ON THE ENTRY OF SEMLIKI FOREST VIRUS INTO BHK-21 CELLS

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ABSTRACT

The pathway by which Semliki Forest virus (SFV), a membrane-containing animal virus, enters BHK-21 cells was studied morphologically and biochemically. After attaching to the cell surface, the majority of viruses was rapidly trapped into coated pits, internalized by endocytosis in coated vesicles, and sequestered into intracellular vacuoles and lysosomes. Direct penetration of viruses through the plasma membrane was never observed.

To assess the possible involvement of lysosomes in the release of the genome into the cytoplasm, the effect of five lysosomotropic agents, known to increase the lysosomal pH, was tested. All of these agents inhibited SFV infectivity and one, chloroquine (the agent studied in most detail), inhibited a very early step in the infection but had no effect on binding, endocytosis, or intracellular distribution of SFV. Thus, the inhibitory effect was concluded to be either on penetration of the nucleocapsid into the cytoplasm or on uncoating of the viral RNA.

Possible mechanisms for the penetration of the genome into the cytoplasm were studied in vitro, using phospholipid-cholesterol liposomes and isolated SFV. When the pH was 6.0 or lower, efficient fusion of the viral membranes and the liposomal membranes occurred, resulting in the transfer of the nucleocapsid into the liposomes. Infection of cells could also be induced by brief low pH treatment of cells with bound SFV under conditions where the normal infection route was blocked.

The results suggest that the penetration of the viral genome into the cytosol takes place intracellularly through fusion between the limiting membrane of intracellular vacuoles and the membrane of viruses contained within them. The low pH required for fusion together with the inhibitory effect of lysosomotropic agents implicate lysosomes, or other intracellular vacuoles with sufficiently low pH, as the main sites of penetration.

KEY WORDS   coated vesicles · lysosomes · endocytosis · membrane fusion · lysosomotropic agents

To infect a cell, a virus must transfer its genome from the extracellular space to the cytosol. This process requires transport through one or more membrane barriers. For animal viruses the mechanisms involved are poorly understood. Data from electron microscopy suggest that penetration oc-

curs either at the plasma membrane or intracellularly after the virus has been taken up by endocytosis (for reviews see references 8 and 35). Penetration at the cell surface has been demonstrated for paramyxoviruses (for reviews see references 7 and 44); the membranes of these viruses fuse with the host cell plasma membrane, and the nucleocapsid is thereby introduced into the cytoplasm. Endocytosis, "viropexis," has been observed for most animal viruses and may play an important role in the infection by many viruses (see reference 8). How the viral genome escapes from the endocytotic vacuoles into the cytoplasm in not clear.

In this paper we have tried to unravel the pathway of infection used by the Semliki Forest virus (SFV). SFV is a simple membrane-containing animal virus belonging to the alphaviruses. The structural properties, the biosynthesis, and the general features of replication have been well characterized. The virus consists of a spherical nucleocapsid containing a single-stranded RNA and 240 copies of a single protein (5, 18). The nucleocapsid is surrounded by a lipid bilayer membrane with glycoprotein spikes (47). Each of these spikes consists of three glycopolypeptides, with apparent mol wt of 48×10^3, 51×10^3, and 10×10^3, respectively (18, 56). The spike proteins are anchored by hydrophobic segments in the lipid bilayer (51). In BHK-21 cells, the cell line used in this study, infection proceeds rapidly (4, 30), penetration occurring within the 1st h after addition of the virus. The viral RNA synthesis is detectable after 1.5 h, and after 2.5 h the first progeny viruses appear in the medium.

Our results suggest the following stages in the entry of SFV into BHK-21 cells: (a) binding to the cell surface, (b) internalization by adsorptive endocytosis in coated vesicles, (c) entry into larger intracellular vacuoles, and (d) fusion of the viral membrane with the vacuolar membrane, whereby the nucleocapsid enters the cytoplasmic compartment.

MATERIALS AND METHODS

Cells and Virus

BHK-21 cells were grown as monolayers in MEM Glasgow medium (Orion Diagnostica, Finland) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 2 mM glutamine, 250 IE/ml penicillin, and 0.1 mg/ml streptomycin sulfate. Plastic cell culture bottles (75 cm², Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), Petri dishes (6 × 1.5 cm, Falcon Labware), 24-well Linbro plates (Linbro Div., Flow Laboratories, Hamden, Conn.), and glass

coverslips (diameter 1.1 cm) in 3-cm diameter petri dishes (Falcon Labware) were used. The cells were passaged twice a week and used on the 3rd d after plating.

A prototype strain of SFV was grown in the BHK-21 cells and purified as described (28). SFV was labeled either with [³⁵S]methionine (Radiochemical Centre, Amersham, England) (0.4 mCi/ml medium), or with [³H]isoleucine, leucine, and valine (0.02 mCi of each per ml medium) (29). Viral protein was determined by the Lowry procedure with 0.1% sodium dodecyl sulfate in the reaction mixture, using bovine serum albumin (BSA) as a standard. In the calculations, we assumed a protein content of 57%, and a viral mol wt of 64×10^6 (34). In the ³²P-labeled virus, ~10% of the activity was extractable by chloroform: methanol:water (8:4:3 vol/vol), indicating that ~90% of the label was in the RNA. The nonradioactive virus, the [³⁵S]methionine-labeled virus, and the ³H-labeled virus were stored at −80°C in 0.05 M Tris-HCl, pH 7.4, containing 0.1 M NaCl and 42% wt/wt sucrose. The ³²P-labeled virus was stored at 4°C and used within 3 d.

Association of [³⁵S]Methionine-labeled SFV to Cells

Confluent cells on 6 × 1.5 cm Falcon dishes were washed twice with R-medium (RPMI 1640 containing 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES, Serva, Germany), pH 6.8, and 0.1% BSA. The dishes were kept either in a CO_2 incubator (37°C) or on ice for 1 h, in the presence or absence of 0.1 mM chloroquine. The medium was removed, and 1 ml of ³⁵S-labeled SFV in R-medium (with or without chloroquine) was added to each dish either at room temperature or in the cold. The cells were then incubated at 37°C or on ice for different periods of time. The medium was removed and the cells were washed twice with 1.0 ml of cold R-medium. The combined medium and washes were centrifuged (5 min, 1,500 rpm, 4°C) and 1.2 ml of the supernate was lyophilized and solubilized in Triton X-100 toluol scintillation fluid. The radioactivity was determined in a Mark III scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). Another 1.2 ml was mixed with an equal volume of 20% TCA. After 1 h at 0°C, the precipitates were collected on 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.) and the radioactivity was determined. The cells were scraped off with a rubber policeman into 1 ml of phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 ml of ice-cold 20% TCA was added. The dishes were washed twice with 1 ml of 10% TCA. After 1 h on ice, the precipitates were collected and assayed for radioactivity. Aliquots of the TCA supernate were dried at 50°C, and radioactivity was determined after solubilization in scintillation fluid.

To synchronize the entry of virus into the cells, the ³⁵S-labeled virus was allowed to bind in the cold for 1 h as described above. Free and loosely bound virus was removed by two washes with 5 ml of cold R-medium. 1 ml of R-medium was added and the dishes were placed on the water surface of a 37°C waterbath or for incubations longer than 20 min into a 5% CO_2 incubator. At various times dishes were removed, cooled, and treated as described above.

Proteinase K Assay for Internalized SFV

Cells treated with radioactive SFV in the cold or at 37°C for varying periods were cooled on ice and washed. 0.5 mg proteinase K (Boehringer, Mannheim, Germany) or 0.5 mg of BSA in 1 ml of PBS was added to duplicate dishes. The dishes were rocked

slowly for 45 min at 4°C, and 1 ml of 1 mM PMSF in PBS, containing 30 mg of BSA, was added. The cells were scraped off, transferred into 15-ml conical glass tubes, vortexed, and diluted to 14 ml with PBS containing 0.2% BSA on ice. After centrifugation (3 min, 2,000 rpm, 4°C) the supernate was discarded, and the cells were washed twice with PBS-BSA. Finally, the cells were dissolved in 10 ml of Triton X-100 toluol scintillation fluid and the radioactivity was measured.

SFV Fusion with Liposomes

Liposomes were prepared daily by dissolving 2.5 μmol each of phosphatidylethanolamine (bovine brain type I, Sigma Chemical Co., St. Louis, Mo.), sphingomyelin (bovine brain, Sigma Chemical Co.), and lecithin (egg yolk, type V, Sigma Chemical Co.), and 3.75 μmol of cholesterol (Merck, Germany, four times crystallized) in 0.5 ml chloroform:methanol (2:1 vol/vol). The mixture was divided in two parts and dried on the walls of glass tubes using a rotary evaporator and was then redissolved twice in diethylether. After a few glass beads were added (1 mm Diam), one of the dried lipid films was suspended by vortexing into 0.5 ml of 0.05 M Tris-HCl, pH 7.2, containing 0.13 M NaCl and 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10 mg/ml pancreatic RNase A (bovine, type IIIA, Sigma Chemical Co.). The other was suspended into 0.02 M HEPES, pH 7.3, containing 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.3% BSA. To remove untrapped enzyme the RNase-containing sample was applied to a 0.7 cm $\times$ 20 cm column of Sephadex G-150 (Pharmacia, Uppsala, Sweden) equilibrated with the HEPES buffer. Fractions (0.31 ml) were collected at room temperature, and the three most opalescent fractions were pooled. To 0.9 ml of this pool, 20 μl of carrier-free ^{32}P-SFV was added (5 to 10 $\times$ 10^5 cpm). Control solutions were prepared as follows: (a) 20 μl of ^{32}P-SFV was mixed with 0.15 ml of the RNase-free liposomes and 0.75 ml of the HEPES buffer containing 0.1 mg/ml RNase, (b) 20 μl of the ^{32}P-SFV was mixed with 0.9 ml of HEPES buffer containing 0.1 mg/ml RNase. Aliquots (100 μl) of each solution were placed in the tip of five collodium thimbles (Sartorius, Göttingen, Germany). The collodium thimbles were dialyzed at 37°C for 20 min against buffers of different pH. The buffers used were 0.02 M HEPES-HCl, pH 7.3, containing 0.13 M NaCl, 0.02 M 2 (N-morpholino)ethane sulphonic acid (MES) with 0.13 M NaCl, adjusted to pH 6.5, 6.0, and 5.5, respectively, and 0.02 M sodium acetate, pH 5.0, containing 0.13 M NaCl. As additional controls, 100-μl aliquots of each starting solution were made 1% in respect to Triton X-100 and incubated for 20 min at 37°C without dialysis, and ^{32}P-SFV (20 μl) was mixed with 0.9 ml of HEPES buffer and kept at 0°C. Immediately after dialysis or incubation, the samples were transferred to 1.5-ml Eppendorf tubes on ice. Aliquots (25 μl) were pipetted onto two 3 MM Whatman paper disks (2.3 cm). One of the disks was allowed to air dry, and the other was immersed in cold 10% TCA. After 1 h at 0°C, it was washed for 15 min with ethanol: diethylether (1:1 vol/vol) and for 15 min in diethylether. After air drying, both paper disks were placed into scintillation vials and the radioactivity was determined using a toluene-based scintillant. The disks that had not been TCA treated gave the total radioactivity, and the disks that had been TCA treated gave the amount of acid-precipitable radioactivity. Identical results were obtained if TCA precipitation was performed in tubes after inactivation and solubilization with 1% sodium dodecyl sulfate at 56°C.

To determine the distribution of acid-soluble nucleotides between the particulate fraction and the medium, a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) was used. To 25-μl aliquots of the dialysates, 0.175 ml of

0.02 M HEPES, pH 7.3, containing 0.13 M NaCl and 0.3% BSA was added. Two 25-μl aliquots of these mixtures were assayed for TCA-soluble and total radioactivity as described above, and the rest were centrifuged for 20 min at 10^5 g at room temperature. From the clear supernate, two 25-μl aliquots were assayed for TCA-soluble and total radioactivity as described above.

Lysosomotropic Agents

Stock solutions (10 mM) were prepared daily of chloroquine-HCl (Sigma Chemical Co.) and tributylamine, 100 mM of NH$_4$Cl and methylamine (Merck-Schuchardt, Hohenbrunn, Germany) and 1 mM of amantadine-HCl (Sigma Chemical Co.) in water. When necessary, the pH was adjusted to neutrality with NaOH or HCl, and appropriate dilutions were then made into culture medium. For experiments involving plaque titrations, BHK-21 cells were grown to confluence in Linbro plates with 2.0-cm^2 wells. They were washed twice with serum-free MEM Glasgow medium and subsequently infected with 0.1 ml of SFV containing serum-free medium with 0.2% BSA in the presence or absence of lysosomotropic agents. After 4.5 h at 37°C with or without inhibitors, the supernate was collected and centrifuged for 4 min at 5 $\times$ 10^3 g (4°C) to remove cell debris. An aliquot (0.1 ml) of the supernate was then added to 0.9 ml of PBS containing 0.2% BSA. Duplicate plaque titrations were performed on BHK-21 cell monolayers as previously described (45). For indirect immunofluorescence microscopy, cells were grown on coverslips in the Linbro wells and infected as above.

Rhodamine and Fluorescein Labeling of SFV and Immunofluorescence Microscopy

SFV was labeled with tetramethyl rhodaminylisothiocyanate Isomer R (TRITC) (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) in the following way: SFV (1 mg of protein) was mixed with a trace amount of [^{35}S]methionine-labeled SFV to give a specific radioactivity of 200–500 cpm/μg protein. The mixture (0.2 ml) was placed in dialysis tubing (Union Carbide Corp., Crystal Products Dept., San Diego, Calif.) and dialyzed for 10 h against 0.1 M sodium bicarbonate and for another 10 h against 10 ml of 0.1 M sodium bicarbonate containing 0.5 mg/ml of TRITC. For fluorescein labeling, 0.4 mg of SFV in 0.2 ml of 1:1 (vol/vol) PBS and saline was made basic by addition of 20 μl of sodium bicarbonate. 2 μg of dichlorotriazinylfluorescein (DTAF, Research Organics, Ohio) was added, and the sample was incubated for 1 h at room temperature before addition of 40 μl of 0.5 M glycine. Both the rhodamine- and the fluorescein-labeled virus were reisolated by sedimentation velocity centrifugation in a sucrose gradient (5–20% sucrose wt/wt in 0.05 M Tris-HCl, pH 7.4, containing 0.1 M NaCl) in the SW50 rotor (Beckman Instruments, Inc., Spinco Div.) (4°C, 35,000 rpm, 30 min). 40–60% of the radioactivity was found as a colored band at the position of control unlabeled SFV, centrifuged in a parallel gradient, the rest as faster sedimenting aggregates. The virus peak was collected and dialyzed overnight at 4°C against R-medium. To determine whether the virus was still intact, aliquots were subjected to sedimentation velocity and isopycnic sucrose gradient centrifugation with an internal standard of added ^{3}H-labeled SFV. The fluorescent virus behaved in the same way as ^{3}H-labeled virus. The virus particles appeared normal after negative-staining electron microscopy, but plaque titration showed that the fluorescein-labeled virus had lost 50% of its infectivity and that the rhodamine-labeled virus was no longer infective. The number of fluorescein and rhodaminyl

groups per virus particle were 200 and 1,000, respectively, as estimated from the absorbances at 495 and 560 nm and the protein content. Both preparations showed the same fluorescence pattern when added to cells, but only the more intensely fluorescent rhodamine-labeled virus could be used for photographic documentation. The fluorescent virus preparations were stable for at least 3 mo at −80°C.

For fluorescence microscopy, cells grown on coverslips were washed with cold medium (RPMI 1640 containing 10 mM MES, pH 6.8 and 0.1% BSA) and incubated with 20 μl of medium containing 0.5 μg of rhodamine or fluorescein-SFV for 1 h on ice. After washing with cold medium, the cells were either fixed for 15 min with 3% paraformaldehyde in PBS or incubated for 1 h at 37°C in medium before fixation.

For indirect immunofluorescence microscopy we used rabbit anti-spike protein serum (55) previously absorbed with 5 × 10^7 BHK-21 cells/ml serum for 1 h at 37°C, and diluted 1:75 with PBS containing 0.1% BSA. Cells on coverslips treated or infected with virus were washed and fixed with 3% paraformaldehyde at room temperature. After washing with PBS containing 0.1 M glycine and PBS alone, the cells were incubated for 30 min with 20 μl of rabbit anti-spike protein serum, washed for 30 min with PBS, and incubated for 30 min with 10 μg/ml fluorescein-conjugated goat anti-rabbit-IgG (Miles, Stoke Poges, England) in PBS. After washing, the cells were mounted in 90% glycerol and viewed in a Zeiss fluorescent microscope using epifluorescence with appropriate filters for fluorescein and rhodamine, and the Neofluotar 25 or the Planapo 63 objective. For photography, Kodak Tri-X film was used. To determine the fraction of infected cells, fields with ~500 cells were randomly photographed with both phase contrast and epifluorescence. The infected cells were brightly fluorescent and could easily be counted.

Electron Microscopy

For thin-section electron microscopy, confluent cells on coverslips were washed with the medium used for immunofluorescence. They were then incubated for 1 h on ice with 0.8 μg of SFV in 10 μl of medium and washed with cold medium. The coverslips either were fixed directly with ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.2, containing 50 mM KCl and 2.5 mM MgCl₂ or they were, before fixation, immersed in 37°C medium for varying times. Dehydration, embedding, sectioning, and uranyl acetate staining were performed as described (24). A Philips 400 or a Siemens Elmiskop 101 electron microscope was used. Negative staining with 2% phosphotungstic acid (pH 7.0) was performed as described (24). Histochemical visualization of acid phosphatase was performed on frozen sections according to Paavola (42).

RESULTS

Morphology of SFV-binding to the Cell Surface

To visualize how SFV interacts with BHK cells, we used fluorescence microscopy to obtain an overall picture and electron microscopy to observe the individual virus particles. Both techniques require a high multiplicity of virus to cells. The amount of viruses used in these experiments was 0.5–1 μg viral protein/10⁶ cells, which is 10² to 10³

times more than normally used for infection studies. To make the SFV visible in the fluorescence microscope, rhodamine or fluorescein was covalently coupled to it (~10³ and 2 × 10² groups, respectively, per virus particle). The fluorescein-labeled virus was not so strongly fluorescent as the rhodamine-labeled virus, but it retained half of its original infectivity. Indirect immunofluorescence with antiserum against the spike proteins and fluorescein-conjugated goat anti-rabbit IgG was also used.

When SFV was allowed to bind to BHK-21 cells in the cold, the patchy distribution shown in Fig. 1 a was seen. The same fluorescence pattern was obtained with rhodamine- and fluorescein-labeled SFV, and with unlabeled SFV stained by indirect immunofluorescence. When the cells coated with rhodamine-SFV in the cold were stained with indirect immunofluorescence using fluorescein-labeled antibodies, full overlap between rhodamine and fluorescein stains was observed, indicating that all the rhodamine-SFV remained on the surface.

Thin-section electron microscopy (Fig. 2) of cells treated with virus in the cold indicated that the viruses bound preferentially to microvilli. A count made of 3 × 10³ SFV particles in thin sections of four different samples showed that 70% were associated with the microvilli. There are 2–4 × 10³ microvilli/cell, and their surface accounts for <40% of the total cell surface area. In the control cells the microvilli projected out from the cell surface; when associated with viruses, they tended to lie flat along the membrane (Fig. 2 b). It is likely that most of the fluorescent patches observed in Fig. 1 a correspond to viruses clustered around microvilli.

Many of the viruses that were not associated with microvilli were located in so-called coated pits (5% of all cell surface viruses) (Fig. 3 a–c), and some (0.5%) were associated with small, flask-shaped indentations devoid of coat (Fig. 3 i). The coated pits are invaginations of the plasma membrane with a characteristic electron-dense bristle coat on the cytoplasmic surface. In BHK-21 cells, the coated pits make up <2% of the total cell surface. Many are localized close to the base of microvilli. Several viruses could be seen in a single coated pit, and virus particles were often located at the edges of the pits. When the pits were highly indented the viruses tended to be close to the neck. Similar observations have been reported for the location of ferritin-labeled low density lipoprotein
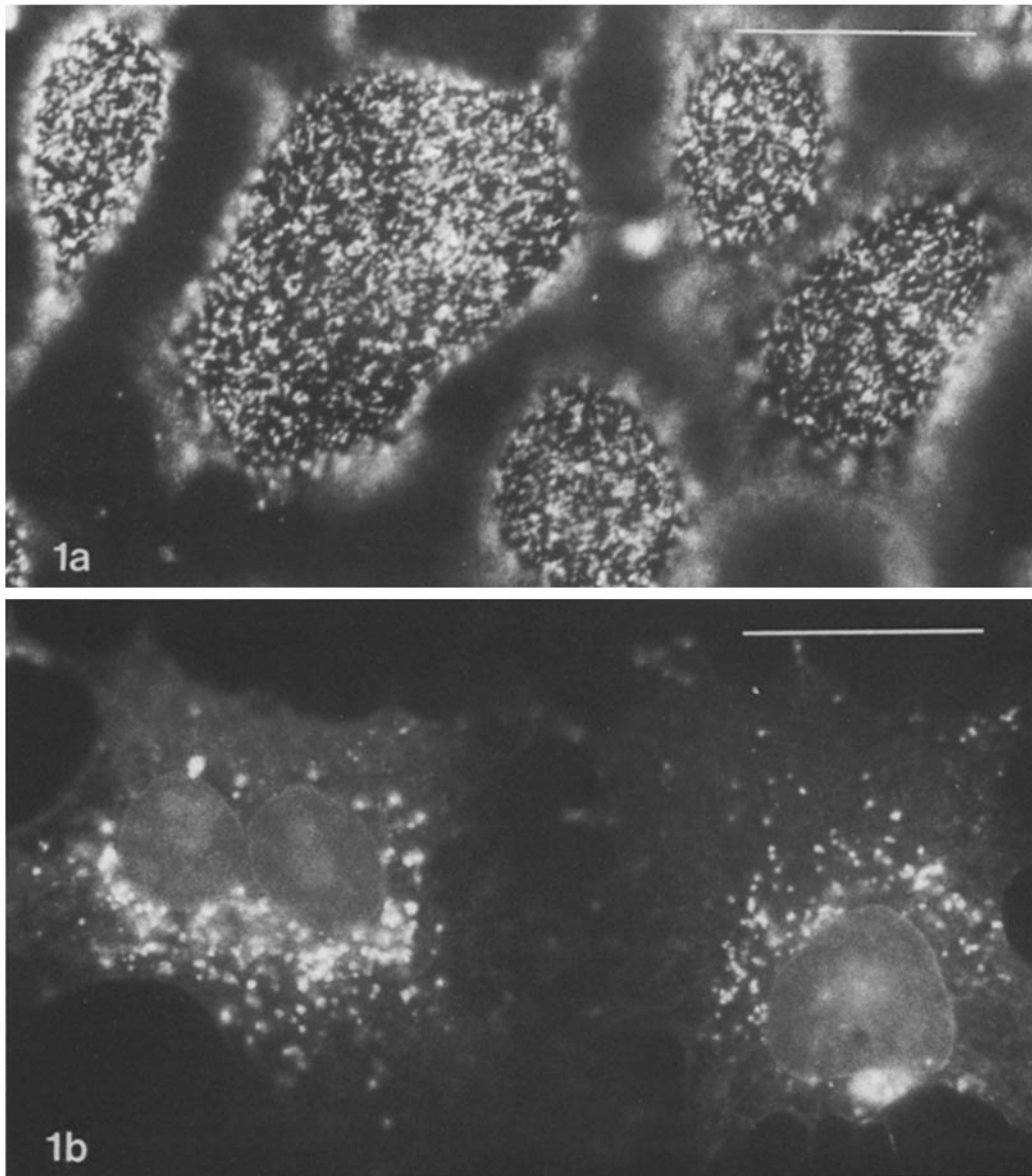
FIGURE 1   Fluorescent virus associated with BHK cells. (a) Rhodamine-labeled SFV (0.5 µg/10⁶ cells) was allowed to bind on ice for 1 h. (b) Rhodamine-labeled virus was allowed to bind on ice for 1 h, and after washing, the cells were incubated for another hour at 37°C. The same distribution was observed with fluorescein-labeled SFV and in the case of a with unlabeled virus after indirect immunofluorescence. Bar, 25 µm. × 1,250.

in coated pits of human fibroblasts (2, 41).

The cell surface distribution of viruses bound at 37°C closely ressembled that at 4°C. We observed, however, in agreement with Anderson et al. (2), that deeply indented coated pits like those seen in Fig. 3 c were relatively scarce at this temperature.

Morphology of SFV Internalization

Coverslips with 10⁶ cells were incubated with virus in the cold for 1 h, washed, transferred to 37°C medium for varying periods of time, and then fixed. The distribution of fluorescence of
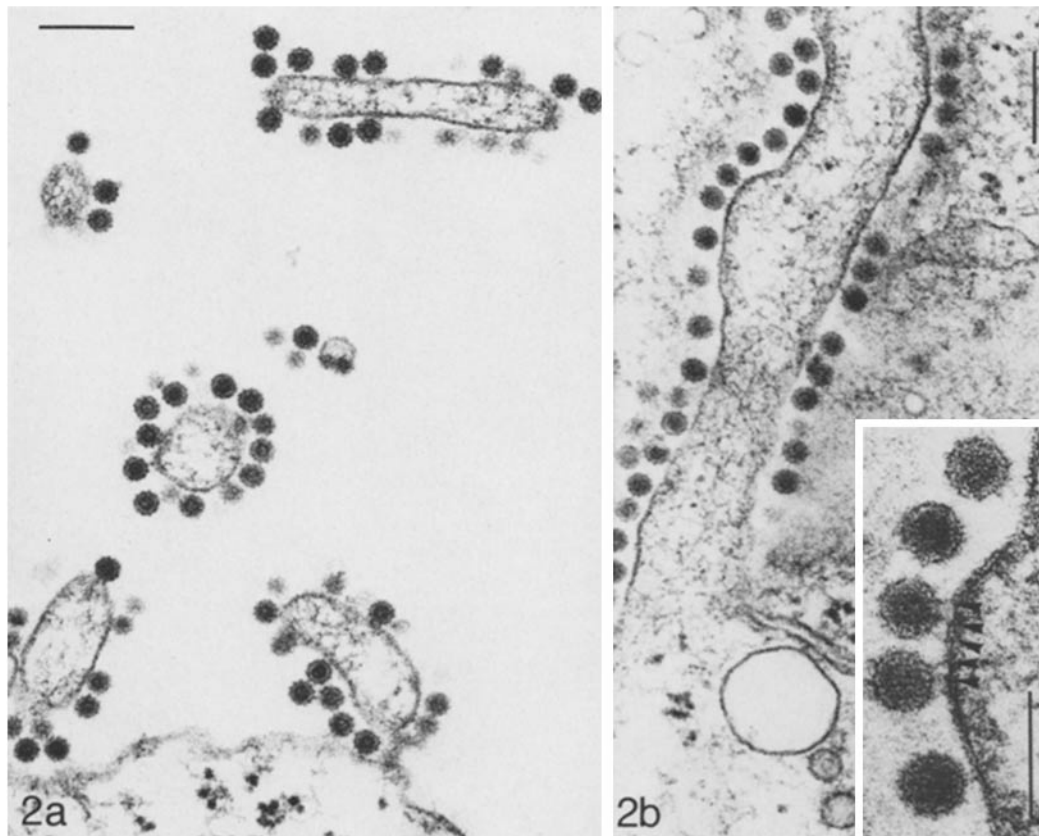
FIGURE 2 Thin-section electron microscopy of BHK cells with SFV bound at 4°C. One million cells on a coverslip were treated with 0.8 μg of SFV for 1 h on ice. The majority of viruses are associated with microvilli (a and b). Microvilli covered with virus were often observed to lie parallel along the membrane (b). Occasionally, multiple contacts between virus spikes and cell surface could be observed (see arrows in inset). Bars (a and b) 0.2 μm; inset, 0.1 μm. × 62,000; inset, × 168,000.

rhodamine-labeled SFV after 1 h at 37°C was that shown in Fig. 1 b. The fluorescence was concentrated in larger patches in the perinuclear region of the cell, a distribution very similar to that of lysosomes as visualized by acridine orange staining (1 μg/ml) (1). The viruses were intracellular as they were no longer accessible to staining by indirect immunofluorescence without breaking the cell membranes.

For thin-section electron microscopy, the virus-coated cells were only briefly warmed to 37°C to see the earliest stages of the internalization process. When the incubation time at 37°C was 15–30 s, some of the viruses had already entered the cell. They were in coated vesicles (Fig. 3 d–h) that were round or ovoid in shape and had an outer diameter of 130–200 nm. As a rule, only one virus was

present in each coated vesicle and it usually remained membrane bound. After 1 min or longer at 37°C, viruses could be observed accumulating in larger vacuoles which were either devoid of bristle coat or had just a small coated region on their surface (Fig. 4 a and b). Other types of vacuoles became prominent after ~5 min of incubation at 37°C (Fig. 4 c–f). These included large vacuoles with a lysosomal appearance. In some of these vacuoles the viruses were bound to the vacuolar membrane, to membranous intravacuolar material, or to each other. Many of the vacuoles could be stained for acid phosphatase activity by the cytochemical reaction of Gomori, confirming their lysosomal nature. The number of viruses at the plasma membrane decreased steadily as the incubation at 37°C proceeded, so that, after 1 h,
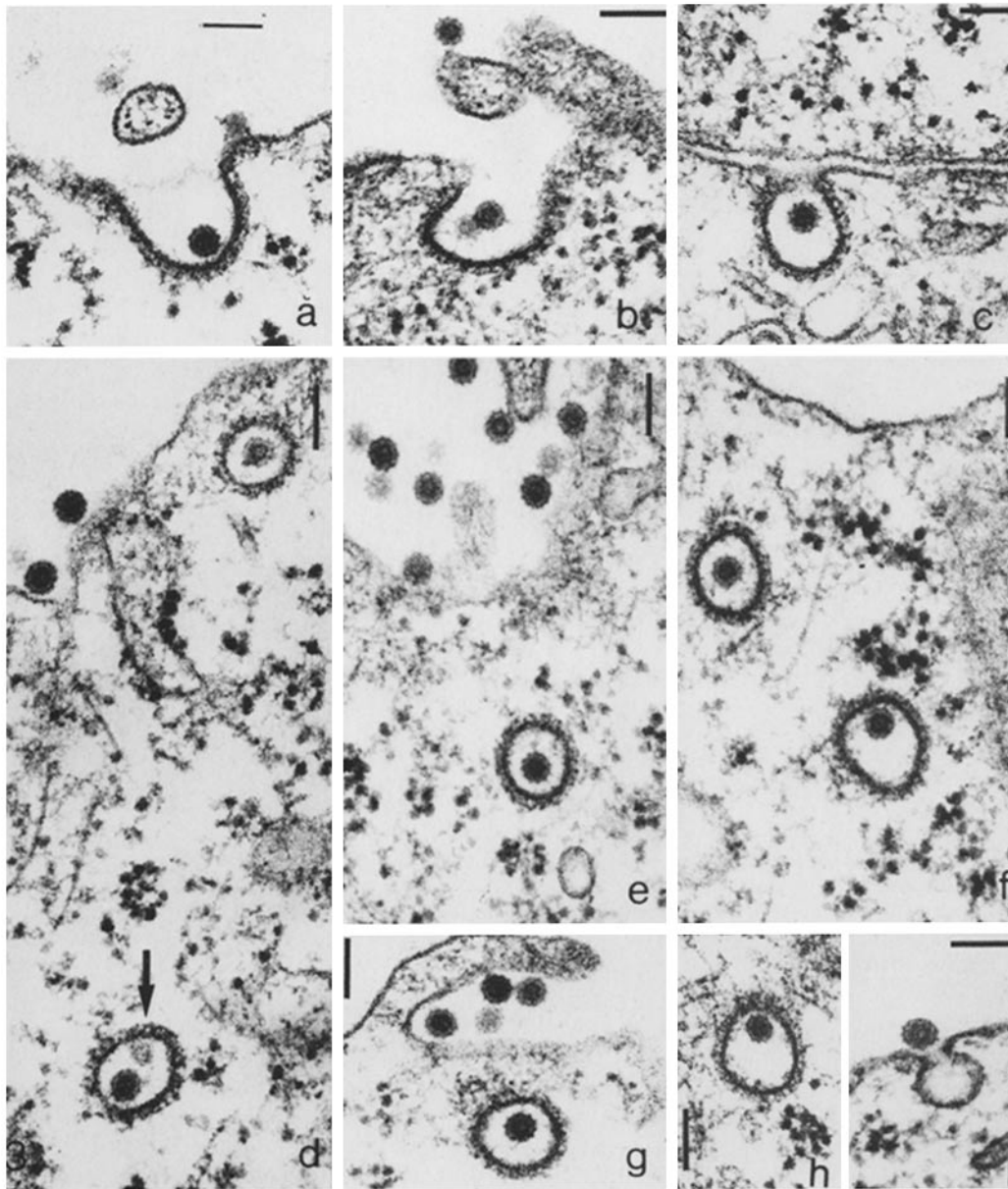
FIGURE 3 Virus associated with coated pits and coated vesicles. Virus was allowed to bind to cells in the cold. The cells were either directly fixed (a–c) or warmed to 37°C for 15 s (g and h), 1 min (d), or 5 min (e and f) before fixation. In i, a virus is seen associated with an uncoated membrane invagination. Although more than one virus could be seen per coated pit (b), only in one case (see arrow in d) have we observed more than one virus in a coated vesicle. Bars, 0.1 μm. × 80,000.

only a few external virus particles could be observed. When experiments were performed at 37°C without prior synchronization of uptake by a temperature shift, the same morphology of virus internalization could be observed but with more

overlap between the various stages.

For a full morphological description of the process of virus uptake, a quantitative analysis is needed. However, from our qualitative study at high multiplicity we can conclude that SFV is
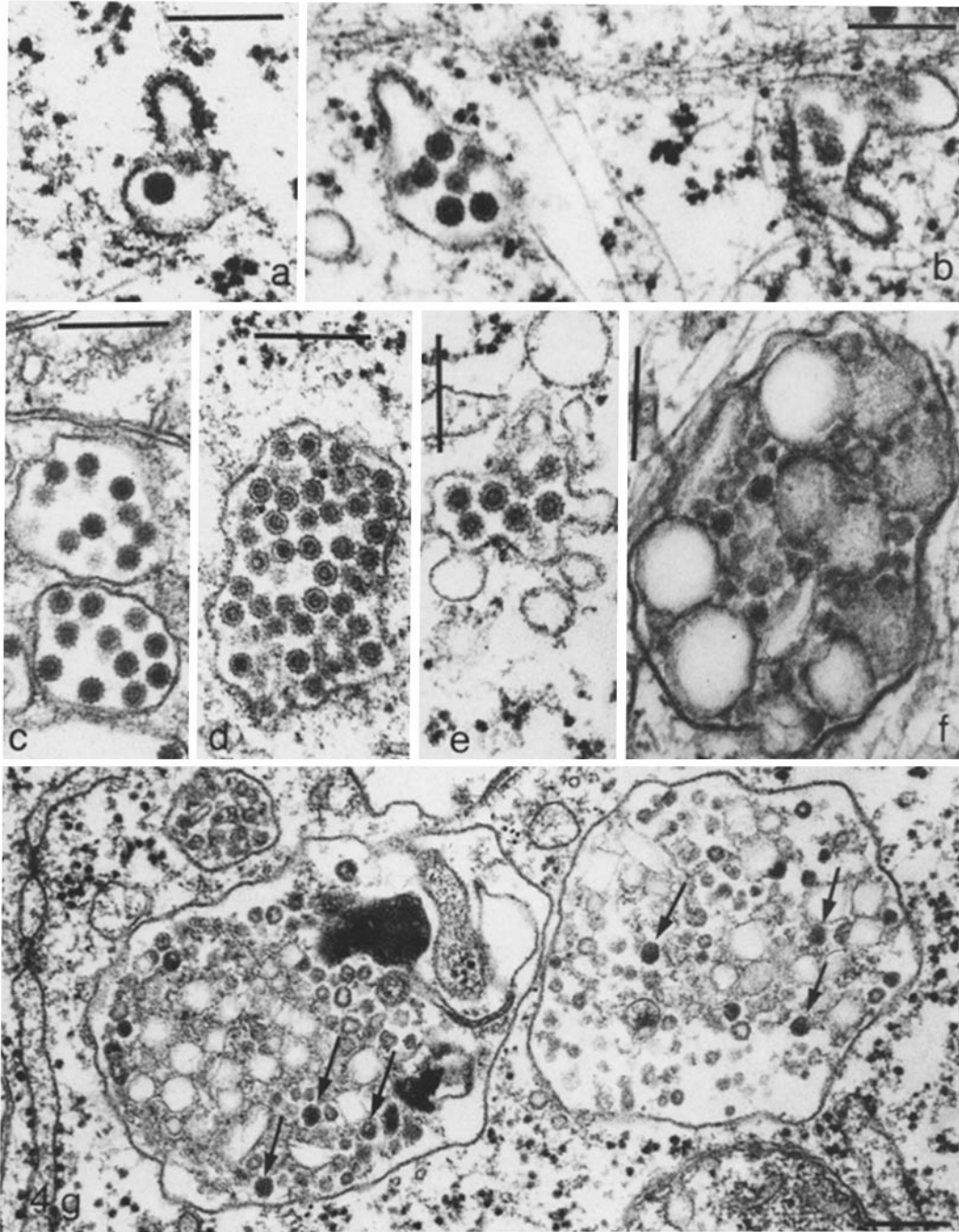
FIGURE 4   SFV in cytoplasmic vacuoles. Virus was allowed to bind to cells in the cold. The cells were washed and warmed at 37°C for 1 min (a and b), 5 min (c), or 30 min (d–f), respectively, before fixation. Virus-containing vacuoles with very different morphology could be seen: partially coated vacuoles with several viruses suggesting fusion of coated vesicles with uncoated vacuoles (a and b); uncoated vacuoles with viruses loosely distributed throughout the interior (c); vacuoles tightly packed with virus particles (d and e); vacuoles with the morphology of secondary lysosomes (f). Panel g shows secondary lysosomes in chloroquine-treated cells containing viruses (arrows). The cells in g were incubated for 30 min at 37°C with virus as above, except that 0.1 mM chloroquine was present. Bars, 0.2 μm. (a–f) × 80,000; (g) × 60,000.

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taken up by adsorptive endocytosis. The uptake occurs almost exclusively by coated vesicles which deliver the virus into intracellular vacuoles, many of which develop into secondary lysosomes.

Internalization of [^{35}S]Methionine-labeled Virus at Low Multiplicity

To follow the fate of the virus at low multiplicity of infection (0.5–1 plaque-forming unit (PFU/cell), we used carrier-free [^{35}S]methionine-labeled virus (1,200 PFU/cpm). Fig. 5 a and b show the kinetics of virus association with cells at 4° and 37°C. At 4°C, the amount of acid-precipitable radioactivity associated with cells reached a constant level of 2×10^3 cpm (1 PFU per four cells) after 1.5 h. Virtually no acid-soluble radioactivity was found associated with the cells or in the medium. At 37°C, the amount of virus associated with the cells reached its maximum (1.3×10^3 cpm) after 1 h and then decreased slowly. After ~30 min, degraded viral protein became detectable in the medium as acid-soluble radioactivity increasing with a rate of 350 cpm/h at least up to 3 h. Little acid-soluble radioactivity was found associated with the cells at any time point.
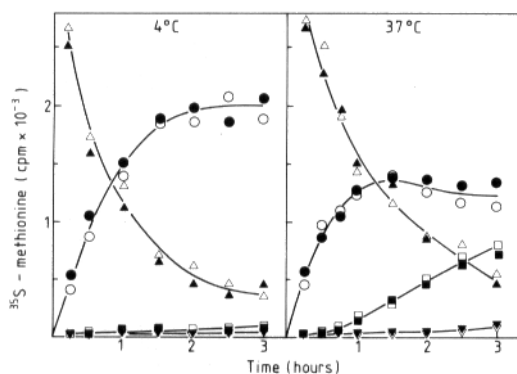

FIGURE 5 Association of carrier-free ^{35}S-SFV with BHK-21 cells at 4° and 37°C. Cells (8 × 10^6 on 6-cm dishes) were incubated with 4,100 cpm ^{35}S-SFV (5 × 10^6 PFU) in R-medium. The acid-soluble and acid-precipitable radioactivities in the medium and in the cells were determined after incubation at 4° or 37°C for different periods of time. Acid-precipitable radioactivity associated with cells in the absence (●) and in the presence (○) of 0.1 mM chloroquine; acid-precipitable radioactivity in the combined medium and cell washes in the absence (▲) and in the presence (△) of chloroquine; acid-soluble activity in the cells in the presence (▼) and in the absence (▽) of chloroquine; acid-soluble activity in the medium in the absence (■) and presence (□) of chloroquine.

To distinguish between the viruses on the cell surface and those inside the cell, we used proteinase K, a nonspecific protease, which has been previously applied to remove proteins external to membranes (55). Indirect immunofluorescence microscopy showed that virtually all virus particles present on the cell surface could be removed by this enzyme without cell lysis. To determine the extent to which SFV was internalized, a double-label experiment was carried out. [^{3}H]isoleucine-, leucine-, and valine-labeled SFV (1,400 cpm/plate and 20 PFU/cell) were allowed to associate with 8×10^6 cells in Petri dishes for 1 h at 37°C. The plates were cooled on ice and washed. [^{35}S]methionine-labeled virus (3,200 cpm/plate corresponding to ~1 PFU/cell) was then allowed to bind for another hour in the cold. The total amount of ^{3}H- and ^{35}S-labeled virus associated with the cells was determined from one dish. Another similarly treated dish was treated with proteinase K in the cold. The cells were washed and the remaining radioactivity was determined. Of the ^{35}S-labeled virus, 94–96% was removed by the protease K treatment; of the ^{3}H-labeled virus, only 20–25% was digested. If ^{35}S-SFV was present during the 37°C incubation and if ^{3}H-SFV was added in the cold, the reverse results were obtained. These data indicated that ~80% of the viruses present at 37°C were internalized during the 1 h at 37°C. As the nucleocapsids contain less than one-fifth of the total radioactivity of SFV, these values cannot be explained as being due to nucleocapsids alone entering the cell. The data also indicate that few, if any, viruses entered the cell at 4°C.

Using proteinase K treatment, we could get an estimate of the rate of virus internalization. In an unsynchronized low multiplicity infection with 0.6 PFU added per cell, the uptake was linear with time (Fig. 6). In a synchronized infection, where the virus was allowed to bind in the cold, the rate of uptake upon warming was initially more rapid (Fig. 7) and the amount of intracellular radioactivity leveled off after about 30 min. At this time, degradation of virus became detectable as acid-soluble radioactivity in the medium (Fig. 7). After 2 h, all the virus radioactivity originally bound to the cell was either in a proteinase-resistant form inside the cell or in acid-soluble form in the medium.

Effect of Lysosomotropic Agents

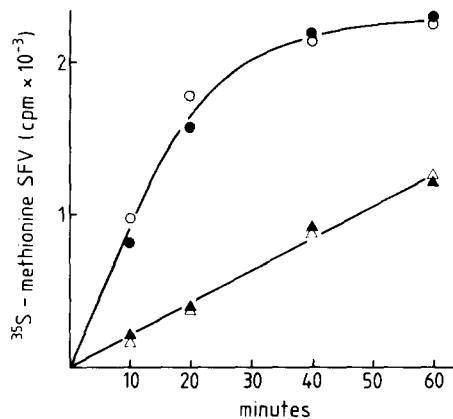To test whether lysosomes might be involved in the infective pathway, we studied the effect of

FIGURE 6 Internalization of carrier-free [35S]methionine SFV. The cells were treated with 35S-virus in the presence or absence of 0.1 mM chloroquine as in Fig. 5 (37°C). After each time point of incubation at 37°C, two dishes were cooled and one was treated with proteinase K (see Materials and Methods). Total cell-associated virus in absence (●) and presence (○) of 0.1 mM chloroquine. Proteinase K resistant virus in absence (△) and presence (▲) of chloroquine.
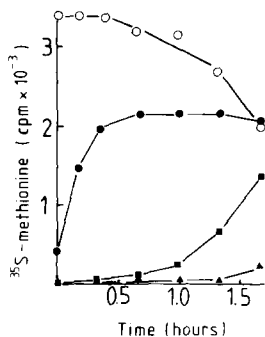

FIGURE 7 Internalization and degradation of [35S]methionine SFV in a synchronized infection. Cells (8 × 10⁶) on Petri dishes were incubated with 10⁴ cpm 35S-SFV corresponding to 1.2 × 10⁷ PFU for 1 h at 4°C. Unbound virus was washed away leaving 3.4 × 10³ cpm bound. The cells were warmed to 37°C and after varying time points the medium was removed and the cells were cooled to 0°C. The total cell-associated radioactivity (○), the proteinase K resistant cell-associated radioactivity (●), and the acid-soluble (■) and acid-precipitable (▲) activity in the medium were determined as described in Materials and Methods.

several lysosomotropic agents on SFV infectivity. The compounds used were weak bases of varied structure which accumulate in lysosomes owing to the low lysosomal pH (11). Their primary effect is to increase the lysosomal pH. Ohkuma and Poole

have recently shown (40) that the change in pH occurs very rapidly and can be as high as 1.7 U. When the agents are removed from the medium, the pH rapidly returns to values close to the original.

All five lysosomotropic compounds tested significantly reduced the titer of virus produced during a single cycle infection (Table I). Chloroquine and NH₄Cl were more effective than methylamine, tributylamine, and amantadine. Not only was the production of virus inhibited but there was also a reduction in the number of cells which expressed viral antigens on their surfaces as determined by indirect immunofluorescence (Table I).

For further studies, we used chloroquine as it was the most effective inhibitor. Although 0.05 mM chloroquine was sufficient to reduce the virus yields to 2% of control values, we used routinely a concentration of 0.1 mM. If chloroquine was present 30 min before virus addition but removed when the virus was added to the cells, no reduction in virus titers after a single cycle infection could be observed. Furthermore, only 30% inhibition was observed if chloroquine was added 1 h after the virus. These results indicate that the chloroquine-sensitive step occurred within the 1st h and that later processes such as viral RNA replication, protein synthesis, and morphogenesis proceeded normally in the presence of 0.1 mM chloroquine. When chloroquine was present during the 1-h

TABLE I

Effect of Lysosomotropic Agents on SFV Infection*

Inhibitor	Inhibitor concn.	PFU/ml × 10⁻⁶	Cells infected
	mM		%
None	—	140	64
Tributylamine	1	66	31
Amantadine	0.5	14	19
Methylamine	10	3.6	10
Chloroquine	0.1	1.2	<1
NH₄Cl	10	0.8	<1

* 1.8 × 10⁶ cells per Linbro well were pretreated for 0.5 h with 0.4 ml of complete medium containing the inhibitors. Virus was added (10 PFU/cell) and allowed to absorb for 1 h. Medium was replaced with 0.5 ml of fresh medium with 0.2% BSA but no serum. After an incubation period of 4.5 h, the cell medium was assayed for virus by plaque titration. In a separate experiment, the fraction of infected cells was determined by indirect immunofluorescence microscopy as described in Materials and Methods. All incubations were carried out at 37°C in 5% CO₂ atmosphere.

period of virus absorption but absent during the rest of the incubation, intermediate titers of virus were obtained. This result suggests that although the infection can be halted by chloroquine, it can proceed with lower efficiency when the inhibitor is removed.

A more exact time point for the chloroquine-sensitive step was obtained by synchronizing the infection by a temperature shift. Viruses were bound to the cell in the cold and allowed to enter by raising the temperature to 37°C. Chloroquine was then added at various times and left in the medium for the rest of the infection. The results in Fig. 8 show that when the inhibitor was added 2.5 min (or earlier) after the temperature shift, the virus yield remained at a low level. Addition at 5 min or later failed to give efficient inhibition. This indicates that in 5 min the first viruses have already proceeded beyond the chloroquine-sensitive phase of the entry pathway.

Chloroquine had no effect on the rate of virus binding to cells or on the rate of endocytosis (Figs. 5 and 6). Furthermore, fluorescence microscopy with rhodamine-labeled virus and thin-section electron microscopy showed that virus was taken up normally into perinuclear vacuoles and lysosomes (Fig. 4g). This led us to conclude that chloroquine most probably inhibited the release of the viral nucleocapsid into the cytoplasm or the uncoating of the RNA from the nucleocapsid.

A direct effect of chloroquine on the virus is unlikely. Treatment of SFV with 10 mM chloroquine for 15 min at pH 6.7 (37°C) before infecting cells had no effect on the infectivity. Two possible indirect mechanisms were considered. Chloroquine may inhibit a lysosomal hydrolase required for penetration, or inhibition could be caused by the elevation in lysosomal pH. Perhaps, acid pH was required to induce the final penetration of SFV through the membrane of some intracellular vacuoles.

Membrane Fusion

To test the role of pH in virus penetration, we used a liposome system where we tried to stimulate the conditions in lysosomes. Our assay relied on the fact that the viral RNA can be digested by pancreatic RNase A only if the enzyme and the RNA are present within the same membrane compartment. In intact SFV, the viral RNA is protected by the viral membrane against RNase, but in the naked nucleocapsid the RNA is sensitive (29). Liposomes were prepared from phosphati-

dylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol in the presence of 10 mg RNase/ml. The external RNase was removed by gel filtration. Carrier-free ^{32}P-RNA-labeled SFV was added to the liposomes, and the pH was adjusted by dialysis to values ranging from 4.5 to 7.3. The amount of ^{32}P-RNA hydrolysed by the intraliposomal RNase in each sample was then determined. Fig. 9 shows that when the pH of the
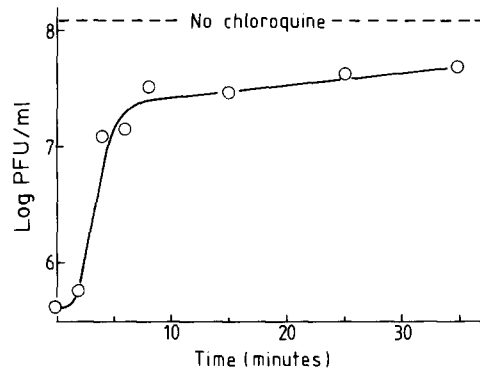

FIGURE 8 Time dependence of chloroquine inhibition. Cells in Linbro plates (1.8 × 10^6/well) were allowed to bind SFV (10 PFU/cell) on ice. After washing in the cold, the cells were warmed to 37°C. Chloroquine (final concentration 0.1 mM) was added at different time points after warming, and after a 4.5-h infection the virus titer in the medium was determined by plaque assay.
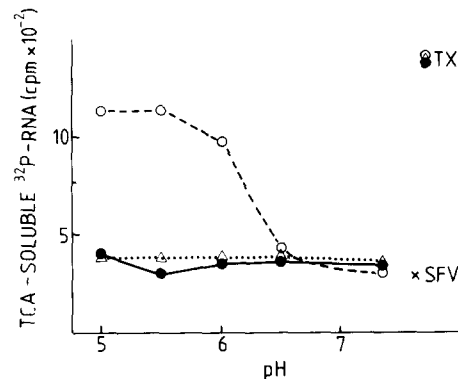

FIGURE 9 RNase action on viral ^{32}P-RNA in the presence of liposomes. The amount of TCA-soluble RNA when the RNase was inside the liposomes (○), when it was outside the liposomes (●), and when RNase was mixed with SFV in absence of liposomes (△). × is the SFV control, and TX stands for the three starting mixtures treated with Triton X-100 and incubated for 30 min at room temperature. For detailed procedure, see Materials and Methods.

dialysis buffer reached values of 6 or lower, the amount of RNA hydrolysed increased from background level (15%) to 75% of total RNA. If RNase was added to the virus in the absence of liposomes or if it was present in the external solution but lacking inside the liposomes, no increase in digestion occurred. To confirm that the digestion of RNA occurred inside the liposomes incubated at pH 5.5, a reaction mixture was centrifuged in a Beckman airfuge. 90% of the acid-soluble nucleotides were in the pellet.

Negative-staining electron microscopy of dialysates containing virus and liposomes devoid of RNase showed that the passage of viral RNA into the liposomes occurred through a process of membrane fusion. After dialysis against pH 5.5 buffer, large liposomal vesicles covered with spikes were seen (Fig. 10a–c). In cases where the stain had penetrated the vesicles, nucleocapsids could be seen. Several could be present in a single vesicle (Fig. 10a). If dialysis was performed at neutral pH, fusion between the viral membrane and the liposomes was not observed (Fig. 10d).

These experiments show that at low pH the viral membrane fuses with liposomes, resulting in the transfer of the viral nucleocapsid into the liposome. The process occurs with high efficiency and without addition of proteins or enzymes.

Infection of BHK-21 Cells in the Presence of Chloroquine

A test was performed to see whether low pH fusion could be induced between the virus and the plasma membrane of BHK cells, and to find out whether such a reaction would lead to infection. BHK cells in a Linbro plate were incubated with SFV (14 PFU/cell) in the cold. Chloroquine (0.1 mM) was present in all solutions to block the normal infection route. The cells were rapidly warmed to 37°C by addition of 2 ml of warm medium of different pH (7.3, 6.5, or 5.5) containing chloroquine into each well. After 90 s, the medium was removed and replaced with normal medium (pH 7.3) containing chloroquine. After 4.5 h at 37°C, the yield of virus and the number of infected cells were determined by plaque titration and indirect immunofluorescence microscopy. The results in Table II show that only at the lowest pH was the chloroquine block on virus infection bypassed. This result suggests that when the pH is low enough, the virus can infect the cell through the plasma membrane.

DISCUSSION

Binding

We have previously shown that BHK-21 cells at 4°C bind about 5×10^4 SFV particles with half-saturation at 10^{-10}–10^{-11} M virus concentrations (17). Binding of the lower affinity was also detected, but it is probably of little consequence during infection at low or moderate multiplicities. We do not yet know the identity of the receptors on BHK cells. However, using mouse and human cells we have shown that the viral spikes have affinity for the major histocompatibility antigens (the H2K and H2D, and the HLA-A and HLA-B antigens) (25). It is likely that homologous proteins occur on BHK-21 cells.

Although SFV enters the cell by endocytosis through the coated regions of the plasma membrane, the microvilli appear to be the preferential binding sites on BHK cells. Only ~5% of the virus particles bind directly to coated pits. This contrasts with the low density lipoprotein of serum whose receptors have been shown to be concentrated in the coated pits (3). Epidermal growth factor, which is also taken up in coated vesicles, has been reported by one group to bind diffusely over the cell surface (37), and by another group to preferentially bind to coated pits (22). As virtually all the viruses, including those bound to the microvilli, became internalized in coated vesicles, the viruses probably move laterally from the site of binding to sites where coated vesicles are formed. Our previous data on the binding of SFV and its isolated glycoprotein (17) suggest that the viruses may bind to multiple receptors (see also Fig. 2, inset). It is conceivable that clustering of surface receptors is sufficient to cause entrapment in coated pits. Alternatively, the association of virus with its receptor molecules might induce specific binding to components of the coated regions.

Internalization

Our morphological and biochemical data at high and low multiplicity indicate that, under normal conditions of infection, SFV enters into the cell by adsorptive endocytosis and not by fusion with the plasma membrane. This conclusion is supported by recent studies of Fan and Sefton (14). They showed that penetration of Sindbis virus (which is closely related to SFV) does not
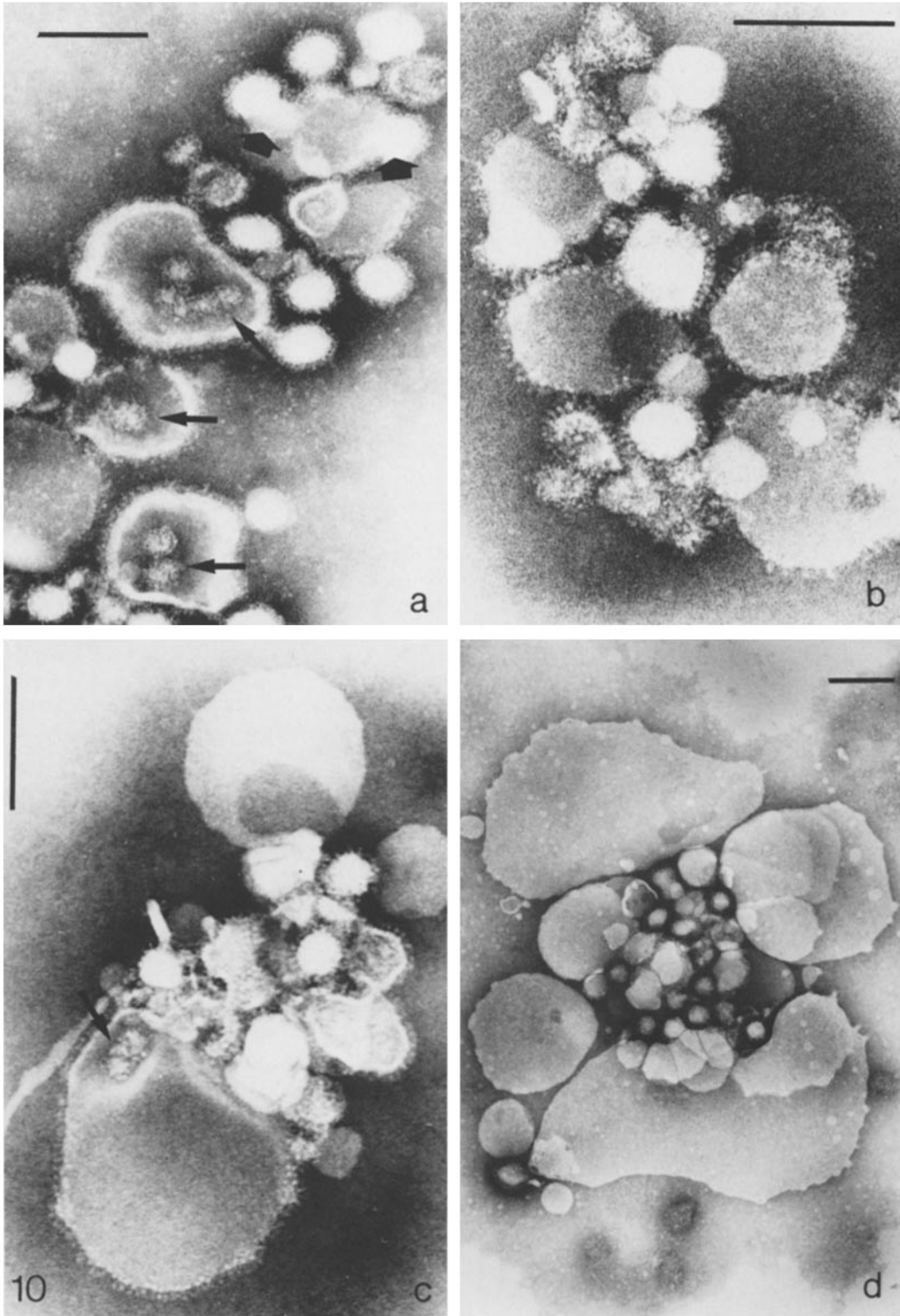
TABLE II

*pH Dependence of SFV Infection in the Presence of Chloroquine**

	pH	Virus titer	Infected cells
		PFU/ml	%
1. No chloroquine	7.3	10×10^5	53
2. Chloroquine, 10^{-4} M	7.3	0.4×10^5	<3
3. Chloroquine, 10^{-4} M	6.5	0.6×10^5	<3
4. Chloroquine, 10^{-4} M	5.5	8.1×10^5	63

* Virus (14 PFU/cell) was allowed to bind in the cold to cells grown in Linbro plates. The cells were then warmed up by addition of 2 ml (37°C) of serum-free medium containing 0.2% BSA and 10 mM MES adjusted to the indicated pH. After 90 s, the media were removed and replaced with 1 ml of neutral serum-free medium containing 0.2% BSA. In samples 2, 3, and 4, all solutions contained 0.1 mM chloroquine throughout the experiments. After incubation at 37°C for 4.5 h the titer of virus was assayed by plaque titration and the percentage of infected cells by indirect immunofluorescence.

introduce viral antigens into the host cell plasma membrane.[1]

The endocytosis of SFV is rapid and efficient. 70–80% of the virus added to the medium can be taken up in 1 h. This is approximately the rate of uptake reported for asialofetuin in isolated hepatocytes (49) and reovirus in L cells (46). It is much faster than the uptake of serum low density lipoprotein into fibroblasts (19). Uptake seems to occur mainly in coated vesicles, but some virus particles may be taken up by other means. Adsorptive endocytosis in coated vesicles has been described as a common mechanism for the internalization of hormones, lipoproteins, and other transport proteins with specific receptors on the cell surface (for review, see references 20 and 21). The virus, there-

[1] We have repeated the experiments of Fan and Sefton (14) using P815 mastocytoma cells and SFV. In agreement with their results with Sindbis virus, we could not detect fusion at the plasma membrane using complement dependent cytotoxicity.

fore, utilizes a complex physiological mechanism which the cell operates for the uptake of macromolecules. The further fate of the endocytosed virus seems very similar to that of low density lipoprotein and most other proteins taken up by this process. After a very short time in the coated vesicles (probably <1 min), the virus is delivered into intracellular vacuoles, part of which then develop into lysosomes. Part of the viral proteins are eventually degraded and released into the medium.

Electron microscope studies with many other viruses have previously shown that endocytosis, "viropexis," is a common phenomenon (8). In some cases, coated vesicles and coated pits containing virus particles have been observed (6, 9, 43, 48). It has been generally accepted that part of the endocytosed virus particles can find their way into lysosomes, but this is usually considered an abberant pathway leading to the destruction of the virus particles (8, 35). An exception is reovirus, a large naked RNA virus. It is taken up into lysosomes with a rate similar to that of SFV (46). In the lysosomes, part of the reovirus protein coat is digested by the lysosomal hydrolases. Being double-stranded, the RNA remains intact. The time-course of endocytosis, proteolytic digestion of coat proteins, and the release of digestion products in the medium is very similar to what we observe for SFV. How the transfer of the reovirus RNA from the lysosome to the cytoplasm occurs is not known.

Penetration

To determine whether lysosomes might be involved in the SFV infection, we tested five lysosomotropic weak bases; they all inhibited SFV infectivity. The inhibition by chloroquine, the most efficient lysosomotropic agent tested, was shown to occur already within the first 5 min after the virus had entered the cell by endocytosis. Chloroquine had no direct effect on the virus particles nor did it inhibit binding, endocytosis, or uptake of SFV into intracellular vacuoles and lysosomes.

FIGURE 10   Fusion of SFV with liposomes. (a–c) SFV was mixed with liposomes and the pH was changed to 5.5 by dialysis as in Fig. 9. Extensive fusion between viruses and liposomes had occurred as judged by the spike protein visible on the liposomal membranes. The nucleocapsids (see arrows in a and c) with a diameter of 30–35 nm were visible in some liposomes. Virus particles in the process of fusing with liposomes are indicated by arrowheads in a. In the control (d) which was dialyzed against pH 7.3 buffer, no fusion was observed. Bars, 0.1 μm. (a) × 150,000; (b) × 240,000; (c) × 180,000; (d) × 88,000.

The lysosomotropic agents we tested, and others similar to them, have been previously reported to inhibit infections by several enveloped viruses including myxo, oncorna, rubella, and paramyxo viruses (10, 13, 15, 26, 27, 32, 36, 39, 50, 54). Where studied, the mechanism of action of these agents appears strikingly similar to that of chloroquine on SFV. They cause no direct damage on the virus particles, little or no effect on binding and, in most cases, small if any effects on internalization (12, 15, 32, 33, 39, 50). In the case of amantadine, most investigators seem to agree that the inhibitory effect is on the final release of the nucleocapsids into cytoplasm or on uncoating of the genome.

Although very different in molecular structure, all these agents are lysosomotropic, i.e., they accumulate in the lysosomes. Moreover, they all tend to increase the intralysosomal pH (and presumably in any organelle with low pH within the cell). The concentrations of chloroquine and NH_4Cl we used in these studies are known to increase the pH of lysosomes of mouse peritoneal macrophages from 4.5 to 6.2–6.4 (40). Amantadine, tributylamine, and methylamine raise the pH somewhat less (to about pH 5.5–6.0). Chloroquine and NH_4Cl which exert the greatest effect on intralysosomal pH were more efficient inhibitors of virus infection than amantadine, tributylamine, and methylamine.

To pursue the possibility of a low pH requirement for SFV infectivity, we developed an in vitro system to study virus penetration through membranes. Using liposomes and isolated viruses, we demonstrated that fusion can occur between the viral membrane and a liposomal membrane, resulting in efficient transfer of the viral nucleocapsid into the liposome. The fusion reaction was strictly pH-dependent; fusion occurred only when the pH was 6 or lower.

The fusogenic activity of SFV (and other Toga viruses) is not limited to our artificial membrane system. Recent data on the interaction of SFV, Sindbis virus, and Rubella virus have shown that they can fuse with erythrocytes from different species. The fusion manifests itself as hemolysis (31, 52, 53) and formation of heterokaryon cells (53). The pH dependence of both hemolysis and cell-cell fusion is identical with that seen in our liposome system. Moreover, Mooney et al. (38) have shown that Sindbis virus binds to liposomes with a pH dependence very similar to that which we observe for SFV fusion with liposomes. Taken together, these data show that, provided the pH is low enough, SFV and related viruses can attach

and fuse with bilayer membranes of widely different origin. An indication that such fusion can cause infection of cells was obtained in an experiment in which BHK cells with SFV bound to them were briefly treated with low pH medium. These cells were thereby efficiently infected in spite of the presence of chloroquine at all stages to block the normal pathway of infection. At low pH, viruses localized on the cell surface probably fused with the plasma membrane and thereby infected the cells directly. Further studies are, however, needed to confirm this interpretation.

Judging by the morphological and immunological data described above, and the pH dependence of fusion, it seems unlikely that fusion of SFV with the plasma membrane can occur under normal conditions. In this respect, SFV differs from Sendai virus (and other paramyxo viruses) which can fuse with the plasma membrane of cells and with artificial lipid bilayers at neutral pH (23). If not the plasma membrane, then what is the site where the SFV genome enters the cytoplasm? Several observations point in the direction of lysosomes (or some other intracellular vacuoles with low pH). These are: (a) the low pH requirement for fusion, (b) the inhibition of uncoating by agents known to accumulate in lysosomes and to increase their pH, (c) the morphological evidence that the endocytosed SFV particles are sequestered into lysosomes and other intracellular vacuoles, and (d) the fact demonstrated by Friedman and Sreevalsan (16) that very rapidly (within 20 min) after SFV has entered chick cells, part of the parental RNA is found in a replicative intermediate form in the mitochondrial-lysosomal organelle fraction. If the lysosomes were the site of entry, why would the virus not be destroyed by the lysosomal hydrolases? Judging by the difference in the speed of infection (Fig. 8) and the rate of appearance of acid-soluble degradation products (Figs. 5 and 7), it seems quite possible that the nucleocapsids may escape into the cytoplasm before the lysosomal enzymes have time to degrade the virus particle. The final penetration of the viral genome into the cytoplasm may, in fact, occur immediately when an endocytotic vacuole containing the virus fuses with a primary lysosome and the pH drops to 6 or below.

The pathway of SFV entry into BHK-21 cells, as suggested by our data, is schematically shown in Fig. 11. More information is needed especially to define the mode of attachment of the virus to the cell membrane, the mechanism of entrapment

PRIMARY LYSOSOME

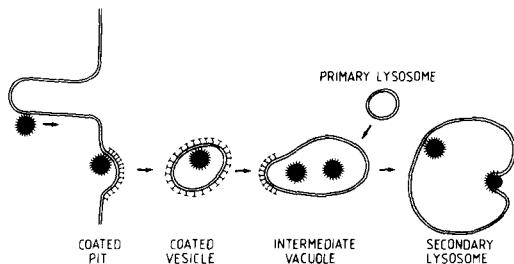COATED PIT   COATED VESICLE   INTERMEDIATE VACUOLE   SECONDARY LYSOSOME

FIGURE 11 Suggested pathway for SFV entry into BHK-21 cells.

in coated pits, the role of intermediate vacuoles and lysosomes, and the mechanism of viral membrane fusion. It will also be of interest to find out to what extent other viruses follow the same pathway. That similarities exist between SFV and other membrane viruses is clear from the available morphological information as well as from the sensitivity to lysosomotropic agents.

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