STRUCTURE AND DEVELOPMENT OF VIRUSES AS OBSERVED IN THE ELECTRON MICROSCOPE

V. Western Equine Encephalomyelitis Virus*

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Plates 25 to 39

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Western equine encephalomyelitis (WEE) virus is 40 to 55 mμ in diameter (1) and apparently contains nucleic acid of the ribose type (2, 3). Although electron microscopic examination of whole mounts of tissue cultures infected with a closely related agent (Eastern equine encephalomyelitis virus) has shown viral particles both within and on the surface of cells (4, 5), no studies employing thin sections have been previously reported. The purpose of this communication is to illustrate and describe the manner in which WEE virus appears to differentiate within, and gain egress from, infected tissue culture cells as revealed in thin sections by the electron microscope. Similarities in development or release among WEE virus, influenza virus, herpes simplex virus, the virus associated with erythroblastosis of chickens, Rous sarcoma virus and a mouse mammary tumor agent will be discussed.

Materials and Methods

Viruses.—Strains of Western equine encephalomyelitis (WEE) virus, generously supplied by Dr. Max Theiler and Dr. William F. Scherer, were passaged intracerebrally in mice; suspensions of freshly harvested mouse brain, suitably diluted, served as the inocula for cell cultures.

Cell Cultures.—

Chicken embryo fibroblasts: Monolayer cultures, prepared from 10-day-old chicken embryos by the method of Dulbecco (6), were inoculated with 10^3 tissue culture infective doses of virus, which caused marked cytopathic changes within 2 days.

Stable human amnionic cells: A stable line of human amnionic epithelium was kindly supplied by Dr. Katherine Sprunt. Replicate cultures were prepared in Leighton tubes without coverslips, using Eagle's basal medium with 10 per cent horse serum. The cultures were inocu-

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lated with 10^3 tissue culture infective doses of virus, marked cytopathic effects being observed within 3 days.

Primary amnionic cells: Fresh human amnionic membranes were washed in Earle's balanced salt solution, minced and suspended in 0.25 per cent trypsin at 37°C. for 16 to 18 hours. The resulting cell suspension was washed and distributed to 8-ounce bottles in mixture 199 containing 10 per cent horse serum. The confluent cell sheets which appeared within 6 to 10 days were trypsinized and the cells were redistributed to tubes in which monolayers formed within a week; these were inoculated with 10^5 tissue culture infective doses of virus, cytopathic effects appearing within 3 days.

Cells were scraped from the glass surface into balanced salt solution and centrifuged at low speed. The pellets of cells were fixed in osmium tetroxide for 20 minutes, dehydrated in graded dilutions of ethyl alcohol, embedded in methacrylate, and sectioned with diamond knives on a Porter-Blum microtome, as previously described (7). Some of the sections were stained with lead by floating them on a saturated solution of lead acetate for 20 minutes, followed by drying and exposure to ammonium hydroxide vapor for 5 seconds, according to the method of Dalton and Zeigel (8).

Since it was observed that WEE virus developed in a similar manner and exerted the same general cytopathic effects in each of the aforementioned cell lines, it appeared unnecessary to burden the presentation of results by repeated references to cell types. It may suffice to say that chicken embryo fibroblasts are shown in Figs. 1 to 8 and 14 to 17, stable human amnionic cells in Figs. 9 to 13, 18 to 21 and 26 to 28, and primary amnionic cells in Figs. 22 to 25.

RESULTS

Fig. 1 illustrates part of an infected cell. The nucleus lies outside the plane of section. Several cytoplasmic extensions, cut at different angles, are evident in the lower portion of the field. The cytoplasm contains numerous granules of variable size and two types of vacuoles: (*a*) irregular clefts which traverse the cytoplasm and often appear to interconnect, and (*b*) single or multiloculated, discrete, oval vacuoles bounded by laminated membranes or rows of dense particles. The definition of the membranes depends upon the angle at which they have been cut. In this field, for example, the lowermost oval vacuoles exhibit sharp, peripheral lamellae where the membranes have been cross-sectioned and diffuse, ill-defined margins where they have been cut obliquely. Four relatively dense lipid droplets are visible, three in a horizontal row near the center, and one at the top. Fig. 2 shows the upper right portion of Fig. 1 at higher magnification. Partially or completely surrounding several of the vacuoles (numbered 1 to 5) are particles which appear larger (averaging 22 mμ in diameter), more dense, more uniform in size, and more sharply defined than the granules that are scattered through the cytoplasm. Where the particles are discrete and exhibit minimal superimposition, a central area of diminished density is visible. Some (indicated by arrows in vacuoles 1 and 5) seem to be incomplete in that they lack part of the dense peripheral zone. Many of the cleft-like vacuoles are interconnected by parallel membranes, thus forming a continuous system. Three of these (marked *A*, *B*, and *C*) are apparent.

Further information can be gained by comparison of serial sections, such as

those illustrated by Figs. 2 to 4. First, some vacuoles which appear to be isolated (as A^1 in Figs. 2 and 3, B^1 in Figs. 3 and 4, or C^1 in Fig. 2), are in reality connected to a vacuolar system. Undoubtedly further interconnections would become apparent if more serial sections were examined. Second, the cleft-like vacuoles do not communicate directly with the oval vacuoles bordered by dense particles. Third, the oval vacuoles exhibit a thin, marginal membrane which separates the dense particles from the lumen, as shown by vacuole 1 in Fig. 2. Such membranes are apparent only when they are perpendicular to the plane of section. Thus, although a membrane is clearly visible at the lower right of vacuole 4 in Fig. 2, it becomes indistinct at the level illustrated by Fig. 4. Presumably the latter micrograph shows a region close to the periphery of the vacuole where the membrane has been cut obliquely. Fourth, two vacuoles (3 and 4 in Fig. 2) exhibit protrusions of the wall into the lumen. The site of attachment of such protrusions may be ill defined (see vacuole 3, Fig. 3) or inapparent depending upon its location with respect to the plane of section. Fifth, when cut at the appropriate level, vacuoles 1, 2, and 3 are seen to contain spherical bodies which possess a core of variable density and a diffuse peripheral coat about 45 mμ in diameter. Their size, shape, and internal structure suggest that such bodies are viral particles, and they will be so designated hereafter.

Fig. 5 illustrates a moderately thick section through a vacuole in another cell. Although the section is too thick for clear delineation of viral structure, the diameter of the majority of viral particles can be accurately measured and is about 45 mμ. In addition to virus, there are smaller particles embedded as aggregates in dense matrix. Whether such aggregates represent protrusions which connect with the wall of the vacuoles at a level removed from the plane of section, or bodies lying free within the lumen, cannot be determined without serial sections. The dense particles lining the periphery exhibit considerable superimposition.

Occasionally vacuoles were encountered which contained not only spherical viral particles, but also well demarcated filaments. Such a vacuole is shown in Fig. 6. It is evident that the width of the filaments closely approximates the diameter of the virus. A cluster of small particles is present near the center of the vacuole. At the periphery the particles vary in size and form a discontinuous lining; where they are lacking the cytoplasm appears to be contiguous to the lumen.

Fig. 7 illustrates a cell with numerous vacuoles, granular, relatively dense cytoplasm and a shrunken, pyknotic nucleus (N). Three oval vacuoles contain virus. Vacuole A was shown at higher magnification in Fig. 5. Vacuole B is surrounded by particles, whereas vacuole C, which is multiloculated, is lined by a membrane devoid of marginal particles. In Fig. 8 the cleft-like vacuolar system is even more extensive than in the preceding micrograph. On the right, in the mid-third, the cellular wall appears to have ruptured and numerous viral parti-

cles are seen in the extracellular space. One vacuole (*A*) exhibits marginal particles but no virus within the lumen; another (*B*), although lacking marginal particles, contains virus. Even at this magnification it is evident that neither in Fig. 7 nor Fig. 8 does the cleft-like vacuolar system communicate with the oval vacuoles.

Fig. 9 shows a cell that has undergone marked condensation. The pyknotic nucleus (*N*) is difficult to distinguish from adjacent cytoplasm, which contains so many dense granules that it is nearly opaque to the electron beam. In the upper third a vacuole contains clusters of virus. Viral aggregates border fragments of cytoplasm which project into the extracellular space at the upper right.

Fig. 10 illustrates a relatively thick section. Characteristic profiles of endoplasmic, reticular membranes traverse the field. In the right half are several altered mitochondria. Near the right margin in the lower third two vacuoles partially surrounded by dense particles appear to have been cut so close to their periphery that the lining membranes are not sharply defined. Near the cellular surface, which runs vertically on the left, is an oval, walled vacuole containing viral particles. Rows and irregular clusters of extracellular virus lie in crypts formed by cytoplasmic extensions.

Fig. 11 illustrates part of a cell with dense, closely packed, cytoplasmic granules and numerous vacuoles, the majority of which are oval in shape. In the lower third of the field several of them contain virus. Along the left margin other vacuoles are separated from the extracellular space by a thin rim of cytoplasm. Virus is clustered on the surface, two large aggregates seeming to lie within ruptured vacuoles. The intracellular vacuole (*A*) just below the center of the field is seen at higher magnification in Fig. 12. Although there has been some displacement, the majority of viral particles are arranged in such regular order as to suggest a crystal and exhibit a center-to-center spacing of approximately 50 mμ. Virus central to the plane of section appears well defined, in a manner analogous to that pointed out in studies of other viral crystals (7, 9, 10). This section was stained with lead thus increasing the contrast of the viral components, which consist of a dense core about 30 mμ in diameter enclosed by an envelope and a peripheral membrane 45 to 48 mμ in diameter. Some cores have a translucent center, whereas others appear uniformly opaque. The membrane of the vacuole seems to be discontinuous and is not lined by small particles of the type illustrated in Figs. 2 to 6. Part of a vacuole containing two viral particles is visible on the left, while just beneath it a cluster of virus appears to lie in a vacuole which has been sectioned peripherally. Fig. 13 also illustrates a portion of Fig. 11. The oval, sharply defined vacuole (*B*) in the lower left of the latter figure is visible at the right margin. Above and to the left of this vacuole the cytoplasm appears to be in process of rupture into the extracellular space, which occupies the left portion of the field. Although most of the virus is distributed at random among cellular fragments, a small cluster of particles near the center exhibits

orderly array. It is evident that this extracellular virus closely resembles the virus contained within the cytoplasmic vacuole shown in Fig. 12.

The cell in Fig. 14 exhibits clumped nuclear chromatin, cytoplasmic granules separated by areas of low density and numerous oval vacuoles. Vacuole *A* contains virus and is devoid of a limiting membrane, the lumen appearing to communicate with the extracellular space. The vacuole above letter *B* is poorly defined, and virus lies within the adjacent cytoplasm. Vacuole *C* at the right margin contains short, irregular, fragmented double membranes. No cellular wall is visible, and at the top of the field cytoplasmic components as well as characteristic viral particles (some of which are marked by arrows) are within the extracellular space.

Fig. 15 illustrates an unusually thin section. Several mitochondria in the upper two-thirds are vacuolated and contain disrupted cristae. Dense particles border seven cytoplasmic vacuoles, and clusters of similar particles (indicated by arrows) are scattered in the cytoplasm. An irregular intercellular cleft containing virus traverses the lower corners of the field diagonally. Fig. 16 shows the lower left portion of the field at higher magnification. The structure of the extracellular virus at the lower left is visible but not sharply demarcated in the absence of lead staining. Two vacuoles on the right are surrounded by dense particles, many of which have typical translucent centers. Above and to the left a vacuole contains virus. It is to be noted that where the margin of this vacuole is adjacent to the virus there are no lining particles. At the lower right a cluster of particles exhibits a regular array. The question naturally arises as to whether this pattern constitutes close packing in two dimensions, such as might be encountered if the plane of section were tangential to a vacuole lined by particles, or whether it constitutes a true crystalline array in three dimensions. By means of serial sections it has been possible to demonstrate that crystals do occur. Fig. 17, for example, shows one of several serial sections through two contiguous crystals. Although the arrays are partially obscured near the center and in the lower portion, probably owing in part to displacement during preparation of the specimen and in part to the effect of superimposition within the section, there is a striking resemblance to the twin crystals encountered in studies of Coxsackie virus (11).

In Fig. 18, illustrating a lead-stained section, characteristic, dense particles are readily identified. Some are scattered in the cytoplasm, whereas others line the opposite surfaces of two concentric lamellae near the mitochondrion. Fig. 19 shows an unstained section. Particles are scattered in the cytoplasm and concentrated on lamellae. At the upper left two parallel membranes appear to change orientation within the section, being sharply defined where they are perpendicular and diffuse where oblique. At the lower left concentric lamellae seem to have been cut at such an angle that neither is well defined. In the upper right corner several particles lie within a vacuole enclosed by double membranes.

Fig. 20 illustrates cytoplasm containing several clusters of dense particles adjacent to double lamellae. Although two lamellae on the left appear to be discontinuous, it is difficult to exclude the possibility that they lie oblique to the section along part of their course. Virus is scattered on the cellular surface, which traverses the upper left corner. Near the left border in the extracellular space a cluster of small particles, resembling those within the cytoplasm, is enclosed by a single membrane. Above, cytoplasmic extensions cut at different angles are visible.

In Fig. 21 the undulant surface of a cell traverses the upper third horizontally. Three dense particles (indicated by arrows) are scattered in the cytoplasm, and four are evident at the surface adjacent to extracellular virus. Fig. 22 shows a cytoplasmic extension projecting upward from the surface of a cell. At the tip the cellular wall appears to be replaced by a dense particle surrounded by a well defined coat. Close examination discloses a thin membrane, which is concave with respect to the cell and separates the cytoplasm from the particle. In Fig. 23 the cellular wall of a small cytoplasmic extension seems to extend outward and to connect with a particle enclosed by a diffuse coat. At the top a characteristic viral particle lies in the extracellular space. Fig. 24 illustrates the convoluted surface of a cell. Near the center of the field viral particles lie at the surface of a large, irregular cytoplasmic extension. In a manner similar to that shown in the preceding micrograph the wall of the cell appears to extend toward, and to be deficient just beneath, several viral particles.

Figs. 22 to 24 are electron micrographs of unusually thin sections. When thicker sections were examined the cellular wall was observed to be so poorly demarcated, owing to variable orientation within the section, that its relationship to virus at the surface became difficult to define, as is illustrated by Fig. 25. In this picture a cytoplasmic extension is visible at the upper left, and another, apparently sectioned obliquely, at the lower right. Viral particles central to the plane of section exhibit characteristic structure, which is accentuated by lead staining (Fig. 26).[1] As previously noted, the 30 mμ core varies in density and is enclosed by a coat averaging 45 mμ in diameter. There is a sharply defined peripheral membrane.

In Fig. 27 the cellular surface extends horizontally across the lower border. In the extracellular space dense particles line the inner aspect of an oval membrane. Above, virus is present in a regular array, the pattern formed by sharply demarcated particles reflecting the orientation of the crystalline lattice with respect to the plane of section. Fig. 28 also illustrates this phenomenon. In the extracellular crystal at the upper right there are rows of well defined particles and zones devoid of clearly delineated virus. Portions of this crystal appear to be in process of dissolution. It is of interest that the closest center-to-center

[1] The authors are indebted to Dr. Richard A. Rifkind for this micrograph.

spacing of particles in the straight rows is about 45 mμ, whereas elsewhere the spacing averages 50 mμ. The cellular wall is deficient where it abuts on the crystal. In the lower right corner another extracellular viral crystal is visible. The cytoplasm of the cell contains dense particles, some of which lie at the surface of double lamellae, resembling those shown previously.

DISCUSSION

Study of cells infected with WEE virus is of unusual interest since somewhat different modes of viral development and release appear to occur simultaneously in the same host cell.

I. Stages of Infection.—Consecutive stages of infection could not be studied at specified intervals, owing partly to the difficulty encountered in infecting a sufficient number of cells with the initial inoculum, while avoiding toxic effects, and partly to the early release of virus (12, 13) with consequent infection of other susceptible cells. It was not difficult, however, to recognize the gross initial cellular response and subsequent degeneration induced by the virus. When these were considered in order, beginning with cells that showed only moderate vacuolization, together with some increase in cytoplasmic granules (as in Fig. 1), and concluding with cells that exhibited marked opacification of the cytoplasm and pyknosis of the nucleus (as in Fig. 9), the sequential stages of viral development and release became evident.

II. Formation of Vacuoles.—The earliest recognizable lesions were walled vacuoles surrounded by dense particles (Figs. 1 to 4). The origin of these vacuoles is obscure. If they arise from saccular dilatation of the channels formed by endoplasmic reticulum, then connection with the reticular network should be encountered at appropriate levels of sectioning. As was pointed out, however, the cleft-like vacuolar system, which is continuous with components of the endoplasmic reticulum (Figs. 1 to 4, 7, and 8), could not be shown even by serial sections to communicate with the oval vacuoles. Moreover, the foregoing hypothesis does not explain the presence of vacuoles lined by double membranes. Another possibility is that the vacuoles result from disintegration of mitochondria. To be sure, mitochondria were encountered in various stages of vacuolization (Figs. 14, 15, and 28), but no more frequently than in control cells undergoing necrosis. Moreover, there was no evident spatial relationship between disintegrating mitochondria and the oval vacuoles, such as should occur if one structure were developing into another. Indeed, vacuoles lined by particles were commonly found adjacent to mitochondria that appeared normal (Fig. 18). Finally, these vacuoles did not contain the compressed or convoluted fragments of cristae (see vacuole *C* in Fig. 14), which often persist when mitochondria become vacuolated (14). In the absence of evidence to the contrary, it is not unreasonable to suggest that the membranes, either singly or in pairs, form *de novo* in the cytoplasm of the host cell. The membranes of the vacuoles in the top left

of Fig. 16 and in the lower left of Fig. 20, for example, may have been in process of formation. Incomplete membranes also have been encountered in the cytoplasm of cells infected with fowl pox and vaccinia (15), ectromelia (16), molluscum contagiosum (17), and Shope fibroma (18) viruses, although they differ from those associated with WEE virus in that each appears to enclose a viral particle.[2]

III. Precursor Particles.—Particles apposed to the membranes could be differentiated readily from the usual cytoplasmic granules, since the former were larger (averaging 22 mμ in diameter), more uniform in size, more consistently spherical with sharply defined margins, and more dense.[3] In sufficiently thin sections the majority exhibited a characteristic central area of diminished opacity. In view of the fact that these particles have a distinctive morphology and were not encountered in uninfected cells, it is believed that they are related to the virus. For reasons that will become apparent, they will be called precursor particles.

IV. Viral Development and Release.—The following hypothesis regarding development and release of WEE virus is consistent with the morphologic data so far obtained. The membranes bordering vacuoles appear to demarcate template sites at which the characteristic precursor particles differentiate. These template sites occupy a specific side of the membrane. When the membrane is single the particles form within a narrow zone on that side away from the lumen of the vacuole (Figs. 2 to 4, 5, 15, and 16), whereas when the membranes are double the particles form on opposite sides (Figs. 18 to 20 and 28), never between membranes.

Further development and ultimate release of the virus is believed to occur by several mechanisms, often found to be operating in the same host cell. In the first of these the precursor particles seem to cross the membrane and enter the lumen of the vacuole, acquiring a peripheral coat as they do so. Presumably, this process occurs quickly for transitional forms are not frequently encountered. In some instances, however, gradations in size from precursor to viral particles are evident at the margin of a vacuole, as illustrated by Fig. 6. Further suggestive evidence in support of the concept that the precursor particle becomes the core of the complete virus is provided by such pictures as Fig. 16, wherein a single layer of particles lining a vacuole is deficient at a site adjacent to a cluster of virus within the lumen. Such an appearance would be expected if the particles had been incorporated into the virus. The absence of precursor

[2] It is of interest that parallel lamellae are occasionally found in the cytoplasm of cells infected with Shope fibroma (18), ectromelia (19), and fowl pox (14) viruses. The origin of these membranes and their relation to the virus have not been established.

[3] The opaque granules which are occasionally encountered in mitochondria (Fig. 20) tend to be small, variable in shape and uniformly dense. They were no more numerous in the infected preparations than in the controls.

particles in cells which show evidence of advanced infection (Figs. 9 and 11 to 13) is also consistent with the belief that they represent an early stage in the development of virus.

Cells were repeatedly encountered with well preserved cytoplasm, vacuoles containing virus, a clearly demarcated cellular wall, and aggregates of extracellular virus lodged between cytoplasmic extensions. These aspects of cell virus relationship were virtually indistinguishable from those encountered in studies of herpes simplex virus (10), suggesting that a similar mechanism of release is operative. It has been postulated (10) that this process resembles morphologically, but in actuality is the reverse of, phagocytosis.[4] As a vacuole approaches the surface the wall and intervening cytoplasm of the cell become attenuated and rupture. The membrane of the vacuole then forms part of the wall of the cell so that ejection of virus results without loss of cellular integrity or dissolution of the cytoplasm. Fig. 10 is believed to illustrate stages in such a process. To the left a vacuole containing virus is near the cellular wall, which bulges slightly. At the surface virus lies between cytoplasmic extensions. Presumably these extensions are the remnants of the attenuated rim of cytoplasm which enclosed vacuoles as they moved outward and ultimately ruptured to release the virus. If the foregoing hypothesis is correct, vacuoles lined by double membranes and containing precursor particles might also be ejected from apparently intact cells. Should this occur to the vacuole shown in Fig. 18, for example, the outer membrane would be incorporated into the cellular wall while the inner membrane, remaining intact, would protect the particles in the lumen. This manner of release would account for the presence of extracellular bodies composed of single membranes enclosing aggregates of precursor particles. One such body is shown near the upper left border of Fig. 20 and another, probably sectioned eccentrically, in the lower third of Fig. 27. Except when enclosed by a membrane, the precursor particles have not been identified extracellularly, possibly because they are unstable and disintegrate outside the host cell.

Frequently precursor particles appeared to have detached from template sites and to be dispersed through the cytoplasm (Figs. 15, 16, 18, 19, and 28), in some instances lining up just beneath the surface of the cell (Fig. 21). Presumably such particles move outward through openings in the wall, acquiring a protective coat and a peripheral membrane as they do so (Figs. 22 to 24). Extrusion of virus at the cellular surface is not a unique phenomenon, having been suggested in the case of influenza virus (20), the virus of erythroblastosis of chickens (21), the virus encountered in studies of spontaneous leukemia in mice (22),

[4] As was pointed out for herpes simplex virus, the direction of the process can only be surmised from indirect evidence. If phagocytosis were occurring and infection thus being initiated, the cell would not exhibit cytopathic changes. In the case both of herpes simplex virus and WEE virus, however, the phenomenon is invariably associated with cells which contain numerous viral particles at various stages of development.

Rous sarcoma virus (23), and an agent associated with mammary tumors of mice (24–28). It is of interest that ejection of virus from vacuoles or extrusion of virus at the surface is encountered in cells which, on morphologic grounds at least, appear to be viable. This observation is consistent with the concept that energy provided by a functioning, intact cell is necessary to carry out these processes of release in a manner analogous to the energy-requiring process of phagocytosis demonstrated in metabolic studies of leukocytes (29).

As was pointed out, the virus exerts a cytopathic effect, generally resulting in death of the host cell. The virulence of the virus with respect to any given cell appears to vary, and consequently necrosis, with rupture and release of virus, may supervene at different stages of infection. Fig. 14, for example, shows a cell which, although vacuolated, has not proceeded to condense. The cytoplasm is obviously in process of dissolution with escape of cellular contents, including virus. Figs. 8, 9, and 11, on the other hand, illustrate cells which have ruptured at more advanced stages of infection. Virus within vacuoles seems to be unaffected by the events of cellular necrosis. The virus shown in Fig. 12 is morphologically intact with clearly delineated, characteristic, internal structure.

V. Crystallization.—A number of viruses have been found to crystallize *in vivo*. Notable examples are adenoviruses (7, 30–32), herpes simplex virus (10), Coxsackie virus (11, 33), poliomyelitis virus (34), reovirus (35), polyoma virus (36) and the virus associated with human verrucae (37). The crystals associated with WEE virus are unusual in three respects. First, some are composed of precursor particles (Fig. 17); second, viral crystals are present in vacuoles (Fig. 12); third, viral crystals are often found in the extracellular space (Figs. 13, 27, and 28). It has been suggested (10, 38) that crystallization results from the differentiation of many particles within a brief interval of time at certain loci. Presumably the crystal shown in the upper portion of Fig. 28 formed at the surface as the result of rapid extrusion of viral particles from one locus. A similar phenomenon may occur in the case of influenza virus (39).

VI. Structure of the Virus.—Sharp *et al.* (1), in examining this virus dried down from purified suspensions, noted a core "of greater density than the enveloping material" and commented that the "enveloping material, though of low density, seems well defined as by a limiting structure." The size of the virus varied between 40 and 55 mμ,[5] depending upon whether it was dried from Ringer solution diluted with water or diluted with 0.023 molar calcium chloride. The foregoing observations agree well with the size and structure of intra- and extracellular virus revealed in thin sections. A dense central core approximately 30 mμ in diameter was observed to be separated by a zone of diminished density from a sharply defined limiting membrane with an average diameter of 45 mμ.

[5] As Beard points out (40), estimates of size by filtration and ultracentrifugation have differed. A major problem affecting results obtained by the latter technique is uncertainty regarding the density of the particles.

The center-to-center spacing in crystalline array varied from 45 to 50 mμ. Although lead staining of the section did not reveal any new structural detail, it increased the density of viral components, particularly the peripheral membrane.

VII. Comparison with Other Viruses.—Certain features of the development and release of WEE virus appear to be shared by a variety of other viral agents. It has been found, for example, that precursor particles, which seem to become incorporated into fully mature viral particles, are associated with the formation of herpes simplex virus (10) and the virus of Lucké renal adenocarcinoma in frogs (41), although differentiation of these viruses occurs in the nucleus rather than the cytoplasm of infected cells. In addition, observations on the mammary tumor agent in mice have led Bernhard (42) to propose that propagation of the virus involves particles which acquire a peripheral layer and membrane as they emerge through the cellular wall. Note should be taken, however, that Moore *et al.* (26) and Lasfargues *et al.* (27) found no evidence for the existence of a true precursor particle. Despite the lack of agreement on this point, it is worthy of mention that relatively large (65 to 70 mμ) particles associated with the mammary tumor agent (24) frequently are seen surrounding cytoplasmic vacuoles in a manner resembling the precursor particles of WEE virus. In this connection, comparison of Fig. 2 and Fig. 15 with Fig. 3 in the paper by Bernhard *et al.* (24) reveals a remarkable similarity.

The release mechanism whereby WEE virus is apparently ejected from cytoplasmic vacuoles without interrupting continuity of the cellular surface has also been described for herpes simplex virus (10) and probably is operative in the case of Rous sarcoma virus (23). In cells infected by herpes simplex virus, however, the walls of the vacuoles seem to originate from nuclear membranes, which envelop the virus as it passes into the cytoplasm, whereas the vacuoles enclosing Rous sarcoma virus appear to arise from the endoplasmic reticulum.

In the paper by Bernhard *et al.* (24, see Fig. 9), and more recently in the papers by Moore *et al.* (26, see Figs. 4, 6, and 7) and Lasfargues *et al.* (27, see Figs. 3 to 5), stages in the emergence of the mammary tumor virus have been illustrated which show a striking resemblance to those shown for WEE virus in Figs. 22 to 24. Moreover, as noted earlier, extrusion through the cellular wall of Rous sarcoma virus (23) and viruses associated with avian erythroblastosis (21) and murine leukemia (22) probably occurs in a similar manner.

Although influenza virus differentiates and is extruded primarily at the surface of cells (20), unpublished studies in this laboratory reveal that virus can also form at the margins of intracytoplasmic vacuoles. Further analogy to WEE virus is provided by the presence of filaments[6] and by the appearance of regular arrays of virus on the surface, suggesting that crystallization may have taken

[6] Filaments have also been observed within aggregates of reovirus (35).

place (39). It should be emphasized, however, that discrete precursor particles have not been encountered in cells infected with influenza virus.

As previously described, necrosis and dissolution of the host cell with release of WEE virus may intervene at any stage of infection. This mechanism of release is also well exemplified in the reproductive cycle of the adenoviruses (7, 43).

VIII. Cytopathic Effect.—WEE virus does not cause a unique, pathognomonic lesion. Pyknosis of the nucleus, condensation and vacuolization of the cytoplasm, disintegration of mitochondria, and rupture of the cellular wall may occur in control cells as well as in cells infected with other viruses (14). Preliminary histochemical studies by Dr. Gabriel C. Godman reveal that many cells infected with WEE virus have abnormally large amounts of ribonucleoprotein in their cytoplasm. This phenomenon is undoubtedly reflected in the electron microscope by the abnormal accumulation of cytoplasmic granules so often encountered.

SUMMARY

Stages in the development and release of Western equine encephalomyelitis virus are illustrated and described. It is suggested that precursor particles 22 mμ in diameter differentiate at template sites close to membranes bordering cytoplasmic vacuoles and that these particles either pass into the lumen of the vacuole, acquiring in the process a coat and peripheral membrane, or are dispersed in the cytoplasm and extruded through the cellular wall, emerging as viral particles on the surface. Although necrosis and dissolution of the cell with release of contents, including virus, may intervene at any stage of infection, ejection of virus from the vacuoles presumably can occur without rupture of the cell. The virus consists of a 30 mμ core separated by a zone of lesser density from a sharply defined peripheral membrane 45 to 48 mμ in diameter. Precursor particles, as well as viral particles, occasionally crystallize, the former in the cytoplasm, the latter in vacuoles and probably on the cellular surface.

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EXPLANATION OF PLATES

PLATE 25

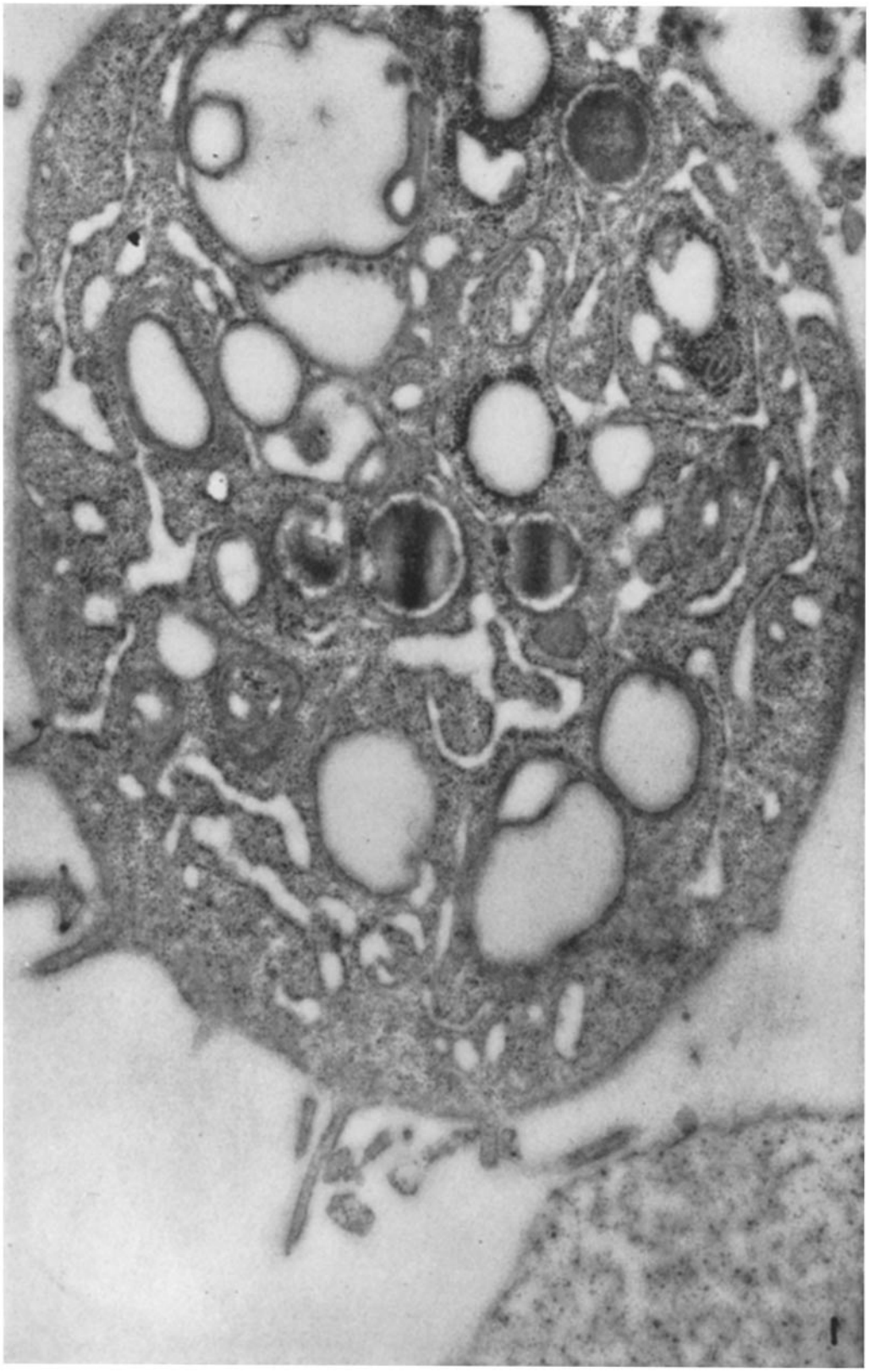
FIG. 1. A relatively early stage of infection. The cytoplasm contains granules and many vacuoles, some of which are elongated, others oval. The latter are lined by laminated membranes or (as in the upper right) surrounded by dense particles. The nucleus lies outside the plane of section. × 34,000.

PLATE 26

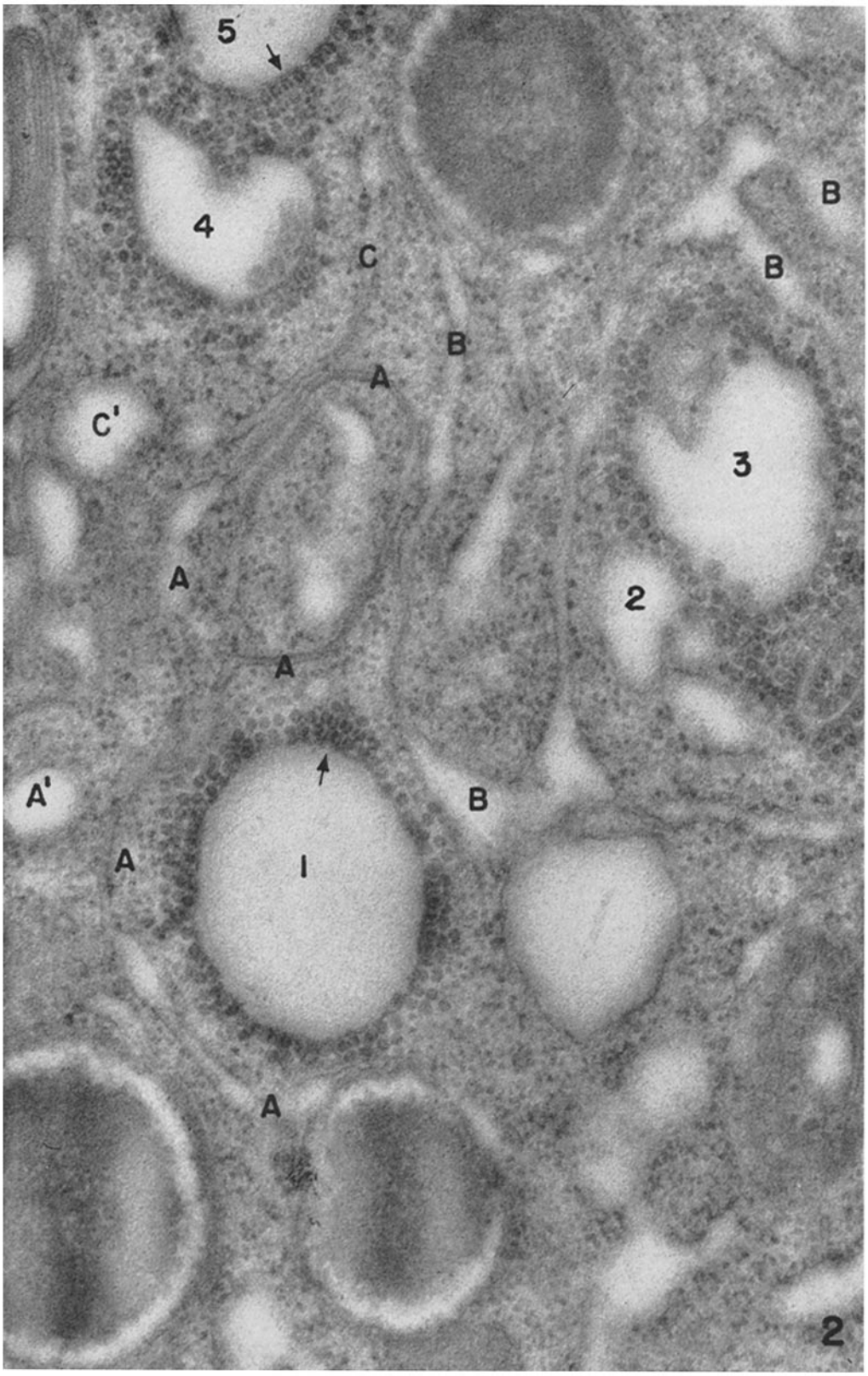
FIG. 2. The upper right portion of the preceding micrograph at higher magnification. The numbered vacuoles are partially or completely surrounded by dense particles, many of which exhibit a translucent center. In the areas indicated by arrows some of the particles appear to be incomplete. There are three distinct vacuolar systems (lettered *A*, *B*, and *C*) of cleft-like spaces interconnected by double membranes. × 84,000.

PLATE 27

FIGS. 3 and 4. Consecutive serial sections of part of the preceding field. Three of the oval vacuoles contain viral particles. Comparison of all three serials reveals that several vacuoles (marked prime), which seem at one level to be discrete, actually communicate with their respective systems. The oval vacuoles bordered by dense particles, however, remain discrete in each section. × 84,000.



(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)



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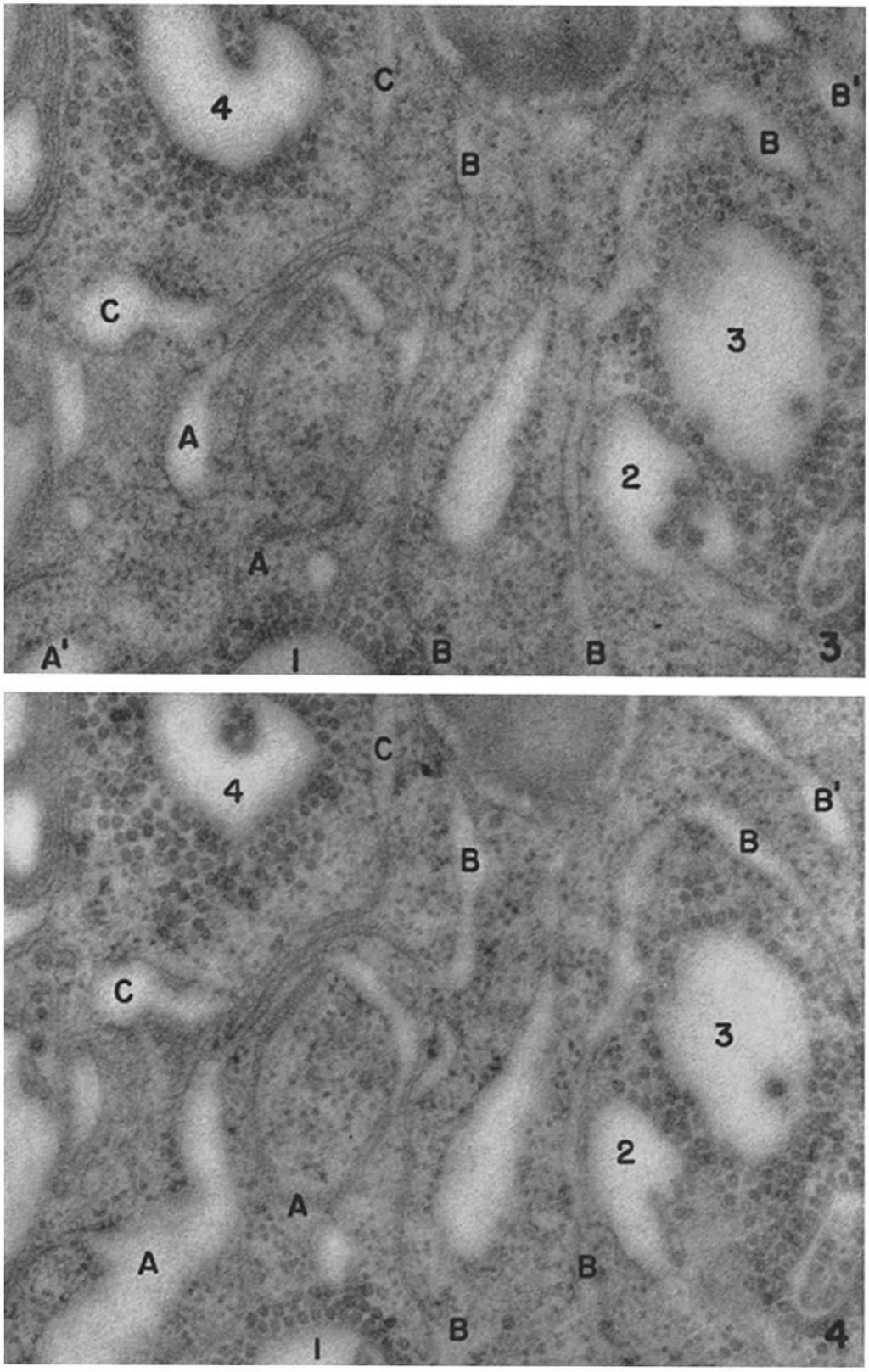
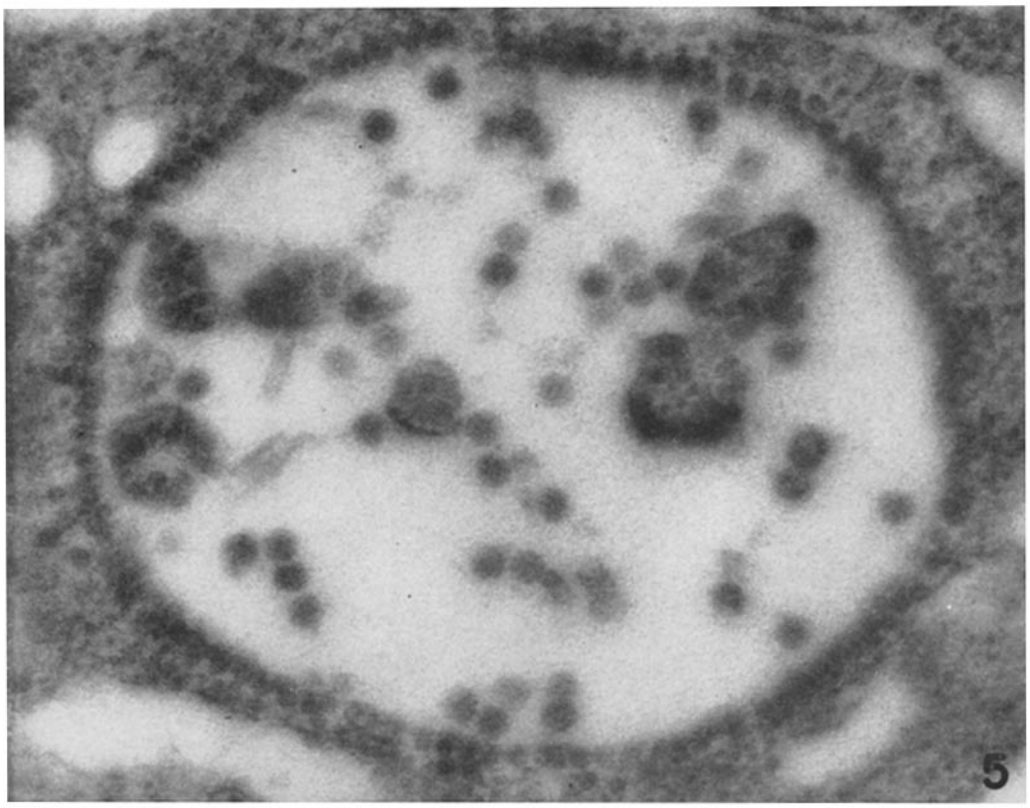


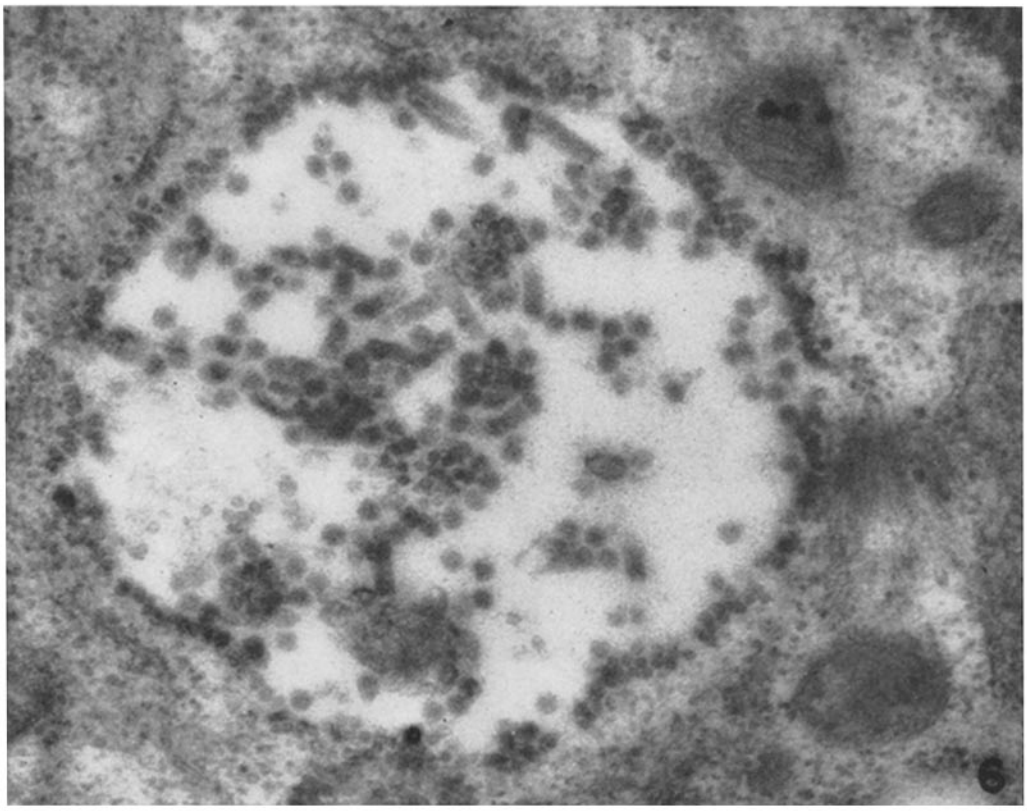
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 28

FIG. 5. A vacuole lined by dense particles and containing virus with an average diameter of 45 mμ. The thickness of the section precludes clear resolution of viral structure. Several forms suggestive of filaments are visible on the left. The surrounding cytoplasm exhibits numerous dense granules. (This cell is shown at low magnification in Fig. 7). × 110,000.

FIG. 6. A large vacuole enclosing virus, short filaments and clusters of small particles. The particles at the margin vary in size and form a discontinuous lining. Three mitochondria and a bundle of fine cytoplasmic filaments are visible on the right. × 67,000.



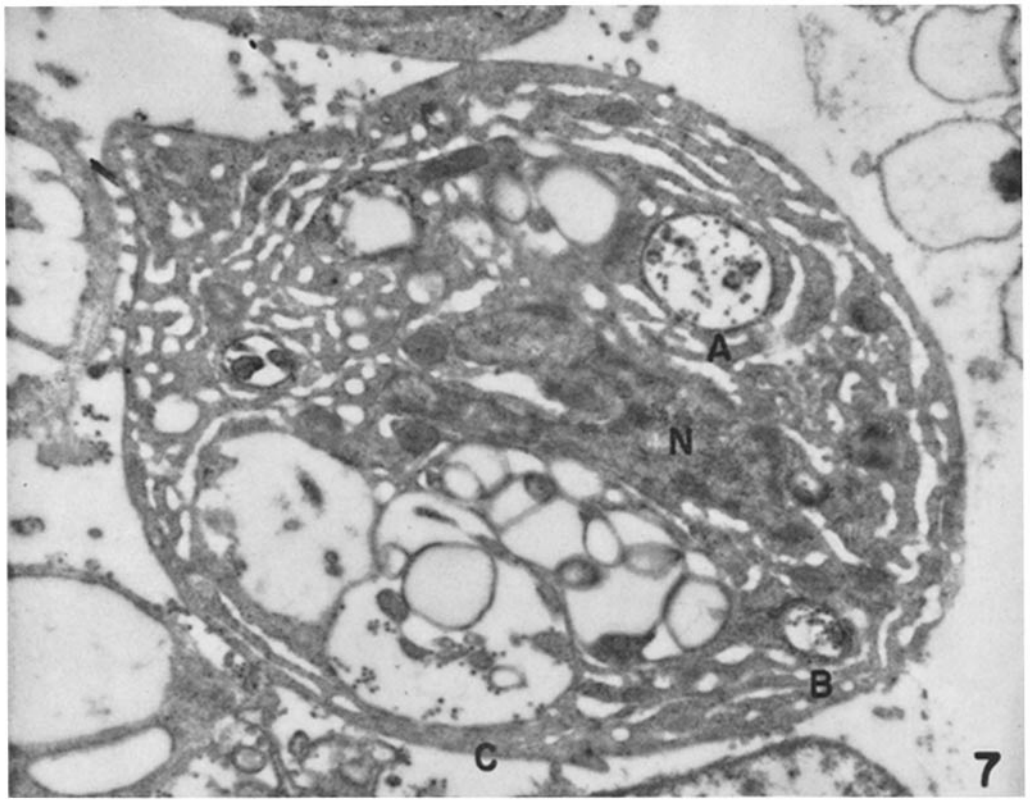


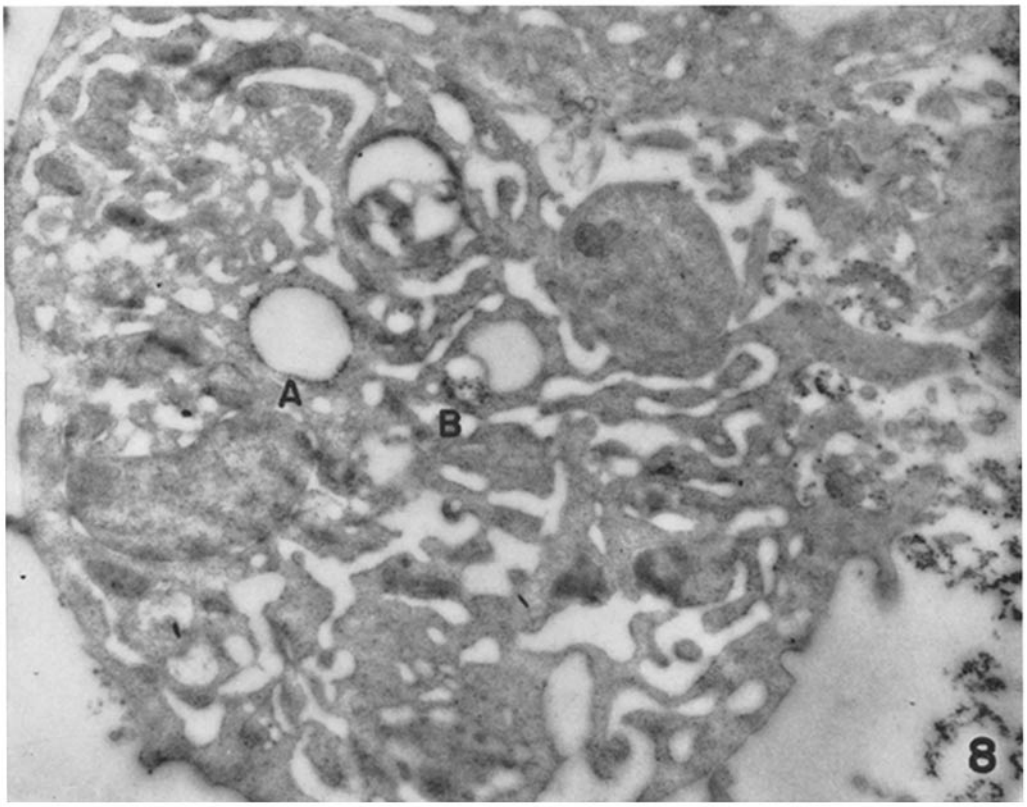
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 29

FIG. 7. A moderately advanced stage of infection. This vacuolated cell exhibits relatively dense cytoplasm and a shrunken, distorted nucleus (marked *N*). The three lettered vacuoles contain virus. Except at one point near the top the cellular wall appears to be intact at this level of sectioning. × 15,000.

FIG. 8. A cell with numerous cleft-like vacuoles and granular cytoplasm. Vacuole *A* is surrounded by dense particles. Vacuole *B* contains virus. The cytoplasm has disrupted on the right and virus is present in the extracellular space. × 15,000.





(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 30

FIG. 9. An advanced stage of infection characterized by very dense, vacuolated cytoplasm and a pyknotic nucleus (*N*). Aggregates of viral particles are visible in one vacuole in the upper third and on the irregular cellular surface at the top. × 15,000.

FIG. 10. Virus between extensions of the cytoplasm on the surface at the left and within a vacuole just beneath the cellular wall. Near the right margin precursor particles surround two vacuoles, which seem to have been cut eccentrically. Mitochondria and components of the endoplasmic reticulum are apparent. × 48,000.

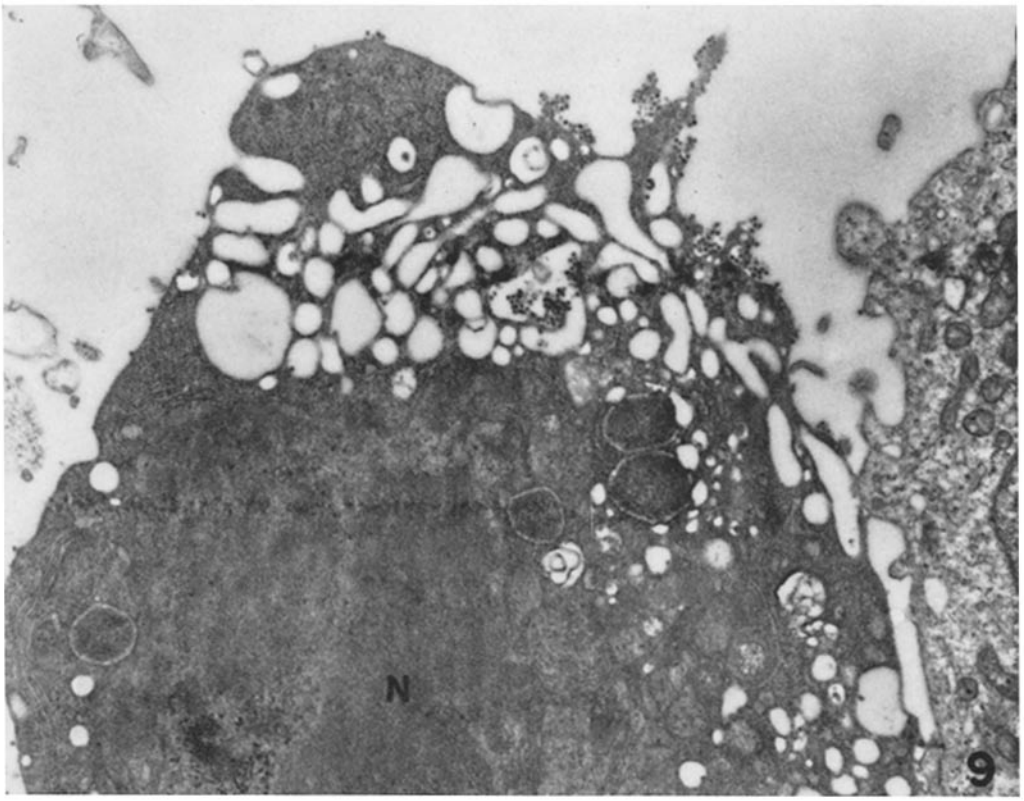
PLATE 31

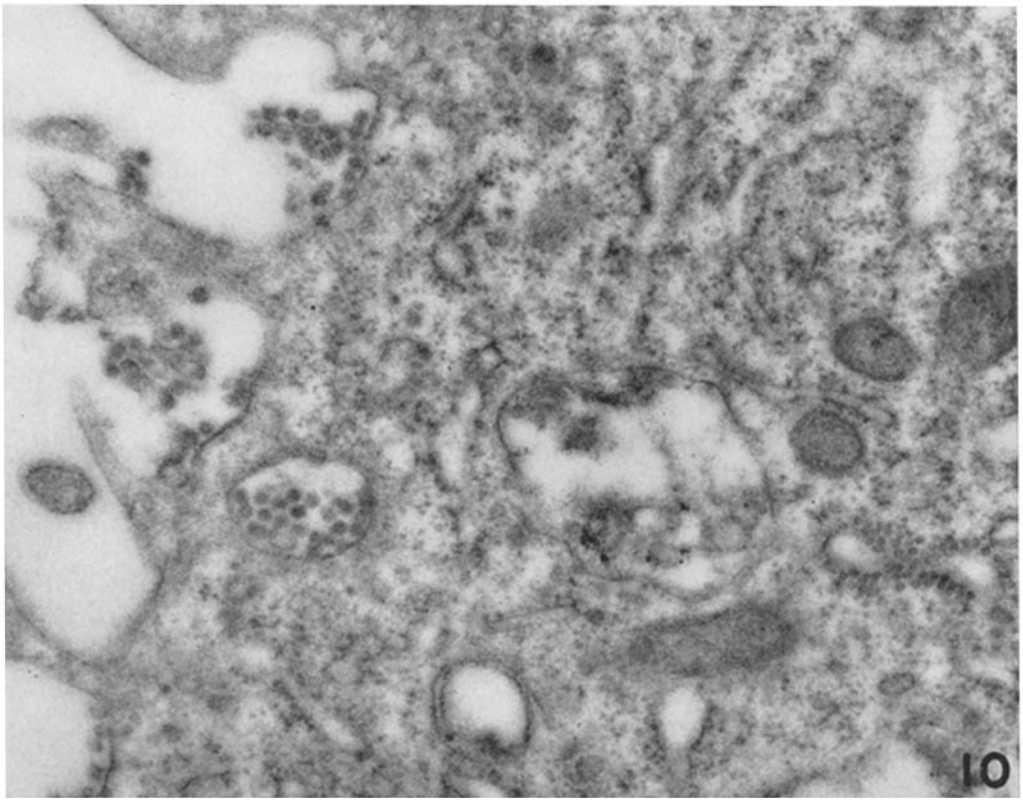
FIG. 11. A cell with granular, vacuolated cytoplasm. Several vacuoles in the lower half enclose virus. Clusters of extracellular viral particles lie within crypts, which appear to have been formed by rupture of vacuoles. Vacuole *A* and part of vacuole *B* are shown at higher magnification in the next two micrographs. This section was stained with lead. × 17,000.

PLATE 32

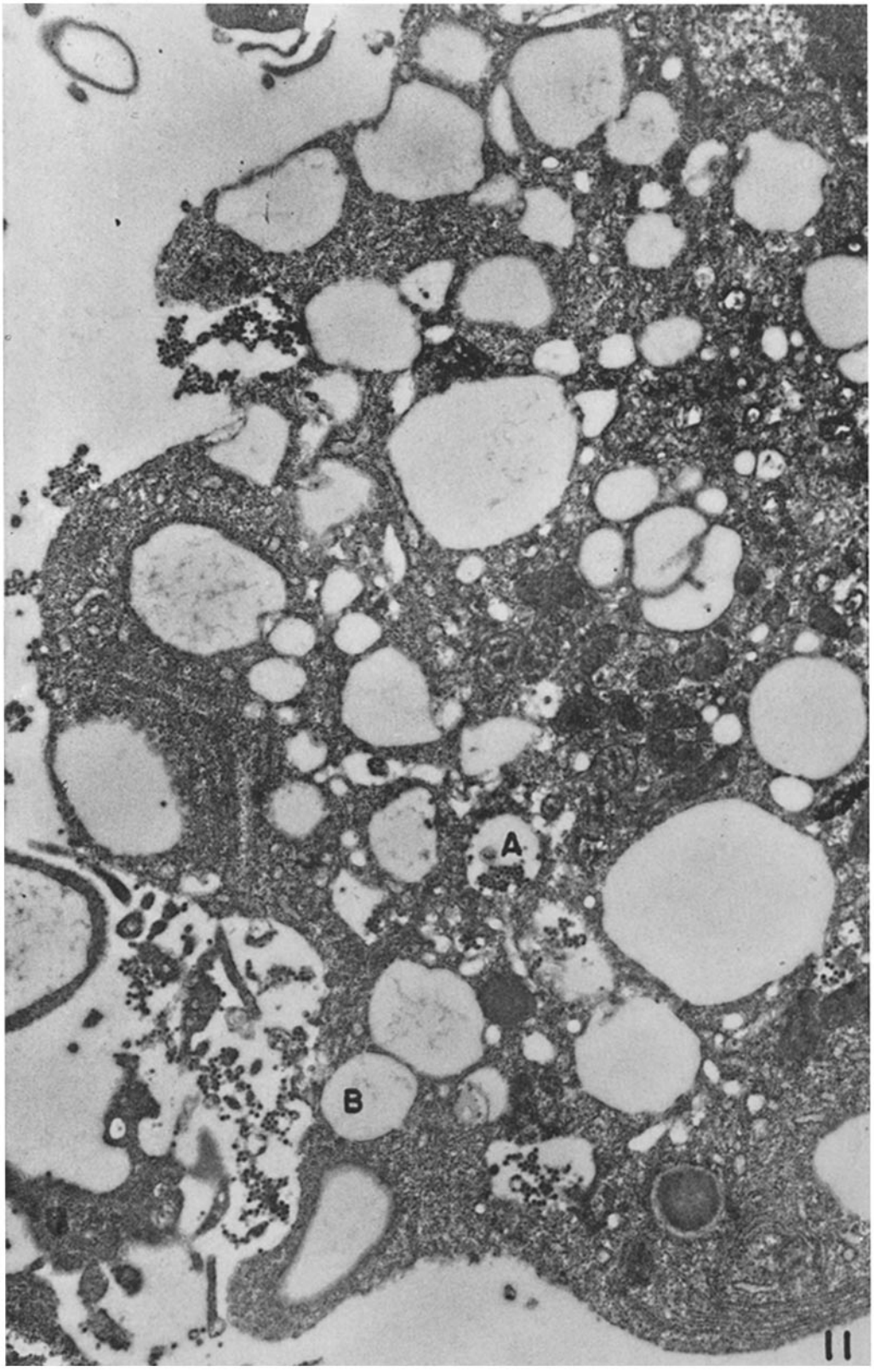
FIG. 12. A viral crystal within a vacuole. Particles central to the plane of section exhibit a 30 mμ core of variable density and a peripheral coat enclosed by a sharply defined membrane 45 to 48 mμ in diameter. × 100,000.

FIG. 13. Extracellular virus. Comparison with the preceding micrograph reveals the similarity in structure of extra- and intracellular virus. Two particles in the small viral crystal (below and to the left of center) appear to have disrupted. × 100,000.

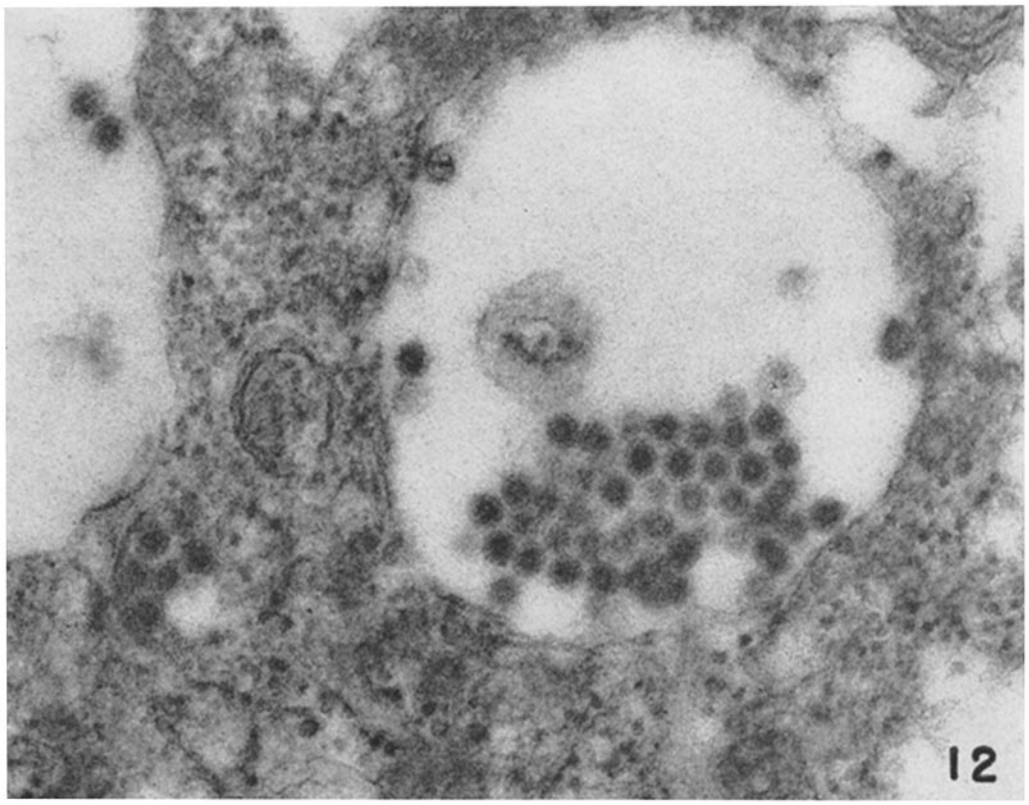


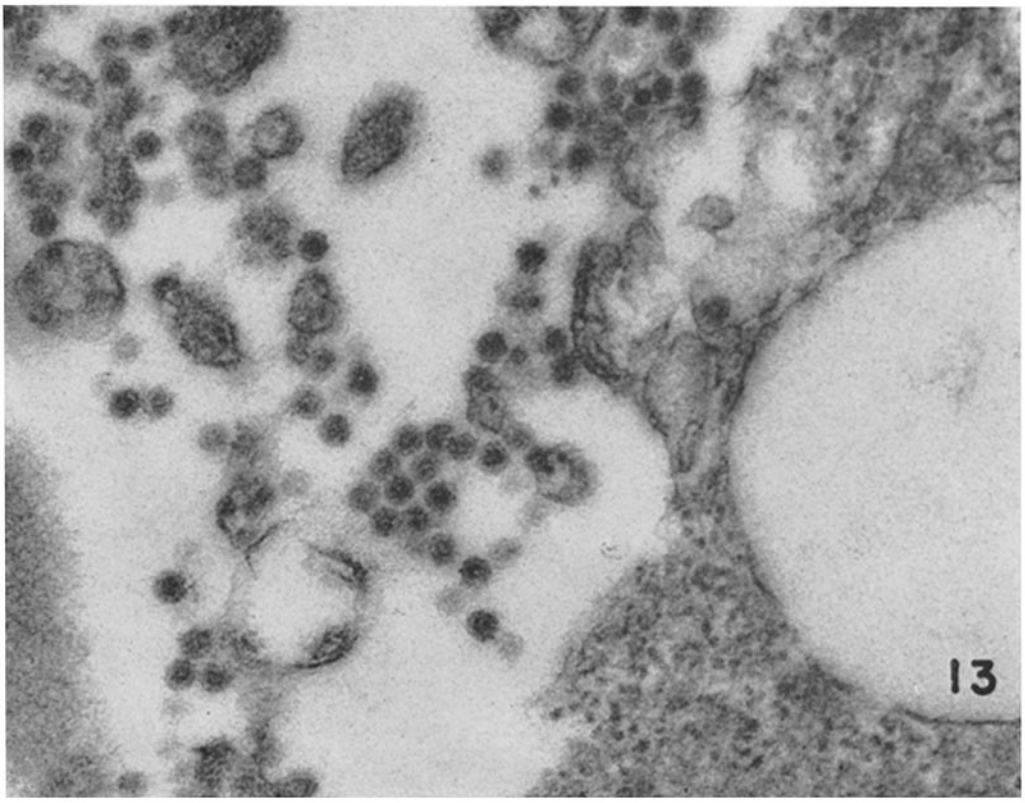


(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)



(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

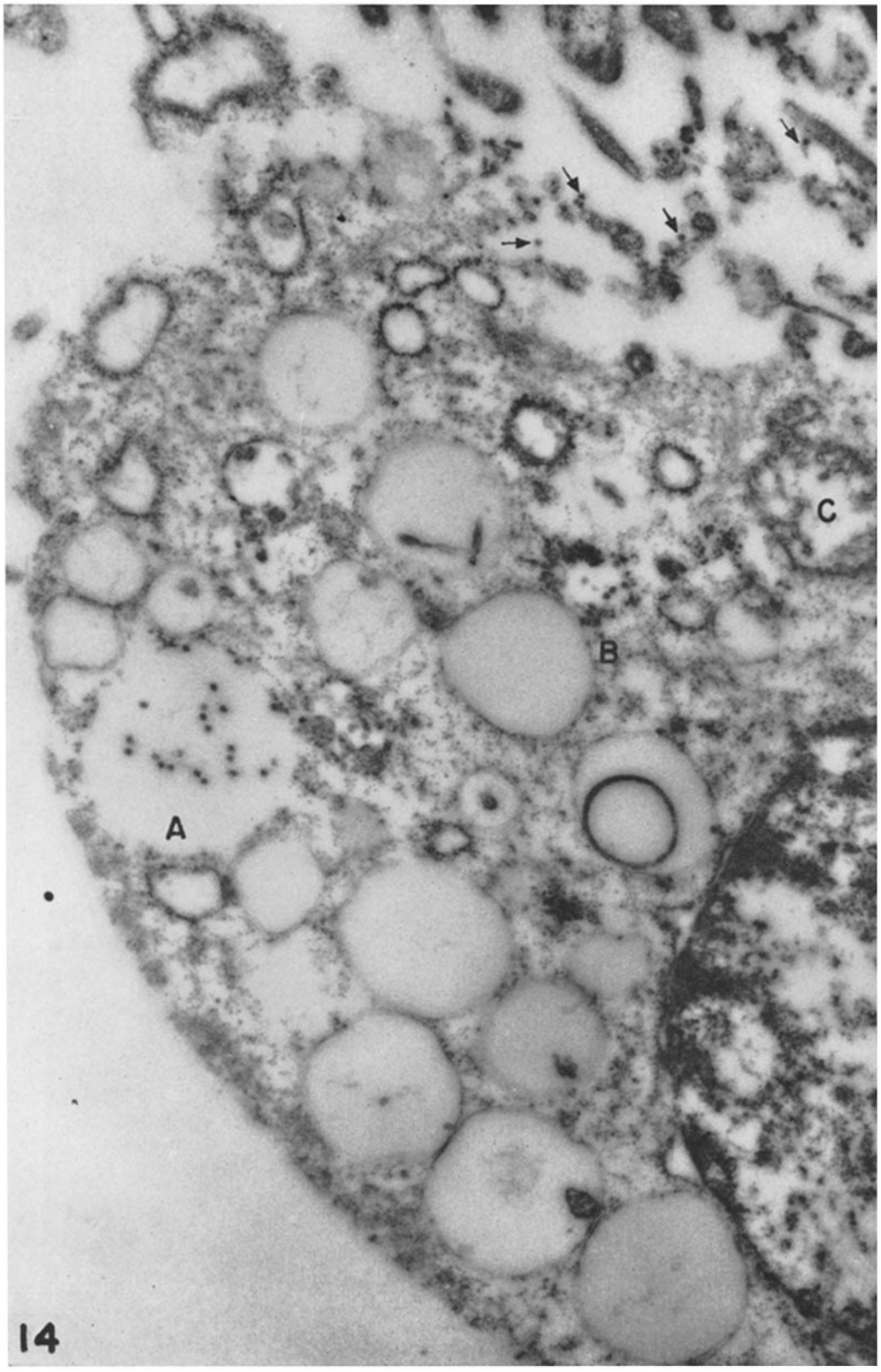




(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 33

FIG. 14. A cell in process of dissolution with release of contents. Extracellular viral particles dispersed between fragments of cytoplasm are indicated by arrows at the top. The lumen of vacuole *A* appears to communicate with the extracellular space, and virus is present in the cytoplasm above the letter *B*. Vacuole *C* (above the nucleus at the right margin) probably represents a vacuolated mitochondrion containing remnants of fragmented cristae. The chromatin of the nucleus is clumped. The nuclear membranes are distorted and, at the top, seem to have ruptured. × 25,000.

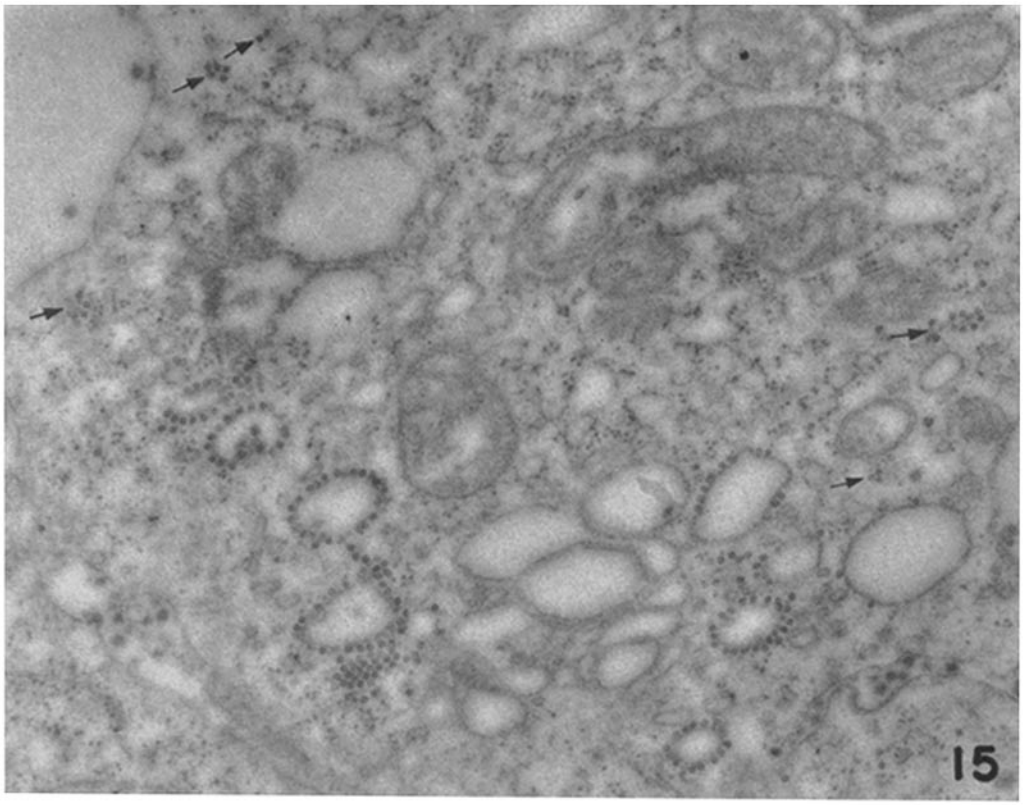


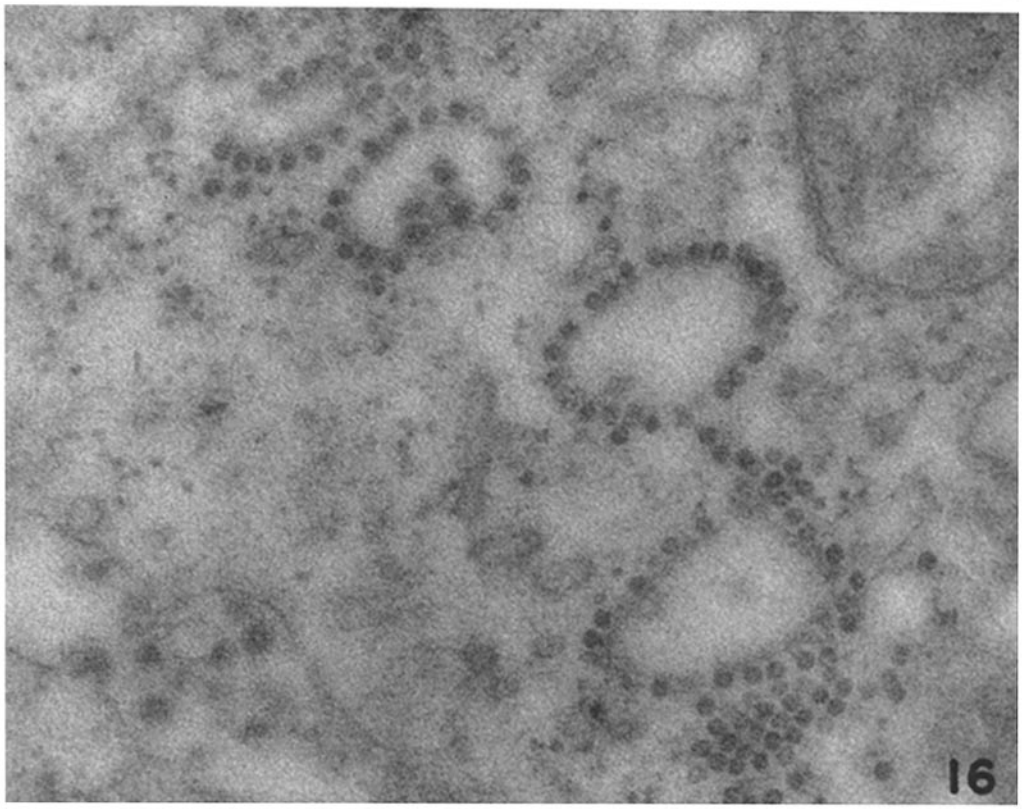
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 34

FIG. 15. Cytoplasm containing characteristic dense particles. Some of these partially or completely surround oval vacuoles, whereas others form scattered aggregates (indicated by arrows) which exhibit no consistent orientation to cytoplasmic components. Virus lies within the intercellular cleft at the lower left and right corners. × 40,000.

FIG. 16. The lower left portion of the preceding micrograph at higher magnification. Particles partially enclose four vacuoles. The vacuole at the top left appears to be open. Another, adjacent to it, is devoid of lining particles in one region contiguous to a cluster of virus within the lumen. Particles in the lower right corner exhibit a regular array. Extracellular virus is visible at the lower left. One particle seems to be in process of emerging from an adjacent cell in a manner analogous to that illustrated in Figs. 22 to 24. × 100,000.





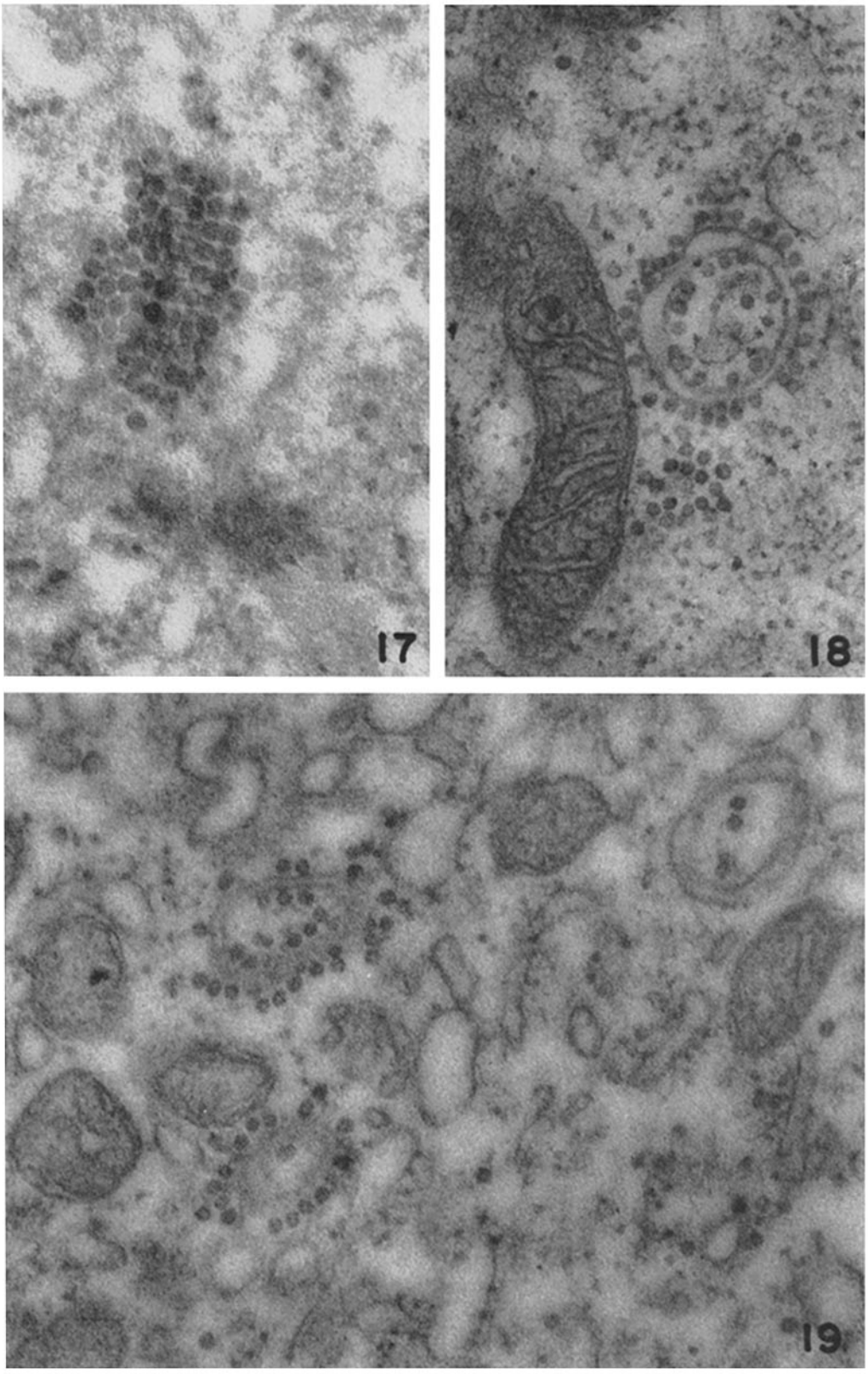
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 35

FIG. 17. Two contiguous crystals composed of primary particles. This micrograph illustrates one of a series of serial sections in which the ordered array was encountered at different levels and thus shown to exist in three dimensions. × 130,000.

FIG. 18. Dense particles scattered in the cytoplasm and concentrated on opposite sides of two concentric lamellae, which lie near a mitochondrion. The section was stained with lead. × 100,000.

FIG. 19. Dense particles bordering concentric lamellae, which have been transected at different angles and hence vary in definition. Several small, distorted mitochondria are visible. Comparison with the preceding picture reveals the relative degree of contrast resulting from lead staining. × 100,000.

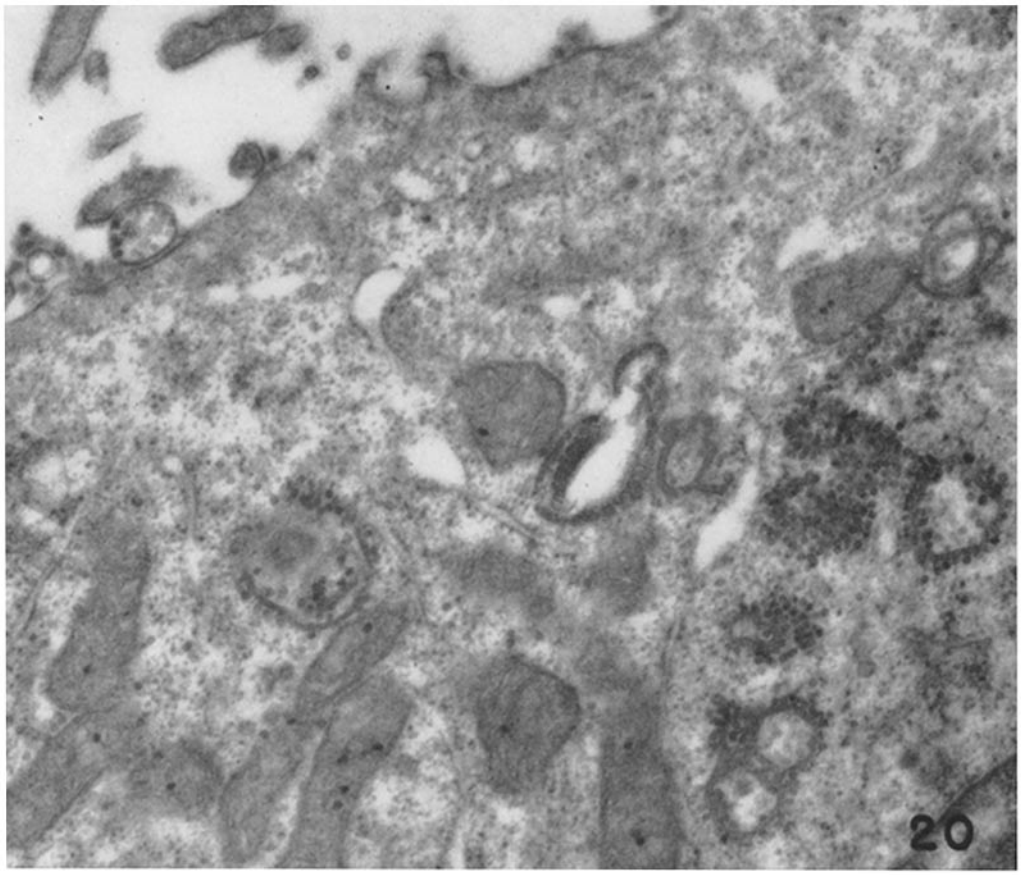


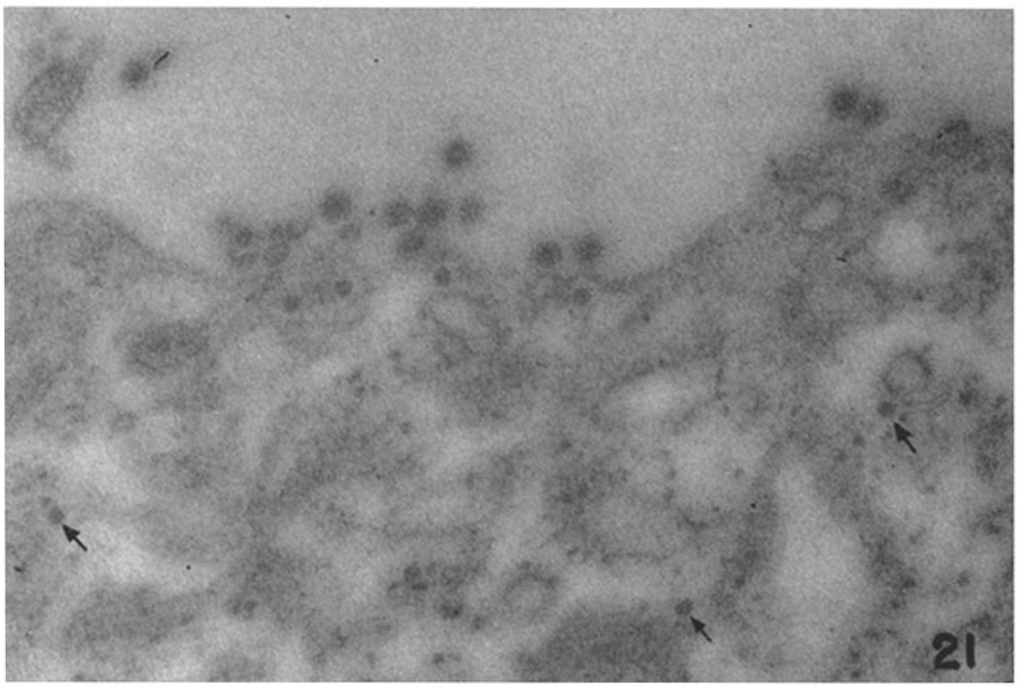
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 36

FIG. 20. Cytoplasm containing mitochondria, scattered granules, and oval vacuoles with double lamellae bordered by dense particles. On the cellular surface at the upper left are viral particles as well as a cluster of small particles enclosed by a sharply defined, single membrane. × 44,000.

FIG. 21. Characteristic particles scattered in the cytoplasm (arrows) and lined up at the surface just beneath extracellular virus. This and the micrographs shown in the next plate illustrate unusually thin sections. × 100,000.





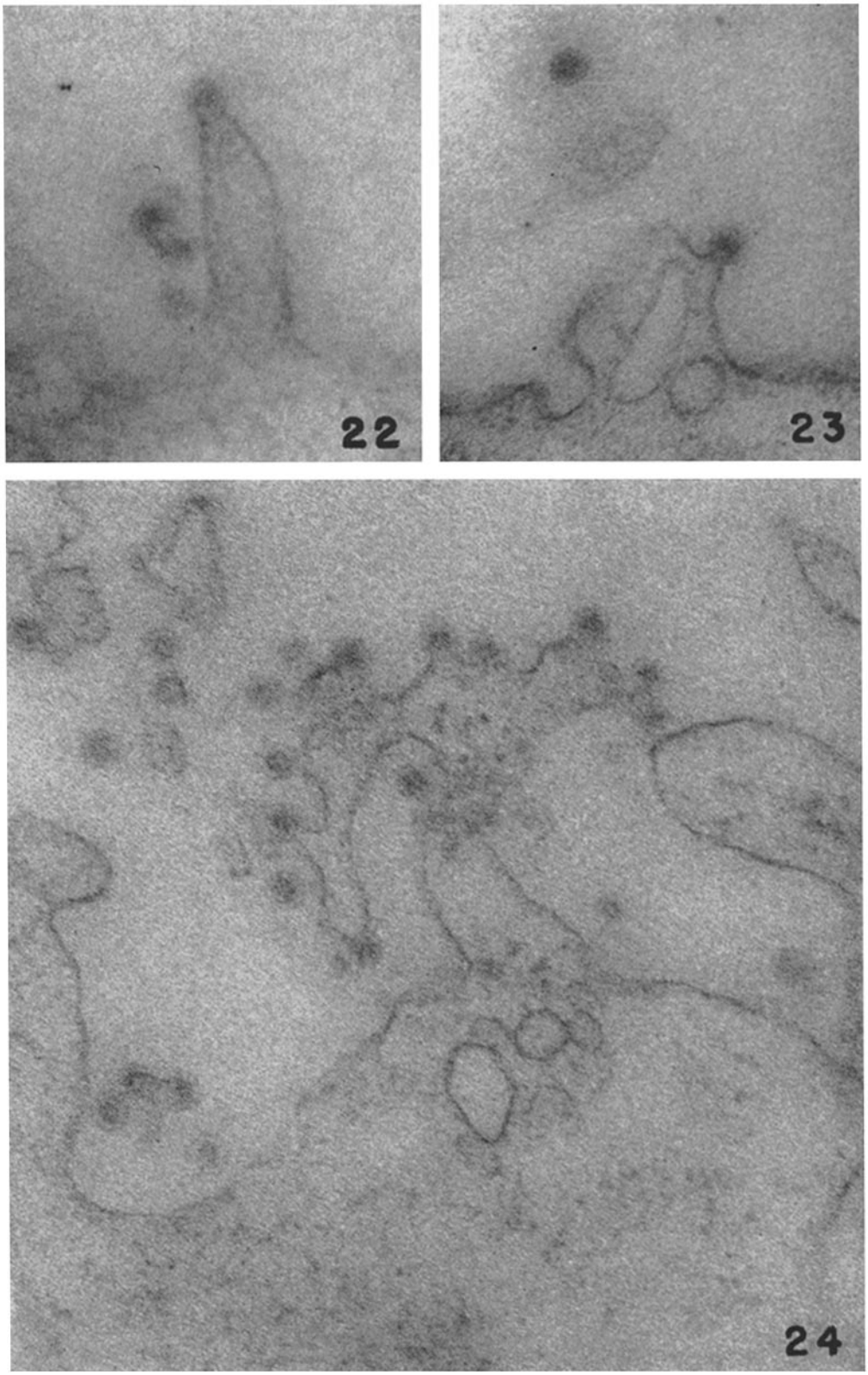
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 37

FIG. 22. A dense particle surrounded by a diffuse coat at the tip of a cytoplasmic extension. A fine membrane, which is concave with respect to the cell, appears to separate the particle from the cytoplasm. × 145,000.

FIG. 23. Virus believed to be in process of emergence. The cellular wall of the irregular cytoplasmic extension appears to have opened. An extracellular viral particle is visible in the upper portion of the field. × 145,000.

FIG. 24. Virus adjacent to a large cytoplasmic extension. Close to several particles the wall is open, extending outward in a manner similar to that shown in Fig. 23. × 145,000.



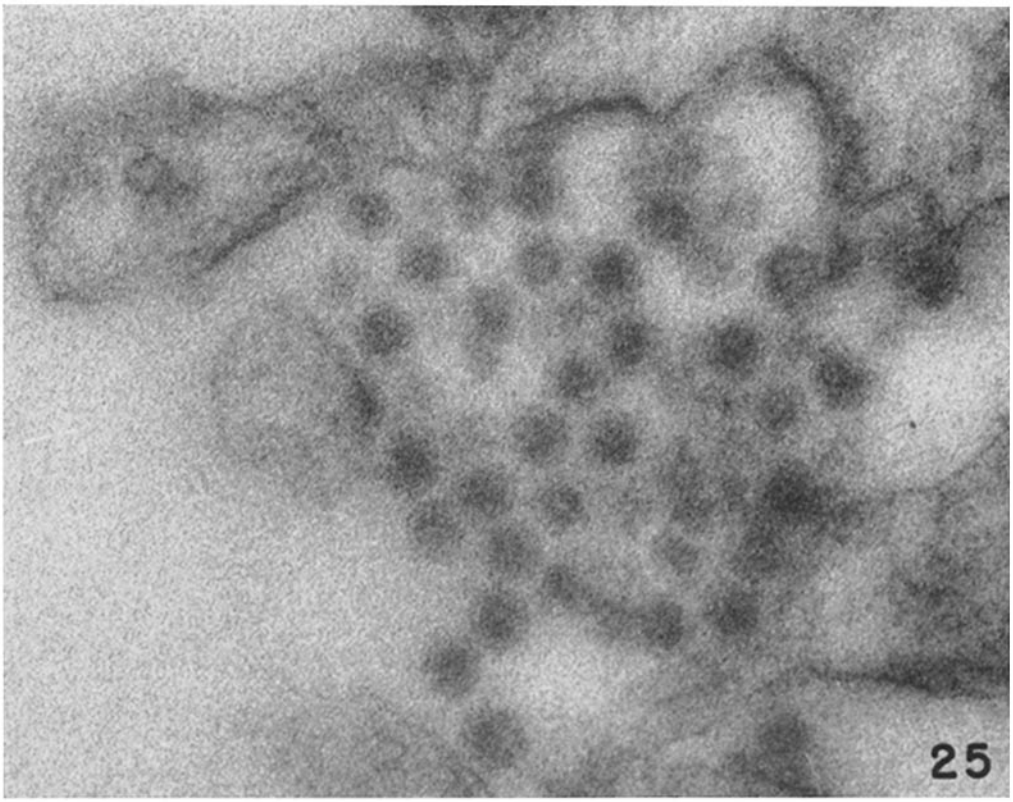
(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

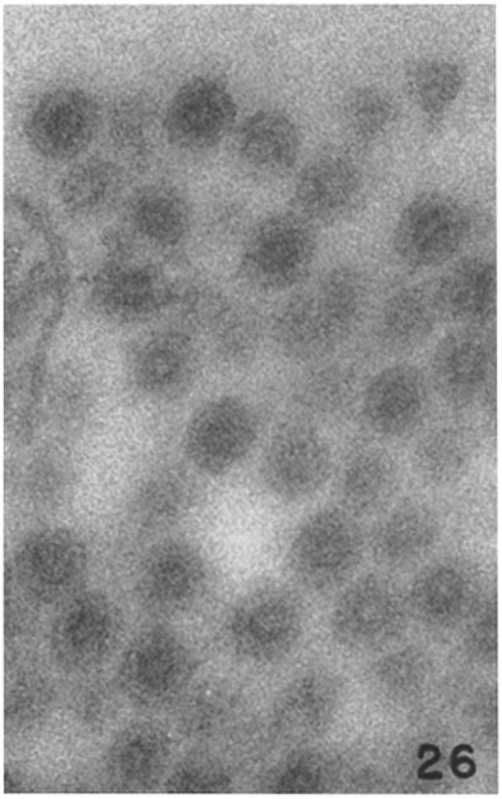
PLATE 38

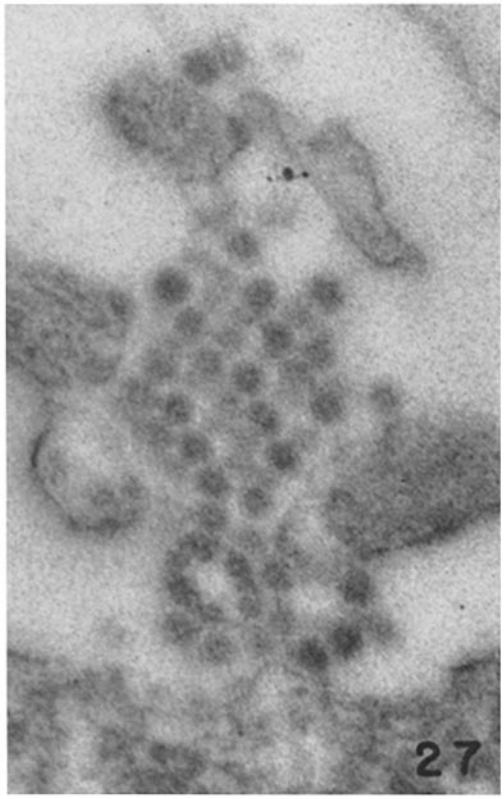
FIG. 25. Extracellular virus near several cytoplasmic extensions which have been cut at different angles. Some particles can be seen to possess a sharply defined peripheral membrane. This is a relatively thick section. × 196,000.

FIG. 26. Extracellular virus in a thin section which has been lead-stained. Particles central to the plane of section exhibit a 30 mμ core of variable density surrounded by a coat and a peripheral membrane 45 to 48 mμ in diameter. × 200,000.

FIG. 27. An extracellular viral crystal. Close to the cellular surface at the bottom is a cluster of particles which appear to line the inner aspect of an oval membrane. × 100,000.



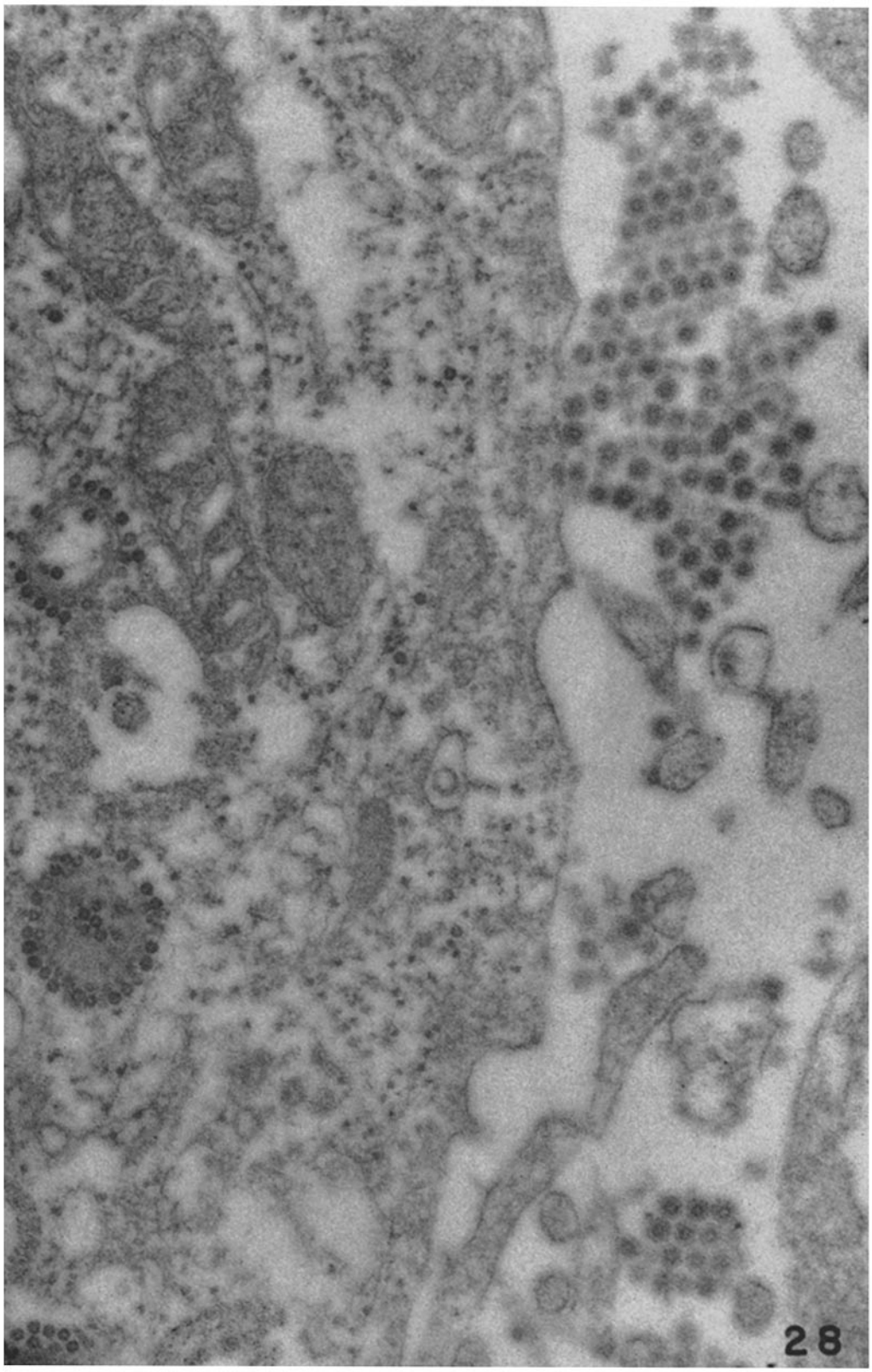




(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)

PLATE 39

FIG. 28. Two extracellular crystals of virus in a lead-stained section. The larger crystal at the top of the field is somewhat distorted, but in the upper portion the regular pattern of distinct particles undoubtedly reflects the angle at which the lattice was cut. The cellular wall is indistinct adjacent to this crystal, suggesting the possibility that virus had emerged in this area. Dense precursor particles are scattered in the cytoplasm and are present on opposite sides of two concentric lamellae near the left border. Below, dense particles indicate by their arrangement that they border lamellae which have been sectioned obliquely and hence are indistinct. × 86,000.



(Morgan *et al.*: Electron microscope study of encephalomyelitis virus)