Entry of Poliovirus Type 1 and Mouse Elberfeld (ME) Virus into HEp-2 Cells: Receptor-mediated Endocytosis and Endosomal or Lysosomal Uncoating

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SUMMARY

Poliovirus type 1 appeared from electron microscope studies to enter HEp-2 cells by receptor-mediated endocytosis. On adsorption the virus was evenly distributed over the cell surface, with some preference for the microvilli and their bases. Invagination of the cell surface membrane with the attached virus commenced at coated pits and led to the formation of virus-containing coated vesicles in the cytoplasm. These coated vesicles fused with intracellular vesicles to form endosomes. When cells infected with poliovirus or Mouse Elberfeld virus were treated with the weak bases chloroquine, NH₄Cl or the ionophore monensin to raise the intraendosomal and intralysosomal pH above 6, virus-directed macromolecular synthesis and production of progeny were prevented. These results suggest that the virus genomes are released to the cytoplasm via endosomes and/or lysosomes by a pH-dependent process.

INTRODUCTION

The early steps of the picornavirus replication cycle comprise (i) virus entry into the cell, i.e. adsorption of the virus at the cell surface and penetration into the cell, and (ii) virus uncoating. Uncoating results in the release of viral capsid proteins from the viral RNA, which is then available for the synthesis of virus-specific RNA and proteins. The individual steps can be distinguished (Crowell & Landau, 1983): for adsorption, reduced recovery of unattached virus and reversibility of virus attachment at the cell surface under certain salt and pH conditions; for penetration, inaccessibility of virus to neutralizing antibodies; for uncoating, loss of photosensitivity of the virus and attainment of RNase sensitivity of the viral RNA.

The pathway of the virus during entry and uncoating, however, has not yet been clearly resolved. For poliovirus, it was suggested from electron micrographs that after adsorption the virus temporarily fuses with the plasma membrane of the infected cell and is subsequently delivered into the cell's interior (Dunnebacke et al., 1969). Other studies suggested that poliovirus is phagocytosed into the cell and subsequently located in intracellular vacuoles (Dales, 1973).

Three sites have been suggested to be the cellular location of virus uncoating: the lysosome for poliovirus (Habermehl et al., 1973); the cytoplasm for echovirus type 12 (Eggers et al., 1979; Rosenwirth & Eggers, 1979); the plasma membrane for coxsackievirus B3 and poliovirus (Roesing et al., 1975; DeSena & Mandel, 1977; Guttmann & Baltimore, 1977).

The entry of many physiologically important macromolecules into cells is regulated by receptor-mediated endocytosis (Goldstein et al., 1979; Brown et al., 1983; Helenius et al., 1983; Pastan & Willingham, 1983). Some peptide hormones, bacterial toxins, low-density lipoprotein (LDL), transferrin and β₂-macroglobulin, and also viruses like Semliki Forest virus (SFV), vesicular stomatitis virus (VSV), and adenovirus interact as ligands with specific receptors at the
cell surface. After clustering of the ligand–receptor complexes in clathrin-coated pits, the internalization of the ligand into the cell results in the formation of coated vesicles in the cytoplasm. These vesicles, liberated from their coat, fuse with intracellular vesicles to form endosomes, from which the internalized ligand can be delivered to secondary lysosomes, the Golgi system or directly into the cytoplasm. The release of the ligand from endosomes and lysosomes can be dependent on the low pH in these organelles. Raising the intraendosomal and intralysosomal pH above 6 by using weak bases entraps the endocytosed particles in endosomes and/or lysosomes (Ohkuma & Poole, 1978; Geisow et al., 1981; Tycko & Maxfield, 1982; Helenius et al., 1982; Marsh et al., 1982; Tartakoff, 1983).

In this work we used ultrastructural studies to show that poliovirus type 1 enters HEp-2 cells by receptor-mediated endocytosis. In addition, we exploited the action of the weak bases NH$_4$Cl, chloroquine and the ionophore monensin, which we have recently shown to interfere with the replication of picornaviruses (Zeichhardt et al., 1983b). We now report that by raising the intraendosomal and intralysosomal pH with these bases the release of the genomes of poliovirus and Mouse Elberfeld (ME) virus to the cytoplasm can be inhibited. These results suggest an uncoating mechanism in endosomes and/or lysosomes.

**METHODS**

_Cells and viruses._ Monolayers of HEp-2 cells were grown in Eagle’s MEM as described by Zeichhardt et al. (1981). Cells were either grown in plastic Petri dishes (diam. 3 cm) or in 24-well cell culture dishes (Nunc). In several experiments the wells contained glass coverslips (diam. 11 mm, one per well: Menzelglaser, F.R.G.). Poliovirus type 1 (strain Mahoney) and ME virus were used throughout these studies. Virus propagation and purification by differential centrifugation or equilibrium centrifugation in CsCl were performed as described previously (Wetz & Habermehl, 1979; Zeichhardt et al., 1981).

_Electron microscopy._ Cells grown on coverslips in multi-well dishes were inoculated with 125 µl highly purified poliovirus in phosphate-buffered saline (PBS) at an m.o.i. of $5 \times 10^2$ p.f.u./cell. Cells and virus were precooled in an ice-bath. Adsorption at 0°C was carried out for 24 h, with the multi-well dishes horizontal in the ice-bath. The cells were then incubated at 37°C for different times (1, 3, 5, 10, 15, 20 and 30 min) by allowing the plates to float in a water-bath. The cells were washed three times with PBS (37°C) and fixed with 4% (w/v) formaldehyde and 0.25% (v/v) glutaraldehyde for 1 h at room temperature. Cells that were not shifted to 37°C were fixed with ice-cold aldehyde. For ultrathin sectioning the cells were fixed with OsO$_4$, post-fixed with tannic acid, dehydrated and flat-embedded in low viscosity epoxy resin (Zeichhardt et al., 1982). Sectioning was performed in a Reichert Ultracut microtome. Micrographs were taken in a Philips electron microscope (EM 301).

_Use of NH$_4$Cl, chloroquine and monensin._ For all experiments stock solution of each of the weak bases were prepared freshly: 1 M-NH$_4$Cl (Merck) and 100 mM-chloroquine-HCl (Sigma) in double-distilled water, 10 mM-monensin (Sigma) in absolute ethanol. The pH of each solution was adjusted to neutrality with NaOH or HCl and appropriate molarities of the bases were achieved by diluting into Eagle’s MEM which contained 20 mM-HEPES pH 7.2 (Serva, Heidelberg, F.R.G.) (Helenius et al., 1982). The culture media contained the bases from 0.5 h before infection and for the whole period of infection. For poliovirus and ME virus the m.o.i. was 20 p.f.u./cell. If not stated otherwise, all steps were carried out at 37°C.

_Virus adsorption and penetration._ Cells in multi-well dishes were pretreated for 0.5 h with 0.3 ml Eagle’s MEM containing one of the weak bases (+ base) and 5% newborn calf serum (NCS; Flow Laboratories). After washing the cells three times with serum-deficient medium (+ base), they were infected with 100 µl of $^3$H-labelled poliovirus in serum-deficient medium (+ base) (RNA labelled with [5,6-$^3$H]uridine, Amersham; sp. act. of virus $4.6 \times 10^{6}$ p.f.u./d.p.m.). As controls L-cells, which are non-permissive for poliovirus, were infected in parallel. Adsorption and penetration were allowed to proceed for 2 h by incubating at 37°C. Cells were washed three times with PBS and removed from the substratum by treatment with 0.1% trypsin (Gibco) 2.5% EDTA (Merck). Radioactivity was determined by solubilizing the cells in Soluene 350, using Dimilume as scintillant (both from Packard) and counting in a Packard Tricarb scintillation counter.

_Activity of viral RNA polymerase in vivo._ The activity in vivo of the RNA polymerases of poliovirus and ME virus was determined by measuring [5,6-$^3$H]uridine incorporation into viral RNA in infected cells in the presence of 2 µg/ml actinomycin D (Boehringer). The method was modified according to Helenius et al. (1982). Cells, grown on coverslips in multi-well dishes, were treated as follows (actinomycin D was present in steps 2 to 7). (1) Pretreatment for 0.5 h with 0.3 ml Eagle’s MEM (+ base), supplemented with 5% NCS, (2) washing three times with serum-deficient medium (+ base), (3) inoculation with 0.3 ml virus for 0.5 h in serum-deficient medium (+ base), (4) washing three times with serum-containing medium (+ base), (5) addition of 0.3 ml medium as in step 4, (6) pulse for 1.5 h with [5,6-$^3$H]uridine (3 µCi/ml) at 3 h post-infection for poliovirus and 4 h for ME virus, (7)
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chase for 0.5 h by transferring the coverslips to a second multi-well dish with 0.3 ml serum-containing medium (+ base), supplemented with 10 mM-uridine (Sigma), (8) washing three times with ice-cold PBS, (9) precipitation of newly synthesized viral RNA by addition of 2 ml ice-cold 10% TCA for 1 h, and (10) determination of radioactivity as described above.

Activity of viral RNA polymerase in vitro. The activity in vitro of membrane-bound RNA polymerases of poliovirus and ME virus in the presence of the weak bases was determined by measuring the conversion of [3H]GTP (Amersham) to an acid-insoluble product as described recently (Zeichhardt et al., 1983a). The system, optimized for the ME virus-specific polymerase, could also be used to assay the polymerase activity of poliovirus.

Virus replication. The kinetics of replication of poliovirus and ME virus were determined in the presence of the weak bases. Cells, grown in plastic dishes (diam. 3 cm), were pretreated and infected as described for measuring the activity in vivo of the viral RNA polymerases (steps 1 to 5). The only exception was that 1 ml of media and inocula were used and actinomycin D was omitted. Cells were harvested 7 h after infection with poliovirus and at 9 h with ME virus. For plaque titration of ME virus the agar was supplemented with DEAE-dextran (0.01% final concentration; Pharmacia) (Zeichhardt et al., 1981).

RESULTS

Receptor-mediated endocytosis of poliovirus

Transmission electron microscopy of ultrathin sections of poliovirus-infected HEp-2 cells revealed that poliovirus entered the cells by endocytosis (Fig. 1). It was deduced that this endocytosis was receptor-mediated because the micrographs showed all the morphological criteria compatible with a mechanism of receptor-mediated endocytosis as reported for epidermal growth factor (EGF), LDL and SFV (Goldstein et al., 1979; Brown et al., 1983; Helenius et al., 1983).

Poliovirus was allowed to adsorb at the surface of HEp-2 cells for 24 h at 0 °C. Adsorption at this low temperature prevented elution and penetration of the virus (Crowell & Landau, 1983). Virus particles could be detected evenly distributed at the cell surface (Fig. 1 a, b). There was some preference for virus adsorption at microvilli (Fig. 1 c) and at their bases (Fig. 1 b) which was in accordance with observations for coxsackievirus B3 (Roesing et al., 1975). When the temperature of such infected cells was shifted to 37 °C for 1 min, most poliovirus particles were located at clathrin-coated pits (Fig. 1 d). The invagination of the plasma membrane with the bound virus commenced at these coated pits 3 min after the temperature shift (Fig. 1 e, f), and in most cases was completed 5 min after the shift when the coated pits became coated vesicles separate in the cytoplasm (Fig. 1 g, h, i). More than one virus particle could be internalized in this way at the same time by one coated pit, and hence two or more could be found in one coated vesicle (Fig. 1 i). After 10 min at 37 °C the virus-containing coated vesicles fused with cytoplasmic vesicles, resulting in the formation of partly coated vesicles (Fig. 1 j, k). A temperature shift of 15 min led to the formation of endosomes (Fig. 1 l). As shown for coated vesicles, the partly coated vesicles and endosomes contained two and sometimes up to four virus particles (Fig. 1 i, l). After 20 to 30 min at 37 °C, virus-containing endosomes were seldom discernible (not shown).

A proportion of the virus particles remained at the cell surface even after 20 min at the raised temperature. In general, these particles were not associated with coated pits (not shown).

Uncoating of poliovirus and ME virus in endosomes or lysosomes

Endosomes are direct precursors of secondary lysosomes (Helenius et al., 1983). In order to investigate whether endosomes and/or lysosomes with their low pH are involved in the uncoating and consequently the release of viral RNA into the cytoplasm, we used three weak bases to raise the pH in these organelles: NH4Cl, chloroquine and the ionophore monensin. We analysed the role of endosomes and/or lysosomes in the uncoating reaction by measuring subsequent RNA synthesis of poliovirus and ME virus as a function of concentration and time of addition of the bases (Fig. 2a to f and 3). Furthermore, we measured adsorption and penetration of the virus as steps prior to uncoating (Table 1), and assayed the effects of the weak bases on the overall virus yield (Fig. 2g, h, i), their direct effect on virus infectivity (Table 2), and on cellular RNA and protein synthesis (Table 3).
Fig. 1. Electron micrographs (ultrathin sections) of poliovirus type 1 entering HEp-2 cells by receptor-mediated endocytosis. Adsorption of poliovirus was performed at 0 °C for 24 h at an m.o.i. of $5 \times 10^3$ p.f.u./cell. For virus entry the samples were shifted to 37 °C for different periods of time (v, virion; cp, coated pits; cv, coated vesicle; e, endosome). (a, b, c) Poliovirus adsorption with even distribution at the cell surface (no temperature shift); some preference for adsorption at microvilli (c) and at their bases (b). (d, e, f) Poliovirus located at coated pits; formation of virus-associated coated pits (1 min of temperature shift) (d); beginning of invagination of the plasma membrane (3 min of temperature shift) (e, f). (g, h, i) Poliovirus located in coated vesicles (5 min of temperature shift); completion of invagination of plasma membrane (g, h); separate coated vesicle containing two virus particles (i). (j, k, l) Poliovirus in partly coated vesicles and endosomes; partly coated cytoplasmic vesicles containing up to four virus particles (10 min of temperature shift) (j, k); endosome containing three virus particles (15 min of temperature shift) (l). Bar marker (j) represents 0.1 μm for each micrograph.

**Synthesis of viral RNA in vivo**

By measuring viral RNA synthesis *in vivo*, we showed that the RNA of poliovirus and ME virus was not released from endosomes and/or lysosomes into the cytoplasm when the weak bases were added. In the presence of both actinomycin D (for inhibition of cellular RNA
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Fig. 2. RNA synthesis and production of infectious progeny of poliovirus (■) and ME virus (○) at different concentrations of monensin, chloroquine or NH₄Cl. (a, b, c) Activity of virus-specific RNA polymerase under in vivo conditions: incorporation of [³H]uridine into newly synthesized viral RNA in pulse-chase experiments in the presence of actinomycin D. The incorporation rate of the virus-specific polymerase in the absence of the bases was taken as 100% (11,300 c.p.m. for poliovirus and 21,200 c.p.m. for ME virus). The basal activities of mock-infected cells at the corresponding base concentrations (1500 to 3100 c.p.m. ) were taken as 0% and used for correcting the virus-specific activities. (d, e, f) Activity of membrane-bound virus-specific RNA polymerase under in vitro conditions in the presence of the weak bases. The membrane-bound RNA polymerases of poliovirus and ME virus were isolated from cells infected in the absence of the weak bases and tested for incorporation of [³H]GMP into newly synthesized viral RNA. Determination of 100% and 0% polymerase activities was performed as for (a) to (c); 100% corresponded to 9000 c.p.m. for poliovirus and 10,600 c.p.m. for ME virus; 0% corresponded to 850 to 1000 c.p.m. for membranes of mock-infected cells. (g, h, i) Production of infectious virus progeny in the presence of the weak bases, present from 0-5 h before virus infection and for the whole length of infection (7 h for poliovirus and 9 h for ME virus). Virus replication was determined by plaque titration.
Fig. 3. Viral RNA synthesis as a function of time of addition of monensin (a), chloroquine (b) and 
NH₄Cl (c). The activity of the RNA polymerases of poliovirus (■) and ME virus (●) was measured in 
the presence of 10 μM-monensin, 0.3 mM-chloroquine or 70 mM-NH₄Cl under in vivo conditions as 
described for Fig. 2(a, b, c). The bases were added 0.5 h prior to infection (−0.5 h), simultaneously with 
the virus (0 h), 0.5, 1, 2 or 3 h after infection.

synthesis) and one of the weak bases (added 0.5 h prior to infection), we measured the 
incorporation rate of [3H]uridine into newly synthesized viral RNA in pulse–chase experiments 
(Fig. 2a, b, c). It could be shown that viral RNA synthesis was progressively diminished by 
increasing concentrations of the weak bases. In the presence of 10 μM-monensin, 0.3 mM-
chloroquine or 70 mM-NH₄Cl, the RNA synthesis of poliovirus and ME virus was reduced by 
more than 90%. NH₄Cl at a low concentration had a pronounced stimulatory effect. In 
comparison with the virus controls without added base, 10 mM-NH₄Cl led to 30% more 
incorporation for poliovirus and 45% more incorporation for ME virus.

The inhibitory effect of the weak bases was dependent on the time of addition (Fig. 3). This 
time dependence was tested at those base concentrations which severely suppressed the RNA 
synthesis of both viruses under the conditions described above (Fig. 2a, b, c): 10 μM-monensin, 
0.3 mM-chloroquine or 70 mM-NH₄Cl were not inhibitory when added 1 h or later after 
infection. For strong inhibition the bases had to be added 0.5 h prior to infection or at least 
simultaneously with the viruses.

Synthesis of viral RNA in vitro

The membrane-bound RNA polymerases of poliovirus and ME virus were isolated from cells 
infected in the absence of the bases. When the in vitro activity of these enzymes was measured by 
[3H]GMP incorporation into acid-insoluble material (Fig. 2d, e, f), none of the three bases 
inhibited the viral RNA polymerases at concentrations that abolished their activity under in vivo 
conditions (Fig. 2a, b, c).

Virus replication

As anticipated from the in vivo RNA synthesis results, the replication of poliovirus and ME 
virus was decreased by each of the bases (Fig. 2g, h, i). The replication kinetics of both viruses in 
the presence of increasing concentrations of the bases revealed that the decrease caused by 
monensin was weak (five- to tenfold), whereas the effects of chloroquine and NH₄Cl were 
strong (>4 log₁₀ units). The two latter bases caused maximum inhibition of reproduction only at 
concentrations higher than those required to block the RNA synthesis in vivo (Fig. 2a, b, c).
Table 1. Effect of monensin, chloroquine and NH₄Cl on the adsorption and penetration capacity of poliovirus*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Virus adsorption and penetration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Monensin</td>
<td>10 μM</td>
<td>122</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0-3 mM</td>
<td>98</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>70 mM</td>
<td>109</td>
</tr>
</tbody>
</table>

* The base concentrations chosen were sufficient to induce maximum inhibition of viral RNA synthesis in vivo (Fig. 2a, b, c). HEp-2 cells were infected with [³H]uridine-labelled poliovirus, and after 2 h of incubation at 37 °C the amount of cell-associated virus was determined. As a control for non-specific virus–cell interactions, poliovirus was incubated with L-cells (non-permissive for poliovirus) in the presence of the weak bases. The amount of virus remaining with L-cells was between 0.13 and 0.25 times of the amount of virus interacting with HEp-2 cells. The values obtained with L-cells were taken as 0% for correcting the results with HEp-2 cells. 100% adsorption and penetration of virus was 2150 c.p.m.

Table 2. Effect of monensin, chloroquine and NH₄Cl on the infectivity of poliovirus and ME virus used for inoculation*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Poliovirus (p.f.u./ml)</th>
<th>ME virus (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>5.1 × 10⁶</td>
<td>4.6 × 10⁶</td>
</tr>
<tr>
<td>Monensin</td>
<td>30 μM</td>
<td>5.5 × 10⁶</td>
<td>3.8 × 10⁶</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>4-8 mM</td>
<td>8.1 × 10⁶</td>
<td>8.5 × 10⁶</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>100 mM</td>
<td>2.9 × 10⁶</td>
<td>6.9 × 10⁶</td>
</tr>
</tbody>
</table>

* Aliquots of poliovirus and ME virus were kept in the presence of the same concentrations of the weak bases as for the infection yield assays (Fig. 2g, h, i) and were plaque-titrated in the absence of the weak bases.

Adsorption and penetration

At drug concentrations that inhibited viral RNA synthesis (Fig. 2a, b, c), poliovirus could still adsorb and penetrate as effectively as in the absence of the weak bases (Table 1).

Viral infectivity and cellular RNA and protein synthesis

In order to test whether the bases inactivated viral infectivity, poliovirus and ME virus were kept in the presence of the bases for the times used for the infection yield assays (7 h for poliovirus and 9 h for ME virus) (Table 2). Even at the much higher concentrations necessary for inhibition of the release of the viral RNA into the cytoplasm (Fig. 2a, b, c), poliovirus and ME virus remained as infective as in the absence of these bases.

Mock-infected cells were tested for cellular RNA and protein synthesis by measuring the incorporation of [³H]uridine and ³H-labelled amino acids into acid-insoluble material in pulse-chase experiments (Table 3). When tested at concentrations causing a strong reduction of RNA synthesis in vivo (Fig. 2a, b, c), the effects of the bases on the cellular RNA synthesis were in all cases weak or negligible. Cellular protein synthesis was affected to different degrees. Monensin and chloroquine allowed protein synthesis of 66% and 75%, respectively. In the presence of 70 mM-NH₄Cl, however, protein synthesis was reduced to 27%.

DISCUSSION

Our data indicate that (i) the entry of poliovirus type 1 into HEp-2 cells is regulated by receptor-mediated endocytosis and (ii) the uncoating of poliovirus and ME virus is a pH-dependent process which presumably occurs in endosomes and/or lysosomes. This indicates that besides glycosylated proteins and viruses (Helenius et al., 1983) non-glycosylated ribonucleoprotein complexes, in this case represented by picornaviruses, can also enter cells by such a mechanism. Although we draw these conclusions, we are aware of the unfavourable ratio of infectious to physical virus particles which may range from 1:10 to 1:1000 (Lonberg-Holm &
Table 3. Effect of monensin, chloroquine and NH₄Cl on cellular RNA and protein synthesis

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>[³H]Uridine incorporation</th>
<th>%</th>
<th>[³H]-amino acid amino acid incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>86800 (100)</td>
<td></td>
<td>20253 (100)</td>
</tr>
<tr>
<td>Monensin</td>
<td>10 µM</td>
<td>93049 (107)</td>
<td></td>
<td>13265 (66)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.3 mM</td>
<td>70134 (81)</td>
<td></td>
<td>15210 (75)</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>70 mM</td>
<td>85932 (99)</td>
<td></td>
<td>5529 (27)</td>
</tr>
<tr>
<td>Actinomycin</td>
<td>2 µg/ml</td>
<td>2777 (3)</td>
<td></td>
<td>ND†</td>
</tr>
<tr>
<td>Dye</td>
<td>10 µg/ml</td>
<td>2187 (11)</td>
<td></td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Mock-infected HEp-2 cells were pulse-chase-labelled in the presence of the weak bases under the conditions described for Fig. 2 (a, b, c). Incorporation of 1 µCi [³H]uridine in the absence of actinomycin D and incorporation of 1 µCi [³H]-labelled amino acid mixture (Amersham) were measured to determine cellular RNA synthesis and protein synthesis, respectively. The inhibitory effects of actinomycin D on the cellular RNA synthesis and of cycloheximide on the cellular protein synthesis were determined as controls.

† ND, Not done.

Philipson, 1974). Our data are compatible with earlier ultrastructural results pointing to a phagocytotic entry of poliovirus (Dales, 1973). Penetration of poliovirus by fusion with the plasma membrane (Dunnebacke et al., 1969) is therefore unlikely.

The involvement of virus-specific cell receptors in endocytosis is most likely, as the ultrastructural studies revealed that coated pits, coated vesicles and endosomes play a role in virus entry (Fig. 1). These structures have been reported to be associated with receptor-mediated endocytosis as shown for EGF, LDL and SFV (Goldstein et al., 1979; Brown et al., 1983; Helenius et al., 1983). Although receptors have not yet been isolated for picornaviruses, several reports imply that the poliovirus-specific receptor is a glycoprotein containing α-mannosyl-like residues, because poliovirus adsorption is inhibited by pretreatment of the cells with concanavalin A, trypsin and elastase (Crowell & Landau, 1983).

The site of picornavirus uncoating has been ascribed to three cellular locations: the plasma membrane for coxsackievirus B3 and poliovirus (Roesing et al., 1975; DeSena & Mandel, 1977; Guttmann & Baltimore, 1977); the cytoplasm for echovirus type 12 (Eggers et al., 1979; Rosenwirth & Eggers, 1979); the lysosome for poliovirus (Habermehl et al., 1973). The latter authors showed for isolated lysosomes of poliovirus-infected cells that the virus capsid was degraded to an extent depending on the period of time after infection. Our results suggesting that the uncoating of poliovirus and ME virus is a pH-dependent process in endosomes and/or lysosomes are consistent with these results. This was determined by the use of the weak bases chloroquine, NH₄Cl and the ionophore monensin as recently presented (Zeichhardt et al., 1983b). A distinction between endosomes and lysosomes was not possible in these investigations, however, as each of the bases influences both acidic organelles by raising their internal pH to above 6 (Helenius et al., 1983). While we were preparing this manuscript, it was reported independently (Madshus et al., 1984) that the entry of the genome of poliovirus type 1 into the cytoplasm of HeLa cells requires a low pH.

Our analysis of RNA synthesis of poliovirus and ME virus in vivo at smooth cytoplasmic membranes (Caliguiri & Tamm, 1970; Zeichhardt et al., 1983a) as a virus-specific reaction that follows the base-sensitive step (Fig. 2a, b, c) indicates that the decrease in RNA synthesis was caused by the blockage in the release of the RNA substrate from endosomes and/or lysosomes into the cytoplasm. The presence of the bases during the very early stages of infection (0-5 h prior to or simultaneous with the infection) was necessary for this inhibition (Fig. 3). The activity of the RNA polymerases in vitro was unaffected in the presence of the bases, at least as far as the chain elongation capacity of the enzymes was concerned. The assay used for measuring the activity of the membrane-bound RNA polymerases in vitro (Fig. 2d, e, f) mainly determined the elongation of preexisting viral RNA strands without reinitiation (Baltimore et al., 1963). The virus functions prior to the uncoating, i.e. adsorption and penetration, were unaltered by the bases (Table 1).

Although all three bases were inhibitory, they exhibited different potencies. We suggest that
monensin acts more selectively on virus entrapment in endosomes and/or lysosomes than chloroquine and NH₄Cl. This was deduced by comparing the effects of the bases on RNA synthesis in vivo (Fig. 2a, b, c) and the overall virus yield (Fig. 2g, h, i). (i) Each of the bases strongly suppressed RNA synthesis, but in addition chloroquine and NH₄Cl were severely inhibitory in the virus yield assays (>4 log₁₀ units) at high concentrations. Monensin, even at high concentrations, did not decrease the virus yield by more than 10-fold. (ii) All three bases allowed substantial virus replication at concentrations even when RNA synthesis in vivo was already decreased to approximately 10%. The residual virus replication at concentrations higher than 3 μM-monensin, 0.3 mM-chloroquine or 60 to 70 mM-NH₄Cl could have been due to the remaining virus having avoided becoming entrapped in endosomes and/or lysosomes. The inhibitory effects of chloroquine and NH₄Cl at higher concentrations on the overall virus yield might be accounted for by additional effects on replication steps following the release of the viral genome. This conclusion is underlined by the observation that NH₄Cl allowed only partial viral RNA synthesis (<50%) when added late in infection (3 h) (Fig. 3).

The mechanism for the uncoating and delivery of the poliovirus and ME virus RNA from the endosomes and/or lysosomes into the cytoplasm remains unclear. A fusion of virus components with the membranes of these acidic organelles as shown for SFV (Helenius et al., 1983) seems improbable, as picornaviruses (unlike SFV) have no envelope or glycosylated proteins (Drzeniek & Bilello, 1974). Analysis of the contribution of lysosomal and endosomal enzymes to virus capsid degradation under different pH conditions should give insight into the uncoating process of picornaviruses.

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