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Further Studies on the Replication of Rabies and Rabies-Like Viruses in Organized Cultures of Mammalian Neural Tissues

S. MATSUMOTO, L. G. SCHNEIDER, A. KAWAI, AND T. YONEZAWA

Institute for Virus Research, Kyoto University, Kyoto, Japan, Department of Pathology, Kyoto Prefectural Medical College, Kyoto, Japan, and Federal Research Institute for Animal Virus Diseases, Tübingen, BRD, West Germany

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Organized cultures of mammalian spinal and dorsal root ganglions were used for a comparative study of the neurocytopathology caused by rabies and so-called rabies-like viruses. Electron microscopy and titration of infectivity revised earlier data in which no difference could be demonstrated between street and fixed-virus infection. In the present study, fixed virus produced inclusion bodies without apparent virus assembly. Sequential electron microscopy revealed that the main sites of virus assembly were the membranes of the Golgi complex. In contrast, rabies-like viruses freshly isolated from wild rodents produced inclusion bodies all of which were associated with virus replication. Electron microscopic evidence has led us to classify these strains as street virus. Nonneural cell elements from cultivated ganglions were susceptible to fixed virus and the cultures yielded higher titers of infectivity as compared to those of rabies-like viruses. Virus budding was shown to occur at the cell surface as well as at intracytoplasmic membranes.

It has generally been accepted that the morphogenesis of rabies virus can be divided into at least two steps: accumulation of nucleocapsids (formation of the inclusion body) followed by envelopment of the virion (budding via the cytoplasmic membrane). This process is subject to slight variation depending upon the virus strain and the type of host cell used (1, 5, 8). In a preceding paper concerned with the in vivo neuropathology of rabies infection, we described the phenomenon that, in spite of high titers of infectious virus, fixed virus particles within inclusion bodies occurred only rarely (8). Similar evidence was obtained with 1-day-old chicken embryos infected with the HEP-Flury virus (A. Kondo, personal communication). Due to the obvious technical difficulties encountered in a sequential study of virus replication in an in vivo system, we have adapted the technique using organized cultures of mammalian ganglion cells (6). It was once again shown by electron microscopy that only a small percentage of inclusion bodies was associated with virus assembly. Up until now this in vitro system has been unsatisfactory due to the low efficiency of infection as compared with nonneural cell culture systems being highly susceptible to the rabies virus. To overcome the relatively slow onset of infection in a limited percentage of neurons, improvement has been obtained by the following: (i) choice of suitable virus strains including rabies-like viruses; and (ii) technical improvement of cultural conditions for viral inoculation. The present paper compares ultrastructural aspects of the neuropathology caused by a number of rabies and rabies-like viruses.

MATERIALS AND METHODS

Viruses. Fixed virus strains used were the challenge virus standard (CVS) strain and the plaque-cloned HEP-Flury strain both adapted to BHK-21 cells. The field strains W56/71 and W131/71, isolated from *Microtus arvalis* in southern Germany, represented the so-called rabies-like viruses (13).

Cell culture. The method used for cultivation of ganglions has been described previously (6). Dorsal and spinal root ganglions from embryos of mice of 15 to 17 days in utero or of hamsters of 13 to 15 days were explanted on collagen-coated round coverslips and incubated with the same medium before and after inoculation. The culture medium consisted of equal volumes of balanced salt solution, Eagle minimal essential medium, calf serum, and saline extract of 9-day-old chicken embryos supplemented with glucose to a final concentration of 6 mg/ml.

Virus inoculation and titration. Explant cultures were washed twice with warm balanced salt solution (BSS) and exposed to 0.05 ml of virus suspended in Eagle minimal essential medium supplemented with 2% calf serum. The virus was allowed to adsorb to the

cultures for 1 h at 37 C. After removal of the inoculum the culture was rinsed three times with BSS, followed by addition of culture medium and incubation at 37 C. Samples for infectivity assay were taken at 3, 6, 12, 24, 48, and 72 h postinfection (hpi). The cell culture supernatant fluid was assayed for the presence of free virus. Cell-associated virus was obtained by suspending two ganglion explants from one culture in 1 ml of BSS containing 2% calf serum and sonicating the suspension for 1 min at 0 C. Cell-associated and free virus were assayed by plaque formation on chicken embryo fibroblasts (HEP-Flury virus) and by intracerebral inoculation of suckling mice (field virus strains).

Light microscopy. Unfixed cultures were observed under the light microscope at a magnification of ×400. The direct immunofluorescent technique was used for identification of virus antigen using infected coverslip cultures. Anti-nucleoprotein (RNP) sera were prepared by immunization of rabbits with cell-derived, purified RNP (14) and labeled with fluorescein isothiocyanate (12).

Electron microscopy. Infected cultures were fixed in situ for 1 h at 4 C with 1% isotonic buffered glutaraldehyde and postfixed in 1% OsO_4 in Millonig phosphate buffer for 1 h (7). This was followed by dehydration in a graded series of alcohol, by immersion in acetone and by embedding in Vestopal W (Fa. Schchhard, Munich, West Germany). Polymerized specimens were placed at −20 C overnight, after which coverslips became ready to remove. Serial sections were cut from the bottom collagen sheet in a parallel direction to the plane of cell growth using a Reichert or Porter-Blum ultramicrotome equipped with a diamond knife. Sections were doubly stained with uranyl acetate and lead citrate, and were photographed using a Siemens 101 or Hitachi HU 11D electron microscope.

RESULTS

Cultural characteristics and kinetics of virus growth. Ganglions usually began to flatten about 24 h after explantation. The first sign of cellular proliferation was the characteristic zigzag evolvement of very fine neurofibrils after 24 h, followed by rapid fibroblast outgrowth at about 48 h. During the early stage of cultivation, individual neurons were indistinguishable among the tightly packed cells except for a few neurons flattened among the outgrowing fibroblasts. Nonneural cells and glial elements, mainly Schwann and satellite cells, were fully developed after 9–14 days, and the majority of neurons could be identified by their characteristic cell body, clear nucleus and nucleolus, and cell processes. Myelin sheeths appeared first in week 2 or 3. Figure 1 shows typical non-infected neurons maintained in vitro for 20 days. About 10% of the neurons were lost within the first 10 days of cultivation. The observed sequential pattern of cell growth depended on the extent of connective tissue growth within the culture.

The efficiency of the rabies virus infection

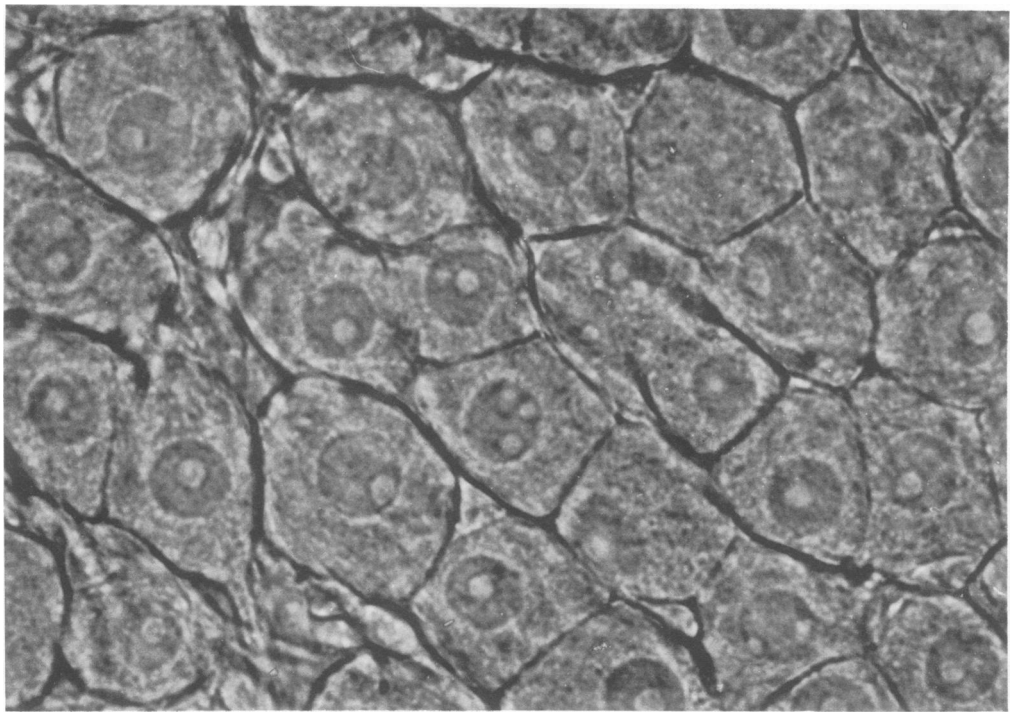

FIG. 1. *A part of unfixed cultivated dorsal root ganglion of mouse embryo. Noninfected neurons maintained in vitro for 20 days.* ×*700.*

was followed by the appearance of a cytopathic effect (CPE) and by demonstration of virus antigen in infected neurons. The typical cytopathic changes, the sequence of which has already been described (6), is shown in Fig. 2. To establish the optimal time for infection of cultivated ganglions, cultures were exposed to HEP-Flury virus at different days after explantation. The degree of CPE and the amount of virus antigen was estimated. Small ganglions from a 15-day-old mouse embryo were selected to minimize the growth of nonneural supportive cells. The results (Table 1) indicate that the efficiency of infection depends on the age of the explant. Therefore, only ganglions cultivated for 2 to 3 days have been used throughout the subsequent experiments.

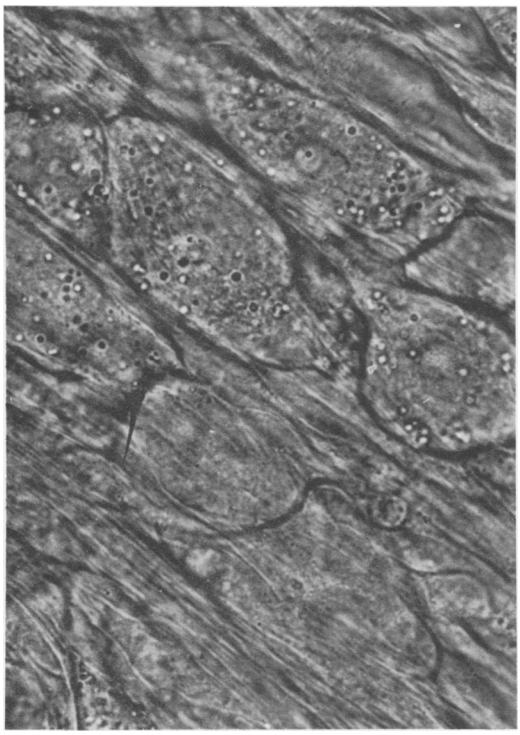

FIG. 2. *Neurons 3 days after infection with rabies-like virus strain W56. Cytoplasmic granulation is seen in most neurons.* ×700.

TABLE 1. *Influence of the age of cultivated ganglions on the efficiency of infection with the HEP-Flury strain of rabies virus*

Days between explantation and virus inoculation	CPE of neurons[a]	FA staining of virus antigen[b]
1	+++	++
5	++	++
10	±	–
14	–	–
20	–	–

[a] Cytopathic effect.
[b] Fluorescent antibody staining.

The growth cycle of a fixed (HEP-Flury) and a field strain (W56) of rabies virus in cultivated ganglion cells is shown in Fig. 3 and 4. It was first shown that a HEP-Flury virus suspension of $10^{7.7}$ PFU/ml was titrated to $10^{7.5}$ mean lethal dose (LD_{50})/ml using suckling mice. Exponential virus growth started at 24 hpi. Cell-associated and free virus of the HEP strain (Fig. 3) reached a maximum of $10^{6.2}$ and $10^{5.3}$ PFU per culture at 72 hpi. Virus yields from the field strain W56 (Fig. 4) were considerably lower and dropped quickly after 72 hpi.

Morphological findings. Light microscopy of ganglion explants infected with different rabies virus strains showed a similar development of the characteristic neuronal CPE. Cytoplasmic granulation appeared at 48 hpi in many neurons located at the periphery of explants. The subsequent degenerative changes took place as described before (6).

By electron microscopy thin sections of ganglion explants infected with four rabies strains, namely W56, W131, CVS, and HEP-Flury were compared. Specimens harvested at the time of maximal CPE (72 hpi) exhibited a varying degree of degeneration within most neurons (Fig. 5–7). The sequence of cytopathic changes can be summarized as follows. Numerous elec-

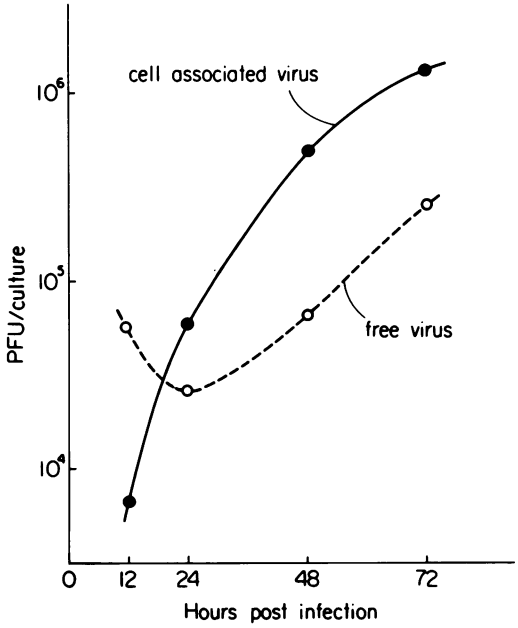

FIG. 3. *Growth curves of fixed rabies virus (HEP-Flury) in cultivated ganglions.* (○) *free virus;* (●) *cell associated virus.*

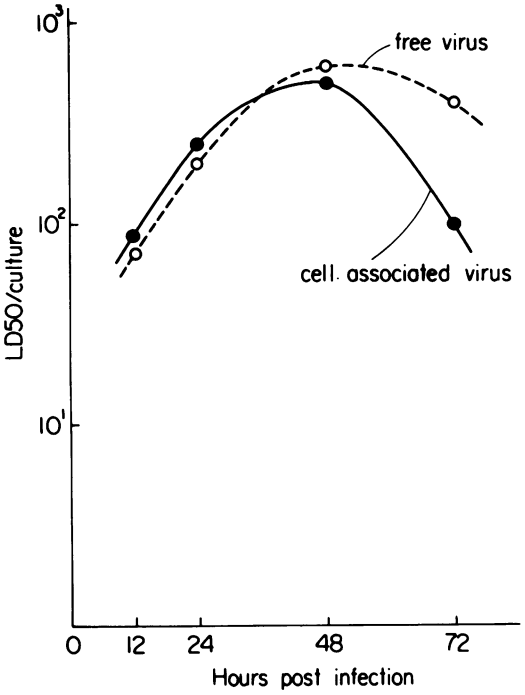

FIG. 4. *Growth curves of rabies-like virus (W56) in cultivated ganglions.* (○) *free virus;* (●) *cell-associated virus.*

tron-dense granules and lucent vacuoles of various size replaced the ground substance of the cytoplasm. The rough-surfaced endoplasmic reticulum, the number of free ribosomes, and Golgi complexes decreased, and the formation of ballooned mitochondria became obvious. Nuclear degeneration was frequently seen in heavily damaged neurons. Finally, the neurons were shrunken and became only identifiable by their complete covering with thin layers of satellite cells (Fig. 7). A small percentage of heavily necrotic neurons contained small-sized inclusion bodies but no virion. Evidence of virus replication associated with the inclusion body was found only in neurons showing slight degenerative changes (Fig. 5 and 6).

Neurons of cultivated ganglions infected with the rodent-origin field virus strains W56 and W131 constantly showed large numbers of virions adjacent to the inclusion body (Fig. 5, 6, 8). At higher magnification (Fig. 8), budding of virions from intracytoplasmic membranes into the lumen of vacuoles is clearly seen. Infection of glial cells was not observed.

Explants infected with the CVS strain examined on day 3 or 4 postinfection contained large inclusion bodies within slightly damaged or apparently intact neurons (Fig. 9). The majority of inclusion bodies did not contain virions and only occasionally was virus replication associated with inclusion bodies of the perikaryon and nerve fiber (Fig. 10). Virions were frequently seen extracellularly between the neuron and its satellite cells (Fig. 11). Figure 11 also shows virus assembly within a satellite cell. However, such neuroglial infection was not commonly encountered.

The ultrastructural features of HEP-Flury virus infection in cultivated ganglions observed from 2 to 4 days postinfection appeared similar to those seen with the CVS strain. Virus assembly, however, occurred in a somewhat different modus. Figure 12 illustrates a representative picture of the early changes at 24 hpi. Two small inclusion bodies are located at the periphery of the perikaryon where many lucent vacuoles are randomly dispersed. Few virions are seen adjacent to the inclusion bodies. At 24 to 48 hpi, numerous small-sized lucent vacuoles appeared in close relation with the Golgi complex. Virus budding occurred mainly into these vacuoles which were not associated with inclusion bodies (Fig. 13 and 14). This modus of replication progressively involved the majority of neurons by 3 days after inoculation.

Fluorescent antibody staining showed that nonneural cells, mostly fibroblasts which filled the outgrowth zone, contained massive aggregates of viral antigen in the case of HEP-Flury virus infection. Electron microscopical observations revealed that the occurrence of inclusion bodies was consistent with that of the viral antigen. Sections of nonneural cells (Fig. 14 and 15) were prepared from peripheral portions of an explant at 72 hpi. Virus budding from the intracytoplasmic and cellular membrane as well as free virions within extracellular spaces were commonly observed.

DISCUSSION

The results presented partially confirmed our previous data (6) and show more elaborately that organized cultures of mammalian neural tissue are a useful tool for the study of rabies virus and host cell interaction. The maintenance of cell topography in vivo is the main advantage of the organ-type culture but it presents difficulties in the analysis of virus infection compared with conventional monolayer cell culture systems. One disadvantage is that thick fibroblastic layers covering the original explant block virus attachment to the neurons. However, this can be overcome by the use of young explants (Table 1) which are readily infected and have proved to be useful for sequential analysis of rabies virus replication within the neuron.

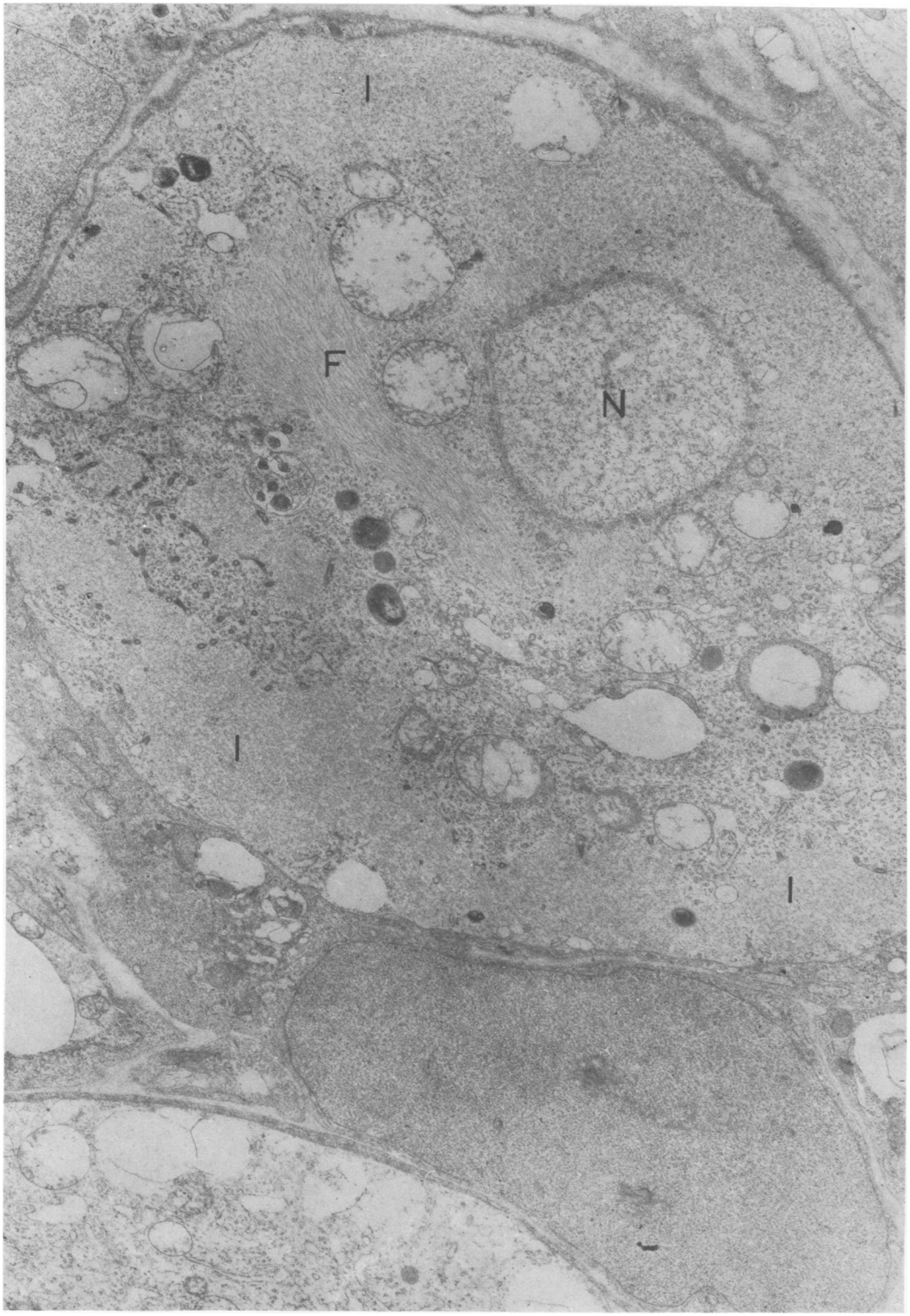

FIG. 5. *Rabies virus (W56) infected neurons. Peripheral cytoplasm of the lower neuron is almost completely replaced by inclusion bodies (I). Virions are located around inclusion bodies. Mitochondria are swollen, lucent vesicles of varying size appear sporadically. Part of a heavily degenerated neuron is seen in lower portion. (F) microfilaments. ×15,000.*

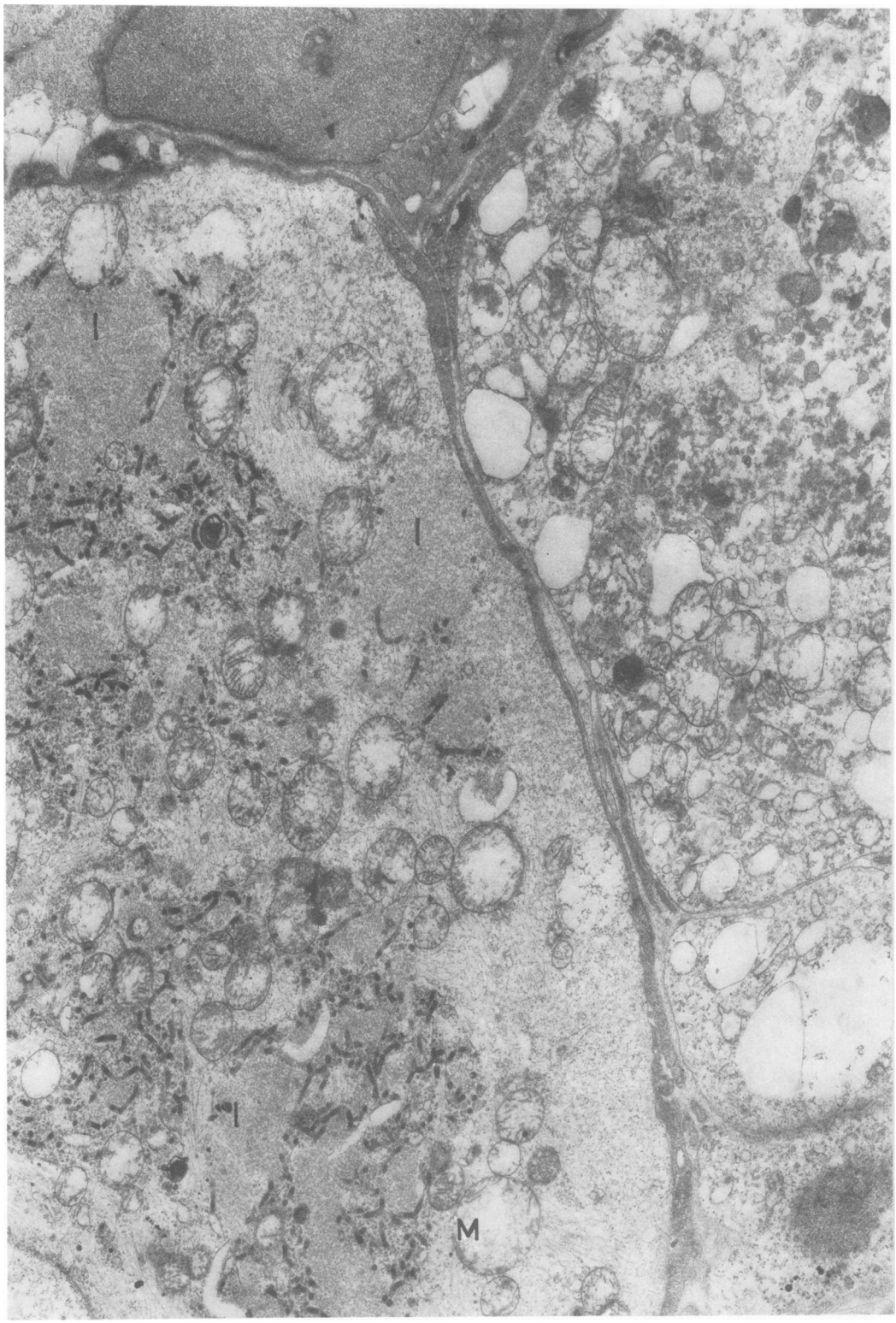

FIG. 6. *Rabies virus (W56)-infected neurons. Numerous virions in association with inclusion bodies (I) are seen throughout the cytoplasm of a slightly degenerated cell (left). (M) mitochondria. Virus assembly is not seen in a markedly damaged neuron (right). Signs of virus infection are not evident within satellite cells. ×18,000.*

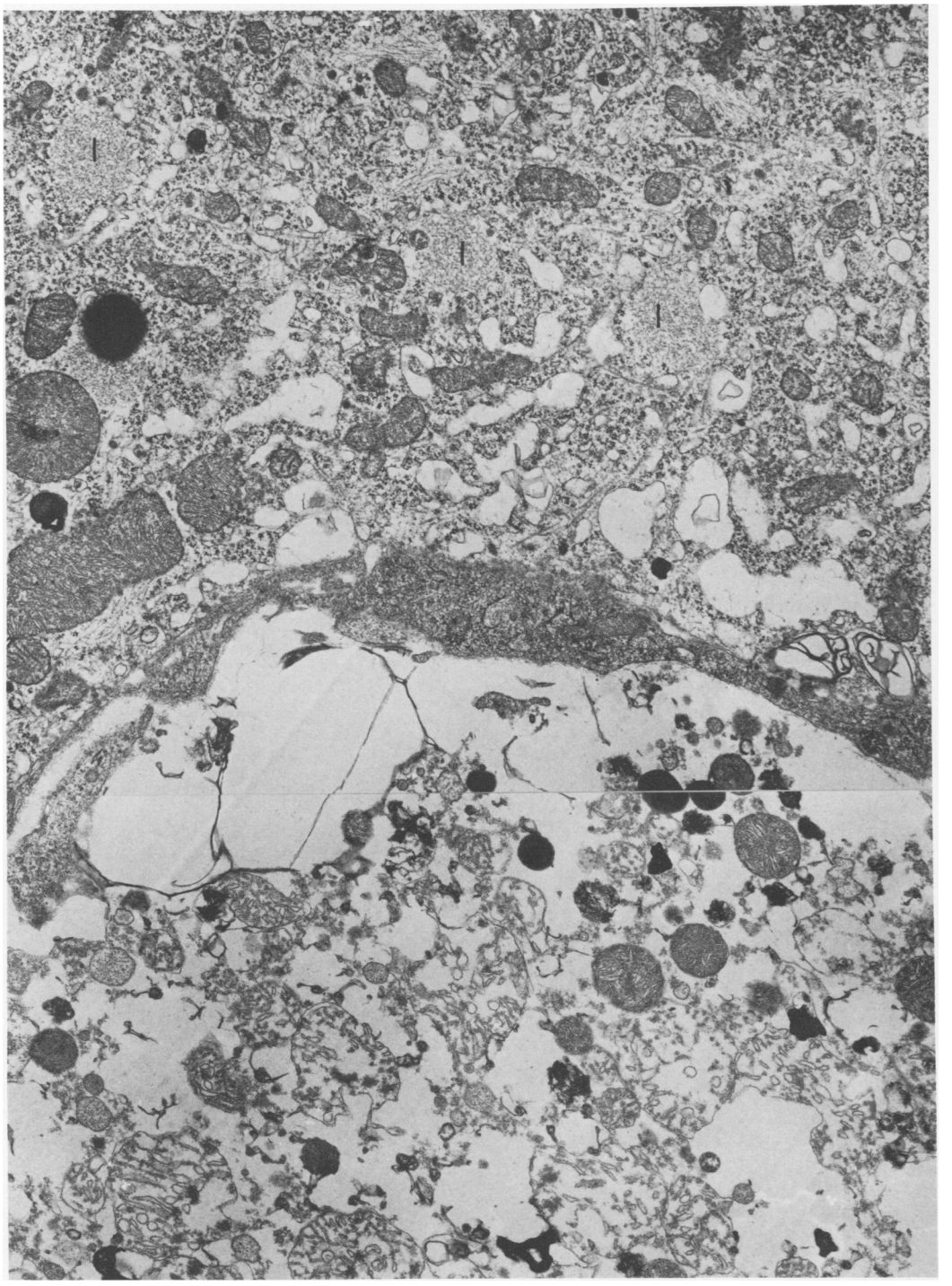

FIG. 7. *Fixed rabies virus (CVS) infected neurons. Cytoplasmic ground substance disppears in the lower neuron. No sign of virus infection is discernible in this heavily damaged cell. Small inclusion bodies (I) are seen within the upper neuron. ×17,000.*

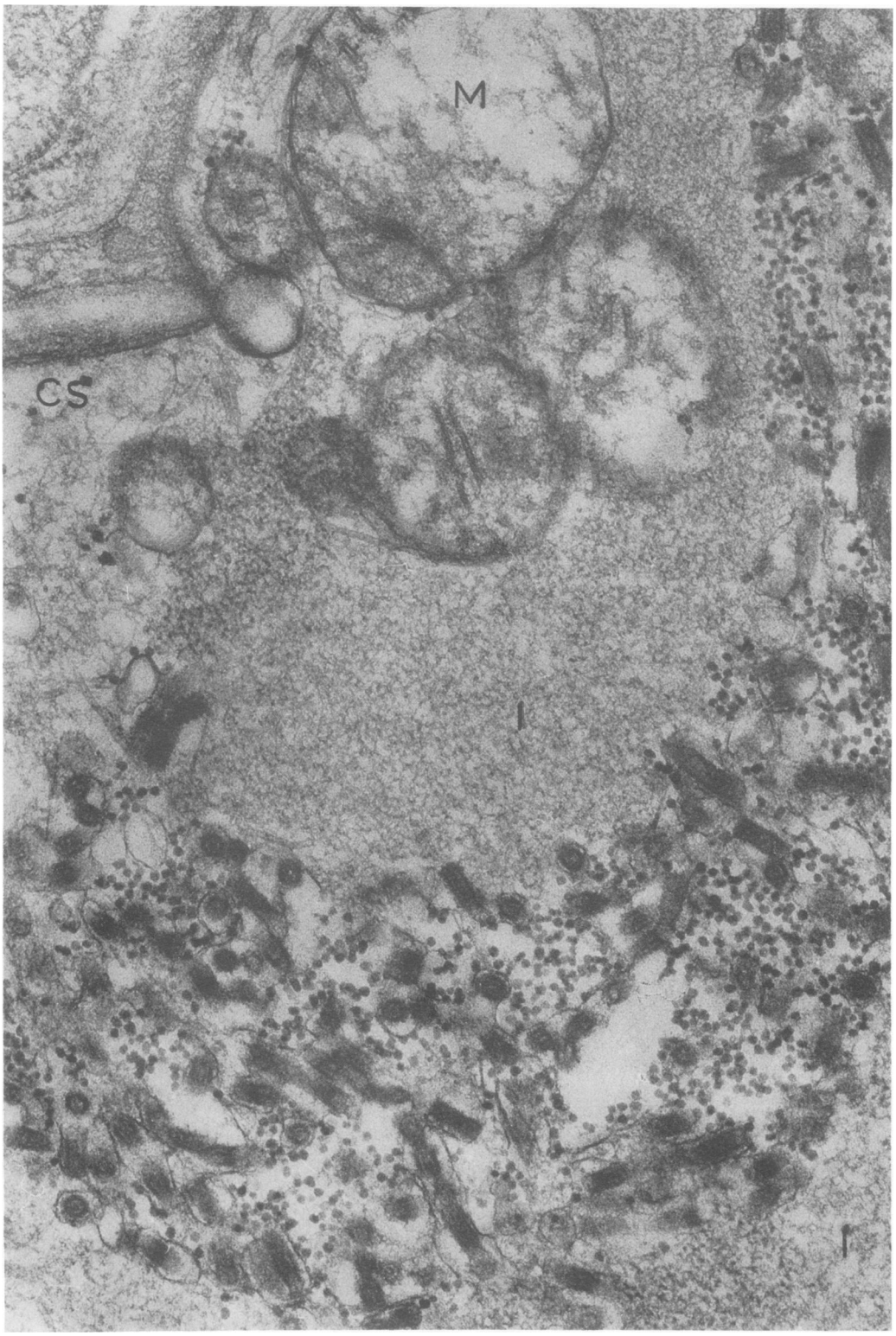

FIG. 8. *Periphery of rabies virus (W56) infected neuron. Virions during the process of budding from membranes surrounding inclusion body (I). Electron dense granules somewhat larger than ribosomes are accumulated at the site of virus assembly. CS; cell surface. M; mitochondria.* ×45,000.

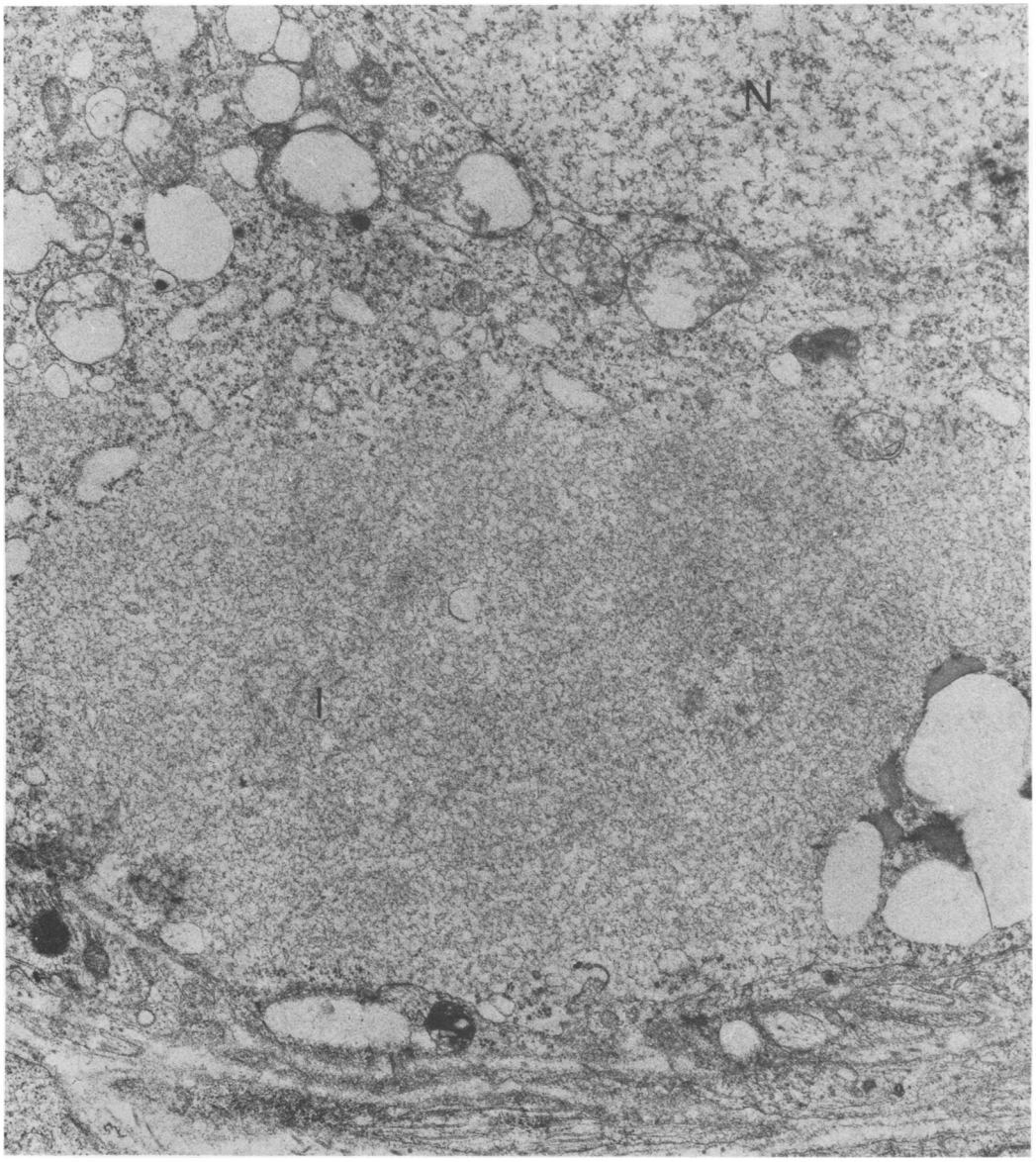

FIG. 9. *Fixed rabies virus (CVS) infected neuron. A large inclusion body (I) without virions. Number of lucent vesicles appears increased.* ×23,000.

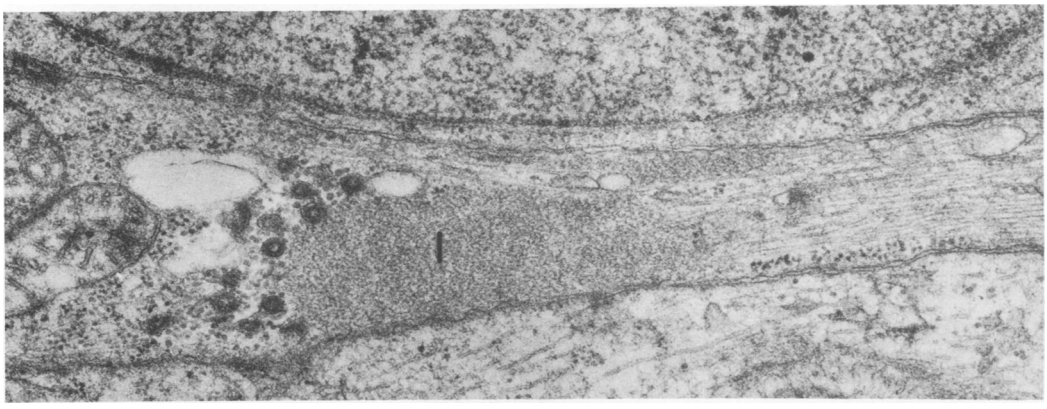

FIG. 10. *Fixed rabies virus (CVS) infected neurofiber cut longitudinally. Virions are located near an inclusion body. Dense granules, as seen in Fig. 8, are found near virions.* ×23,000.

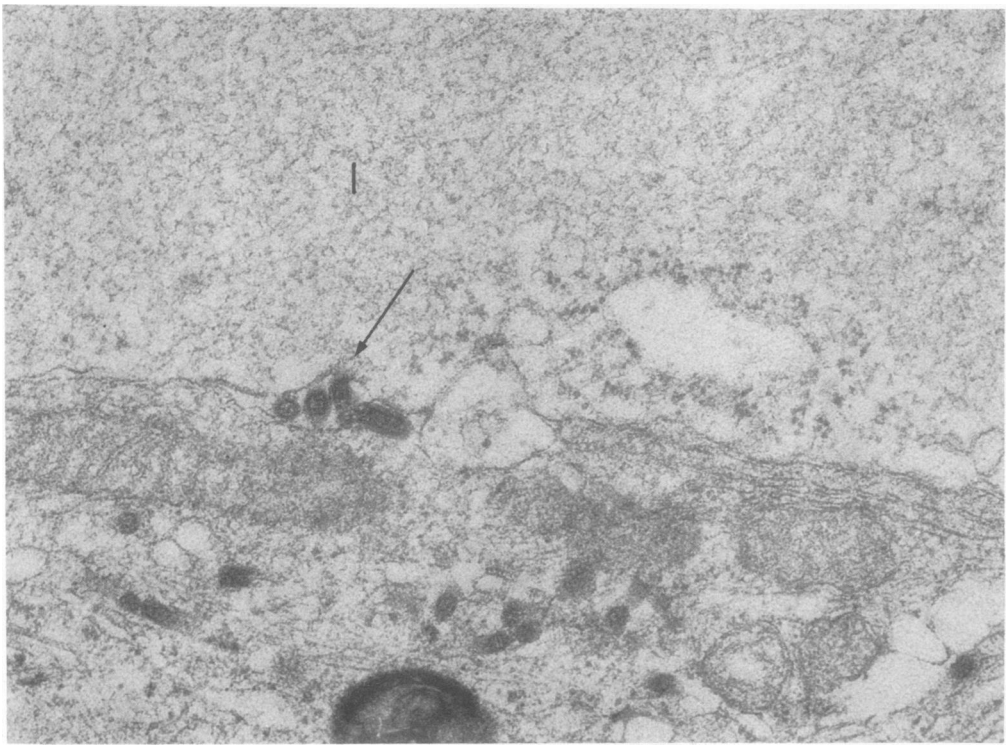

FIG. 11. *Fixed virus (CVS) infection. Arrow indicates extracellular virions. Upper half of figure is occupied by an inclusion body (I). Virions in lower half of figure are located within a satellite cell.* ×33,000.

Similar degenerative changes within neurons were observed after infection with different virus strains. Ultrastructural findings of virus morphogenesis, however, differed markedly. After fixed-virus infection with the CVS and HEP-Flury strains, a large percentage of inclusion bodies was not associated with virus assembly. This evidence, being inconsistent with the actual yield of infectious progeny virus, has repeatedly been obtained by in vivo and in vitro experiments with fixed rabies virus (6, 8). In the present study this observation was so prominent that chronological electron microscopical studies of the HEP-Flury virus replication within cultured neurons were undertaken. The results indicated an unusual mode of rabies virus morphogenesis. It was noted that, in addition to the occurrence of few inclusion bodies associated with virions (Fig. 12 and 16), the main sites of virus assembly were the vesicular membranes of the Golgi complex. Infected neurons were easily recognized by an increasing number of small vesicles throughout the perikaryon. Virions were individually formed and packed within these structures. An increase of Golgi complex-associated small vesicles has also been observed in mouse brain neurons infected with fixed virus, but virus budding from these membranes was not observed (8). Our recent findings lead us to speculate that virus assembly may also occur at the Golgi vesicles during the in vivo infection but this is not demonstrable due to the rapid degenerative alterations of infected neurons, possibly a consequence of autodigestive processes induced by the increased number of lysosomes. The involvement of the Golgi complex in the replication of enveloped viruses, especially arboviruses, has been reported before (2, 11). Arboviruses budding from the Golgi vesicles accumulated in membranous enclosures, whereas HEP-Flury virus particles were mostly contained individually.

Striking differences were observed after infection of cultivated ganglions with rodent-origin field viruses. Numerous virions which budded from intracytoplasmic membranes were located within inclusion bodies. The accumulation of virions within brain neurons in vivo has been emphasized as the main characteristic of the street rabies virus infection (3, 5, 8, 9, 10). The similarity of virus morphogenesis within the neuron in vitro and in vivo suggests that the rabies-like viruses examined here may be classified as street viruses. In an earlier study (6) we

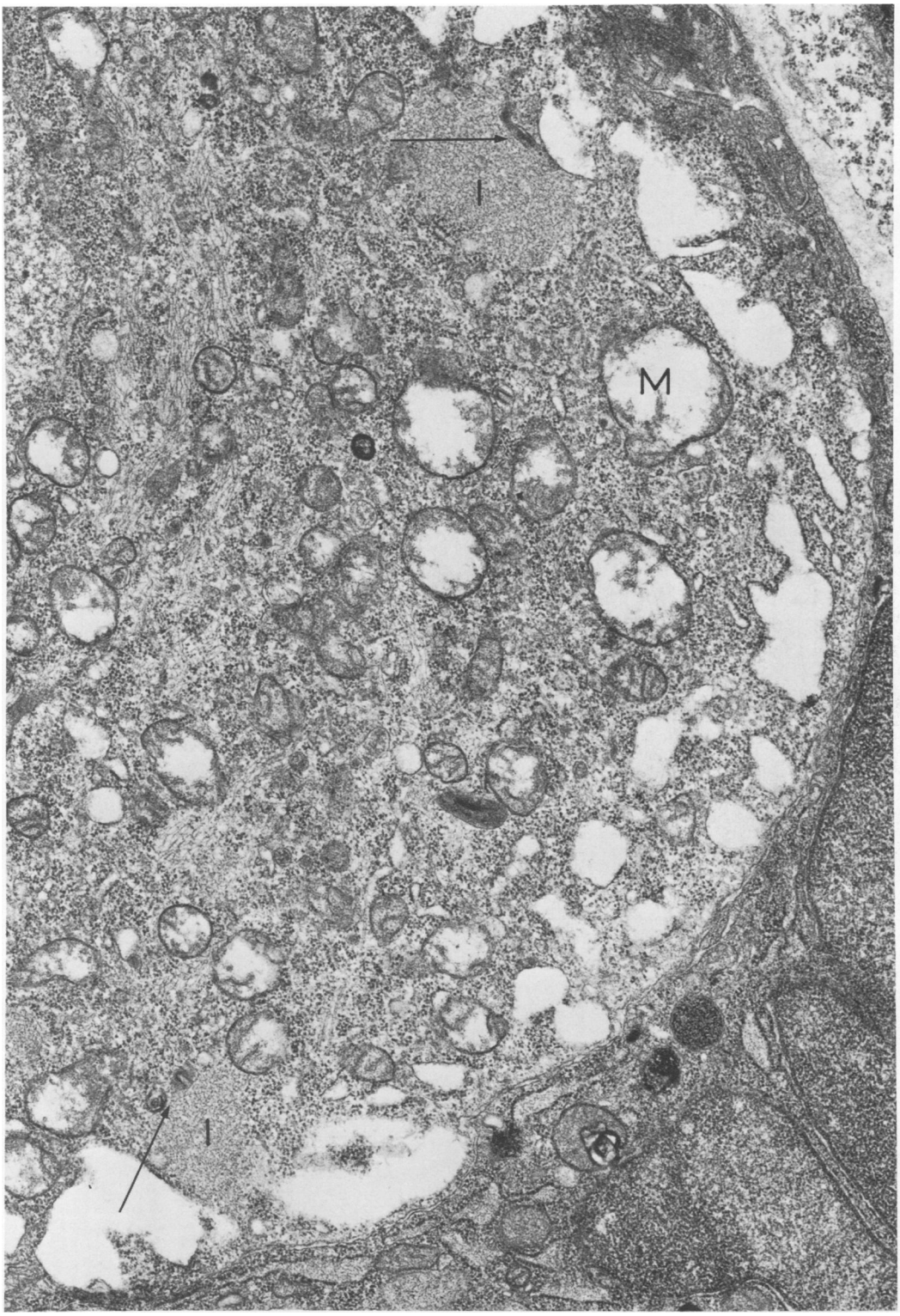

FIG. 12. *Fixed virus (HEP-Flury) infected cell at 24 hpi. Lucent vesicles occur at the periphery of a neuron. Some mitochondria (M) are ballooned but ground substance of the cytoplasm remains intact. Two small inclusion bodies show peripheral virus budding (arrows).* ×*20,000.*

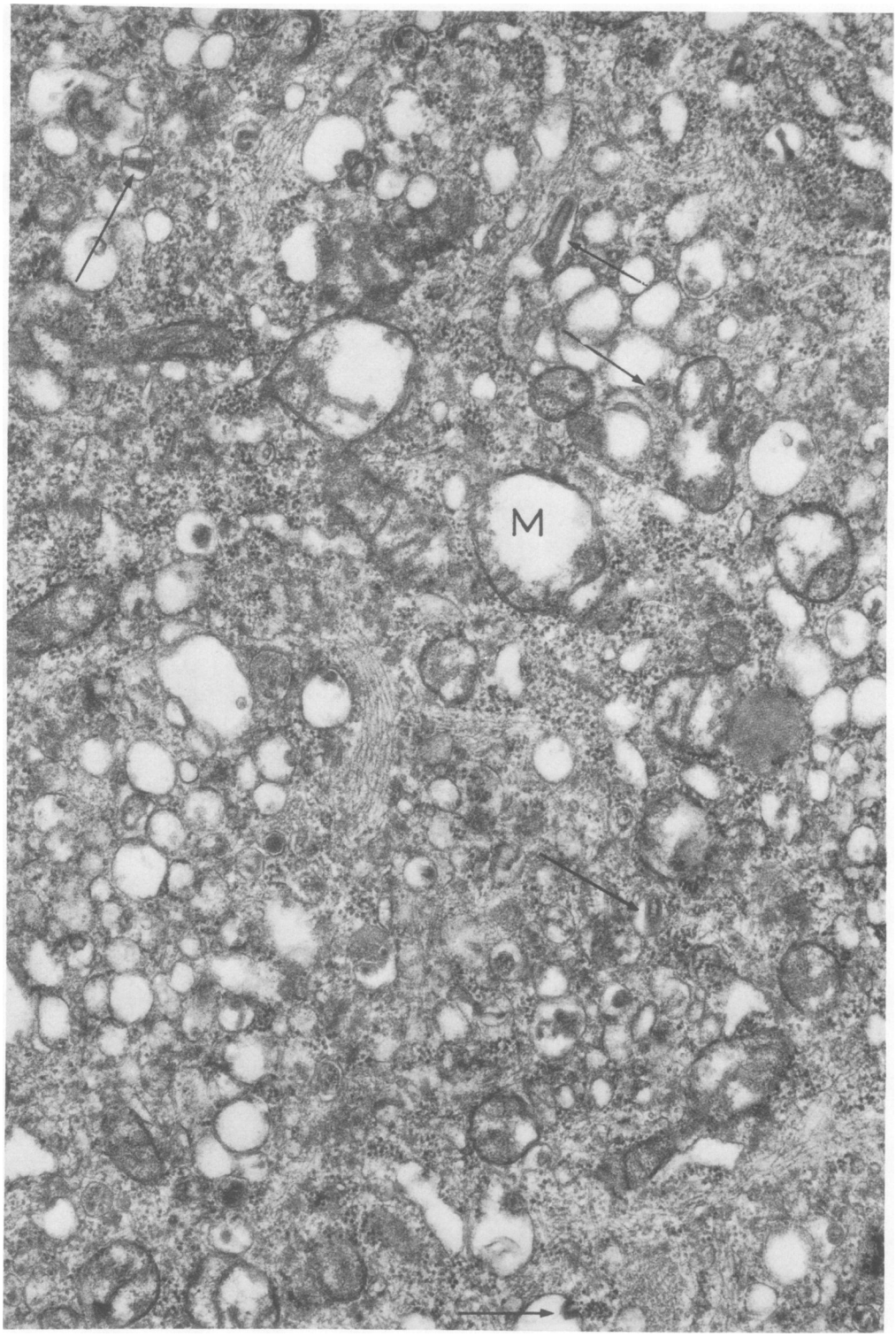

FIG. 13. *Fixed virus (HEP-Flury) infected neuron at 48 hpi. Numerous small vesicles are distributed in the cytoplasm. Virus budding from vesicular membranes is indicated by arrows. Inclusion bodies are not present in this figure.* ×35,000.

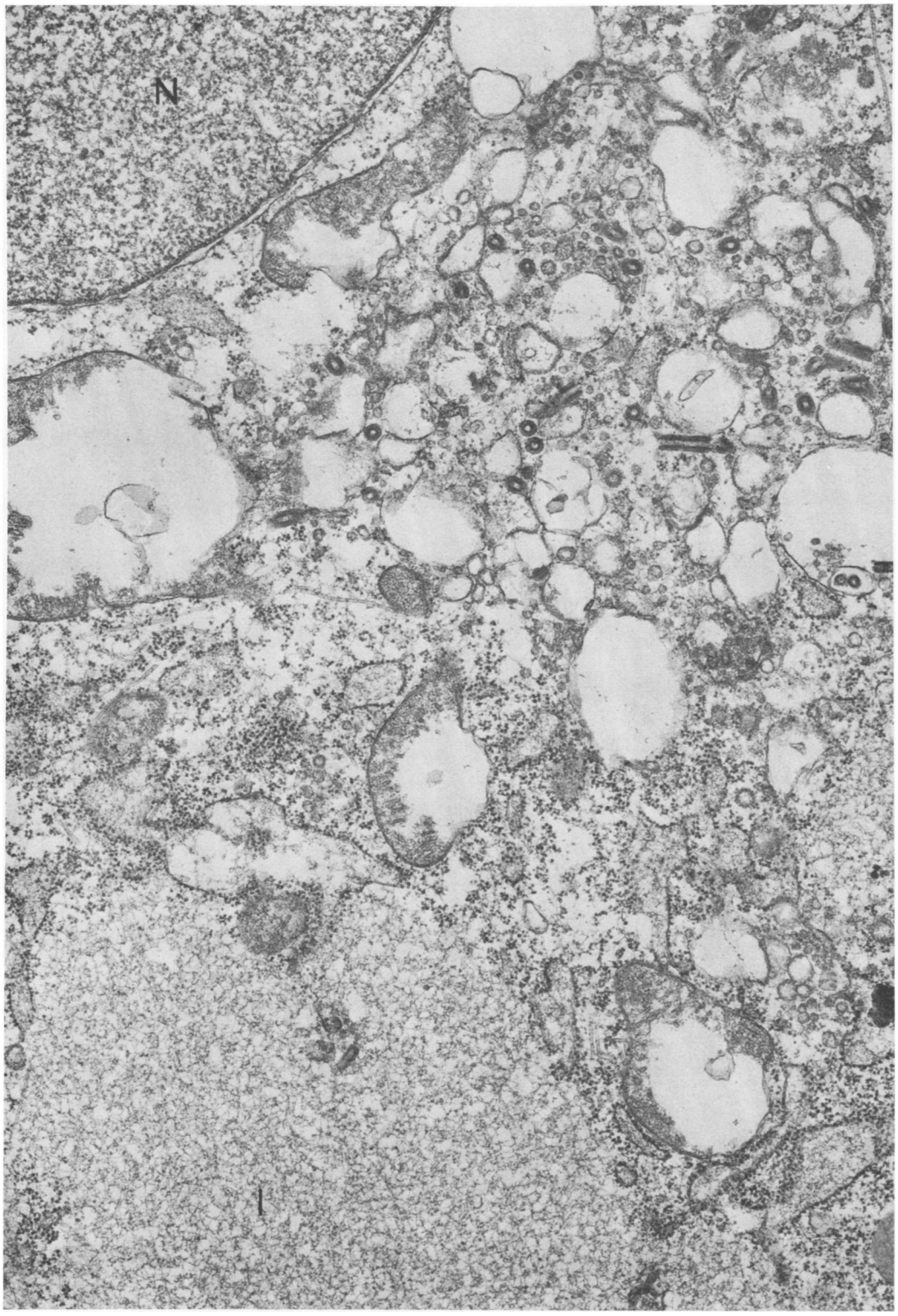

FIG. 14. *Fixed virus (HEP-Flury)-infected fibroblast at 72 hpi. Virus assembly is independent from inclusion body (I). Cytoplasmic membranes from which virus budding occurs are not clearly demonstrated at this magnification.* ×*24,500.*

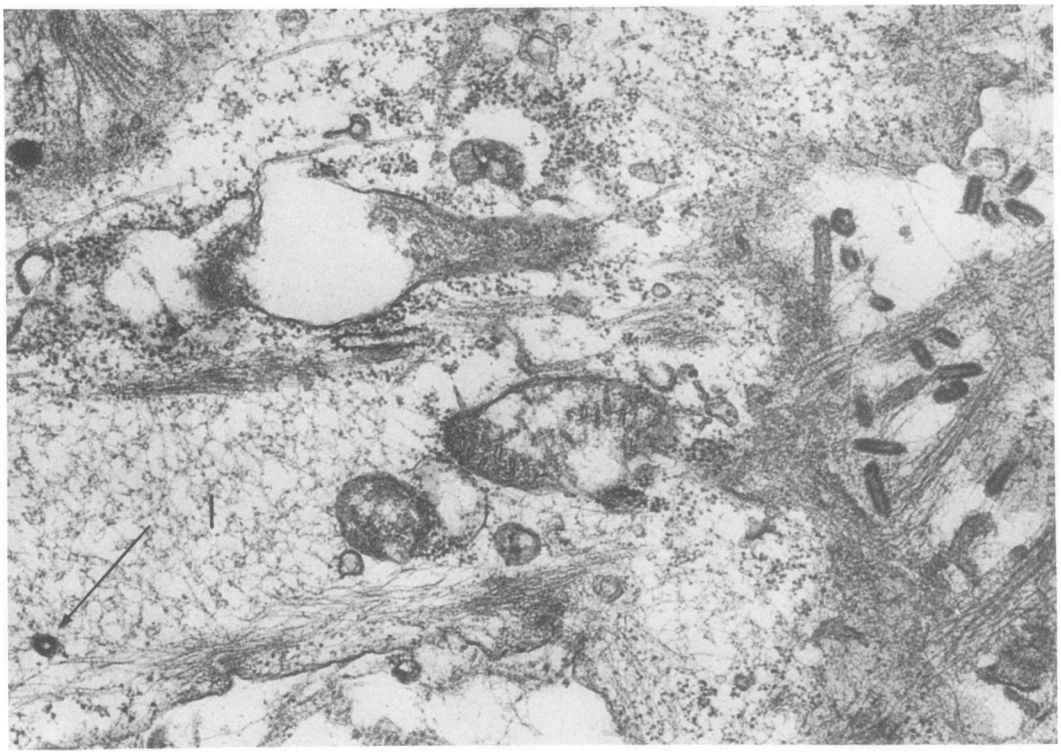

FIG. 15. *Extracellular accumulation of virions (HEP-Flury). Arrow shows a intracellular virion adjacent to the inclusion body (I) within a fibroblast.* ×*31,000.*

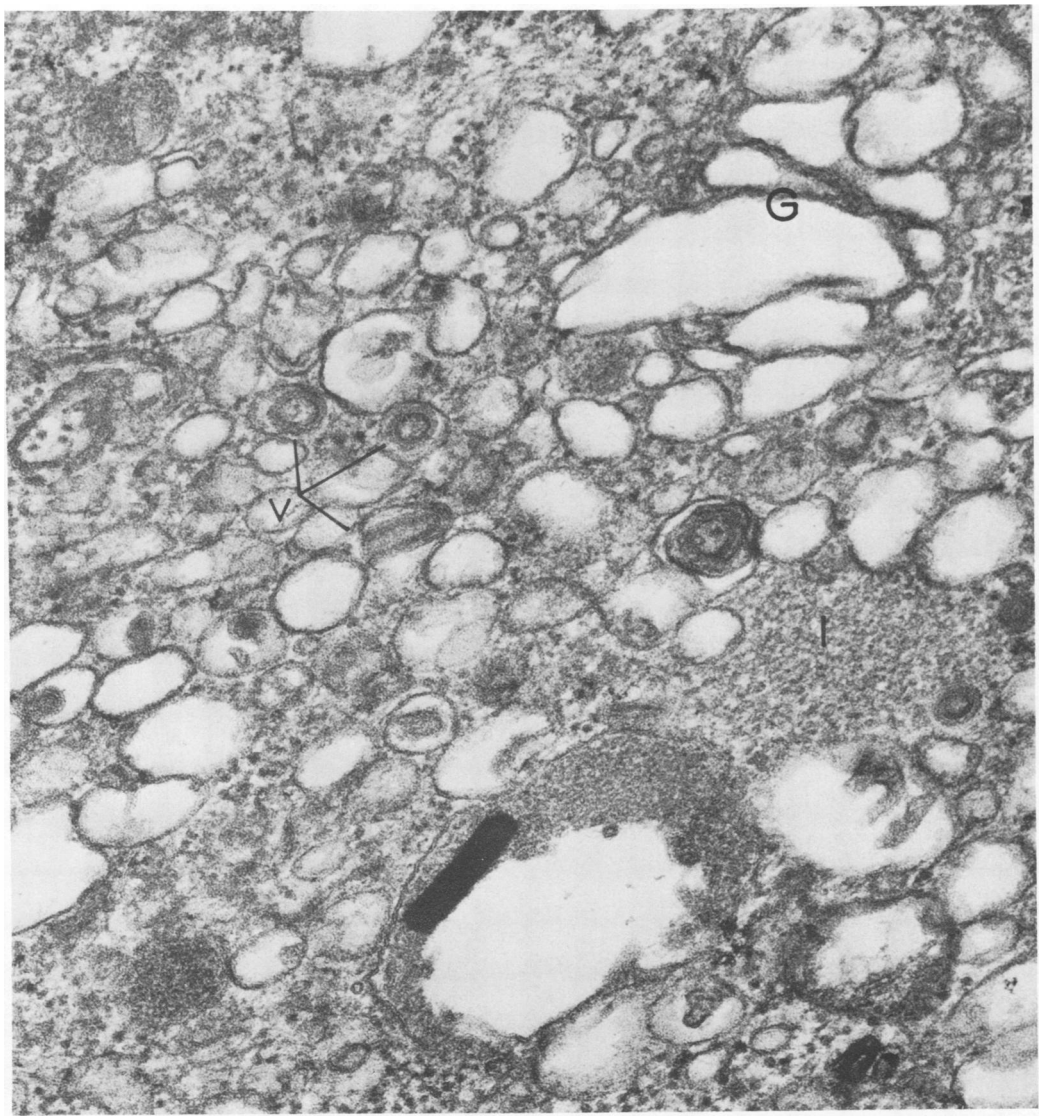

FIG. 16. *Fixed virus (HEP-Flury)-infected neuron at 24 hpi, showing development of small vesicles from Golgi complex (G). Virions are found within vesicles (arrows) and in a small inclusion body (I). ×76,000.*

have shown that a small percentage of inclusion bodies was regularly associated with virus assembly, regardless of whether the infecting virus was of street or fixed-virus origin. The reason for these different findings has not yet been determined. Variation of virus during numerous passages in mice may be one reason, technical aspects or inadequate virus attachment to the target cells may be another.

Growth curves of fixed (HEP-Flury strain) and field (W56 strain) viruses differed quantitatively and qualitatively. The HEP-Flury virus continued to multiply exponentially until 72 hpi (Fig. 3 and 4). Its peak titers were several-thousandfold higher than those of the W56 virus. The latter virus reached its peak titer at 48 hpi and then decreased progressively. The different behaviour indicates that the HEP-Flury virus replicates not only within the neuron but also within other supportive cells of the organized neural culture. Electron microscopy confirmed the productive infection of fibroblasts and satellite cells (Fig. 11, 14, and 15).

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LITERATURE CITED

1. Iwasaki, Y., T. J. Wiktor, and H. Koprowski. 1973. Early events of rabies virus replication in tissue cultures. Lab. Invest. **28**:142-148.
2. Lyons, M. J., and J. Heyduk. 1973. Aspects of the developmental morphology of California encephalitis virus in cultured vertebrate and arthropod cells and in mouse brain. Virology **54**:37-52.
3. Matsumoto, S. 1963. Electron microscope studies of rabies virus in mouse brain. J. Cell Biol. **19**:565-591.
4. Matsumoto, S. 1970. Rabies virus. Advan. Virus Res. **16**:257-301.
5. Matsumoto. S., and A. Kawai. 1969. Comparative studies on development of rabies virus in different host cells. Virology **39**:449-459.
6. Matsumoto, S., and T. Yonezawa. 1971. Replication of rabies virus in organized culture of mammalian neural tissues. Infect. Immunity **3**:606-616.
7. Millonig, G. 1961. Advantages of phosphate buffer for OsO_4 solution in fixation. J. Appl. Physics **32**:1637.
8. Miyamoto, K., and S. Matsumoto. 1967. Comparative studies between pathogenesis of street and fixed rabies infection. J. Exp. Med. **125**:447-456.
9. Murphy, F. A., S. P. Bauer, A. K. Harrison, and W. C. Winn. 1973. Comparative pathogenesis of rabies and rabies-like viruses. Viral infection and transit from inoculation site to the central nervous system. Lab. Invest. **28**:361-376.
10. Murphy, F. A., A. K. Harrison, W. C. Winn, and S. P. Bauer. 1973. Comparative pathogenesis of rabies and rabies-like viruses. Infection of the central nervous system and centrifugal spread of virus to peripheral tissues. Lab. Invest. **29**:1-16.
11. Murphy, F. A., A. K. Harrison, and S. G. Whitfield. 1973. Bunyaviridae; morphologic and morphogenetic similarities of Bunyamwera serologic subgroup viruses and several other arthropod-borne viruses. Intervirology **1**:297-316.
12. Schneider, L. G. 1973. A rapid method for fluorescein labelling of rabies antibodies, p. 336-338. *In* M. M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies, 3rd ed. World Health Organization, Geneva.
13. Schneider, L. G., and U. Schoop. 1972. Pathogenesis of rabies and rabies-like viruses. Ann. Inst. Pasteur **123**:469-476.
14. Schneider, L. G., B. Dietzschold, R. E. Dierks, W. Matthäus, P. J. Enzmann, and K. Strohmaier. 1973. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. J. Virol. **11**:748-755.