OBSERVATIONS ON THE FINE STRUCTURE OF MATURE HERPES SIMPLEX VIRUS AND ON THE COMPOSITION OF ITS NUCLEOID

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Plates 1 to 5

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In recent work with adenovirus and the Rous virus, specific enzymic digestions were applied to samples of purified pelleted particles, and the resulting changes caused within the particles were then observed in thin sections with the electron microscope; in this way the site and nature of viral nucleic acid were determined (1–5). Since the accuracy of the findings was confirmed in each case by concomitant specific staining of the purified material for nucleic acids (2, 4), it was considered that the results of applying enzyme digestions and electron microscopy alone to virus particles unseparated from cells might safely be relied upon, such preparations being, of course, unsuitable for the additional checking of observations cytochemically.

An approach of this kind seemed particularly appropriate in relation to herpes virus, for the mature particles can be readily recognized and their tendency to accumulate at the cell surface is well known (6–9). Furthermore, although cytochemical investigations of the nuclear inclusions arising in herpes infections have demonstrated that they contain much deoxyribonucleic acid (DNA) (10) little is known about the composition of the virus. It has sometimes been assumed that the latter also includes nucleic acid of this type (11), but even though excess DNA is in fact synthesized by cells very soon after infection with herpes virus (12), no connection has been established between this material and the constituents of the agent itself.

Experiments were accordingly undertaken in which herpes-infected HeLa cells were examined in thin sections with the electron microscope so that the mature virus particles around them might be studied after treatment with various fixatives, specific nucleases, and in different embedding media, both with and without electron staining. Tests for the biological activity of the cultures examined were included in the first part of the work. The results obtained in this investigation are now reported.

Materials and Methods

Virus Strain.—The HFEM strain of herpes virus has been used; it was received frozen at −70°C in infected tissue culture fluid from the 46th HeLa cell passage (13) (through the

kindness of Professor M. G. P. Stoker and Dr. P. Wildy) and has subsequently been passed in cultures of such cells.

Stock Virus Seed.—Culture medium harvested from a HeLa cell culture showing marked cytopathic effects 2½ days after infection with a heavy dose of virus was diluted with an equal volume of a suspending fluid, described elsewhere (14), and was stored at −70°C in sealed hard glass ampoules in 1 ml volumes until required.

Maintenance of HeLa Cell Cultures.—The cells were grown in flat 8 ounce glass bottles by methods already reported (15, 16); when confluent sheets of cells developed they were either used as a source of fresh cultures or were infected to provide material for the experiments.

Infection of HeLa Cell Cultures.—For each experiment the culture fluid was removed from one or two confluent cultures and was replaced by 2 to 3 ml of stock virus seed, either neat or diluted with an equal volume of medium designated RI; the latter was similar to the growth medium (medium I) of Pereira and Kelly (15) except that rabbit serum was substituted for human serum and the sodium bicarbonate was increased to a *w/v* concentration of 0.025 per cent. The virus inoculum was left in contact with the cell sheet as a thin covering film for 3 to 4 hours at 32°C, after which about 13 ml of medium RI were added. The infected cultures were then incubated for between 2 and 3½ days either at 37°C or, less frequently, at 32°C. The use of this temperature both for infecting the cells and during their subsequent incubation was suggested by the findings of Farnham and Newton (17).

Preparation of Cells for Electron Microscopy.—(*a*) *Collection:* When cells showed cytopathic changes they were collected in a manner similar to that described previously (18). Since, however, the infected cells were always starting to detach from the glass they were either loose enough to be shaken into trypsin solution at once without incubation in the enzyme, or else so loose that enzyme was not added at all, 3 ml of culture medium being left in place to serve as collecting fluid instead.

(*b*) *Fixation:* Cells were fixed either in osmium tetroxide by the same methods as in earlier work (18), or in potassium permanganate (19) applied in an identical way.

(*c*) *Dehydration and embedding:* Osmium-fixed material was dehydrated and embedded in *n*-butyl methacrylate as already reported (18), and the same procedure was used after permanganate fixation except that treatment with the two changes of iced 30 per cent ethanol recommended by Luft (19) was included at the start of dehydration.

In addition, material fixed with both osmium and permanganate was embedded in aquon (20), this water-miscible fraction of the commercial epoxy resin epikote 812 (Shell Chemical Co., Ltd., London) being prepared by the methods of Gibbons (20); British-made epikote 812 is said by the manufacturers to be identical with American epon 812. Specimens were passed through increasing concentrations of the cold resin (20) and were then embedded in a mixture of 10 ml aquon resin, 19 ml dodecenyl succinic anhydride, 3 ml hexahydrophthalic anhydride, and 0.3 ml benzyldimethylamine (the three latter reagents also from British sources). Curing at 60°C was continued for 4 to 5 days.

(*d*) *Microtomy and microscopy:* Sections were cut with glass knives or a diamond knife (21, 22) on a Porter-Blum microtome, and were mounted on carbon-coated grids (23) for examination in a Siemens Elmiskop I electron microscope fitted with self-heating dished form 20 μ objective apertures. Sections showing pale yellow or silver interference colours were studied, both with and without lead hydroxide staining (24, 25).

Enzyme Digestions.—Ribonuclease (RNase), deoxyribonuclease (DNase) and control media were applied by methods reported elsewhere (4).

Assay of Virus.—Where assays were to be made of the mature virus free in infected cultures chosen for electron microscopy, a sample was taken from the medium when this was removed just before the cells were collected for fixing and embedding. The samples were diluted in serial tenfold steps and titrated on the chorioallantoic membranes of eggs; this was done exactly as in earlier work (14) as were the calculations of the results.

EXPERIMENTAL PROCEDURE

In the first experiments samples of osmium and permanganate-fixed cells from each herpes-infected culture were embedded in methacrylate and examined with the electron microscope in order to study the structure of the mature extracellular virus. At the same time the amount of this virus free in the medium from each culture, was assayed.

In further experiments, the structure of the virus was investigated, as before, after both types of fixation but using material embedded in aquon.

For a final set of experiments infected cells fixed with permanganate were treated with nucleases or control fluid before embedding in methacrylate; the effects of the enzymes on the morphology of the virus were then assessed with the electron microscope.

RESULTS

Where herpes-infected cells have been examined with the electron microscope after osmium fixation, it has been found that the mature extracellular agent measures between 110 and 180 mμ in diameter and consists of an electron-opaque nucleoid surrounded by two membranes (6–9). Particles with three membranes have also been described (9) but these were within cells and would appear to have been the usual form closely surrounded by membranous cellular components; a few specimens fixed with formalin, either alone or followed by osmium vapour, have shown the dense nucleoid but little else (9). In addition, surface staining methods (26) have shown the virus to possess hollow polygonal subunits surrounding a central "core" and forming a particle 100 mμ in diameter (13). When such particles were found lying within an outer "envelope" the whole structure measured 180 mμ across (13).

In the present work all the infected cultures were similar in that a high proportion of their cells were surrounded by numerous particles of about 120 to 165 mμ in diameter (Figs. 1 to 5, 7, 10 to 12), depending on the preparation methods used.

The aquon preparations did not seem to undergo any shrinkage during embedding, and sections of this material rapidly expanded in the knife trough to the full size of the block face from which they were cut. In the absence of staining, aquon-embedded preparations were of low electron density and showed poor contrast (Fig. 3).

Combined Electron Microscopy and Titrations.—The extracellular particles contained a nucleoid, were uniform in structure, and were usually close to the cell surface in association with slender microvilli about 100 mμ or less in width (Figs. 1 to 3, 5, 7, 9 to 12). The microvilli were longer and more profuse than in uninfected cells (18); when sectioned transversely they appeared as circular profiles without inner structure (Figs. 1 to 3, and 5), and could be readily distinguished from the mature virus particles.

Medium from the infected cultures showed considerable biological activity (a lesion dilution 50 per cent per ml of between $10^{-5.5}$ and $10^{-6.8}$, Table I) even though the cells were not disturbed while the medium was being taken off.

Fine Structure of the Virus.—After embedding in methacrylate the virus particles were sometimes round in profile but more often oval (Figs. 1, 4, 5, and 7); in contrast, aquon-embedded particles were usually spherical or very nearly so (Figs. 3, 6, 8, and 9). Wherever oval particles were found their long axes were always parallel to one another (Figs. 4 and 5) and at right angles to the direction of cutting.

TABLE I

The Biological Activity of Culture Fluids from Infected HeLa Cultures Whose Cells Were Used for Electronmicroscopy (Titration on Egg CAM)

Experiment No.	Dilutions of sample and result*					Lesion dilution 50 per ml. of sample
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
1	3/3	4/4	3/4	2/4	0/4	$10^{-6.8}$
2	3/3	2/4	1/4	0/3	0/4	$10^{-5.5}$
3	3/3	2/2	4/4	1/4	0/4	$10^{-6.7}$
4	3/3	2/4	1/4	0/4	0/4	$10^{-5.5}$
5	2/2	4/4	1/3	0/4	0/2	$10^{-5.8}$
6	3/3	2/2	1/4	0/4	0/4	$10^{-5.7}$

* Denominator = No. of live eggs harvested.
Numerator = No. of live eggs with lesions.

The size of the mature virus varied with the embedding medium. It was estimated by measuring the diameter of round virus profiles or by averaging the longest and shortest measurements across those which were oval. In methacrylate preparations the mature virus averaged about 120 mμ in diameter, there being some scatter in size range, most marked after osmium fixation. On the other hand, the round particles in the aquon preparations showed almost no variation in their dimensions, measuring about 165 mμ across irrespective of the fixative used.

The particles were enclosed by an outer limiting membrane which was readily visible when fixed with osmium (Figs. 1, 3, 4, and 7) and showed a triple layered structure after fixation in permanganate (Fig. 5), particularly striking where this was combined with aquon embedding (Figs. 6, 8, and 9); it consisted of two electron-opaque layers about 5 mμ apart with a less dense layer in between (Figs. 5, 6, 8, and 9), corresponding exactly with the triple layering of the cell membrane in permanganate-fixed material (Figs. 2, 5, and 9). The viral limiting membrane covered a relatively dense outer zone some 30 mμ wide which, in turn, surrounded a central area of low electron opacity (Figs. 4 to 9) in which

lay the nucleoid, and which measured about 90 mμ across.The junction between the dense outer zone and the central area was sharply defined and corresponds to the site of the innermost of the two membranes said to characterize mature herpes particles (6–9) when seen in osmium-fixed methacrylate material at low magnification (Fig. 1). However, no such inner membrane was found after permanganate fixation (Figs. 2, 5, 6, 8, and 9) nor after osmium where aquon-embedding was used (Fig. 3), and even in conventional osmium methacrylate preparations high magnifications did not always reveal any indication of it (Figs. 4 and 7) except in swollen or distorted particles (Fig. 7).

The dense nucleoid was eccentrically placed within the central area of the virus both in permanganate-fixed material (Figs. 2, 5, 6, 8, and 9) and also after osmium where this was followed by aquon embedding (Fig. 3). Only in osmium methacrylate preparations did the nucleoid appear to fill the centre of the particle, but here lessening of density at the centre of the nucleoid itself (Figs. 1, 4, and 7) suggested distortion. The shape of the nucleoid was either round, oval, or like a short round ended rod (Figs. 3, 6, 8, and 9), no doubt according to its relation to the angle of sectioning, since its width was always constant at about 35 mμ. The finding of particles in which no nucleoid could be seen (Fig. 9) was clearly also due to the chances of sectioning, the plane of section passing through the virus in these instances in such a way as not to include the eccentric nucleoid.

When embedded in aquon, the nucleoid was noticeably electron-opaque by comparison with other structures whose contrast was poor (Fig. 3) and this was paralleled by its ability to take up considerable amounts of lead even in the thinnest sections (Figs. 6, 8, and 9).

Effects of Nucleases on Virus Morphology.—All the preparations which were incubated in watery medium before embedding appeared rather ragged and extracted when examined in the electron microscope (Figs. 10 to 12). Nevertheless, the control preparations treated with enzyme-free DNase medium always contained numerous particles with well marked nucleoids (Fig. 10); similarly, particles in material exposed to RNase also contained dense nucleoids (Fig. 11). In marked contrast, the nucleoids were removed from those particles which had been subjected to DNase digestion, leaving an empty space in the central area (Fig. 12).

DISCUSSION

The extracellular particles examined in the present work resemble similar bodies observed in all previous morphological investigations of herpes-infected cells (6–9), and the results of the titration experiments provide at least some evidence for their viral nature, since the cultures which contained the particles in profusion were all active when tested biologically for herpes virus (Table I). In fact, the titres recorded are quite striking when it is remembered that in

taking samples of the culture fluids, efforts were made not to disturb the cells in order to keept them intact for electron microscopy. The sampling method thus worked against releasing the virus trapped between and around the cells (Figs. 1, 5, 7, 10 to 12) and if this is taken into account when considering the virus free in the fluids (Table I), the total virus content of the whole cultures must have been high. It is of interest to note in contrast, that in preliminary experiments with a different strain of herpes yielding much lower titres when grown in HeLa cultures, recognisable virus particles could not be found in thin sections of the cells by electron microscopy. That high yields of virus of the HFEM strain of herpes were obtained in the present experiments clearly reflects not only the successful adaptation of this strain to HeLa cells after 46 passages, but also the appropriateness of the conditions obtaining when the cultures were infected, thus confirming the conclusions of Farnham and Newton (17) whose methods were followed.

Others have pointed out (20, 27) that the possibility of dispensing with dehydrating agents when embedding for electron microscopy has the great theoretical advantage of avoiding the undesirable leaching out of components and the sometimes drastic shrinkage which occur when biological specimens are dehydrated by passage through the usual series of alcohols. The use of water-miscible aquon for embedding overcomes these defects and also other sources of specimen shrinkage and distortion, for, as reported here, the resin does not appear to contract during polymerization and gives sections free of knife compression; both these attributes are common to other epoxy resins (27).

The structure of mature herpes simplex virus as revealed after embedding in aquon is therefore considered to approximate closely to that present in life. The size of the virus, about 165 mμ in diameter, was remarkably constant throughout the preparations after two types of fixation and fell well within the extremes reported from observations made on specimens embedded in methacrylate. Although rather greater than the average figure of 120 to 130 mμ usually obtained with methacrylate material (6–9) and confirmed here, the difference would be consistent with the avoidance of shrinkage during dehydration, an artefact which probably also accounts for the wide range of size encountered where methacrylate has been used. Furthermore, a diameter of 165 mμ agrees very well with the figure of 180 mμ obtained for particles with "envelopes" (13) in surface stained preparations, if allowance is made for some flattening and deformation as these dried.

It is also clear that the virus is spherical (Figs. 3, 8, and 9), oval particles (Figs. 4 and 5) being the result of knife compression. This and other distortions would likewise appear to account for the so-called inner membrane and the diffuse central nucleoid seen sometimes in osmium-fixed preparations embedded in methacrylate (Fig. 7), for these features were never present after aquon embedding (Figs. 6, 8, and 9) even where osmium fixation preceded it (Fig. 3),

nor in methacrylate specimens which had been fixed with permanganate (Fig. 3 and 5).

The striking triple-layered structure of the outer viral limiting membrane found after permanganate fixation (Figs. 5, 6, 8 to 12) has not hitherto been recorded. It resembles the limiting membrane around similarly prepared mature vaccinia virus (14) and Rous virus (28) both in appearance and dimensions and the close correspondence of its form with the triple layering of cell membranes (Figs. 5 and 9) (29–31) appears to have considerable significance in relation to the mechanism whereby herpes particles are released from their host cells and become surrounded by such an enclosing structure (32). The presence of the laminated limiting membrane indicates that the dense outer zone, or viroplasm, of the agent is covered by a series of molecular layers of different composition, such as presumably form the basis of all the so-called membranes (33) found when sectioned biological material is examined by electron microscopy. Some viruses, for example the adenovirus, are without a covering of this type (34) having instead a viroplasm which extends right to the surface, and it is interesting to note that only in smaller animal viruses where this arrangement occurs have regular surface subunits been shown so far (35–37) by the surface staining technique of Brenner and Horne (26). In the case of herpes virus, subunits, though present, lie within the mature particle covered by an "envelope" (13) which appears to correspond to the triple-layered outer membrane and the underlying dense outer zone found here; if this is so, then the boundary between the dense zone and the central zone might represent the site of the outer face of these internal subunits (13).

It is tempting to speculate on the possibility that the larger viruses which have a covering membrane may differ fundamentally from those without, not only with regard to this structure, but also in those of their functions which may be influenced by it, namely, attachment to, and release from, the cells they infect. The latter processes must surely be linked to the presence of an outer viral membrane which is perhaps identical with that surrounding the susceptible cell (Figs. 5 and 9).

The noticeable density of the eccentric, round ended, rod-shaped nucleoid when embedded in aquon (Fig. 3) and its parallel ability to take up lead in sections of this material (Figs. 6, 8, and 9) are at first sight unusual. However, it has already been suggested by Gibbons on theoretical grounds (20) that aquon might possibly have a special fixing and preserving action on nucleic acids, and the digestion experiments reported here show clearly that the nucleic acid of herpes virus is localized in the nucleoid. For, despite the general extraction effects present in all the incubated preparations, it was the nucleoid alone which was removed by the action of a specific nuclease (Fig. 12) whilst remaining untouched by control fluid (Fig. 10) or an inappropriate enzyme (Fig. 11). In the light of Gibbons' contention (20), the demonstration of nucleic

acid within the viral nucleoid could well explain the density of this structure when embedded in aquon. The finding from irradiation studies that the radiosensitive zone of herpes virus has a diameter of 38 mμ (38) constitutes further evidence relevant to this point since the dimensions of the nucleoid found here in aquon preparations (Figs. 3, 6, 8, and 9) fit this figure exactly.

Besides locating the site of the viral nucleic acid the digestion experiments have also been able to establish that it is of deoxyribose type, this observation being supported unequivocably by the specificity of the DNase, the negative results of the control experiments which included a related enzyme, and the known reliability of the methods used (2, 4).

That DNase was able to act directly on the nucleoid of herpes virus without some preliminary pretreatment of the particle was in marked contrast to earlier findings with the adenovirus, an agent without an outer limiting membrane (34); in that case the nucleoid was only digested by enzyme when the protein of the viral outer coat had first been denatured with mild alkali (4). On the other hand, the nucleoid of the Rous virus which has an outer limiting membrane (28) like that of herpes, was susceptible to RNase digestion immediately after fixation (2). Differences such as these could depend on a number of factors, but they might well reflect a further effect of the possession or lack of a limiting membrane, already discussed above. Without a limiting membrane the protein units are closely packed together, as in the adenovirus (35), and nuclease penetration is not possible after fixation alone (4), whereas where membranes are present, as in the Rous (2) and herpes viruses (Figs. 1 to 9), nucleases can penetrate straight away both the fixed membrane, as with cells, and the protein units within. That this can happen means that some different arrangement of protein units occurs in cases where they lie under membranes, perhaps looser packing and greater hydration. Morphological evidence for looser packing has been reported by Wildy and his coworkers (13), whilst the present observations on particle size after avoiding dehydration shrinkage by aquon embedding, strongly suggest that herpes virus exists in a state of considerable hydration.

The various advantages of combined electron microscopy and specific enzymic digestions for determining the nature and whereabouts of substances within minute structures have recently been discussed (39, 40). In the past the methods have been applied successfully to viruses using preparations of isolated particles (1–5); the present investigation, besides yielding information on the structure and composition of herpes virus, demonstrates the further suitability and usefulness of such combined techniques for viruses *in situ*, in cases where they can be recognized without having to be separated from the cells they infect. The findings of the present study are also considered to provide a practical example of the merits of a water-miscible embedding medium whose advantages have hitherto been advocated on purely theoretical grounds.

SUMMARY

The fine structure and composition of mature herpes virus have been investigated in thin sections by electron microscopy. The virus was grown in cultured HeLa cells and was collected with them. Tests for the biological activity of the infected cultures were included in the first half of the work. Preparations were fixed with both osmium and permanganate, and were embedded either in methacrylate or in aquon, a water-miscible fraction of a commercially available epoxy resin. In further experiments material fixed with permanganate was subjected to the action of specific nucleases or control medium before embedding.

All the preparations showed numerous uniform particles around and between the cells and this was paralleled by considerable biological activity where tests were made on samples of the culture fluids.

Mature herpes virus has been found to be round and to measure, when dehydration shrinkage is avoided, about 165 mμ in diameter. The particle contained an eccentric round-ended rod-shaped, electron-opaque nucleoid lying in an inner zone of low density. A dense outer zone or viroplasm surrounded this, no membrane being present between the two zones. After permanganate fixation the particle was found to have an outer limiting membrane showing a triple-layered structure morphologically indistinguishable from that of the plasma membrane of the HeLa cells.

The results of the digestion experiments show that herpes virus contains nucleic acid of deoxyribose type and that this is localized in the dense nucleoid.

Both the findings and the methods used are discussed.

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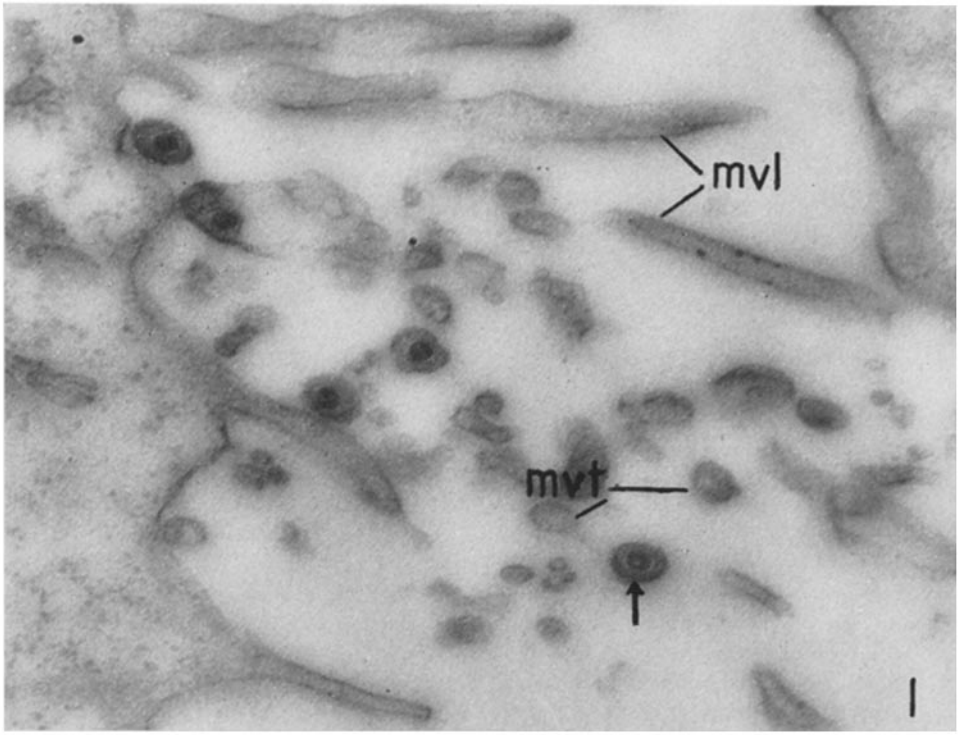
EXPLANATION OF PLATES

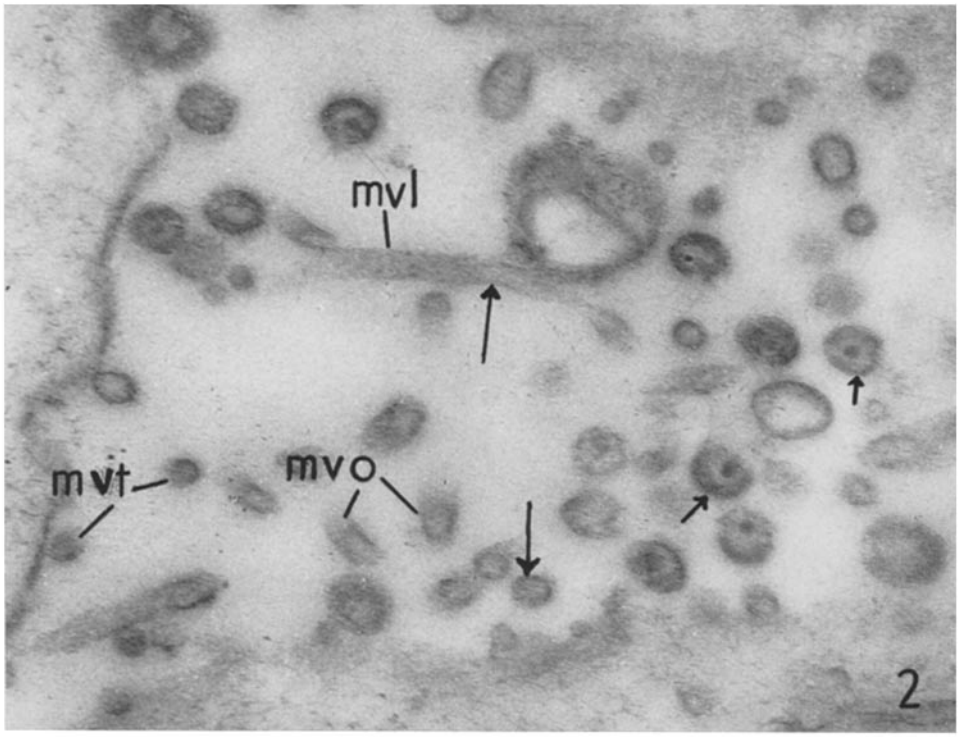
All the figures are electron micrographs of thin sections cut through mature extracellular herpes simplex virus particles lying close to infected HeLa cells.

PLATE 1

FIG. 1. Survey picture of an intercellular space. The surface of one cell is included on the left of the field whilst that of another crosses the upper right hand corner. Numerous slender microvilli project from the cells in an irregular manner and have been cut in various planes ranging from longitudinally (*mvl*) to transversely (*mvt*) in which case they present structureless circular profiles. Mature virus particles lie between the microvilli and can be distinguished by their size, about 120 mμ, and their inner structure. This consists of an outer membrane over an electron-opaque outer zone, and an inner zone almost filled by a central, dense nucleoid which in some particles shows a decreased density at its own centre (*arrow*); there is also the suggestion of a membrane between the outer and inner zones. Osmium fixation; methacrylate embedding; unstained. × 60,000.

FIG. 2. Intercellular space with the surface of cells on the left and below, the former sectioned at right angles to its cell membrane, the latter very obliquely. Microvilli sectioned longitudinally (*mvl*), obliquely (*mvo*) and transversely (*mvt*), can be seen with virus particles between them. In this type of preparation the viral nucleoid is small and eccentric and there is no sign of a membrane between the outer and inner zones of the particle. There is, however, an indication that the cell membrane bounding the microvilli is triple-layered (*long arrows*) and this structure seems to be repeated in the outer limiting membrane of the virus (*short arrows*). Permanganate fixation; methacrylate embedding; unstained. × 60,000.





(Epstein: Fine structure of mature herpes simplex virus)

PLATE 2

FIG. 3. Intercellular space with cells above and below; many microvilli fill the centre of the field with virus particles amongst them. Prepared as here, the virus is round and about 160 mμ in diameter. It can be seen to have an eccentric round, oval, or even slightly rod-shaped nucleoid of great density as compared to other structures present whose contrast is uniformly poor. The viral outer limiting membrane is clear, as is the sharp demarcation between the dense outer zone and the inner zone containing the nucleoid. Osmium fixation; aquon embedding; unstained. × 45,000.

FIG. 4. Detail of virus particles lying just outside the surface of a cell whose plasma membrane crosses the top of the field. The particles are oval and their long axes are parallel to one another; they were also at right angles to the direction of sectioning. A decrease in electron opacity can be seen at the centre of two of the nucleoids (*arrows*) as can the demarcation between the inner and outer zones of the particles. Osmium fixation; methacrylate embedding; unstained. × 120,000.

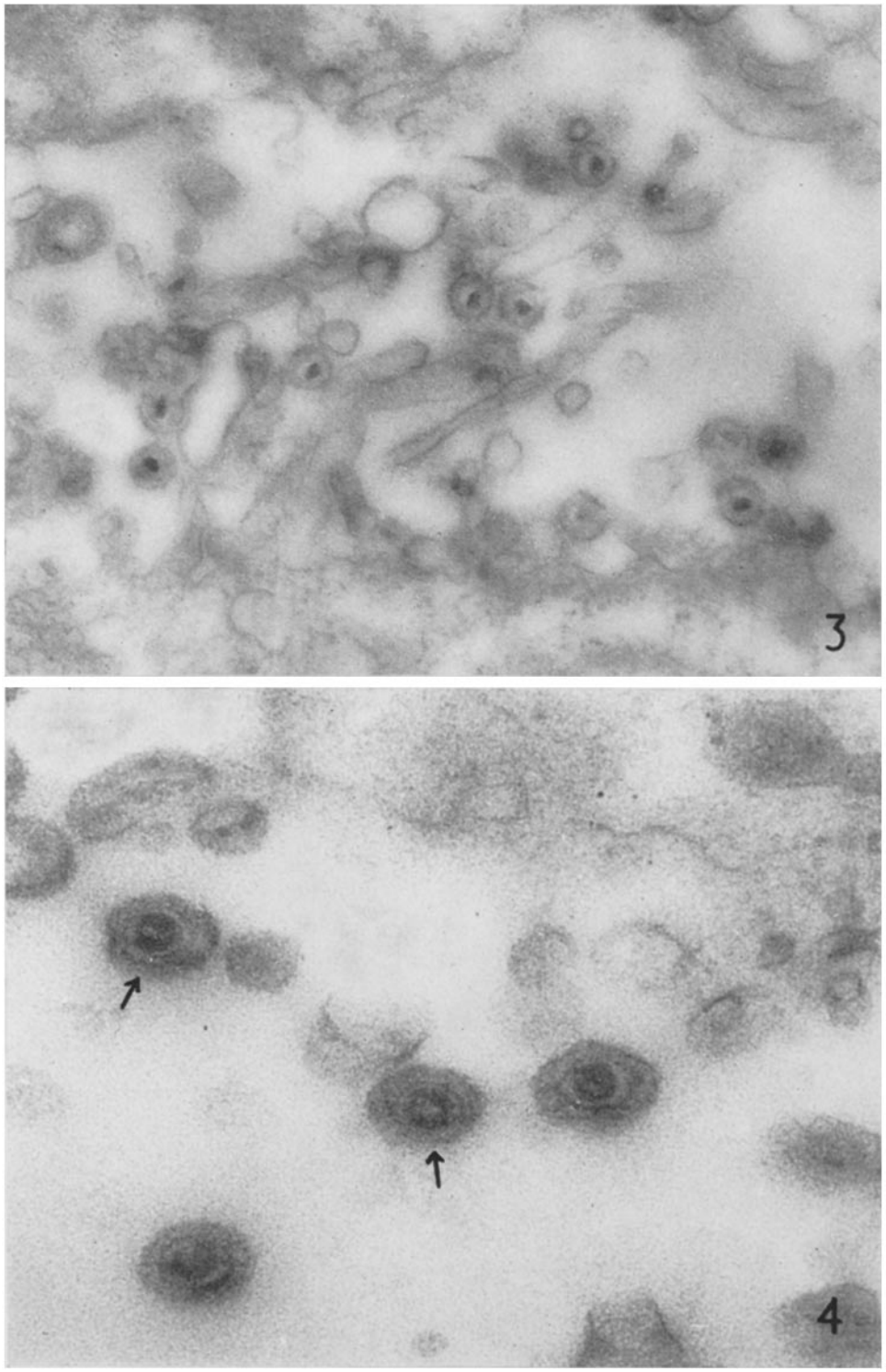


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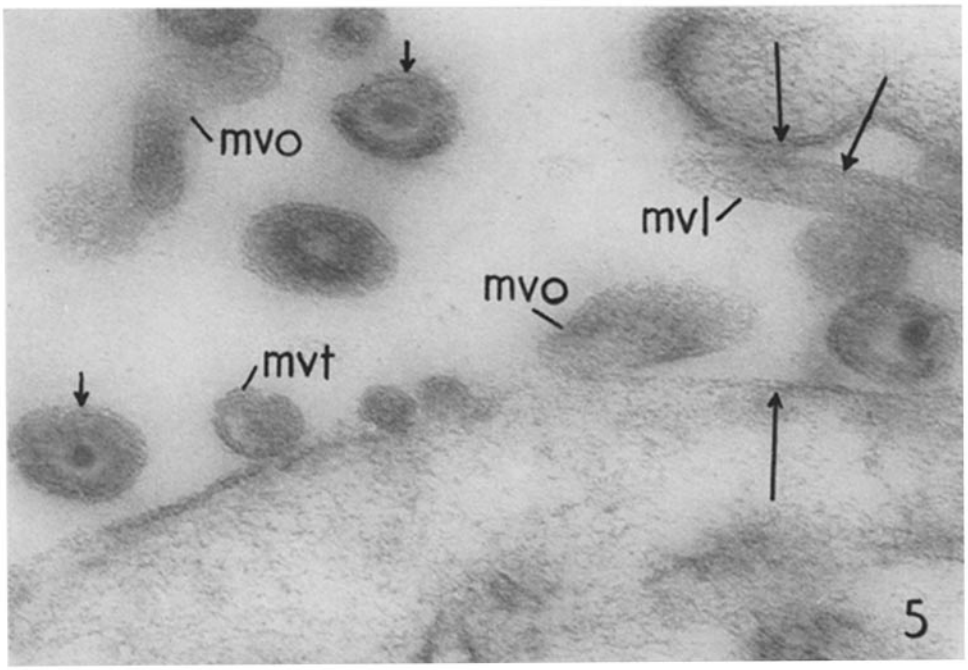
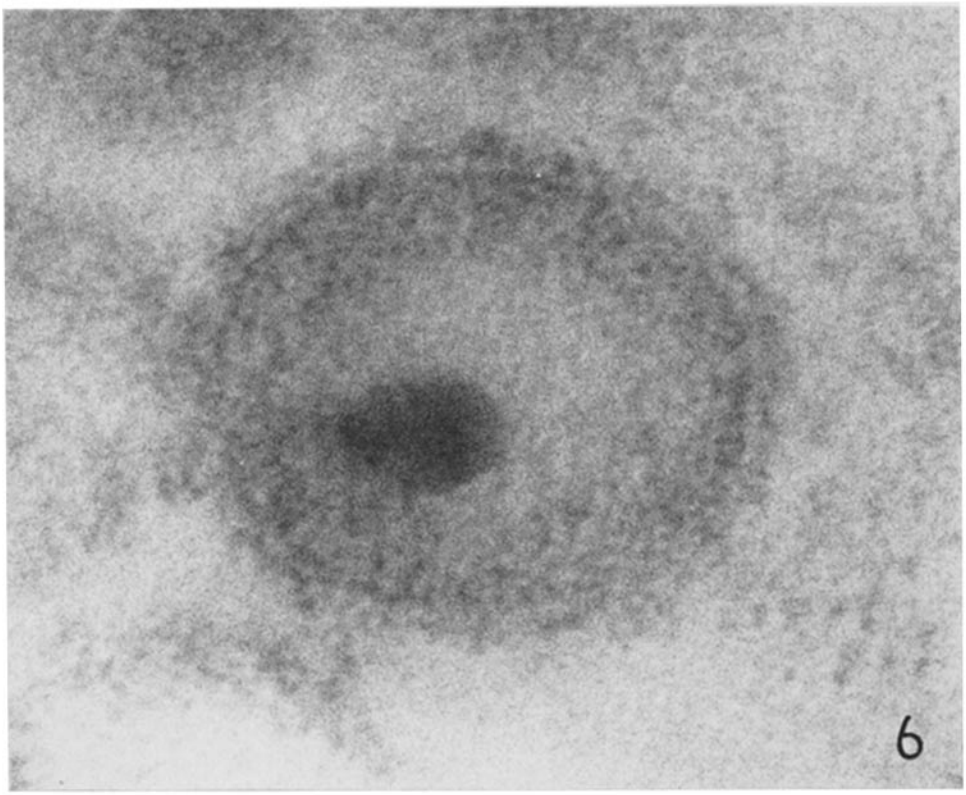
PLATE 3

FIG. 5. Virus particles close to a cell which fills the lower part of the field and the upper right hand corner. Microvilli cut longitudinally (*mvl*), obliquely (*mvo*) and transversely (*mvt*) are present together with virus particles. The preparation method shows both the triple-layered structure of the cell membrane (*long arrows*) and the morphologically identical form of the outer viral limiting membrane (*short arrows*). The small eccentric round or oval viral nucleoid is also evident; there is no membrane between the outer and inner zones of the particles. Permanganate fixation; methacrylate embedding; unstained. × 120,000.

FIG. 6. Detail of mature herpes virus. The particle measures about 165 mμ across and has two zones. The inner one of low density and 90 mμ in diameter contains the eccentric, oval, electron-opaque nucleoid which is about 35 mμ across its smallest dimension; the outer denser zone is some 30 mμ wide. Although the demarcation between the two zones is sharp it is not marked by a membrane. The whole particle is surrounded by a triple-layered limiting membrane consisting of two electron-opaque layers 5 mμ apart with a less dense layer in between. Permanganate fixation; aquon embedding; lead hydroxide staining. × 400,000.





(Epstein: Fine structure of mature herpes simplex virus)

PLATE 4

FIG. 7. Virus particles showing some distortion. The particles lie near a cell which crosses the top of the field; the particle on the right has a large nucleoid filling its inner zone and showing loss of density at the centre. The oval particle on the left (*arrow*) also shows this as well as considerable compression and separation of its zones giving the appearance of an inner membrane. No such structure can be seen in either of the other particles which are less compressed. Osmium fixation; methacrylate embedding; unstained. × 90,000.

FIG. 8. Detail of mature virus showing all the features illustrated in the particle of Fig. 6 except that the nucleoid is round. Permanganate fixation; aquon embedding; lead hydroxide staining. × 240,000.

FIG. 9. Detail of particles cut in various planes and showing the same structures as those of Figs. 6 and 8. The rod-shaped eccentric nucleoid of the upper left particle has been included within the thickness of the section whereas that of the lower left one has not. The particle on the right has been cut tangentially and only a thin surface sliver is present; the obliquity of the cut has masked the outer limiting membrane which is very evident in the other two cases. In the upper right hand corner of the field a microvillus passes obliquely through the plane of the section; the triple-layered nature of its cell membrane (*long arrow*) is indistinguishable from the outer viral membranes (*short arrows*). Differences in nucleoidal shape (*cf.* Figs. 3, 6, and 8) are consistent with the sectioning at various angles of a short, round ended rod 35 mμ wide. Permanganate fixation; aquon embedding; lead hydroxide staining. × 160,000.

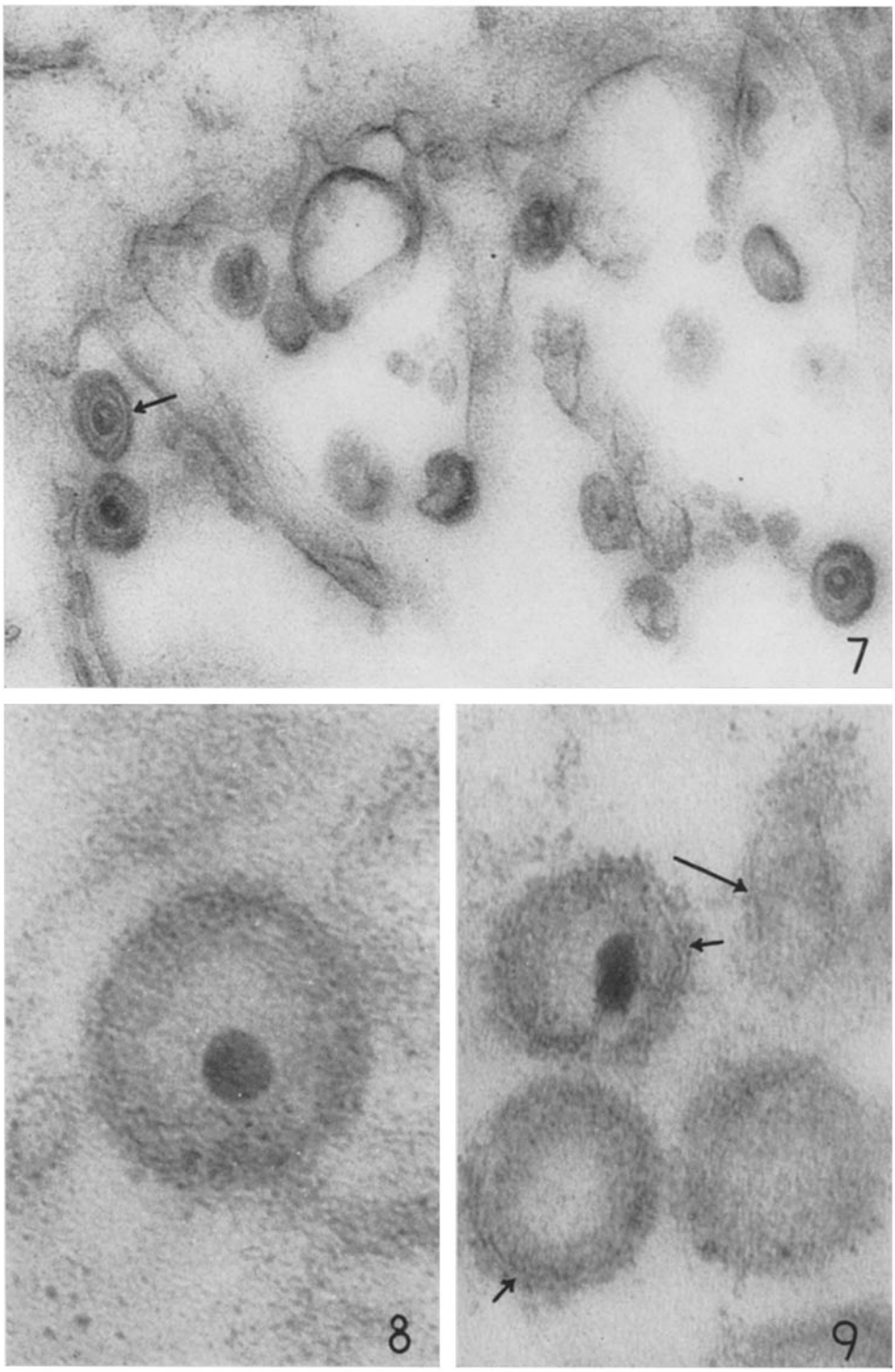


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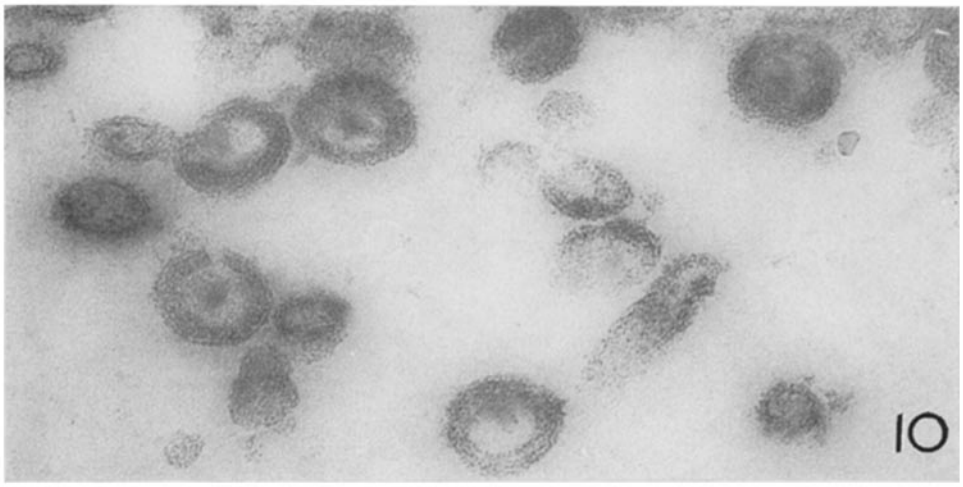
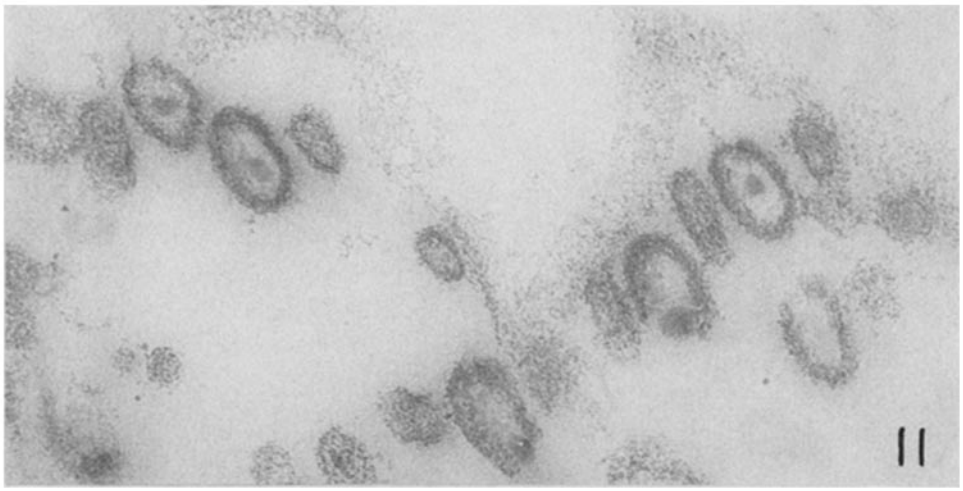
PLATE 5

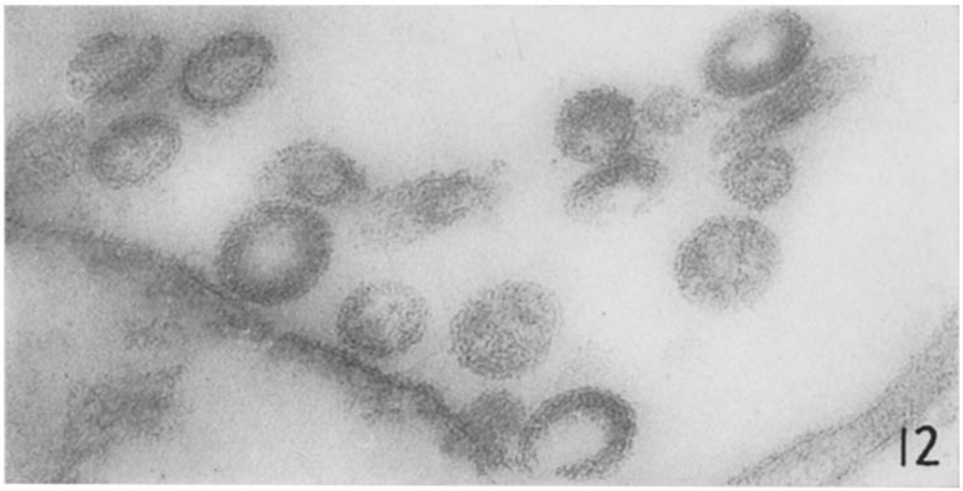
FIG. 10. Mature virus particles lying between microvilli at the surface of a cell. Control preparation incubated in enzyme-free medium before embedding; general extraction effects have occurred but the dense viral nucleoids are nevertheless present. Permanganate fixation; methacrylate embedding; unstained. × 100,000.

FIG. 11. Particles and microvilli. This preparation was incubated with RNase before embedding. Here too, the nucleoids are intact despite extraction effects. Permanganate fixation; methacrylate embedding; unstained. × 100,000.

FIG. 12. Cell surface with microvilli and neighbouring particles. The preparation was exposed to DNase before embedding and it can be seen that the nucleoids have all been removed. Permanganate fixation; methacrylate embedding; unstained. × 100,000.







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