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VIRAL CRYSTALLINE ARRAYS IN FL CELLS INFECTED WITH HERPES SIMPLEX VIRUS[1,2]

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SUMMARY FL cells infected with two laboratory strains (HF and -GCr Miyama) and three freshly isolated strains (Ko, Iwa and Watanabe) of herpes simplex virus at an adsorbed multiplicity of approximate 10 were fixed at late stages of infection, (i.e. 40–63 hr after infection) and ultra-thin sections were prepared from them. The appearances and arrangement of viral crystals were then examined with an electron microscope and the following observations were made. (1) Viral crystals were seen in FL cells infected with all strains but they were not seen very often. They were seen most easily in cells infected with the HF strain which caused syncytial formation in the cell monolayers. (2) All the crystals found with the five strains were located in the nucleus, except for one which was in the cytoplasm of a cell infected with the Ko strain. (3) Some of the crystals observed were hexagonal in shape and no pentagonal crystal-like structure was detected.

INTRODUCTION

Intranuclear crystals of herpes simplex virus observed with an electron microscope were first reported by Morgan et al. (1958). These workers suggested that crystallization was a property of a particular strain of virus, rather than a state imposed by the host cell, and this idea seems to be supported by some other investigators (Shipkey et al., 1967; Asayama, 1967; Cook and Stevens, 1968).

Time sequential studies on the development of the Miyama strain of herpes simplex virus in FL cells infected with a high multiplicity of the virus revealed that crystallization occurred only at late stages of infection (Nii et al., 1968). With BHK cells and Earle's L cells infected with approximately the same dose of virus, crystals were rarely detected and their presence seemed to depend upon the duration of infection before death of the cells (Nii et al., 1966).

Some crystals observed by Morgan et al. (1958 and 1959) were hexagonal in shape. More recently, however, a pentagonal dipyramidal crystal-like structure was found by Melnick et al. (1968) in the nucleus of a liver cell of a mouse with hepatitis caused by herpes simplex virus.

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2 This work was presented at the 16th Annual Meeting of the Society of Japanese Virologists in Fukuoka, on October 9, 1968.

This study was on crystallization and the morphology of crystals in FL cells infected with two laboratory strains and three freshly isolated strains of herpes simplex virus.

MATERIALS AND METHODS

1. *Virus and cells*

Two laboratory strains of herpes simplex virus, the -GCr Miyama and HF strains, were used. The former strain was isolated in this laboratory and causes a proliferative type of cytopathic change in FL cell monolayers (Nii and Kamahora, 1961). The latter strain was obtained from Dr. Yoshino (Institute for Medical Sciences, Tokyo University). He has adapted this strain to grow well in HeLa cells and it induces syncytial formation in FL cell monolayers.

Three freshly isolated strains, the Ko, Iwa and Watanabe strains, all isolated in this laboratory from patients with *herpes labialis*, were also used. FL cells were used for isolation and subsequent passages of these strains. A proliferative type of cytopathic change was induced by the Ko and Watanabe strains in the cell monolayers and this was predominantly observed on infection with the Iwa strain (results to be published). For electron microscopic examinations cell cultures were infected with these three strains at early levels of passage (i.e. the 2nd and 3rd passage levels).

2. *Infection*

All five strains produced titers of 10^7—10^8 PFU/ml in the extracellular culture fluid at advanced stages of infection, so after a low speed centrifugation the supernatant fluid of infected cultures was used as virus material for inoculation. FL cell monolayers prepared in Leighton tubes or occasionally in 200 ml square glass bottles were infected with one of these strains at an adsorbed multiplicity of approximate 10. After adsorption for 1 hr the inoculum was replaced by maintenance medium: Eagle's Minimum Essential Medium (MEM) supplemented with 5% calf serum. The infected cultures were removed from the incubator 40–63 hr after infection for fixation.

3. *Preparation of material for electron microscopy*

The maintenance medium was discarded from the infected cultures and replaced by an appropriate amount of MEM. The cells were scraped in the medium and centrifuged at low speed. The resulting pellet was fixed for 1 hr in 1% glutaraldehyde, washed well, fixed for 30 min in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Sections were made with a glass knife in an LKB microtome and stained with uranyl acetate and lead citrate. A Hitachi 11 B electron microscope was used.

RESULTS

Fig. 1 and 2 show crystals found in the nuclei of FL cells 40 hr after infection with the —GCr Miyama strain of herpes simplex virus. The one shown in Fig. 1 is hexagonal. It consists of especially well-shaped arrays and many capsids in the crystal are each seen to be closely surrounded by six neighbouring capsids.

The cores of viral particles in Fig. 1 and 2 are mostly small and only the few particles shown by arrows in Fig. 2 have large ring-shaped cores.

The finding of viral crystallization with the Miyama strain confirms the previous report that this strain can form crystals (Nii et al., 1968).

Fig. 3–6 illustrate the nuclei of FL cells 43 hr after infection with the HF strain. Crystalline arrays are clearly observed in Fig. 3, 4 and 5, while crystal formation is not clear in Fig. 6 where aggregates of viral particles are seen. The virus cores have various morphological forms in these figures. Some particles have cores of low electron density most of which have a translucent center, as seen with particle b″ in Fig. 5. Some particles seem to be empty. Electron dense cores are ring-shaped, ribbon-like, or ovoid.

In Fig. 3 some hexagonal particles are arranged in rows fitting angle to angle next to each other as seen with the two neighbouring particles, A and B.

In the nucleus shown in Fig. 4 three or more crystals are seen in contact with one another,

suggesting that crystallization occurs in multiple foci. At the upper right-hand corner there is one particle with an envelope just outside the nucleus.

In the crystal shown in Fig. 5 distinct particles which are at, or near the plane of section are seen to have a periodic arrangement, (i.e. particles a, b, a′, b′, a″, b″ and a‴, b‴).

In some nuclei aggregates of viral particles without any definite crystalline arrays were observed. Fig. 6 shows numerous scattered particles in the nucleus. It is unknown whether these aggregation of viral particles leads to crystal formation.

Fig. 7, 8, 9 and 10 illustrate crystals in FL cells 62 hr after infection with the Ko strain. The viral particles in both Fig. 7 and 8 are arranged in beautiful crystalline arrays. Namely, the capsids are arranged in such a way that each hexagon in the crystals may be surrounded by six neighbouring ones by side to side contact. At the upper left-hand end of the crystal in Fig. 7 there are aberrant shaped particles shown by an arrow (Nii et al., 1968) and at the bottom of the picture one particle is almost completely enveloped. Proliferative changes of the nuclear membranes are seen in Fig. 8 and more clearly in Fig. 7.

One crystal found in the cytoplasm is shown in Fig. 9. Fig. 10 illustrates the same crystal at higher magnification. Similar granules to those which were widely distributed in the cytoplasm and which are thought to be ribosomal particles are also interspersed among the virus particles of the crystal. Therefore, presumably crystallization occurred at the site where this crystal was found. In other words this crystal was not extruded from the nucleus and may have been formed in an infected mitotic cell as an extreme rarity.

Fig. 11 shows one crystal in the nucleus of an FL cell 62.5 hr after infection with the Iwa strain, while Fig. 12 illustrates that found in the nucleus of an FL cell 63 hr after infection with the Watanabe strain. A part of the crystal in Fig. 11 is delineated and the rows are lettered. In each row inside the line one distinct and two indistinct particles are seen arranged alter nately. The three particles shown by arrows are incomplete, suggesting that they are in the process of formation.

DISCUSSION

Morgan et al. (1958) first suggested that crystallization of viral capsids of herpes simplex was a property of some particular strains of the agent. This idea seems to be supported by some other investigators (Shipkey et al., 1967; Asayama, 1967; Cook and Stevens, 1968). However, it does not seem easy to conclude definitely from electron microscopic observations whether any given virus strain has the property of inducing crystals after infection. Moreover, especial caution is required before concluding that some strain does not produce crystals. With the MP variant crystals were not found by Cook and Stevens (1968) but were found by Schwarz and Roizman (1969) although infrequently. Therefore, discrepant results on the occurrence of crystallization of herpes simplex virus seems to mainly due to limitation in the extent of electron microscopic observations.

Time sequential electron microscopic studies on FL cells infected with a high multiplicity of the Miyama strain of herpes simplex virus revealed that viral crystals appeared at late stages of infection (Nii et el., 1968) but they

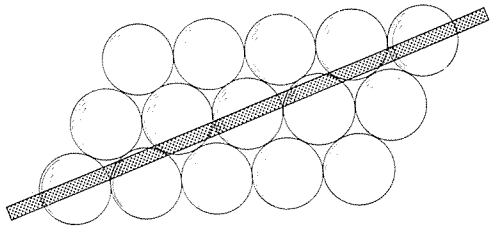

FIGURE 13. *Planar view of a layer of fifteen spherical particles in a hexagonal close packing arrangement. The shadowed area represents a section through the centers of three particles and peripheral parts of four particles. This cross section explains the periodic appearances of one distinct and two indistinct particles in rows of viral particles in part of the cystal shown in Fig. 11.*

were rarely seen with BHK cells and Earle's L cells under similar experimental conditions (Nii et al., 1966). Cell destruction after infection occurred earlier in the latter two cell lines than in FL cells and the appearance of viral crystals seems to depend upon the duration of infection before death of the cells. In other words a sufficient duration of infection seems to be necessary for retention of enough virus particles or their precursor materials in the nucleus to induce crystallization. Thus the kind of host cell seems to be another factor determining the appearance of crystals.

Based on the above considerations we tested the abilities of five strains to induce formation of crystals in infected FL cells and we also studied the morphological appearance of these crystals. Four of the five strains were isolated in this laboratory from patients with *herpes labialis*. The Miyama strain was isolated in 1958. During its in vitro passage three substrains were obtained (Nii and Kamahora, 1961), and one of these, the −GCr strain, was examined in this study. The other laboratory strain, HF was obtained from Dr. Yoshino. It had been adapted to HaLa cells and was the only strain used which induced syncytial formation in FL cell monolayers. The three freshly isolated strains were each used within three in vitro passages. Crystal formation was seen with all five strains, but crystals were not very numerous. They were most easily detected with the HF strain. This finding is compatible with the fact that the +GC variant of the Miyama strain, which also induces syncytial formation in cell monolayers, caused formation of crystals more frequently than the −GCr variant causing a proliferative type of cytopathic change (Nii, unpublished data). This might be explained as follows. In large syncytia where many nuclei are aggregated close together egress of intranuclear viral particles into the cytoplasm may be somewhat inhibited and this restriction may result in a more frequent appearance of crystals.

In contrast to our results, Shipkey et al. (1967) did not find crystals in HEp–2 cells infected with the HF strain. It is unknown whether this discrepancy was due to a difference in the passage histories of the HF strains used by Shipkey et al. and by us.

Schwarz and Roizman (1968) reported that viral crystals were observed in all nuclei of cells infected with the F strain, a freshly isolated type 1 herpes simplex virus, but were rarely seen in cells infected with laboratory strains. They interpreted this as follows: numerous passages of virus in HEp–2 cells result in selection of virus variants with an increased ability to multiply in these cells while freshly isolated strains are partially restricted in HEp–2 cells. However, we did not find viral crystals very frequently with three freshly isolated strains.

Crystallization itself dose not seem to be dependent upon the genetic properties of certain strains of herpes simplex virus and all strains of the virus seem to have the ability to induce crystallization. Namely if a number of virus particles of capsids are produced and concentrated in one locus of an infected cell, they will consequently become arranged in crystalline arrays. If this is so, only the frequency of appearance of crystals seems to depend upon the properties of the strain.

The capsid of herpes simplex virus is an icosahedron consisting of 162 capsomers (Wildy et al., 1960). In Fig. 13 particles of capsids are drawn diagrammatically as spheres, with a closely packed hexagonal arrangement. The shadowed area indicates a section with a thickness of approximate 200 A and three single particles are cut through their centers while four particles are cut through peripheral parts. This sectioned plane explains the alternate arrangement of distinct and indistinct particles seen in rows of viral particles in a crystal, such as that seen in Fig. 11.

Morgan et al. (1956) using electron microscopy observed intranuclear viral crystals in HeLa cells infected with adenovirus types 3, 4 and 5 and suggested that viral particles were packed in crystals in a cubic-body centered lat-

that each particle was surrounded by eight equidistant particles. This arrangement was supported by Low and Pinnock (1956). Morgan et al. (1960) also observed a hexagonal viral crystal in the nucleus of a cell infected with type 5 adenovirus. A hexagonal crystalline arrangement is the best method of packing a large number of viruses or macromolecules into a small space and some of the crystals observed in the present investigation appeared hexagonal. Pentagonal crystals such as those reported by Melnick et al. (1968) could not be found.

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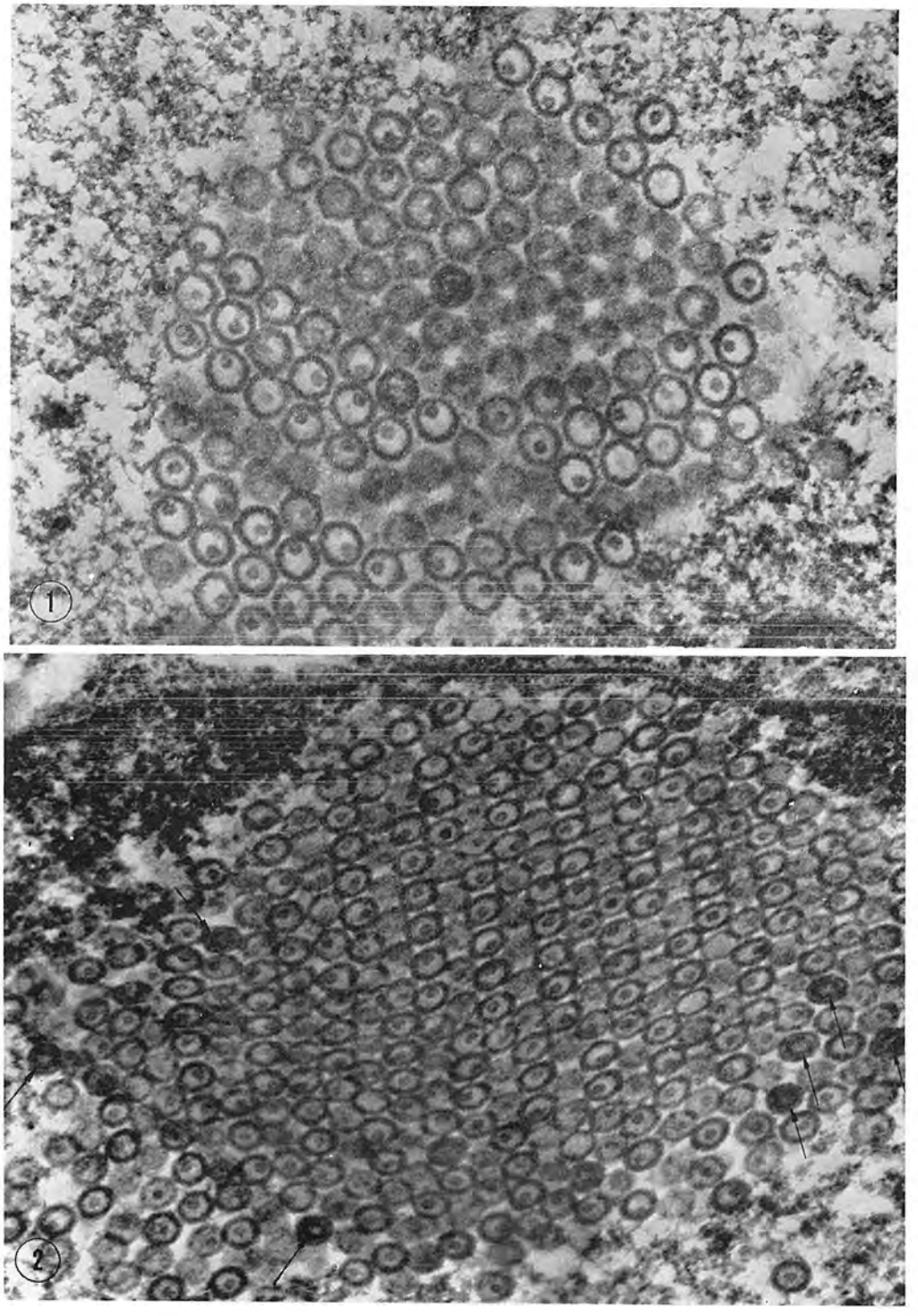
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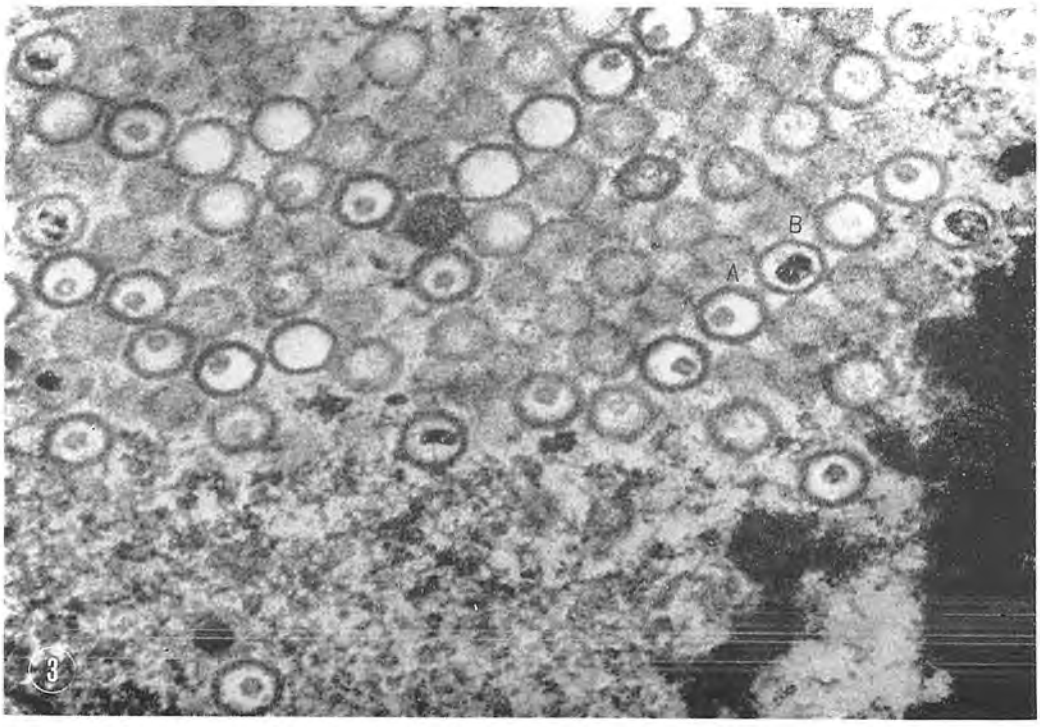


FIGURES 1 and 2. *Viral crystals found in the nuclei of FL cells 40 hr after infection with the -GCr Miyama strain of herpes simplex virus. The crystal in Fig. 1 is hexagonal.*
Fig. 1 ×64,000 *Fig. 2* ×60,000

FIGURES 3 and 4. *Viral crystals found in the nuclei of FL cells 43 hr after infection with the HF strain of herpes simplex virus. In Fig 3 two neighbouring particles, A and B, are seen adjacent to each other, angle to angle.*

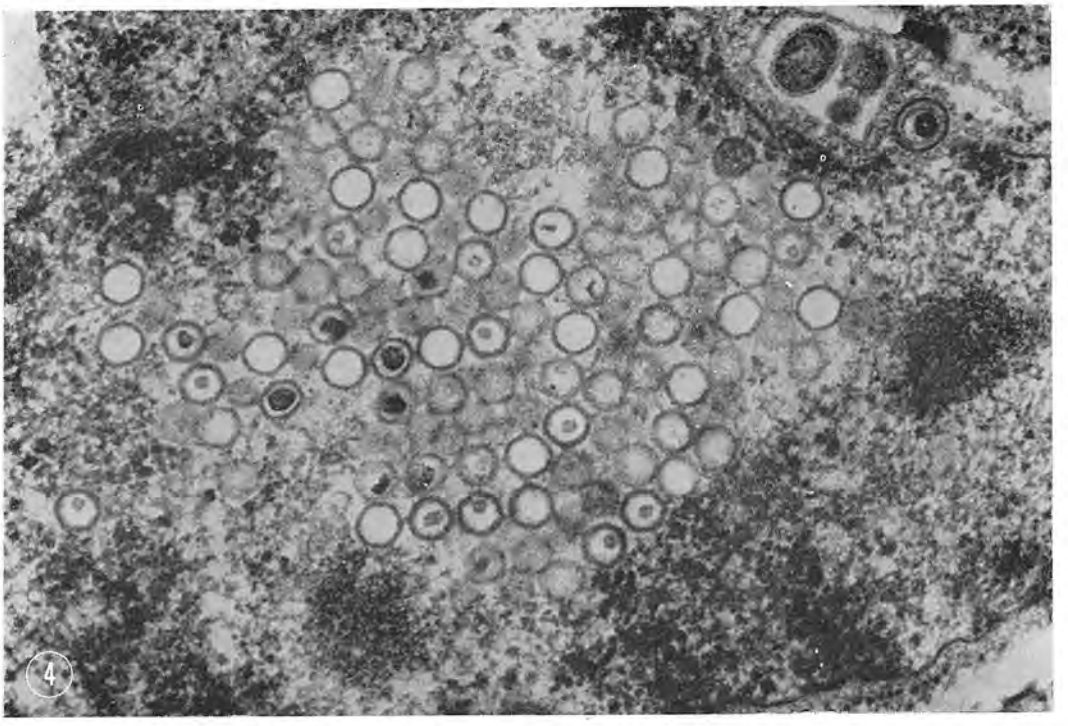
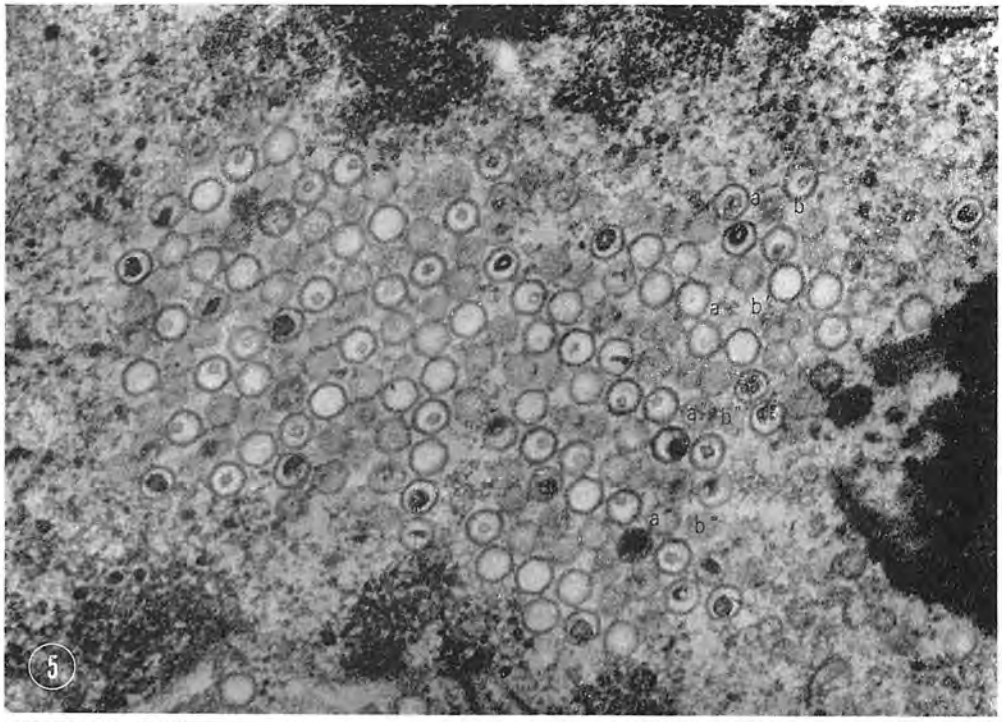


Fig. 4 shows viral crystals in the nucleus and one enveloped particle just outside it.
Fig. 3 ×93,000 Fig. 4 ×67,000

FIGURE 5. *Viral crystals in the nucleus of an FL cell 43 hr after infection with the HF strain of herpes simplex virus. Distinct particles have a periodic arrangement as seen with particles a, b; a′, b′; a″, b″ and a‴, b‴. Virus cores of various shapes are also seen. Some particles have cores of low electron density, and usually a translucent center, as seen in particle b″. Some particles appear empty. The other particles have electron dense cores which are ring-shaped, ribbon-like or ovoid. ×60,000*

FIGURE 6. *Viral aggregates in the nucleus of an FL cell 43 hr after infection with the HF strain of herpes simplex virus. No definite viral crystal was detected. ×43,000*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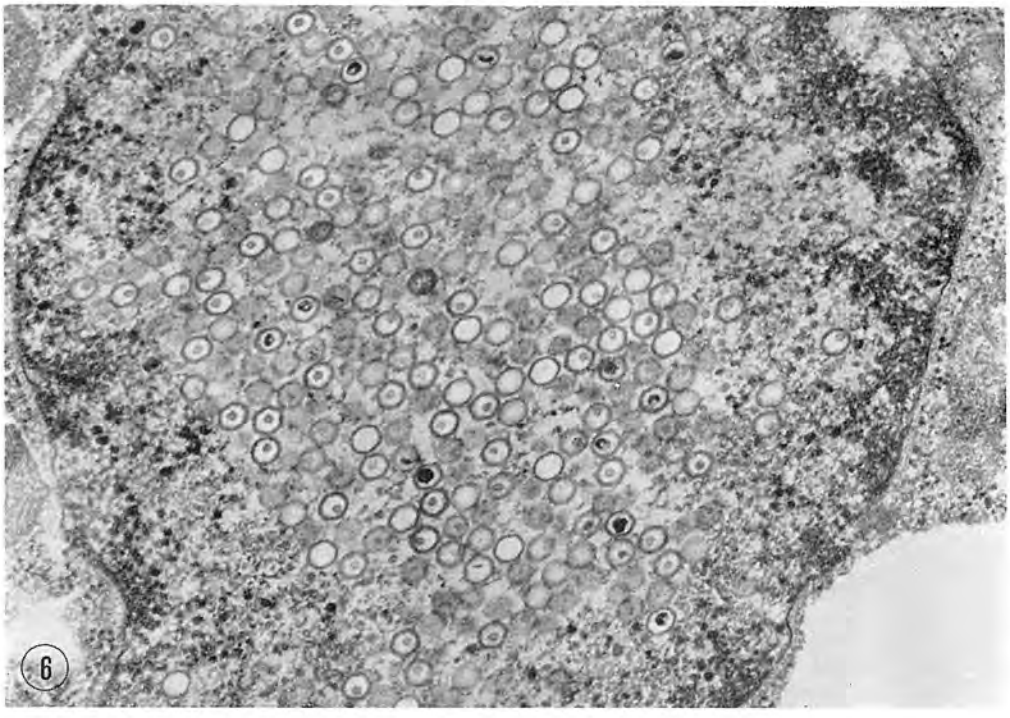
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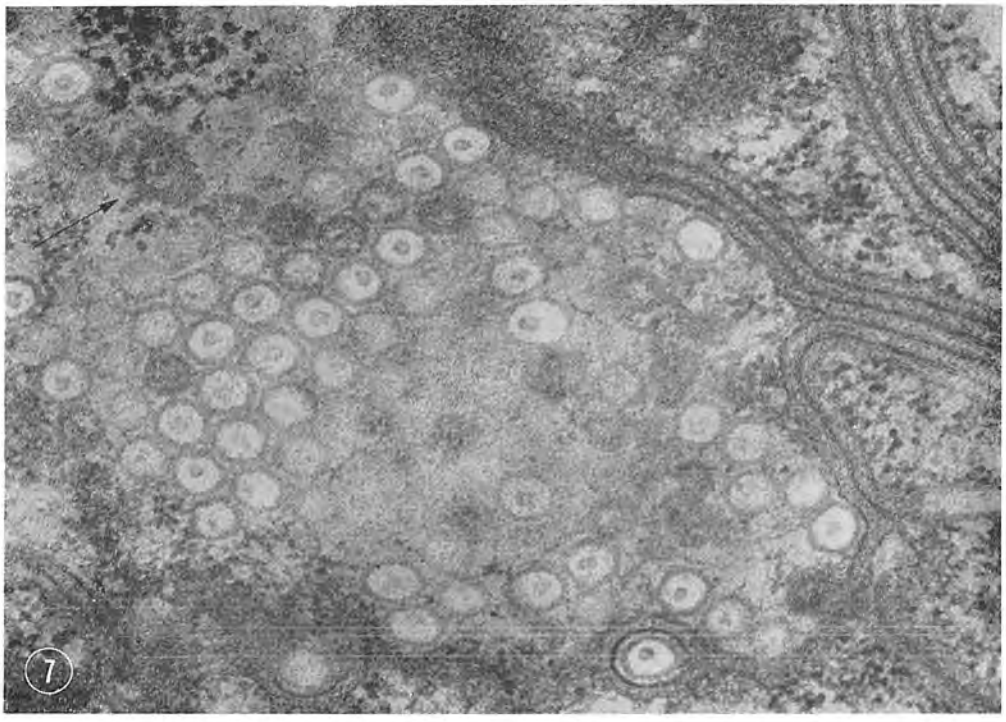
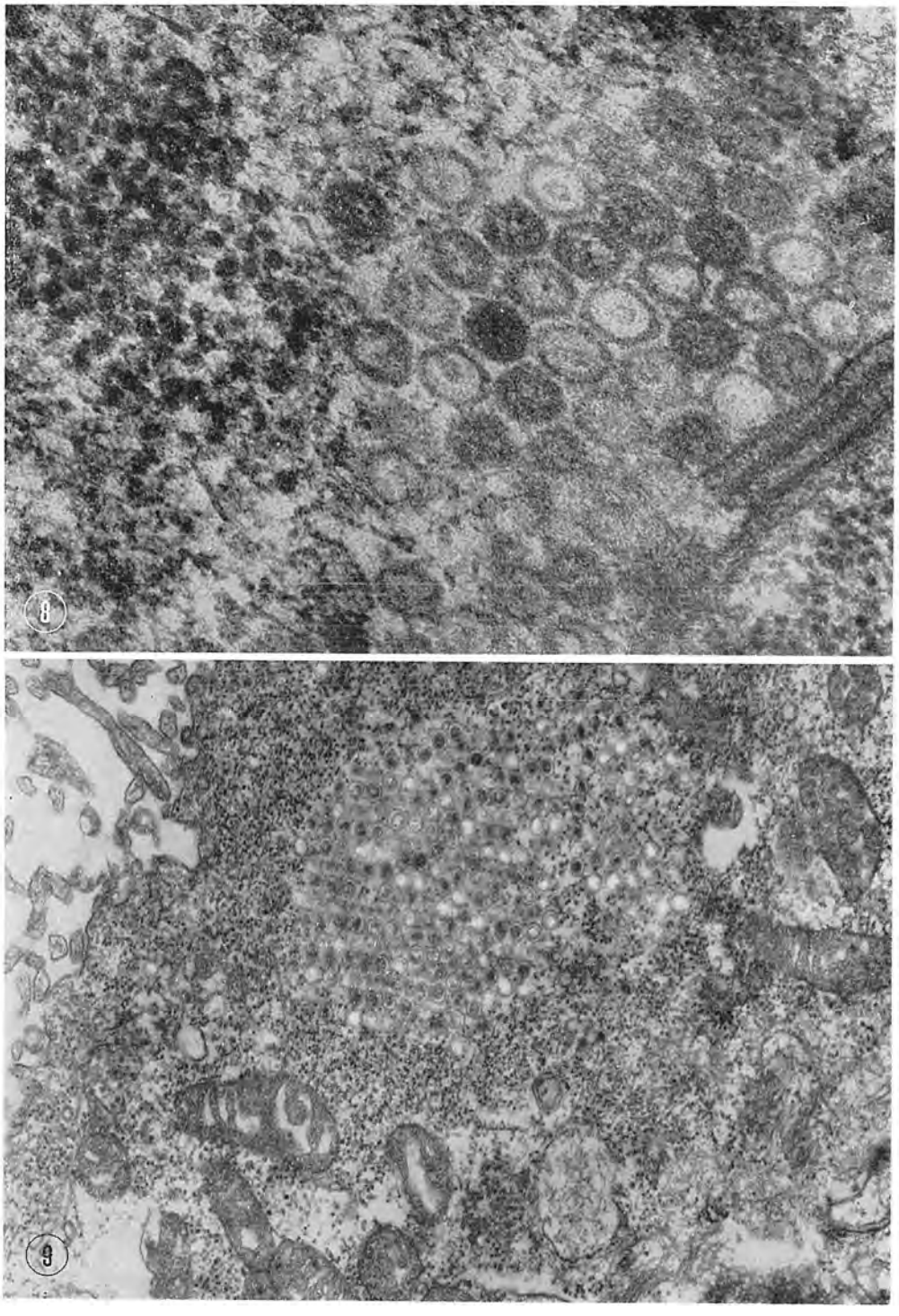


FIGURES 7 and 8. *Viral crystals in the nuclei of FL cells 62 hr after infection with the Ko strain of herpes simplex virus. Reduplication of the nuclear membrane is also seen. At the bottom of Fig. 7 one particle has an almost complete envelope.* An arrow shows aberrant shaped particles.
Fig. 7 ×*85,000* *Fig. 8* ×*97,000*

FIGURE 9. *A viral crystal found in the cytoplasm of an FL cell 62 hr after infection with the Ko strain of herpes simplex virus.*
Fig. 9 ×*28,000*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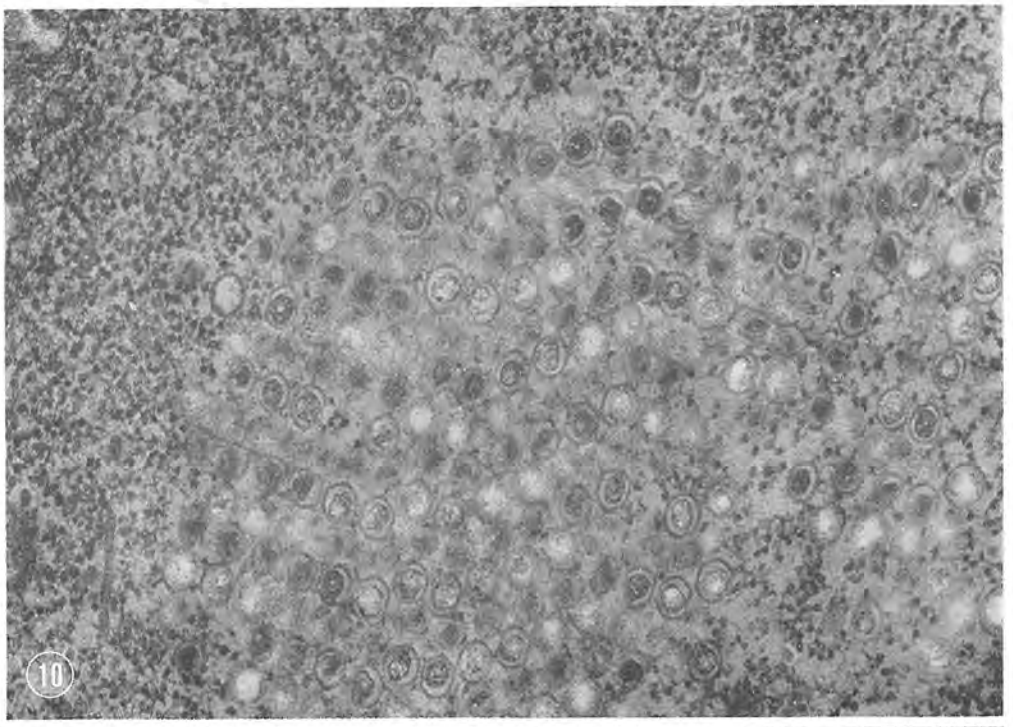
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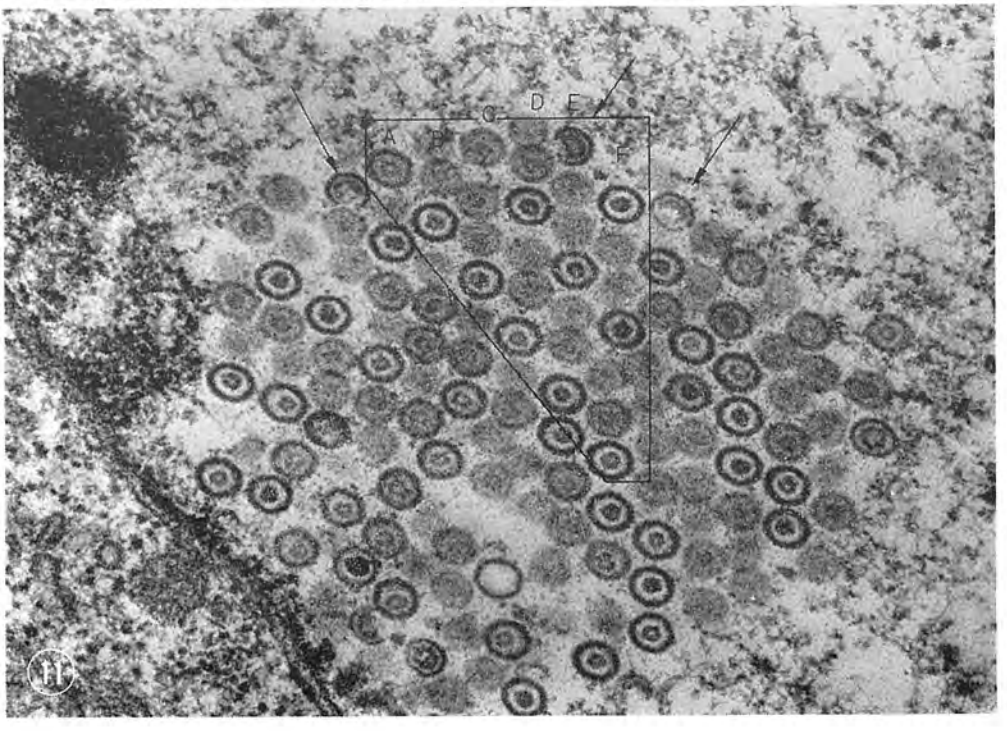
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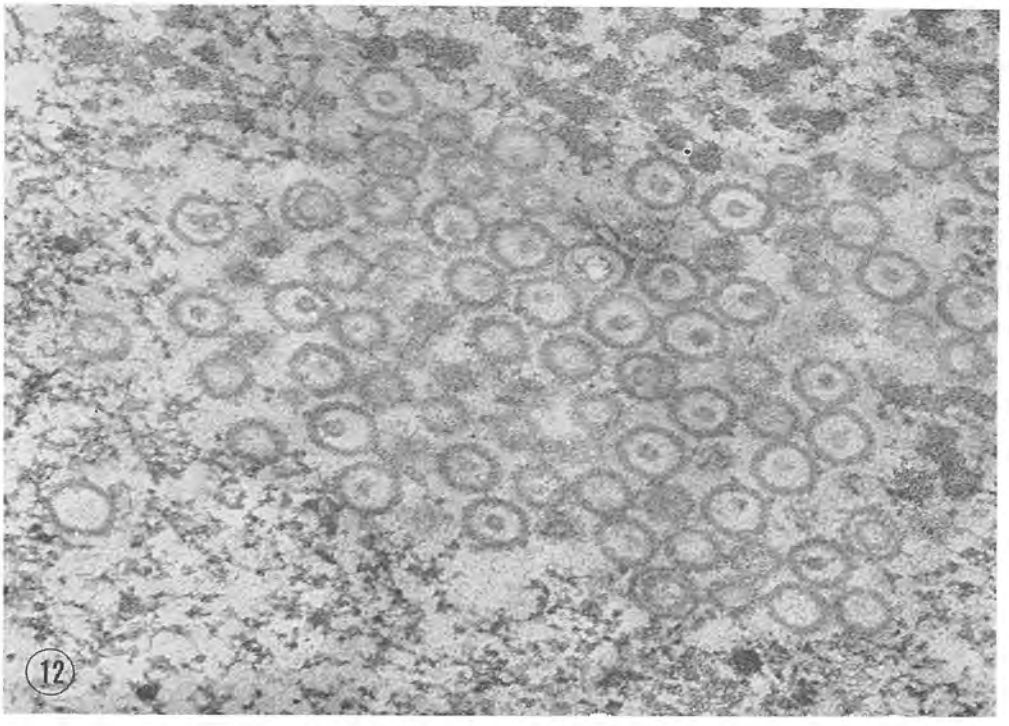

FIGURE 10. *A viral crystal found in the cytoplasm of an FL cell 62 hr after infection with the Ko strain of herpes simplex virus.* ×58,000

FIGURE 11. *A viral crystal found in the nucleus of an FL cell 62.5 hr after infection with the Iwa strain of herpes simplex virus. Part of the crystal is delineated, and one distinct and two indistinct particles are seen alternately in each lettered row.* ×60,000

FIGURE 12. *A viral crystal in the nucleus of an FL cell 63 hr after infection with the Watanabe strain of herpes simplex virus.* ×88,000