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Kinetics of Poliovirus Uncoating in HeLa Cells in a Nonacidic Environment

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Lysis of HeLa cells infected with poliovirus revealed intact virus; 135S particles, devoid of VP4 but containing the viral RNA; and 80S empty capsids. During infection the kinetics of poliovirus uncoating showed a continuous decrease of intact virus, while the number of 135S particles and empty shells increased. After 1.5 h of infection conformational transition to altered particles resulted in complete disappearance of intact virions. To investigate the mechanism of poliovirus uncoating, which has been suggested to depend on low pH in endosomal compartments of cells, we used lysosomotropic amines to raise the pH in these vesicles. In the presence of ammonium chloride, however, the kinetics of uncoating were similar to those for untreated cells, whereas in cells treated with methylamine, monensin, or chloroquine, uncoating was merely delayed by about 30 min. This effect could be attributed to a delay of virus entry into cells after treatment with methylamine and monensin, whereas chloroquine stabilized the viral capsid itself. Thus, elevation of endosomal pH did not affect virus uncoating. We therefore propose a mechanism of poliovirus uncoating which is independent of low pH.

In recent years, cell biology studies have revealed the importance of receptor-mediated endocytosis as an internalization route for many ligands (18, 31, 42), thereby improving our knowledge about modes of virus entry into permissive host cells. Ultrastructural studies have shown that nonenveloped viruses, such as adenovirus (4), reovirus (37), and picornaviruses (46), and enveloped viruses, such as Semliki Forest virus (17), vesicular stomatitis virus (26), and influenza virus (44), may enter cells via endosomal vesicles. The recent identification of the receptor for poliovirus (28) pointed to receptor-mediated endocytosis as the mode of entry of this virus species. The pathway of poliovirus entry and the mechanism of uncoating, however, have not yet been clarified. Adsorption of the virus to its specific binding site and subsequent clustering of receptor-ligand complexes in coated pits are suggested to be early steps of particle internalization. Coated pits give rise to endosomes which become acidic soon after their formation (14, 38). The low endosomal pH causes dissociation of receptor-ligand complexes (6, 16), thus allowing the further processing of internalized ligands. Evidence exists that endosomes possess proteolytic activity (9), indicating that acid proteolysis is not confined to lysosomal function, but may also contribute to protein processing at the endosomal level during receptor-mediated endocytosis (10). The nucleocapsid of some enveloped viruses is released into the cytoplasm by fusion of the viral envelope with the endosomal membrane at low pH (25). Furthermore, an acidic environment is known to be required for the proteolytic digestion of outer capsid proteins of the nonenveloped reovirus (37).

Similarly, uncoating of picornaviruses has been suggested to require a low-pH environment, but the mechanism that might cause the release of the viral genome has not been elucidated. The supposition of the critical role of low pH during initiation of picornavirus infection was deduced mainly from experiments investigating the effects on viral replication of reversing endosomal acidification. In fact, the yield of virus progeny from infected cells, treated with lysosomotropic agents to elevate endosomal pH values, was substantially reduced in experiments with different picornavirus species (2, 46). According to these results, it has been proposed that low pH is required for uncoating of poliovirus (24).

We attempted to test this hypothesis more thoroughly and analyzed the generation of poliovirus eclipse products in the presence and absence of several lysosomotropic agents to gain insight into the poorly described mechanism of poliovirus uncoating (5). We observed two types of conformational altered subviral particles during cell-mediated eclipse, in accordance with previous reports (21). The transition of intact virions to particles with altered sedimentation rates was accompanied by the release of the viral genome from the capsid. On the basis of the kinetics of poliovirus uncoating, found to be unchanged in a nonacidic environment, and the morphological characteristics of the intermediate particles generated, we propose an uncoating mechanism independent of low pH.

MATERIALS AND METHODS

Cells and viruses. Monolayers of HeLa cells were grown in Eagle minimal essential medium (MEM) in plastic Petri dishes (diameter, 6 cm; Nunc, Roskilde, Denmark). Poliovirus type 1 (Mahoney) was used throughout these experiments. Virus propagation, purification, and plaque titration were performed as described previously (39, 40). Radioactively labeled virus was grown in the presence of [^{3}H]leucine (3.5 μCi/ml), [^{35}S]methionine (4.2 μCi/ml), [^{3}H]myristic acid (2.5 μCi/ml), or [^{3}H]uridine (6.3 μCi/ml); all radiochemicals were from Amersham International, Brunswick, Federal Republic of Germany.

Treatment with lysosomotropic amines, monensin, and glutathione. Solutions of each compound to be tested were prepared. These are 20 mM methylamine (E. Merck, AG, Darmstadt, Federal Republic of Germany), 100 μM chloroquine (Sigma, Munich, Federal Republic of Germany), 100 mM ammonium chloride (Merck), 10 μM monensin (Sigma), and 10 mM glutathione (Sigma); they were obtained by

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dilution of stock solutions into Eagle MEM. The pH of each solution was adjusted to neutrality with NaOH or HCl.

Synchronized infection of HeLa cells. HeLa cell monolayers were washed twice with ice-cold Eagle MEM and incubated for 20 min at 37°C in Eagle MEM with or without the different compounds to be tested. The cell monolayers were rechilled to 2°C for 10 min, and ^{35}S-labeled poliovirus at a multiplicity of infection (MOI) of 100 PFU per cell was added. Adsorption was allowed to proceed for 1 h. Thereafter, the monolayers were washed three times with Eagle MEM, with or without one of the weak bases, to remove unbound particles and further incubated at 37°C for the times indicated in the Figure legends. Finally, eluted particles were removed by an additional two washings.

Sucrose gradient centrifugation. The infected cells were lysed with 0.5 ml of 0.25% Nonidet P-40 (Sigma) and 0.25% sodium dodecyl sulfate (SDS; Serva, Heidelberg, Federal Republic of Germany). The mixture was held for about 3 min at room temperature until a clear solution had formed. The extract (0.5 ml) was layered onto a 10 to 30% (wt/wt) sucrose gradient in phosphate-buffered saline (PBS) and centrifuged for 2 h at 40,000 rpm in an SW 41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 5°C. Fractions of approximately 200 μl were collected and counted for radioactivity.

Recovery of adsorbed virus by low-pH treatment. HeLa cell monolayers were preincubated with Eagle MEM or 100 mM methylamine in Eagle MEM for 20 min at 37°C and rechilled to 2°C for 10 min. ^{35}S-labeled virus was added at an MOI of 100 PFU per cell and allowed to adsorb for 1 h. After removal of unbound particles, the temperature was shifted for the times stated in the legend to Fig. 2. Then the samples were immediately rechilled to 2°C and overlaid for 2 min with glycine buffer at pH 1.5 (50 mM glycine in 0.9% NaCl, adjusted with HCl to pH 1.5). Neutral pH was rapidly restored by washing after the low-pH treatment with Eagle MEM. The radioactivity recovered by acid treatment and subsequent five washings was counted. The cells were lysed with 1% SDS in double-distilled water to determine cell-associated radioactivity.

Preparation of neutralizing ^{3}H-labeled polyclonal IgG. Preparation of antisera in rabbits against poliovirus type 1 (Mahoney) has been described previously (40). Poliovirus-specific immunoglobulin G (IgG) antibodies were isolated by affinity chromatography. Activated Affi-Gel 10 (10 ml; Bio-Rad Laboratories, Munich, Federal Republic of Germany) was washed once with 50 ml of propanol and then three times with 20 ml of double-distilled water in the cold. The gel was treated with 3.5 ml of poliovirus type 1 (Mahoney) in 0.3 M triethanolamine hydrochloride (pH 8.2) in the cold overnight. The virus solution contained 71 A_{260} units, equivalent to 9.6 mg of virus. The gel was washed in the cold until it was free of unbound virus. From measurements of the supernatant, it was calculated that about 50% of the input virus was covalently bound to the gel. The gel was transferred to a column and treated overnight in the cold with 3 ml of poliovirus antiserum which had been previously inactivated by heating to 56°C for 30 min. The column was washed with PBS until extinction at 280 nm reached zero. Antibodies were eluted with 0.5 M acetic acid into 1 M Tris hydrochloride (pH 8.7) and immediately brought to neutral pH. After dialysis against PBS the yield was 5.1 mg of IgG, using $A_{280}^{1\%}$ = 13.5 as standard. Radio-labeling of IgG with *N*-succinimidyl-[2,3-^{3}H]propionate (Amersham International) has been described previously (39). The antibody was proved by SDS-polyacrylamide gel electrophoresis to be pure IgG and neutralized virus efficiently. The specific activity of [^{3}H]IgG was 2.59 × 10^{4} dpm/pmol.

Antibody binding to adsorbed virus. HeLa cell monolayers were preincubated for 20 min at 37°C in Eagle MEM with or without monensin at concentrations of 10 and 20 μM. The cells were rechilled for 10 min at 2°C, and virus at an MOI of 100 PFU per cell was added. Adsorption proceeded for 2 h, after which unbound virions were removed. After the stated time of incubation at 37°C, the cells were rechilled instantaneously to 2°C and polyclonal anti-poliovirus [^{3}H]IgG was allowed to bind for 1 h. Unbound antibody was removed by washing five times, and cell-associated radioactivity was counted. The amount of nonspecific binding of antibodies was determined by counting mock-infected samples and was subtracted from the radioactivity obtained in the different probes.

Treatment with RNase A. [^{3}H]uridine-labeled virus and isolated [^{3}H]uridine-labeled 135S particles were treated with 0.1 mg of RNase A (Sigma), and 0.5 mg of bovine serum albumin (Sigma) was added. The preparations were held for 15 min at room temperature. The RNA was precipitated by adding 10% trichloroacetic acid, and the probe was centrifuged at low speed. The pellet was washed three times by resuspension in PBS. Finally, the supernatants and the sediments were counted for radioactivity.

SDS-polyacrylamide gel electrophoresis. Fractions containing [^{3}H]leucine-labeled 135S particles were collected from sucrose gradients and dialyzed against PBS for 5 h to lower the sucrose concentration. The particles were disrupted in a boiling solution of 1% mercaptoethanol and 5% SDS. The polypeptides were electrophoresed in 12.5% cylindrical gels prepared as described elsewhere (20). The gel was sliced, and each fraction was incubated at 60°C for 6 h with 200 μl of 30% H_2O_2 to dissolve the gel and counted for radioactivity.

RESULTS

Time course of poliovirus-specific alteration in early stages of infection. Poliovirus bound to permissive host cells at low temperature and allowed to penetrate and eclipse by incubation at 37°C rapidly loses its structural integrity. The distribution pattern of viral particles in different functional and morphological states at various times after infection indicates efficiency and kinetics of uncoating of the viral RNA. These patterns were obtained by sucrose gradient analysis of viral material recovered from infected cells. Infection of HeLa cells was synchronized by treatment with ^{35}S-labeled poliovirus at an MOI of 100 PFU per cell for 1 h at 2°C, with subsequent temperature shift for different periods. The cells were disrupted by detergents, and the extract was analyzed by sucrose gradient centrifugation. After 15 min of incubation at 37°C (Fig. 1A, curve a), the bulk of internalized virus sedimented at 156S and a small peak of viral material sedimenting at 135S was discernible. After 30 min (curve b) an additional peak at 80S appeared. The number of intact particles was substantially reduced after 1 h at 37°C (curve d), and the corresponding peak at 156S was completely absent from material recovered from samples held for 90 min at 37°C (curve e). Since altered particles were isolated from cells by using 0.25% SDS and 0.25% Nonidet P-40, these detergents could be responsible for the breakdown of small amounts of subviral degradation products to very slowly sedimenting material, which could be recovered from the top of the gradients. The kinetics were reproducible and were the results of several experiments. At the MOI

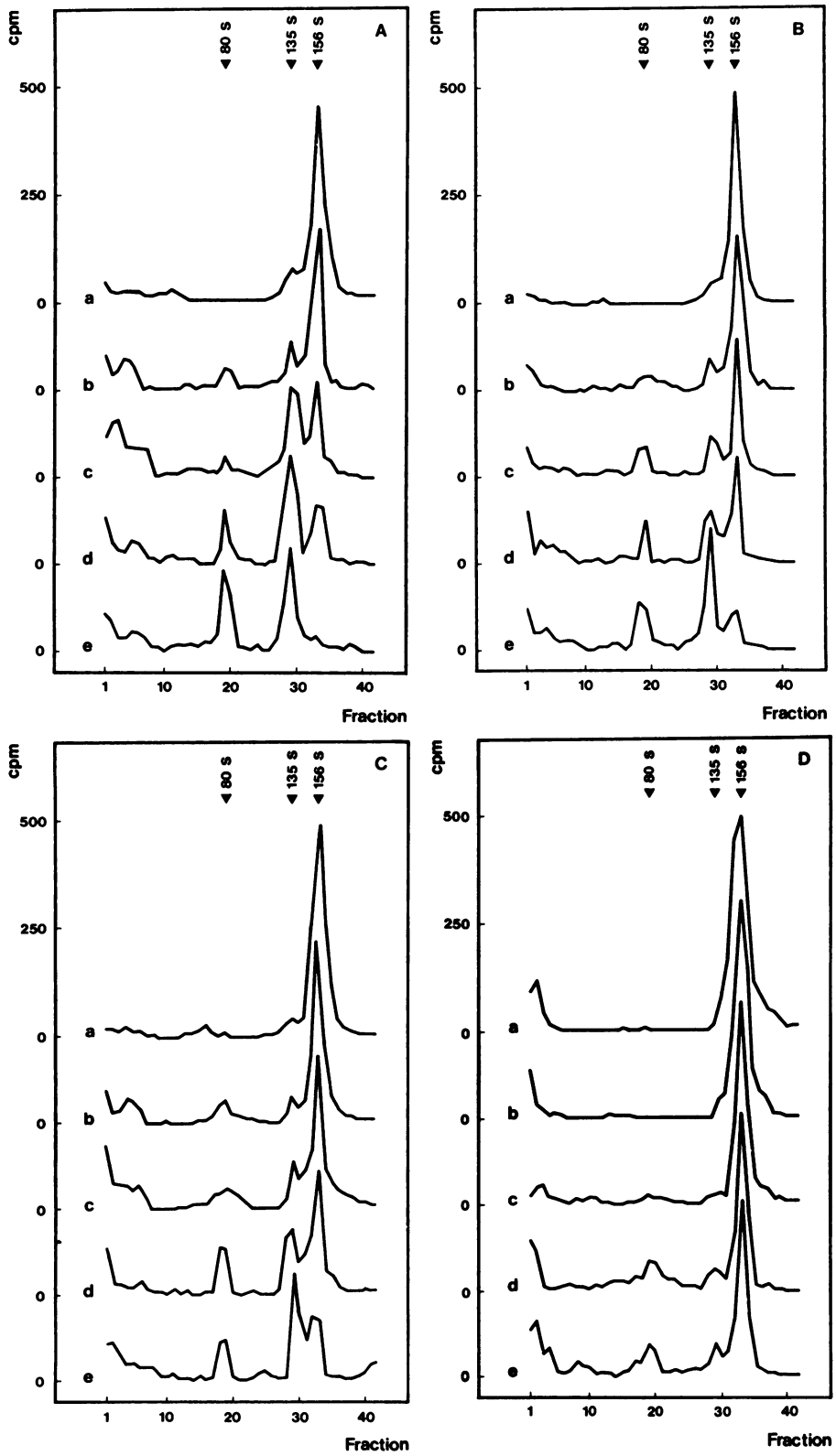

FIG. 1. Kinetics of poliovirus uncoating in cold-synchronized infection of HeLa cells as determined by sucrose gradient centrifugation in the absence (A) or presence of lysosomotropic agents: 20 mM methylamine (B), 10 μM monensin (C), or the antiviral agent 10 mM glutathione (D). Cells were lysed 15 min (a), 30 min (b), 45 min (c), 60 min (d), or 90 min (e) after temperature shift. Sedimentation is from left to right. For details, see Materials and Methods.

used, the virus adsorption rate of a maximum of 4% of the input limited the yield of subviral particles.

Effect of lysosomotropic weak bases on poliovirus-specific alteration. Endosomal vesicles are continuously acidified by an ATP-dependent proton pump during receptor-mediated endocytosis (38). The most common method of studying these effects relies on the use of lipophilic weak bases which are membrane permeable when uncharged at neutral pH and relatively membrane impermeable once protonated. Thus, they will accumulate within these vesicles, keeping the pH near neutrality by trapping protons (35). By using fluorescein-labeled ligands, pH changes and their perturbation by lysosomotropic agents can be quantitatively monitored, since the intensity and excitation spectrum of fluorescein fluorescence are titratable functions of pH (27, 30). Since in previous reports low endosomal pH has been conjectured to be a prerequisite for picornavirus uncoating (24), one would expect inhibition of poliovirus uncoating in the presence of lysosomotropic bases.

To test this hypothesis, we analyzed the sedimentation patterns of viral material recovered from cells infected with poliovirus in the presence of these compounds. Experiments were carried out as described for Fig. 1A, using Eagle MEM containing the compound to be tested in the appropriate molarity. In the presence of 20 mM methylamine (Fig. 1B), considerable degradation occurred. As judged by comparing the ratios of intact particles to particles sedimenting at 135S, in the presence (Fig. 1B) and absence (Fig. 1A) of methylamine, the transition of virions to subviral degradation products was delayed by approximately 30 min when methylamine was present. If poliovirus uncoating required endosomal low pH, complete prevention of alteration would be expected to persist even after 90 min, since it is well known that the endosomal pH, in the permanent presence of lysosomotropic amines in the concentration range used, remains elevated for at least 2 h (32). The fact that in the presence of 20 mM methylamine, after 90 min at 37°C (Fig. 1B, curve e) the bulk of intact particles had undergone conformational alteration to 135S particles and 80S empty shells reflects virus uncoating in a nonacidic environment. The kinetics of virus uncoating were similarly affected in the presence of 10 μM monensin. Uncoating was not prevented and virus degradation did occur with the delayed appearance of subviral particles (Fig. 1C), compared with control data (Fig. 1A). Nor did 100 μM chloroquine lead to a block of virus uncoating (data not shown); the degradation kinetics were merely delayed by about 30 min. After 90 min at 37°C, the amount of intact virus was invariably reduced under conditions of elevated endosomal pH.

When 10 to 100 mM ammonium chloride was used, the resulting sedimentation patterns were indistinguishable from those obtained in the absence of lysosomotropic agents (data not shown). Ammonium chloride, which is known to elevate endosomal pH values like the other agents tested, with comparable potency (35), did not have any inhibitory effect on the time course of poliovirus-specific degradation, even at concentrations up to 100 mM. Evidence exists, therefore, that low pH is not required for poliovirus uncoating, since lysosomotropic weak bases did not influence the appearance of subviral degradation products at all (in the case of ammonium chloride) or caused a simple delay in the time course of degradation steps. None of the compounds tested blocked the generation of particles with altered sedimentation rate in a manner that would parallel their effect on the endosomal pH. Protection against poliovirus uncoating has been suggested to occur upon treatment with 10 mM glutathione, because this compound exerts capsid-stabilizing effects against heat-induced degradation (12). In the presence of glutathione, even after 90 min at 37°C, the virus was almost resistant to cell-mediated alteration (Fig. 1D). Glutathione did not influence virus adsorption and internalization, but virus progeny was strongly reduced (K. Wetz, unpublished data). Hence, a clear distinction can be made between delay of virus uncoating effected by lysosomotropic agents and uncoating block achieved by treatment with glutathione.

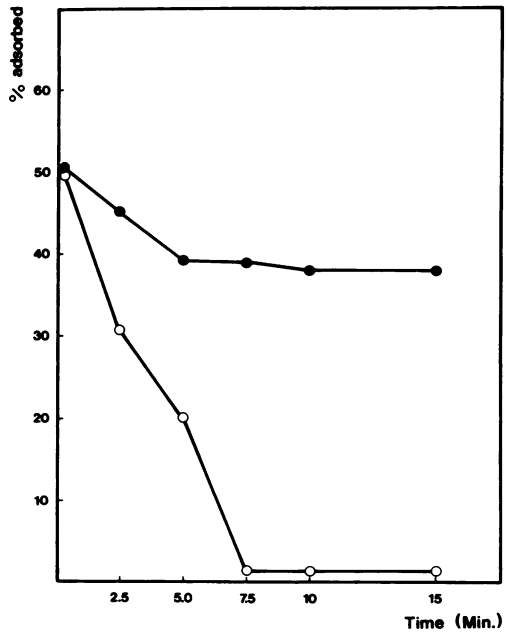

FIG. 2. Recovery of cell-adsorbed virus by low-pH treatment. After adsorption of virus at 2°C for 1 h and different times of incubation at 37°C, cells were treated with glycine buffer at pH 1.5 to release preadsorbed particles. Cells were pretreated with 100 mM methylamine (●) or not treated (○). Total radioactivity recovered by acid treatment and five subsequent washings was measured.

Effects of lysosomotropic weak bases not associated with endosomal pH elevation. The question was raised about which properties of methylamine, monensin, and chloroquine could account for their delaying effects on poliovirus uncoating steps. Ammonium chloride is known to be a lysosomotropic agent almost devoid of pharmacological side effects, whereas methylamine, chloroquine, and monensin are highly active pharmacological substances with various effects on cellular compartments other than the endosomal apparatus (7, 35). For this reason we considered that compounds that influenced viral uncoating rates exerted their effects through properties not associated with their lysosomotropic character. Two basic mechanisms, possibly resulting in retardation of degradation steps, were proposed: (i) inhibitory action on events occurring before uncoating, e.g., inhibition of the internalization mechanism; and (ii) stabilizing effects, protecting the capsid from destabilization and further structural degradation, analogous to the action of glutathione or the WIN compounds (36).

Influence of methylamine on virus internalization. Figure 2 shows the kinetics of recovery of cell-adsorbed virus at pH 1.5 in samples infected in the presence or absence of 100 mM methylamine. The virus was allowed to adsorb for 1 h at 2°C, and unbound virus was removed. The temperature was shifted to 37°C for different periods, and the cells were

overlaid with ice-cold glycine buffer at pH 1.5 for 2 min to remove loosely bound particles (45). Ninety percent of cells remained viable after the pH 1.5 treatment, as determined by their ability to exclude the dye trypan blue. Without temperature shift, about 50% of adsorbed particles could be recovered by acid treatment (Fig. 2). The amount of virus released at low pH diminished rapidly with increasing time of incubation at 37°C, and the virus had completely passed beyond the acid-sensitive state after 7.5 min at 37°C. In the presence of 100 mM methylamine, even after 15 min at 37°C, subsequent acid treatment led to disruption of 38% of receptor-ligand complexes (Fig. 2), although the amount of virus bound to cells at 2°C was constant whether the cells were pretreated with 100 mM methylamine or not. Thus, early events of virus entry conferring resistance to adsorbed particles against low-pH treatment were affected by methylamine. After incubation for 30 min at 37°C in the presence of the compound, virus was no longer recoverable by acid treatment (data not shown), indicating that virus internalization is not completely blocked by methylamine, but is delayed by about 25 min. This is in agreement with the observations shown in Fig. 1B. The prolonged appearance of intact particles in sucrose gradients reflects the delayed internalization of these particles in the presence of methylamine. Adsorbed virus remained at the cell surface, resistant to degradation and uncoating. The operational criterion of acid recovery distinguishes between loosely bound particles released in a low-pH environment and particles in a more complex and stable state of virus-cell interaction and hence measures early steps of virus internalization.

Influence of monensin on virus internalization. Different approaches had to be used to demonstrate the distinct effects on virus internalization of methylamine and monensin, because the acid recovery test did not show an inhibitory effect of monensin (see Discussion). Therefore, another operational criterion was adopted to investigate internalization efficiency in the presence of monensin: the loss of antibody sensitivity (19). Virus was allowed to adsorb to HeLa cells at 2°C in the presence of 10 or 20 μM monensin or in the absence of the compound. After a temperature shift for the times stated, the samples were rechilled and incubated with equimolar amounts of ^{3}H-labeled polyclonal anti-poliovirus IgG at 2°C. After 1 h the cells were washed thoroughly to remove unbound or nonspecifically bound antibody, disrupted with detergents, and counted for radioactivity. In control experiments, preadsorbed virus had almost entirely lost sensitivity to antibody binding by about 30 min of incubation at 37°C. At this time, specifically bound antibody corresponded to 9% of the adsorbed particles (Fig. 3). However, in samples incubated for 30 min in the presence of 10 μM monensin, only 30% of prebound virus proceeded to states of the internalization pathway no longer admitting antibody binding, while 70% remained accessible to antibodies. Even after 60 min of incubation, only about 50% of adsorbed virus had escaped the binding reaction in cells treated with 10 μM monensin. When the concentration of monensin was increased to 20 μM, the effect observed was still more pronounced, totally inhibiting eclipse events for about 20 min at 37°C. Any reaction of the antibody with subviral degradation products, namely, 135S and 80S particles, could be excluded by immunoprecipitation analysis (data not shown). Hence, the data shown clearly demonstrate virus internalization to be delayed by 30 to 45 min in the presence of monensin. This correlates well with the delay of virus degradation observed during sucrose density gradient analysis in the presence of monensin (Fig. 1C).

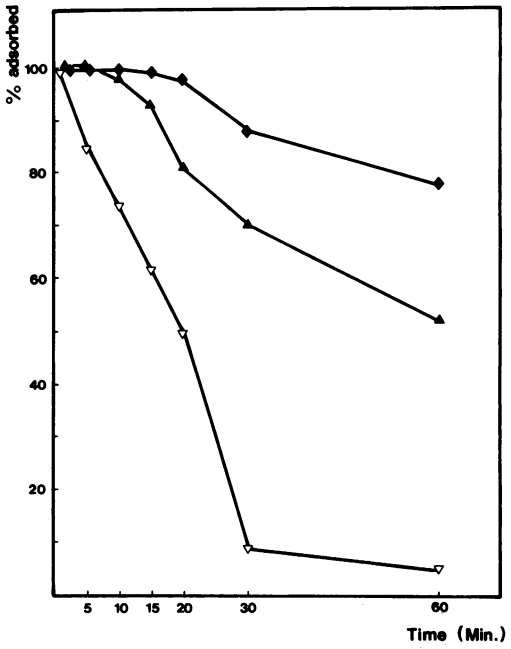

FIG. 3. Loss of antibody sensitivity of cell-adsorbed virus. Virus was allowed to adsorb to cells at 2°C. After different times of incubation at 37°C, infected cells were treated with ^{3}H-labeled anti-poliovirus IgG. Radioactivity associated with the cells was determined in samples treated with 20 μM (◆) and 10 μM (▲) monensin or without the compound (▽). The proportion of virus adsorbed to cells was constant, whether the cells were pretreated with monensin or not.

Capsid-stabilizing effect of chloroquine. Chloroquine did not exert any inhibitory effect on the internalization mechanism, as determined by applying both criteria described above. We tested whether the compounds used in this work could impair virus-uncoating efficiency by stabilizing the capsid against degradation. Virus was heated for 1.5 min at 48°C in the presence of one of the weak bases to be tested at different molarities and was then analyzed by density gradient centrifugation. Monensin, methylamine, or ammonium chloride, even in high concentrations, did not have any capsid-stabilizing effect against heat-induced degradation to 80S empty shells. Since it has been shown that chloroquine is very effectively accumulated in acidic vesicles, achieving concentrations which exceed the concentration in the medium several thousand-fold (41), we used 20 mM chloroquine to test capsid-stabilizing effects. Heat treatment of labeled virus in the presence of 20 mM chloroquine resulted in partial protection of virus from degradation: approximately one third of the radioactivity sedimented at 156S as intact poliovirus, whereas in the absence of the compound virus was completely degraded to 80S particles (Fig. 4). Additionally, according to the degradation pattern observed in sedimentation velocity analysis of infected cell extracts, subviral particles of 135S appeared as a distinct peak and only one third of input virus was converted to 80S particles.

Characterization of subviral particles generated in vitro. The characterization of virus particles in different conformational states was facilitated by the possibility of generating altered particles in vitro. Virus and 135S and 80S particles were obtained by density gradient centrifugation of chloroquine-treated virus heated as described above. The peak fractions from the density gradient corresponding to intact

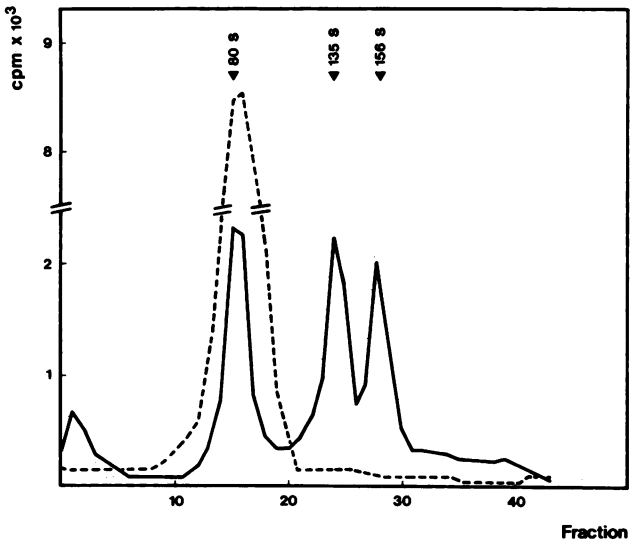

FIG. 4. Sedimentation velocity analysis of poliovirus after heat treatment for 1.5 min at 48°C in the presence of 20 mM chloroquine (——) or in its absence (------). For conditions of centrifugation, see Materials and Methods. Sedimentation is from left to right.

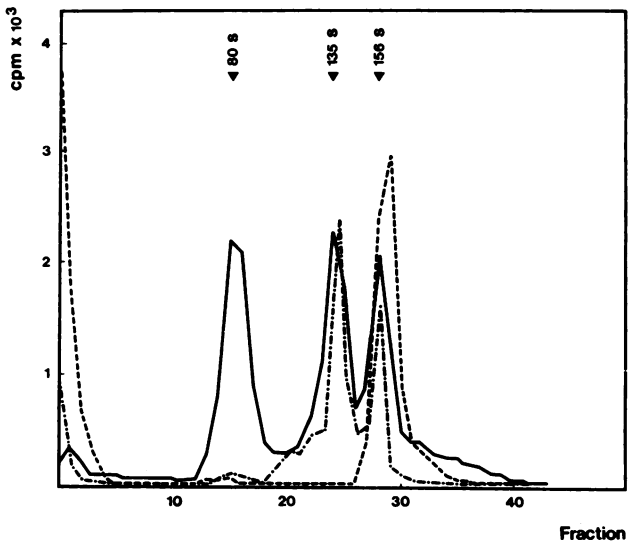

FIG. 6. Sedimentation velocity analysis of poliovirus after heat treatment for 1.5 min at 48°C in the presence of 20 mM chloroquine. [^{3}H]uridine-labeled poliovirus (–·–·–), [^{3}H]myristate-labeled poliovirus (-------), and [^{35}S]methionine-labeled poliovirus (——) were used. Sedimentation is from left to right.

virus and 135S particles were further analyzed by SDS-polyacrylamide gel electrophoresis. The 156S peak revealed the complete set of four structural proteins, whereas the 135S peak consisted of the three major structural proteins, lacking VP4 (Fig. 5). The presence or absence of VP4 in the different particle types could be conclusively demonstrated by using poliovirus specifically labeled with [^{3}H]myristic acid in VP4. Sedimentation velocity analysis of heat-treated virus in the presence of 20 mM chloroquine revealed that

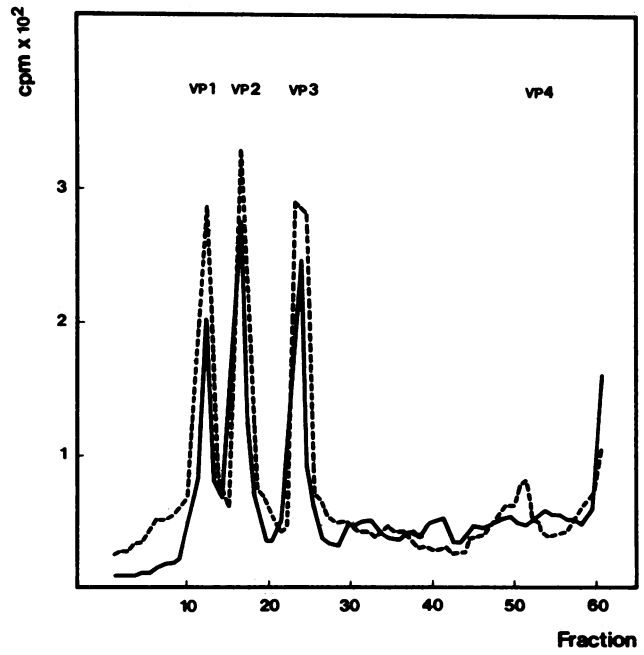

FIG. 5. SDS-polyacrylamide gel electrophoresis of intact virus (-------) and 135S particles (——) generated by heat treatment of virus at 48°C for 1.5 min in the presence of 20 mM chloroquine. See Materials and Methods for details.

radioactivity was exclusively associated with intact particles, reflecting the lack of VP4 from particles sedimenting at 135S and 80S (Fig. 6). Since the protein composition of 80S particles was identical to that of 135S particles (data not shown), the different sedimentation rates of the two subviral particles were ascribed to the absence of the viral RNA in the former. This assumption was confirmed in an experiment with chloroquine-treated virus labeled with [^{3}H]uridine in the RNA (Fig. 6). We tested the possibility of whether 135S particles are intermediate subviral degradation products, from which the RNA is released, leaving a residual empty capsid sedimenting at 80S. When 135S particles were generated by heat exposure of chloroquine-treated [^{3}H]uridine-labeled virus and tested for RNase sensitivity, 90% of the radioactivity became trichloroacetic acid soluble, while intact virus obtained from the same sucrose gradient was resistant to RNase treatment. Since the RNA in 135S particles is accessible to degrading enzymes, it is suggested that these particles attained a conformational state, favoring RNA release from the capsid.

Analysis of the sedimentation patterns of viral material recovered from cells infected with [^{3}H]myristic acid- and [^{3}H]uridine-labeled poliovirus preparations showed that the subviral particles generated during cell-mediated eclipse were similar to those produced in vitro with regard to sedimentation rate, lack of VP4, and RNA content (data not shown).

DISCUSSION

In this work we have shown the kinetics of poliovirus uncoating in HeLa cells and provided evidence that this process occurs similarly in a nonacidic environment. Poliovirus enters HeLa cells as intact virions which are continuously converted into 135S particles and 80S empty shells. Since these two kinds of particles differ in their content of viral RNA, the generation of 80S particles is assumed to be caused by the spontaneous release of the RNA from 135S particles. A similar time course for processing of infectious

poliovirus particles in HeLa cells has recently been observed (43). It has been argued that different pathways of poliovirus internalization and release of viral RNA to initiate the infectious cycle, or the virus itself, could be responsible for the unfavorable ratio of infectious units to the number of viral particles. However, we suggest that this phenomenon is due mainly to early interactions of the virus with cell membranes. In our studies, a maximum of 4% of input virus was adsorbed to HeLa cells at low temperature. Although about 60% of these particles were internalized after temperature shift, the others were rejected as altered particles (12). Altogether, roughly 1% of the total input virus was recovered after lysis of the cells. If it is correct that only 1 of 100 particles is internalized, virus uncoating and initiation of infection would occur with high efficiency. For foot-and-mouth disease virus it has been shown by microinjecting RNA that, indeed, a single RNA molecule can infect a cell (1).

There is evidence that picornaviruses enter cells by receptor-mediated endocytosis (29, 46). The mechanism of uncoating, however, remains unclear. Therefore, we investigated the role of an acidic environment in endosomes for poliovirus uncoating. Counteracting endosomal acidification with different lysosomotropic agents, we did not observe a block of virus uncoating. Uncoating could be prevented only in the presence of the capsid-stabilizing agent glutathione, a compound without any effects on lysosomal pH values. Although ammonium chloride completely failed to influence the kinetics of uncoating, methylamine, monensin, and chloroquine impeded particle alteration, delaying conformational transition by about 30 min. This delay was obviously not caused by inhibition of pH-dependent mechanisms, because even after 2 h of incubation, pH elevation in the presence of lysosomotropic weak bases is known to be preserved (32). None of these agents affected the nature of the different particles generated, as deduced from sedimentation velocity analysis. It is therefore assumed that uncoating is independent of low pH. The effects of methylamine and monensin on the kinetics of poliovirus uncoating could be attributed to a delay of virus internalization. Methylamine has been shown to reversibly block steps of receptor-mediated endocytosis, possibly by inhibition of clustering of receptor-ligand complexes in coated pits (8); this might be the reason for the delay of poliovirus entry into HeLa cells. Furthermore, the formation of primary endosomes, or receptosomes, from coated pits was suggested to be disturbed in the presence of the carboxylic ionophore monensin (34), which likewise could influence poliovirus entry. Since the modes of action of methylamine and monensin affect different, yet subsequent stages of the endocytosis process, different operational criteria for virus internalization had to be adopted. Receptor-bound particles lost sensitivity to acid treatment after only 7.5 min at 37°C; hence, only inhibition of early steps of endocytosis, e.g., clustering of receptor-ligand complexes, could be measured by using this experimental tool. The inhibiting effect of monensin on later endosome generation was more appropriately demonstrable with the operational criterion of antibody sensitivity, because attached particles lost sensitivity to neutralizing antibodies only after 30 min at 37°C.

The high concentration of chloroquine in endosomes, due to accumulation of the compound in these organelles, is demonstrated to lead to delayed appearance of subviral degradation products in the kinetics of uncoating, since increased resistance against particle alteration by heat treatment in the presence of chloroquine is observed. Thus, we demonstrated that those lysosomotropic agents influencing early steps of infection affect virus internalization or capsid disintegration by effects not associated with their lysosomotropic, pH-elevating action. The interference of these agents with steps of viral infection which do not depend on low endosomal pH has been demonstrated (3).

In previous work, a model of poliovirus uncoating has been presented, based on the decisive role of low endosomal pH (23). It was proposed that particle alteration is induced by low pH. This has been established in vitro for rhinovirus (22), but there exists no experimental evidence indicating the possibility of generating altered particles by low-pH treatment of poliovirus. Others favored the hypothesis that acid hydrolases are responsible for the initiation of uncoating, a mechanism which occurs during reovirus infection (37). A similar mechanism has been proposed for poliovirus (11), but this has not been confirmed by our results. However, it is impossible to exclude proteolytic cleavage of capsid proteins as being responsible for uncoating, since we failed to isolate 135S particles from infected cells in reasonable numbers for proper analysis of the protein composition. Analysis of 135S particles generated in vitro by heat treatment of poliovirus in the presence of chloroquine revealed the three major proteins, whereas VP4 was lost. Since the S values of in vitro- and in vivo-generated particles were similar, both contained the RNA, and both lacked VP4, they are suggested to be identical. This is in accordance with other reports concerning 135S particles produced in vitro by different methods (15, 21, 22).

It is suggested that 135S particles are expanded virus particles, because their mass differs from that of native virus only in VP4, whereas the sedimentation in a centrifugal field is clearly slower. This implies that the slower-sedimenting particle has a larger diameter (40). The conformational transition of intact virions to 135S particles appears to permit the escape of the viral RNA from the perforated capsid, since 135S particles have lost the compact, stable architecture, conferring RNase resistance to intact virus. Our findings are consistent with an uncoating model, analogous to the expansion of structurally similar plant viruses (33). X-ray analysis of poliovirus revealed the structural basis for conformational rearrangements, leading to an expansion-equivalent state of poliovirus particles (13).

Therefore, the transition of intact virus to expanded 135S particles, possibly with a critical role for divalent cations, would occur independently of low pH. At present, we are attempting to improve experimental procedures to optimize the yield of altered particles from infected cells. Further analysis of the protein composition of 135S and 80S particles generated in vivo would help to determine the structural and functional relationship between (i) cell-mediated eclipse products, (ii) 135S particles eluted during infection (12), and (iii) subviral particles produced in vitro.

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