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Uptake of Minute Virus of Mice into Cultured Rodent Cells

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The uptake of minute virus of mice into cells in tissue culture was examined biochemically and by electron microscopy. Cell-virus complexes were formed at 4°C, and uptake of virus was followed after the cells were shifted to 37°C. The infectious particles appeared to enter cells at 37°C by a two-step process. The first and rapid phase was measured by the resistance of cell-bound virus to elution by EDTA. The bulk of the bound virus particles became refractory to elution with EDTA within 30 min of incubation at 37°C. The infectious particles became resistant to EDTA elution at the same rate. The second, slower phase of the uptake process was measured by the resistance of infectious particles to neutralization by antiserum. This process was complete within 2 h of incubation at 37°C. During this 2-h period, labeled viral DNA became progressively associated with the nuclear fraction of disrupted cells. The uptake of infectious virus could occur during the G_1 phase of the cell cycle and was not an S phase-specific event. The uptake process was not the cause of the S phase dependence of minute virus of mice replication. In electron micrographs, virus absorbed to any area of the cell surface appeared to be taken into the cell by pinocytosis.

In many instances, the primary interaction between virus and cell occurs at a specific cell surface component present in limited quantities (1). These virus receptors may have a limited distribution among the tissues of a susceptible organism, and, in some cases, the receptors appear at specific times during development (3). The tissue specificity of virus infection by poliovirus and the adenoviruses is controlled to some extent by the presence or absence of these virus receptors (1). The nondefective parvoviruses also exhibit a degree of cytotropism (6), and a specific virus receptor appears to be involved in infection by the parvovirus minute virus of mice (MVM) (5). The cell receptors for MVM saturate at about 5×10^5 virus particles bound per cell, and the presence of these saturable binding sites has been correlated with susceptibility to infection (5). Examination of the cell-virus complexes with an electron microscope suggested that the virus receptor is randomly located on the cell surface (5).

In this study we examined the early steps in virus infection beyond the initial binding reaction. After binding [^{3}H]thymidine-labeled virus to cells at 4°C, we used biochemical techniques to measure the synchronous internalization of virus at 37°C. We also examined the uptake of virus by electron microscopic techniques in an attempt to further characterize the early steps of the infection.

The replication of the nondefective parvoviruses, including MVM, requires host cell functions which occur during S phase (7). It is not clear exactly which step in MVM replication is subject to this cell cycle control. We examined the uptake process in synchronized cells and showed that it is not restricted to S phase but may occur early in G_1.

MATERIALS AND METHODS

The cell lines used were the A-9 mouse line and the RT-7 rat brain tumor cell line (8). A-9 cells were grown in F-11 minimal essential medium (Grand Island Biological Co.) supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in suspension at densities of from 5×10^4 to 5×10^5 cells per ml. RT-7 cells were grown in monolayers in the above media. Suspensions of mitotic RT-7 cells were prepared as previously described by manually shaking culture flasks containing monolayers at near confluency (8). Cells prepared in this fashion are virtually all in mitosis at the time of detachment (8).

Virus. Plaque-purified MVM was originally obtained from Peter Tattersall. MVM and MVM labeled in its DNA with [*methyl*-^{3}H]thymidine were grown in RT-7 cells and purified as previously described (5). The concentrations of particles in the purified viral preparations were calculated from absorption at 280 nm (10).

Binding assay. Binding of radiolabeled virus to cells was performed in suspension as described previously (5). Briefly, a small volume of virus was added to cells and incubated at 4°C, and then the suspension was filtered through a membrane filter (Nuclepore

Corp.). The amount of cell-bound virus was determined by measuring the radioactivity retained on the washed filters by scintillation counting.

Separation of nuclei and cytoplasm. Isolation of nuclei was performed by a modified version of the method of Wray (11). Cells suspended in media were centrifuged at 4°C and 2,000 rpm for 5 min and resuspended at 10^6 cells per ml in buffer containing 50 μM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 1 mM $CaCl_2$, and 0.5 M hexylene glycol (2-methyl-2,4-pentanediol; Eastman Organic Chemical Div., Eastman Kodak Co.) at pH 6.5. The suspension was incubated at 37°C for 5 min and then passed twice through a 25-gauge needle to burst the swollen cells. Nuclei were pelleted as described above, and the supernatant was collected. The pellet was resuspended in 1 ml of isolation buffer and centrifuged at 1,000 rpm. The supernatants of this and the previous centrifugation were combined as the cytoplasmic fraction. The nuclear pellet of the final centrifugation was resuspended in 1 ml of isolation buffer. These nuclei are 90 to 95% free of cytoplasmic tags (8). Acid-insoluble radioactivity in each fraction was measured by precipitating the material with 10% trichloroacetic acid at 4°C for 1 h, using 50 μl of a 1:10 dilution of fetal calf serum as carrier. Precipitates were collected by filtration on Whatman GFC filters and solubilized with Soluene-100 (Packard). Radioactivity was measured by scintillation spectrophotometry in toluene-based scintillation cocktail.

Electron microscopy. Cells were embedded for electron microscopy in Luft Epon as previously described (5). Ultrathin sections were cut perpendicular to the plane of growth, mounted on 400-mesh copper grids, and stained with uranyl acetate and lead citrate as described previously (5). Micrographs were taken with either a Zeiss EM-9A electron microscope at a 40-kV acceleration voltage or a Jeol JEM 100 B electron microscope at a 60-kV acceleration voltage.

RESULTS

Elution of virus particles from the cell surface by EDTA. We have previously shown that MVM bound to A-9 cells at 4°C can be eluted from the cell surface by washing with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS) containing EDTA (5). The early stages of the uptake process were examined by following the elution of labeled virus from the cell surface using this wash procedure. To observe the uptake process in the largest number of cells, the cell-virus complexes were formed at 4°C and subsequently incubated at 37°C (1). Most of the MVM bound at 4°C is at the cell surface (5).

As Fig. 1 shows, virtually all of the virus particles bound to A-9 cells at 4°C became resistant to EDTA elution within 30 min of incubation at 37°C.

Elution of infectious virus from the cell surface by EDTA. In these virus preparations, the ratio of infectious particles to total particles ranged from 1:600 to 1:3,000. The interactions of the labeled particles with the cell surfaces did

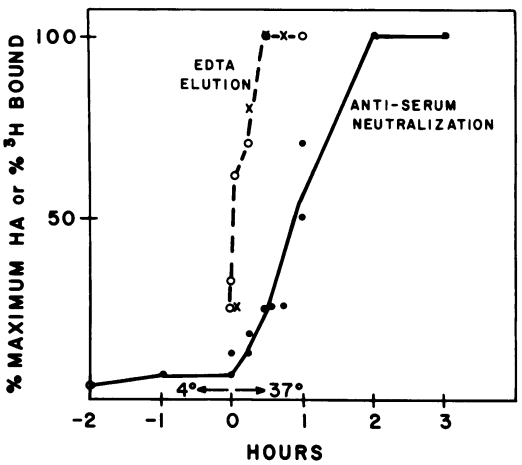


FIG. 1. *Comparison of the rate at which cell-bound virus becomes resistant to elution by EDTA with the rate at which the bound particles become resistant to inactivation by antiserum. Symbols: ○, rate at which labeled virus bound to randomly growing A-9 cells became resistant to elution by EDTA. A-9 cells (10^6 cells in 1 ml of serum-free F-11 medium) were reacted with [^{3}H]thymidine-labeled virus (2.4 × 10^{10} particles) for 1 h at 4°C. After the incubation at 4°C, the cell suspensions were incubated at 37°C for the indicated times. Suspensions were then filtered as described in the text and washed for 5 min at 0°C with Ca^{2+}-Mg^{2+}-free PBS containing 1 mM EDTA. The cell-associated radioactivity, after cold incubation and washing with normal PBS, was defined as 100% (22,500 cpm) for comparison with EDTA-PBS-washed samples. The input quantity of virus contained approximately 23,000 cpm of acid-precipitable radioactivity. ×, Rate at which infectious virus bound to synchronized RT-7 cells became resistant to elution by EDTA. RT-7 cells were synchronized and allowed to attach to T flasks for 2 h at 37°C. The monolayers were placed at 4°C and exposed to virus for 2 h at 4°C. The cell-virus complexes were shifted to 37°C, and at intervals cell samples were released from the surface of the flask with PBS-EDTA solution. The cells were rinsed twice in PBS-EDTA by centrifugation and were replated at 37°C in medium. After 2 days the cells were disrupted, and the amount of viral protein produced was determined by HA titration. The data are presented as percentages of the maximum HA titer obtained (4,096). ●, Rate at which infectious virus bound to synchronized RT-7 cells became resistant to neutralization by antiserum. Synchronized RT-7 cells were allowed to attach to T flasks and exposed to MVM at 4°C as described above. At intervals during the 4°C absorption period and during the subsequent 37°C incubation, all samples were exposed to 19 hemagglutination inhibition units of rabbit anti-MVM antiserum. After 4 h of exposure to antiserum, the monolayers were rinsed with fresh medium without antiserum and incubated in antiserum-free medium for 2 days. The cell cultures were then harvested, and the extent of infection was determined by HA titration. The data are presented as percentages of the maximum HA titer obtained (8,192).*

not necessarily reflect the interactions of the infectious particles. To study the fate of the infectious particles after the cell-virus complexes were shifted to 37°C, we examined the rate at which the infection process became resistant to EDTA elution. In these experiments, synchronized RT-7 cells were used to determine whether uptake of infectious virus could occur during the G_1 phase. RT-7 cells in mitosis had approximately the same number of viral receptors per cell (5×10^5) as randomly growing A-9 cells (Fig. 2).

Synchronized RT-7 cells were exposed to virus at 4°C and shifted to 37°C, and at intervals cell samples were washed with 1 mM EDTA. This procedure removed loosely bound virus as well as detaching the cells from the surface of the flask. The cells were washed and replated into T flasks at 37°C. After 48 h, the cell samples were harvested, and the accumulated viral protein was titrated by hemagglutination (HA). As Fig. 1 shows, the rate at which the total viral particle population (radioactivity) became resistant to EDTA after the shift to 37°C was approximately the same as the rate at which the infectious particles (HA production) became elution resistant.

Movement of infectious virus into the cell. Resistance of bound virus to elution from the cell surface by EDTA does not necessarily result from internalization of the virus particles. To measure internalization, we examined the rate at which bound virus became resistant to inactivation by antiserum after incubation of the cell-virus complexes at 37°C.

When the cell-virus complexes were exposed to antiserum before incubation at 37°C, the cultures were infected, but only at a low level, indicating that the bulk of the infectious particles remained vulnerable to neutralization at the cell surface at 4°C (Fig. 1). However, after incubation at 37°C, the infection process became progressively more resistant to the antiserum until, after 2 h, the antiserum no longer inhibited infection.

The bulk of the virus particles, as well as the minor fraction of infectious particles, became resistant to EDTA elution from the cell surface at the same relatively rapid rate. However, resistance of infectivity to antiserum proceeded at a slower rate and was maximal only after a 2-h incubation of the cell-virus complexes at 37°C. The formation of a virus-receptor complex stable to EDTA elution appeared to represent a rapid initial step which occurred at 37°C before internalization of the infectious virus.

Association of viral DNA with the nuclear fraction of disrupted cells. Do the bulk of the virus particles enter the cell at the same

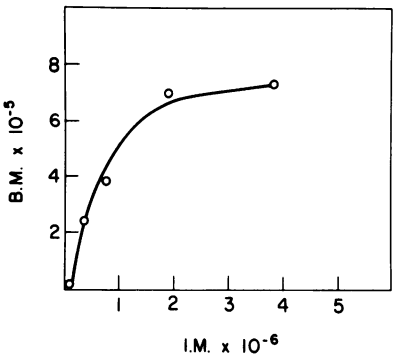


FIG. 2. *Binding of MVM to mitotic RT-7 cells. A total of 2×10^5 RT-7 cells harvested by mechanical detachment of mitotic cells were reacted with the indicated multiplicities of [^{3}H]thymidine-labeled MVM particles per cell (input multiplicity [I.M.]) for 2 h at 4°C in 1 ml of PBS. The bound multiplicity (B.M.) was determined after filtration, as described in the text.*

rate that the infectious particles are taken up? To answer this question, we determined the rate and extent to which input viral label became associated with the nuclear fraction of infected cells after in vitro disruption. Association of viral label with the nuclear fraction is taken simply as an indication that the viral particles have entered the cell. Nuclear association does not indicate whether viral particles have actually penetrated to the nucleus of the intact cells nor does it give any information on the physical state or infectivity of the virus particle which contains the label. In these experiments, the cell-virus complexes were again formed at 4°C; the cells were then incubated at 37°C to allow viral uptake, and at intervals the cells were disrupted and separated into nuclear and cytoplasmic fractions. The proportions of the bound viral label in the nuclear and cytoplasmic fractions were determined from the acid-precipitable radioactivity. In randomly growing A-9 cells, roughly 27% of the cell-bound viral label became associated with nuclei after 2 h at 37°C, and further increases occurred at a relatively slow rate (Fig. 3).

We take the rate of association of viral label with the nuclear fraction as representing the rate of entry of the viral particles into the cell. The initial rate of entry of the labeled particles was not markedly different from the rate of internalization of the infectious particles (Fig. 1 and 3).

Nucleus-associated viral label is in viral DNA. In the experiments described above, the bulk of the [^{3}H]thymidine-labeled viral material which was found associated with the cell nuclear fraction resided in intact single-stranded viral

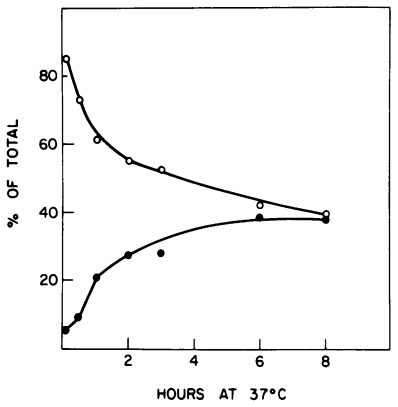


FIG. 3. *MVM uptake and compartmentalization in A-9 cells. A total of 1.4 × 10⁶ A-9 cells in 1 ml of serum-free F-11 medium were reacted with 4.8 × 10¹⁰ [³H]thymidine-labeled virus particles for 1 h at 4°C. After the cold incubation, cell suspensions were incubated at 37°C with agitation for the indicated times. After the warm incubation, suspensions were transferred to new siliconized glass tubes at 0°C, and the cells were pelleted. The nuclear and cytoplasmic fractions were isolated as described in the text. Trichloroacetic acid-precipitable radioactivity was measured in the supernatant (unbound) fraction as well as the nuclear and cytoplasmic fractions (see text). Trichloroacetic acid precipitation of the input quantity of virus yielded a total of 41,000 to 46,000 cpm. The total radioactivity present in samples for each time point (100%) was between 38,000 and 47,000 cpm. Symbols: ●, nucleus; ○, cytoplasm.*

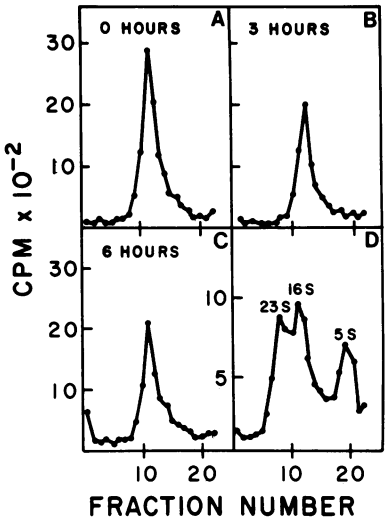


FIG. 4. *Size of labeled viral DNA recovered from infected cells. A-9 cells were exposed to [³H]thymidine-labeled MVM at 4°C for 2 h. The cell-virus complexes were shifted to 37°C, and at zero time and 3 and 6 h total cellular nucleic acid was purified by protease K digestion and phenol-chloroform extraction. A portion of each sample was then centrifuged on a neutral 15 to 30% sucrose gradient (SW40, 16 h at 25,000 rpm). The labeled material was located by scintillation counting a sample from each fraction. The size of the labeled viral material was determined relative to ³H-labeled Escherichia coli ribosomal RNA run in a parallel gradient (D).*

DNA. A-9 cells were infected with labeled virus at 4°C. At intervals after incubation at 37°C, the total DNA was extracted from the cells. Over 95% of the cell-bound label was recovered as 16S DNA after sucrose gradient centrifugation (Fig. 4). DNA from each sample was digested with single-strand-specific S_1 nuclease under conditions which did not digest double-stranded DNA (9). None of the DNA was resistant to S_1 digestion; the DNA fragments resulting from S_1 digestion were 5S or smaller in size (Fig. 5).

Thus, at times during the infection when 25 to 35% of the viral label was associated with the nuclear fraction, virtually all of the cell-associated viral label was in single-stranded DNA of MVM genome length.

Association of viral DNA with the nuclear fraction of synchronized cells. The association of viral label with the nuclear fraction of the cells was used to examine the movement of the bulk of the bound virus into synchronized cells. As with the A-9 cells, the rate of association of viral label with the nuclear fraction was maximal during the first 2 h of incubation at 37°C (Fig. 6).

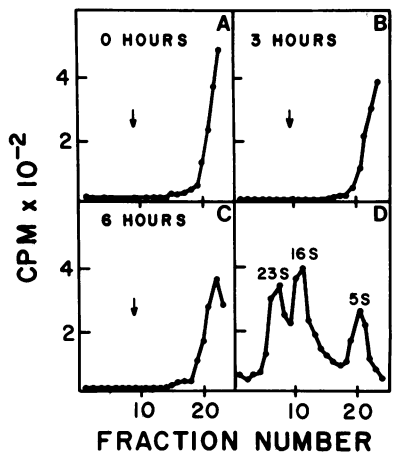


FIG. 5. *S_1 nuclease sensitivity of labeled viral DNA recovered from infected cells. A portion of the samples from the experiment described in the legend to Fig. 4 was digested with S_1 nuclease (1 h, 37°C) and then centrifuged on neutral sucrose gradients (see legend to Fig. 4). Samples of each gradient fraction were directly assayed for radioactivity by scintillation counting. Labeled E. coli ribosomal RNA was run in a parallel gradient (D).*

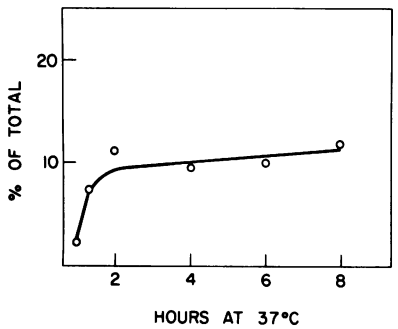


FIG. 6. *Accumulation of viral label in the nucleus of RT-7 cells. A total of 4.4 × 10^5 mechanically detached mitotic RT-7 cells in 1 ml of serum-free F-11 medium were reacted with 6.2 × 10^{10} [^{3}H]thymidine-labeled virus particles for 1 h at 4°C. After the cold incubation, the cell suspensions were transferred to 25-cm^2 Falcon T flasks and incubated for the indicated times at 37°C. The nuclei were isolated from these cultures as described in the text, and the trichloroacetic acid-precipitable nucleus-associated radioactivity was measured.*

Changes in infectivity of viral particles during the uptake process. We observed that a percentage of the virus bound at 4°C spontaneously eluted from the cell surface at 37°C. For example, in the experiment shown in Fig. 3, 9% of the input label did not bind to cells and was found free in the supernatant after absorption at 4°C. When the cells were incubated at 37°C, 17% of the input label was recovered in the supernatant after 30 min. Between 8 and 10% of the bound virus eluted from the cells when the temperature was raised. The eluted virus apparently remained free from the cell during the subsequent incubation at 37°C, as the percentage of unbound virus remained relatively constant once the temperature was raised. Virus which eluted at 37°C retained its ability to bind to cells and was as infectious as the original virus (data not shown). These results may indicate that the binding of virus to the cell surface at 4°C does not inactivate the particles.

It has been shown that the interaction of poliovirus and adenovirus with the cell surface at 37°C results in loss of infectivity, and this may be the initial step in the uncoating process for these viruses (1, 2, 4). In the case of MVM, we showed that the particles absorbed to the cell at 4°C became tightly bound within 30 min after the cells were shifted to 37°C. Does the formation of this stable cell-virus complex disrupt the bound viral particles? As Table 1 shows, incubation of the complex at 37°C had very little effect on the infectivity of the bound virus. In these experiments, virus was recovered from the infected cells at intervals up to 4 h after the complexes were shifted to 37°C, when the uptake process was essentially complete. The specific infectivity of the recovered virus decreased by only about 50%, indicating that the formation of a tight complex with the cells at 37°C does not result in a loss of infectivity for the bound particles. Although those experiments did not necessarily examine the infectious process, the kinetics of inactivation of the bound particles roughly follow the uptake of labeled particles. It appears that the bound viruses lose infectivity during or after their internalization. Whether this loss of infectivity is related to viral uncoating requires further investigation.

Electron microscopy of virus uptake. Because of the low ratio of infectivity to particles in our MVM preparations, it was not possible to distinguish the infectious interactions from the fate of the bulk of the virus particles by electron microscopy. However, the experiments described above indicate that there are no gross differences in the rate at which the mass of the virus particles enter the cell and the rate of entry

TABLE 1. *Infectivity of cell-bound virus during the uptake process*[a]

Time	Infectivity ($TCID_{50}$)	Radioactivity (total cpm)	Specific infectivity ($\times 10^{-4}$)	% Specific infectivity
5 min	6.5×10^7	5,400	1.2	100
30 min	5.6×10^7	5,900	0.9	75
1 h	3.8×10^7	5,100	0.8	67
2 h	2.0×10^7	3,200	0.6	50
3 h	2.8×10^7	4,000	0.7	58
4 h	4.4×10^7	8,800	0.5	42

[a] RT-7 cells were synchronized by mitotic detachment and allowed to attach to the surface of T flasks for 2 h at 37°C. Labeled virus was absorbed to cells for 2 h at 4°C, the inoculum was removed, and the monolayers were rinsed twice in 4°C serum-free medium and placed at 37°C. At intervals after the shift to 37°C, the cells were removed from the surfaces of the T flasks in PBS–EDTA solution, which also removed loosely bound virus. The cells were washed twice in serum-free medium, resuspended in 0.01 M Tris, pH 9.0, and frozen. The virus was extracted from the cells by Dounce homogenization, DNase digestion (1 h at 37°C, 50 μg of DNase per ml, 1 mM $MgCl_2$), and Freon extraction. Samples of the extracted virus were assayed for radioactivity and infectivity. The 50% tissue culture infective dose ($TCID_{50}$) is the reciprocal of the dilution of the extracted virus which gives a positive HA titer in 50% of triplicate infected cultures (5). The specific infectivity is the $TCID_{50}$ divided by the acid-precipitable counts per minute present in the sample of viral extract used for the $TCID_{50}$ assays. At the time of extraction from the infected cells, the input virus preparation had a specific infectivity of 1.1×10^4 (10^8 $TCID_{50}$ per 9,100 cpm).

of the infectious particles. On this basis, it remains possible that the cell handles the uptake of both kinds of particles in the same fashion, and electron micrographs of the uptake of the bulk of the particles might also represent the uptake process for the infectious particles.

To visualize the process of virus uptake, A-9 cell monolayers were incubated with approximately 10^6 particles per cell at 4°C. The cultures were then incubated at 37°C for 10 min and processed for electron microscopy. In Fig. 7 and 8 the uptake of MVM into A-9 cells appears to occur in several ways. Single virions, as well as groups of virus, are taken into coated vesicles via endocytotic clefts. We have shown previously that MVM binds to these clefts at 4°C in the absence of uptake (5). The clefts bubble into the cell and pinch off to form free cytoplasmic vesicles (Fig. 7). The submembranous thickening of the original cleft now forms the rough coating of the vesicle. These coating vesicles are generally seen close to the cell surface.

Virus particles are also taken up in smooth vesicles. In Fig. 7a and d and 8a, the virus particles appear to act as multivalent ligands, binding adjacent regions of the plasma membrane together. Filipodia often become ligated to the cell surface in such a manner. Often a filipodium completely coated with virus appears to be internalized by the cell (Fig. 7d and 8b).

Virus-filled channels running into the cytoplasm are frequently seen (Fig. 8a) and may be the product of virus-mediated interconnection of membranes. Such channels may pinch off and fuse with other vesicles to form large vacuoles lined on their inner surfaces with viruses. Such large virus-lined vacuoles are frequently seen (Fig. 7 and 8), usually located deeper in the cytoplasm than the coated vesicles. Smaller smooth vesicles containing viruses are also frequently seen at varying depths in the cytoplasm (Fig. 7 and 8).

In Fig. 7b, small vesicles containing single virus particles are seen. However, intermediate stages of single particle uptake (monocytosis) were not observed, and these vesicles with single virus particles could represent cross sections of the channels of virus described above. The fact that active monocytosis was not observed may be due to the difficulty of accurately identifying single particles of this size at the cell surface.

Double-walled, smooth vesicles were often observed. These vesicles appeared to be channels of virus which had circularized. The central region of such vesicles could either be empty, as a vacuole, or enclose a matrix very similar to the surrounding cytoplasm (Fig. 8b and e).

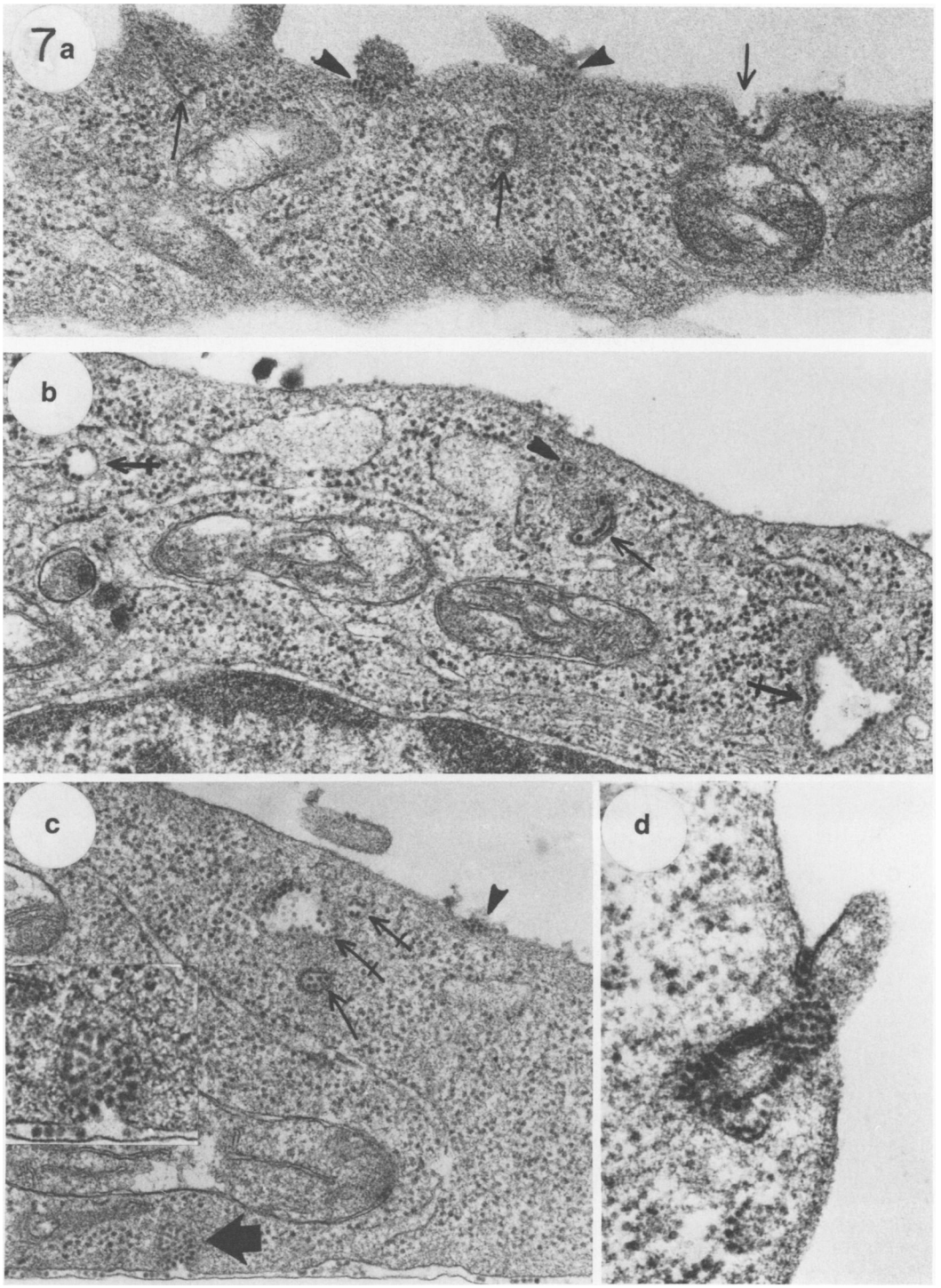
When virus binding and uptake were allowed to proceed simultaneously at 37°C, the uptake process was not noticeably different from that observed with prior viral absorption at 4°C (data not shown).

Uninfected A-9 cells were embedded in monolayers and sectioned perpendicular to the plane of growth (data not shown). The cell surface was clear of particulates. The plasma membrane of these cells frequently exhibited thickened regions which appeared to give rise to coated vesicles. In the absence of virus these regions and vesicles were free of particles.

DISCUSSION

In this study, we examined some aspects of the uptake of MVM by cultured rodent cells. The uptake of the infectious particles appears to occur by a two-step process. The majority of the particles, as well as the infectious particles, first form an EDTA-resistant complex at the cell surface at 37°C. Formation of this initial complex is relatively rapid and is complete by 30 min. The infectious particles then leave the cell surface and become resistant to antiserum inactivation at a slower rate. Infectious particles leave the cell surface at approximately the same rate that viral label becomes associated with the nuclear fraction of disrupted infected cells. From these data, the infectious particles appear to be

FIG. 7. *MVM uptake by A-9 cells. Virus was applied to A-9 cells (10^6 particles per cell) in a monolayer at 4°C for 1 h. Approximately 2×10^5 particles were bound per cell, as measured by a parallel binding assay. The cultures were incubated at 37°C for 10 min to allow viral uptake and were then processed for electron microscopy. (a) Arrows indicate stages of virus uptake into coated vesicles from invagination (right and left) to autonomous vesicle (center). Arrowheads show patches of virus continuous with filipodial and flat surface plasma membrane. ×65,000. (b) A portion of a coated vesicle containing virus (arrow) is seen adjacent to an apparent monoendocytotic vesicle (arrowhead). Crossed arrows indicate a large, smooth vesicle coated on its inner surface with virus and a smaller, smooth vesicle only partially coated on its inner surface with virus. ×54,000. (c) Small arrow indicates a coated vesicle containing virus fairly deep in the cytoplasm. This represents as deep a penetration of the cytoplasm as was ever observed for coated vesicles. Crossed arrows indicate a large vesicle and a smaller, smooth vesicle that may actually be continuous with each other. The large arrow at the bottom (also see inset) indicates a smooth vesicle still in the process of invagination. Virus completely fills the resultant vesicle. Virus in a patch is also evident at the cell surface (arrowhead). ×59,000. (d) A filipodium, apparently coated with virus. The filipodium seems to be bound to the adjacent surface membrane via its coating of virus. ×68,000.*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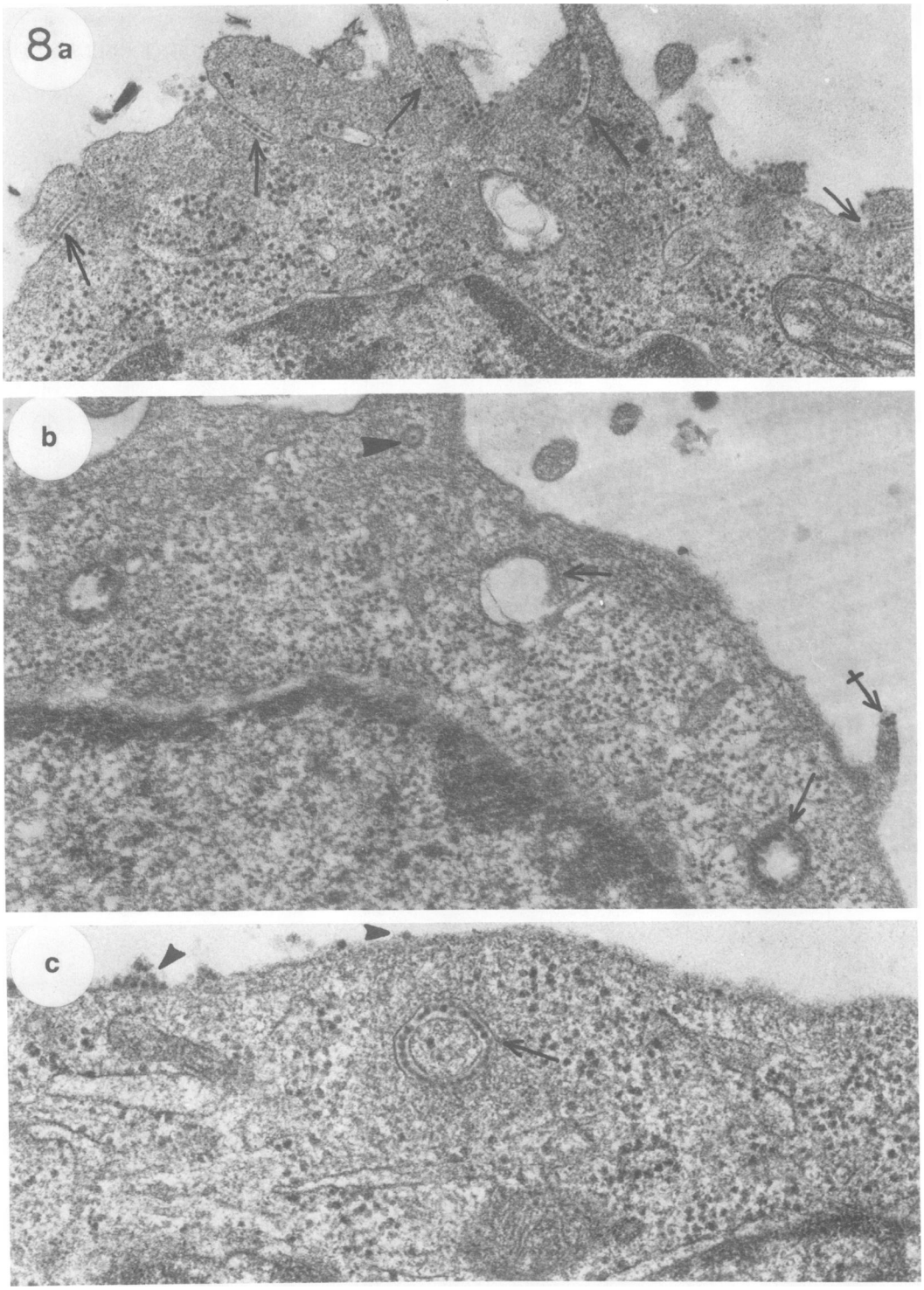

FIG. 8. *MVM uptake into A-9 cells. The experimental conditions are the same as those described in the legend to Fig. 7. (a) Arrows indicate several places where channels of virus have been formed by the apposition of membrane surfaces. Filipodia are involved in some of these. ×30,000. (b) Arrows indicate several large vesicles with smooth surfaces containing numerous virus particles. One such vesicle (center) appears to consist of two membrane surfaces interconnected with virus, forming a circular channel. The crossed arrow indicates a small patch of virus that has not been internalized. The arrowhead indicates a coated vesicle possibly containing a single virus particle. ×42,000. (c) Arrow indicates a circular channel of membrane-bound virus. This may be a cross section through the base of a filipodium that is sunken into the cytoplasm. Arrowheads indicate viruses that have not yet been internalized. ×80,000.*

taken into the cells as rapidly as the bulk of the viral particles which do not initiate infection.

The uptake of infectious virus occurs in the early G_1 phase of the cell cycle and is not limited to the S phase. Therefore, neither the binding nor the uptake of virus is the cause of the S phase restriction of MVM replication.

Spontaneous elution of virus from the cell surface has been observed with adenoviruses (1) and picornaviruses (2, 4). In these cases, virus bound to the cells at 4°C and eluted at 37°C is no longer capable of binding again to the cell due to some physical change in the viral particles (1, 2). Approximately 8 to 10% of the MVM particles bound to A-9 cells at 4°C elute at 37°C and remain unbound even after an additional 8 h of incubation at 37°C. However, binding of parvovirus to cells at 4°C does not appear to alter the particles in any significant way, as the eluted virus remains fully infectious. In addition, the formation of the initial stable cell-virus complexes at 37°C does not disrupt the bound virus. Virtually all of the bound virus has formed an EDTA-resistant complex within 30 min at 37°C, and the viruses recovered from the cells at this time have lost little of their infectivity. On the other hand, there is a progressive decline in the infectivity of virus recovered from cells during the internalization process. It remains to be seen whether this loss of infectivity in the particles taken into the cells can be related to an infectious uncoating process.

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