

# Mechanism of Entry into the Cytosol of Poliovirus Type 1: Requirement for Low pH

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**ABSTRACT** The effect of a number of drugs and culture conditions on the entry into cells of a strain of poliovirus 1 (Brunende) was tested. The cells were exposed in the dark to light-sensitive, neutral red-containing virus, in the presence of the drug to be tested. Then the cells were exposed to light, transferred to normal medium, and incubated overnight. Cytopathogenic effect was measured as inhibition of [<sup>3</sup>H]leucine incorporation. Compounds that dissipate proton gradients across membranes, like monensin, protonophores, and amines, and compounds that inhibit the acidification process, such as *N,N'*-dicyclohexylcarbodiimide (DCCD) and tributyltin, inhibited the entry of virus, but not virus binding. This was also the case with metabolic inhibitors that deplete cells for ATP. The same compounds also inhibited the cell-induced alteration of the virus particles. When cells with surface-bound virus were exposed to low pH, the virus entered efficiently, even in the presence of monensin and DCCD. The results indicate that acidification somehow facilitates the entry of the virus RNA into the cytosol and that under normal conditions the entry occurs from intracellular acidic vesicles.

Until recently, little was known about the mechanism by which virus particles and their genomes enter into the cytosol of eucaryotic cells. It now appears that the genome of viruses containing a lipid membrane is introduced into the cytosol by fusion of the lipid bilayer envelope with membranes of the target cell. In most cases low pH is required for this fusion to occur and therefore it usually takes place inside acidified endocytic vesicles (1). The entry mechanism of viruses that lack a lipid bilayer coat has so far been unknown.

In the case of the picornaviruses, infection is ensured if an intact copy of the positive-strand RNA that constitutes that genome penetrates into the cytosol (2). It is conceivable that the genome of picornaviruses enters cells in a manner similar to that of many protein toxins that act on target molecules inside the cell. These toxins, which comprise the plant toxins abrin, modeccin, ricin, and viscumin and the bacterial toxins diphtheria toxin and Shigella toxin, are first bound to cell surface receptors and then one of their constituent peptide chains is transferred to the cytosol (for review, see reference 3). Entry of a single copy of the enzymatically active moiety may be sufficient to kill a cell (4, 5).

In attempts to elucidate the mechanism of entry of picornaviruses into cells, we have studied here the penetration of a strain of poliovirus 1 into HeLa S<sub>3</sub> cells and measured the

effect on this process of a variety of drugs and medium conditions previously found to affect the uptake of the plant and bacterial toxins (6–10).

## MATERIALS AND METHODS

**Cell Cultures:** HeLa S<sub>3</sub> cells were used both for production and titration of virus. The cells were maintained as monolayer cultures in minimal essential medium (Gibco Laboratories Inc., Glasgow, Scotland) with 10% fetal calf serum (11). In experiments with antibodies against poliovirus, the serum was preincubated at 56°C for 30 min to inactivate complement. The cells were transferred to disposable trays with 24 wells (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) the day before the experiment. In most experiments about 5 × 10<sup>6</sup> cells per well were used.

**Virus Preparation:** A strain (Brunende) of poliovirus type 1 was obtained from The National Institute for Public Health, Oslo. The virus was propagated in HeLa S<sub>3</sub> cells and purified as described by Cole et al. (12) with CsCl-gradient centrifugation as the last purification step. The virus concentration was estimated from the absorbance at 260 nm; 1 OD<sub>260</sub> was taken to be 9.2 × 10<sup>12</sup> virus particles per milliliter (13). One plaque forming unit (pfu), measured as described below, corresponded to approximately 700 virus particles. Virus labeled with [<sup>35</sup>S]methionine and <sup>32</sup>P was prepared in HeLa S<sub>3</sub> cells infected with virus in normal medium, and after 2 h it was transferred to medium containing either 7 nM methionine and 0.25 mCi/ml [<sup>35</sup>S]methionine or 0.25 mCi/ml Na<sub>3</sub><sup>32</sup>PO<sub>4</sub> and no unlabeled phosphate. The labeled virus was sedimented by centrifugation and isolated by sucrose gradient centrifugation. CsCl-gradient centrifugation of the labeled virus showed that the radioactivity banded together with the virus.

**Preparation of Light-sensitive Virus:** The procedure is essentially as described by Mandel (14). HeLa S<sub>3</sub> cells growing in medium containing 10 µg/ml of neutral red were infected with virus (1 pfu per cell) and incubated overnight. The next day the cells were disrupted by three cycles of freezing and thawing. We added a small sample of the virus-containing medium (1 pfu per cell) to new bottles of HeLa S<sub>3</sub> cells in medium with neutral red, and the cells were incubated and disrupted as above. Finally the cell debris was removed by centrifugation. The supernatant was stored frozen until use. All work with virus containing neutral red was carried out in the dark. The light-sensitive virus suspension used contained  $1.4 \times 10^5$  pfu/µl.

**Virus Titration:** A plaque assay used to titrate the virus was carried out as follows: Dilutions of virus (500 µl) were added to Petri dishes (9 cm diam) containing a monolayer of HeLa S<sub>3</sub> cells and incubated at 37°C for 1 h. Then 5 ml 1% agar in serum-free medium was layered over the cells. Finally, 10 ml of medium was layered over the agar and the plates were incubated at 37°C for 48 h. The agar was then carefully removed and the plates were stained with May-Grünwald solution.

In most experiments virus was titrated by measuring the virus-induced inhibition of protein synthesis in HeLa S<sub>3</sub> cells. Virus and the compound to be tested were added to cells growing in disposable trays with 24 wells ( $5 \times 10^4$  cells per well in 1 ml of medium) as described in the legends to figures, and the plates were incubated for 18 h, when not otherwise indicated. We terminated the experiments by measuring the incorporation of [<sup>3</sup>H]leucine into trichloroacetic acid-insoluble material during 1 h as earlier described (11).

When light-sensitive, neutral red-containing virus was used, the cells were incubated with the virus in the dark for the indicated period of time. The cells were then exposed for 5 min to the light from a 60 W bulb at a distance of 15 cm. The cells were then incubated over night and their ability to incorporate [<sup>3</sup>H]leucine was measured.

**Antiserum to Virus:** Rabbits were infected intravenously with  $10^{12}$  virus particles weekly for 4 wk. A booster dose was given 4 wk later and the antiserum was collected 1 wk after the last injection. The serum was stored at -20°C. Before use the serum was incubated at 56°C for 30 min to inactivate complement.

**Analysis of Virus Particle Alteration by Sucrose Gradient Centrifugation:** HeLa S<sub>3</sub> monolayer cultures in 24-well disposable trays ( $10^5$  cells per well) were preincubated at 37°C for 15 min with 100 µl per well of HEPES medium, with and without the different compounds to be tested. Then the cells were chilled to room temperature, and radioactively labeled virus (~ $10^5$  cpm per well in 20–30 µl) was added. The plate was placed in a moist chamber and gently agitated. The virus was allowed to bind for 1 h, after which the cells were washed three times to remove unbound virus, medium with the different compounds to be tested was added to a total volume of 100 µl per well, and the incubation was continued for an additional 1–2 h at 37°C. The cells were then dissolved by adding 0.5% Triton X-100 to each well. The nuclei were removed by centrifugation for 3 min in an Eppendorf centrifuge. SDS (0.2% wt/vol) was added to the supernatant which was then layered on top of sucrose gradients (15–30% wt/vol) in 0.14 M NaCl, 10 mM Na-phosphate, pH 7.4 (15). The gradients were centrifuged for 1 h at 234,000 g in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). Fractions were collected and the radioactivity in 150-µl aliquots was measured.

**Virus Binding:** After 15 min of preincubation at 37°C with the compound to be tested, the cells were chilled to 0°C and [<sup>35</sup>S]methionine-labeled virus (10,000–70,000 cpm) was added to each well. The cells were kept at 0°C for 2 h and then washed three times to remove unbound virus. Finally, the cells were dissolved in 0.2 ml of 0.1 M KOH and the bound radioactivity was measured.

**Measurement of Cellular ATP:** Cells in 24-well disposable trays were incubated with and without drugs as described. Then the medium was removed and 250 µl 2% (wt/vol) ice-cold perchloric acid was added to each well. After 30 min the perchloric acid extract was collected and centrifuged in an Eppendorf centrifuge for 10 min. 200 µl were taken from the supernatant and mixed with 800 µl of 0.1 M Tris-EDTA, pH 7.7. Dilutions from this solution were made in the same buffer and aliquots were taken and added to luciferase-luciferase mixture according to the procedure described by the company (LKB, Bromma, Sweden). The emitted light was measured in an LKB Wallac luminometer 1250. A standard curve obtained by measuring samples with known amounts of ATP was used to estimate the ATP content in the sample.

**Chemicals:** Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, 2-deoxyglucose, 2,4-dinitrophenol, FCCP (carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone),<sup>1</sup> CCCP (carbonylcyanide *m*-

<sup>1</sup> Abbreviations used in this paper: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone.

chlorophenylhydrazone), DCCD (*N,N'*-dicyclohexylcarbodiimide), procaine, chloroquine, monensin, and methylamine were obtained from Sigma Chemical Co. (St. Louis, MO). Tri-*N*-butyltin chloride was obtained from Aldrich, Belgium, Br-X-537A was obtained from Hoffmann-LaRoche (Inc., Nutley, NJ), and neutral red from George T. Gurr, Ltd., London.

## RESULTS

### Characterization of the Screening System

To assay the effect of a large number of compounds and medium conditions on the entry of poliovirus RNA into the cytoplasm, we required a simple and quantitative test system to measure the rate of virus entry. When increasing amounts of virus were added to HeLa S<sub>3</sub> cells in 24-well microtiter plates, protein synthesis was inhibited in a dose-dependent manner (Fig. 1). In most cases it was convenient to measure the residual rate of protein synthesis after 18 h. Under these conditions the cytopathogenic effect of the internalized virus is amplified because cell lysis and reinfection occurs during this incubation period. As shown in Fig. 1, protein synthesis under these conditions was 50% inhibited in wells that had received  $10^7$  virus particles per well (~0.3 pfu per cell) and no antiserum. When the cultures were incubated for 48 rather than 18 h,  $10^4$  virus particles per well (~0,0003 pfu per cell) were sufficient to reduce protein synthesis to half the control value.

If antiserum was added after exposure to the virus for 90 min at 37°C, the amount of virus required to achieve 50% inhibition in the course of 18 h was 100 times higher than in the absence of antiserum. Under these conditions the virus produced was unable to infect new cells and propagate the infection. If, however, the cells were thoroughly washed to remove excess antibodies, the inhibition of protein synthesis after 18 h was found to be the same as when antiserum was not added (data not shown).

Many of the compounds we chose to test for their ability to interfere with virus uptake are toxic to cells. To be able to limit the exposure of the cells to toxic compounds to the period of actual virus entry, we took advantage of the observation that poliovirus grown in the presence of neutral red is sensitive to visible light as long as the RNA remains inside

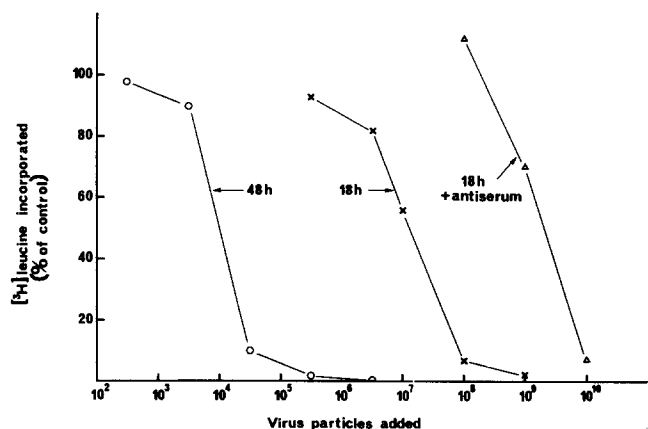


FIGURE 1 Titration of cytopathogenic effect by measuring protein synthesis inhibition. Increasing amounts of virus were added to HeLa S<sub>3</sub> cells growing in 24-well microtiter plates and the cells were incubated at 37°C. In one set of incubations, 10 µl of antipoliovirus serum was added after 90 min and then the incubation was continued. After the indicated period of time, [<sup>3</sup>H]leucine was added and the ability of the cells to incorporate radioactivity during 1 h was measured.

the neutral red-containing capsid (14). However, once released, e.g., into the cytosol, the neutral red diffuses away and the RNA is no longer sensitive to light. Therefore, when cells are exposed in the dark to light-sensitive virus for a certain period of time and then exposed to light, all virus that has not released its RNA into the cytosol is inactivated.

The virus-induced inhibition of cellular protein synthesis can be used as a measure of virus entry. The data in Fig. 2 show that no infection occurred if the cells were exposed to light immediately after addition of the virus. However, with increasing time of incubation with virus at 37°C before exposure to light, increasing inhibition of protein synthesis occurred. When the cells were exposed to light after 60 min, the extent of protein synthesis inhibition was essentially the same as in cells kept in the dark overnight, indicating that most of the virus entry occurred during the first 60 min.

### Ability of Metabolic Inhibitors and Compounds Inhibiting Vesicle Acidification to Inhibit Virus Entry

To assay the ability of various compounds to inhibit virus entry, we exposed cells to virus for 40 min in the presence and absence of the compounds to be tested. The cells were then exposed to light and transferred to normal medium. The extent of protein synthesis inhibition was measured 18 h later.

Previously we have found that treatment of cells with a combination of 2-deoxyglucose, which inhibits glycolysis, and  $\text{NaN}_3$ , which uncouples oxidative phosphorylation, prevented the entry of all protein toxins we tested (3, 8–10). When cells were exposed to light-sensitive virus in the presence of 2-deoxyglucose and  $\text{NaN}_3$ , very little infection was obtained (Fig. 3A). The results in Table I show that such treatment

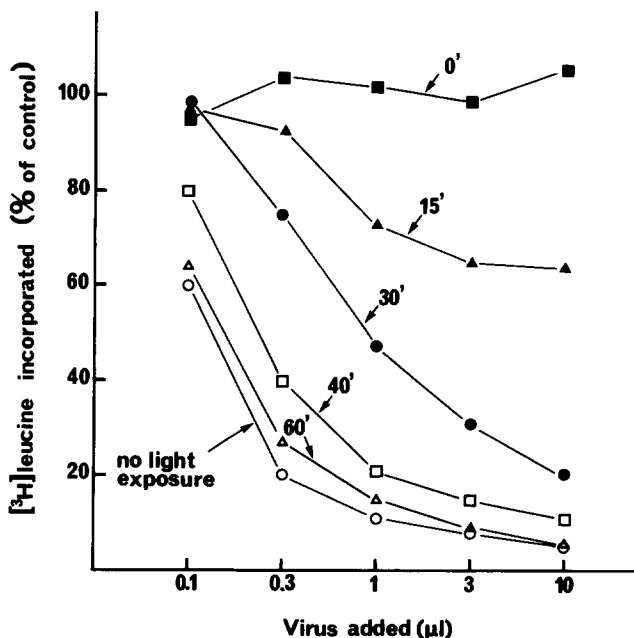


FIGURE 2 Effect of exposure time on the entry into cells of virus containing neutral red. Increasing amounts of virus were added in the dark to HeLa  $S_3$  cells in 24-well microtiter plates that were incubated in the dark at 37°C for the indicated period of time and then exposed to light, as described in Materials and Methods. In one case the cells were not exposed to light. The cells were further incubated at 37°C for 18 h and their ability to incorporate [ $^3\text{H}$ ]leucine was measured.

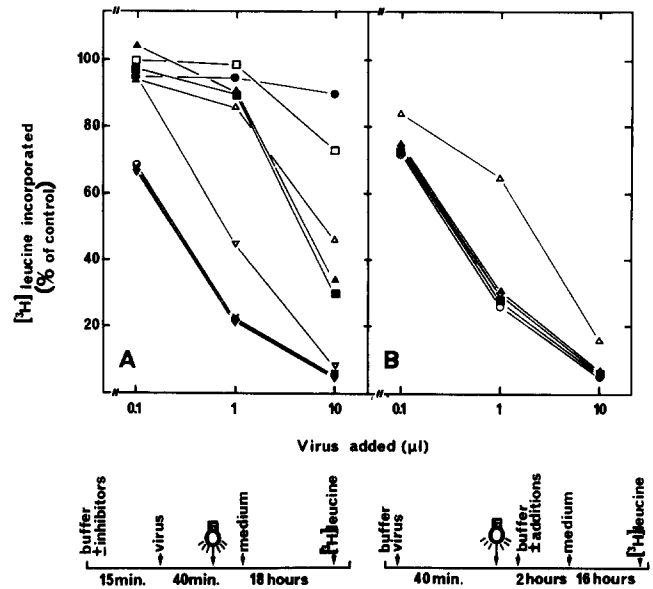


FIGURE 3 Effect of different compounds on virus entry and on virus multiplication. The virus-induced inhibition of protein synthesis was measured when the compounds were added before (A) or after (B) the uptake had been stopped by illumination. (A) The indicated compounds were added to HeLa  $S_3$  cells in 24-well disposable trays and, after 15 min, increasing amounts of virus containing neutral red were added. After incubation in the dark for 40 min at 37°C, the cells were exposed to light and then transferred to normal medium and incubated overnight. (B) Cells in medium without additions were exposed to virus for 40 min and then exposed to light, and the various compounds were added as indicated. After 2 h at 37°C the cells were transferred to normal medium and incubated overnight. The rate of [ $^3\text{H}$ ]leucine incorporation was measured the next day. The additions in A were (▼) 10 mM  $\text{NaN}_3$ ; (x) 50 mM 2-deoxyglucose; (●) 50 mM 2-deoxyglucose and 10 mM  $\text{NaN}_3$ ; (▽) 20  $\mu\text{M}$  FCCP; (■) 10  $\mu\text{M}$  CCCP; (△) 30  $\mu\text{M}$  DCCD; (▲) 1  $\mu\text{M}$  tributyltin; (□) 1  $\mu\text{M}$  monensin; (○) control, no addition. In B the additions were as follows: (●) 50 mM 2-deoxyglucose and 10 mM  $\text{NaN}_3$ ; (▽) 20  $\mu\text{M}$  FCCP; (■) 10  $\mu\text{M}$  CCCP; (△) 30  $\mu\text{M}$  DCCD; (▲) 1  $\mu\text{M}$  tributyltin; (□) 1  $\mu\text{M}$  monensin; (○) control, no addition.

strongly reduced the level of cellular ATP.  $\text{NaN}_3$  alone and 2-deoxyglucose alone were less efficient in reducing cellular ATP (Table I) and did not protect against virus (Fig. 3A). The data therefore indicate that one or more ATP-requiring processes are necessary for the entry of poliovirus.

A number of enveloped viruses as well as diphtheria toxin and modeccin require low pH for entry, and cells can be protected against infection and intoxication by compounds that dissipate pH gradients across membranes (1, 6, 7, 9). We therefore tested if such compounds also protect against poliovirus. The data in Fig. 3 and Table I show that the ionophore monensin, which exchanges protons for monovalent cations, protected strongly against poliovirus. This was also the case with the protonophore CCCP, while FCCP was less efficient. The data indicate that low pH is required for poliovirus entry.

Endocytic vesicles are apparently acidified by ATP-driven proton pumps in the membrane (16–18). These pumps can be inhibited by tributyltin and DCCD which both inhibit various ATP-ases. As shown in Fig. 3A, these compounds also protected against poliovirus.

It must be kept in mind that in the test system used to estimate the rate of virus entry, the cytopathogenic effect is amplified by two to three cycles of lysis and reinfection. It

TABLE I  
Ability of Different Compounds and Conditions to Inhibit Infection of Cells by Poliovirus

Compound*	Concentration mM	Cytopathogenic effect†		Virus binding‡	Cellular ATP§
		% of control	% of control		
NaN <sub>3</sub>	10	100			67.8
2-deoxyglucose	50	100			13.8
2-deoxyglucose + NaN <sub>3</sub>	50 } 10 }	<1	122		3.2
2,4-dinitrophenol	1	100			
NH <sub>4</sub> Cl	20	25			
Monensin	0.0001	20			
	0.001	<1			
	0.01	<1	106		89.6
Br-X-537A	0.0001	50			
	0.001	1			
Tributyltin	0.0005	2	106		111.3
Chloroquine	0.2	2			
Methylamine	20	5			
Procaine	10	3			
FCCP	0.02	20	133		112
CCCP	0.01	2	130		
DCCD	0.03	1	112		86.1

\* The indicated compounds were added to cells growing in HEPES medium, pH 7.8, in 24-well disposable trays. After 15 min at 37°C, light-sensitive virus was added and the cells were treated as in Fig. 3.

† Cytopathogenic effect was measured as reduced protein synthesis in the same way as in Fig. 3. The virus concentration required to reduce protein synthesis to half the control value (ID<sub>50</sub>, median infective dose) was estimated. The data are expressed as ID<sub>50</sub> in the control sample divided by ID<sub>50</sub> in the presence of the compound tested × 100.

‡ [<sup>35</sup>S]methionine-labeled virus was added to cells in 24-well disposable trays and binding was measured as described in Materials and Methods. The data are expressed as percent of the amount bound in the absence of added compounds.

§ The amount of cellular ATP was measured with the luciferase-luciferase method as described in Materials and Methods.

was therefore necessary to see whether the different treatments interfered with the amplification cycles as such. To study this, we added the compounds to be tested after the virus had presumably entered the cells. These were first infected for 40 min at 37°C in normal medium with light-sensitive virus. Then the cells were exposed to light, the different compounds were added, and the incubation was continued for 2 h more. Subsequently the cells were transferred to normal medium and incubated overnight, and the rate of protein synthesis was measured. As shown in Fig. 3B, NaN<sub>3</sub>, 2-deoxyglucose, the ionophores, and tributyltin did not protect under these conditions, indicating that their effect is limited to inhibition of virus entry. DCCD had some protective effect in this system, indicating that this compound has an effect in addition to that on virus entry. When NH<sub>4</sub>Cl, procaine, and methylamine were tested in the same way, they were found to increase the cytotoxic effect (Fig. 4B). This suggests that the exposure to weak amines somehow increases the virus production.

Weak bases like NH<sub>4</sub>Cl have been found to protect against a number of enveloped viruses and toxins (1, 6, 7, 19, 20), apparently because free NH<sub>3</sub> is able to penetrate membranes and can therefore enter intracellular acidic vesicles. There it becomes protonated and thus increases the pH of the vesicles. The data in Fig. 4A show that NH<sub>4</sub>Cl protected only slightly against poliovirus. Better protection was obtained with other amines like procaine, methylamine, and chloroquine. Since the amines appear to increase virus production, the data in

Fig. 4A do not adequately reflect the inhibition of virus entry.

A possible reason for the protection against poliovirus observed under different conditions could be that these inhibit virus binding to the cells. However, the data in Table I show that none of the conditions tested inhibited the binding of [<sup>35</sup>S]methionine-labeled virus to the cells.

### Effect of Protective Conditions on Particle Alteration

When cells with bound picornavirus are incubated at 37°C, the virus is altered in the sense that it loses one of the capsid proteins, VP4, and sediments more slowly than intact virus (21–24). Possibly, this alteration is required for entry of the RNA. To study this question we measured the ability of cells to alter poliovirus under different protective conditions. Virus labeled with <sup>32</sup>P or [<sup>35</sup>S]methionine was incubated with cells, the cells were lysed, and the released virus was analyzed by sucrose gradient centrifugation. When the incubation was carried out at 23°C, the released virus sedimented largely as a single peak of intact virus (Fig. 5A), but after incubation at 37°C for 60 min additional peaks appeared. The peak denoted A was found both in samples labeled with Na<sub>3</sub><sup>32</sup>PO<sub>4</sub> (Fig. 5B) and in those labeled with [<sup>35</sup>S]methionine (data not shown) and apparently represents altered particles containing RNA (13, 24). At the location indicated by B, a prominent peak was found in [<sup>35</sup>S]methionine-labeled samples incubated at 37°C. The peak probably represents empty capsids (24). Virus titration of the different fractions showed that only the most rapidly sedimenting peak, denoted V, was infective and thus represents intact virus.

When cells with bound <sup>32</sup>P-labeled virus were incubated at 37°C for 1 h in the presence of those compounds shown above to protect cells against virus, it was found that these also inhibited the alteration of the virus particles (Fig. 5, C–I). Thus in the presence of procaine, the combination of 2-deoxyglucose and NaN<sub>3</sub>, or monensin, FCCP, DCCD, tributyltin, or CCCP, the amount of altered particles formed was reduced. Only in the presence of FCCP, which was least efficient in protecting the cells (see Fig. 3A), was a definite peak of altered particles present (peak A in Fig. 5F). If the

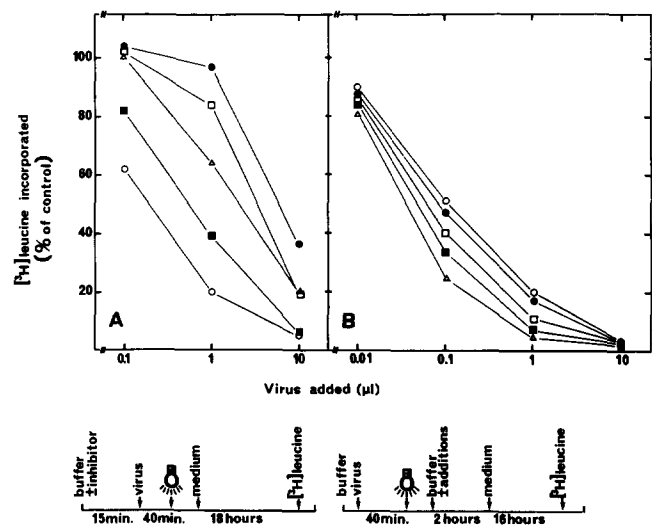


FIGURE 4 Effect of different amines on virus entry (A) and on virus multiplication (B). Conditions are the same as in Fig. 3. The additions were (■) 20 mM NH<sub>4</sub>Cl; (△) 20 mM methylamine; (□) 10 mM procaine; (●) 0.2 mM chloroquine; (○) control, no additions.

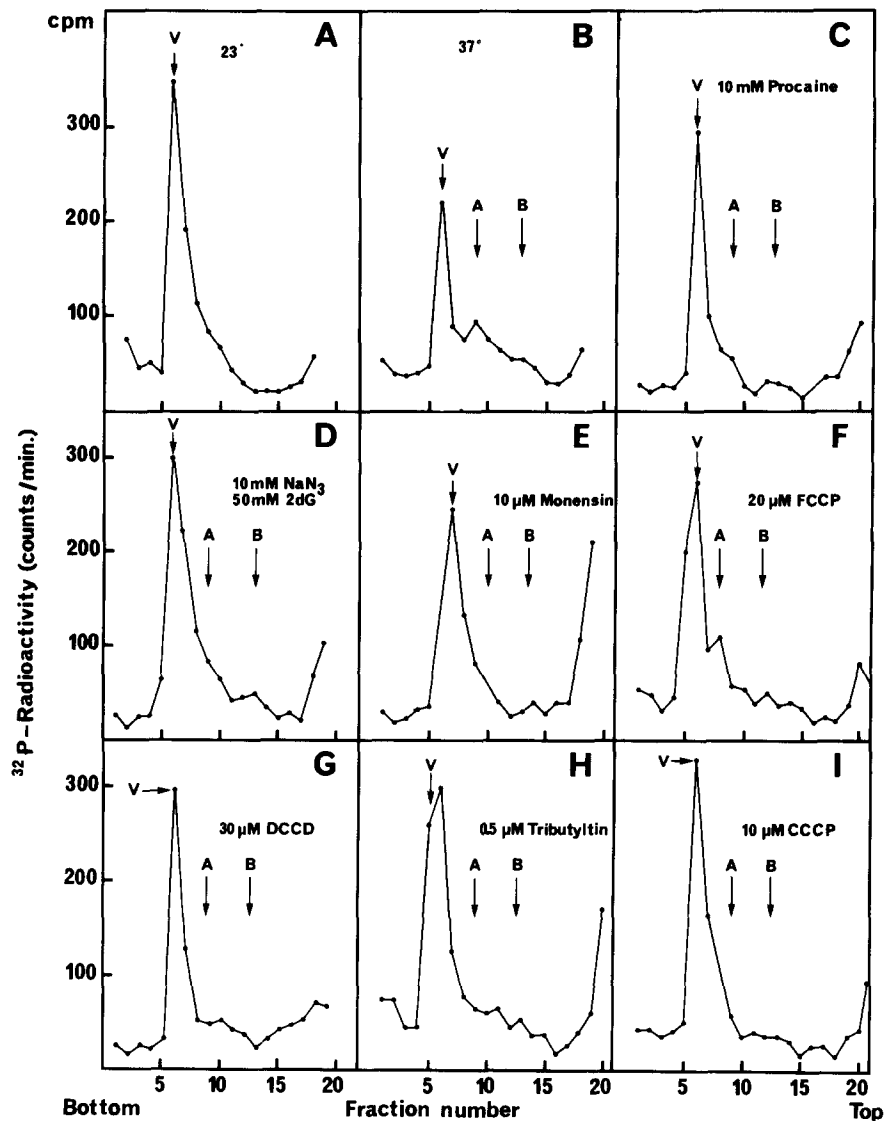


FIGURE 5 Sucrose gradient centrifugation of  $^{32}\text{P}$ -labeled virus after incubation with cells in the presence and absence of protective compounds. Cells were incubated with  $^{32}\text{P}$ -labeled virus at  $23^\circ\text{C}$  for 1 h and then washed. One sample (A) was treated with Triton X-100 whereas the other samples were incubated for 1 h at  $37^\circ\text{C}$  in the absence (B) and presence (C-I) of protective compounds as indicated. Finally the cells were treated with Triton X-100 and analyzed by sucrose gradient centrifugation.

incubation was prolonged to 2 h, altered particles were formed in most cases. Only in the presence of 2-deoxyglucose and  $\text{NaN}_3$  was the alteration inhibited even under these conditions (data not shown). The data indicate that conditions that prevent vesicular acidification inhibit the formation of altered particles.

#### Induction of Poliovirus Entry by Exposure of the Cells to Low pH

In the case of a number of enveloped viruses (1, 19, 20) and diphtheria toxin (6), the acidic vesicles can be bypassed if cells with surface-bound virus or toxin are exposed to low pH. Under these conditions entry apparently occurs directly from the cell surface. To test if this is also the case with poliovirus, light-sensitive virus was bound to cells in the cold and then the cells were exposed to medium containing monensin or DCCD at pH 5.5 and 7.5. (In the presence of monensin, pH <5.5 is toxic). After 20 min the cells were exposed to light and incubated for 36 h, and the ability of the cells to incorporate [ $^3\text{H}$ ]leucine was measured. As shown in Fig. 6, A and B, the entry in the presence of monensin and DCCD was much more efficient at pH 5.5 than at pH 7.5. Efficient entry also occurred at pH 6.5 (not shown). In the

absence of monensin the cells were slightly more sensitive at pH 5.5 than at pH 7.5, but the difference was much smaller than in the presence of monensin. These results indicate that low pH is indeed required for the entry of poliovirus.

The ability of cells to alter poliovirus in the presence of monensin was strongly increased at low pH. As shown in Fig. 7, exposure of monensin-treated cells with surface-bound virus to pH 5.5 for 90 min induced considerable alteration of the virus, whereas this was not the case at pH 7.8. Only cell-bound virus is altered at low pH. Thus, exposure of virus to pH 5.5 at  $37^\circ\text{C}$  in the absence of cells did not induce alteration of the particles (not shown). Altogether the data indicate that particle alteration brought about by low pH is a necessary step in virus entry.

#### DISCUSSION

The main finding in this paper is that a strain of poliovirus type 1 requires low pH for injection of its genome into the cytosol. Thus chloroquine, monensin, protonophores, DCCD, tributyltin, and other compounds known to inhibit the acidification of intracellular vesicles (16, 25) protected against the virus. This suggests that under normal conditions the entry of poliovirus occurs from acidic vesicles. The fact

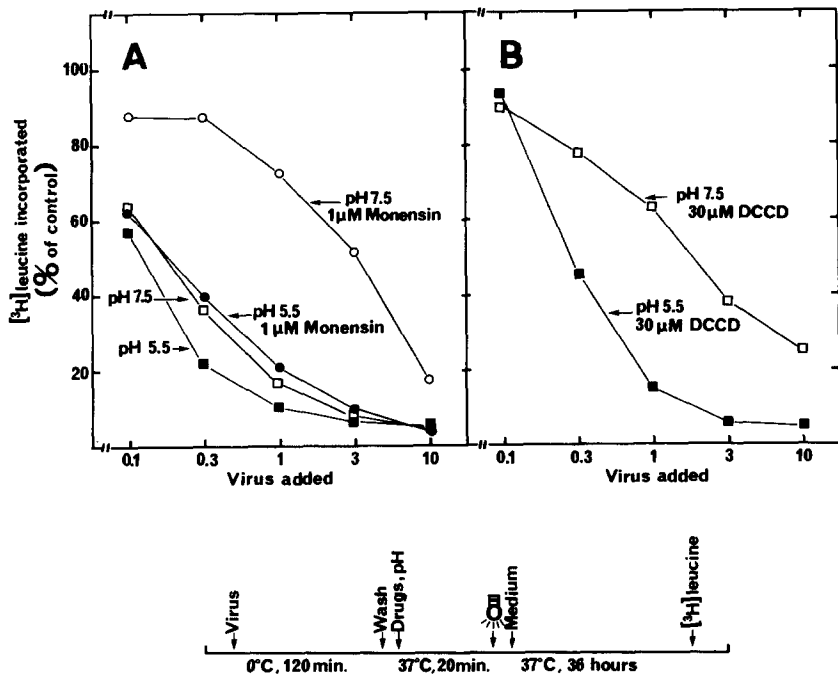


FIGURE 6 Ability of low pH to counteract the protective effect of monensin (A) and DCCD (B) on virus entry. Increasing amounts of light-sensitive virus were added to HeLa S<sub>3</sub> cells in 24-well microtiter plates and the virus was allowed to bind at 0°C for 2 h. The cells were then washed and HEPES medium containing 1 μM monensin or 30 μM DCCD adjusted to pH 5.5 or 7.5, and prewarmed to 37°C, was added. After 20 min the cells were exposed to light, normal medium was added, and the cells were incubated for 36 h. Finally, the ability of the cells to incorporate [<sup>3</sup>H]leucine was measured.

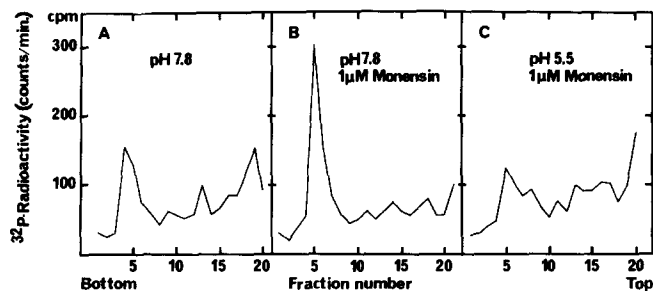


FIGURE 7 Effect of pH on the sedimentation pattern of <sup>32</sup>P-labeled virus, incubated with cells in the absence and presence of monensin. Cells were incubated with <sup>32</sup>P-labeled virus at 0°C for 2 h and then washed. HEPES medium with and without monensin, and with pH as indicated, was added and the cells were incubated at 37°C for 90 min. Then the cells were treated with Triton X-100 and analyzed by sucrose gradient centrifugation as in Fig. 5. (A) pH 7.8, no monensin; (B) pH 7.8, 1 μM monensin; (C) pH 5.5, 1 μM monensin.

that the different compounds protect to different extents may be due to different abilities to increase the pH of intracellular vesicles sufficiently to prevent infection. Apparently the entry of poliovirus that occurs even at pH 6.5 requires less acidification than the entry of Semliki Forest virus (1) and of diphtheria toxin (8).

When cells are depleted of ATP by treatment with 2-deoxyglucose and NaN<sub>3</sub>, infection did not occur, indicating that virus did not enter. Similar findings were earlier made with poliovirus type 2 (15). Most likely the protective effect of ATP-depletion is due to inhibition of the endocytic uptake of the virus and inhibition of vesicle acidification.

Several authors (13, 24, 26, 27) have shown that an isolated membrane fraction from poliovirus-sensitive cells is able to alter virus particles *in vitro*. The alteration resembles that occurring *in vivo*, in the sense that particles lacking VP4 were produced. However, the RNA was not released. The alteration in this system occurs only at low osmotic strength and it occurs most readily at slightly alkaline pH (27), whereas we

find that cell-bound virus is altered in isotonic buffer at acidic pH.

The finding that low pH is required for virus entry indicates that under normal conditions poliovirus does not inject its RNA at the cell surface, but that this takes place in acidic intracellular vesicles. Further evidence that endocytosis is required for poliovirus entry was reported by Lonberg-Holm (28), who found that treatment of cells with concanavalin A reduced the ability of the cells to alter poliovirus type 2 and rhinovirus type 2. It had previously been found by Okada and Kim (29) that concanavalin A protected against a number of viruses. One effect of concanavalin A is to inhibit endocytosis (30). Also ultrastructural data suggested that endocytosis is involved in the entry of poliovirus (31).

The main advantage of the test system used here to measure virus entry, which involves the use of light-sensitive virus, is that it allows the time of exposure of the cells to the test conditions to be limited to 30–40 min. This is important since prolonged exposures to several of the drugs and conditions tested here are lethal to the cells. Further advantages of the test system are that it permits testing of a large number of drugs and conditions and that it does not require large amounts of virus.

From experiments *in vitro* there is good evidence that the virus is light sensitive as long as the RNA remains inside the intact capsid (14, 32). It is not known if the light sensitivity persists even in altered particles that appear to represent intermediates in the internalization. The altered particles still contain RNA, but their structure is less compact and it is therefore conceivable that the neutral red can diffuse out of the altered particles. Also it is not known if exit of RNA from the capsid is directly coupled to transfer across the membrane into the cytosol. Clearly, the uncoating could occur in vesicles before the RNA transfer across the membrane takes place. Also it is not excluded that the whole particle is transferred across the membrane and that uncoating takes place in the cytosol. It is therefore important to stress that what we have measured with the light-sensitive virus are effects on those steps that occur until the RNA becomes light-resistant. This

time period may not necessarily comprise the whole process of uncoating and RNA penetration into the cytosol.

The finding that poliovirus appears to enter from acidic vesicles is similar to that previously made with a number of enveloped viruses (1, 19, 20). Also, the toxins enter the cytosol from endocytic vesicles and usually not from the cell surface. Thus diphtheria toxin (6–8) and probably modeccin (9) and *Pseudomonas aeruginosa* exotoxin A (33) require low pH for entry, conditions encountered in intracellular vesicles. The plant toxins abrin, ricin, and viscumin also enter the cytosol from endocytic vesicles although acidification is not required in these cases (9, 10).

Diphtheria toxin appears to enter the cytosol as soon as the endosome containing the toxin is acidified. It can also, like poliovirus, enter directly from the cell surface if cells with surface-bound toxin are exposed to low pH (6, 8). The entry of diphtheria toxin is strongly inhibited by  $\text{NH}_4\text{Cl}$  whereas that of poliovirus is not, perhaps because a higher extent of acidification is required for entry of diphtheria toxin than for poliovirus.

Upon exposure of diphtheria toxin to low pH a hidden hydrophobic region is exposed and may insert itself into the membrane (8). A similar mechanism may apply in the case of poliovirus. Thus Lonberg-Holm et al. (34) found that altered virus particles have lipophilic properties. Possibly, upon alteration, the virus particles insert themselves into the membrane and release their RNA into the cytosol. A low efficiency of this process could be why half of the bound viruses are released from the cells as altered particles, while the majority of the remaining virus is degraded (35). Only ~5% of the cell-associated and uncoated virus RNA remains acid insoluble during the 2–2.5 h that proceed from virus attachment until synthesis of new virus RNA becomes detectable (35).

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