JOURNAL OF VIROLOGY, Nov. 1971, p. 786-795
Vol. 8, No. 5

Printed in U.S.A.

Entry of Vesicular Stomatitis Virus into L Cells

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Received for publication 11 June 1971

Early stages of the entry of vesicular stomatitis (VS) virus into L cells were followed by electron microscopy with the aid of ferritin antibody labeling. Cells which were infected at 0 C and incubated for 10 min at 37 C were reacted first with anti-viral-antiferritin hybrid antibody and then with ferritin or fluorescein-labeled apoferritin. Extensive ferritin labeling of the cell surface was detected by both electron and fluorescence microscopy. The labeled regions of the cell surface were continuous with and indistinguishable from the rest of the host cell membrane, suggesting incorporation of viral antigens into the cell surface during viral penetration. Fusion of parental viral membrane with host cell membrane was further demonstrated by examining the localization of ^{3}H-labeled viral structural proteins in cells infected at 0 C and incubated for short periods at 37 C. Viral nucleoprotein was found in a soluble fraction of the cells which was derived primarily from the cytoplasm, whereas a particulate fraction from the cells was enriched in viral envelope proteins. Cytoplasmic membrane was isolated from these cells, and this membrane contained viral envelope proteins. These results suggest that penetration by VS virus occurs by fusion of the viral and cellular membranes followed by release of nucleoprotein into the cytoplasm.

Recent electron microscopic studies by Morgan and his associates (8, 12, 13) have indicated that membrane-enclosed viruses such as influenza virus and Sendai virus can enter cells by fusion of the viral and host cell membranes followed by release of the nucleocapsid core directly into the host cell cytoplasm. In an electron microscopic study with vesicular stomatitis (VS) virus, fusion of viral and host cytoplasmic membranes was observed within minutes after infection, suggesting a similar method of penetration for this virus (6). However, Simpson et al. (22), using somewhat different conditions for infection, observed VS virus only in phagocytic vacuoles and suggested that penetration occurs by phagocytosis followed by intracellular uncoating of the virus.

The observation by electron microscopy that viral and host cell membranes can fuse in early stages of infection is not sufficient evidence to implicate virus-cell fusion as the predominant mode of viral entry or to rule out entry by phagocytosis followed by lysosomal uncoating. There are two limitations to the use of electron microscopy for answering this question. First, only a small and not necessarily representative fraction of the input virions can be detected at the extremely high multiplicities of infection needed for electron microscopy. Second, only the initial stage of infection can be observed by electron microscopy. It is likely that the tightly packed nucleocapsid helix of VS virus (14) unfolds upon entry into the cytoplasm, resulting in loose coils of nucleoprotein (14, 21) which would be impossible to identify against the background of the normal cellular constituents. No empty shells of VS virus were observed by electron microscopy of infected cells (6). The morphologically distinct nucleoprotein serves to identify the virus in sectioned preparations and in its absence the viral envelope cannot be distinguished from host cell membrane.

Two approaches were used to circumvent these technical limitations. The first entails the electron microscopic observation of infected cells labeled with antibody that reacts with electron-dense ferritin to determine if viral antigens remain on the cell surface after viral penetration. The second involves determining the fate of radioactively labeled virus proteins during viral penetration. The combination of results obtained with these techniques and the previous results by electron microscopy (6) strongly indicate that viral entry can occur by fusion of host and viral membranes.

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MATERIALS AND METHODS

Virus, cells, and media. The Indiana serotype of VS virus was used throughout. Virus was grown in confluent monolayers of L cells, and virus titers were determined by counting virus particles by electron microscopy (3, 5). Suspension cultures of L cells were grown in medium 199 supplemented with 10% fetal calf serum to a final culture density of about 10^6 cells per ml.

Eagle's basal medium (BME) prepared without leucine and tyrosine was used for radioactive labeling. Infected monolayers of L cells growing in Falcon plastic flasks (75 cm^2 surface) were covered 1 hr after virus adsorption with 5 ml of this medium containing 10 μCi of ^{3}H-tyrosine per ml (and 10 μCi of ^{3}H-leucine per ml). After 8 hr, an additional 5 ml of complete BME containing 2% fetal calf serum was added. The medium from the cultures containing released virus was harvested after 16 to 18 hr, and the virus was purified by the method of McSharry and Wagner (10). After centrifugation of the medium at low speed to remove cell debris, the virus was precipitated with polyethylene glycol 6000 (9) and further purified by diethylaminoethyl (DEAE) cellulose column chromatography (10). The virus eluted from the DEAE column was pelleted by centrifugation, and the pellet was resuspended in 0.5% lactalbumin hydrolysate medium (LA) and stored at −70 C.

Virus used for the preparation of rabbit antiserum was grown in monolayers of chick embryo fibroblasts in LA supplemented with 2% fetal calf serum. Virus was purified by the procedure outlined above for ^{3}H-labeled virus. Separate columns were used for purifying viruses grown in L cells and chick embryo cells to prevent cellular antigen cross-contamination.

Culture media were purchased from Grand Island Biological Co., Grand Island, N.Y.

Reagents. ^{3}H-leucine (10.3 to 14.7 Ci/mmole) and ^{3}H-tyrosine (28.2 to 43 Ci/mmole) were obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Pepsin was obtained from Worthington Biochemical Corp., Freehold, N.J. Ferritin was obtained from Polyscience Inc., Rydale, Pa. Purified immunoglobulin G (IgG) from rabbits hyperimmunized with ferritin and fluorescein-labeled apoferritin were obtained from Gerald Goldstein of this department.

Preparation of hybrid antibody. Purified VS virus grown in chick embryo cells was used for all immunizations. Rabbits were given intramuscular injections into each flank at weekly intervals for 3 weeks. Each injection consisted of 10^{10} virions suspended in 0.5 ml of Freund's incomplete adjuvant. After a rest period of 2 months, each rabbit received two additional intravenous injections 1 week apart, each consisting of 10^{10} virions in 0.5 ml of LA. The rabbits were bled 7 days after the last injection. Serum was pooled from 10 rabbits. The pooled antiserum diluted 1:256,000 neutralized about 90% of VS virus plaque-forming units.

IgG was isolated from the rabbit antiserum by the method of Reif (17). The IgG was precipitated with cold 1.8 M ammonium sulfate, and the precipitate was dissolved in 0.15 M NaCl and dialyzed overnight against 0.01 M phosphate buffer (*p*H 8.0). Contaminating serum proteins remaining in this preparation were removed by absorption with Whatman DE-52 cellulose. A 4.4-g amount of wet DE-52 equilibrated against 0.01 M phosphate buffer (*p*H 8.0) was added per ml of antiserum, and this suspension was allowed to stand for 1 hr with occasional stirring. The suspension was filtered through a Buchner funnel, and the residue was washed three times with small volumes of buffer. The combined filtrate was dialyzed overnight against 0.15 M saline containing 0.01 M sodium phosphate buffer, *p*H 7.4 (PBS), and concentrated by vacuum dialysis to a final concentration of 20 mg of protein per ml. The purity of this preparation was tested by immunoelectrophoresis (18), which revealed a single IgG precipitin line with goat antiserum to whole rabbit serum.

The combination of purified rabbit antiviral IgG with similarly purified rabbit antiferritin IgG to give hybrid antibody is based on a principle first suggested by Nissonoff and Rivers (15). Reduction and reoxidation of a mixture of antiviral and antiferritin Fab fragments of IgG should result in random recombination to form bivalent 5*S* molecules, a considerable proportion of which should have one viral antigen-combining site and one ferritin antigen-combining site. Preparation of hybrid antibody was based on the method described by Hammerling et al. (4) as modified by Graziano and Goldstein (*manuscript in preparation*) for studying microbial antigens. Wagner et al. (23) have described the application of this technique for determining the kinetics and cellular sites of antigen synthesis.

The purified IgG preparations were dialyzed overnight against a buffer (*p*H 4.5) containing 7.0 g of anhydrous sodium acetate and 6.4 ml of glacial acetic acid per liter, and the $(Fab')_2$ peptides were obtained by incubation in the same buffer with pepsin (0.02 mg/mg of IgG) for 20 hr at 37 C. The *p*H was adjusted to 8.0 with NaOH, and the preparation was dialyzed at 4 C against several changes of 0.5 M sodium acetate buffer (*p*H 5.0) to remove small peptide fragments. Antiviral $(Fab')_2$ and antiferritin $(Fab')_2$ prepared in an identical manner were mixed in a ratio of 1:4 and reduced under N_2 at 37 C for 1 hr after the addition of 2-mercaptoethylamine hydrochloride to give a final concentration of 0.015 M. The mercaptoethylamine was removed by passage over an Amberlite IR-20 column, and the protein eluted from the column was adjusted to *p*H 8.0 with NaOH. The preparation was then reoxidized by stirring under an O_2 atmosphere for 6 hr. The protein solution was concentrated to 4 to 6 ml by vacuum filtration and applied to a Sephadex G-100 column equilibrated with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 8.6) containing 0.2 M NaCl. The fractions containing the first protein peak eluted from the column (5*S* recombinants) were pooled, concentrated to 5 mg/ml by vacuum filtration, and dialyzed against PBS. The 5*S* peak represented 47% of the protein applied to the column. The hybrid antibody preparation was sterilized by membrane

filtration (Millipore Corp., Bedford, Mass.) and stored at 4 C.

Electron and ultraviolet light microscopy of antibody-labeled infected cells. L cells grown in suspension culture were infected at 4 C and incubated at 37 C for periods as long as 10 min as previously described (6). The capillary tubes were chilled in an ice bath and the cells were removed, centrifuged, and washed once with PBS containing 0.25 M sucrose. The antiviral-antiferritin hybrid antibody was added to a suspension of the cells in PBS containing sucrose to give a final concentration of 0.25 mg of antibody protein per 10^6 cells in a volume of about 0.1 ml. The suspension was allowed to stand for 20 min on ice, and the cells were washed twice with PBS.

One set of duplicate samples treated as described above was incubated for 30 min at 0 C in PBS containing fluorescein-labeled apoferritin at a final concentration of 0.03 mg per 10^6 cells in a volume of about 0.1 ml. These cells were then washed twice as above and examined for surface fluorescence under a Leitz ultraviolet light microscope with a 5840/K2 filter. Photographs were taken at a magnification of 95×.

A duplicate set of cell samples exposed to hybrid antibody was incubated with native ferritin in PBS at a concentration of 0.125 mg per 10^6 cells for 30 min at 0 C. These cells were washed twice as above, fixed for electron microscopy by centrifugation in Beckman microfuge tubes partially filled with buffered glutaraldehyde, and processed for electron microscopy as previously described (6).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Maizel (11) with minor modification as described by Wagner et al. (25). Samples of as much as 250 μliters were layered on gels (0.5 by 10 cm) containing 7.5% acrylamide, 0.5 M urea, and 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer (*p*H 7.2). Gels preserved by freezing and storing at −20 C were sliced into 1.25-mm sections and solubilized for liquid scintillation counting by a modification (19) of the method of Basch (1).

Enzyme assays. Acid phosphatase was measured by the method of Fishman and Lerner (2) in an assay system containing 5.5 mM *p*-nitrophenylphosphate and 0.1% Triton X-100 in 0.5 M citrate buffer (*p*H 4.8). The reaction was stopped after 15 min at 37 C by the addition of excess NaOH and read at 405 nm. Glucose-6-phosphatase was assayed spectrophotometrically by a modification of the procedure of Noltman et al. (16). Samples were preactivated by treatment with the detergent Lubrol WX (20). The assay system contained 0.01 M glucose-6-phosphate, 0.01 M $MgSO_4$, and 0.2 mM nicotinamide adenine dinucleotide phosphate (NADP) in 0.04 M HEPES (*N*-2 hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid) buffer (*p*H 8.0). The change in absorbance was followed at 340 nm after the addition of the NADP.

RESULTS

Localization of viral antigen on the surface of infected cells. Figures 1 and 2 show the appearance of free virus particles observed in a virus-cell complex which was not incubated at 37 C after virus adsorption but which was incubated at 0 C with antiviral-antiferritin hybrid antibody followed by incubation with ferritin. The heavy ferritin labeling evident in these micrographs was completely absent if the hybrid antibody was omitted (Table 1) or if normal bivalent antiferritin antibody was used in place of the hybrid antibody. Figures 3 to 7 show the appearance of the ferritin label on the surface of cells incubated at 37 C after virus adsorption at 4 C. Figure 3 shows a cellular process which is heavily labeled with ferritin. Ferritin labeling was often observed to be particularly extensive on cellular processes or pseudopodia. Figure 4 illustrates a typical infected cell as seen by flouorescence microscopy, showing the characteristic "ring" of surface fluorescence. Figures 5 through 7 are representative micrographs of cells chosen to illustrate the range of ferritin staining which was observed. Figures 5 and 6 show examples of spotty labeling, where the ferritin label is found in small patches on the surface of the cells, whereas Fig. 7 shows a more generalized labeling of a larger area of the cell surface. In all cases, the ferritin-labeled areas were continuous with and indistinguishable from adjacent, unlabeled areas of the cell surface. No intact virions were observed fused to the surface of cells stained with ferritin by the hybrid antibody technique. This probably did not result from the antibody treatment but from the prolonged incubation at 0 C and from the repeated washing necessary for the ferritin labeling procedure. In the previous study in which virions were observed fused to the cell surface (6), the virus-cell complex was fixed rapidly within a few minutes after incubation at 37 C.

Figure 8 shows a portion of the cell surface of an uninfected cell. No ferritin labeling was observed with uninfected cells or with other control preparations (Table 1). The results summarized in Table 1 indicate that the ferritin label is quite specific, since labeling of the cell surface was observed only with virus-cell complexes incubated at 37 C and only in the presence of the antiviral-antiferritin hybrid antibody.

Uncoating of virions after incubation with L cells as measured by release of ^{3}H-labeled proteins. If virions penetrate cells by fusion of viral and host cell membranes, nucleoprotein should appear in the cytoplasm after incubation of virus-cell complexes. If virions penetrate cells by phagocytosis followed by fusion of lysosomes with phagocytic vesicles, virion proteins should be contained in particulate organelles until degradation of the virions occurs. It is not possible to distinguish between these possibilities if the procedure employed for cell breakage results in breakage of

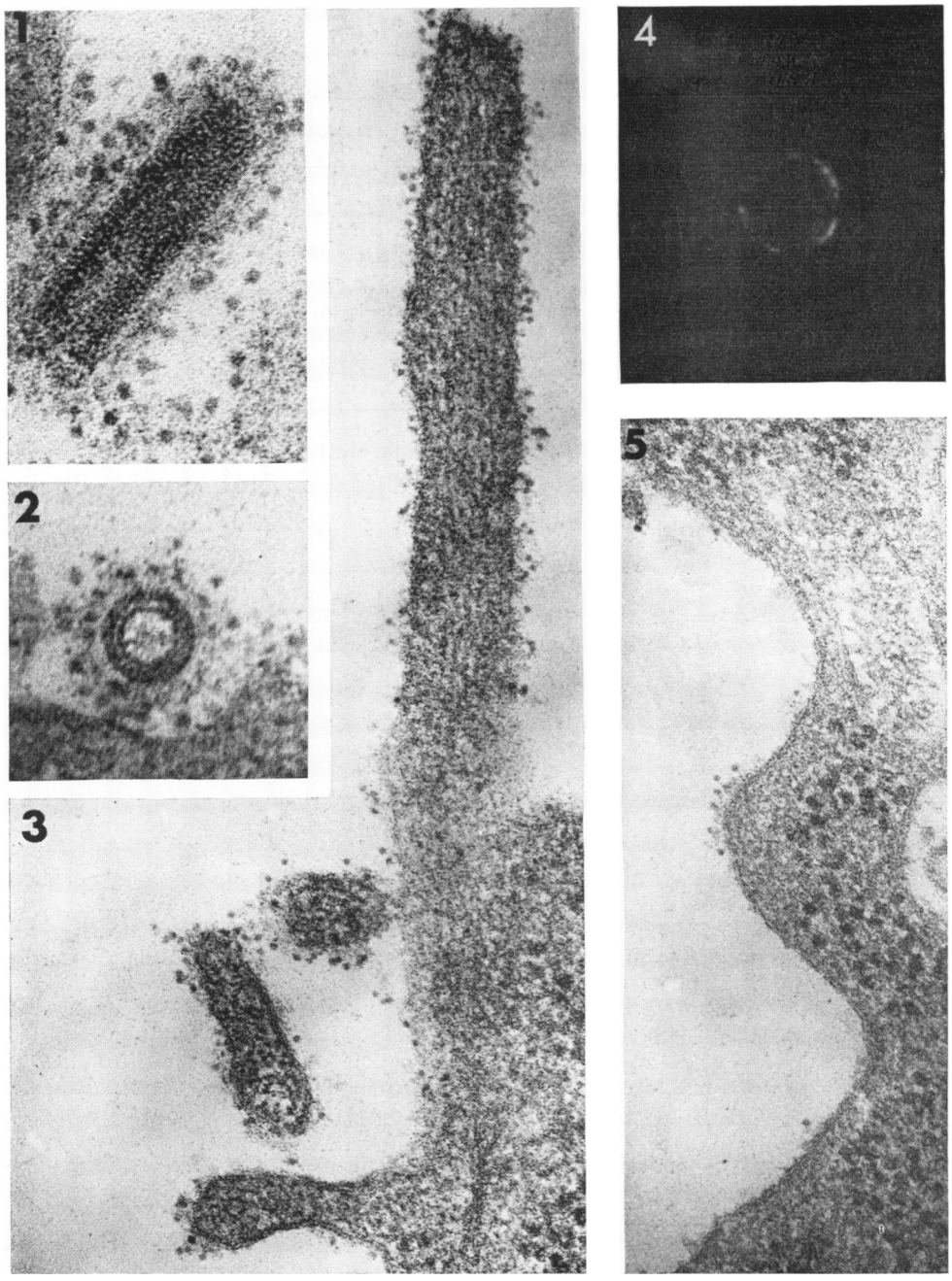


FIG. 1. *Longitudinal section of VS virus particle showing ferritin labeling on the viral surface. This particle was a free virus which was observed in a preparation of infected cells which was incubated at 0 C after infection and exposed to antiviral-antiferritin hybrid antibody.* ×*230,000.*

FIG. 2. *Transverse section of VS virus particle from the same preparation as Fig. 1.* ×*230,000.*

FIG. 3. *Cellular process (pseudopodium) from an infected cell incubated at 37 C for 10 min after infection. The cell surface shows extensive ferritin labeling.*

FIG. 4. *Fluorescence micrograph of a typical cell showing moderate surface fluorescence. This cell is from a preparation which was incubated at 37 C for 10 min after virus adsorption at 4 C followed by incubation with antiviral-antiferritin hybrid antibody and fluorescein-labeled apoferritin.* ×*200.*

FIG. 5. *Infected cells incubated at 37 C for 10 min after virus adsorption at 4 C showing spotty ferritin labeling.* × *103,500.*

TABLE 1. *Specificity of ferritin labeling with the antiviral-antiferritin hybrid antibody method*[a]

Sample	Incubation temp (C)	Hybrid added[b]	Indicator added	Results[c]
Virus-cell complex	0	aV + aF	Ferritin	Free virus +, cells −
Uninfected cells	37	aV + aF	Ferritin	cells −
Virus-cell complex	37	aV + aF	Ferritin	Free virus +, cells +
Virus-cell complex	37		Ferritin	Free virus −, cells −
Virus-cell complex	0	aF + aF	Ferritin	Free virus −, cells −
Virus-cell complex	37	aV + aF	Fluorescein, apoferritin	99
Virus-cell complex	37		Fluorescein, apoferritin	Less than 1

[a] Cells were infected at 4 C by the sedimentation inoculation method (3) at a multiplicity of 10^3 virions per cell. The cells were then transferred to capillary tubes and incubated for 10 min at the temperatures indicated above. At the end of the incubation period, the capillary tubes were chilled and the cells were removed, treated with antibody and ferritin or fluorescein-labeled apoferritin, and processed for microscopy.

[b] Antiviral-antiferritin (aV + aF) hybrid IgG was compared with homologous 5*S* antiferritin IgG (aF + aF).

[c] For the first five samples, results represent location of ferritin label as determined by electron microscopy. For last two samples, results represent per cent of cells showing surface fluorescence by ultraviolet microscopy.

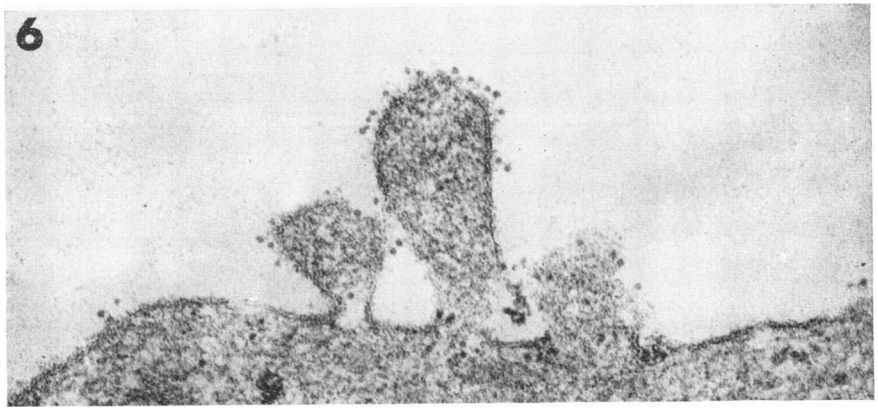
subcellular organelles as well. To follow the fate of virion proteins after infection, it was necessary to develop a gentle method of breaking the cells, one which results in maximum rupture of plasma membrane with minimum damage to lysosomes and other subcellular organelles.

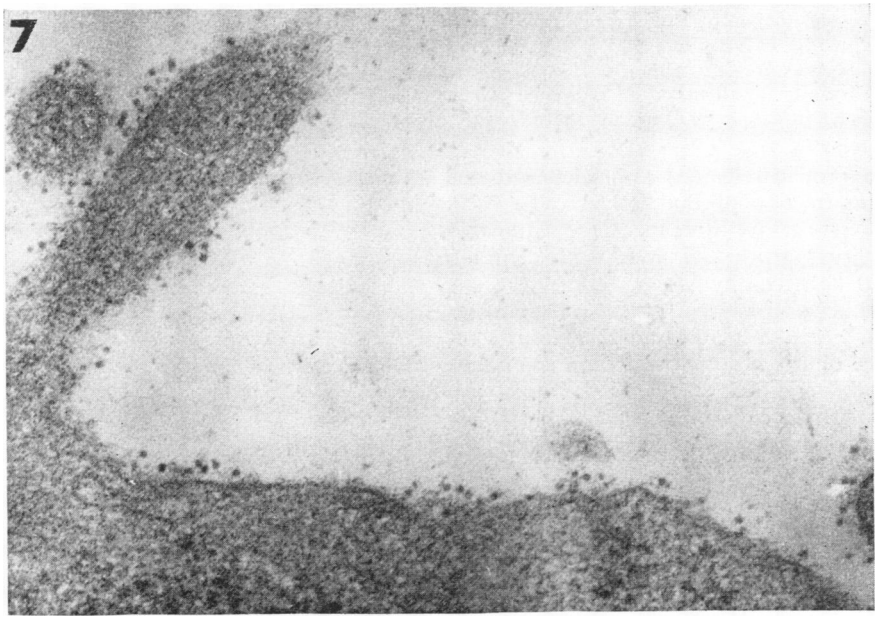
The best method for rupturing cells was determined by comparing the release of cytoplasmic and lysosomal enzymes from the sedimentable fraction of the cells. Normal and infected L cells (10^6 cells per test) propagated in suspension culture were suspended in 2.5 ml of cold medium containing 0.22 M D-mannitol, 0.07 M sucrose, and 0.5 mg of bovine serum albumin per ml in 2 mM HEPES buffer, *p*H 7.4 (20). The cells were ruptured by three cycles of freezing and thawing, by treatment in the microchamber of a Sorvall Omnimixer for 2.5 min at a speed setting of 5, or by rapid decompression by release from a N_2 bomb (27) after an equilibration period of 20 min at 0 C and 880 psi. Preliminary experiments with the N_2 bomb indicated that these conditions were optimum for producing maximal cell breakage and minimal lysosomal breakage.

Table 2 shows the distribution of the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, and the lysosomal enzyme, acid phosphatase, in the soluble and particulate fractions obtained after breaking the cells by the three methods outlined above. Decompression from a N_2 bomb was the best breakage method, since it resulted in essentially complete release of glucose-6-phosphate dehydrogenase with only 38% of the acid phosphatase released into the soluble fraction. No difference in the release of acid phosphatase was observed with infected cells, indicating that viral infection did not cause increased lysosomal fragility.

To determine the fate of virion proteins during penetration, cells were infected with ^{3}H-labeled virus at a multiplicity of 40 virions per cell at 4 C by the sedimentation inoculation method (3); the virus-cell complex was then incubated for 10 to 15 min at 37 C. The incubated cells were then suspended in cold sucrose-mannitol medium as above and ruptured with the N_2 bomb. The suspension recovered from the bomb was fractionated by centrifugation at 40,000 × *g*; the distribution of virion proteins in the pellet and supernatant fractions was determined by polyacetylamide gel electrophoresis. The results of this experiment are shown in Table 3. The three predominant structural proteins of the virion are designated by the scheme of Wagner and Schnaitman (24), in which the G and S proteins are viral envelope proteins of molecular weights of 81,500 and 34,000 daltons, respectively, and the N protein is the nucleoprotein with a molecular weight of 59,000 daltons.

Very little virus protein was solubilized when the virus-cell complex was incubated at 0 C. The activity found in the supernatant fluid probably resulted from damaged virus particles, since free virus particles are generally pelleted by centrifugation under the conditions employed. When the virus-cell complex was incubated at 37 C after infection, a substantial amount of nucleoprotein was solubilized, whereas almost all of the





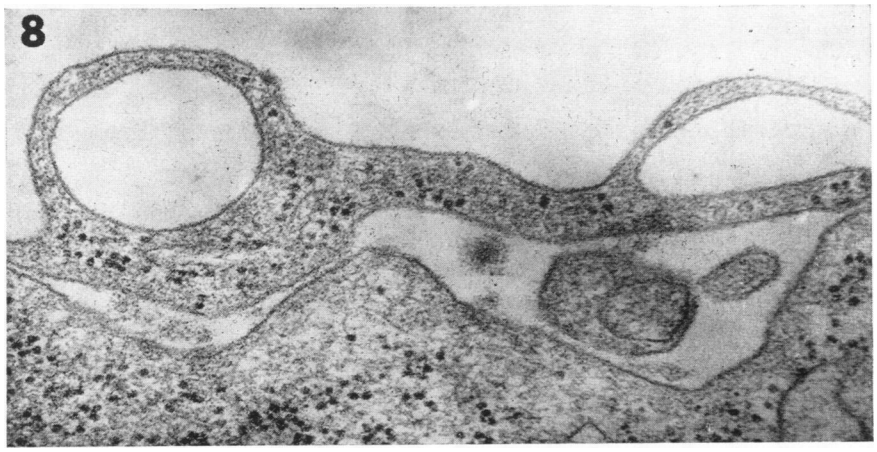


FIG. 6. *Infected cells incubated at 37 C for 10 min after virus adsorption at 4 C showing spotty ferritin labeling.* × *103,500.*

FIG. 7. *Infected cell, as in Fig. 5, but showing more ferritin labeling.* ×*119,500.*

FIG. 8. *Uninfected cell treated as in Fig. 4 to 7. No ferritin labeling was observed.* ×*119,500.*

TABLE 2. *Release of lysosomal and cytoplasmic marker enzymes from particulate fraction of cells broken by various methods*[a]

Method of breakage	Per cent of acid phosphatase activity				Per cent of glucose-6-PO_4 dehydrogenase activity			
	Uninfected cells		Infected cells[b]		Uninfected cells		Infected cells[b]	
	Supernatant fluid	Pellet	Supernatant fluid	Pellet	Supernatant fluid	Pellet	Supernatant fluid	Pellet
Freeze-thawing	54.0	45.7			>95	<5		
Oximixer treatment	60.0	40.0			>95	<5		
Decompression from N_2 bomb	38.0	62.0	39.9	59.8	>95	<5	>95	<5

[a] Cells were broken by the various methods above in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES buffer (*p*H 7.4), and 0.5 mg of bovine serum albumin per ml. After breakage, the cell suspensions were centrifuged at 27,000 × *g* for 15 min. The activities are expressed as per cent of total recovered activity. In all cases, the recovery was essentially 100% as compared to unfractionated cells.

[b] Cells were infected at 4 C and incubated for 10 min at 37 C before breakage.

G and S protein remained associated with the particulate fraction of the broken cells. As a control for the above experiment, the sedimentation of virus particles disrupted with digitonin exactly as described by Wagner et al. (26) was examined. The ^{3}H-labeled proteins of digitonin-fragmented virus remained in the supernatant fluid when centrifuged under the same conditions used for the fractionation described above.

Parental virion proteins associated with plasma membrane isolated from infected L cells. The experiments described above suggest that viral membrane becomes associated with host cell cytoplasmic membrane after incubation of virus-cell complexes. To demonstrate this more conclusively, a preparation of cytoplasmic membrane was isolated from virus-cell complexes incubated at 37 C and examined for the presence of ^{3}H-labeled viral envelope proteins by polyacrylamide gel electrophoresis. The isolation of cytoplasmic membrane was facilitated by the fact that L cells will actively ingest polystyrene latex beads. When beads with a diameter of 0.714 μm are added to cells either in suspension or by cosedimenting the beads and cells onto a flat agar surface, the beads are taken up singly and appear in the cytoplasm enclosed in individual vacuoles of cytoplasmic membrane. The membrane-enclosed beads can be released from the cells by breakage with an N_2 bomb and isolated by flotation on sucrose gradients. The basic details of this method for the isolation of cytoplasmic membrane of L cells are described elsewhere (7). Although this method produces a relatively small yield of cytoplasmic membrane, it has the advantage that the cytoplasmic membrane isolated by this procedure is quite pure, since the density of the membrane-enclosed beads is determined by the density of the polystyrene and is quite different from the density of contaminating membrane fragments of subcellular organelles (28).

TABLE 3. *Distribution of labeled viral proteins in soluble and particulate fractions of infected cells*[a]

Viral protein	Distribution (%) of labeled viral proteins under different incubation conditions			
	15 min at 0 C		15 min at 37 C	
	Supernatant fluid	Pellet	Supernatant fluid	Pellet
G	0.4	99.5	5.2	94.8
N	0.8	99.2	32.7	67.3
S	0.4	99.6	3.3	96.7

[a] Results are expressed as the percentage of total recovered radioactivity of each of the major viral structural proteins. Cells were infected at 0 C, incubated as indicated above, and broken by decompression from a N_2 bomb. After breakage, the cells were centrifuged at 500 × *g* for 10 min, and the pellet was discarded. The supernatant fluid was centrifuged at 40,000 × *g* for 20 min; the proteins extracted from the pellet and supernatant fluid were analyzed by polyacrylamide gel electrophoresis.

Flat agar surfaces were prepared by adding 50-ml samples of 2% agar to 250-ml round bottom centrifuge tubes and centrifuging at 3,000 rev/min in a Sorvall HS-4 swinging bucket rotor until the agar solidified. L cells grown in suspension culture were then sedimented onto the agar surface (2.5×10^6 cells per tube) by centrifugation at 3,000 rev/min for 10 min. The supernatant fluid was decanted, and 0.5 ml of virus labeled with ^{3}H-amino acids (a multiplicity of 60 virions per cell) was

added to each tube and centrifuged at 0 C for 20 min at 9,500 rev/min to sediment the virus onto the cells. A 10-ml amount of prewarmed medium 199 was added to each tube, and the tubes were incubated in a water bath for 10 min at 37 C. The tubes were cooled in an ice bath, and a suspension of 0.714-μm polystyrene beads was added at a multiplicity of 10^3 beads per cell. The beads were sedimented onto the cells by centrifugation at 0 C for 10 min at 9,000 rev/min. The tubes were again incubated at 37 C for 30 min to permit optimum uptake of the beads. After this incubation, ten tubes were again cooled on ice, and the infected cells containing the beads were washed off the surface of the agar, centrifuged, and resuspended in cold PBS containing 0.25 M sucrose, 0.5 mg of bovine serum albumin per ml, and 10^{-3} M ethylenediaminetetraacetic acid (EDTA). The cells were ruptured by decompression from a N_2 bomb as described previously (7).

The fractionation scheme shown in Fig. 9 was used to isolate both the membrane-enclosed beads and the other fractions from the cells. Membrane-enclosed beads were sedimented by centrifugation at 25,000 $\times$ g for 15 min and purified by sucrose gradient flotation (7). This is designated in Fig. 9 as fraction 1. The supernatant fluid from the first 25,000 $\times$ g centrifugation was then centrifuged at 40,000 $\times$ g for 20 min to sediment any small membrane fragments remaining after removal of the beads; proteins extracted from both the pellet (fraction 3) and the supernatant fluid (fraction 2) were analyzed by polyacrylamide gel electrophoresis. The protein from the membrane-enclosed beads (fraction 1) was solubilized for gel electrophoresis by the same procedure which was used for the other fractions (*see above*). The beads were removed by centrifugation at 10,000 $\times$ g for 15 min after solubilization of the protein.

Electropherograms of these fractions are shown in Fig. 10. Figure 10A shows the electrophoretic profile of the proteins extracted from intact, purified virions. Figure 10B shows the profile of radioactive proteins in the cytoplasmic membrane isolated by the latex bead method (fraction 1). Both the G and S proteins are present in this fraction in roughly the same proportions as in the intact virion, but the nucleoprotein is essentially absent from this fraction. This fraction represents a rather small proportion of the total recovered virion protein (about 3% of the G and S proteins), but it does show that a relatively pure preparation of cytoplasmic membrane contains primarily viral envelope proteins.

The distribution of viral proteins in the other two fractions (Fig. 10C and 10D) is quite consistent with the earlier results shown in Table 3, in that the soluble fraction (fraction 2) contains predominantly nucleoprotein, whereas the particulate fraction (fraction 3) contains all three structural proteins but is enriched in the G and S proteins.

DISCUSSION

A preliminary electron microscopic study of L cells infected with VS virus showed that fusion of viral and cellular membranes occurred within a very short incubation period (2 to 4 min) and that this fusion required incubation at 37 C after infection at 4 C (6). Although this observation suggested fusion of the viral membrane with the

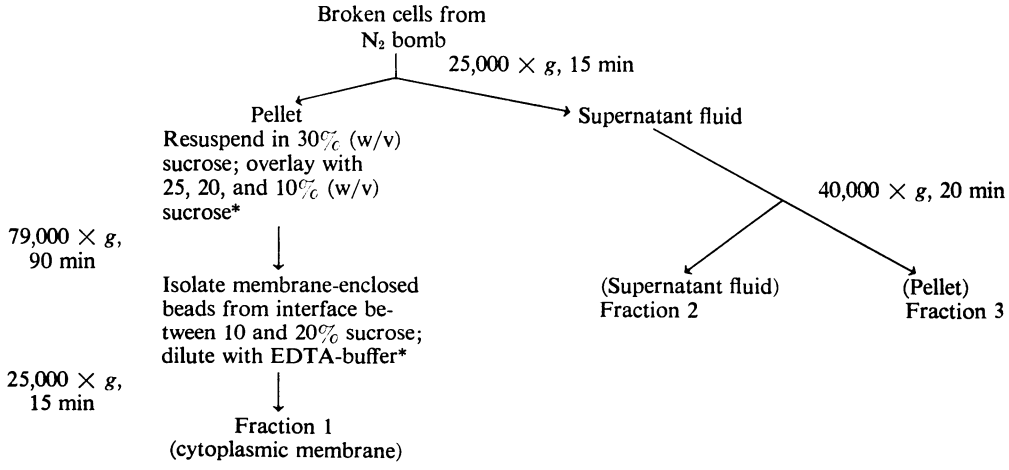


FIG. 9. *Fractionation of infected cells containing polystyrene beads. Sucrose solutions were prepared in a buffer containing 10^{-3} M EDTA and 0.02 M Tris-hydrochloride, pH 6.8.*

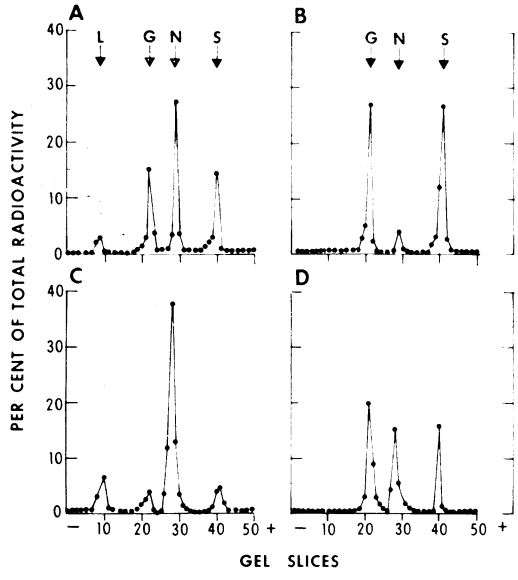


FIG. 10. *Electropherograms of a purified preparation of VS virus and of the various fractions of infected cells obtained by the procedure illustrated in Fig. 9. The cells were infected with ^{3}H-labeled virus at a multiplicity of 60 virus particles per cell, followed by incubation for 10 min at 37 C in the absence of polystyrene beads and incubation for 30 min at 37 C after the addition of the beads. To facilitate comparison, all of the results are expressed as the percentage of total radioactivity recovered from each individual gel. In all cases, the major peaks represent 200 to 1,000 counts/min above background. (A) Electropherogram of ^{3}H-labeled proteins of purified VS virus. (B) Electropherogram of cytoplasmic membrane fraction solubilized from membrane enclosed polystyrene beads (fraction 1, Fig. 9). The G and S protein peaks each represent approximately 200 counts/min. The counts recovered in this fraction represent about 3% of the total G and S proteins recovered. (C) Electropherogram of the supernatant fraction (fraction 2, Fig. 9). This fraction contains predominantly nucleoprotein (protein N). The counts recovered in this fraction represent 16% of the total recovered activity or 35% of the total recovered nucleoprotein. (D) Electropherogram of the microsome fraction (fraction 3, Fig. 9) from infected cells. This fraction is somewhat enriched in the G and S proteins and represents 83% of the total recovered activity.*

host cytoplasmic membrane and possible release of nucleoprotein into the cytoplasm, neither of these phenomena could be tested directly. The release of nucleoprotein into the cytoplasm could not be observed by electron microscopy because of the density of the cytoplasm. No empty shells of virions were observed; therefore, viral membrane on the surface of cells could no longer be detected after the release of the nucleoprotein.

The present study provides several additional kinds of evidence pertaining to the mode of entry of this virus. First, staining of cells with a hybrid antibody specific for VS virus and ferritin has permitted the visualization of viral antigen on the surface of infected cells. The ferritin label was widely distributed on the surface of the cell, as would be expected from the high multiplicity of infection employed in this experiment. The labeled regions on the cell surface were indistinguishable from adjacent unlabeled areas of plasma membrane. This cannot be attributed to newly synthesized viral antigen because of the brief period of incubation at 37 C which was used.

In studies with labeled virus, adsorption of virus followed by incubation for short periods of time resulted in a substantial release of nucleoprotein into the cytoplasmic fraction when cells were broken by a technique which resulted in minimal lysosomal breakage. The particulate fraction of the infected cells, which contained both membrane fragments and unbroken cell organelles, was enriched by a corresponding amount in the viral envelope proteins. A preparation of cytoplasmic membrane isolated from phagocytized latex beads was found to contain primarily the envelope proteins of the virus. The membrane isolated by phagocytosis of latex beads should, by the nature of the technique, consist of a rather pure preparation of cytoplasmic membrane (28).

All of these experiments strongly point to fusion of the virus and host cell membranes followed by release of the nucleoprotein into the cytoplasm as the likely method of viral entry. If a pinocytotic mechanism followed by lysosomal uncoating were invariably involved in viral uptake, it is difficult to understand why viral antigen should be located on the surface of infected cells after brief incubation periods. In a similar fashion, it is difficult to explain the selective release of viral nucleoprotein into the cytoplasm by a mechanism of pinocytosis followed by lysosomal uncoating. Rupture of lysosomes or phagocytic vesicles containing partially degraded virions during the breakage process before cell fractionation cannot account for the selective release of nucleoprotein into the soluble fraction, since partially degraded virions should yield both envelope proteins and nucleoproteins in the soluble fraction. The isolation of a cytoplasmic membrane preparation from infected host cells which contained both envelope proteins of the virion also supports the idea that membrane fusion has occurred and is similarly difficult to reconcile with phagocytosis and lysosomal degradation as a mode of viral entry.

Cohen et al. (1*b*) have recently shown that VS

virus envelope proteins from the cytoplasm of infected cells can bind to plasma membrane isolated from uninfected cells. Such a nonspecific binding of envelope proteins to the cell membrane after degradation of intact virions cannot be strictly ruled out by the experiments described above. However, the conditions employed by Cohen et al. for the in vitro binding of envelope proteins to membrane fragments are quite different from the conditions which we have employed in studying the fate of virus protein during infection and, furthermore, these authors found binding only of the "S" protein to the membrane fragments, whereas we observed in all cases that both the "G" and "S" proteins were found associated with the membrane in proportions quite similar to the proportions in which these proteins are found in the intact virions.

It is difficult to argue that the conditions used in these studies did not allow phagocytosis to take place, since the dells readily took up latex beads. It is possible that phagocytosis does play a role in viral penetration by maintaining virus in close proximity to the cytoplasmic membrane in phagocytic vesicles, thereby increasing the efficiency of the fusion of viral and host cell membranes. Such a role of phagocytosis in the infectious process cannot be ruled out by the experiments described herein.

ACKNOWLEDGMENTS

This investigation was supported by American Cancer Society grants E-617 and T-515, National Science Foundation grant GB-8142, Public Health Service grant CA-10387, from the National Cancer Institute, and Research Career Development Award (to C. A. S.) GM-22053 from the National Institute of General Medical Sciences.

We thank Robert R. Wagner for many helpful discussions.

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