

# Rapid Inhibition of Pinocytosis in Baby Hamster Kidney (BHK-21) Cells following Infection with Vesicular Stomatitis Virus

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**ABSTRACT** Infection of baby hamster kidney cells with vesicular stomatitis virus (VSV) caused a reduced rate of pinocytosis (as judged by the uptake of horseradish peroxidase) after 1 h, and maximum inhibition (60–80%) was observed at 4–6 h. This inhibition occurred 2–3 h before release of virus or changes in cell morphology. Analytical cell fractionation of homogenates of VSV-infected cells indicated that the horseradish peroxidase taken up by pinocytosis was transferred to lysosomes. The inhibition of pinocytosis required viral gene expression: little or no inhibition was detected in cells infected with UV-irradiated virus, wild-type virus in the presence of cycloheximide, or a temperature-sensitive mutant which failed to synthesize viral proteins. When cells were infected with temperature-sensitive viruses with mutations in the five VSV genes, an inhibition of pinocytosis was observed only when the viral transmembrane glycoprotein was present on the surface of the cells.

Fluid phase endocytosis can result in the internalization of a major fraction of the cell surface (37, 38). Experiments that followed binding, internalization, and return to the surface of anti-plasma membrane antibodies (33, 42) and the exchange of labeled proteins between phagolysosomal and cell surface compartments (27) provided evidence for a reutilization or recycling of membrane components. However, the intracellular pathway followed by internalized membrane in its return to the surface is poorly understood; one approach we are developing is the use of inhibitors of fluid phase endocytosis as an aid to understanding membrane recycling (43).

Cells infected with an enveloped virus release large quantities of the phospholipid bilayer from the host plasma membrane during the budding of viral particles. It seemed possible that this release of plasma membrane could perturb the normal membrane flow within the cell. Therefore, we studied the effect of vesicular stomatitis virus (VSV)<sup>1</sup> infection on membrane flow.

VSV is the prototype of the rhabdovirus group and contains a single-stranded RNA genome that encodes five viral proteins

(7), all of which are components of the mature virus particle. Three proteins, N, NS, and L, are components of the nucleocapsid and are required for viral RNA synthesis; M, the matrix protein, plays a role in the association of the nucleocapsid with the host cell plasma membrane, at sites which contain the transmembrane glycoprotein, G.

We show here that VSV infection of baby hamster kidney (BHK) cells causes a rapid inhibition of fluid phase pinocytosis. Analysis of cells infected with temperature-sensitive (ts) mutants of VSV indicates that the inhibition is detected only when the viral membrane protein, G, is expressed on the cell surface.

## MATERIALS AND METHODS

**Materials:** Horseradish peroxidase (Type VI [HRP]) and fluorescein isothiocyanate-dextran (average molecular weight, 73,100) were obtained from Sigma Chemical Co (St. Louis, MO); Affigel 102 and 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride were obtained from Bio-Rad Laboratories (Richmond, CA); [<sup>35</sup>S]methionine (sp act 450 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and all other reagents were purchased from commercial sources and were of analytical grade or higher purity.

**Cells:** BHK-21 cells were cultured at 37°C in 5% CO<sub>2</sub> in Eagle's minimum essential medium, supplemented with 7% calf serum, 10% tryptose phosphate, and 100 U/ml penicillin and 100 µg/ml streptomycin (44).

<sup>1</sup> *Abbreviations used in this paper:* BHK, baby hamster kidney; HRP, horseradish peroxidase; ts, temperature-sensitive; VSV, vesicular stomatitis virus.

**Viruses:** Wild-type VSV (Indiana strain) was propagated in BHK cells (11). Temperature-sensitive mutants ts G 11, ts G 22, and ts G 41 (31) and mutants ts O 23 and ts O 45 (9) were obtained from Dr. R. R. Wagner (University of Virginia School of Medicine); mutant T-1026 R1 (8, 36) was obtained from Dr. C. P. Stanners (University of Toronto, Ontario Cancer Institute). The ts mutant viruses were propagated in BHK cells at 34°C and monitored routinely to ensure that they had not reverted (9, 31). The efficiency of plating of the ts mutants at the nonpermissive temperature (39.5°C) was  $<10^{-5}$  of that at the permissive temperature (34°C). The virus particle concentration was determined by negative staining (26), and the plaque-forming unit to particle ratio for wild-type VSV was 0.06. When required, viruses were irradiated with UV light as described elsewhere (17). Cells were infected with virus by two procedures. In the first, which was always used for ts mutants, cells were incubated with viruses in complete medium for 1 h at 2–4°C, at a multiplicity of infection indicated in the text (usually 10); inoculum was then removed, fresh medium without virus was added, and the cells were returned to culture. In the second procedure, viruses were added in complete medium at 37°C; after 1 h, the inoculum was removed and the culture continued in fresh medium. Zero time was defined in the first procedure as the time at which cells were returned to incubation at 37°C, and in the second as the time at which viruses were added to the culture. The time course of the effects on pinocytosis and protein synthesis and also virus production was similar when cells were infected with wild-type VSV by the two procedures, i.e., inhibition of HRP uptake was detected 1 h after the cells were warmed at 37°C.

**Pinocytosis:** The uptake of HRP was analysed by the technique of Steinman and Cohn (37), with minor modifications as described elsewhere (43). In most experiments, cells were incubated in duplicate or triplicate with 1 mg/ml HRP for 20 and 40 min. To measure the uptake of fluorescein dextran, we incubated cells with the marker and washed in the same manner as for the uptake of HRP. The cells were then solubilized in 1% SDS, 20 mM Tris-Cl, pH 8.5, and samples were assayed for protein (22) and for fluorescence, using a Perkin-Elmer Corp. (Norwalk, CT) 650-10S fluorescence spectrophotometer (28).

**Protein Synthesis:** The rate of protein synthesis during viral infection was determined by adding methionine-free medium containing 20  $\mu$ Ci/ml [ $^{35}$ S]methionine to infected cell monolayers, grown in 16-mm multiwell plates, and incubating for 30 min at 37°C. The medium was removed and the cells were solubilized in 1% SDS. The cell lysates were precipitated with trichloroacetic acid, and the acid insoluble radioactivity was measured by liquid scintillation counting using ACS scintillant (Amersham Corp., Arlington Heights, IL) and a Beckman LS-7500 (Beckman Instruments, Inc., Palo Alto, CA). To characterize the proteins synthesized by cells infected with different VSV mutants, we labeled cells for 1 h, beginning 3 h postinfection. The cells were solubilized in Laemmli sample buffer (18), and samples containing the same amount of protein, as opposed to radioactivity, were analysed by SDS gel electrophoresis and fluorography (18, 21).

**Na<sup>+</sup> and K<sup>+</sup> Content of Cells:** Total cell Na<sup>+</sup> and K<sup>+</sup> were determined as described by Smith and Rozengurt (35).

**Preparation of Antiviral Antibodies:** Antiserum to VSV was prepared in rabbits (6). As judged by immunoprecipitation analysis, 90% of the antiviral antibodies were directed against the viral membrane protein G. Two procedures were employed to obtain antibodies for binding assays. In the first, the antibody was adsorbed to intact VSV (5 mg protein), and iodinated with 1 mCi  $^{125}$ I using chloramine-T (24). The VSV-anti-VSV complex was sedimented (160,000 g for 15 min), and washed five times with PBS by resuspension and resedimentation; it was then dissociated by suspension in 2 ml of 0.25 M acetic acid, 0.5 M sodium chloride, and the virus was removed by sedimentation. The antibody solution was neutralized by the addition of solid NaHCO<sub>3</sub> and dialysed against 0.15 M sodium chloride 10 mM HEPES, pH 7.5; the yield was ~1 mg and the sp act  $1.1 \times 10^4$  cpm/ $\mu$ g.

In the second procedure, the viral G protein was isolated (25), solubilized in 0.1% Triton X-100, and conjugated to Affigel 102 by the carbodiimide procedure described in the Bio-Rad catalog. Anti-G antibodies were adsorbed to the matrix from an anti-VSV antiserum, eluted with 0.25 M acetic acid, 0.5 M sodium chloride, and neutralized as described above.

**Binding of Antiviral Antibodies to Intact Cells:** Cells were grown and infected in 16-mm multiwell plates. At appropriate time intervals after infection they were washed twice with HEPES-saline containing 10% newborn calf serum (42) and cooled to 4°C in the same medium. In experiments analysing the binding of  $^{125}$ I anti-VSV, the medium was removed and replaced with 0.4 ml of a medium containing (per ml) 100  $\mu$ l of newborn calf serum, 75  $\mu$ l of anti-VSV antiserum and 20  $\mu$ g of  $^{125}$ I anti-VSV antibody in HEPES-saline. As determined by binding competition studies, the anti-VSV antiserum contained 3.3 mg/ml anti-G-antibody. This antibody concentration, as analysed in preliminary experiments, resulted in 80% of the saturation binding to the cells, with minimal nonspecific binding (to uninfected cells). After 90 min, at

which time binding was maximum, the cells were washed twice at 2°C with 1 ml of HEPES-saline containing 10% newborn calf serum, and then washed at room temperature, twice with 1 ml of the same medium and three times with 1 ml of HEPES-saline. The cells were solubilized in 1% SDS, boiled for 2 min, and appropriate samples were taken for determination of radioactivity and protein (22).

In experiments that analysed the binding of affinity-purified anti-G IgG, the antibody was added to HEPES-saline containing 10% newborn calf serum so that the concentration was 200  $\mu$ g/ml; the cells were incubated with antibody, and washed as described above. Anti-G IgG bound to the cells was then analysed by binding  $^3$ H-F(ab')<sub>2</sub> fragments of sheep anti-rabbit F(ab')<sub>2</sub> (40  $\mu$ g/ml in HEPES-saline containing 10% newborn calf serum) as described elsewhere (42).

**Light Microscopic Techniques:** Cells were cultured on 22-mm glass coverslips in 35-mm culture dishes. For studies using phase-contrast microscopy, the cells were washed three times with PBS, fixed in 2.5% glutaraldehyde in PBS, washed five times with PBS, and mounted in 50% glycerol in PBS. They were photographed with a Leitz microscope.

To study the uptake of fluorescein dextran, we cultured cells for 4 h in medium supplemented with 2.0 mg/ml fluorescein dextran. These conditions permitted the maximum uptake with minimum toxicity to the cells. They were then fixed for 10 min in 10% formalin in PBS, washed five times with PBS, and mounted in a medium (13) designed to minimize fading of fluorescein. Cells were photographed with an AO-Reichert microscope provided with epifluorescence and interference-contrast optics.

To analyse the fraction of cells infected by VSV, we infected cells for 4 h; the coverslips were washed six times by immersion in a series of beakers of PBS warmed to 37°C. The cells were fixed for 10 min in 10% formalin in PBS, washed five times with PBS, immersed in acetone at -20°C for 2 min, and air dried. The cells were then incubated successively with anti-VSV antibodies (6) and fluorescein-conjugated goat anti-rabbit IgG (14).

## RESULTS

### Inhibition of Pinocytosis in BHK Cells Infected with VSV

An inhibition of pinocytosis, estimated by the uptake of HRP, was detected 1 h after VSV was added to confluent monolayers of BHK-21 cells maintained at 37°C (Fig. 1); the inhibition was ~60% at 2 h and increased only slightly (to 75%) during the next 6 h. This effect on pinocytosis was

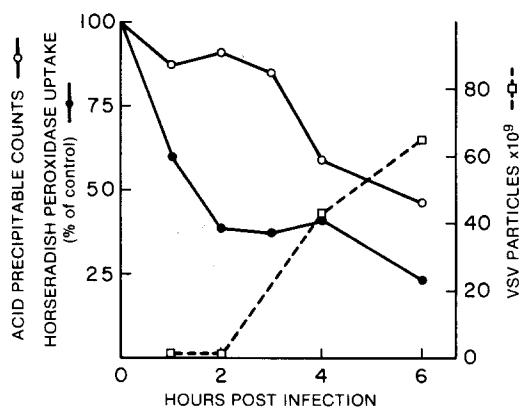


FIGURE 1 Inhibition of pinocytosis following VSV infection. BHK cells were infected with the Indiana strain of VSV at a multiplicity of 20. The virus was adsorbed to the cells in medium for 1 h at 37°C; the virus inoculum was removed and the cells were then returned to culture at 37°C in fresh medium. At the times indicated after the initial addition of virus, cells were analysed for HRP uptake and [ $^{35}$ S]methionine incorporation and the medium was analysed for viral particles as described in Materials and Methods. The uptake of HRP by control cells ranged from 1.9 to 2.1 ng/min per mg cell protein; ● denotes HRP uptake (values from five experiments), ○ denotes acid precipitable counts incorporated after labeling with [ $^{35}$ S]methionine for 30 min (two experiments) and □ denotes VSV particles released into the medium (two experiments).

almost at its maximum before release of virus into the medium was detected and preceded the inhibition of the incorporation of [<sup>35</sup>S]methionine into protein by the cells by ~3 h (Fig. 1).

In uninfected cells the uptake of both HRP and fluorescein dextran was linear with respect to time (Fig. 2) and the concentration of the marker in the medium (not shown). The uptake of HRP (1.4 ng/min/mg protein with 1 mg/ml in the medium) was greater than that of fluorescein dextran (0.75 ng/min/mg protein with 1 mg/ml in the medium), a finding which is consistent with reports (32) that different fluid phase markers are taken up at different rates. At 4 h after infection with VSV, there was a marked inhibition of the uptake of both markers, although the kinetics of uptake remained linear (Fig. 2). The magnitude of the inhibition (69% for HRP and 78% for fluorescein dextran) was similar.

### Effects of VSV Infection on Cell Morphology and Plasma Membrane Integrity

To discover whether there was a correlation between the inhibition of pinocytosis and gross morphological changes in the cells. We studied BHK cells infected with VSV by phase-contrast microscopy (Fig. 3). Control cells (Fig. 3A) and cells 2 h after infection (Fig. 3B) could not be distinguished. By 4 h (Fig. 3C), some rounding of the cells was noticeable, and this was more pronounced at 6 h (Fig. 3D).

Since the inhibition of pinocytosis could have been caused by changes in the permeability of the plasma membrane, the permeability of the plasma membrane to trypan blue, and the sodium and potassium ion concentrations in the cells were studied. In agreement with the results of Gray et al. (10), no change was detected during the first 6 h after infection. At 8 h, a few cells took up trypan blue, and the Na<sup>+</sup>/K<sup>+</sup> ratio increased slightly (not shown).

### Effects of the Inhibition of Host-Cell RNA and Protein Synthesis on Pinocytosis

Host-cell RNA and protein syntheses are inhibited rapidly following infection with VSV (5, 40). To examine the possibility that the inhibition of pinocytosis might be a consequence of the inhibition of host gene expression, we studied the effects of actinomycin D and cycloheximide on pinocytosis. Actinomycin D, at a concentration of 5 μg/ml, inhibited host RNA synthesis by 95% and caused a minimal effect on pinocytosis (Fig. 4), which varied from 5 to 20% in different

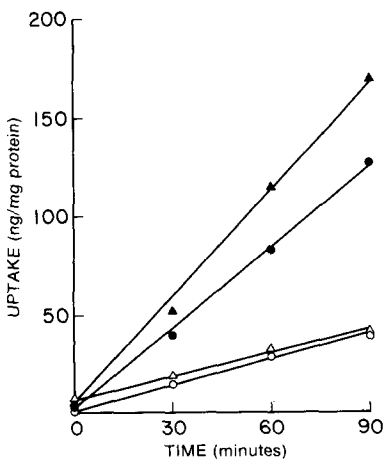


FIGURE 2 Uptake of HRP and fluorescein dextran by VSV-infected cells. BHK cells were incubated for the times indicated with either 1 mg/ml HRP (circles) or 2.5 mg/ml fluorescein dextran (triangles) as described in Materials and Methods. Closed symbols denote uninfected (control) cells, and open symbols, cells 4 h after infection with VSV.

experiments. When cells were incubated with 200 μg/ml cycloheximide, incorporation of amino acids into protein was inhibited by 98%. In some experiments this treatment caused no change in the rate of uptake of HRP; in others (Fig. 4) there was a slight inhibition. Inhibition of host-cell RNA and protein syntheses thus had little or no effect on pinocytosis during the 6 h time-period analysed in these experiments.

Additional evidence was obtained that the inhibition of pinocytosis was not a consequence of the cytopathic effects produced by VSV. When cells were infected with mutant T-1026 R1, a virus that causes minimal cytopathology by 6 h (Fig. 3E), the time course of the inhibition of pinocytosis was indistinguishable from that in cells infected with wild-type VSV (Fig. 4).

### Effects of VSV Infection on Pinocytosis by Individual Cells

The inhibition of HRP uptake was never complete, and varied from 60 to 80% in different experiments. To determine whether this could be explained by a significant number of uninfected cells in the population, we stained cells with anti-VSV antibodies 4 h after infection. This time was selected since it represents the onset of release of virus from the cells, and a time at which changes in cell morphology are not very pronounced (Fig. 3). When about 500 cells from three different preparations of infected cells were analysed, the number of infected cells ranged from 87 to 93%. This value may actually represent a minimum estimate of the number of infected cells, since some of the cells from VSV-infected cultures were released from the coverslips during the numerous washings involved in the staining procedure.

The incomplete inhibition of pinocytosis might have been related to heterogeneity in the cell population with respect to the rate of pinocytosis. Cells were incubated with fluorescein dextran and examined by fluorescence microscopy; all the control cells took up the marker although a few cells were more intensely fluorescent than the majority (Fig. 5, A and C). VSV-infected cells appeared very similar to uninfected cells (Fig. 5, B and D), except that the intensity of fluorescence was diminished. There was no evidence for a population of cells in which pinocytosis was inhibited completely, and the heterogeneity in the uptake by individual infected cells was no greater than that exhibited by uninfected controls.

### Requirement of Viral Gene Expression for the Inhibition of Pinocytosis

Cells were infected with VSV in the presence of 200 μg/ml cycloheximide, or with virus that had been irradiated with UV light under conditions which decreased infectivity by a factor of >10<sup>9</sup>. In both cases, there was a slight inhibition of pinocytosis (~20%) within 2 h, which did not increase during the next 4 h (Fig. 6).

The next approach employed ts viruses with mutations in each of the five genes of VSV to identify the specific viral gene(s) involved in the inhibition of pinocytosis. The mutants studied were: ts G 11 (mutant in L), which produces no VSV mRNA at 39.5°C; ts G 22 (mutant in NS) and ts G 41 (mutant in N), which fail to replicate viral RNA, but which allow the primary transcription of viral mRNA from the infecting RNA and its polymerase complex at the nonpermissive temperature; ts O 23 (mutant in M) and ts O 45 (mutant in G), both of which exhibit defective virus assembly at 39.5°C (30).

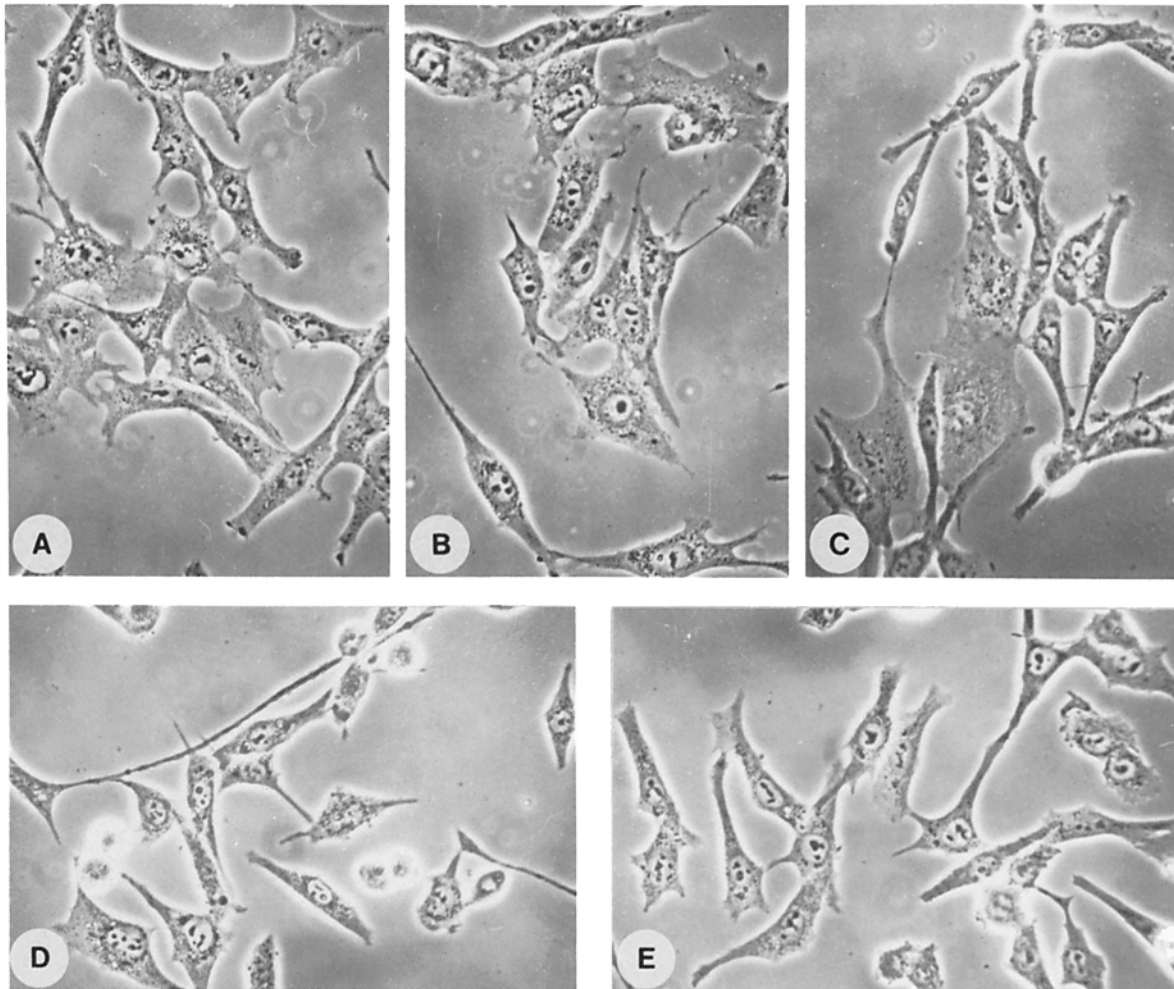


FIGURE 3 Phase-contrast micrographs of BHK cells. Cells were grown and processed for microscopy as described in Materials and Methods, and infected with virus at 37°C at a multiplicity of 10. Representative field of control cells (A); cells infected with wild-type VSV 2 h postinfection (B), 4 h postinfection (C); and 6 h postinfection (D); cells infected with mutant T-1026 R1 6 h postinfection (E).  $\times 260$ .

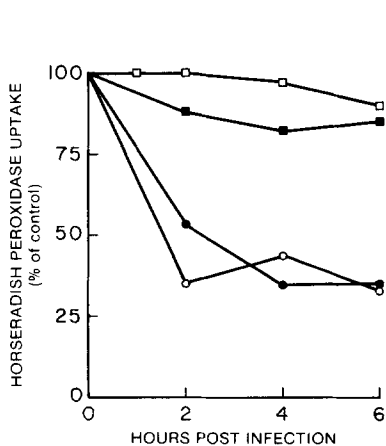


FIGURE 4 Relationship between host protein and RNA syntheses and pinocytosis. BHK cells were incubated with 200  $\mu\text{g}/\text{ml}$  cycloheximide (■) or 5  $\mu\text{g}/\text{ml}$  actinomycin D (□), or were infected with wild-type VSV (○) or the T-1026 R1 mutant (●) at a multiplicity of 10. Experimental procedures were as described in Fig. 1, and the rate of HRP uptake by control cells was 2.2 ng/min per mg protein.

The proteins synthesized by these mutants at the nonpermissive temperature (39.5°C) were analysed (Fig. 7). There was no detectable viral protein synthesis in cells infected with ts G 11 (Fig. 7, lane 4), whereas cells infected with ts G 22, ts G 41, ts O 23, and ts O 45 all exhibited a relatively normal complement of viral proteins (Fig. 7).

The effect of these mutants on HRP uptake, determined at

the nonpermissive temperature, is shown in Table I. As expected from the results in Figs. 6 and 7, there was only a slight inhibition of HRP uptake in cells infected with ts G 11; the inhibition was much greater in cells infected with ts G 22 and ts G 41, although in both cases the inhibition was less than that observed in cells infected with wild-type virus. Cells infected with ts O 23 showed the same inhibition of HRP uptake as cells infected with wild-type virus, whereas there was very little inhibition in cells infected with ts O 45. The inhibition in cells infected with ts G 11 and ts O 45 was very similar to that observed in cells infected with UV-irradiated virus (Table I).

The results indicated that viral gene expression was required for the inhibition of pinocytosis, and suggested that the G protein might play a role. To determine the amount of G on the surface of cells infected with the ts mutants, we reacted the cells with affinity-purified anti-G IgG, and the anti-G bound was estimated by the subsequent binding of  $^3\text{H}$ -sheep-anti-rabbit F(ab')<sub>2</sub>. G protein was detected on the surface of cells infected with wild type, ts G 22, ts G 41, and ts O 23 virus. When cells were infected with ts G 11 and ts O 45, the level of surface G was similar to that found on cells infected with UV-irradiated virus (Table I).

Cells were infected with wild-type, ts G 11, and ts O 45

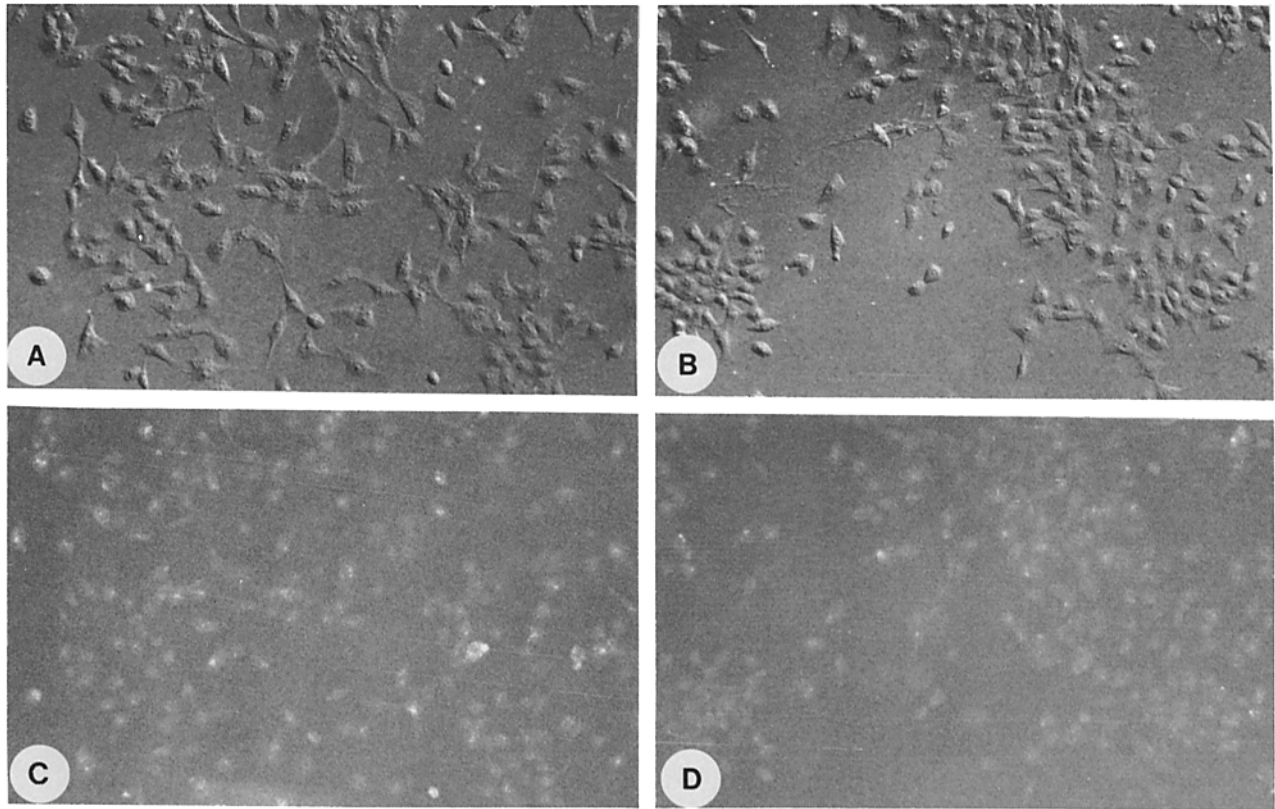
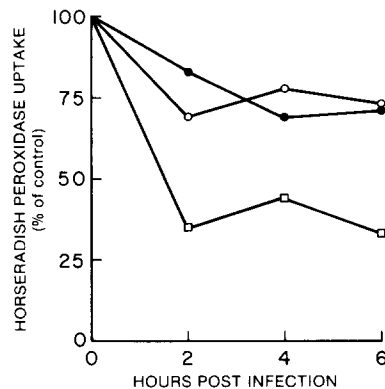


FIGURE 5 Uptake of fluorescein dextran by VSV-infected cells. Cells were incubated with 2 mg/ml fluorescein dextran for 4 h (A and C) or infected with wild-type VSV at a multiplicity of 10 and then incubated for 4 h with 2 mg/ml fluorescein dextran, beginning 2 h after infection; the figure illustrates representative fields studied both by interference-contrast microscopy (A and B) and by fluorescence microscopy (C and D) as described in Materials and Methods. In D, exposures were adjusted to permit visualization of the much lower levels of fluorescence in the cells. Note that all the cells seem to have taken up fluorescein dextran.  $\times 90$ .

FIGURE 6 Relationship between viral protein and RNA syntheses and endocytosis. BHK cells were infected with wild-type VSV in the presence of 200  $\mu\text{g}/\text{ml}$  of cycloheximide (●) or wild-type VSV that had been irradiated with UV light (○) as described in Materials and Methods. (□) denotes uptake in cells infected with wild-type VSV.



The multiplicity was 20 in each case. HRP uptake was determined as in Fig. 1 and the rate for control cells was 1.95 ng/min per mg protein.

virus at the permissive temperature (34°C). In each case, there was an inhibition of the uptake of HRP, and G was detected on the cell surface (Table I).

#### Time Course of the Appearance G on the Cell Surface and the Inhibition of Pinocytosis

To determine the time at which inhibition of HRP uptake could be detected first, we added HRP and VSV to cells together, and HRP uptake was followed as a function of time

(Fig. 8). Cells infected with UV-irradiated VSV showed a slight inhibition which remained constant for 2 h; this inhibition was even detected at 15 min, the earliest time point that was tested (not shown). Cells infected with wild-type VSV exhibited the same uptake as cells infected with UV-irradiated VSV for the first 60 min; during the next 60 min, HRP uptake decreased progressively (Fig. 8).

The binding of anti-G to the cell surface was also analysed as a function of time after infection (Table II). There was virtually no binding to uninfected cells, and a similar low value was obtained when wild-type VSV was added to cells for 2 min at 2°C before assaying the binding (not shown). Cells infected with UV-irradiated VSV exhibited the same binding at 1 h (not shown) as was observed after 4 h (Table I). After 1 h, cells infected with wild-type VSV bound approximately the same amount of antibody as cells infected with UV-irradiated VSV. There was a marked increase in binding at the end of the second hour, and during the next 2 h, when there was essentially no change in the rate of HRP uptake (Fig. 1), there was a further increase in binding (Table II).

The two assays employed for binding produced very similar results when cells infected with wild-type VSV at the nonpermissive temperature were analysed (Table II). In addition, the time course of the appearance of G on the cell surface was the same for cells infected at 39.5°C with ts G 22, ts G 41, and ts O 23 as for cells infected with wild-type virus, and virtually no G was detected at any time point when cells were infected with ts O 45 (not shown).

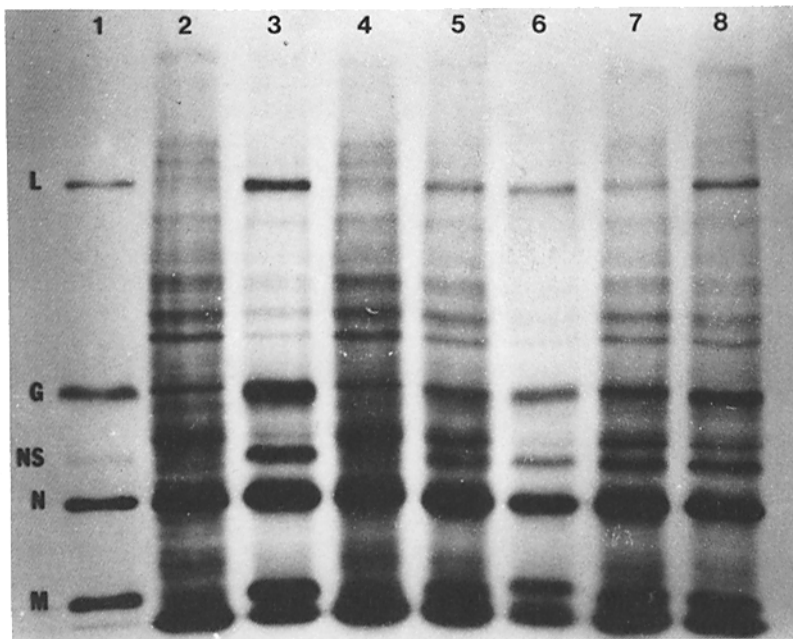


FIGURE 7 Characterization of the proteins synthesized by BHK cells infected at 39.5°C with ts mutants of VSV. Cells were infected with viruses at a multiplicity of 10 and incubated at the non-permissive temperature (39.5°C) for 3 h and then for 1 h at the same temperature in medium supplemented with [<sup>35</sup>S]methionine; proteins were analysed by SDS gel electrophoresis and fluorography as described in Materials and Methods. L, G, NS, N, and M on the left of the figure denote the position of the five virally encoded proteins. Lane 1, purified [<sup>35</sup>S]methionine-labeled VSV run as a standard; lane 2, uninfected cells; lane 3, cells infected with wild-type virus; lane 4, cells infected with ts G 11 (L mutant); lane 5, cells infected with ts G 22 (NS mutant); lane 6, cells infected with ts O 23 (M mutant); lane 7, cells infected with ts G 41 (N mutant); lane 8, cells infected with ts O 45 (G mutant).

TABLE I  
HRP Uptake and Binding of Anti-G Antibody to BHK Cells  
Infected with ts Mutants of VSV

Cells*	HRP uptake (ng/min/mg protein)		<sup>3</sup> H antibody bound (ng sheep anti-rabbit F(ab') <sub>2</sub> /mg protein)*	
	39.5°C	34°C	39.5°C	34°C
	Control, uninfected	1.82	1.68	55
Wild-type VSV	0.65	0.66	3,350	2,085
UV-irradiated VSV	1.67	ND <sup>§</sup>	135	ND
ts G 11	1.70	0.79	115	1,160
ts G 22	1.05	ND	1,950	ND
ts G 41	0.93	ND	2,670	ND
ts O 23	0.72	ND	3,710	ND
ts O 45	1.64	0.71	145	1,505

\* Cells were infected with viruses at a multiplicity of 10. HRP was added to the cells 4 h after culture at 39.5 or 34°C, and uptake was analysed after 20 and 40 min at the same temperature. Antibody binding was analysed at 4°C after 4 h at 39.5 or 34°C.

<sup>§</sup> Cells were incubated at 4°C, first with affinity purified anti-G IgG and then with <sup>3</sup>H-F(ab')<sub>2</sub> fragments of sheep anti-rabbit F(ab')<sub>2</sub> as described in Materials and Methods.

<sup>§</sup> ND, denotes not determined.

## DISCUSSION

Our results demonstrate a rapid inhibition of fluid phase pinocytosis in BHK cells infected with VSV. There were apparently two aspects to the inhibition: first, a slight and variable inhibition (5–20%), which occurred in the absence of viral gene expression; and second, more pronounced inhibition (60–80%), which required viral gene expression. Since the inhibition was almost at its maximum before virus could be detected in the medium (Fig. 1), the inhibition was clearly not a direct consequence of the release of phospholipid bilayer from the cells that accompanies the release of virus.

The inhibition of pinocytosis preceded the decrease in the rate of protein synthesis in infected cells: incorporation of amino acids remained at or near control levels for at least 3 h after infection, whereas HRP uptake decreased after 1 h (Fig. 1). Drugs that inhibited host cell RNA or protein syn-

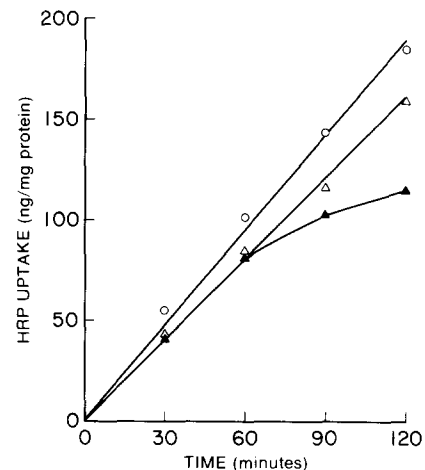


FIGURE 8 Time course of HRP uptake by BHK cells infected with VSV. Cells were transferred at zero time to medium containing 1 mg/ml HRP (O), 1 mg/ml HRP and UV-irradiated VSV (multiplicity of infection = 10) (Δ), or 1 mg/ml HRP and wild-type VSV (multiplicity of infection = 10) (▲). HRP taken up by the cells at the times indicated was analysed as described in Materials and Methods.

thesis also had little or no effect on the rate of pinocytosis during the time period studied (Fig. 4). In addition, the T-1026 R1 mutant, which has an impaired ability to inhibit the synthesis of host proteins (8, 36), caused an inhibition of pinocytosis which was essentially the same as that observed for wild type virus (Fig. 3).

There was no correlation between the inhibition of pinocytosis and cytopathic effects on the cells: 2 h after infection, cell morphology was normal (Fig. 3), whereas the inhibition was almost at its maximum (Fig. 1). While there are several reports (see reference 2) that VSV can cause cytopathic changes as soon as 1 h after the addition of virus, such changes have only been detected when virus was added at multiplicities of approximately 100. Concentrations of VSV which are five- or tenfold higher than those employed here may well produce a different response in the cells. It is also likely that the inhibition of pinocytosis is not responsible for the gross cy-

TABLE II  
Binding of Anti-G Antibodies at Different Times after Infection  
with VSV

Cells*	Antibody bound		
	<sup>125</sup> I Anti-VSV <sup>†</sup>		<sup>3</sup> H-sheep- anti-rabbit F(ab') <sub>2</sub> <sup>‡</sup>
	Cells cul- tured at 37°C	Cells cul- tured at 39.5°C	
(ng/mg protein)			
Control, uninfected	25	30	45
Wild-type VSV at 1 h	130	90	190
Wild-type VSV at 2 h	1,255	755	650
Wild-type VSV at 4 h	5,525	3,745	3,170

\* Cells were infected as described in Table I.

<sup>†</sup> Cells were cultured at 37 or 39.5°C and then incubated at 4°C with <sup>125</sup>I anti-VSV antibodies, as described in Materials and Methods.

<sup>‡</sup> Cells were cultured at 39.5°C and then incubated at 4°C first with affinity purified anti-G IgG and then with <sup>3</sup>H-F(ab')<sub>2</sub> fragments of sheep anti-rabbit F(ab')<sub>2</sub> as described in Materials and Methods.

topathic changes seen following VSV infection: cells infected with the T-1026 R1 mutant exhibited the same inhibition as cells infected with wild-type virus, although their morphology was much more normal (Fig. 4).

The results suggest that the inhibition of pinocytosis is the consequence of a reduced rate of internalization of the fluid phase. Most agents that have been shown to inhibit pinocytosis, for example chloroquine, ammonium chloride, or monensin (19, 39, 43), seem to influence intracellular events in plasma membrane recycling, and cause extensive formation of vacuoles and inhibit the transfer of pinocytosed material to lysosomes (34). Cells infected with VSV were not extensively vacuolated (Fig. 3) and preliminary experiments suggest that the transfer of HRP to lysosomes is not affected (unpublished results). This indicates that the fusion of pinocytic vesicles with lysosomes occurred normally in VSV-infected cells, and that the inhibition is the consequence of an effect on the plasma membrane at the level of the formation or stabilization of pinocytic vesicles.

Although a variety of changes has been observed in plasma membranes of virally infected cells (29), they occur at relatively late stages after infection. Gray et al. (10) showed recently, in experiments where the time course of the effect of VSV on host protein synthesis and viral production was very similar to that described here, that an increase in 2-deoxy-D-glucose transport could be detected 2–3 h after infection. The effect on pinocytosis, then, seems to be the earliest effect on plasma membrane function to have been described so far.

While we have not established the mechanism by which pinocytosis is inhibited, our findings are consistent with an essential role for the viral membrane protein G. There was very little inhibition in the absence of viral protein synthesis (infection in the presence of cycloheximide [Fig. 6] or with ts G 11 [Fig. 7, Table I]), and the decreased inhibition in cells infected at 39.5°C with ts G 22 and ts G 41 was consistent with the effects of these mutations on viral gene expression (30). Cells infected at 39.5°C with ts O 23 showed a normal inhibition, suggesting that the M protein did not play a major role.

In contrast, HRP uptake in cells infected at 39.5°C with ts O 45 was the same as in cells infected with UV-irradiated

virus, and virtually no G was detected on the cell surface (Table I). This confirms, by a different technique, the finding of Knipe et al. (16), who showed that G in cells infected with ts O 45 at the nonpermissive temperature was inaccessible to iodination at the cell surface; other studies have shown that the mutant G is blocked at the rough endoplasmic reticulum (4, 16). Since G synthesized at the nonpermissive temperature still reacts with antibody to G prepared against the protein isolated from wild-type virus (4), it is reasonable to conclude that, at 39.5°C, essentially no G reaches the cell surface. In addition, the onset of the inhibition of pinocytosis (Fig. 8) correlated with the appearance of G on the surface (Table II).

There was not, however, a linear relationship between G on the surface and inhibition of pinocytosis: there was a marked increase in surface G between 2 and 4 h postinfection, and cells infected with ts G 22 and ts G 41 contained more G on the surface than seemed to be needed for maximum inhibition (Tables I and II). It will be of obvious interest to test the effect of incorporating G directly into the plasma membrane, since techniques that could achieve this have been developed for VSV and other membrane proteins (3, 41).

It is also possible that effects of virus infection on the host cytoskeletal network (20) might contribute to the inhibition of pinocytosis. Preliminary experiments (K. Fujiwara and C. C. Widnell, unpublished results) suggest that there is a change in the distribution of actin-containing filaments 4 h after infection with VSV. The availability of mutants which affect pinocytosis to different extents should facilitate the analysis of other factors that contribute to the inhibition.

The slight inhibition of pinocytosis observed in the absence of viral gene expression might also be related to the presence of G on the cell surface. When VSV enters the cell the viral membrane fuses with the membranes of endocytic vesicles (12). The presence of a viral membrane protein in the endocytic vesicles, or its transport to the surface as a result of membrane recycling (33, 42), could then result in an inhibition of pinocytosis. Traces of G were always detected on the surface of cells infected with UV-irradiated virus, although the possibility that this might simply represent adsorbed virus has not been excluded.

It is also possible that this inhibition could be related to displacement of the normal volume of pinocytic vesicles as a result of the internalization of virus particles originally bound to the cell surface. This effect has been demonstrated for Semliki Forest virus by Marsh and Helenius (23), employing virus concentrations about 200-fold higher than used here. Such a mechanism, however, could not explain the component of the inhibition that requires viral gene expression since even at such high concentrations of virus the inhibition of fluid-phase uptake did not exceed 20%.

The inhibition of pinocytosis was not only observed in BHK cells infected with VSV. In preliminary experiments we found that HRP uptake was inhibited by 65% within 2 h when we infected BHK cells with the enveloped virus, vaccinia. When VSV and vaccinia were used to infect L-cells, pinocytosis was inhibited by 60% and 90%, respectively, at 2 h. In contrast, encephalomyocarditis virus, a nonenveloped virus, had essentially no effect even after 6 h (unpublished results). Further studies will be required, however, to determine whether inhibition of pinocytosis is a specific property of enveloped viruses.

It is clear from our results that the intracellular transport of newly synthesized plasma membrane protein (G) is not

coupled directly to the recycling of membrane involved in pinocytosis. At 4 h after VSV infection, the transport of G to the cell surface occurs at a rate which is similar to that of plasma membrane proteins in uninfected cells (1, 15), whereas pinocytosis, and therefore membrane recycling, is inhibited by at least 60%. This finding complements the results from previous experiments (43), which indicated that membrane recycling was not affected for at least 3 h after the inhibition of intracellular transport of secretory protein by monensin.

In addition to the effects on fluid-phase pinocytosis described here, we found that infection with VSV also inhibits adsorptive endocytosis of viruses (40a). If, in future experiments, it is possible to determine the nature of the interactions between viral proteins and the host membrane that result in the inhibition of pinocytosis, these viral proteins may represent useful inhibitors for the analysis of pinocytosis.

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