JOURNAL OF VIROLOGY, Mar. 1981, p. 1060-1065
0022-538X/81/031060-06$02.00/0

Vol. 37, No. 3

Effects of Chloroquine and Cytochalasin B on the Infection of Cells by Sindbis Virus and Vesicular Stomatitis Virus

KEVIN COOMBS, ERIC MANN, JUDY EDWARDS, AND DENNIS T. BROWN*

Cell Research Institute and Department of Microbiology, University of Texas at Austin, Austin, Texas 78712

The effects of cytochalasin B and chloroquine on the process of endocytosis of Sindbis virus particles and polystyrene spheres were determined by electron microscopy. The effects of these agents on the process of infection (attachment, penetration, and uncoating) of BHK-21 cells by Sindbis virus and vesicular stomatitis virus were also determined. Cytochalasin B completely blocked ingestion of Sindbis virus particles or latex spheres by BHK cells but had no effect on the ability of Sindbis virus or vesicular stomatitis virus to infect or replicate in BHK cells. Chloroquine did not inhibit the ingestion of either latex spheres or virus particles but greatly reduced the yields of virus produced. These data suggest that endocytosis is not essential for the infection of cultured cells by Sindbis virus or vesicular stomatitis virus.

The events related to the penetration of cells by viruses represent some of the more controversial and experimentally difficult aspects of contemporary virology. The literature contains a wealth of scientific reports on investigations of this process (1, 3-5, 9, 10, 16, 19). These studies have generated two basic concepts of how this process may occur. One concept envisions viruses entering cells by a process morphologically similar to endocytosis (4, 10). After attachment to the cell surface, virions are ingested into coated pits and are internalized in membrane-limited vesicles. Virions may either escape from the vesicles early after their formation or the virus-containing phagosome may fuse with lysozomal membranes. In the latter case it has been proposed that the low pH of the lysosome induces the rapid fusion of membrane containing viruses and the lysosomal membranes with the subsequent release of virus core into the cell cytoplasm (10).

A second model suggests that viruses may enter cells by direct penetration of the plasma membrane (4). This hypothesis holds that membrane containing viruses fuse with the cell surface through the interaction of the virus envelope and the cell plasma membrane (1, 9). Data have been published suggesting that nonenveloped viruses follow a similar pathway through the cell plasma membrane (3, 5). The mechanism by which this may occur is obscure.

Experiments reported to support either of these models fall prey to a common criticism: for a given population of animal viruses, it is not possible to experimentally demonstrate a one-to-one correspondence between the number of physical particles and the number of PFU. In the best of circumstances, less than 20% of the virus population can be demonstrated to be infectious; in most instances, this percentage is much lower. Thus, in any morphological or biochemical assay of virus penetration, the majority of data are derived from virions which may not be capable of successfully infecting cells. It has also been suggested that cells may be capable of uncoating only a limited number of infecting viruses (19). It is not known whether cells discriminate between infectious and noninfectious particles at the point of uncoating, with infectious agents following one pathway and noninfectious agents another.

We have attempted to evaluate the role endocytosis may play in the infection of cells by enveloped viruses by inhibiting this process through treatment of cells with cytochalasin B, an inhibitor of microfilament formation and phagocytosis (15, 23). We have compared the effects of this agent with the effects of chloroquine which has been demonstrated to block receptor-mediated endocytosis and the replication of membrane containing viruses without affecting phagocytosis (3).

MATERIALS AND METHODS

Cells, virus, and media. BHK-21 cells were grown as monolayers in Eagle minimal essential medium (6) supplemented with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), 10% tryptose phosphate broth, and 1 mM glutamine as described previously (18). *Aedes albopictus* cells were cultured as described previously (18). The heat-resistant strain of Sindbis virus and its propagation and titration have been described (18).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ^{35}S-labeled polypeptides were re-

solved in 11% polyacrylamide slab gels (14 by 12 by 0.15 cm) (12). Samples were prepared for electrophoresis by boiling for 5 min in a sample buffer containing 1% sodium dodecyl sulfate and 1.5% dithiothreitol in 0.24 M Tris (pH 6.8) and then subjected to electrophoresis for 3.5 h at a constant power of 4.0 W per gel slab. The slabs were fixed in 20% trichloroacetic acid and impregnated with diphenyloxazole by the method of Bonner and Laskey (2). The gels were dried and then subjected to autoradiography (X-Omat RP X-ray film; Eastman Kodak, Rochester, N.Y.).

Electron microscopy. Subconfluent BHK-21 cell monolayers, to which either latex beads or virus particles had been added, were fixed for 12 h at 4°C in 2.5% gluteraldehyde with 0.05 M sodium cacodylate buffer (pH 7.2). A 15-min fix in 2% osmium tetroxide followed, after which monolayers were scraped from the plastic substrate and pelleted in 2% agarose. Specimens were stained for 12 h at 4°C en bloc in 0.5% aqueous uranyl acetate. After an ethanol dehydration series, specimens were imbedded in Araldite 502 (13). Thin sections were made by using a Sorvall MT-2 Ultramicrotome; sections were mounted on Parlodion-coated grids, and poststained in 0.5% aqueous uranyl acetate and lead citrate (20). Specimens were examined and photographed with an Hitachi HS-8 electron microscope operated at an accelerating voltage of 50 kV.

RESULTS

Electron microscopy of endocytosis of latex spheres and viruses by cells treated with cytochalasin B or chloroquine. BHK-21 cells (80% confluent) were treated with either 0.1 mM chloroquine (10, 16) or 10 μg of cytochalasin B per ml for a period of 30 min before infection with Sindbis virus or treatment with polystyrene beads (average diameter, 0.7 μm; Sigma Chemical Co., St. Louis, Mo.). A concentration of 10 μg of cytochalasin B per ml is higher than generally employed in experiments examining the effects of this agent on cell motility (15). This concentration has been demonstrated in this laboratory (data not shown) and in the laboratories of others (11, 23) as adequate for enucleation of cultured cells. In all experiments, the pH of the medium was carefully maintained in the range of 7.2 to 7.6.

Cells were infected with Sindbis virus at a multiplicity of 5,000 PFU per cell in the presence of drug for a period of 30 and 60 min at room temperature. Identical cultures of cells were treated with a concentrated solution of beads in the presence of drug to produce a ratio of beads to cells of 5,000 to 1. Control experiments were conducted in both cases without drug. Figure 1 shows representative electron micrographs of chloroquine- and cytochalasin B-treated cells exposed to either Sindbis virus or beads. Only the 60-min samples are shown. The images presented by the 30-min samples were qualitatively identical. Both partially ingested and completely internalized virus or beads could be readily detected in chloroquine-treated cells (Fig. 1). Basically, these results were identical to those obtained with cells not treated with drug but treated with beads (data not shown), although ingested virus particles were more apparent in chloroquine-treated cells than in control cells.

Internalization of either beads or virus was completely inhibited when cells were treated with cytochalasin B (Fig. 1). The association of beads with cells was greatly reduced, perhaps due to the lack of ingestion and accumulation of the beads. Sindbis virus was not found in coated pits or internal vesicles in the presence of cytochalasin B (Fig. 1B), although the virions could be seen atttached to the cell surface (Fig. 1B). The distribution of virus on the surface of the cell appeared more organized than did the surface distribution of virus in chloroquine-treated cells (Fig. 1A) or cells not treated with drug (not shown). Virions were frequently found clustered together in patches on the cell surface (Fig. 1B).

These observations demonstrate that cytochalasin B blocks the internalization by endocytosis of particulate material such as virus or polystyrene spheres. Internalization was either enhanced or took place normally in the presence of chloroquine.

Infection of cells and virus production in the presence of cytochalasin B or chloroquine. The effects of chloroquine or cytochalasin B on the replication of vesicular stomatitis virus or Sindbis virus in BHK-21 cells and Sindbis virus in *A. albopictus* (mosquito) cells were examined under conditions of infection and drug treatment similar to those employed in the electron microscopy study described above. Identical monolayers of BHK-21 cells were treated with either chloroquine (0.1 M), cytochalasin B (10 μg/ml), or neither (control). Monolayers were then infected with either vesicular stomatitis virus or Sindbis virus at a multiplicity of 50 PFU per cell for a period of 1 h in the presence (experiment) or absence (control) of drugs at the concentrations given above. Drug-treated cultures were maintained in the drug throughout a subsequent 12-h incubation period. At the end of this period, the medium was titrated by plaque assay to determine the amount of virus released. A similar experiment was conducted with cultured *A. albopictus* cells and Sindbis virus to determine the generality of any effects seen with BHK-21 cells. The results of these experiments are presented in Table 1.

These data show convincingly that chloroquine reduces the yields of either vesicular stomatitis virus or Sindbis virus from BHK-21 cells when the drug is present throughout the infec-

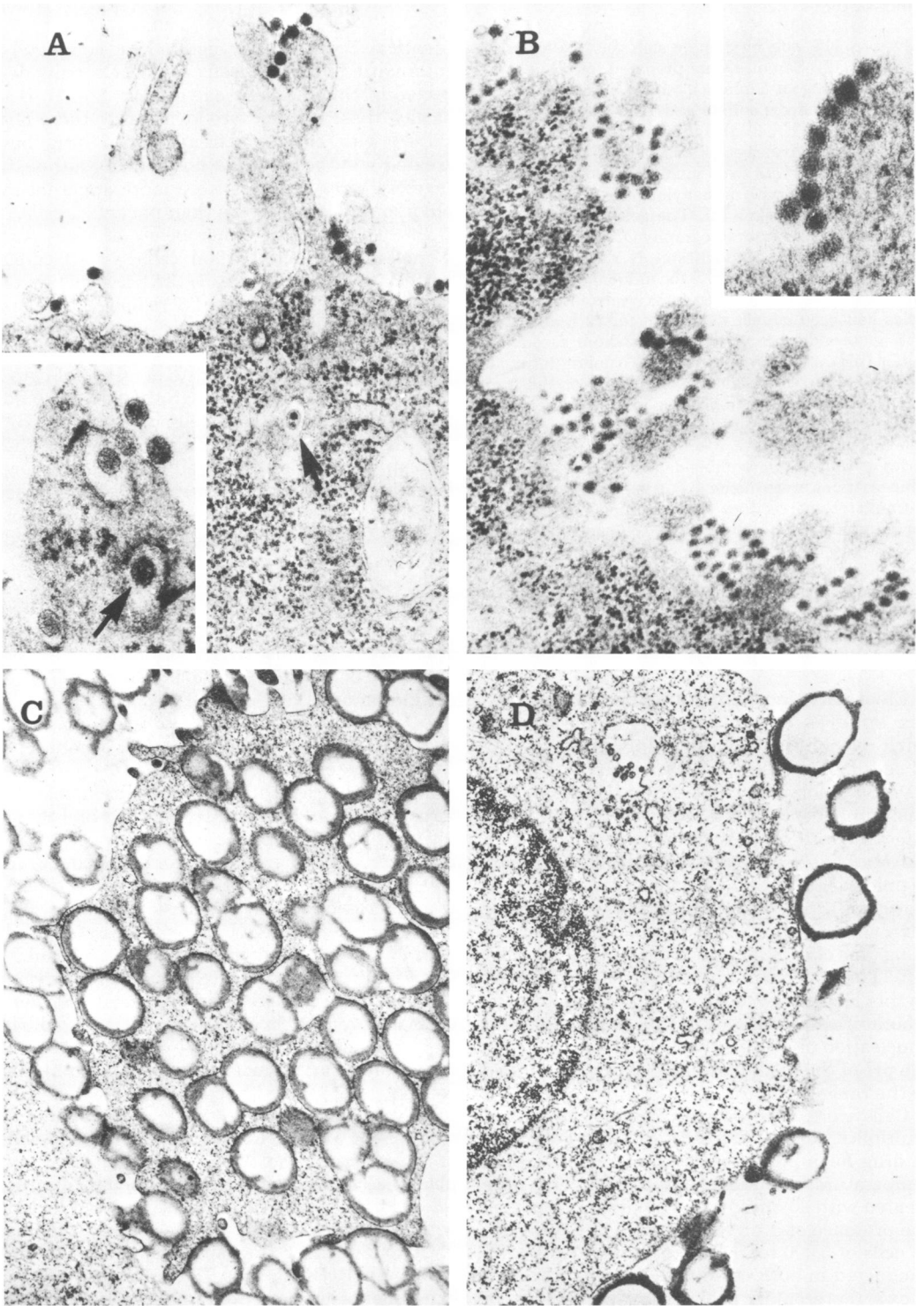

FIG. 1. *Electron micrographs of cultured BHK-21 cells treated with Sindbis virus particles (A and B) or polystyrene beads (C and D) in the presence of chloroquine (A and C) or cytochalasin B (B and D). Cells treated with chloroquine have partially ingested and completely ingested virus particles or beads (arrows); internalized virus particles are not found in cytochalasin B-treated cells (B). Polystyrene beads were not found internalized in cytochalasin B-treated cells. Magnification: A and B, ×39,600 (×90,000 for the inserts); C, ×13,000; and D, ×15,500.*

TABLE 1. *Effect of chloroquine on the replication of vesicular stomatitis virus and Sindbis virus in cultured BHK-21 and A. albopictus cells*[a]

Expt	Yield of virus[b]		
	No drug (*control*)	Chloroquine	Cytochalasin B
Sindbis virus in BHK-21[c]	1.5×10^8	2.5×10^6	2.0×10^8
Vesicular stomatitis virus in BHK-21[c]	1.2×10^9	4.9×10^6	2.8×10^8
Sindbis virus in *A. albopictus*[d]	7.0×10^8	7.1×10^8	5.0×10^8

[a] See text for protocol.
[b] Values represent an average of three experiments.
[c] Incubation at 37°C for 12 h in Eagle medium.
[d] Incubation at 28°C for 36 h in M+M medium (18).

tious cycle. This observation agrees well with other data derived from experiments in which vesicular stomatitis virus- or Sindbis virus-infected cells were treated with chloroquine. Chloroquine had no effect on Sindbis virus production in cultured mosquito cells. Cytochalasin B did not affect the production of vesicular stomatitis virus or Sindbis virus, in either BHK or mosquito cells.

Protein synthesis in Sindbis virus-infected chloroquine-treated cells. Identical monolayers of BHK-21 cells were treated with either actinomycin D (4 μg/ml) or actinomycin D (4 μg/ml) plus chloroquine (0.1 mM) for a period of 60 min before infection with Sindbis virus (50 PFU per cell). The drug was maintained in the medium throughout the period of infection and subsequent incubation at 37°C. At 5 h postinfection, the cells were labeled with [^{35}S]methionine (20 μCi/ml) in methionine-free medium for a period of 2 h. At the end of the labeling period, the monolayers were dissolved in sample buffer, and the cell-associated proteins were analyzed by polyacrylamide gel electrophoresis as described above. The distribution of the label in the gels is shown in Fig. 2. Actinomycin D treatment alone produced the expected profile of virus proteins B (100,000 daltons), PE_2, E_1, E_2, and C (Fig. 2, track 1). Chloroquine plus actinomycin D-treated cells also contained the B protein PE_2, E_1, and C (Fig. 2, track 2): however, E_2 protein could not be detected. The combination of chloroquine and actinomycin D resulted in the sustained synthesis of host proteins (Fig. 2, tracks 2 and 3) normally terminated by the combination of actinomycin D and Sindbis virus infection (Fig. 2, track 1). This result probably reflects the fact that in the presence of chloroquine only a small percentage of the cells are successfully infected (10) and that many cells continue production of host proteins in the absence of virus-mediated host cell shutoff (Fig. 2, track 3). The absence of E_2 protein in chloroquine-treated Sindbis virus-infected cells suggests that chloroquine treatment may inhibit Sindbis virus in the cells that become infected in the presence of chloroquine by blocking some late step in maturation (22).

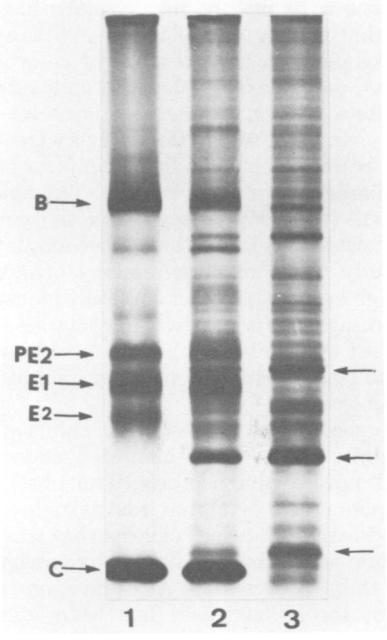

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gels of proteins associated with cells infected with Sindbis virus in the absence and presence of the drug chloroquine. Track 1, cells pretreated with actinomycin D and infected with Sindbis virus; track 2, cells pretreated with actinomycin D plus chloroquine and infected with Sindbis virus; and track 3, uninfected actinomycin D-treated cells. All cells were prepared as described in the text. The positions of the proteins PE_2, E_1, E_2, and C were determined by a marker virus preparation. The unlabeled arrows (track 3) indicate the positions of prominent host proteins seen also in track 2.*

The effect of chloroquine upon Sindbis virus maturation in BHK-21 cells, was found to be dependent upon the age of the cells used. In cells that were actively growing (70 to 80% confluent), addition of chloroquine at any time, before or after virus infection, inhibited virus production. In older cells (1 to 2 days after cells become confluent), the addition of chloroquine later than 30 min postinfection has no significant effect on virus production (data not shown).

DISCUSSION

We have confirmed the previously published observation that chloroquine treatment of cultured cells can inhibit replication of vesicular

stomatitis virus or Sindbis virus. We have also found that endocytosis of Sindbis virus by cells can take place in the presence of chloroquine. We have demonstrated that the endocytosis of particulate material such as virus or latex spheres can be blocked effectively by treatment of cells with cytochalasin B, an inhibitor of actin microfilament formation (15, 23). The ability of this agent to block phagocytotic activity of cells at concentrations lower than those employed in this study has been described by others (23). Although cytochalasin B completely blocked the internalization of virus by phagocytosis, it did not affect the ability of the cells to be successfully infected by either vesicular stomatitis virus or Sindbis virus.

Recent evidence suggests that chloroquine is an inhibitor of receptor-mediated endocytosis (7) but not of phagocytosis. The observation that endocytosis of alphavirus particles is not affected by chloroquine suggests that this internalization may be mediated by phagocytes rather than by receptors. As of now, no specific receptor for alphaviruses has been identified (17), a circumstance which may be in keeping with the extensive host range of alphaviruses. That the endocytosis of alphaviruses seen in the electron microscope is the result of phagocytosis is further demonstrated by the observation that this process is blocked by cytochalasin B. These data suggest that alphaviruses enter cells by some mechanism which is not dependent upon ingestion of the particles by the cells and that in high multiplicity infection, such as that employed in electron microscope studies, this route of successful infection is taken by a nondetectable minority of virions. It is possible that this route of entry is via the direct fusion of the virion membrane with the surface of the host cell. Chloroquine may block this fusion by altering characteristics of the cell plasma membrane. Chloroquine was found to have no effect on Sindbis virus infection of mosquito cells, an observation which may result from the differences in the lipid composition of the invertebrate membrane relative to BHK-21 cells (14). The lack of effect on virus maturation in mosquito cells could likewise result from differences in the composition of cellular membranes as well as from differences in the basic process of virus maturation (8).

Although these data alone cannot resolve the basic controversy regarding the pathway by which viruses infect cells, it is clear that replication of vesicular stomatitis virus and Sindbis virus was not affected under conditions which prevented cellular functions related to endocytosis. This observation is compatible with the model for direct interaction of enveloped virus with the cell plasma membrane, as suggested by Heine and Schnaitman (9). Our data are incompatible with the concept that internalization of virus by phagocytosis, followed by an interaction of lysosomal structures with the resulting phagosomes, is essential for the infection of cells.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI-14710 from the National Institutes of Allergy and Infectious diseases and grant F-717 from the Robert A. Welch Foundation.

We thank Bob Slocum for expert technical assistance.

LITERATURE CITED

1. **Bachi, T., and C. Howe.** 1972. Fusion of erythrocytes by Sendai virus studied by electron microscopy. Proc. Soc. Exp. Med. Biol. **72**:141–149.
2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
3. **Brown, D. T., and B. T. Burlingham.** 1973. Penetration of host cell membranes by adenovirus 2. J. Virol. **12**: 386–396.
4. **Dales, S.** 1973. Early events in cell-animal virus interactions. Bacteriol. Rev. **37**:103–135.
5. **Dunnebacke, Th. H., J. D. Levinthal, and R. C. Williams.** 1979. Entry and release of poliovirus as observed by electron microscopy of cultured cells. J. Virol. **4**:505–513.
6. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
7. **Fitzgerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor mediated internalization of Pseudomonas toxin by mouse fibroblasts. Cell **21**:867–873.
8. **Gliedman, J. B., J. F. Smith, and D. T. Brown.** 1975. Morphogenesis of Sindbis virus in cultured *Aedes albopictus* cells. J. Virol. **16**:913–926.
9. **Heine, J. W., and C. A. Schnaitman.** 1971. Entry of vesicular stomatitis virus into L cells. J. Virol. **8**:786–795.
10. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
11. **Kos, K. A., B. A. Osborne, and R. A. Goldsby.** 1975. Inhibition of group B arbovirus antigen production and replication in cells enucleated by cytochalasin B. J. Virol. **15**:913–917.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Luft, J. H.** 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**:409.
14. **Luukkonen, A., C. H. von Bonsdorff, and F. Renkonen.** 1977. Characterization of Semliki Forest virus grown in mosquito cells. Comparison with the virus from hamster cells. Virology. **78**:331–335.
15. **MacLean-Fletcher, S., and T. D. Pollard.** 1980. Mechanism of action of cytochalasin B on actin. Cell **20**:329–341.
16. **Miller, D. K., and J. Lenard.** 1980. Inhibition of vesicular stomatitis virus infection by spike glycoprotein. J. Cell Biol. **84**:430–437.
17. **Oldestone, M. B. A., A. Tisthon, F. J. Dutko, S. I. T. Kennedy, J. J. Holland, and P. W. Lampert.** 1980. Does the major histocompatibility complex serve as a specific receptor for Semliki Forest virus? J. Virol. **34**: 256–265.
18. **Renz, D., and D. T. Brown.** 1976. Characteristics of Sindbis virus temperature-sensitive mutants in cultured

BHK-21 and *Aedes albopictus* (mosquito) cells. J. Virol. **19**:775–781.

19. **Rosenwirth, B., and H. J. Eggers.** 1979. Early processes of echovirus 12-infection: elution, penetration and uncoating under the influence of rhodanine. Virology **97**: 241–255.
20. **Sato, T.** 1968. A modified method for lead staining of thin sections J. Electron Microsc. **17**:158–159.
21. **Schlesinger, M. J., and S. Schlesinger.** 1973. Large-molecular-weight precursors of Sindbis virus proteins. J. Virol. **11**:1013–1016.
22. **Smith, J. F., and D. T. Brown.** 1977. Envelopment of Sindbis virus: synthesis and organization of proteins in cells infected with wild type and maturation-defective mutants. J. Virol. **22**:662–678.
23. **Tanenbaum, S. F.** 1978. Cytochalasins: biochemical and biological aspects. North-Holland Publishing Co., Amsterdam.