OBSERVATIONS ON THE MODE OF RELEASE OF HERPES VIRUS FROM INFECTED HeLa CELLS

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ABSTRACT

The development of a well adapted strain of herpes virus has been studied in HeLa cells using thin sectioning techniques for electron microscopy. Particular attention was directed to events in the cytoplasm and certain new features were observed. Profuse immature particles with a nucleoid and single limiting membrane were present in the nuclei of infected cells, often in crystalline array; morphologically indistinguishable immature particles were also found very frequently in the cytoplasm. Cells with such particles were intact and well preserved, and contained smooth vacuoles apparently derived from the Golgi component of the endoplasmic reticulum. The cytoplasmic particles escaped from the cells by bulging out as buds through the cell membrane or through that of the cytoplasmic vacuoles until they were attached only by a pedicle and then became free. During this process the particles were gradually enclosed by the membrane through which they passed and carried a coat of it with them as they matured. After permanganate fixation the triple-layered structure of the cell membrane and vacuolar membranes was evident and was identical with that of the outer coat of the mature virus. These findings are discussed both in relation to different types of virus structure and to function in the endoplasmic reticulum and cell membrane.

Early morphological studies on herpes simplex infection using thin sectioning methods for electron microscopy (1–3) gave results which agreed on two important points. Firstly, the virus formed in the nucleus of infected cells, and appeared there as a particle having a single membrane around a dense nucleoid; and secondly, the mature extracellular virus was larger, and apparently possessed two membranes. However, the maturation process and mode of release of the agent remained obscure.

The maturing particle was at first believed to acquire its second coat in the cytoplasm (1), but forms with this coat were subsequently observed within nuclei (2, 3), in groups close to the nuclear envelope, and surrounded by a membrane derived perhaps from the latter by invagination (3). More recently, elaborate infoldings and reduplications of the nuclear envelope have been found in infected cells (4, 5) and these are, at present, thought to provide a means for the virus to pass out of the intact nucleus (4), possibly by budding (5), and to be the source both of the second membrane of the agent and of the sacs known to surround it in the cytoplasm (4, 5).

In the course of an investigation into the structure and composition of mature extracellular herpes virus (6), immature particles were frequently seen lying free in the cytoplasm of undamaged, morphologically intact, infected HeLa cells. This unusual finding did not seem to fit in with current views on the developmental cycle of the agent (4, 5) and it was therefore considered

that further study of the phenomenon might be of value, particularly as regards its cytoplasmic stages and the mechanism whereby the mature virus escapes from the cell. A line of HeLa cells whose usual fine structure was known (7) was accordingly infected with a well adapted strain of herpes virus and then examined in thin sections with the electron microscope; the observations made on this material are reported in the present communication.

MATERIALS AND METHODS

Maintenance and Infection of HeLa Cultures

HeLa cells were grown in flat glass bottles by methods already described (8, 9), and when confluent sheets of cells developed they were infected with a heavy dose of a stock seed of the HFEM strain of herpes virus exactly as in earlier work (6).

Preparation of Cells for Electron Microscopy

In each experiment the cultures were harvested when they showed advanced cytopathic changes 2 to 3½ days after infection. The cells were collected, fixed in osmium tetroxide or permanganate (10), embedded in *n*-butyl methacrylate or Aquon (11), sectioned, and examined in the electron microscope, using techniques reported elsewhere (6, 7). Most of the work was done with osmium-fixed, methacrylate-embedded material, the permanganate preparations being used to confirm and amplify the findings. Permanganate-Aquon sections were stained with lead hydroxide (12, 13).

OBSERVATIONS

General Features

When examined between 2 and 3½ days after infection under the conditions used, the cultures were starting to detach from the glass and were found to contain large numbers of intact cells together with degenerating cells and debris. At this stage almost all the cells were associated with virus, a few having large intranuclear viral crystals (Fig. 1), and most others profuse particles in various sites or very close to their plasma membranes (Figs. 2 to 10).

As has already been reported, the infected cells possessed many long microvilli (6), and often showed considerable development of cytoplasmic membranous elements (4), in contrast to the uninfected state (7). These cytoplasmic membranes were either rough cisternae of the endoplasmic reticulum in abundant parallel arrays, or elaborate forms of paired cytoplasmic cisternae (7), or large smooth vacuoles concentrated in the Golgi zone and apparently derived from this component of the endoplasmic reticulum (Figs. 2 to 6, and 10). Some infolding and reduplication of the nuclear envelope (4, 5) was also observed. A number of unusually large cells could be found in each infected culture.

Intranuclear Particles

Particles with a dense nucleoid and single limiting membrane were very frequently present

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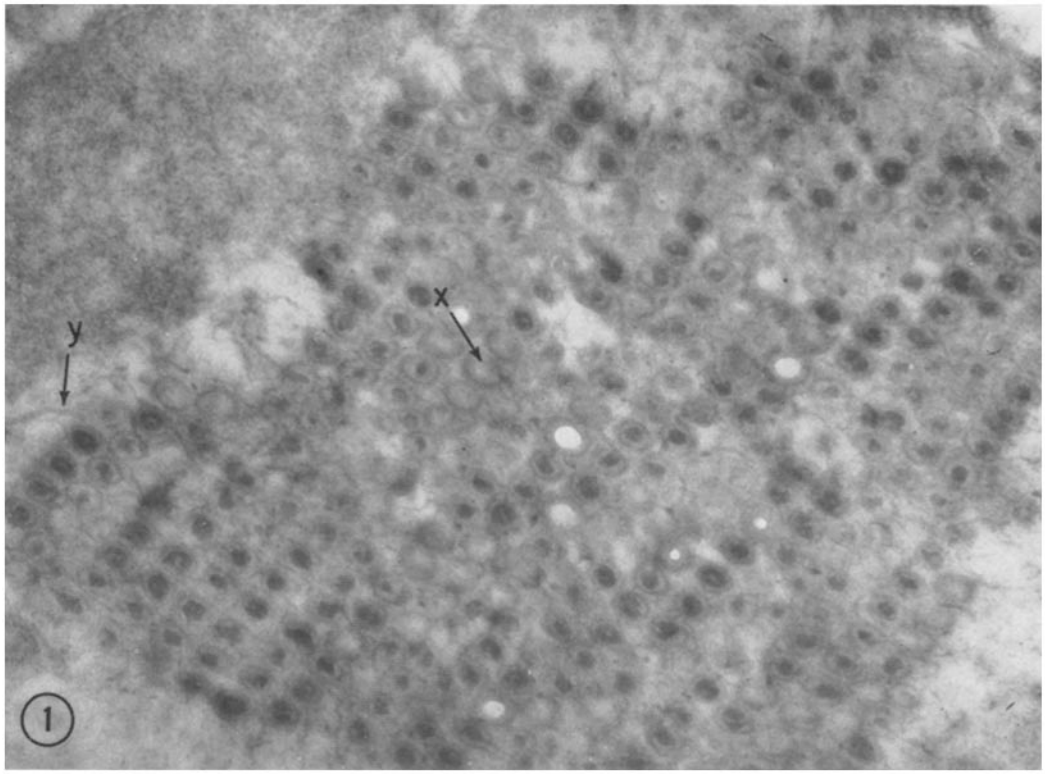
All the figures are electron micrographs of thin sections of HeLa cells from cultures infected with herpes simplex virus.

FIGURE 1

Small area of nucleus containing a mass of immature virus particles packed in orderly, crystalline array. The particles consist of an electron-opaque nucleoid surrounded by a single membrane; empty virus membranes can be seen at *x* and there are irregular membranes running between the particles as at *y*. Part of the nuclear envelope lies just within the upper left corner of the field. Osmium fixation; methacrylate embedding; unstained. × 56,000.

FIGURE 2

Portion of nucleus and adjacent cytoplasm. The nucleus (*n*) lies on the right bounded by a normal, unbroken, double membrane. Within the nucleus immature virus particles and empty viral membranes (*x*) are evident. The cytoplasm contains morphologically identical immature particles together with mitochondria (*m*) and smooth surfaced membrane-bounded vacuoles (*v*). Osmium fixation; methacrylate embedding; unstained. × 56,000.


y
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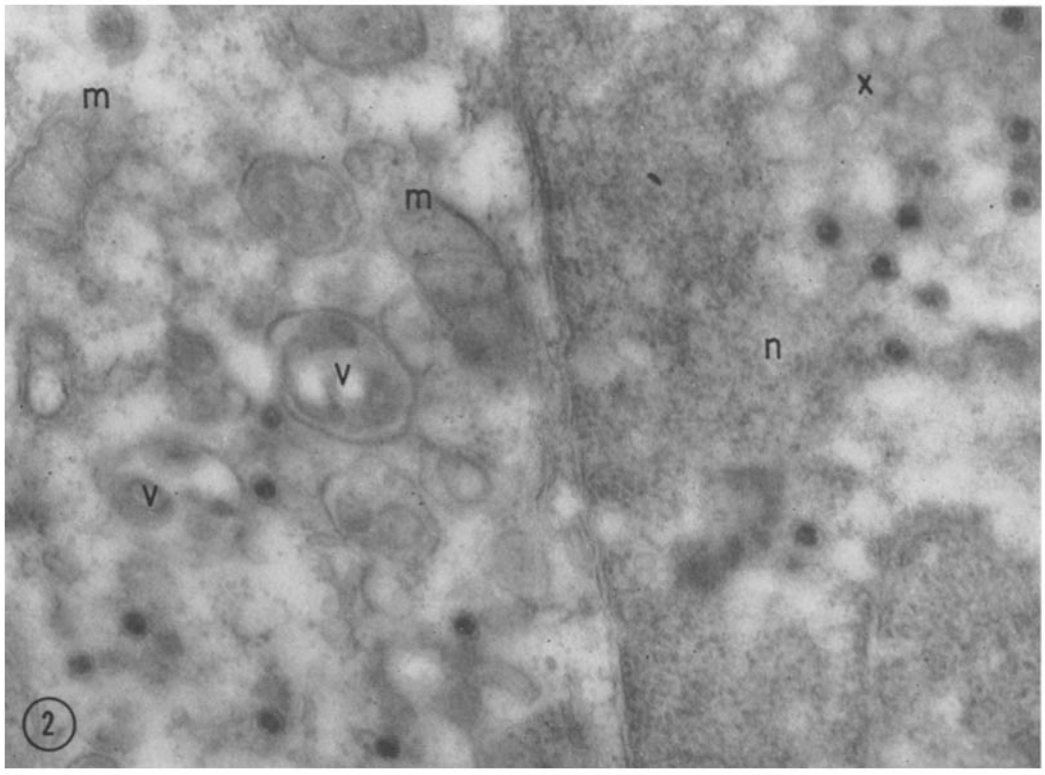
1


m
m
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2

in the nuclei, sometimes in the crystalline arrays mentioned above (Fig. 1), but more often in irregular masses or scattered less densely through a particular region (Figs. 2 and 3). Empty viral limiting membranes (Figs. 1 to 3), dense granules, and irregular membranes between or close to the particles (Fig. 1), such as have all been reported by Morgan *et al.* (4), were regularly encountered within nuclei. In addition, particles with an outer coat and the morphology of maturity (6) were also seen; they were rare, always near the nuclear envelope, and always in small groups enclosed by a membrane.

Cytoplasmic Particles

Well preserved cells with intact nuclear envelopes and immature particles free in the cytoplasm (Figs. 2 and 3) were a constant feature of the infected cultures. The particles were surrounded by a single limiting membrane (Figs. 2 to 8), were indistinguishable morphologically from immature virus found in the nucleus (1–5) (Figs. 1 to 3), and were often seen in the neighbourhood of smooth surfaced cytoplasmic vacuoles (Figs. 2 to 6). Where this association occurred individual particles could be found very close to the vacuolar membrane (Fig. 3), partly surrounded by the membrane and bulging into the interior of the vacuole (Fig. 4), or even lying within the vacuole almost entirely surrounded by its membrane except for a pedicle (Figs. 5 and 6). Apart from the pedicle, such particles were structurally identical with the mature form of the virus (1–6), numbers of which accumulated within the vacuoles (Figs. 3 and 10).

Other immature cytoplasmic particles were sometimes observed close to the surface of the cells (Fig. 7); here they could be seen beneath the cell membrane and partly surrounded by it as they bulged towards the exterior of the cell (Fig. 8), or merely attached by a pedicle (Fig. 9).

After permanganate fixation a triple-layered structure was evident in the vacuolar membrane (Fig. 10) precisely like that already observed in the cell membrane (6). The exact correspondence of the triple layering of the cell membrane with that of the outer viral membrane has already been noted (6) and a similar correspondence was found in the case of the vacuolar membrane (Fig. 10).

FIGURE 3

Part of the nucleus (*n*) above (right) and neighbouring cytoplasm. An immature virus particle and empty membranes are present in the nucleus and the intact nuclear envelope is well seen. Immature cytoplasmic virus, indistinguishable from that in the nucleus, tends to lie close to smooth surfaced vacuoles (*v'*, *v''*) some of which (*v''*, *v'''*) contain mature particles. Osmium fixation; methacrylate embedding; unstained. × 52,000.

FIGURE 4

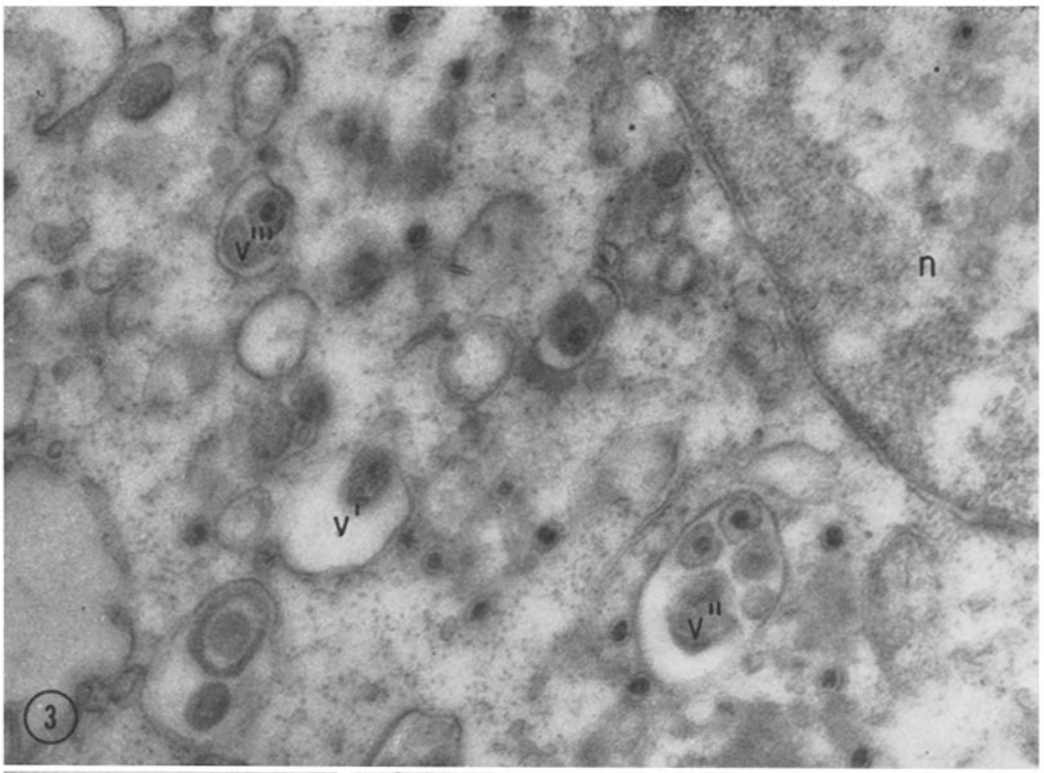
Small area of cytoplasm showing an immature particle beside a smooth surfaced vacuole (*v*). The particle bulges slightly into the vacuole and is half surrounded by the vacuolar membrane. Osmium fixation; methacrylate embedding; unstained. × 100,000.

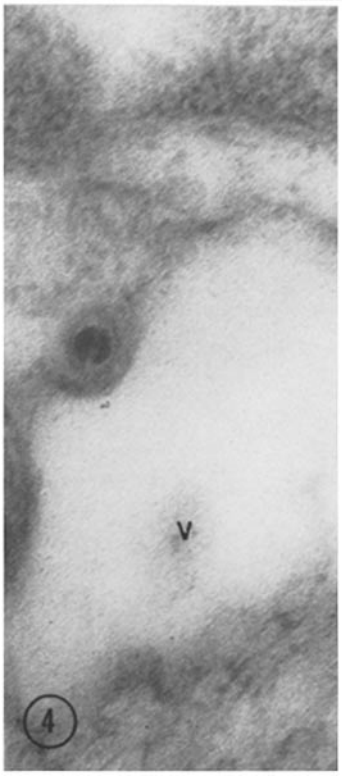
FIGURE 5

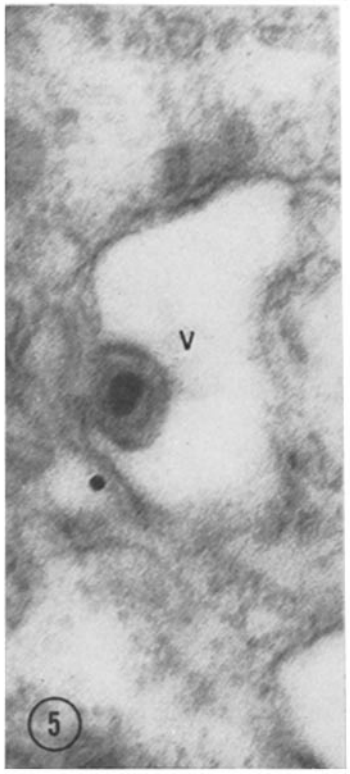
Field similar to that of Fig. 4. Here the particle protrudes farther into the vacuole (*v*) and is more completely surrounded by the vacuolar membrane. Osmium fixation; methacrylate embedding; unstained. × 100,000.

FIGURE 6

Area of cytoplasm like that shown in the two preceding figures. A maturing particle above is in the same stage as that of Fig. 5, whilst another, below, lies within a vacuole (*v*) attached only by a small pedicle; it is almost entirely enclosed by the vacuolar membrane and resembles the mature form of the virus. Osmium fixation; methacrylate embedding; unstained. × 90,000.


V'''
n
V'
V''
3


V
4


V
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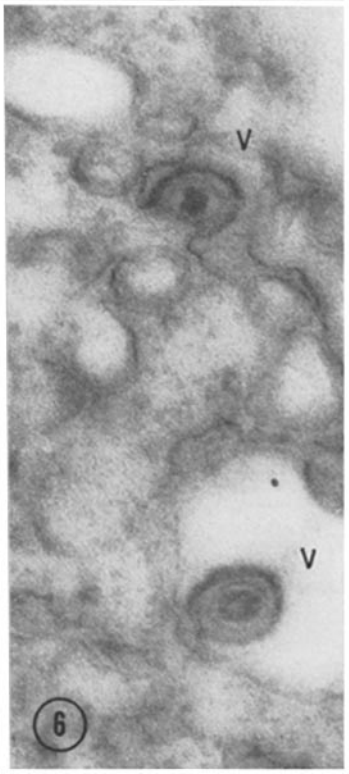
V
V
6

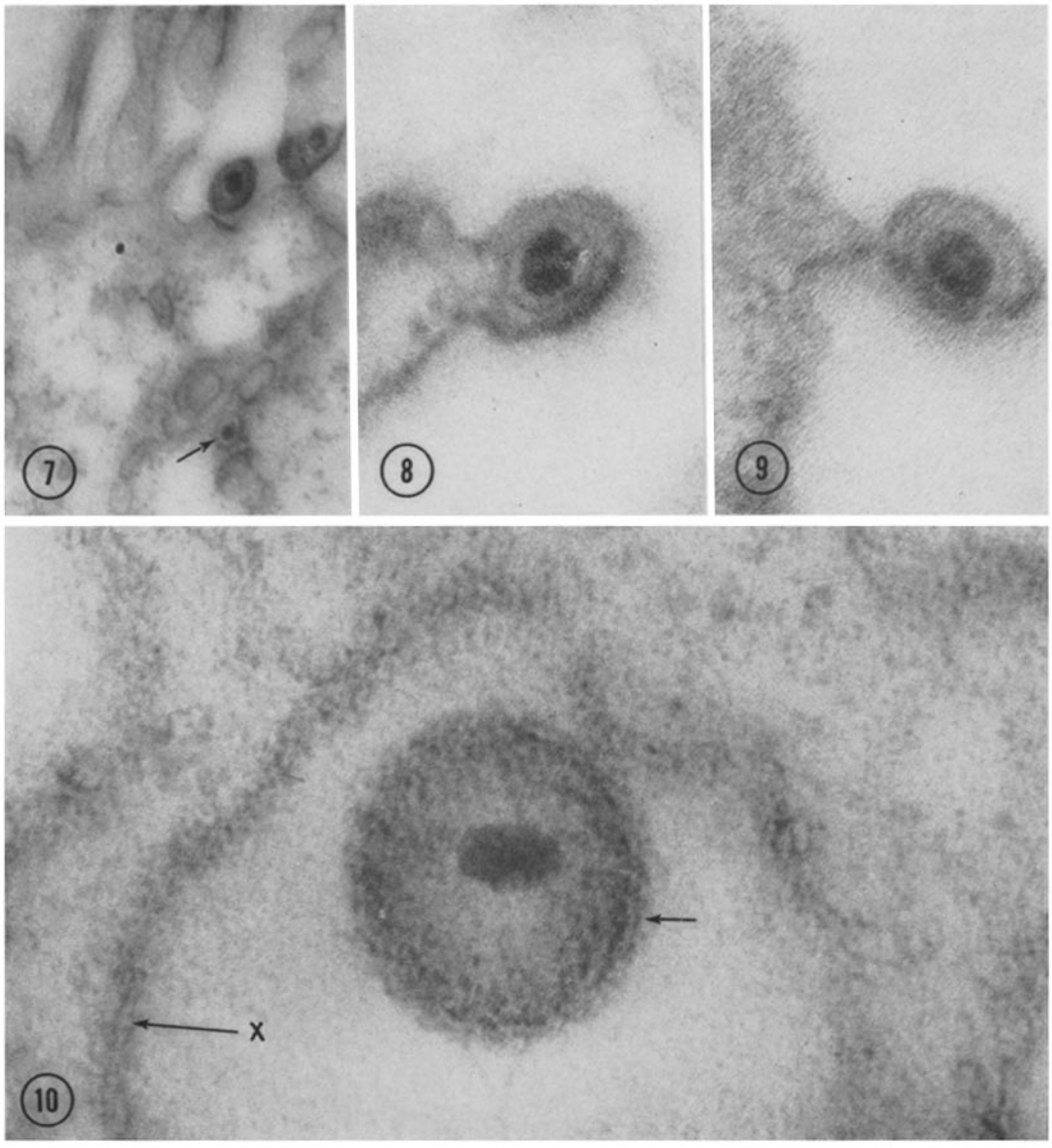

FIGURE 7

Detail of peripheral cytoplasm. The cell wall crosses the centre of the field and includes a number of slender microvilli. An immature particle lies in the cytoplasm below (arrow) close to the cell surface, and a mature extracellular form of the virus lies above. Osmium fixation; methacrylate embedding; unstained. × 60,000.

FIGURE 8

Detail of cell surface with an immature particle bulging out from the cell and almost entirely surrounded by the cell membrane. Osmium fixation; methacrylate embedding; unstained. × 160,000.

FIGURE 9

Cell surface with a maturing particle attached only by a small pedicle. Apart from the latter the particle has the appearance of the typical mature extracellular virus. Osmium fixation; methacrylate embedding; unstained. × 160,000.

DISCUSSION

In the present experiments mature herpes particles in osmium-methacrylate preparations have shown the characteristic appearance observed in all previous investigations of such material (1–5) and are, accordingly, described as having two membranes. However, work reported elsewhere (6) suggests that the second inner membrane of the mature extracellular virus only appears as a result of methacrylate embedding following the osmium and represents the site of an interface, for it was not present after permanganate fixation nor in osmium-treated particles embedded in Aquon. Where permanganate was used here, the absence of the membrane has now been confirmed in mature particles within vacuoles (Fig. 10).

Viral crystals in the nuclei of herpes-infected cells have been described by Morgan and his co-workers (4, 14). The present findings provide a second example of a herpes virus causing this phenomenon (Fig. 1), and the fact that the HFEM strain was well adapted to the HeLa cells in which it was grown (15, 6) supports the suggestion (4) that crystallization is probably a function of the profuse and rapid differentiation of virus particles at a given site.

Both reduplication of the nuclear envelope (4, 5) and occasional groups of apparently mature virus in sacs at the periphery of the nucleus (2, 3) have been found with the cell-virus system used. These phenomena would seem to be linked to the mechanism whereby developing virus escapes from the intact nucleus, but the actual manner in which the escape takes place could not be determined; nor could support be found for the suggestion that this might be by a combination of budding and fusion of viral membranes with those of the nuclear envelope (4, 5). Thus, in the absence of information, the way in which immature particles with one membrane reach the cytoplasm of intact cells (Figs. 2, 3, and 7) remains obscure, but the frequency with which they were seen there makes it clear that this was accomplished by an orderly process and not through cell damage or degeneration. Although it is theoretically possible that the cells with cytoplasmic particles were disrupted in areas close to, but not included in, the sections examined, if disruption had really been the mechanism whereby the particles entered the cytoplasm, signs of it should have been present at least in some instances.

No evidence has been obtained regarding the origin of the smooth surfaced cytoplasmic vacuoles in which mature herpes virus accumulates (4) (Figs. 3 and 10), although their constant association with the Golgi components of the endoplasmic reticulum suggests that they may form as dilatations of this structure rather than from the nuclear envelope (4, 5) or from invaginations of the cell membrane. Nevertheless, however these vacuoles arise, it is clear that they play an exactly similar role to that of the cell membrane in the release of immature virus from the cytoplasm, for in both cases the particles pass a membrane by budding and mature by deriving their final outer coat from it in the process (Figs. 4 to 9). The arbitrary arrangement of static images in a sequence to illustrate an active process has obvious dangers, but it is considered justified in the present context since other examples of viral release by budding have been recognised (16–18) and, furthermore, the triple-layered outer membrane of mature herpes particles (6) is clearly acquired in this way (Figs. 4 to 9).

The fact that herpes virus leaves the cytoplasm through both the cell membrane and the vacuolar membrane is of interest in several ways. From the point of view of viral release, either process leads to the cell exterior, the one directly, and the other presumably indirectly through an opening after fusion of the vacuolar walls with the cell wall, as occurs in various physiological processes (19, 20). From the wider point of view of cell organization, the phenomenon implies a similarity in behaviour between these smooth vacuoles

FIGURE 10

Mature particle lying within a vacuole after permanganate fixation and embedding in Aquon. The virus shows the dimensions and inner structure characteristic of such preparations (6) with eccentric rod-shaped nucleoid, absence of inner membrane, and triple-layered outer membrane (short arrow). At *x* the vacuolar membrane has been sectioned transversely and shows the same triple layering. Lead hydroxide staining. × 240,000.

of the endoplasmic reticulum and the cell membrane. It has been thought for some time that the membranes of the endoplasmic reticulum, the cell membrane, and the nuclear envelope all form part of a single morphological entity (21, 22), and in support of this both a structural continuity (23) and a functional continuity (24) have been demonstrated; in addition, all the membranes involved are composed of three layers (25, 6) arranged in an identical manner. The present findings show that widely separated parts of this continuous entity, namely, the cytoplasmic vacuoles and the cell membrane, can perform identical functions (Figs. 4 to 9).

The release mechanism of herpes virus is significant in yet another way. Closely similar budding has only been found with tumour viruses which escape slowly and continuously from infected cells (16–18); herpes virus resembles these agents not only in its mode of release, but also to some extent in the slowness with which this is achieved (26, 27). It has already been suggested that viruses differ in the fundamentals of their structure, depending on whether or not they possess a triple-layered outer limiting membrane (6), and the observations reported here indicate that this, in turn, is conditioned by the way in which a virus grows in, and leaves, the cell it parasitizes. Slow growing tumour viruses such as the Rous virus possess a triple-layered membrane (28) like that of herpes, whereas the adenovirus which bursts out of disrupted cells (29) does not (30). It might well be that the presence or absence of an outer membrane can be taken as a guide to a given agent's mode of release.

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