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# Morphological Aspects of the Uptake of Simian Virus 40 by Permissive Cells

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After exposure of permissive cells to simian virus 40 (SV40), single particles were engulfed by the cell membrane and transported to the nucleus. The cell membrane closed tightly around the particles, increasing their diameter from 40 to 55 nm. The cell membrane was lost during interaction with the nuclear membranes, and particles of the original size were found in the nucleus 1 hr after infection. Uncoating of these nuclear particles occurred rapidly, and none could be found 4 hr after infection. Viral progeny appeared 24 hr after infection.

The morphological aspects of the replication of simian virus 40 (SV40) in permissive cells is well documented (6, 7, 13-15). The preceding events, however, i.e., the mode of uptake by the cells and the intracellular transport of the virus to the nuclear sites of replication, have not been studied. Thus, it is not known whether the SV40 particles lose their protein coat in the cytoplasm while the genome enters the nucleus or whether the particles reach the nucleus morphologically unaltered and the necessary uncoating occurs at nuclear sites of replication.

In the present study, which was designed to investigate the uptake process, highly purified SV40 was used, and susceptible cells were exposed to the virus at a high input multiplicity. The results show that virions penetrate the cellular and nuclear membranes and can be found in the nucleus as early as 1 hr after infection. The mode of uptake and the intracellular pathways of SV40 are also described.

## MATERIALS AND METHODS

**Cells.** Primary African green monkey kidney cells (AGMK) were cultured in 30-ml plastic flasks at 37 C. CV-1 cells, a continuous cell line isolated from AGMK cells (9), were grown in 1-liter Blake bottles in an atmosphere of 4% $CO_2$. The growth and maintenance media used for both cell types were described previously (5).

**Virus.** The LP 4 large-plaque variant of the RH 911 strain of SV40 was used in all experiments. The preparation of virus pools in monolayer cultures of CV-1 cells and the assay of the virus by the plaque technique were described previously (4, 5). Virus pools used in the present study contained an average of $2 \times 10^8$ plaque-forming units (PFU) per ml.

**Purification and concentration of virus.** SV40 was concentrated and purified by a procedure outlined before (5), except that treatment with trypsin was omitted because it was found to decrease the infectivity of the preparation. To separate fully infectious SV40 from noninfectious, incomplete viral forms (16-18) and from coreless viral shells (2), the purified virus was centrifuged to equilibrium in CsCl solution with an average density of 1.30 g/cc in the SW 50 rotor of a Spinco centrifuge at 5 C and 130,000 × g for 24 hr. The band of fully infectious virus located at a density of 1.34 g/cc was collected and freed from CsCl by dialysis against 0.13 M NaCl-0.05 M tris-(hydroxymethyl)aminomethane (Tris) at *p*H 7.8 (NT buffer). The infectivity of the purified virus was stabilized by the addition of 5% fetal calf serum, and all preparations were used within 24 hr for infection of AGMK cells. The infectivity titers of these preparations ranged from $9 \times 10^{10}$ to $19 \times 10^{10}$ PFU/ml. The purified virus had a 260/280 nm optical density ratio of 1.37 to 1.39, indicating the absence of coreless virus shells (10). It was previously determined that 1 mg of purified SV40 corresponds to 3.85 $OD_{260}$ units (10) and that the molecular weight of the virus is $17.3 \times 10^6$ daltons (1). By using these data, it was calculated that the purified virus preparations used in the present study contained 10 to 20 virions per PFU.

**Infection of cell cultures and electron microscopy.** Cultures of AGMK cells were infected at a multiplicity of about 1,000. At the intervals indicated below, the inoculum was decanted and the cultures were washed three times with phosphate-buffered saline (PBS). Subsequently, the infected monolayers were fixed in situ, dehydrated, and embedded as described previously (8). The infection of the cultures was interrupted at 10, 30, and 45 min, and at 1 and 2 hr. After removal of inoculum and washing, medium was added to some cultures which were harvested at 4, 6, 24, and 48 hr.

Thin sections were doubly stained with uranyl ace-

tate and lead citrate and were viewed in an electron-microscope at a magnification of 10,500.

## RESULTS

For the purpose of illustrating the results of this investigation, the 10-min, 2-hr, and 24-hr intervals were selected because they demonstrated the pertinent features. At 10 min after infection, virus particles were found attached to the plasmalemma and inside the cytoplasm. In Fig. 1 the attachment of the virus particles on the cell membrane can be seen. They are aligned along the plasmalemma; aggregates were rarely observed. Actual uptake of the virus by the cell occurred by two mechanisms: (i) engulfment of single virus particles and (ii) formation of pinocytotic vesicles containing several of them. The first mechanism, or monopinocytosis, can be seen in Fig. 2 and 3. After engulfment, the cell membrane seemed to close tightly behind the particle, and the newly acquired membrane increased the diameter of the virus by 15 nm. Such a particle is demonstrated in Fig. 4. It should be pointed out that the efficacy of uptake was low. Numerous particles were consistently found still attached to the plasma membrane many hours after exposure.

The second mechanism of uptake is demonstrated in Fig. 4 and 5. In the particular cell system under investigation, it was rarely seen. Several virus particles were engulfed by the cell membrane, forming pinocytotic vesicles. The beginning of the process can be seen in Fig. 4 and the end result in Fig. 5. Whether particles can escape from these vesicles into the cytoplasm by

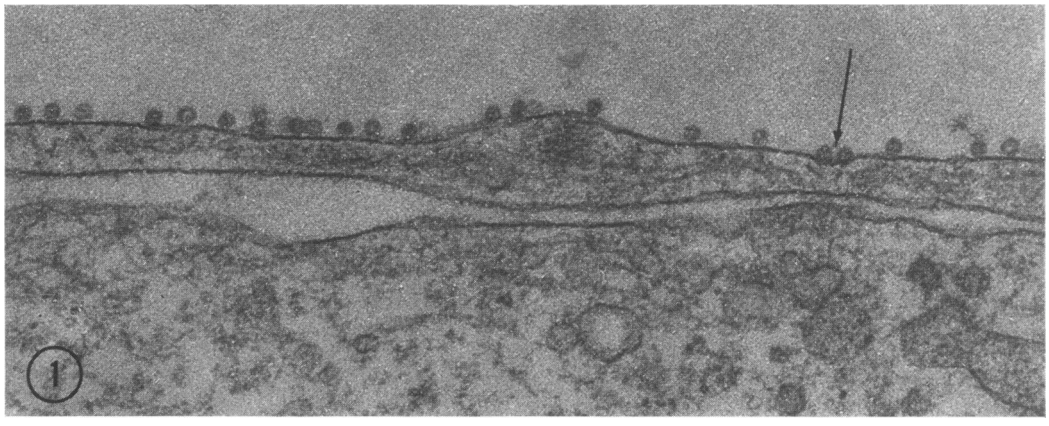


FIG. 1. *SV40 particles attached to the plasma membrane at 10 min after infection. Beginning pinocytosis is marked by the arrow.* × *73,500.*

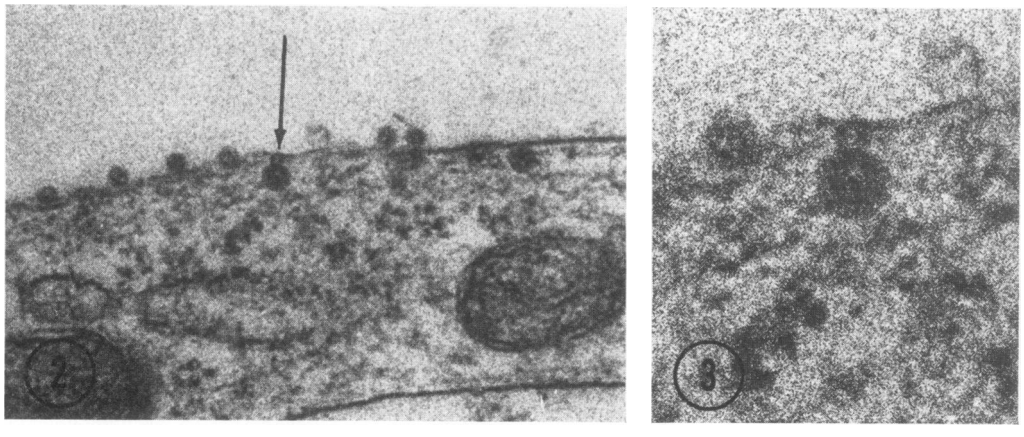


FIG. 2 and 3. *Single SV40 particle near completion of pinocytosis at 10 min after infection. The cell membrane envelops the particle tightly and is in the process of closing behind it. Fig. 2,* × *73,500. Fig. 3,* × *210,000.*

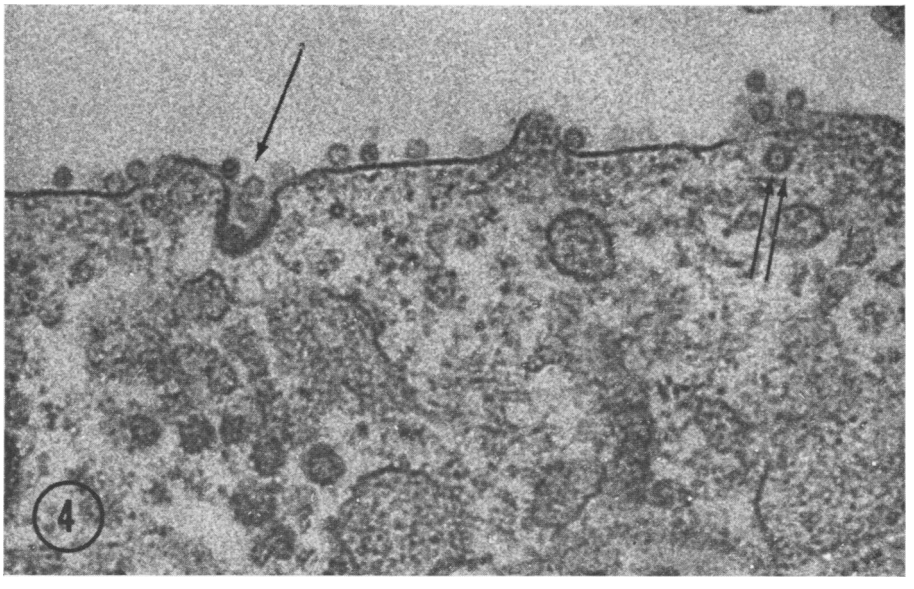


FIG. 4. *Modes of SV40 uptake at 10 min after infection. Pinocytosis of several particles on the left (single arrow), and completed uptake of single particle on the right (double arrow). The diameter of this particle has increased from 40 to 55 nm.* × *73,500.*

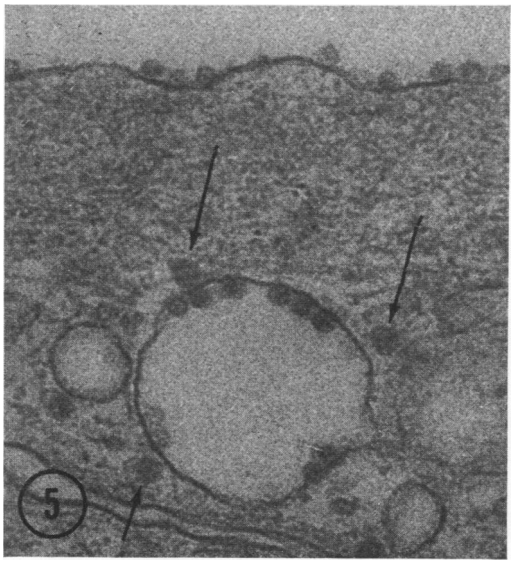


FIG. 5. *Pinocytotic vesicle containing several SV40 particles at 10 min after infection. Release of single particles from vesicle into the cytoplasm was not observed, but particles larger in diameter can be seen close to the vesicle membrane (arrows).* × *73,500.*

the monopinocytotic mechanism responsible for the uptake could not be observed directly, but several particles with increased diameters were evident in the cytoplasm near the membrane of the vesicle in Fig. 5. Enveloped single virus particles were found occasionally in the proximity of the nucleus at 10 min after infection.

Virus particles devoid of the membrane acquired at the initial uptake were found in the nucleus at 1 hr after infection, although they were seen most frequently at the 2-hr interval. The actual mechanism of passage through the nuclear membranes was difficult to determine. It seems, however, that the acquired membrane fused with the outer nuclear membrane, as can be seen in Fig. 6 and 7, and that only the original virus particle proceeded into the nucleus to cause morphological alterations of the nuclear membranes. All particles found in the nucleus had the same diameter as the original infecting virus, namely, 40 nm. In Fig. 8, two virus particles in the nucleus seemed to have passed the nuclear membrane barrier recently. In both cases, the nuclear membranes are disturbed in proximity to the particles. Occasionally, groups of virus particles were found in the nucleus (Fig. 9 and 10). The process of uncoating seems to start very early; at least, some morphological evidence of that phenomenon can be seen in Fig. 9 and 10. Morphologically discernible virus particles could not be found in the nucleus at 4 hr after infection.

Morphological evidence of viral replication became evident at 24 hr after infection, as is demonstrated in Fig. 11. All further events of virus development proceeded as described previously (14).

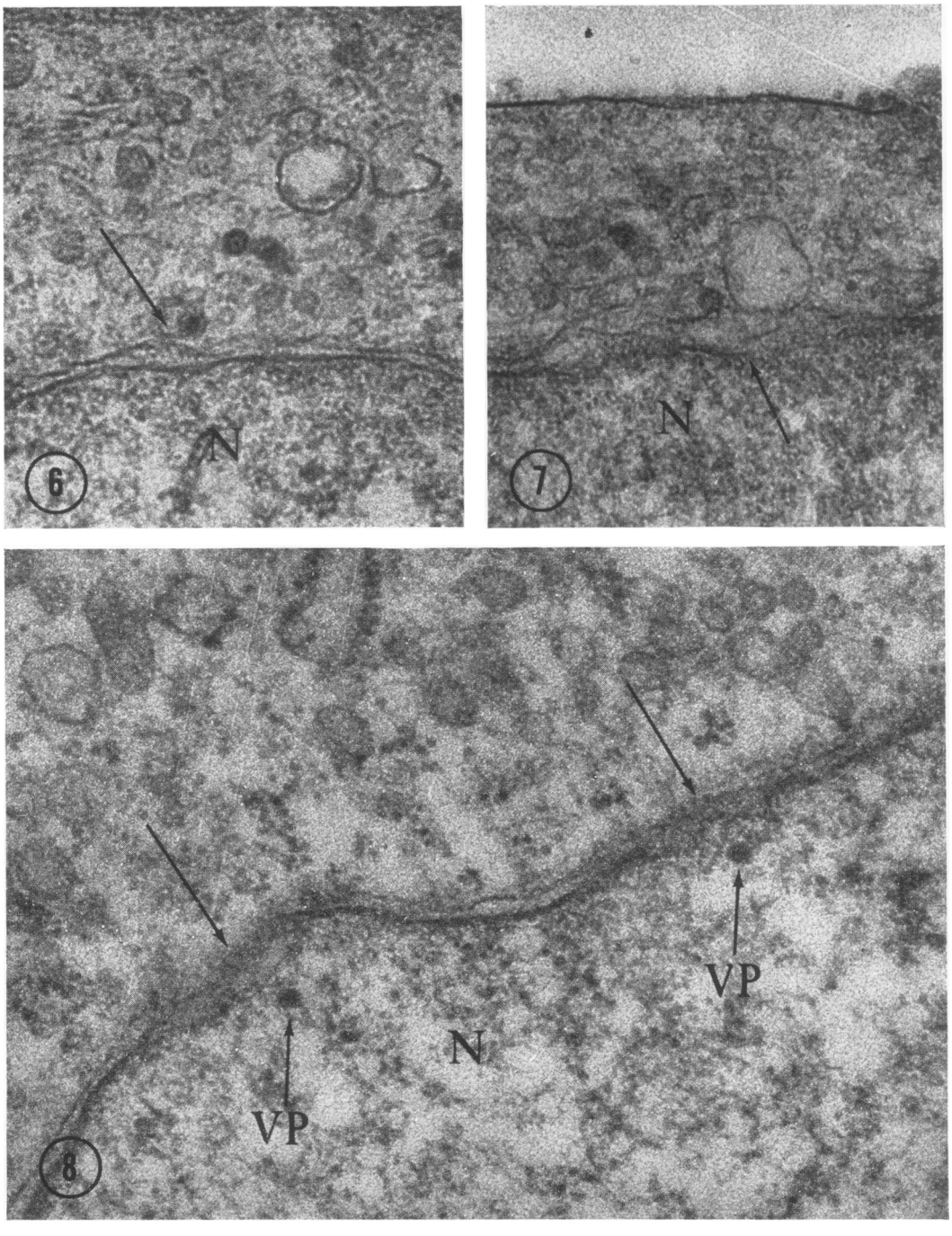


FIG. 6. *Interaction of engulfed SV40 with outer nuclear membrane at 2 hr after infection. Particle attached to or fused with outer nuclear membrane (arrow), losing its cell membrane in the process. Structure of outer nuclear membrane becomes disturbed. N, nucleus.* × *73,500.*

FIG. 7. *Same as in Fig. 6. Both nuclear membranes show alteration of their fine structure.*

FIG. 8. *Two SV40 particles in the nucleus after penetration of the nuclear membrane, 2 hr after infection. Morphology of the nuclear membranes is altered at point of penetration (arrows), and the particles have regained their original diameter, 40 nm, indicating loss of cell membranes during process of penetration. N, nucleus; VP, virus particles.* × *73,500.*

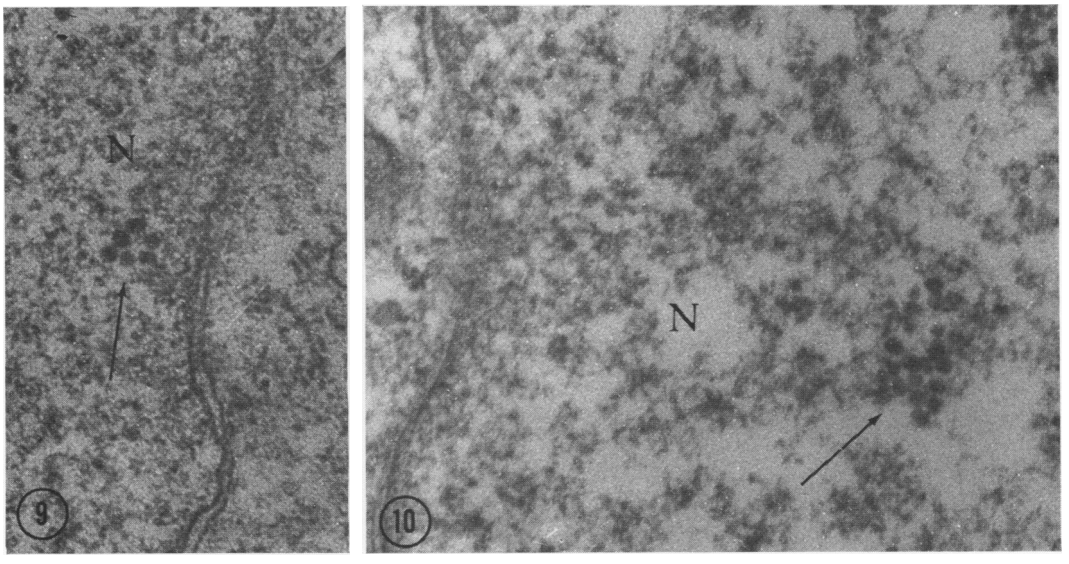


FIG. 9 and 10. *Groups of SV40 particles in the nucleus at 2 hr after infection. Some particles appear less electron-dense than others, possibly indicating the beginning of the uncoating process.*

## DISCUSSION

To facilitate morphological studies on the process of infection of permissive cells by SV40, highly purified virus, from which the bulk of noninfectious, incomplete particles had been eliminated, was used at a high multiplicity of input. Under those circumstances, virus attachment to the plasma membrane seemed to occur almost immediately, all input virus showing a remarkable affinity for the cell membrane. Only a relatively small fraction of the attached particles entered the cell. The remaining virus stayed attached to the plasma membrane and could be observed in this position hours after the initial contact, the time when most of them seemed to undergo a process of degeneration.

The most prevalent mode of entry was a pinocytosis of single particles, or monopinocytosis. After this had been accomplished, the virus particles were easily recognized in the cytoplasm by the addition of the extra membrane adhering closely to the particle itself. This process resulted in an increase of diameter from 40 to 55 nm. Pinocytoses of several particles into larger vesicles were seen rarely in the system under investigation. The uptake of single virus particles from these vesicles into the cytoplasm, however, cannot be excluded, and indirect evidence pointed to its occurrence. Thus, such particles may also contribute to the infectious process. Particles without the additional membrane were never found in the cytoplasm, and, therefore, direct transfer of SV40 through the plasmalemma without engulfment seemed not to be a likely mechanism of infection.

The engulfed particles passed into the nucleus by fusion of their cell-acquired coat with the nuclear membranes and subsequent injection into the nucleus with loss of the acquired cell membrane in the process. These mechanisms were similar to those found by Mattern et al. (12) with polyoma virus, except that polyoma virus was found only in the nuclear membrane interspace. The failure to observe polyoma virus in the nucleus may have been a result of the relatively low input multiplicity used, because the efficiency of transfer of SV40 from the cytoplasm to the nucleus was low even at high multiplicities of infection.

Invasion of the nucleus was seen as early as 1 hr after infection. Preliminary radioautographic experiments with $^3$H-thymidine-labeled SV40 also indicated the presence of viral deoxyribonucleic acid (DNA) in the nucleus at this time (*unpublished observations*). The greatest number of intranuclear SV40 was found at 2 hr after infection, and morphological evidence indicated that uncoating of the virus began almost immediately upon arrival at the nuclear uncoating site. This is further supported by the fact that at 4 hr after infection intranuclear invading virus was no longer found. Biochemical studies on the uptake of SV40 by permissive cells and the intracellular uncoating were in agreement with these morphological findings (2).

The efficiency with which parental virus is

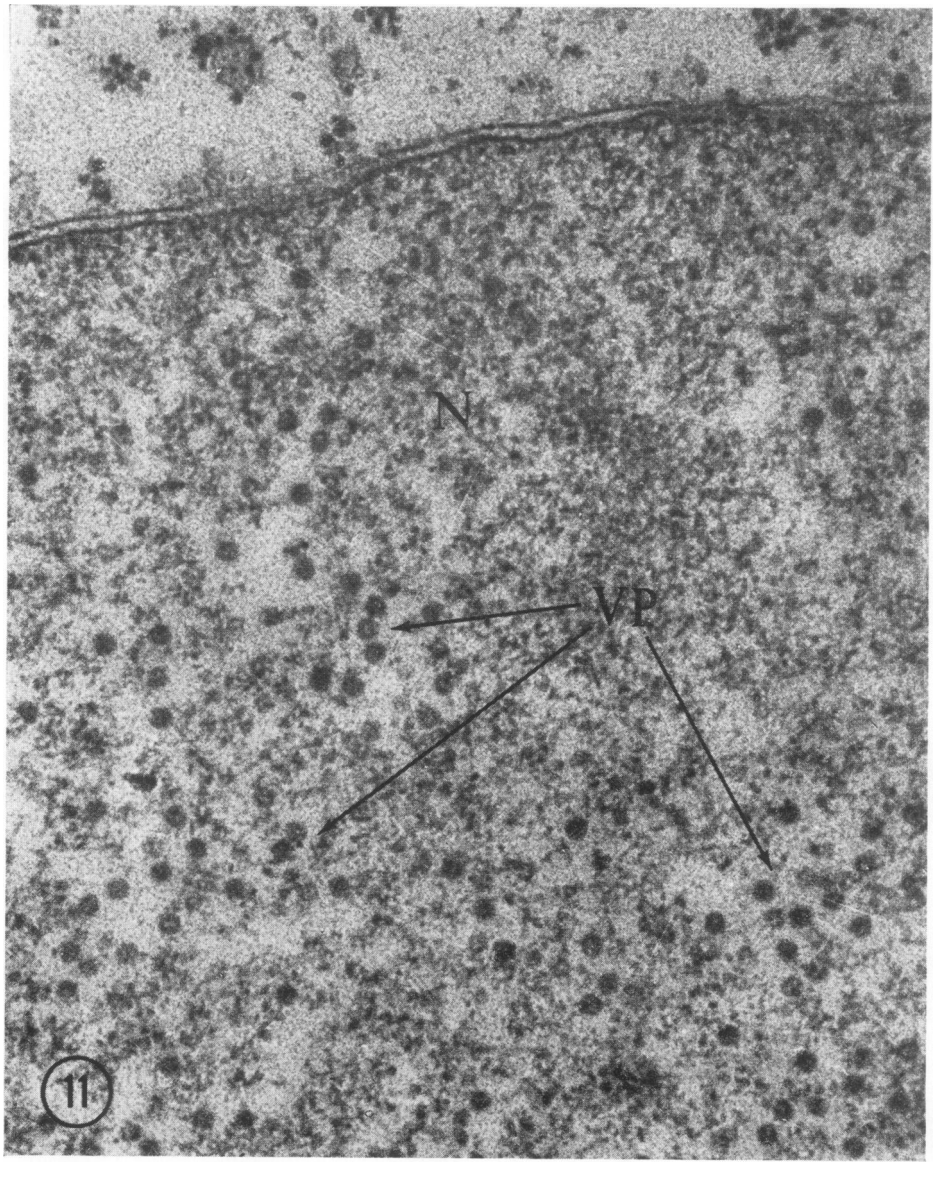


FIG. 11. *Nucleus of cell infected with SV40 24 hr previously. Viral progeny is apparent. N, nucleus; VP, virus particles.* × *73,500.*

seemingly uncoated in the nucleus raises the question why the progeny virus is impervious to this process. This may be due to an inhibition of the synthesis, or activity, of nuclear uncoating enzymes after the start of viral DNA replication, or the progeny virus may have a protein coat sufficiently different from the infecting particles to protect it against uncoating enzymes. The observations by Levinthal et al. (11) and Oshiro et al. (15) that virus-specific ferritin-labeled antibody did not tag intranuclear virus but was readily bound to intracytoplasmic virus particles would speak for a different coat antigen on nuclear progeny virus, which is either lost or changed upon leaving the nucleus. Thus, the different antigenic surface or configuration of the viral coat may protect progeny virus against uncoating enzymes in the nucleus.

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