THE ENTRY AND DISTRIBUTION OF HERPES VIRUS AND COLLOIDAL GOLD IN HeLa CELLS AFTER CONTACT IN SUSPENSION

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PLATES 19 TO 23

Although much has been known for some time about the mechanism whereby phage particles penetrate susceptible bacteria (1), relatively little comparable information is available concerning this process in the case of animal viruses and the cells they infect.

It has been shown recently that vaccinia, adenovirus, and influenza virus are all taken into host cells by phagocytosis (2–5), and the fact that this is common to three agents differing so widely in size and in the fundamentals both of their structural type and of the biological behaviour determining it (6), suggests that such a mode of entry may be shared by many, or even all, animal viruses.

As Dales has pointed out (3), virus entry by phagocytosis, or during pinocytosis, might simply be a special example of the general method whereby cells in a wide variety of normal situations take up particulate objects. But under normal conditions phagocytosis is usually directed to the concentration of assimilated material in membrane-bounded vacuoles or inclusions connected with digestive disposal processes (7, 8), and for infection by ingested virus particles to occur at such sites, some additional unphysiological element would seem to be necessary. In the case of vaccinia and adenovirus there is indeed evidence that special pathways are followed after entry into cells through cytoplasmic membrane systems, since both agents have been observed lying free deep in the cytoplasmic matrix (3) in regions related to their multiplication sites. Although it is not known how these viruses pass to their destinations through the vacuolar membranes which normally enclose ingested particles, their doing so appears to constitute an abnormal phenomenon directly related to the presence of an infective, as opposed to an inert, particle.

These early steps in the penetration of cells by animal viruses are clearly of the greatest importance for establishing infection, and it was therefore con-

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sidered that a study which might help to clarify those aspects of the process which are obscure, or indicate the type of cellular mechanisms involved, would be of value.

Experiments were accordingly undertaken in which the cells of a stable *in vitro* system were mixed either with an inert type of particle or with a virus particle, so that a comparison could be made of differences in their mode of entry and subsequent intracellular distribution. For this work, HeLa cells of a strain whose fine structure had already been defined (9) were placed in suspension cultures for various brief periods either with particles of colloidal gold or with mature herpes simplex virus. The latter was selected because it was not one of the very few animal viruses which have been observed during cell penetration and because it has significant structural and functional differences (6) from those which have been observed (2–5), whilst the choice of colloidal gold for comparison was determined by the fact that HeLa cells have long been known to take it up from suspension in culture fluid (10). In addition, the availability of a preparation containing gold particles varying considerably in size, permitted any possible effects of this factor on uptake to be checked.

After contact with the particles, the HeLa cells were embedded and examined in thin sections in the electron microscope in order to study the whereabouts of ingested material. The present communication reports the results of the observations made.

Materials and Methods

Virus Strain.—HeLa cell–adapted herpes simplex virus of the HFEM strain (11) has been used for the experiments.

Stock Virus.—Ampoules of stock virus were prepared and stored just as in earlier work (12). When titrated in eggs following freezing to −70°C under the conditions used for storage, the virus seed was found to have a 50 per cent end point per ml of $10^{-6.5}$; the assay was done by methods already described (12).

Colloidal Gold.—A suspension of colloidal gold in 1 per cent aqueous gelatin was kindly prepared and supplied by Mr. H. E. F. Notton of Crookes Laboratories Ltd., London. On direct examination in the electron microscope the majority of particles were found to have a diameter of 15 to 20 mμ, but in addition, appreciable numbers were either as small as 10 mμ across or measured between 30 and 35 mμ. The gold was present in a concentration of 6 mg per ml.

Maintenance of HeLa Cells.—HeLa cells (13) were grown in flat glass bottles as reported previously (14, 15), and whenever confluent cell sheets developed they were used as a source of new cultures.

Suspension Cultures of HeLa Cells.—For the experiments, culture bottles were selected where the individual cells were dispersed on the glass singly but close together. The culture medium was removed and was replaced by 0.5 ml rabbit serum with which the cells were washed from the glass by gentle pipetting; the serum was inactivated before use by heating to 56°C for 30 minutes. As the cells collected in the serum, sample counts were made in a Fuchs Rosenthal haemocytometer, and pipette washing was continued until the serum contained a total of about 350,000 cells. On one occasion medium I of Pereira and Kelly (14) was used instead of serum.

The cell suspension was placed at 37°C in a 15 ml capacity weighing bottle with ground glass stopper and was kept as a suspension by means of a ⅛ inch length of steel wire rotated on a magnetic stirrer at 250 RPM. At the start of stirring, test particles, either stock virus or colloidal gold, were added to the cells in sufficient fluid to dilute the serum to 30 per cent of the final volume.

Preparation of Cells for Electron Microsocpy.—When the cells in a suspension culture were ready for harvesting, they were drawn into a syringe prewarmed to 37°C, squirted into 2 ml phosphate-buffered osmium tetroxide with glucose (16), pelleted in a tapered 8 ml polythene centrifuge tube (J. E. Frankle Company, Philadelphia) while being fixed in osmium textroxide, and then cut from the pellet into 1 mm cubes in 50 per cent alcohol exactly as in earlier work (9, 17). The cubes of pelleted cells were dehydrated, embedded in epikote 812, sectioned, and contrast-stained in the section with uranyl acetate, as already described elsewhere (18). In some instances sections were stained with phosphotungstic acid (PTA) (19); this technique did not usually work well when applied to material embedded in epikote and for the experiments where it was used a few of the cubes were embedded in araldite as recommended by Glauert and Glauert (20).

EXPERIMENTAL PROCEDURE

In a first set of experiments HeLa cells were mixed in suspension with mature herpes simplex virus at a high virus to cell ratio, and were exposed to the particles during stirring for 15, 30, 60, or 120 minutes. At the end of these periods the cells were prepared for electron microscopy, and the morphological aspects of their interactions with the virus were studied with special reference to the duration of exposure.

In further experiments, cells which had been in contact with the virus for short periods were examined after staining in the section with PTA.

For a final group of experiments HeLa cells were stirred in suspension cultures with particles of colloidal gold for 30, 60, or 120 minutes and were then prepared for examination in the electron microscope; observations on the mode of uptake of this inert material were made for comparison with that of the virus.

RESULTS

General Observations.—HeLa cells harvested from suspension cultures after the brief periods used in the experiments showed no significant unusual features of fine structure. However, there was a tendency for such cells to be both more rounded and very much more uniformly covered with microvilli than those which had been attached to a flat surface (9), and chains of micropinocytosis vesicles extending inwards from the cell membrane (9) were very common (Figs. 4, 8, and 12).

Uptake of Herpes Virus.—After 15 minutes of stirring in suspension with herpes virus almost all the cells in a section showed mature particles close to the outer aspect of their surfaces but separated from the cell membrane by a narrow gap (Figs. 1, 2, and 3). The profuse distribution of these particles was evident in sections which cut the cells tangentially so as to include a thin surface sliver in full-faced view (Fig. 2). The particles were structurally intact (12), were often in close relation to microvilli (Fig. 1), and the latter sometimes seemed to be bending over to enclose them.

Where the cells collected after 15 minutes with virus were stained in the

section with PTA, no spiny projections were detected between the cell surface
and nearby virus particles (Fig. 3). In rare instances, however, the PTA stain-
ing revealed an amorphous extraneous coat lying against the plasma membrane
and filling the gap between adsorbed particles and the surface of the cell (Fig.
3).

Cells which had been exposed to the virus for 30 minutes also had particles
at the surface. But in addition to this it was now more characteristic for the
particles to be present within vacuoles; the latter were situated in the pe-
ripheral cytoplasm below microvilli or surface processes and were frequently
linked to the exterior by chains of pinocytosis vesicles (Figs. 4 to 6). The way
in which the vacuoles formed and virus became included in them was indicated
where particles were seen partially ingested at the bottom of deep invaginations
between cell processes (Fig. 4) at a stage beyond the surface location found after
15 minutes (Fig. 1) but before a fully completed vacuole had become dis-
connected from the plasma membrane (Figs. 4 and 8).

Many of the virus particles lying both in invaginations and within vacuoles
were often strikingly different in structure from those usually encountered
outside cells (12) (Fig. 1) in that their outer layers were either split or in various
phases of detachment (Figs. 5 to 8). At higher magnifications it was evident
that the detached layers consisted of the coverings which the virus acquires
from the cell membrane and underlying cytoplasm during release and matura-
tion by budding (21, 22) (Figs. 5 to 8). In each case stripping could be seen to
occur along the plane where these outer coverings lay against the subunits
surrounding the viral nucleoid (Figs. 5 to 8); such subunits lie deep within
mature herpes virus (23, 12), and their outer aspect appears to form the surface
of the immature particle (21-23, 12). Objects morphologically indistinguishable
from immature herpes (24, 21) and thus also from the central portion of the
mature virus off which the covering layers stripped away in the vacuoles
(Figs. 5, 6, and 8), were very occasionally found, at this stage, lying free in the
juxtanuclear cytoplasm (Fig. 7).

The appearances encountered in cells after 60 minutes of contact with the
virus were very similar to those after 30 minutes but with some difference in
the relative frequency with which virus was seen in various situations. Particles
were less common at the cell surface and more common in vacuoles than at the
earlier stage, and detachment of the outer coverings of those which had been
ingested was a frequent finding (Fig. 8). Completely stripped central portions
of such virus were also more frequent than before in the cytoplasmic matrix
close to the nuclear envelope; in addition, they were observed for the first time
within vacuoles (Fig. 9), but no transition between these two sites could be
detected.

All the foregoing features of particle uptake were present in cells examined
after 120 minutes' stirring in suspension with herpes virus, but considerably

more rarely than before. Despite this, many of the cells showed increased pinocytosis and vacuole formation in the peripheral cytoplasm immediately below the plasma membrane.

Uptake of Colloidal Gold.—Cells mixed in suspension cultures for 30 minutes with colloidal gold were found to have small numbers of electron-opaque particles lying close against the outer aspect of their plasma membranes, particularly in the region of surface projections. Particles were also seen in the early stages of ingestion, the larger ones of 30 to 35 mμ diameter being either in invaginations between microvilli or within superficial vacuoles (Fig. 10), and the smaller, in micropinocytosis vesicles. After contact for this period of time, gold was not present at any other site in the cell.

Following exposure for 60 minutes, gold particles were more frequent at the surface of the cells, as was uptake both by vacuole formation (Fig. 11) and *via* the smallest vesicles (Fig. 12). In addition, particles were found singly or in twos and threes within membrane-bounded spaces deeper in the cytoplasm (Fig. 13), and in considerable numbers in large paranuclear digestive vacuoles (Figs. 13 and 14) which sometimes contained other ingested material such as crystals (9, 25) (Fig. 14).

When cells were examined after stirring with the gold for 120 minutes, the surface phenomena had become more rare, and increased numbers of particles were found concentrated in digestive vacuoles.

DISCUSSION

Preliminary observations on high titre herpes suspensions (kindly supplied by Dr. P. Wildy) made from experimentally disrupted infected cells showed that particles without outer layers were present as well as intact mature virus, and that both were ingested by test cells. Although there is some indication that incomplete naked particles may sometimes be biologically active (26), they would not appear usually to take part in the transmission of herpes infection since all past morphological studies have shown without exception that only the mature virus ever occurs outside cells (27–29, 24, 30, 12, 21, and 22); mature membrane-bounded particles thus constitute the natural infecting agent. In order to follow cell penetration during this normal type of herpes infection and avoid confusion arising from the presence of morphologically incomplete virus, only suspensions of mature particles in fluid from undisturbed infected HeLa cultures were used in the present investigation.

With this material it has been found that mature herpes virus enters susceptible HeLa cells by a series of steps, normal and non-specific to begin with but more distinctive later.

The first stage clearly consists of viral attachment, since cells sampled following 15 minutes' exposure to the agent were found, even after spinning down during preparation, with many particles around them (Figs. 1 to 3). The

mechanism of this attachment is not based on the presence of spiny projections on the particles such as those which hold myxoviruses close against the plasma-lemma of susceptible cells (4, 5), for PTA staining failed to reveal such structures in the present case (Fig. 3) although known to be capable of showing them in other systems (19). However, the finding of a narrow gap between adsorbed herpes virus and the cell surface (Figs. 1, 3, and 7) suggests that an extraneous coat on the outer aspect of the plasmalemma (31, 32) as was sometimes revealed by PTA staining (Fig. 3), may here play the principal role in holding the particles in position. Under identical conditions inert colloidal gold attaches in a similar way (Figs. 10 to 13), and in each case the process closely resembles that operating normally with other particles at the surface of several types of cell (32–34).

Once attached, mature herpes particles were engulfed in surface invaginations and phagocytosed into vacuoles in the peripheral cytoplasm (Figs. 4 to 8) just as was reported for the uptake of vaccinia, adenovirus, and influenza virus (2–5). But this stage in cell penetration also appears to be non-specific since the larger colloidal gold particles were ingested in the same manner (Figs. 10 and 11), and the whole process is exactly comparable to physiological uptake where the particulate matter is of a similar or slightly larger size (35). In contrast, the smaller gold particles were quite clearly taken into the cells in a different way, namely, by inclusion in micropinocytosis vesicles (Fig. 12), suggesting that size alone may determine the method of uptake. The fact that another small particle, ferritin, also enters cells like this in tiny vesicles (8, 36) lends some support to this idea, and although vacuole and micropinocytosis ingestion seem to be closely related, their divergence in behaviour with particles of different sizes appears to imply definite functional differentiation.

Having been engulfed, the inert colloidal gold travelled across the cytoplasm within smooth surfaced spaces (Fig. 13) to the juxtanuclear region, where, after 60 minutes of mixing with the cells, it was found collecting in the vacuoles (Figs. 13 and 14) in which it has long been known to accumulate (10). This kind of vacuole was already thought to have digestive functions early in electron microscope studies (37), a view supported in the case of HeLa cells by the finding there of unusual ingested extraneous material such as asbestos micro-crystals (9, 25) (Figs. 7 and 14), and hydrolytic activity has actually been demonstrated recently in the comparable vacuoles of phagocytic cells in rat liver (38).

Asbestos and gold, unlike organic matter, are not susceptible to digestion and therefore remain in the vacuoles (9, 10). But with herpes particles, the outer coverings derived from host cell plasma membrane and subjacent cytoplasm during virus release by budding (21, 22) begin to strip off (Figs. 4 to 8) and digest away (Fig. 9) even as vacuoles are forming from surface invaginations (Fig. 4), suggesting that as the cell membrane changes location its functions

rapidly alter to acquire digestive properties. Another example of such a quick functional change in the plasmalemma of HeLa cells has recently been reported with adenosine triphosphatase activity (39), and the important problems such findings present regarding the siting and activation of enzymes on the constantly flowing membrane systems of cells (40, 37, 41–43) have already been discussed in that context (39).

The intravacuolar removal of the outer layers of mature herpes virus appears therefore to result from normal cellular digestive functions. But the stripped central portion of the virus must then quickly leave the vacuoles since it was only seen there rarely (Fig. 9) whilst being relatively common in the cytoplasm near the nucleus (Fig. 7). Non-viral material does not pass out of vacuoles, so that at this stage an abnormal pathway is being followed; a comparable phenomenon has been reported with ingested adenovirus (3), but just as here, escape from the vacuoles could not be observed (3), and its nature remains obscure. However, it must involve some stimulus causing the cell to treat virus in a special way even before the viral nucleoid has left the particle (Figs. 7 and 9) (3) and become available to act on cellular functions.

The findings of the present experiments thus provide a further example of a virus being ingested by non-specific phagocytic processes, and to that extent increase the possibility that "viropexis," which was merely hypothetical when first proposed (44), may indeed be the basic general mechanism whereby animal viruses penetrate susceptible cells. In the case of herpes virus it is certainly considered that this ingestion was part of infection rather than mere disposal of particles, since the virus leaves the usual intracellular sites connected with digestion of extraneous matter and follows abnormal pathways into the paranuclear cytoplasmic matrix.

In addition to the question of cell entry, the results obtained are of further interest in relation to subsequent happenings. These are significant in that they follow a pattern apparently determined by the structure of the herpes particle. The pattern is also of interest because it contrasts with that which follows the uptake of viruses having a different morphology. Like herpes virus, adenovirus is phagocytosed and comes to lie in cytoplasmic vacuoles (3); but this agent is released from cells without a membranous covering (45) and being of naked type (6) from the outset with a surface of tightly-packed protein subunits (46), it is not digested in the vacuoles and passes from them unchanged deeper into the cell (3). Influenza virus on the other hand forms at the surface of cells where its components are surrounded by cell membrane as the particle is released by budding (47, 48). Influenza virus is thus of the membrane-bounded type (6), and when taken into the vacuoles of susceptible cells its outer coverings are digested and it disintegrates (4, 5); the genetic material liberated from the virus in this way appears to enter the cell directly from the vacuole (5).

Although herpes virus is also released from cells by the budding mechanism

(21, 22) it is not assembled at the cell surface. It replicates first in the nucleus to form immature particles (27, 24) and it is these, as preformed structures which escape through cellular membranes in buds (21), maturing in the process by acquiring their final outer membranous covering from the cell. Thus, on ingestion the outer layers of the particle are destroyed in vacuoles (Figs. 4 to 9) as occurs with the membrane-bounded influenza particle (5), but the stripped central portion of the agent then passes out of the vacuoles morphologically intact (Fig. 7) and comes to lie free in the cytoplasm in the same way as the adenovirus (3).

It has already been suggested that biological and functional factors, particularly those connected with virus release, play an important role in determining the structure of the mature particles of different viruses (6). The present findings appear to indicate that particle structure in its turn influences the functional behaviour of susceptible cells when they take up and are penetrated by viruses of different morphology.

SUMMARY

The way in which herpes virus of a well adapted strain penetrates susceptible HeLa cells has been investigated using thin sectioning techniques for electron microscopy. Mature virus particles and cells were mixed together in suspension cultures for 15, 30, 60, or 120 minutes so that the stages in virus uptake could be followed in sequence. The ingestion of particles of colloidal gold by HeLa cells under similar conditions was studied for comparison in parallel experiments.

After 15 minutes' contact, the mature virus was found adsorbed on the surface of the cells but separated from them by a narrow gap in which phosphotungstic acid staining was sometimes able to reveal an extraneous coat which appeared as an amorphous layer on the outer aspect of the plasma membrane. When mixing continued for longer the particles were present in deep invaginations or actual cytoplasmic vacuoles, with their outer layers in various stages of stripping and digestion. The stripped, naked, central portion of the virus was occasionally found in these vacuoles but was more commonly free in the cytoplasmic matrix; the mode of transition between these sites could not be determined. Where contact continued for 2 hours these phenomena were much less frequently observed.

The larger particles of colloidal gold were ingested in the same way as the virus, but smaller ones were taken up in micropinocytosis vesicles. The gold passed through membrane-bounded cytoplasmic spaces to accumulate in vacuoles from which, in contrast to herpes particles, it did not escape.

These findings are discussed, and considered with particular reference to their bearing on the initiation of infection, the uptake and disposal of particles by cells, and the influence on the latter of virus morphology.

Note Added in Proof.—Since this communication went to press, I. H. Holmes and D. H. Watson have reported "An electron microscope study of the attachment and penetration of herpes virus in BHK21 cells" (*Virology*, 1963, **21**, |112); this work shows herpes uptake by ingestion into vesicles and thus supports the early stages in penetration found in the present experiments.

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EXPLANATION OF PLATES

All the figures are electron micrographs of thin sections of HeLa cells collected from suspension cultures after various periods of exposure to mature herpes virus or particles of colloidal gold. Epoxy resin embedding was used, and all the sections were contrast stained with uranyl acetate, except for that shown in Fig. 3.

PLATE 19

FIG. 1. Portion of cell surface and underlying cytoplasm after 15 minutes contact with virus. The cell membrane crosses the right side of the field from top to bottom and is thrown up into processes and microvilli. A mature herpes particle (arrow) lies close against the cell but separated from it by a narrow gap. Fine filaments (as at x), Golgi elements (g), and free particles (p), can be seen in the cytoplasm. × 40,000.

FIG. 2. Part of a cell after 15 minutes with virus, shown in a tangential section which has grazed the exterior and included a thin surface sliver within its thickness. With this full-faced view the profuse distribution of attached herpes virus (arrows) can be seen. × 30,000.

FIG. 3. Detail of cell surface following similar exposure to the foregoing but after staining with phosphotungstic acid (19). The cell membrane runs across the centre of the field; with this preparation method the gap between it and the attached particle on the left appears to be occupied by an extraneous coat. Internal viral morphology is obscured by the staining. × 100,000.
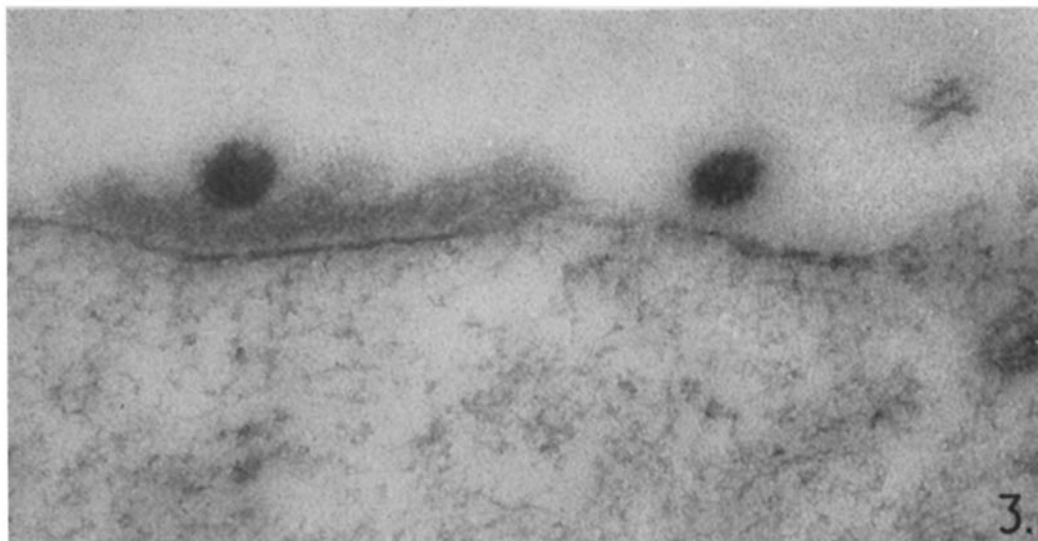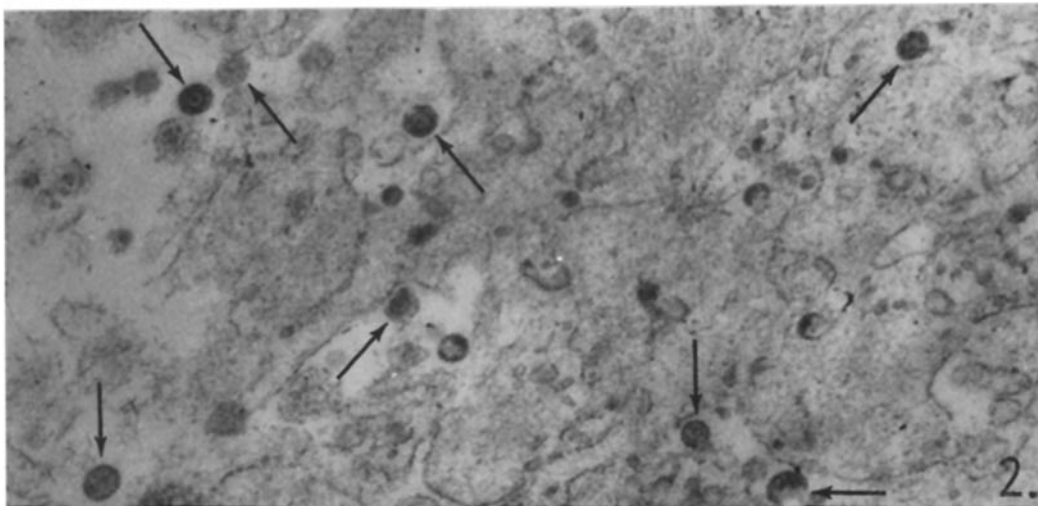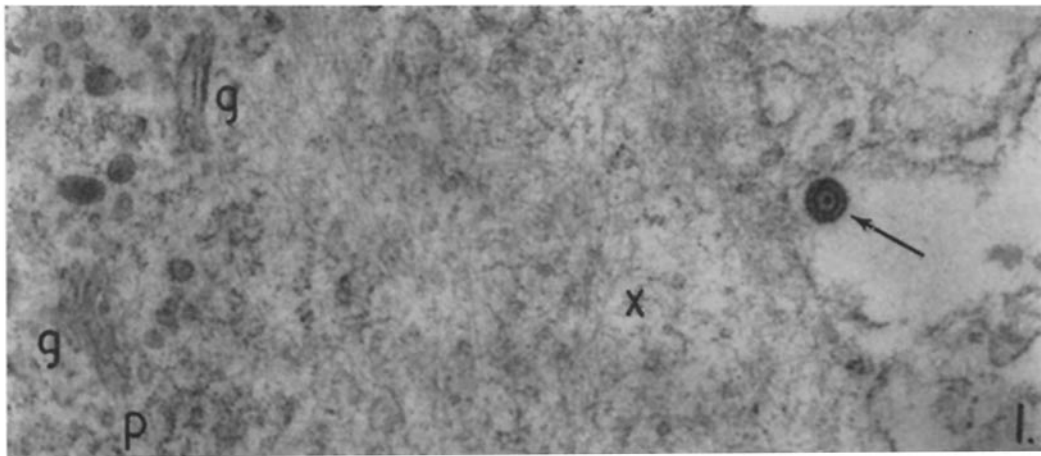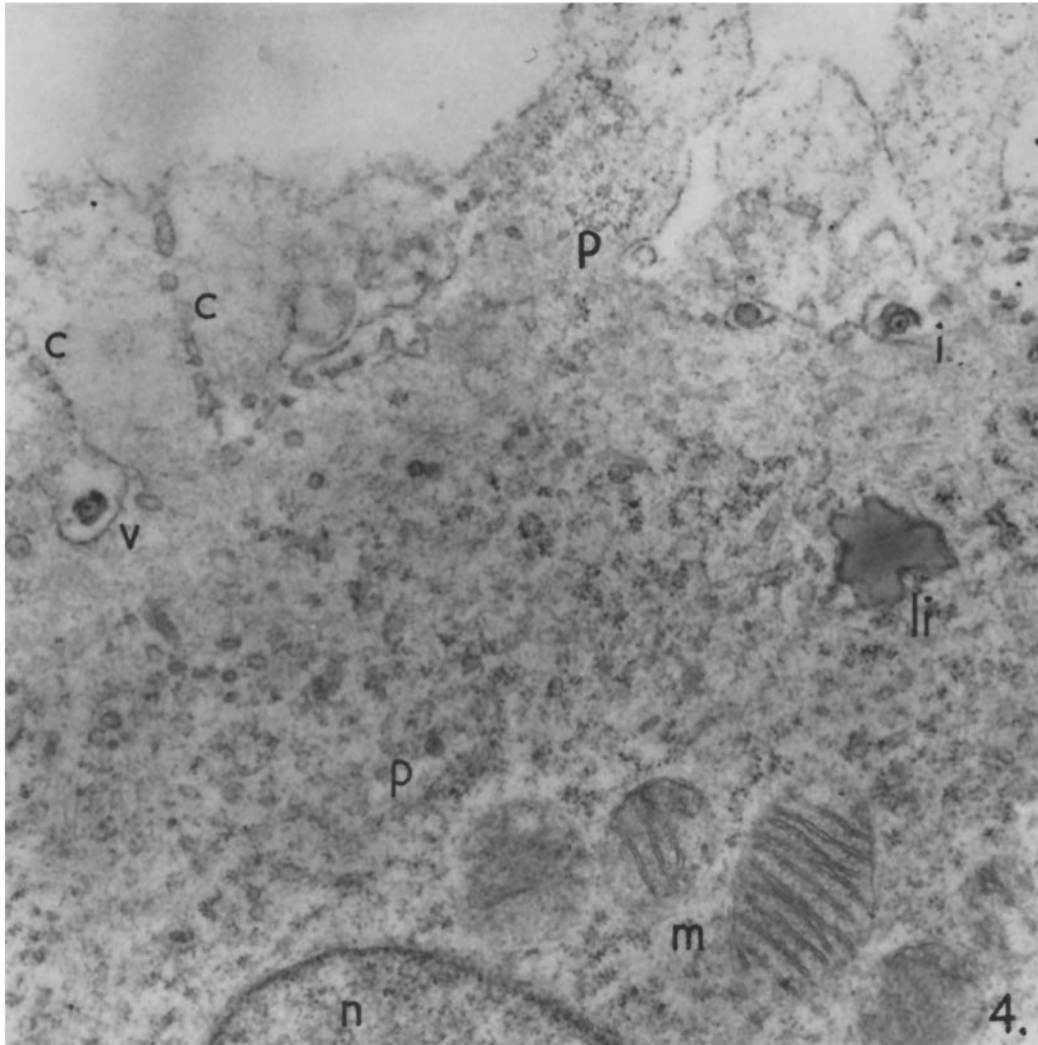
(Epstein *et al.*: Uptake of herpes virus and gold by HeLa cells)

PLATE 20

FIG. 4. Survey picture of cytoplasm with a small area of the nucleus (*n*) below, from a cell exposed to herpes virus for 30 minutes. The cell membrane crosses the top of the field and forms several processes on the right. Between two of these, at *i*, there is a deep invagination with a virus particle at the bottom, whilst a fully engulfed particle is present in a vacuole (*v*) on the left of the field; in both cases the outer viral coats are in the process of removal. A lipid body (*li*), mitochondria (*m*), cytoplasmic particles (*p*), and chains of pinocytosis vesicles (*c*) can all be recognised; filaments are present in the cytoplasmic matrix. × 30,000.

FIGS. 5 and 6. Small areas of cytoplasm from the foregoing preparation showing herpes particles within membrane-bounded spaces. Stripping of the outer viral coats can be clearly seen. × 120,000.
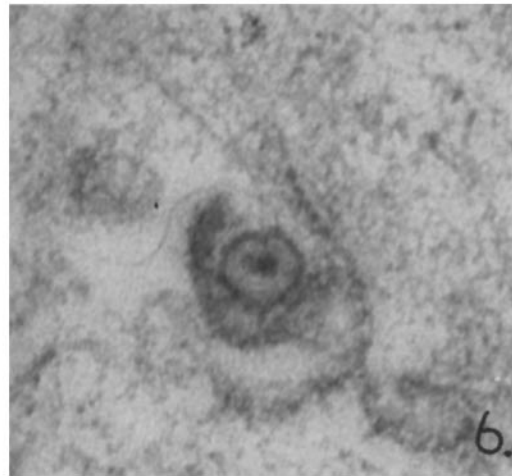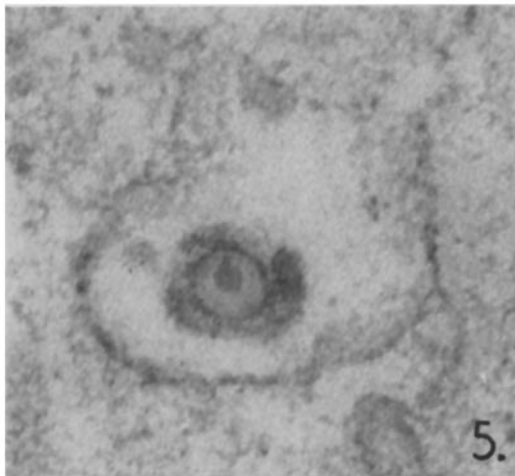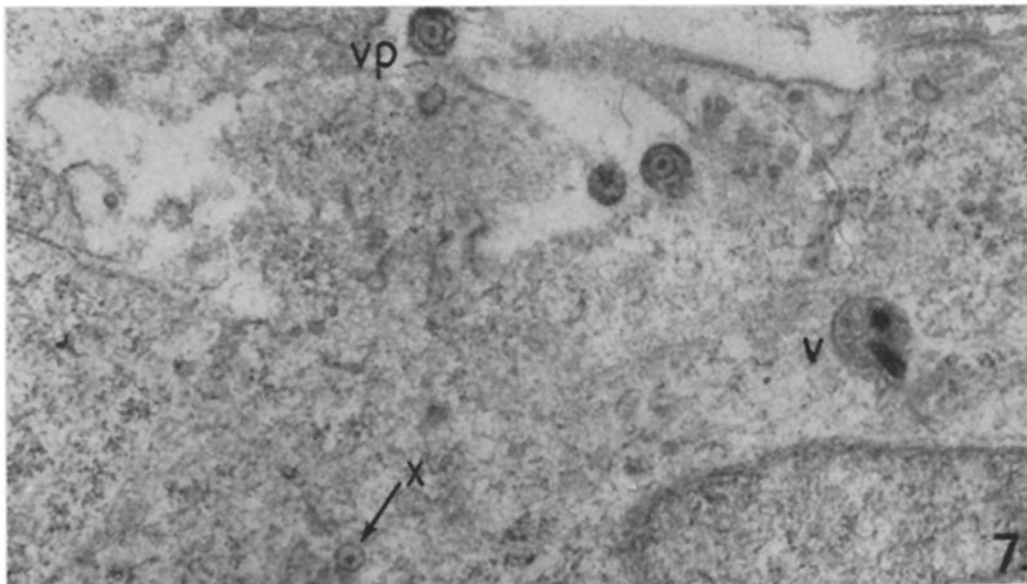
PLATE 21

FIG. 7. Peripheral cytoplasm in a cell mixed with virus for 30 minutes. The convoluted surface of the cell is at the top of the field and a small area of the nucleus in the bottom right-hand corner. An adsorbed extracellular particle (*vp*) is present close to the plasma membrane, with two other particles below and to the right lying in an enclosed space; one of the latter appears small as it has been cut tangentially and, the other shows stripping of its outer layers. A digestive vacuole (*v*) contains ingested asbestos crystals, while at *x*, the stripped, naked, central part of a herpes particle lies free in the cytoplasmic matrix. × 40,000.

FIG. 8. Small area of cytoplasm below the surface of a cell mixed with virus for 60 minutes. The cell membrane is above, with many invaginations and chains of pinocytosis vesicles (*c*) running in from it. Two intravacuolar herpes particles (*hv*) show disintegration of their outer layers. × 50,000.

FIG. 9. Detail of nucleus (*n*) and adjacent cytoplasm following 60 minutes contact with herpes virus. The completely stripped central part of a virus particle is present inside a cytoplasmic vacuole (arrow). × 60,000.
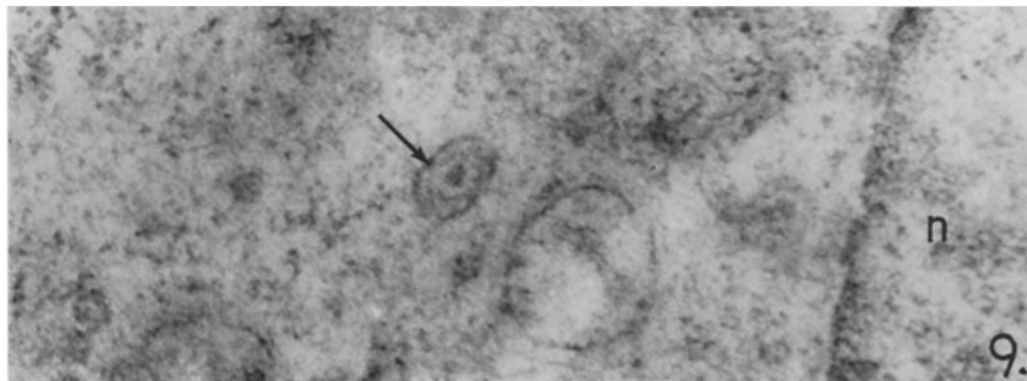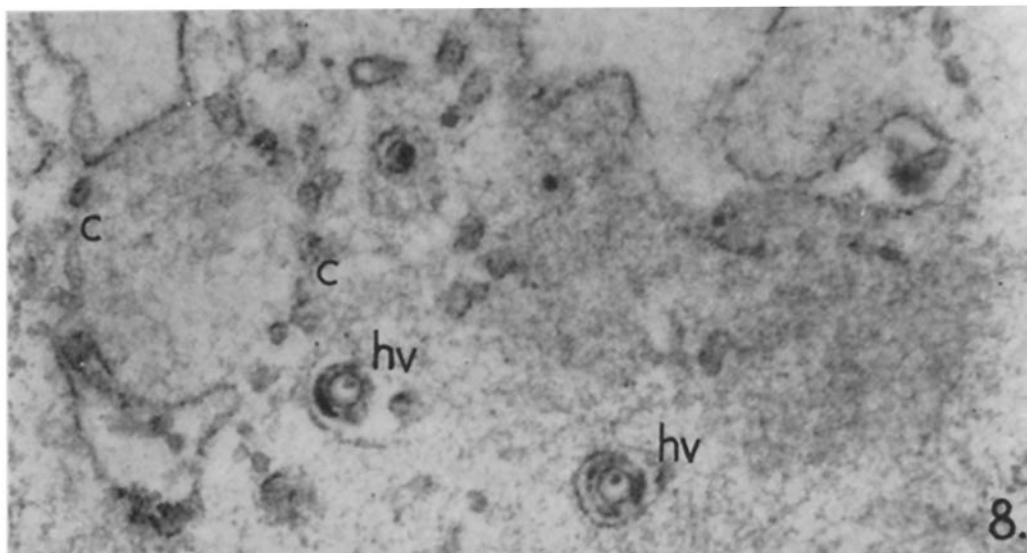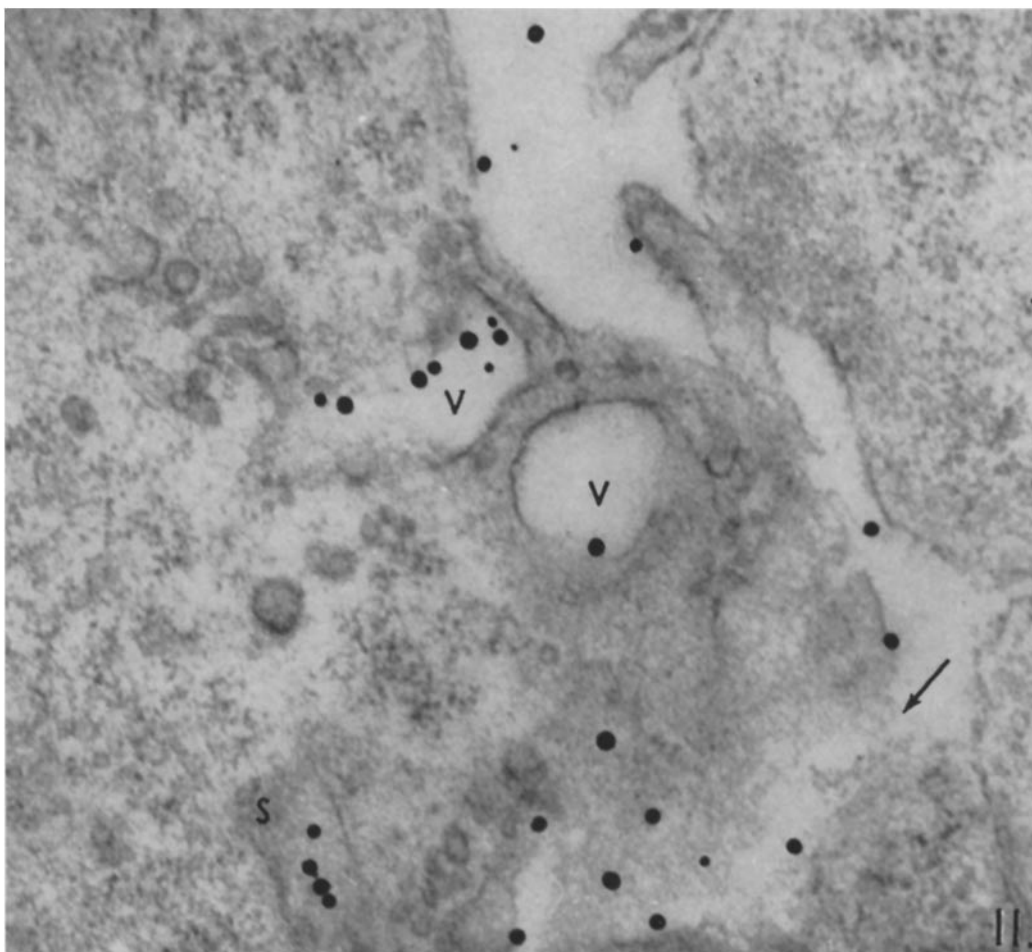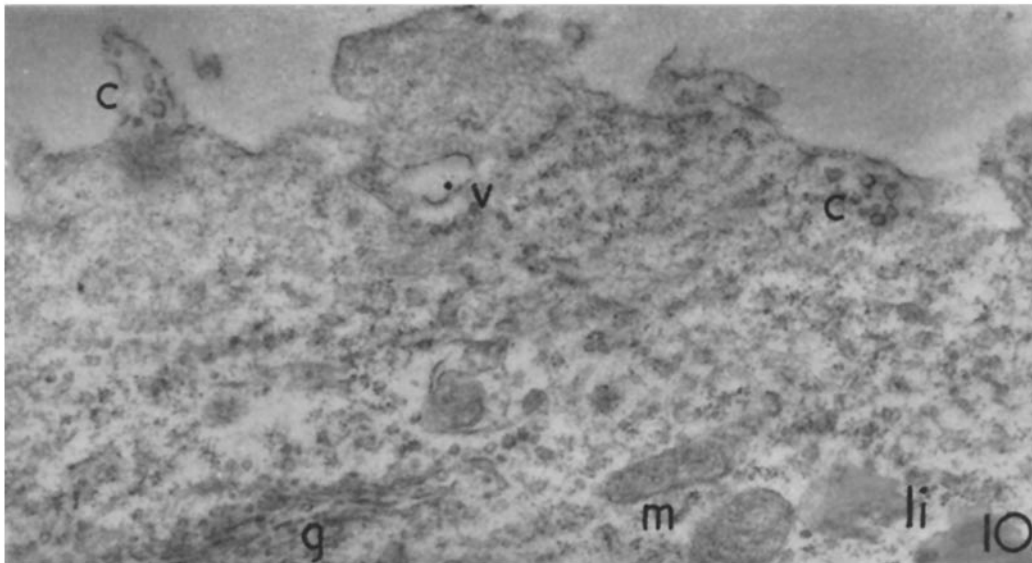
(Epstein *et al.*: Uptake of herpes virus and gold by HeLa cells)

PLATE 22

FIG. 10. Peripheral cytoplasm of a cell placed with colloidal gold for 30 minutes, showing the plasma membrane crossing the top of the field; one of the larger gold particles of about 30 mμ diameter has been engulfed in a vacuole at v. Golgi components (g), mitochondria (m), lipid bodies (li), and micropinocytosis vesicles (c) can be seen. The cytoplasmic matrix also contains free ribonucleoprotein particles and fine filaments. $\times$ 30,000.

FIG. 11. Detail of the surfaces of two adjacent cells which had been in contact with gold for 60 minutes. The intercellular space runs from the top to the lower right corner of the figure where an obliquely sectioned invagination (arrow) dips into the cytoplasm of the cell on the left. Gold particles having diameters mostly greater than 30 mμ are present close to the cell surface as well as engulfed in superficial vacuoles (v) and a membrane-bounded cytoplasmic space (s). $\times$ 60,000.
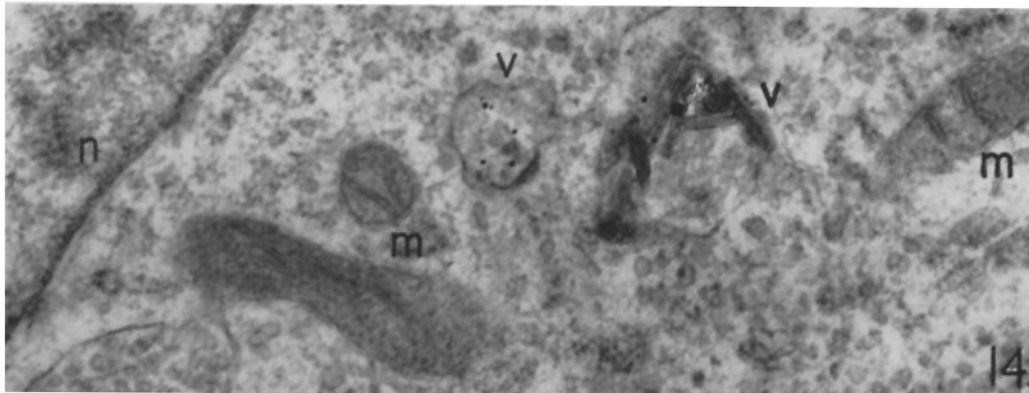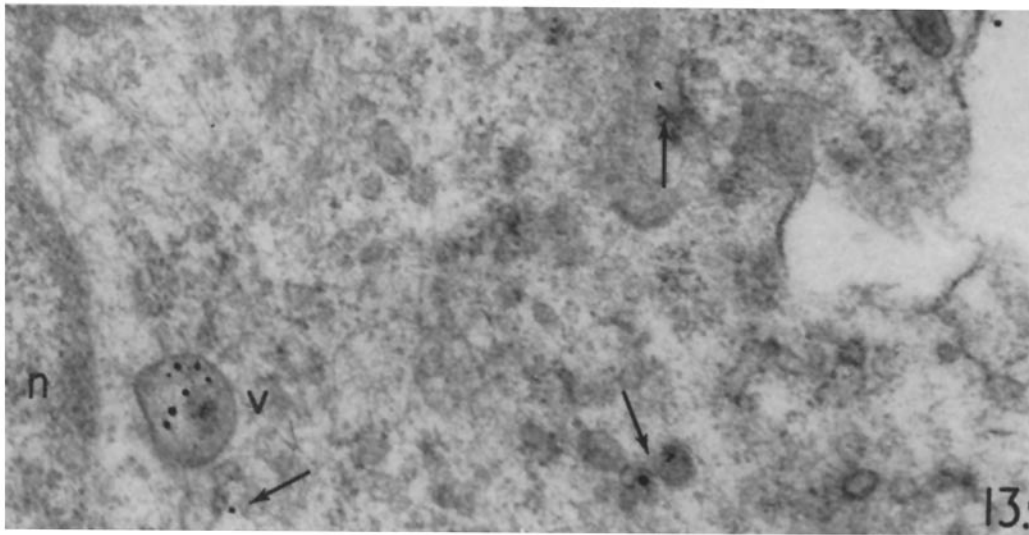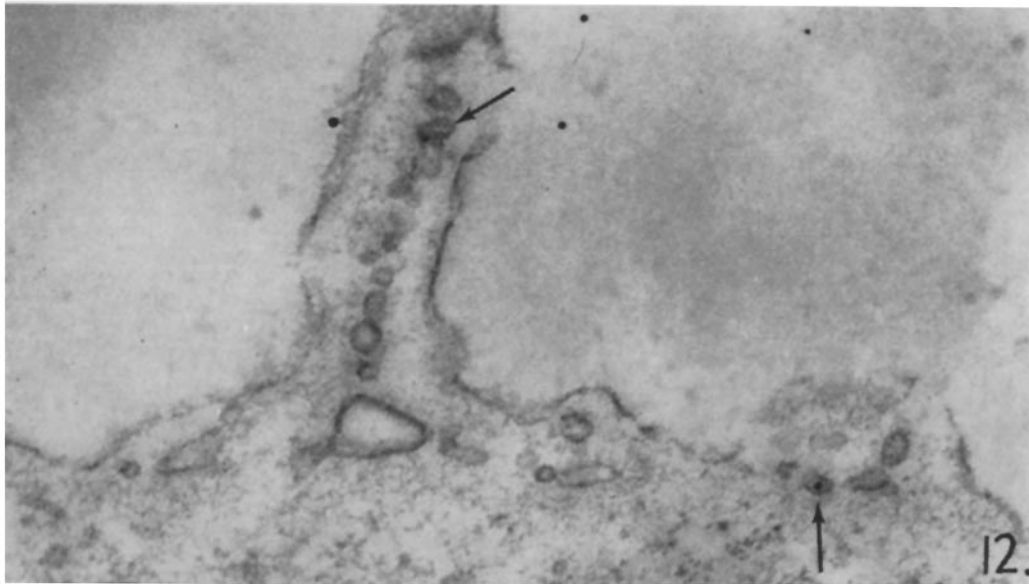
(Epstein *et al.*: Uptake of herpes virus and gold by HeLa cells)

PLATE 23

FIG. 12. Cell surface with small gold particles between 10 and 15 mμ in diameter. Stirring in suspension was for 60 minutes. Chains of micropinocytosis vesicles extend from the cell membrane, especially in the centre of a microvillus which protrudes up towards the top of the field. A gold particle lies against the left side of the microvillus whilst two others, which have been ingested, are present within tiny vesicles (arrows). × 62,500.

FIG. 13. Part of the cytoplasm between the nucleus (n) and the cell surface, lying on the right, following 60 minutes contact with colloidal gold. An extracellular particle is present close to the plasma membrane in the upper right corner of the figure; particles which have been taken into the cell can be seen accumulating in a paranuclear digestive vacuole (v) or crossing the cytoplasm from the surface in membrane-bounded spaces (arrows). The cytoplasmic matrix contains abundant fine filaments. × 55,000.

FIG. 14. Portion of nucleus (n) and adjacent cytoplasm from a preparation exactly like the foregoing. Large and small gold particles have collected in two digestive vacuoles (v) close to the nucleus; the concentration of phagocytosed asbestos crystals in the right hand vacuole emphasises the role of such structures in the disposal of ingested material. Mitochondria (m) and many smooth vesicles can also be seen. × 42,000.

(Epstein *et al.*: Uptake of herpes virus and gold by HeLa cells)