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Compartmentalization of Pseudorabies Virus and Subvirion Components in BHK-21 Cells and in the Extracellular Fluid

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The intracellular and extracellular localization of pseudorabies virions and subvirion components was determined at various stages in the replicative cycle. It was discovered that infectious pseudorabies virus appears first in the nucleus of the infected cell early in the infectious cycle but later accumulates in the cytoplasm. Subvirion components (nucleoids and nucleocapsids) are restricted to the nucleus, and only the complete virion is released from the infected cell.

It was shown previously (10) that pseudorabies virions, as well as subvirion components (nucleoids and nucleocapsids), could be stabilized with formaldehyde and separated in preformed CsCl gradients. Five bands were found upon centrifugation of whole infected-cell extract labeled with ^{3}H-thymidine. These bands were isolated and identified as follows: band A contained aggregates of virions, empty capsids, and nucleocapsids; band B contained virions; band C contained a mixture of capsids and nucleocapsids; and bands D and E contained small nucleoid particles. Pulse-chase studies indicated that the subvirion components were precursor products of the complete virion.

Since formaldehyde fixation offers an excellent tool with which to study the subvirion components, it became of interest to characterize these particles further. The purpose of this paper was to determine the intracellular and extracellular location of pseudorabies virions and their subvirion components during various stages in the replicative cycle.

MATERIALS AND METHODS

Solutions and media. TEA was 0.02 M triethanolamine plus 0.05 M KCl and 0.001 M $MgCl_2$ at a *p*H of 7.08 (6). TEA-F consisted of TEA with 28% formaldehyde. Saline-sodium citrate (SSC) was prepared as described by Marmur (3). PBS-G was phosphate-buffered saline prepared according to the method of Dulbecco and Vogt (1) and containing 0.1% glucose and 1% fetal calf serum. Growth medium was Eagle's (2) minimal essential medium (MEM) plus 5% fetal calf serum. Maintenance medium consisted of MEM plus 1% fetal calf serum. Plaque overlay medium was medium 199 containing 1% fetal calf serum and 1% methyl cellulose.

Cells. Starter cultures of BHK-21 cells were obtained from Microbiological Associates, Inc. The cells have been carried in this laboratory as monolayer cultures grown in 32-oz (0.95-liter) bottles or in roller bottles.

Virus. The properties of the pseudorabies virus (PSV) strain used in this study have been described previously (10).

Radiochemicals. Thymidine-*methyl*-^3H (specific activity, 6.7 Ci/mmole) and thymidine-*2*-^{14}C (55 mCi/mmole) were purchased from New England Nuclear Corp.

Plaque assay. Virus samples were diluted in PBS-G, and duplicate 1.0-ml portions of the appropriate dilutions were placed in monolayer cultures of BHK-21 cells grown in plastic bottles (Falcon Plastics). After a 2-hr adsorption at 37 C, the inoculum was aspirated and replaced with 7 ml of plaque overlay medium; the bottles were then incubated at 37 C for 40 hr. The medium was removed, and the cells were fixed with methanol and stained with Giemsa stain. The plaques were then counted.

Preparation of nuclear and cytoplasmic extracts. PSV-infected or mock-infected BHK-21 cells were scraped from the glass and centrifuged at 800 × *g* for 5 min. The cells were resuspended in 3 ml of water and placed in a Dounce homogenizer. The cytoplasmic membranes were disrupted by 20 strokes with a tight-fitting pestle, and the nuclei were sedimented by centrifugation at 1,000 × *g* for 5 min. The supernatant (cytoplasmic extract) was removed. The pellet (nuclear extract) was washed two times to remove adhering cytoplasmic debris and then was suspended in 3 ml of water.

These procedures were found to yield cytoplasmic extracts with less than 2% nuclear contamination. Approximately 85% of intact nuclei were recoverable and were shown by direct phase-contrast microscopy to be free from whole-cell contamination and adhering cytoplasmic "tabs." This procedure is only valid for about the first 12 hr of infection; after that time,

the cells become too fragile and the nuclei begin to disrupt.

Preparation and centrifugation of formaldehyde-fixed cell lysates. The procedures have been described in detail previously (10). Briefly, whole cell homogenates or cytoplasmic and nuclear extracts of infected or mock-infected BHK-21 cells labeled with ^{3}H-thymidine were adjusted with TEA and TEA-F to give a final concentration of 3% formaldehyde and 0.02 M TEA. The fixed lysates were kept at 4 C for 16 hr, and then 0.8-ml samples were carefully layered onto preformed CsCl gradients prepared in SW 41 centrifuge tubes. The tubes were centrifuged at 210,000 × *g* for 20 hr at 15 C. The bottoms of the tubes were punctured, and 4-drop fractions were collected directly into tubes containing 1.0 ml of SSC. Trichloroacetic acid was added to each tube to give a final concentration of 5%, and the precipitates formed were collected on HA type membrane filters (Millipore Corp.). The filters were washed with trichloroacetic acid and 70% ethyl alcohol, air-dried, and immersed in toluene-base scintillation fluid for determination of acid-precipitable radioactivity in a Packard liquid scintillation spectrometer.

RESULTS

Intracellular location of PSV. The following experiment was performed to determine the kinetics of the development of infectious PSV during the first 12 hr of the infectious cycle. Monolayer cultures of BHK-21 cells were infected with PSV at a multiplicity of 50 plaque-forming units/cell, and the virus was allowed to adsorb for 2 hr at 37 C; the inoculum was then removed and replaced with maintenance medium. At various times after infection, the cells were harvested, and nuclear and cytoplasmic extracts were prepared and assayed for infectivity as described in Materials and Methods. The results of three experiments are summarized in Fig. 1. At 6 hr after infection, 85% of the total infectious virus recovered was associated with the nuclear extract. By 12 hr after infection, this percentage decreased to 26%. Concurrently, the amount of infectious virus found in the cytoplasmic extracts increased from 15% at 6 hr to 74% at 12 hr after infection.

It was possible that the virus being measured in the nuclear extract was actually cytoplasmic virus which had become attached to the nuclear membrane during the extraction procedures. To rule out this possibility, the following experiments were performed. First, PSV was mixed with uninfected BHK-21 cells, nuclear and cytoplasmic extracts were prepared as described, and both samples were assayed for infectious virus. It was found that 99% of the infectivity was recovered from the cytoplasmic extract. Second, PSV was added directly to a nuclear extract from uninfected cells; the nuclei were washed again as described and were assayed for infectivity. Less

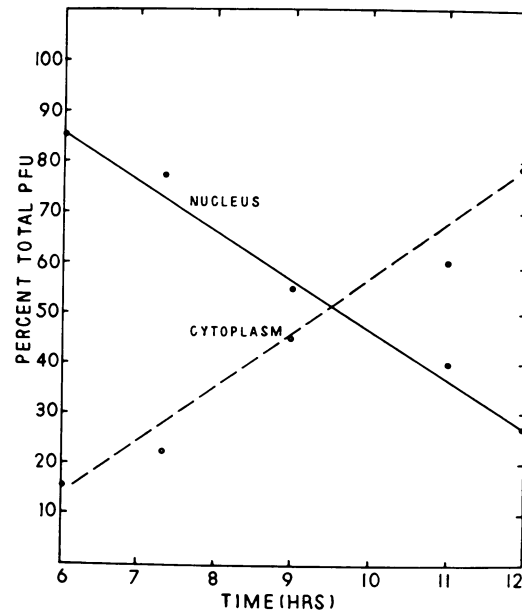

FIG. 1. *Percentage of total plaque-forming units from cytoplasmic and nuclear fractions of PSV-infected cells at various times after infection. BHK-21 cells were infected with PSV; at the times indicated, the cells were scraped and separated into nuclear and cytoplasmic fractions as described in the text. Plaque assays were performed in monolayer cultures of BHK-21 cells under a methyl cellulose-medium 199 overlay. The cell sheet was stained with Giemsa after 2 days of incubation at 37 C, and the plaque-forming units were counted.*

than 1% of the total infectivity was recovered from the washed nuclei. Third, 10-hr infected BHK-21 cells were scraped from the glass and suspended in water; ^{3}H-thymidine-labeled PSV was added. Nuclear and cytoplasmic extracts were prepared and assayed for acid-precipitable radioactivity. Less than 2% of the total acid-precipitable radioactivity was found associated with the nuclear extract. These results confirm that the PSV being measured in the nuclear ex-extracts in Fig. 1 was intimately associated with the nuclei and was not an artifact of the isolation procedure.

Intracellular location of viral products in PSV-infected cells. Monolayer cultures of BHK-21 cells in 32-oz bottles were mock-infected or were infected with PSV. After a 2-hr adsorption period, 40 ml of maintenance medium containing 30 μCi of ^{3}H-thymidine was added to each bottle. Infected cultures were extracted at 6 and 10 hr after infection, and the uninfected culture was extracted 8 hr after the addition of label. For both infected and uninfected cultures, nuclear and

cytoplasmic extracts were prepared; samples of each were fixed with formaldehyde, centrifuged in preformed CsCl gradients, and analyzed as described in Materials and Methods.

In the 6-hr infected-cell culture, all five infected-cell bands (bands A to E) were detected in the nuclear extract, whereas only the virion band (band B) could be found in the cytoplasmic extracts (Fig. 2). Moreover, 83% of the total radioactivity associated with the virion band was located in the nuclear extract. In the 10-hr infected-cell sample, again only the virion band (band B) was found in the cytoplasmic extract; all five bands were found in the nuclear extract. The total amount of radioactivity associated with the virion band in the nuclear extract had decreased to 58%. The amount of radioactivity associated with band B in the cytoplasmic extracts increased from 17% at 6 hr to 42% at 10 hr after infection. The uninfected control showed a single band of radioactivity, at a density of 1.40 g/cc, associated with the nuclear extract. As described previously (10), this material undoubtedly accounts for some of the material in band D of the infected cell, but virus-specific antigens and deoxyribonucleic acid are also associated with this band.

These results are in direct accord with the infectivity assay, and they indicate that the subvirion components of PSV are restricted to the nucleus of the infected cell at least through the first 10 hr of the infectious cycle. The entire infectious cycle in this system is 16 hr (*unpublished results*).

The patterns obtained in the nuclear and cytoplasmic extracts also indicate the degree of purity of the cytoplasmic extracts used. If any of the nuclei were broken during the extraction process, one would expect to find small amounts of bands A, C, D, and E in the cytoplasmic samples. That this was not the case indicates that the cytoplasmic extracts were not significantly contaminated with nuclear material.

It has been suggested previously (7) that the extent of formaldehyde interaction with a given sample is dependent upon the ionic concentration of the sample. To rule out the possibility of selective formaldehyde fixation of any radioactive products in the cytoplasmic and nuclear samples due to different ionic environments, the following experiment was performed. Prior to the extraction procedures, the uninfected and infected cell samples were mixed with equal amounts of uninfected, unlabeled BHK-21 cells, and then nuclear and cytoplasmic extracts were prepared and analyzed as described. The results obtained were identical to those shown in Fig. 2, indicating that

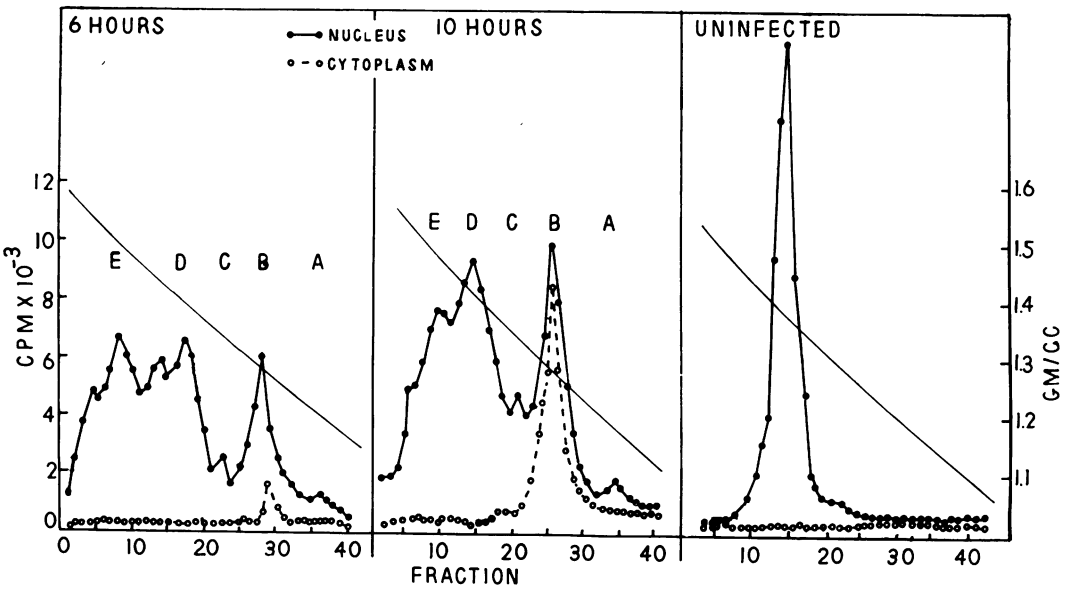

FIG. 2. *Counts per minute of ^{3}H-thymidine incorporated into acid-precipitable material extracted from nuclear and cytoplasmic fractions of 6- and 10-hr infected cells and from uninfected cells labeled for 10 hr. Cells were mock-infected or infected with PSV, and ^{3}H-thymidine was added. The infected cells were extracted at 6 and 10 hr after infection, and the uninfected sample was extracted after 10 hr of labeling. The cells were divided into nuclear and cytoplasmic fractions; each fraction was fixed with formaldehyde and centrifuged in preformed CsCl gradients as described. Symbols: ●, nuclear extract; ○, cytoplasmic extract. The contents of bands A, B, C, D, and E are described in the text.*

no preferential formaldehyde fixation of any virus-specific products occurred in either the nuclear or cytoplasmic sample.

Sedimentation of nuclear and cytoplasmic virus in CsCl. Spring and Roizman (7) reported that nuclear and cytoplasmic preparations of herpes simplex virus sedimented at different rates in sucrose gradients, suggesting that the nuclear virus was not fully enveloped. It therefore became of interest to determine whether any difference in density could be detected between nuclear and cytoplasmic samples of PSV centrifuged in CsCl. Six monolayer bottles of BHK-21 cells were infected with PSV. After a 2-hr adsorption period, the inoculum was decanted and maintenance medium containing either 100 μCi of ^{3}H-thymidine or 10 μCi of ^{14}C-thymidine was added to each bottle. At 10 hr after infection, the cells were scraped, and nuclear and cytoplasmic extracts were prepared from the ^{14}C- and ^{3}H-thymidine-labeled infected cells. The extracts were then fixed with formaldehyde. Nuclear and cytoplasmic extracts containing different radioisotopes were mixed and centrifuged in CsCl gradients as described. The results of one such dual-labeling experiment are shown in Fig. 3. The sedimentation patterns of the ^{14}C- and ^{3}H-thymidine-labeled virions in band B were not coincidental; i.e., the nuclear virus was slightly denser than the cytoplasmic virus. The peak density of the cytoplasmic virus was 1.280 g/cc, whereas the peak density of the nuclear virus was 1.295 g/cc.

Intracellular and extracellular location of viral products. The demonstration above that the subvirion components of PSV are associated with the nuclei of infected cells made it of interest to determine what virus-specific products were released from the infected cell during the infectious cycle. Monolayer cultures of BHK-21 cells in roller bottles (2.8×10^{8} cells per bottle) were infected with PSV, and 50 ml of maintenance medium containing 100 μCi of ^{3}H-thymidine per roller bottle was added after a 2-hr adsorption period. After 24 hr, the cells were gently shaken off the glass, and the cell suspensions were centrifuged at 800 $\times$ g for 5 min. The supernatant (extracellular fluid) was decanted, and the cell pellet (intracellular virus) was suspended in water. The cells were sonically disrupted, the cell debris was removed by centrifugation at 800 $\times$ g for 5 min, and then both the extracellular fluid and intracellular virus samples were centrifuged at 45,000 $\times$ g for 1 hr. The pellets were suspended in water, and both samples were then fixed with formaldehyde and centrifuged in CsCl gradients as described. The results of one such experiment are shown in Fig. 4. As would be expected, the intracellular sample contained all five infected-cell bands (A to E).

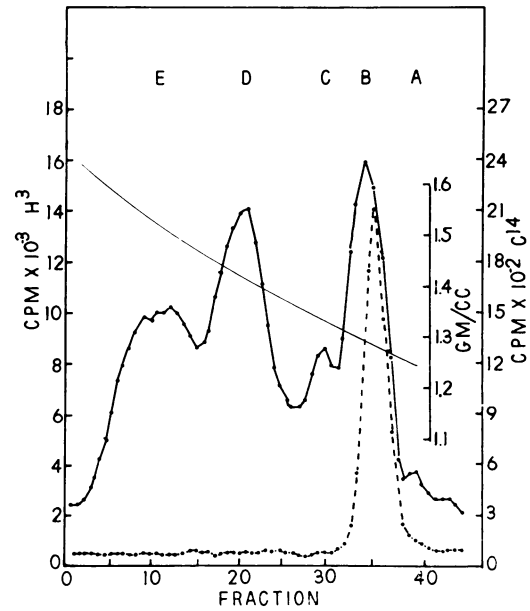

FIG. 3. *Co-sedimentation of ^{14}C-thymidine-labeled cytoplasmic extract and ^{3}H-thymidine-labeled nuclear extract of PSV-infected cells in a CsCl gradient. BHK-21 cells were infected with PSV and labeled with either ^{14}C-thymidine or ^{3}H-thymidine. At 10 hr after infection, cytoplasmic and nuclear extracts were prepared and fixed with formaldehyde. Samples of ^{14}C-thymidine-labeled cytoplasmic extract and ^{3}H-thymidine-labeled nuclear extract were mixed, layered onto a preformed CsCl gradient, and centrifuged as described in the text. The amount of radioactivity of each fraction was determined in a liquid scintillation system set up for dual-channel counting, and the appropriate corrections for spillover of the ^{14}C channel were made. The dashed line represents ^{14}C-labeled cytoplasmic extract; the solid line, ^{3}H-labeled nuclear extract.*

However, the material extracted from the extracellular fluids contained only the virion band (band B).

It was possible that subvirion components were being released from the infected cells but, because of the ionic conditions in the extracellular fluids, were either degraded or failed to become fixed with formaldehyde. To eliminate these possibilities, the following two experiments were performed. First, samples of extracellular fluid prepared as described above were mixed with unlabeled uninfected cell lysates and then were fixed with formaldehyde. Controls received media only prior to fixation. Upon centrifugation in CsCl gradients, no differences in the radioactive patterns of the two samples were observed, and the patterns were identical to those shown for extracellular fluids in Fig. 4.

Second, samples of intracellular virus and extra-

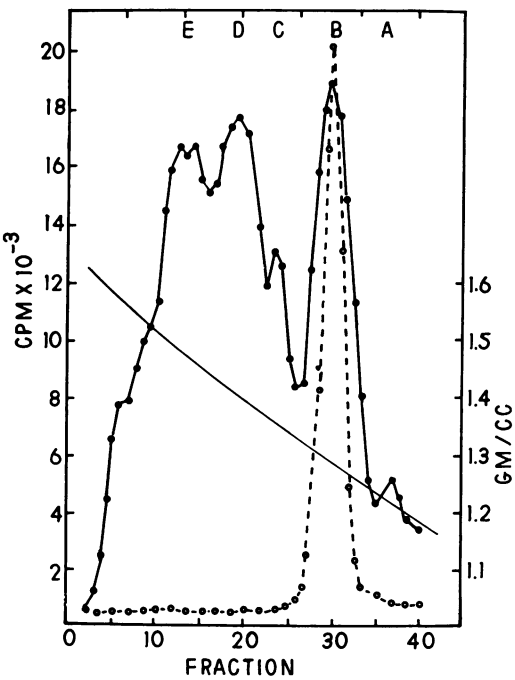

FIG. 4. *Distribution of ^{3}H-thymidine-labeled virions and virus-specific products in cells infected for 24 hr with PSV and in the extracellular fluids. Cells were infected with PSV, ^{3}H-thymidine was added, and 24 hr after infection the extracellular fluids were removed. The cells, containing the intracellular virus, were disrupted, and the cell debris was removed by centrifugation. Both the intracellular and extracellular samples were then centrifuged at 19,500 rev/min in a no. 30 rotor for 1 hr; the resultant pellets were resuspended, fixed with formaldehyde, and centrifuged in CsCl gradients as described. Solid line, intracellular samples; dashed line, extracellular fluids. Contents of bands A, B, C, D, and E are described in the text.*

cellular fluid identical to those described above were centrifuged, and the pellets were suspended in extracellular fluid from a 24-hr infected-cell culture to eliminate any ionic differences in the intracellular and extracellular samples. One portion of each sample was placed in ice; the other was immersed in a water bath at 37 C for 0.5 hr. All four portions were then fixed with formaldehyde and centrifuged in CsCl gradients as described. No differences in the patterns could be observed with either the intracellular or extracellular samples when they were heated for 0.5 hr at 37 C. These results indicate (i) that the ionic conditions in the extracellular fluids would permit the fixation of subvirion particles in the sample and (ii) that the subvirion particles are heat-stable for at least 0.5 hr and thus unlikely to be completely degraded in the extracellular fluid.

Finally, to eliminate the possibility that subvirion components were being released from the infected cell at an early stage in the infectious cycle and not in the later stages, an experiment similar to that outlined in Fig. 4 was performed, except that the extracellular fluid was examined at 12 hr after infection rather than at 24 hr. No subvirion components were found in the extracellular fluids.

DISCUSSION

The results presented in this paper offer direct biochemical and biological confirmation of previous electron microscopic observations on the intracellular and extracellular location of herpesviruses during the infectious cycle (4, 5, 8, 9).

Thus, it appears that subvirion components of PSV are restricted to the nucleus of the infected cell for at least the first 10 to 12 hr of the 16-hr growth cycle. Since no subvirion components could be detected in the extracellular fluids even after treatment with formaldehyde, it appears that these precursor particles are restricted to the nucleus throughout the entire growth cycle. The heat stability of these precursor particles in extracellular fluids from infected cells eliminates the possibility that they are being released from the cell and then rapidly inactivated in the extracellular milieu.

The data also show that infectious PSV first appears in the nuclei of infected cells. This is in direct accord with the previous studies of Spring and Roizman (7), who showed that 67% of herpes simplex virus was in the nucleus at 8 hr after infection. The excellent correlation of the infectivity data (Fig. 1) with the biochemical analysis of formaldehyde-fixed nuclear and cytoplasmic samples (Fig. 2) shows that infectious PSV (band B) is present in the nucleus of the infected cell early in the infectious cycle. The density differences noted between nuclear and cytoplasmic virions (Fig. 3) support the findings of Spring and Roizman (7) that the virus found in the nucleus is not fully enveloped.

From the data in Fig. 4, it appears that only complete virions are released from infected cells. Since it also has been shown that only complete virions are present in the cytoplasm of the infected cells (Fig. 2), it appears that the partially enveloped virions formed in the nucleus become fully enveloped upon passage through the nuclear membrane. These fully enveloped virions accumulate in the cytoplasm and are released from the infected cell during the later stages of the infectious cycle.

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LITERATURE CITED

1. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167–182.
2. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130:432–437.
3. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208–218.
4. Morgan, C. H., M. Rose, M. Holden, and E. P. Jones. 1959. Electron microscopic observations on the development of herpes simplex virus. J. Exp. Med. 110:643–656.
5. Nii, S., C. Morgan, and H. M. Rose. 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. J. Virol. 2:517–536.
6. Spring, S. B., and B. Roizman. 1967. Herpes simplex virus products in productive and abortive infection. I. Stabilization with formaldehyde and preliminary analyses by isopycnic centrifugation in CsCl. J. Virol. 1:294–301.
7. Spring, S. B., and B. Roizman. 1968. Herpes simplex virus products in productive and abortive infection. III. Differentiation of infectious virus derived from nucleus and cytoplasm with respect to stability and size. J. Virol. 2:979–985.
8. Spring, S. B., B. Roizman, and J. Schwartz. 1968. Herpes simplex virus products in productive and abortive infection. II. Electron microscopic and immunological evidence for failure of virus envelopment as a cause for abortive infection. J. Virol. 2:384–392.
9. Stoker, M. G. P., K. M. Smith, and R. W. Ross. 1958. Electron microscopic studies of HeLa cells infected with herpesvirus. J. Gen. Microbiol. 19:244–249.
10. Sydiskis, R. J. 1969. Precursor products found in formaldehyde-fixed lysates of BHK-21 cells infected with pseudorabies virus. J. Virol. 4:283–291.