesicles and vacuoles in the vicinity of the Golgi cisternae often showed precursor and mature viral particles (Fig. 11, 15).

Blood vessels. No alterations were noted in the cytoplasm of endothelial cells. Blood vessels of mice sacrificed 30 to 48 hr after inoculation showed 45-nm particles in their lumen (Fig. 3); these particles were formed by a dense core of 30 nm surrounded by a halo of 5 nm and by an envelope 10 nm in thickness (Fig. 14, 17).

Acid phosphatase. Acid phosphatase activity in control mice was present at the lysosomes and some Golgi cisternae of some nerve cells. In mice inoculated with VEE virus, increased acid phosphatase activity was found in the tortuous cisternae, vesicles, and vacuoles of the Golgi complex (Fig. 21, 22). In some cisternae and vesicles, viral particles were seen among precipitated crystals of lead acetate (Fig. 23).

There was acid phosphatase activity in the endoplasmic reticulum (Fig. 23) and perinuclear cisternae of some neurons, as well as in the Golgi area and lysosomes. In neurons with osmiophilic and granular cytoplasm, lead deposits indicating enzyme activity were extensive.

DISCUSSION

The development of VEE virus in tissue-cultured cells was described by Mussgay and Weibel (12); they proposed that virus particles are detached from the cytoplasmic membranes during the course of their maturation and then migrate through the cytoplasm to the surface membrane of the cell where they are released. I could verify and complete this proposed hypothesis with my

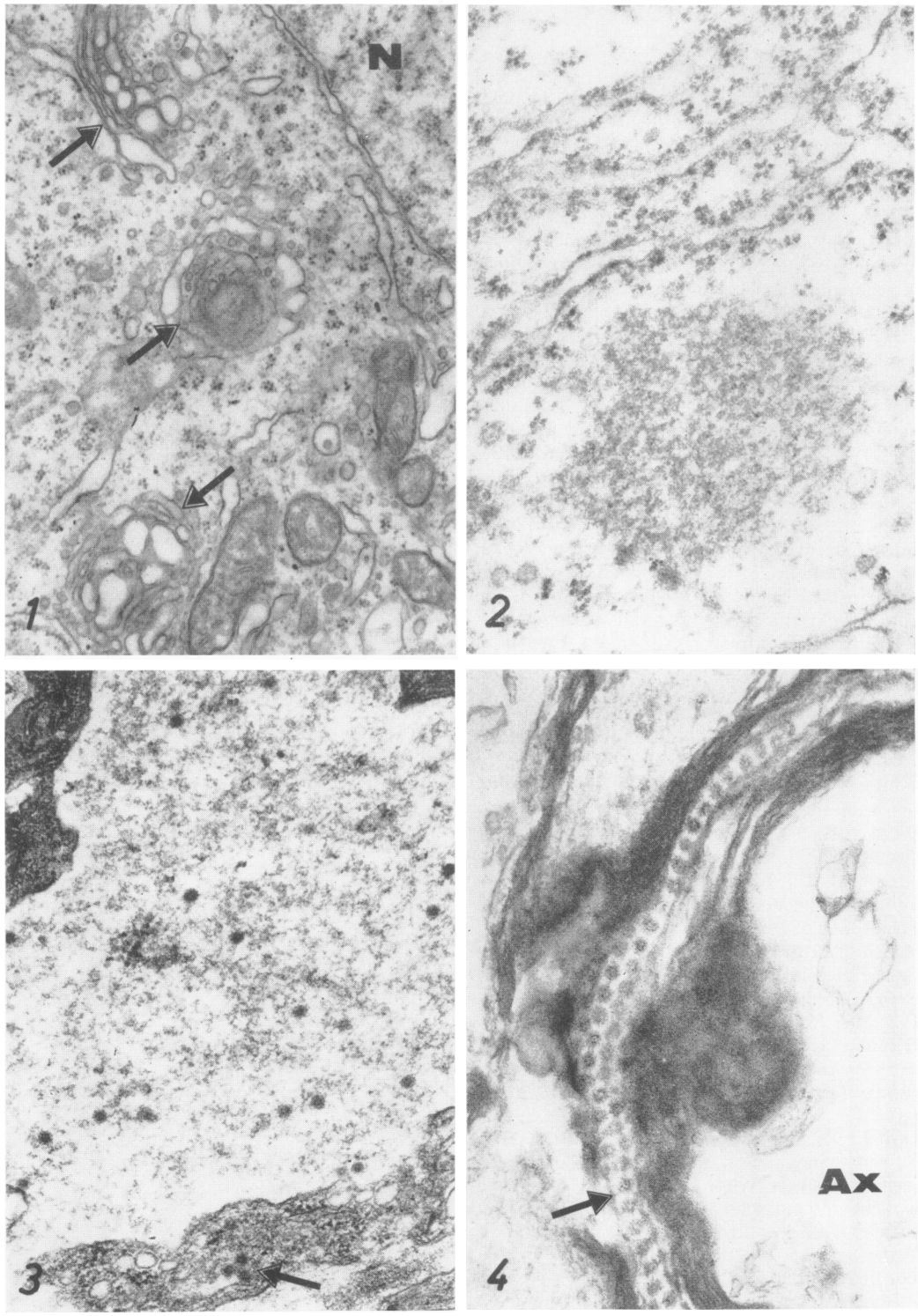


FIG. 1. *Proliferation of Golgi complex (arrows) in a nerve cell. The nucleus (N) and perinuclear cistern are noted. At 24 hr after inoculation. ×55,200.*

FIG. 2. *Aggregation of granular material in the cytoplasm. At 24 hr after inoculation. ×72,000.*

FIG. 3. *Numerous viral particles in the lumen of a blood vessel. The endothelium shows vesicles and viral particles (arrow) within a vacuole. ×50,000.*

FIG. 4. *Myelinated axon (Ax), showing VEE virus particles in the periaxonal space. ×60,000.*

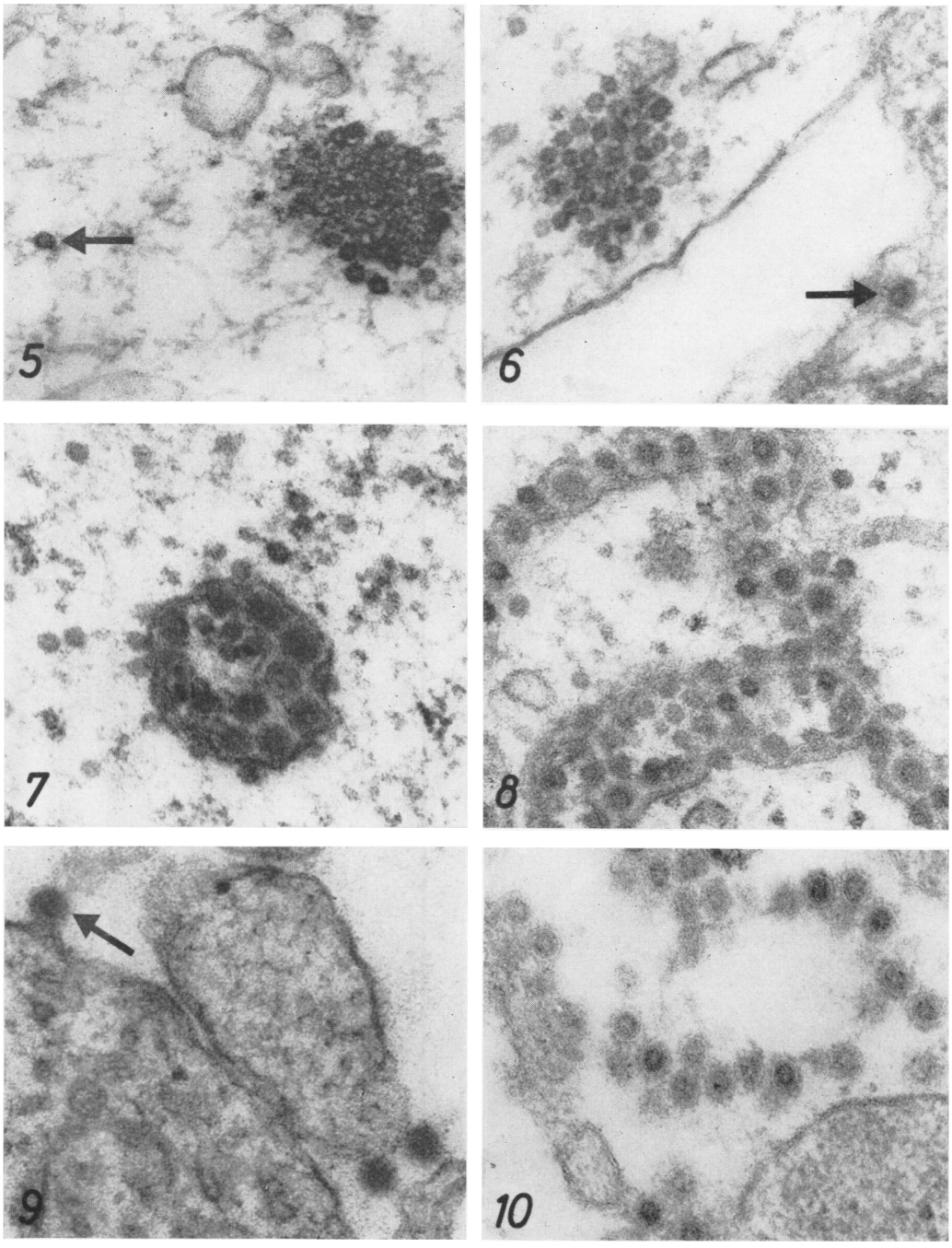


FIG. 5. *Mass of viroplasm and precursor particles (arrow).* ×*106,250.*
FIG. 6. *Precursor and mature VEE virus particles (arrow).* ×*106,250.*
FIG. 7. *Penetration of precursor particles into a cytoplasmic vacuole.* ×*106,250.*
FIG. 8. *Mature VEE virus and precursor particles in the lumen of Golgi cisternae.* ×*106,250.*
FIG. 9. *Budding of VEE virus at the plasma membrane.* ×*106,250.*
FIG. 10. *Mature VEE virus particles in the extracellular space.* ×*106,250.*

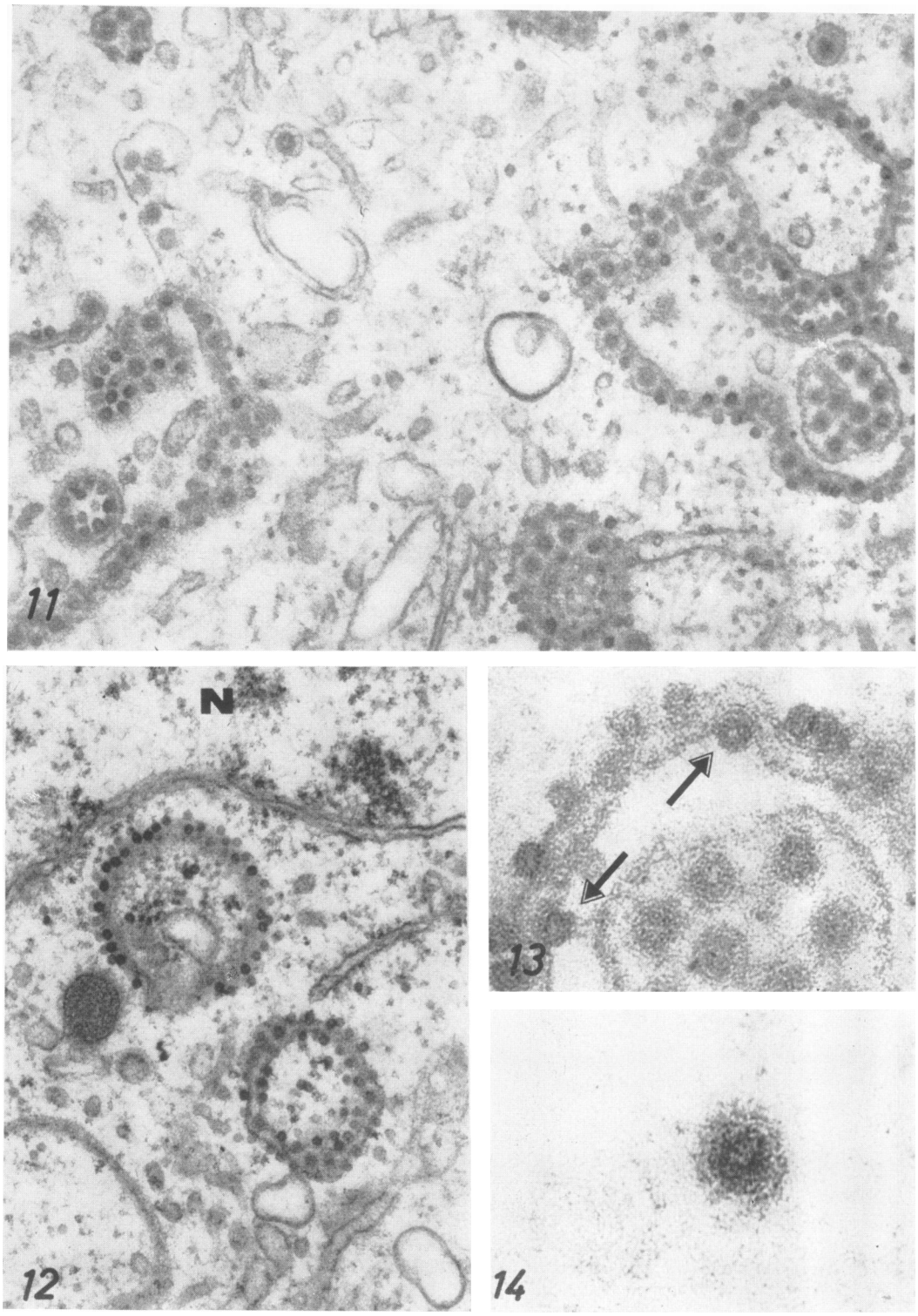
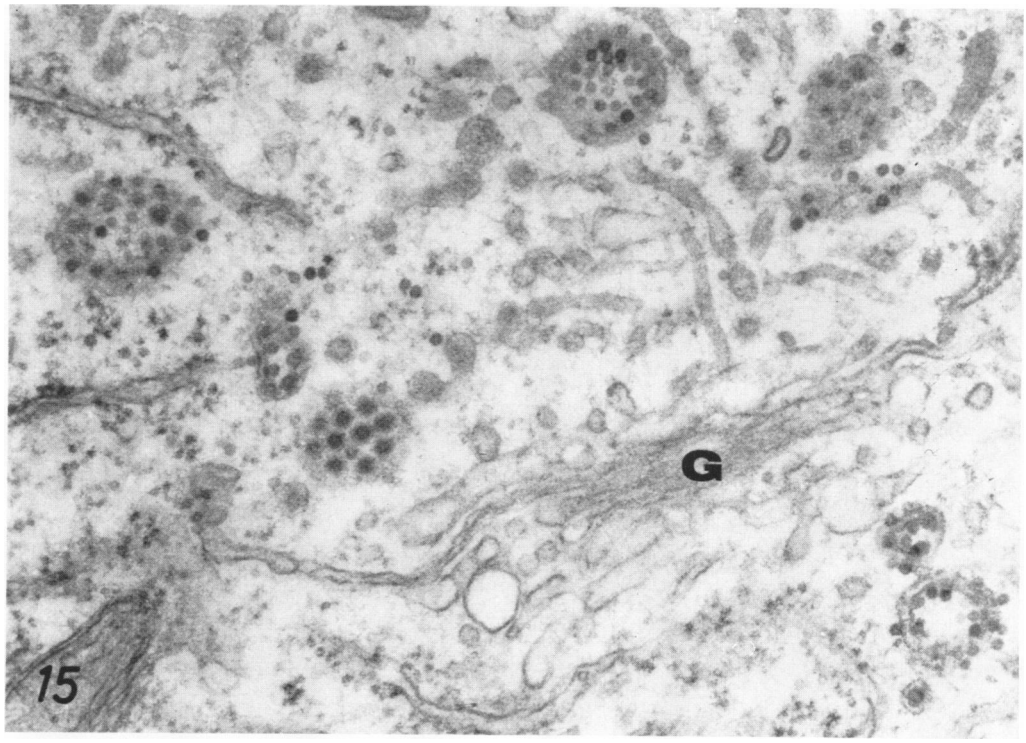


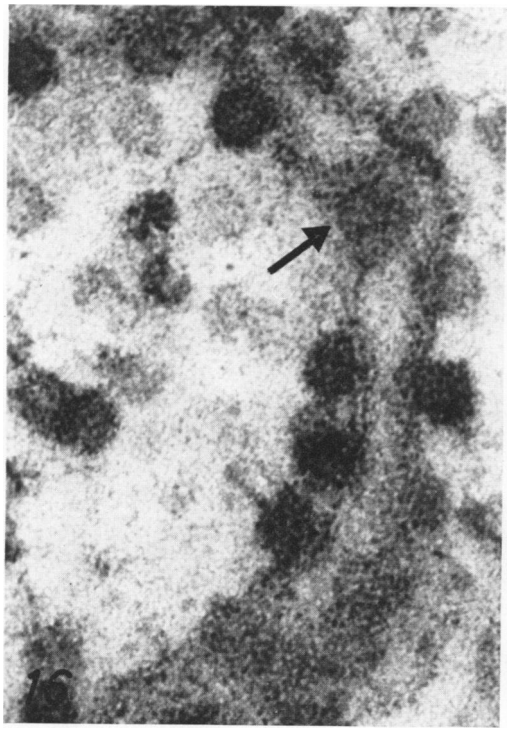
FIG. 11. *Tortuous Golgi cisternae and vacuoles with precursor and mature VEE virus particles.* ×*60,000.*

FIG. 12. *Nucleus (N) and the cytoplasm of a nerve cell with ring-shaped Golgi cisternae showing precursor particles.* ×*55,200.*

FIG. 13. *Arrows point to precursor particles budding into a Golgi cisternae.* ×*110,400.*

FIG. 14. *VEE viral particle.* ×*292,500.*





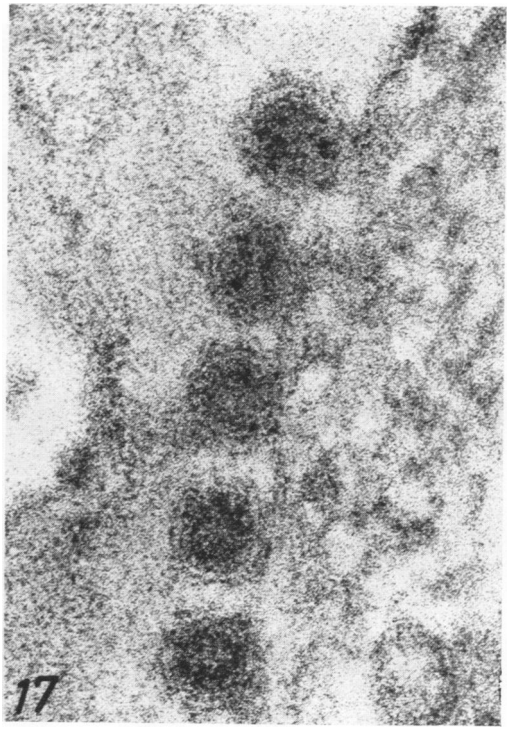
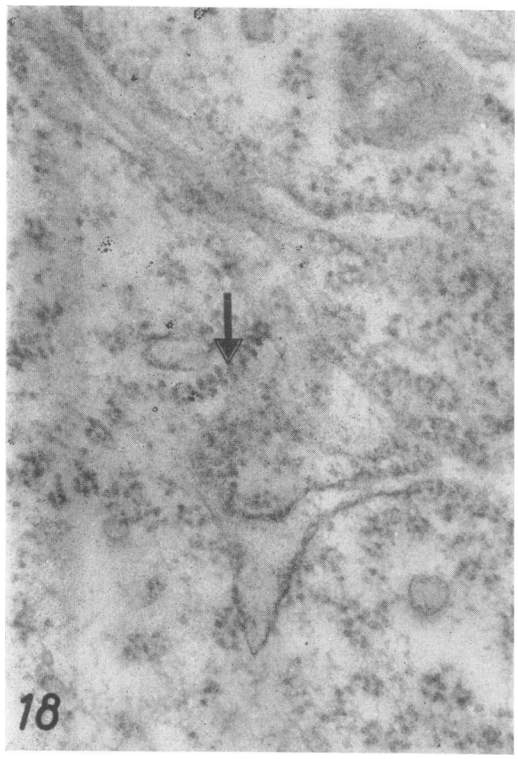
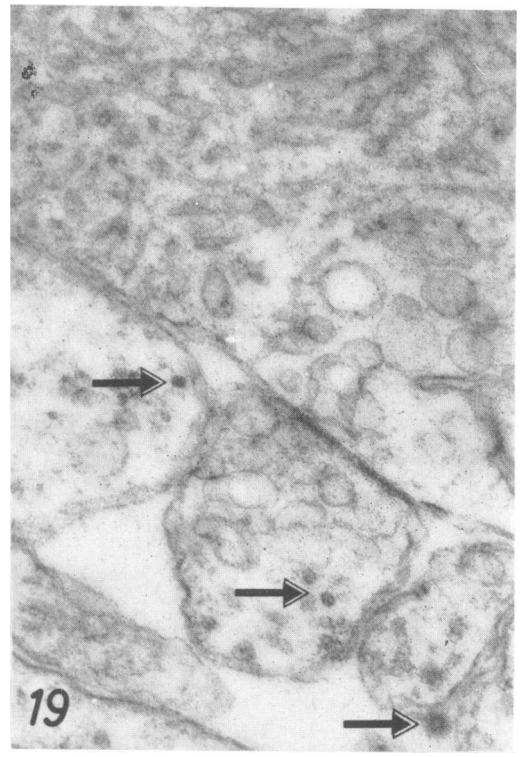


FIG. 15. *Golgi complex (G) and virus particles in the cisternae and vacuoles.* ×*60,000.*
FIG. 16. *Precursor particles and budding (arrow) into a Golgi cisternae.* ×*250,000.*
FIG. 17. *Mature VEE virus particles.* ×*250,000.*





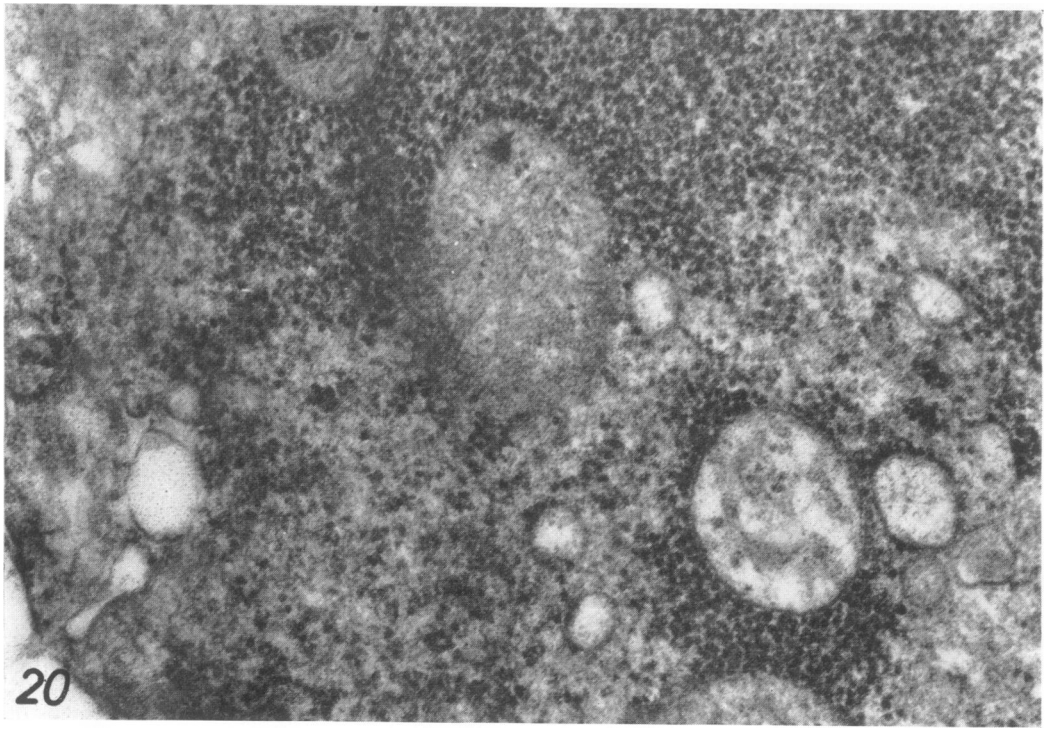


FIG. 18. *Ribosomes organized with helicoidal arrangement.* ×72,000.

FIG. 19. *Synaptic end, with dilated vesicles. The arrows point to precursor particles and to a mature VEE virus in the extracellular space.* ×60,000.

FIG. 20. *Vacuolated nerve cell with clumping of ribosomes.* ×72,000.

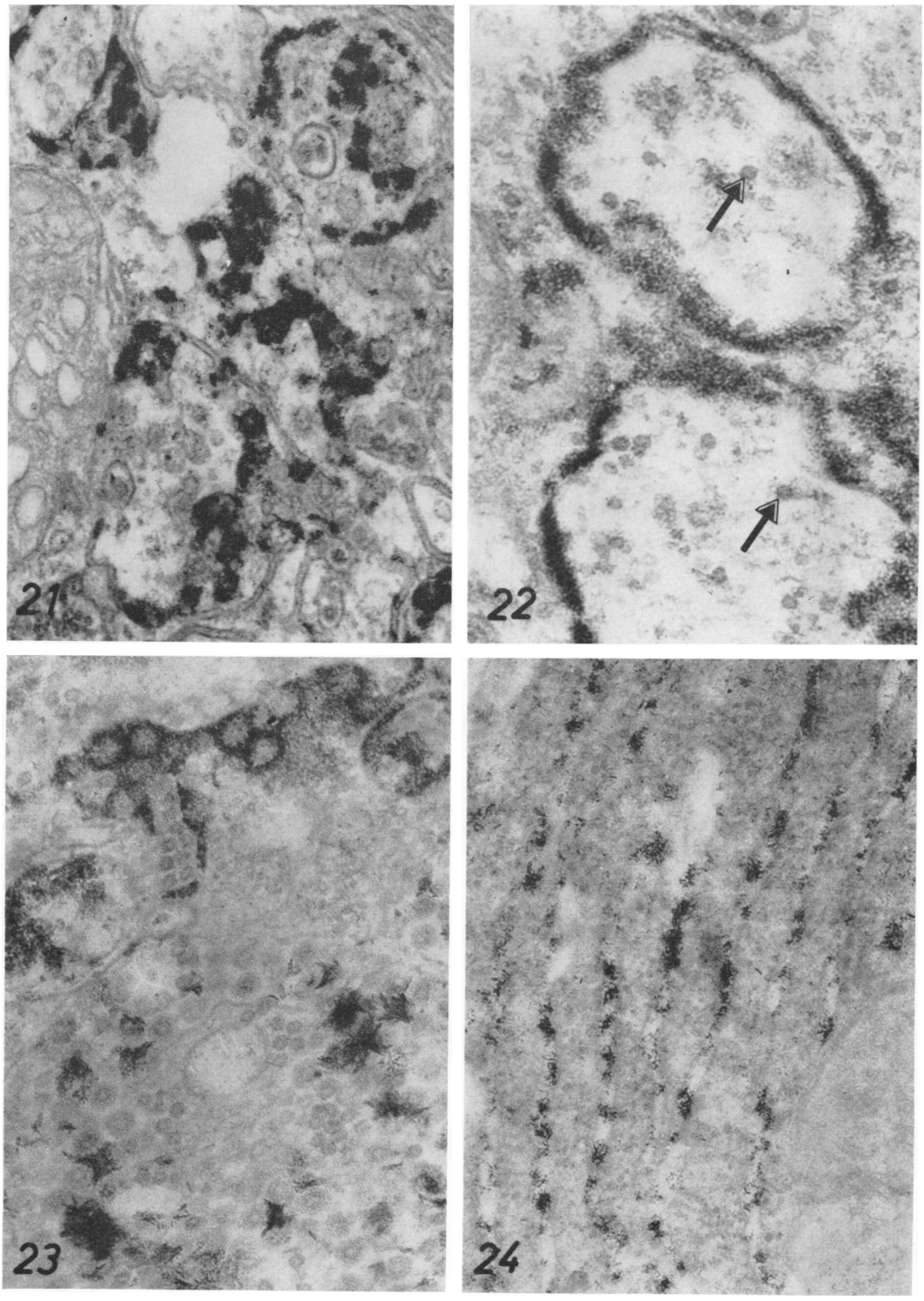


FIG. 21. *Acid phosphatase activity in the Golgi cisternae.* ×*60,000.*

FIG. 22. *Golgi cisternae showing acid phosphatase activity and some precursor particles (arrows) in the cytoplasm.* ×*106,250.*

FIG. 23. *Mature and precursor particles and acid phosphatase activity in a nerve cell.* ×*106,250.*

FIG. 24. *Acid phosphatase activity in the rough endoplasmic reticulum cisternae.* ×*60,000.*

findings in the central nervous system of newborn mice.

Furthermore, observations of nervous tissue indicated the formation of precursor particles in masses of viroplasm, their migration to the membrane of the Golgi complex, penetration of precursor particles in the lumen of Golgi cisterns, and vesicles becoming transformed into mature viral particles. Penetration of precursor particles into cytoplasmic vacuoles and budding at the cell surface are in agreement with changes previously described in other group A arbovirus infections (1, 6, 10).

Viroplasmic foci, similar to those observed in the nerve cells of VEE-inoculated newborn mice, have been rarely reported in cells infected by an arbovirus (8, 15); however, masses of viroplasm were recently described in tissue-cultured cells infected with VEE virus (4). The development of Eastern equine encephalitis virus (11) and Semliki Forest virus (5) in mouse brain has been recently described; these observations are similar to my results; in spite of this, no masses of viroplasm in the cytoplasm of nerve cells were demonstrated.

The role of the Golgi complex in the formation of VEE (14) and Aura (8) viral particles has been previously stressed; however, budding of group A arboviruses at the Golgi cisterns has only been reported in Semliki Forest virus infection of mouse brain (5). Budding of VEE virus at the tortuous cisterns, sacs, and vesicles of the Golgi area have been described; these structures also revealed acid phosphatase activity. The presence of the enzyme in the cisterns indicates that they belong to the Golgi complex and indicate a new way of intracellular maturation of group A arboviruses (5).

VEE viral particles in the lumen of blood vessels were frequently seen; I have no knowledge of a previous report of this finding in VEE or other arbovirus infections. Viral particles in the blood stream must be considered as morphological evidence for the severe viremia of VEE-inoculated newborn mice. The presence of VEE virus in the periphery of the myelin sheath of some axons is similar to the observations reported in Japanese B encephalitis virus infection of mice (16). These findings may bring into consideration a possible periaxonal route for virus dissemination in the central nervous system.

Histochemical findings demonstrated increased acid phosphatase activity in the Golgi complex and lysosomes of infected nerve cells. Previous light microscopic studies have revealed acid phosphatase activity in tissue culture cells infected with several viruses (2). Augmentation of acid hydrolytic enzymes was considered to be a defensive reaction of the cells to digest viral proteins (8). Acid phosphatase activity in the endoplasmic reticulum and perinuclear cistern of nerve cells in VEE virus infection suggested increased production of this enzyme in the ribosomes.

The presence of acid hydrolytic enzymes in the nervous tissue of VEE-inoculated newborn mice may represent a response of the lysosomal system of the nerve cells infected by the virus. It is likely that increase production of acid phosphatase may also correspond to a direct action of VEE virus on the enzymatic system of the nerve cells.

More detailed investigations of the ultrastructure and histochemistry of nervous tissue infected by VEE virus are necessary to understand the morphological and physicochemical basis of cell-virus interaction.

ACKNOWLEDGMENTS

I would like to express my gratitude to Pedro Iturbe for his encouragement and continued support during this investigation. This study could not have been accomplished without the collaboration of Gernot Bergold and the personnel from the Section of Virology and the Department of Microbiology of IVIC. The technical assistance of J. Vivas, G. Leslie, E. Murcia, and H. Prieto is gratefully acknowledged. The author is also indebted to Mevlyn Semprun and Aída B. Prieto for their assistance in typing the manuscript.

LITERATURE CITED

1. Acheson, N., and I. Tamm. 1967. Replication of Semliki Forest virus: an electron microscopic study. Virology 32: 128–143.
2. Allison, A. C., and L. Mallucci. 1965. Histochemical studies of lysosomes and lysosomal enzymes in virus infected cell cultures. J. Exp. Med. 121:463–477.
3. Barka, T., and P. J. Anderson. 1963. Histochemistry: theory, practice and bibliography, p. 238–242. Hoeber Medical Div. Harper & Row Publishers, New York.
4. Bykosvsky, A. F., F. I. Yershov, and V. M. Zhdanov. 1969. Morphogenesis of Venezuelan equine encephalomyelitis virus. J. Virol. 4:496–504.
5. Grimley, P. M., and R. M. Friedman. 1970. Development of Semliki Forest virus in mouse brain: an electron microscopic study. Exp. Mol. Pathol. 12:1–13.
6. Higashi, N., A. Matsumoto, K. Tabata, and Y. Nagamoto. 1967. Electron microscopic study of chinkungunya virus in green monkey stable (VERO) cells. Virology 33:55–69.
7. Horzinek, M., and K. Munz. 1969. Structural constituents of Venezuelan equine encephalitis virus. Arch. Gesamte Virusforsch. 27:94–108.
8. Lascano, E. F., M. I. Berría, and J. G. Barrera Oro. 1969. Morphogenesis of Aura virus. J. Virol. 4:271–282.
9. Mallucci, L., and A. C. Allison. 1965. Lysosomal enzymes in cells infected with cytopathic and non-cytopathic viruses. J. Exp. Med. 121:477–485.
10. Morgan, C., C. Howe, and H. M. Rose. 1961. Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. J. Exp Med. 113: 219–234.
11. Murphy, F. A., and S. G. Whitfield. 1970. Eastern equine encephalitis virus infection: electron microscopic studies

of mouse central nervous system. Exp. Mol. Pathol. 13: 131–146.

12. Mussgay, M., and J. Weibel. 1963. Electron microscopic and biological studies on the growth of Venezuelan equine encephalitis virus. Virology 16:52–62.
13. Mussgay, M., and J. Weibel. 1963. Electron microscopic demonstration of purified Venezuelan equine encephalomyelitis virus. Virology 19:109–112.
14. Nuñez Montiel, O., M. Mussgay, and J. Weibel. 1962. Citopatologia de la infeccion con reovirus y virus de la EEV en células cultivadas. Rev. Latinoamer. Anat. Patol. 6:1–17.
15. Southam, C. M., F. H. Shipkey, B. I. Babock, R. B. Bailey, and R. A. Erlandson. 1964. Virus biographies. I. Growth of West Nile and Guaroa viruses in tissue culture. J. Bacteriol. 88:187–199.
16. Yasusumi, C., and I. Tsubo. 1965. Analysis of the development of Japanese B encephalitis virus. II. Electron microscopic study of neurons infected with JBE virus. J. Ultrastruct. Res. 12:314–316.