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Intracellular Digestion of Reovirus Particles Requires a Low pH and Is an Essential Step in the Viral Infectious Cycle

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Lysosomotropic drugs such as NH_4Cl have been useful for studying the role of low pH in early events in virus infection. NH_4Cl blocks the production of infectious progeny virus in mammalian reovirus-infected cells. The inhibitory effect of NH_4Cl is mediated by an inhibition of intracellular digestion of reovirus outer capsid proteins. In vitro digestion of viral outer capsid proteins produces infectious partially uncoated particles, called intermediate subviral particles, which are no longer inhibited by the presence of NH_4Cl. These results indicate that proteolytic processing of reovirus outer capsid proteins takes place in a low pH compartment of the cell and is an essential step in the viral infectious cycle.

A wide variety of biologically active molecules, including plasma transport proteins, hormones, asialoglycoproteins, and certain toxins, are taken into cells by a receptor-mediated endocytic pathway (18, 47). In addition, a number of enveloped (19, 20) and nonenveloped (10, 16, 30, 31) viruses also enter cells by receptor-mediated endocytosis. Such viruses are thought to bind to specific receptors on the cell surface. After binding, the viruses are internalized in clathrin-coated vesicles which are uncoated and acidified before fusion with other endocytic vesicles and eventually with lysosomes. In some cases the membrane of the enveloped virus undergoes fusion with the membrane of an intracellular vesicle, resulting in delivery of the viral nucleocapsid to the cytoplasm. This fusion reaction requires exposure of the virus to low pH at some stage of the entry pathway, e.g., in prelysosomal vesicles. To understand the importance of low intravesicular pH for virus penetration, lysosomotropic agents have been used to alter the pH in these vesicles. One such lysosomotropic agent is ammonium chloride (NH_4Cl), a weak base that has been shown to reversibly raise the pH in endocytic vesicles (33) and lysosomes (36).

While much effort has gone into studying the role of pH in the entry and penetration of enveloped viruses, fewer studies have addressed the role of low pH in entry and penetration of nonenveloped viruses (7, 8, 30, 31, 41). Our laboratory and others are studying the icosahedral nonenveloped mammalian reoviruses as models to understand the interactions between nonenveloped viruses and their host cells. It has been shown that reoviruses enter into mouse L cells in endocytic vesicles and are subsequently sequestered in lysosomes (45). Within the cell, viral outer capsid proteins are digested to produce partially uncoated particles called subviral particles (SVPs) (6, 44). SVPs are later converted by the cell to transcriptionally active particles. A role for low pH in reovirus infection was suggested by the fact that growth of reovirus serotypes 1 and 3 in L cells is inhibited by the presence of 10 mM NH_4Cl (7).

In this report we describe further the effect of NH_4Cl on the production of infectious progeny reovirus and identify the stage at which NH_4Cl blocks reovirus growth. Increasing the intravesicular pH of cells by treating virus-infected cells with NH_4Cl prevents virus growth by inhibiting digestion of the viral outer capsid proteins. Proteolytic digestion of the outer capsid proteins in vitro before infection produces an intermediate SVP (ISVP) that is insensitive to growth inhibition by NH_4Cl. These results demonstrate that intracellular digestion of reovirus outer capsid proteins requires a low pH and is an essential step in the viral infectious cycle.

MATERIALS AND METHODS

Cells and viruses. Cells used in these studies were either mouse L cells maintained in our laboratory or mouse L929 cells obtained from the American Type Culture Collection, Rockville, Md. L cell suspensions or monolayer cultures were maintained in Joklik's modified Eagle minimal essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 5% fetal calf serum (FCS)–1% penicillin–1% streptomycin–1% glutamine. L929 cell monolayers were maintained in modified Eagle minimal essential medium with 10% FCS–1% penicillin–1% streptomycin–1% glutamine. Unless otherwise indicated, these were the media used throughout these studies. The virus used in these studies was reovirus serotype 1 (T1) (strain Lang) plaque purified twice on L-cell monolayers. The origin of T1 (strain Lang) has been described (37). The particle/PFU ratio for purified intact T1 was typically 150 to 200:1.

Virus purification. L cells were pelleted and suspended at a concentration of 5×10^6 per ml in 25 ml of Puck's medium supplemented with 1% FCS–20 mM $MgCl_2$. Reovirus T1 was added at a multiplicity of infection (MOI) of 5 and allowed to adsorb at room temperature for 30 min. Cells were diluted 10-fold with medium and maintained in suspension at 35°C for 72 h.

Virus was purified from pelleted cells, as described previously (13), except that isopycnic centrifugation of virus in CsCl was done using an SW27.1 rotor at 23,000 rpm (100,000 × *g*) for 5 h. The banded virus was collected and dialyzed extensively against 5 mM Tris (pH 7.4)–150 mM NaCl–7.5 mM $MgCl_2$.

Radiolabeled virus was prepared as above except that the cells were pelleted after 17 h of incubation at 35°C. They

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were suspended in 250 ml of medium containing one-half the normal amount of methionine and 500 μCi of [^{35}S]methionine (approximately 1,100 Ci/mmol) (New England Nuclear Corp., Boston, Mass.). Virus was purified from the cells after an additional 55 h of incubation at 35°C.

Preparation of T1 ISVPs. T1 ISVPs were prepared by incubating 2.5×10^{11} purified reovirus T1 particles in 0.1 ml of 1× SSC (0.015 M sodium citrate, 0.15 M NaCl) containing 10 μg of chymotrypsin (CHT) for 60 min at 37°C. Under the digestion conditions used here, σ1 and δ were stable after 0 to 24 h of digestion. The particle/PFU ratio of T1 ISVPs was typically about 85:1.

Growth curves. Cells were plated in 2-dram (7.4-ml) glass vials at 2×10^5 cells per vial and were incubated overnight to allow adsorption and spreading to occur. The cells were chilled to 4°C. The medium was removed, and after a wash with cold phosphate-buffered saline (PBS) (pH 7.4), cells were inoculated with 0.1 ml of purified reovirus T1 or T1 ISVPs (MOI, 5 to 10) diluted into Puck's medium supplemented with 1% FCS–20 mM $MgCl_2$. Virus was allowed to adsorb for 1 to 2 h on ice before unbound inoculum was removed by washing cells with 1 ml of cold PBS (pH 7.4). Cells were overlaid with 1 ml of medium and placed in a 5% CO_2 incubator at 37°C. Time points were taken by placing the appropriate vials at −70°C. Before titration samples were frozen, and later they were thawed and subjected to sonication for 15 s at a setting of 3 by using the microprobe of a Head Systems-Ultrasonics, Inc., model W-225R sonicator. All points were done in duplicate. The plaque assay used to titrate the virus was as described previously (42).

One study (see Fig. 10) was performed with and without pretreatment of cells with NH_4Cl. The results were the same either way.

For studying the effect that time of addition of the drug has on reovirus growth (see Fig. 2), 1 ml of prewarmed (37°C) medium containing 40 mM NH_4Cl was added to each 2-dram (7.4-ml) vial (containing 1 ml of medium) at the specified times.

Determination of the extent of intracellular cleavage of T1. Cell monolayers were drained of overlying medium, chilled to 4°C, washed with cold PBS (pH 7.4), and infected with [^{35}S]methionine-labeled reovirus T1 (10^5 particles per cpm) at an MOI of 100. Virus was allowed to adsorb for 1.5 to 2 h at 4°C before unbound inoculum was removed with three cold PBS washes. Cells were overlaid with medium containing 0 to 20 mM NH_4Cl and were incubated for a specified period at 37°C. By use of a rubber policeman, cells were harvested in cold PBS (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and were collected at 4°C by centrifugation at 77 × *g* for 20 min. Cells were suspended in lysis buffer (10 mM Tris [pH 7.4], 0.14 M NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40) and maintained for 6 to 10 min in an ice slurry before nuclei were collected by centrifugation at 940 × *g* for 10 min. The resulting postnuclear supernatant was diluted 10-fold into ice-cold acetone, and precipitated protein was sedimented at 9,770 × *g* for 20 min. After the supernatant fluid was decanted, the residual acetone was carefully removed with a cotton swab. The protein pellet was suspended in Laemmli sample buffer and boiled. Equal numbers of cell equivalents were loaded onto each lane, and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide). The gel was subjected to fluorography, dried, and exposed to preflashed film (X-Omat RP-1; Eastman Kodak Co., Rochester, N.Y.) for autoradiography. Protein bands were quantitated by using a Joyce Loebl double-beam recording microdensitometer. Each lane was scanned four times; the peaks were cut out and weighed, and the averages were determined.

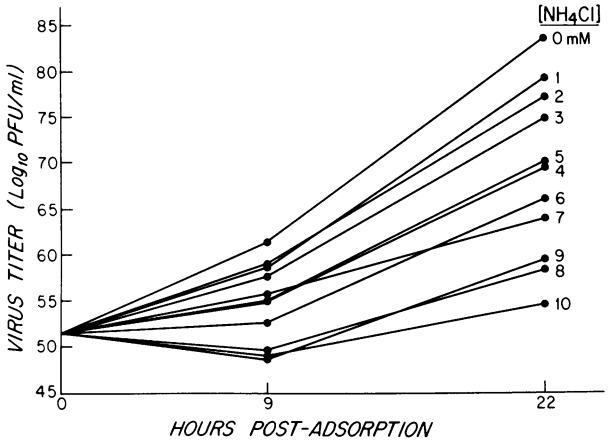

FIG. 1. Effect of NH_4Cl concentration on virus growth. Virus was adsorbed to cells for 1.5 h at 4°C (MOI, approximately 5); the inoculum was removed, and the cells were washed once before addition of medium containing 0 to 10 mM NH_4Cl. Incubation was at 37°C in 5% CO_2. Virus growth was interrupted at 0, 9, or 22 h by placing samples at −70°C. Samples were thawed, sonicated, and titrated to determine infectious yield. All points represent the average of duplicate samples.

Thin-section electron microscopy. Cells were fixed with 2% glutaraldehyde, dehydrated, and embedded in Epon. Thin sections, stained with uranyl acetate and lead citrate, were examined in a JEOL 100B electron microscope. The microscope was operated at 80 kV, and 50-μm condenser and objective apertures were used.

RESULTS

Effect of different doses of NH_4Cl on yield of reovirus T1. The addition of NH_4Cl to cultured mouse peritoneal macrophages (36) or to canine kidney cells (49) causes a rapid and reversible concentration-dependent rise in intralysosomal pH. We have previously reported that growth of reovirus serotypes 1 and 3 is inhibited in the presence of 10 mM NH_4Cl (7). To determine the effect lower concentrations of NH_4Cl and, therefore, milder perturbations of intraendosomal and -lysosomal pH have on reovirus growth, we studied the growth of reovirus T1 in mouse L cells overlaid with medium containing various concentrations (1 to 10 mM) of NH_4Cl. We chose to study reovirus T1 since the analysis of various intermediates of reovirus indicates that, under the conditions used, T1 SVPs are more stable than T3 SVPs (5; L. J. Sturzenbecker, unpublished observation).

Concentrations of NH_4Cl as low as 1 mM had an adverse effect on growth of reovirus T1, and increasing NH_4Cl concentrations had an increasingly inhibitory effect on T1 growth (Fig. 1). Viral yield at 22 h postinfection (p.i.) in 10 mM NH_4Cl-treated cells was reduced more than 1,000-fold compared with that of control cells. These results suggest that low intravesicular pH is important for reovirus growth and that the more intravesicular pH is raised above normal, the greater is the inhibitory effect on reovirus growth. Subsequent experiments showed that 20 mM NH_4Cl inhibits virus growth more completely than 10 mM NH_4Cl, and thus some experiments were done using the higher concentration of drug.

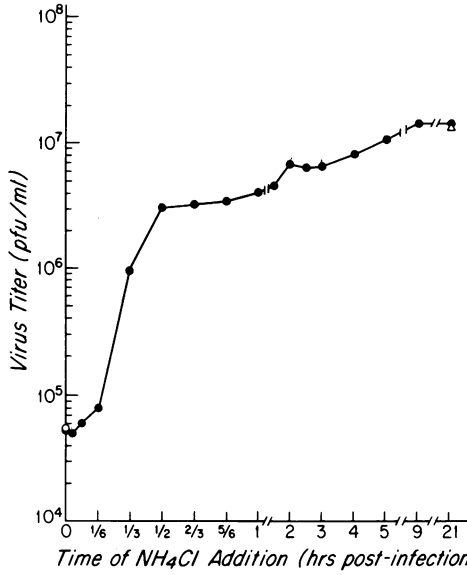

FIG. 2. Effect of time of addition p.i. of NH_4Cl on virus growth. After adsorption of virus for 1 h at 4°C (MOI, approximately 3), the inoculum was removed, and cells were washed once before the addition of NH_4Cl-free medium. Samples were incubated at 37°C in 5% CO_2. At the indicated times, prewarmed medium containing NH_4Cl was added to the samples such that the final NH_4Cl concentration was 20 mM. The cells remained in 20 mM NH_4Cl medium throughout the remainder of the incubation. At 24 h p.i. samples were frozen at −70°C. They were subsequently thawed, sonicated, and titrated for infectious yield. All points represent the average of duplicate samples. ○, Titer of samples frozen before incubation at 37°C. The titer was the same whether or not the samples were made 20 mM NH_4Cl before freezing. △, Virus titer in samples incubated for 24 h at 37°C in the absence of NH_4Cl, i.e., maximal viral yield.

Basis for NH_4Cl inhibition of reovirus growth. To understand the basis for the block to reovirus growth imposed by high concentrations of NH_4Cl, we studied the effect of NH_4Cl during reovirus replication. We first determined whether NH_4Cl itself altered the infectivity of virus particles. Reovirus T1 particles were incubated in 1 to 100 mM NH_4Cl in dialysis buffer (see Materials and Methods) for 2 h at 37°C and then assayed for infectivity in non-NH_4Cl-treated cells. Virus particles preincubated with NH_4Cl showed no change in infectivity (data not shown). Thus, NH_4Cl itself does not alter the infectivity of reovirus particles. We also tested the possibility that high concentrations of NH_4Cl might cause L-cell death and thereby prevent virus growth. In the presence of 20 mM NH_4Cl we found that L cells continued to divide for at least 72 h, although at a reduced rate compared with that of control cells. Furthermore, we found that after 72 h in 20 mM NH_4Cl more than 85% of L cells remained viable (as determined by trypan blue exclusion). Thus, death of the host cell due to NH_4Cl could not account for the inability of reovirus to grow in NH_4Cl-treated cells.

Effect of time of addition of NH_4Cl on yield of reovirus. To determine when NH_4Cl inhibits reovirus growth, we added NH_4Cl to virus-infected cultures at various times p.i. and determined the virus yield of each culture at 24 h, i.e., after a single cycle of virus replication. We observed that to prevent virus growth entirely, NH_4Cl had to be added at the time infection was initiated (Fig. 2). Allowing the infection to progress even 30 min before the addition of NH_4Cl allowed a 60-fold increase in infectious yield compared with a 270-fold increase in yield in control cells. Addition of NH_4Cl later than 30 min had only a minor inhibitory effect on viral yield. These results demonstrate that NH_4Cl inhibits a step that occurs early in the viral replication cycle.

Electron microscopy studies of the early stages of reovirus infection. Having shown that the NH_4Cl effect occurred early in the viral replication cycle, we attempted to define the specific step affected by NH_4Cl. Since NH_4Cl was added to cells after virus was already bound (see Materials and Methods), prevention of virus binding could not have been the basis for NH_4Cl inhibition. However, it was possible that NH_4Cl was preventing uptake of virus particles by the cell. To determine if this was true we used electron microscopy to study thin sections of virus-infected cells in the presence or absence of NH_4Cl.

In the first few seconds after initiation of endocytosis in control cells, virus particles were found in clathrin-coated pits (Fig. 3B). At later times, virus particles were found in vesicles, some of which appeared to be clathrin coated. These vesicles generally contained one or only a few virus particles (Fig. 3D). At 30 min p.i. virus was located in perinuclear vesicles, each containing numerous virus particles (Fig. 3F). The vesicles in which reovirus particles accumulate have previously been identified as lysosomes (45). In our studies of control cells, virus accumulated in vesicles which contained densely staining material and which, therefore, resembled lysosomes.

In NH_4Cl-treated infected cells the endocytosis of reovirus particles was not inhibited. Virus particles were taken into cells in what appeared to be clathrin-coated pits and vesicles (Fig. 3A and C), as in control cells. Furthermore, virus particles accumulated in vesicles in which densely staining material could often be seen (Fig. 3E). Additionally, we observed that vesicles inside NH_4Cl-treated cells were usually swollen.

To quantitatively compare endocytosis of reovirus particles in NH_4-Cl-treated and control cells, we counted several hundred virus particles in electron micrographs of thin sections of infected cells prepared 5 and 30 min p.i. and scored them for location either outside or inside the cell (Table 1). We observed that uptake of reovirus particles by NH_4Cl-treated cells was quantitatively similar to uptake by control cells. We conclude, therefore, that NH_4Cl inhibition of viral growth cannot be explained by an inhibition of viral entry into cells. In addition, our studies suggest that reovirus particles enter L cells via the same pathway in the presence and absence of NH_4Cl and that this pathway is receptor-mediated endocytosis.

Effect of NH_4Cl on intracellular cleavage of viral proteins. Soon after reovirus particles enter the cell the proteins of the outer capsid begin to undergo digestion (presumably in endocytic vesicles or lysosomes). This digestion process takes place during the first several hours of the infection (45). We analyzed viral proteins and polypeptides harvested from infected cells which had been treated or not treated with NH_4Cl to determine whether NH_4Cl inhibits proteolytic processing of the parental virus.

In control cells we observed that digestion began within the first half hour of the infection and was complete by 3 h p.i. (Fig. 4). Outer capsid protein σ3 was digested first, followed by processing of outer capsid protein μ1c to its δ-intermediate form. μ1, the in vivo precursor to μ1c, was also digested to an intermediate form, a polypeptide which migrated under SDS-PAGE between μ1c and δ (14; M. Nibert, unpublished observation). Other viral proteins, in-

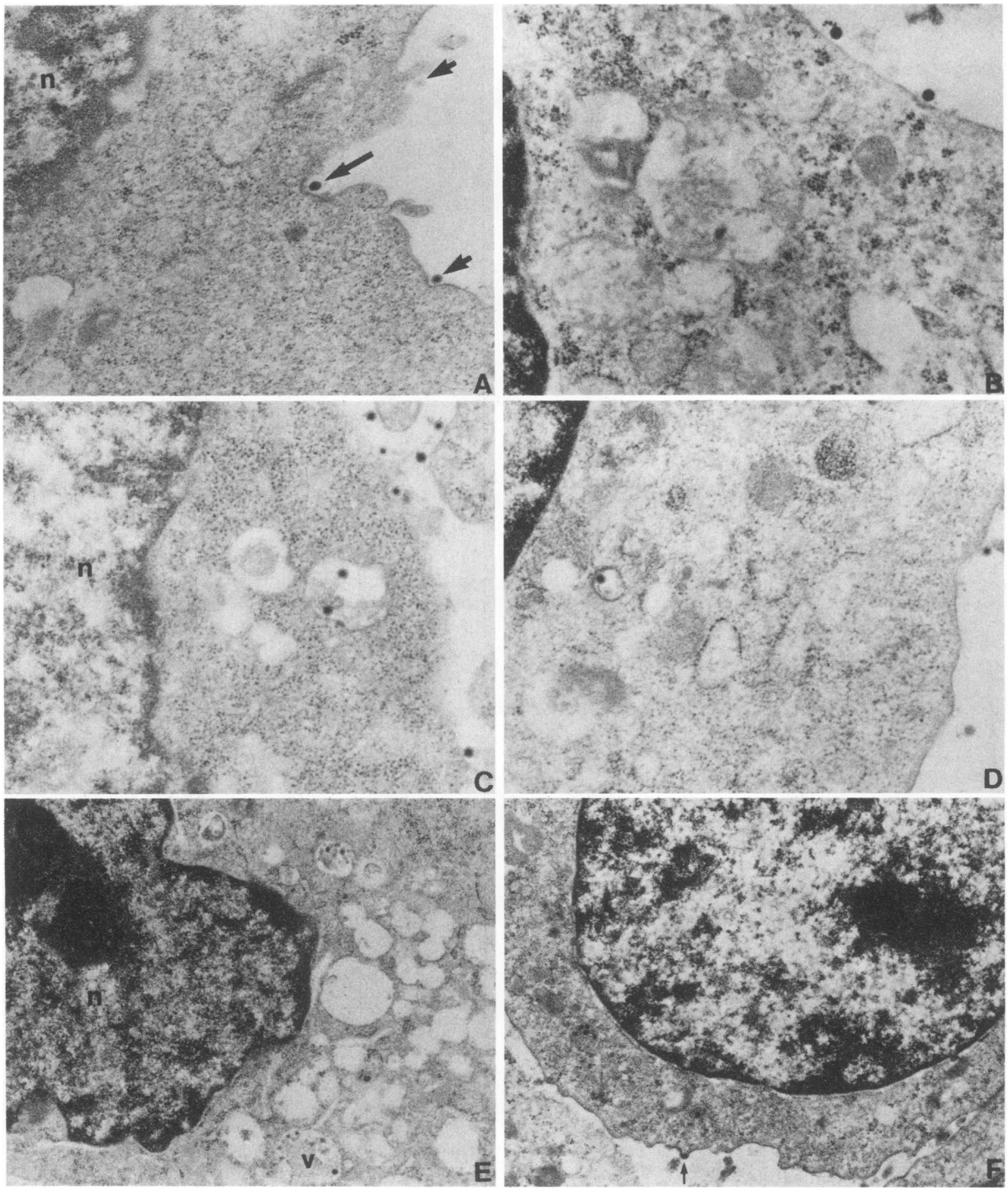

FIG. 3. Thin-section electron micrographs of the early stages of infection of monolayer L cells by intact T1 particles. Cells were incubated in the presence of 10 mM NH_4Cl (panels A, C, and E) or in the absence of NH_4Cl (panels B, D, and F). Cells were harvested 20 sec (panels A and B), 5 min (panels C and D), and 30 min (panels E and F) p.i. (A) Arrows identify reovirus particles; long arrow identifies a virus particle in what appears to be a clathrin-coated pit. Note virus particles in coated pits (panels A and B), coated vesicles (panel D), and in perinuclear vesicles (v) containing amorphous, densely staining material (panels E and F). n, Nucleus. (F) Arrow indicates a budding C-type particle endogenous to L cells. Magnification, ×30,000 (panels A through D); ×15,000 (panels E and F).

cluding the outer capsid protein σ1, appeared to remain undigested during the course of the experiment. Virus particles that have been processed by the cell have been termed SVPs.

In sharp contrast to the extensive processing of virus particles which occurred in control cells, virus particles in cells treated with NH_4Cl showed reduced processing of outer capsid proteins even after 7 h of infection (Fig. 5). The

TABLE 1. Location of reovirus T1 particles in control and NH_4Cl-treated cells at various times p.i.

Virus	NH_4Cl concn (mM) in medium	Time (min) p.i.	Particle location			
			Outside cell		Inside cell	
			No.	%	No.	%
T1	0	5	578	90	67	10
T1	0	30	135	19	581	81
T1	10	5	493	90	57	10
T1	10	30	158	27	417	73
ISVP	0	5	535	92	47	8
ISVP	0	30	116	18	526	82
ISVP	10	5	506	86	82	14
ISVP	10	30	45	8	518	92

greater the concentration of NH_4Cl used to treat cells, the greater was the inhibition of cleavage. These results are shown quantitatively in Fig. 6. Thus, NH_4Cl prevents intracellular processing of viral outer capsid proteins, i.e., the formation of SVPs. The concomitant inhibition of production of infectious progeny virus suggests that the generation of SVPs inside the cell is an essential step in the life cycle of the virus.

Kinetics of intracellular digestion of outer capsid proteins in various concentrations of NH_4Cl. We thought it likely that NH_4Cl at low doses was not blocking but rather slowing down intracellular digestion of outer capsid proteins in a concentration-dependent manner and, thereby, slowing down rather than blocking virus growth. To test this possibility we analyzed the kinetics of intracellular processing of viral proteins in various concentrations of NH_4Cl (Fig. 7). As expected, we found that the presence of low concentrations of NH_4Cl reduced the rate of viral outer capsid processing. Virus particles from cells treated with 2 mM NH_4Cl showed a slight reduction in the rate of outer capsid digestion compared with those from control cells but eventually achieved close to complete outer capsid cleavage. Virus in cells treated with 6 mM NH_4Cl showed a more marked reduction in rate of digestion and did not show

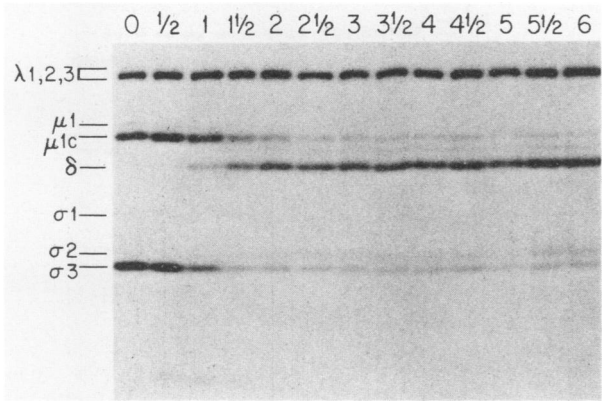

FIG. 4. Kinetics of in vivo digestion of reovirus outer capsid proteins. After adsorption of T1 virus ([^{35}S]methionine labeled) to cells at 4°C, unbound virus was washed off, and cells were overlaid with medium containing no NH_4Cl. Cells were harvested 0 to 6 h p.i., and samples were prepared for SDS-PAGE, as described in Materials and Methods. The fluorographed dried gel was exposed to film for autoradiography. Numbers at the top of each lane represent the time (hours p.i.) at which cells were harvested. Reovirus structural proteins or their derivatives are indicated at the left.

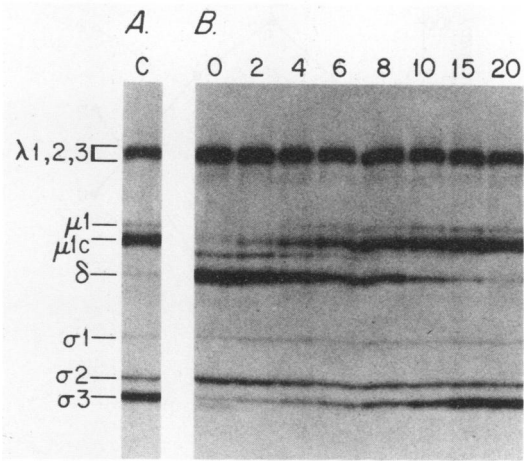

FIG. 5. In vivo cleavage of reovirus outer capsid proteins in cells treated with varying concentrations of NH_4Cl. After adsorption of T1 virus ([^{35}S]methionine labeled) to cells at 4°C, unbound virus was washed off, and cells were overlaid with medium containing 0 to 20 mM NH_4Cl. Cells were harvested immediately (panel A) or after 7 h of incubation at 37°C (panel B) and prepared for SDS-PAGE. The fluorographed dried gel was exposed to film for autoradiography. Numbers at the top of each lane represent the concentration of NH_4Cl in the medium. Reovirus structural proteins or their derivatives are indicated at the left.

complete processing after 20 h of infection. Virus from cells treated with 20 mM NH_4Cl showed little processing over the course of the experiment. Thus, NH_4Cl affects the rate of outer capsid cleavage. The greater the concentration of drug,

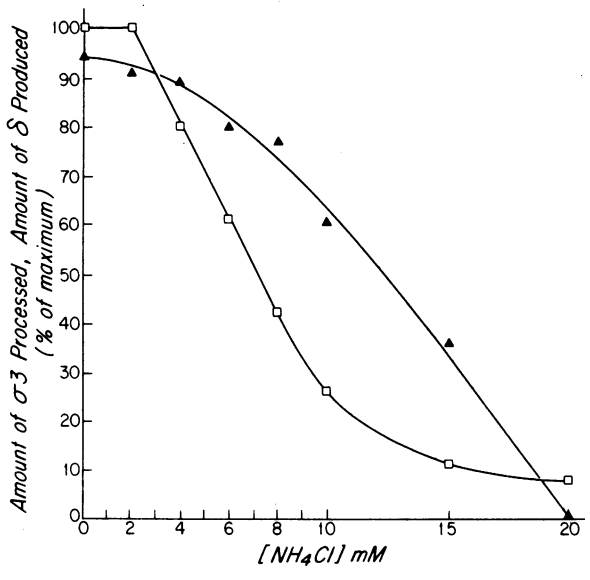

FIG. 6. Quantitation of the amount of viral outer capsid cleavage occurring in various concentrations of NH_4Cl. The polypeptide bands in the autoradiograph shown in Fig. 5 were quantitated by densitometry. The amount of protein in each lane was standarized by using the σ2 protein. ▲, Amount of σ3 processed. Maximum σ3 (100%) was that amount present at the start of infection (Fig. 5, lane C); all other σ3 calculations were made relative to this amount. □, Amount of δ produced. Maximum δ (100%) was that amount present in control cells at 7 h p.i. (Fig. 5, lane 0); all other δ calculations were made relative to this amount.

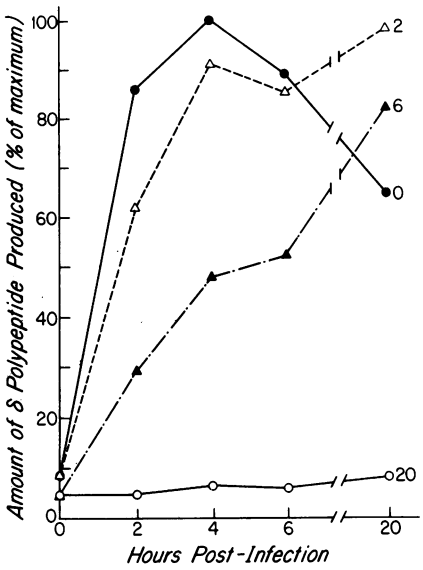

FIG. 7. Effect of NH_4Cl concentration on the kinetics of cleavage of outer capsid protein μ1c to the δ polypeptide. Experimental protocol was the same as that described in the legend to Fig. 4, except that the overlying medium contained no (●), 2 (△), 6 (▲), or 20 mM (○) NH_4Cl. Cells were harvested at 0, 2, 4, 6, and 20 h p.i., and samples were prepared for SDS-PAGE as described in Materials and Methods. The resultant gels were fluorographed, dried, and exposed for autoradiography. Autoradiographs (not shown) were scanned by densitometer to determine the intensity of each polypeptide band present at each time point. The amount of protein in each lane was standardized using the λ-proteins band. Maximum δ (100%) was that amount present at 4 h p.i. in control cells. All other δ calculations were made relative to this amount.

the greater the reduction in rate of digestion. High concentrations of NH_4Cl reduced the rate of digestion so much that inhibition of processing was essentially complete over the course of our studies.

We have shown that low concentrations of NH_4Cl simply slowed outer capsid processing. If the degree of processing is, as we believe, closely associated with the amount of virus growth achieved, then low concentrations of NH_4Cl should also slow down but not block virus growth. Indeed, viral yields in cells treated with 2 to 6 mM NH_4Cl did, after 3 to 5 days, reach the same levels as viral yields in control cells (Table 2). The greater the concentration of drug added to cells, the greater was the time required for maximum yield to be achieved. In contrast, doses of 15 mM NH_4Cl or greater allowed only a low level of virus growth. Maximum viral yield in the presence of 15 or 20 mM NH_4Cl was reached early in infection and did not increase upon further incubation. Thus, low concentrations of NH_4Cl reduced both the rate of outer capsid processing and the rate of infectious progeny production, whereas high doses blocked outer capsid processing and significantly reduced viral yields.

TABLE 2. Viral yield in reovirus T1-infected cells treated with various concentrations of NH_4Cl

NH_4Cl concn (mM)	Maximum virus titer[a] (PFU/ml)	Day p.i. at which maximum titer reached
0	6.3×10^8	3
2	6.3×10^8	3
4	5.1×10^8	4
6	6.2×10^8	5
8	$(2.2 \times 10^8)^b$	na[c]
10	$(1.0 \times 10^8)^b$	na
15	3.3×10^7	2
20	1.2×10^7	2

[a] Initial titer, 1.7×10^5 PFU/ml.
[b] Titer at 5 days p.i.
[c] na, Steady state not achieved by 5 days p.i.

Direct evidence that the NH_4Cl block is through inhibition of cleavage: preprocessing of outer capsid proteins creates particles which are no longer NH_4Cl sensitive. The analysis of reovirus particles harvested from cells treated with high concentrations of NH_4Cl showed that cleavage of viral outer capsid proteins was inhibited. The outer capsid proteins of reovirus particles can be digested in vitro with proteolytic enzymes. Knowing this, we reasoned that we could determine whether the NH_4Cl inhibition of cleavage is the critical block to virus growth by processing viral outer capsid proteins in vitro and testing the processed virus for sensitivity to growth inhibition by NH_4Cl. Since most prior studies involving reovirus SVPs have used T3 virus, we wished to investigate the in vitro digestion kinetics of T1 and to determine whether the processed particles were structurally stable and could be used to infect cells.

In vitro production and analysis of T1 ISVPs. SVPs generated in vitro have been termed ISVPs. To produce T1 ISVPs, we used proteolytic cleavage with CHT (13, 23). Upon digesting T1 virus particles with CHT we observed that outer capsid protein σ3 was cleaved to transient intermediate-sized polypeptides which were rapidly digested (Fig. 8). After initiation of σ3 digestion, the small amount of remaining μ1 in the reovirion was cleaved, and μ1c was digested to its δ-intermediate form, a polypeptide some 14,000 M_r smaller than μ1c (M. Nibert, unpublished observation). A limit digest was essentially achieved after 30 min of digestion. Continued digestion had no apparent effect on ISVPs, and they were structurally stable even after 24 h of digestion (data not shown). The pattern of CHT digestion

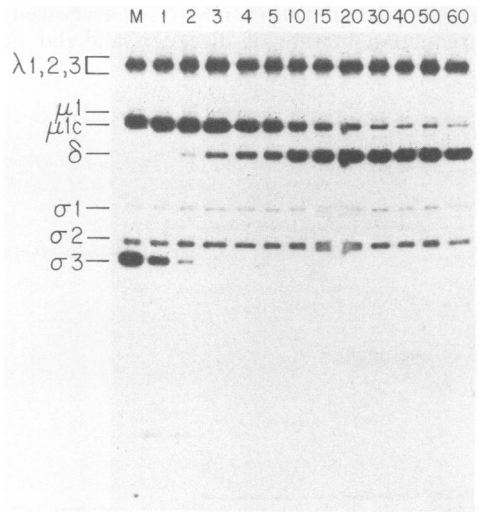

FIG. 8. Kinetics of in vitro digestion of reovirus T1 with CHT. Virus particles ([^{35}S]methionine labeled) were incubated with CHT at 37°C for the indicated times (minutes). Lane M is a mock digestion which received 1× SSC instead of enzyme and was incubated at 37°C for 30 min. Immediately after termination of digestion, samples were diluted into Laemmli sample buffer, boiled, and subjected to SDS-PAGE. The fluorographed dried gel was exposed to film for autoradiography.

that we observed for T1 was similar to that previously described for T3 (3). Furthermore, this digestion pattern was the same as that observed when virus particles were digested intracellularly (Fig. 4).

To determine whether in vitro-generated T1 ISVPs are similar to the SVPs produced by the cell, we purified radiolabeled parental T1 SVPs from L cells according to standard procedure after 7 h of incubation at 37°C and compared them with T1 ISVPs which were produced in vitro with CHT and subjected to the same purification. SDS-PAGE revealed that ISVPs and SVPs had very similar protein compositions (Fig. 9); both had lost most or all of σ3 and had δ as their major form of the μ1 protein. In addition, both particles had σ1 intact. The buoyant density of the SVPs produced in vitro and in vivo was between 1.356 and 1.373. The only difference we detected was that the SVPs produced in the cell contained slightly more undigested σ3 than did ISVPs. Overall, the T1 ISVPs produced in vitro were very similar in protein composition and density to the SVPs generated by the cell.

We additionally tested the possibility that the presence of NH_4Cl could inhibit the in vitro digestion of reovirus outer capsid proteins. We observed that the presence of 1 to 100 mM NH_4Cl had no effect on in vitro CHT digestion of reovirus particles (data not shown). These results suggested that the NH_4Cl-mediated inhibition of outer capsid digestion in the cell is indirect.

Capacity of T1 ISVPs to bypass the NH_4Cl block. To determine whether digestion of the reovirus outer capsid in vitro produces an SVP whose growth in L cells is no longer inhibited by NH_4Cl, we infected NH_4Cl-treated cells with ISVPs and monitored viral yield over a 24-h period. Viral growth curves constructed from these data (Fig. 10) revealed the following.

First, the number of infectious units present at the start of the infection was the same for both intact T1 particles and T1 ISVPs. This suggested that binding of T1 particles was not significantly altered when they were converted in vitro to ISVPs. This is consistent with the fact that the cell attachment protein σ1 (28) was not digested or removed upon generation of ISVPs. Second, reovirus T1 grown in the absence of NH_4Cl for 24 h yielded a titer of infectious virus 1,000-fold greater than that initially present, while in the presence of 10 mM NH_4Cl, there was only a small increase in titer between 0 and 24 h. Third, in contrast to the results with intact T1, reovirus T1 ISVPs grew as well in the presence as in the absence of 10 mM NH_4Cl. By 24 h, ISVPs in the presence or absence of NH_4Cl reached a titer which was fourfold higher than that achieved by intact virus in the absence of the drug. Finally, in addition to the higher yield of virus from ISVPs, the kinetics of growth of ISVPs was different; the eclipse phase was shortened. After 8 h of incubation in the absence of NH_4Cl, T1 showed no increase in titer, whereas T1 ISVP titers in both the presence and absence of NH_4Cl increased 100-fold. An additional 4 h were required for T1 (without NH_4Cl) to achieve a 100-fold increase in titer. This shortened eclipse phase for ISVPs has been documented for T3 as well (6, 11).

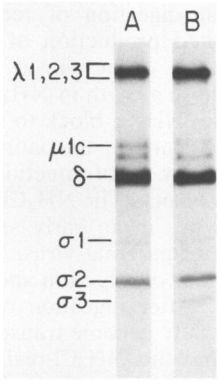

FIG. 9. SDS-PAGE of purified [^{35}S]methionine-labeled T1 ISVPs and T1 SVPs. (A) T1 ISVPs produced in vitro by digestion with CHT for 60 min at 37°C. L cells were added to the digestion mix before ISVP purification. (B) T1 SVPs harvested from L cells 7 h p.i. ISVPs and SVPs were purified by the procedure used to purify intact T1 virus from suspension L cells.

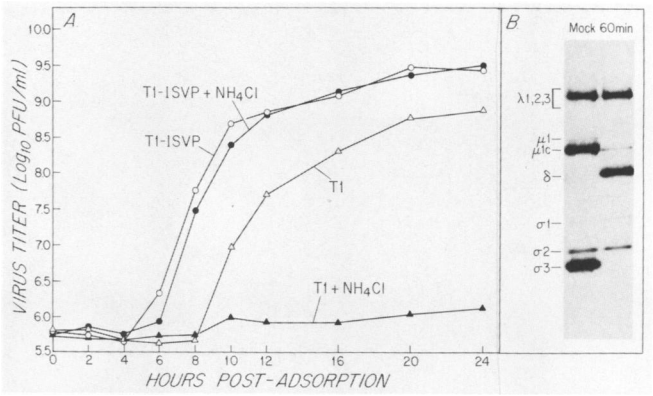

FIG. 10. (A) Growth curves of reovirus T1 and T1 ISVPs in control and NH_4Cl-treated L cells. After adsorption of virus in the cold, unbound virus was washed off, and cells were overlaid with medium containing no or 10 mM NH_4Cl. After incubation at 37°C for the indicated times, samples were frozen at −70°C and later thawed, sonicated, and titrated. All points represent the average of duplicate samples. (B) Autoradiograph of a SDS-polyacrylamide gel (10% acrylamide), resolving the polypeptides of mock digested and chymotryptically digested (60 min at 37°C) reovirus T1. The sample in the lane labeled 60 min is the T1 ISVPs used in panel A.

The above results showed that in vitro cleavage of the T1 outer capsid proteins creates an SVP that can generate infectious progeny even in the presence of high concentrations of NH_4Cl. This demonstrates that NH_4Cl does not block steps in the viral infectious cycle which occur subsequent to outer capsid processing and proves directly that digestion of viral outer capsid proteins is essential to viral growth.

Pathway of entry used by T1 ISVPs. T1 ISVPs were able to bypass the block to virus growth imposed by NH_4Cl, but whether they were able to do this by using the normal pathway of entry into the cell or an alternate one was not known. Electron microscopic studies of thin sections of T1 ISVP-infected cells (Fig. 11) revealed that, in both the presence and absence of NH_4Cl, T1 ISVPs were found in the same cellular locations as intact T1 particles (Fig. 3). At early times p.i., virus particles in ISVP-infected cells were located in what appeared to be clathrin-coated pits (Fig. 11A) and vesicles (Fig. 11B), each of which generally contained only one virus particle. At later times, virus was concentrated in vesicles which resembled lysosomes in shape and electron density (Fig. 11C and D). These results are consistent with the notion that intact T1 particles and T1 ISVPs enter cells by the same pathway and hence that T1

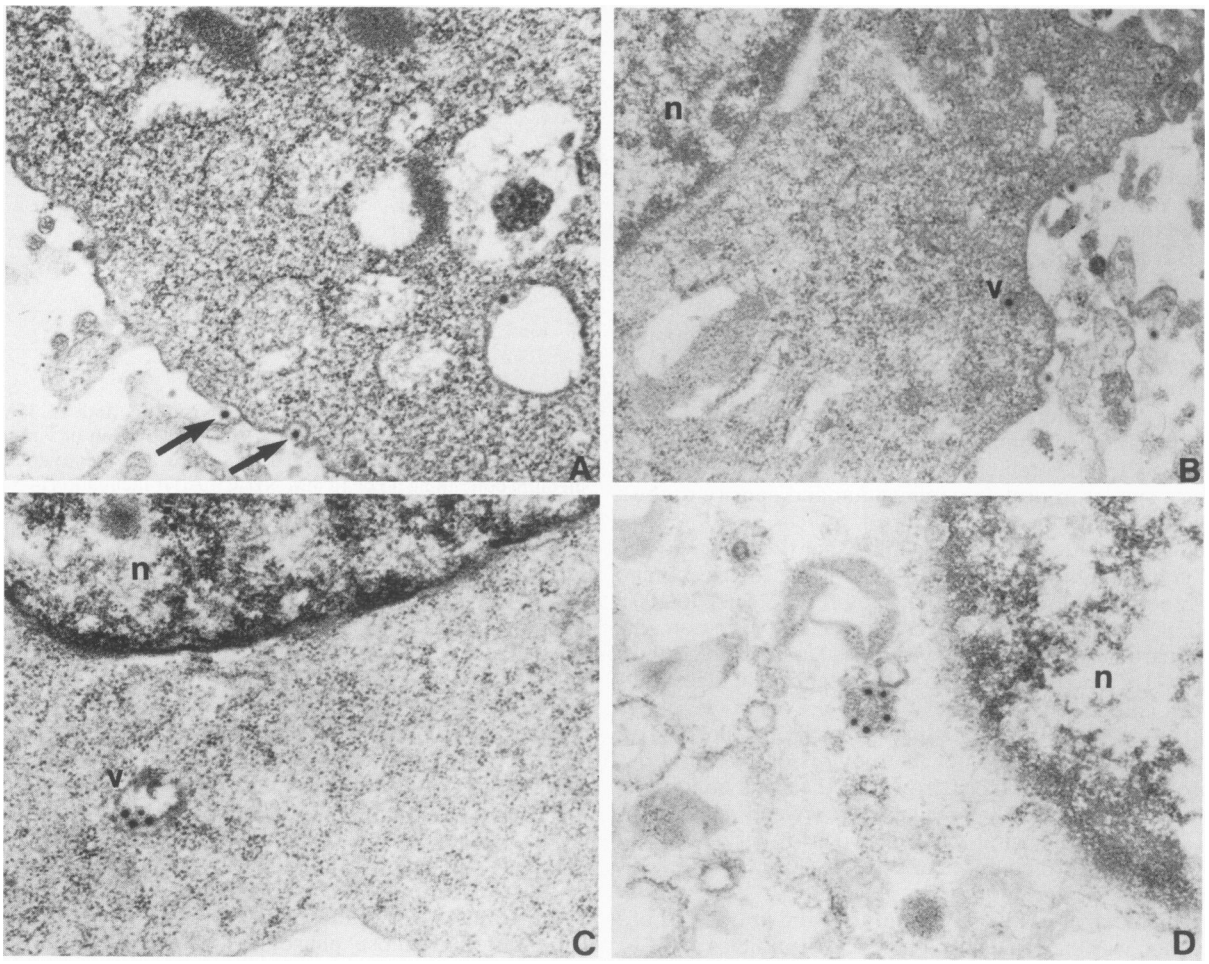

FIG. 11. Thin-section electron micrographs of the early stages of infection of monolayer L cells by T1 ISVPs. Cells were incubated in the presence of 10 mM NH_4Cl (panels A and C) or in the absence of NH_4Cl (panels B and D). Cells were harvested at 5 min (panels A and B) and 30 min (panels C and D) p.i. Arrows (used in panel A only) identify reovirus particles. Note virus particles in what appear to be clathrin-coated pits (shown by arrows in panel A; also see panel B), clathrin-coated vesicles (panel B), and in uncoated vesicles (v) containing amorphous, densely staining material (panels C and D). n, Nucleus. Magnification, ×30,000.

ISVPs bypass the NH_4Cl block to infection while following the normal pathway of entry into the cell.

Quantitative analysis of the locations of virus particles at various times p.i. revealed that in infected control cells, 10 and 8% of cell-associated T1 particles and T1 ISVPs, respectively, were located inside cells at 5 min p.i. (Table 1). At 30 min p.i., the numbers increased to 81 and 82% for T1 particles and ISVPs, respectively. Furthermore, the presence of NH_4Cl in the medium did not appear to significantly alter the rate of endocytosis of either type of virus particle. At 5 min p.i. 10 and 14% of T1 particles and ISVPs, respectively, were located inside NH_4Cl-treated cells. At 30 min p.i. the numbers increased to 73 and 92%, respectively. Thus, the rate of uptake of intact T1 particles and T1 ISVPs by control or NH_4Cl-treated cells appears to be similar. These results support the conclusion that intact particles and ISVPs enter cells by the same pathway.

DISCUSSION

In this report we show that intracellular digestion of the reovirus T1 particle requires a low pH and is an essential step in the viral infectious cycle. Low concentrations of the lysosomotropic amine NH_4Cl reduced the rate at which the reovirus outer capsid was digested inside the cell and altered the kinetics of virus growth. High concentrations of NH_4Cl inhibited intracellular digestion of reovirus outer capsid proteins and prevented production of infectious progeny virus. Digestion of the outer capsid in vitro, however, produced an SVP whose growth in NH_4Cl-treated cells was uninhibited. Thus, the NH_4Cl block to reovirus growth lies at the stage of virus particle uncoating, and this step is critical for the establishment of infection.

Nature of the NH_4Cl block. The NH_4Cl block to production of progeny reovirus lies in an early step in the virus life cycle, the uncoating of parental virus. This process begins within the first half hour of infection and continues until 2.5 to 3 h p.i. (Fig. 4). After digestion of the outer capsid, reovirus SVPs eventually become transcriptionally active in the cell. The fact that the NH_4Cl-resistant T1 ISVPs are transcriptionally inactive at the time of infection (data not shown) suggests that activation of viral transcription in these ISVPs takes place even in the presence of NH_4Cl. This suggests that the changes that occur in the SVP to make it transcriptionally active can take place in the NH_4Cl-altered pH of the endosome or lysosome.

How might the NH_4Cl block be mediated? The addition of NH_4Cl to cultured cells causes a rapid and reversible rise in intraendosomal and -lysosomal pH (12, 36, 49). The amount by which the pH is increased is determined by the concentration of NH_4Cl used. For example, the intralysosomal pH of macrophages in 0, 0.1, 1, and 10 mM NH_4Cl was approximately 4.75, 4.8, 5.4, and 6.2, respectively (36). Although we did not measure intralysosomal pH in mouse L cells, it is likely that NH_4Cl has the same effect on these cells and that incrementally increasing the concentration of NH_4Cl from 0 to 20 mM causes incremental increases in intravesicular pH. The fact that the kinetics of both outer capsid digestion and virus growth decrease with increasing NH_4Cl concentration suggests that there is an intravesicular pH optimum for outer capsid processing and virus growth. The greater the deviation from the normal intravesicular pH toward the alkaline level, the greater the inhibitory effect on the virus.

Further evidence that low intravesicular pH is important in reovirus infection comes from studies which showed that other agents known to raise intravesicular pH also have an inhibitory effect on reovirus growth in L cells (data not shown). Methylamine functions like NH_4Cl, showing a concentration-dependent inhibition of virus growth and reducing viral yield to the same degree as NH_4Cl when used at similar concentrations. Monensin (1 μM) and chloroquine (50 μM) reduced viral yield at 20 h p.i. by 50 and 90%, respectively.

How might the increased intravesicular pH inhibit reovirus outer capsid digestion? Increased pH may inactivate the enzyme(s) that is involved in digestion of the reovirus outer capsid. Such an enzyme may be located in acidic vesicles in the cells. Indeed, lysosomes contain proteases such as the cathepsins which are enzymatically active at low pH (2). Alternatively, the enzyme which digests reovirus outer capsid proteins may be a component of the virus particle. Perhaps both cellular and viral proteases are involved. The possibility that high pH might prevent the outer capsid proteins from assuming a conformation necessary for cleavage to occur by preventing proper exposure of a cleavage site is unlikely since reovirus particles are readily cleaved in vitro at pHs above that likely to be achieved in intracellular compartments in cells treated with high concentrations of NH_4Cl.

The possibility that NH_4Cl grossly alters the pathway of entry used by the virus was eliminated when virus-infected cells were examined by electron microscopy of thin sections. We cannot, however, exclude the possibility that more subtle changes in the endocytic pathway might have taken place. Changes such as inhibition of endosomal-lysosomal fusion could result in the virus losing access to essential lysosomal proteases. Although our results suggest that delivery of virus to secondary lysosomes occurs in the presence of 10 mM NH_4Cl, we cannot say with certainty that all reovirus particles reach the lysosome and, therefore, lysosomal enzymes in the presence of the drug.

Importance of processing to initiation of reovirus infection. The digestion of the reovirus outer capsid proteins to form an SVP is termed outer capsid processing. Our results indicate that outer capsid processing is essential to virus growth. This seems logical given that transcriptionally inactive virus particles enter the cell in endosomes and subsequently get sequestered inside lysosomes. From within the endosome or lysosome the virus must activate its transcriptase and successfully deliver its transcribed mRNA to the cytoplasm. Processing of the outer capsid proteins may be necessary to accomplish each of these tasks. Indeed, activation of the viral transcriptase in vitro requires extensive digestion of the outer capsid proteins (1, 43, 46), and activation of the transcriptase in vivo probably requires outer capsid processing as well (9, 44, 45).

How reovirus delivers its mRNA to the cytoplasm is not currently understood. One possibility is that mRNA delivery requires translocation of the entire SVP across a cellular membrane to the cytoplasm. This mechanism appears to be utilized in adenovirus entry, in which large numbers of virus particles are found in the cytoplasm soon after initiation of infection (16). The same mechanism has been proposed for reovirus (4). In contrast to the findings of Borsa et al. (4), however, our electron microscopy studies indicate that the vast majority, if not all, of reovirus particles found inside the cell are in membrane-bound vesicles. We cannot, however, eliminate the possibility that small numbers of particles become free in the cytoplasm.

An alternate mechanism for delivery of reovirus mRNA to the cytoplasm requires penetration of a cellular membrane (most likely that of the lysosome) by only a portion of the reovirus SVP. Both the complete- and partial-penetration mechanisms require an intimate association between the SVP and a cellular membrane. It is likely that outer capsid processing is necessary to enhance hydrophobicity or allow conformational changes in the SVP which allow it to associate with the membrane.

Comparison with other viruses and some bacterial toxins. Certain lysosomotropic agents, including NH_4Cl, chloroquine, amantadine, and monensin, are effective in protecting cells from infection by Semliki Forest virus (19, 21), influenza virus (24, 26), vesicular stomatitis virus (32, 35), poliovirus, and rhinovirus (30, 31), as well as from intoxication by diphtheria toxin (DT) (38), *Pseudomonas* exotoxin (15), and anthrax lethal toxin (17). For Semliki Forest (21), influenza (24), and vesicular stomatitis viruses (32), the block to viral reproduction is in fusion of the viral membrane with that of the endosome or lysosome, i.e., release of the viral nucleocapsid into the cytoplasm. Drugs such as monensin inhibit poliovirus infection at or before the stage of release of parental RNA into the cytoplasm (30). Monensin also inhibits the production of poliovirus SVPs in infected cells, a step which may be necessary for release of parental RNA (30). Drugs which inhibit intoxication by *Pseudomonas* exotoxin block mainly at the level of clustering and internalization of toxin-receptor complexes (15). The inhibition of DT toxicity imposed by NH_4Cl and chloroquine can be bypassed by incubating toxin-treated cells briefly at low pH. This allows toxin to penetrate the plasma membrane directly and obviates the need for low intravesicular pH (38). Semliki Forest virus (48) and poliovirus (31) can also be led to fuse with the plasma membrane by low pH treatment of virus-infected cells. Thus, as for reovirus, exposure of each of these viruses and toxins to low pH is an important early step in infection or intoxication.

The requirement for cleavage of viral proteins, as demonstrated here for reovirus, is also true of enveloped viruses. Indeed, both the hemagglutinin of influenza virus (25, 27) and the membrane glycoprotein F of Sendai virus (22, 40) must be cleaved from their precursor forms for the viruses to be infective and for Sendai virus to have cell fusion activity (40). Similarly, nicking of DT is a requirement for its direct penetration of the plasma membrane (39). While the cleavage of reovirus proteins takes place inside the cell, cleavage of Sendai and influenza virus proteins occurs before virus entry into the cell.

We have shown that processing of the reovirus particle is a critical step in the viral pathway of entry into the cell and

does not merely serve to degrade the virus particle. It has been suggested (29, 34) that unnicked DT may be cleaved and thereby activated in the lysosome. Conclusive evidence for a requirement for intracellular cleavage of DT is still lacking, however.

Overview. Although the mechanism of membrane penetration by a nonenveloped virus such as reovirus still remains an enigma, much has been learned about the early steps in infection leading up to penetration. As for many enveloped viruses, contact with a low pH environment at early times p.i. is an essential occurrence in the reovirus infectious cycle. For the enveloped viruses studied to date, low pH has been shown to be necessary to induce conformational changes in viral proteins which result in fusion of the viral and cellular membranes. Our data indicate that for reovirus infection low pH is essential in allowing intracellular processing of viral outer capsid proteins.

Outer capsid processing during receptor-mediated entry of reovirus particles may serve an important early regulatory role for the virus; it may ensure that the viral transcriptase is activated only in the proper environment (14). In addition to viruses, it may be discovered that other polypeptides or other more complex protein structures use proteolytic digestion during endocytosis as an important step in determining subsequent intracellular events.

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LITERATURE CITED

1. **Banerjee, A. K., and A. J. Shatkin.** 1970. Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase. J. Virol. **6**:1–11.
2. **Barrett, A. J.** 1972. Lysosomal enzymes, J. T. Dingle (ed.), p. 46–135. *In* Lysosomes, a laboratory handbook. American Elsevier Publishing Co., New York.
3. **Borsa, J., D. G. Long, M. D. Sargent, T. P. Copps, and J. D. Chapman.** 1974. Reovirus transcriptase activation *in vitro*: involvement of an endogenous uncoating activity in the second stage of the process. Intervirology **4**:171–188.
4. **Borsa, J., B. D. Morash, M. D. Sargent, T. P. Copps, P. A. Lievaart, and J. G. Szekely.** 1979. Two modes of entry of reovirus particles into L cells. J. Gen. Virol. **45**:161–170.
5. **Borsa, J., M. D. Sargent, T. P. Copps, D. G. Long, and J. D. Chapman.** 1973. Specific monovalent cation effects on modification of reovirus infectivity by chymotrypsin digestion in vitro. J. Virol. **11**:1017–1019.
6. **Borsa, J., M. D. Sargent, P. A. Lievaart, and T. P. Copps.** 1981. Reovirus: evidence for a second step in the intracellular uncoating and transcriptase activation process. Virology **111**: 191–200.
7. **Canning, W. M., and B. N. Fields.** 1983. Ammonium chloride prevents lytic growth of reovirus and helps to establish persistent infection in mouse L cells. Science **219**:987–988.
8. **Carrillo, E. C., C. Giachetti, and R. H. Campos.** 1984. Effect of lysosomotropic agents on the foot-and-mouth disease virus replication. Virology **135**:542–545.
9. **Chang, C.-T., and H. J. Zweerink.** 1971. Fate of parental reovirus in infected cell. Virology **46**:544–555.
10. **Chardonnet, Y., and S. Dales.** 1970. Early events in the interaction of adenovirus with HeLa cells. 1. Penetration of type 5 and intracellular release of the DNA genome. Virology **40**:462–477.
11. **Cox, D. C., and C. W. Clinkscales.** 1976. Infectious reovirus subviral particles: virus replication, cellular cytopathology, and DNA synthesis. Virology **74**:259–261.
12. **DeDuve, C., T. DeBarsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoot.** 1974. Lysosomotropic agents. Biochem. Pharmacol. **23**:2495–2531.
13. **Drayna, D., and B. N. Fields.** 1982. Activation and characterization of the reovirus transcriptase: genetic analysis. J. Virol. **41**:110–118.
14. **Ewing, D. D., M. D. Sargent, and J. Borsa.** 1985. Switch-on of transcriptase function in reovirus analysis of polypeptide changes using 2-D gels. Virology **144**:448–456.
15. **FitzGerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. Cell **21**:867–873.
16. **FitzGerald, D. J. P., R. Podmanabhan, I. Pastan, and M. C. Willingham.** 1983. Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell **32**:607–617.
17. **Friedlander, A. M.** 1986. Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261**:7123–7126.
18. **Goldstein, J. L., R. G. W. Anderson, and M. S. Brown.** 1979. Coated pits, coated vesicles and receptor mediated endocytosis. Nature (London) **279**:679–685.
19. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
20. **Helenius, A., M. Marsh, and J. White.** 1980. The entry of viruses into animal cells. Trends Biochem. Sci. **5**:104–106.
21. **Helenius, A., M. Marsh, and J. White.** 1982. Inibition of Semliki Forest virus penetration by lysosomotropic weak bases. J. Gen. Virol. **58**:47–61.
22. **Homma, M., and M. Ohuchi.** 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural differences of Sendai viruses grown in eggs and tissue culture cells. J. Virol. **12**:1457–1465.
23. **Joklik, W. K.** 1972. Studies on the effect of chymotrypsin on reovirions. Virology **49**:700–715.
24. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plaque virus by I-adamantanamine hydrochloride. Virology **37**:632–641.
25. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza viruses by trypsin treatment. Virology **68**: 426–439.
26. **Koff, W. C., and V. Knight.** 1979. Inhibition of influenza virus uncoating by rimantadine hydrochloride. J. Virol. **31**:261–263.
27. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the haemagglutinin polypeptide. Virology **68**:440–454.
28. **Lee, P. W. K., E. C. Hayes, and W. K. Joklik.** 1981. Protein sigma-1 is the reovirus cell attachment protein. Virology **108**: 156–163.
29. **Leppla, S. H., R. B. Dorland, and J. L. Middlebrook.** 1980. Inhibition of diptheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. **255**:2247–2250.
30. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Mechanism of the entry into the cytosol of poliovirus type 1: requirement for low pH. J. Cell Biol. **98**:1194–1200.
31. **Madshus, I. H., S. Olsnes, and K. Sandvig.** 1984. Different pH requirements of entry of two picornaviruses, human rhinovirus 2 and murine encephalomyocarditis virus. Virology **139**:346–357.
32. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1982. Pathway of vesicular stomatitis virus entry leading to infection. J. Mol. Biol. **156**:609–631.
33. **Maxfield, F. R.** 1982. Weak bases and ionophores rapidly and reversibly raise the pH in endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. **95**:676–681.
34. **Middlebrook, J. L., and D. L. Leatherman.** 1982. Differential

sensitivity of reticulocytes to nicked and unnicked diphtheria toxin. Exp. Cell Res. **138**:175–182.
35. **Miller, D. K., and J. Lenard.** 1980. Inhibition of vesicular stomatitis virus infection by spike glycoprotein. J. Cell Biol. **84**:430–437.
36. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75**: 3327–3331.
37. **Ramig, R. F., R. K. Cross, and B. N. Fields.** 1977. Genome RNAs and polypeptides of reovirus serotypes 1, 2, and 3. J. Virol. **22**:726–733.
38. **Sandvig, K., and S. Olsnes.** 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. **87**:828–832.
39. **Sandvig, K., and S. Olsnes.** 1981. Rapid entry of nicked diphtheria toxin into cells at low pH. J. Biol. Chem. **256**:9068–9076.
40. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:475–490.
41. **Seth, P., D. J. P. Fitzgerald, M. C. Willingham, and I. Pastan.** 1984. Role of a low-pH environment in adenovirus enhancement of the toxicity of a *Pseudomonas* exotoxin-epidermal growth factor conjugate. J. Virol. **51**:650–655.
42. **Sharpe, A. H., and B. N. Fields.** 1982. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. Virology **122**:381–391.
43. **Shatkin, A. J., and J. D. Sipe.** 1968. RNA polymerase activity in purified reovirus. Proc. Natl. Acad. Sci. USA **61**:1462–1469.
44. **Silverstein, S. C., C. Astell, D. H. Levin, M. Schonberg, and G. Acs.** 1972. The mechanisms of reovirus uncoating and gene activation *in vivo*. Virology **47**:797–806.
45. **Silverstein, S. C., and S. Dales.** 1968. The penetration of reovirus RNA and initiation of its genetic function in L-strain fibroblasts. J. Cell Biol. **35**:197–230.
46. **Skehel, J. J., and W. K. Joklik.** 1969. Studies on the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. Virology **39**:822–831.
47. **Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn.** 1983. Endocytosis and the recycling of plasma membrane. J. Cell Biol. **96**:1–27.
48. **White, J., J. Kartenbeck, and A. Helenius.** 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. **87**:264–272.
49. **Yoshimura, A., and S.-I. Ohnishi.** 1984. Uncoating of influenza virus in endosomes. J. Virol. **51**:497–504.