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Requirements for entry of poliovirus RNA into cells at low pH

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HeLa S_3 cells were protected against infection by poliovirus type I by the presence of monensin and N,N'-dicyclohexylcarbodiimide (DCCD), compounds elevating the pH of acidic intracellular compartments. The protection was fully overcome by exposing the cells to pH 5.5 and lower, and at ~pH 6.1 it was reduced by half. Measurements of the ability of the virus to enter the detergent phase under conditions where Triton X-114 was separated from water indicated that the virus is hydrophilic at neutral pH, and that it exposes hydrophobic regions at low pH. When the cells were pretreated with acetic acid, which reduces the intracellular pH, virus entry was inhibited, indicating that a pH gradient across the membrane is necessary for infection. Under all conditions which induced infection, the virus particles were altered to more slowly sedimenting material. Also, virus bound to aldehyde-fixed cells was altered when exposed to low pH at 37°C. The data indicate that poliovirus bound to receptors on cells exposes hydrophobic regions at low pH, and that at physiological temperature it undergoes alteration. This alteration may be a necessary, but not sufficient requirement for infection.

Key words: poliovirus/endocytosis/acidic vesicles/Triton X-114

Introduction

A number of enveloped viruses (Helenius *et al.*, 1980; White *et al.*, 1981; Matlin *et al.*, 1982; Sato *et al.* 1983) as well as certain toxic proteins (Draper and Simon, 1980; Sandvig and Olsnes, 1980, 1981, 1982; FitzGerald *et al.*, 1980; Sandvig *et al.* 1984a), enter the cytosol from acidified vesicular compartments in the cell. Recently we have presented evidence that low pH is necessary also for entry of poliovirus RNA into the cytosol (Madshus *et al.*, 1984). To elucidate the requirements for poliovirus entry, we decided to study in more detail the effect of pH and temperature on this process. We have also attempted to elucidate the role of virus alteration in the entry process by correlating the extent of virus alteration under different conditions with the concurrent infection.

Results

Effect of pH on the entry of virus at 37°C

To study the pH requirements for entry of poliovirus into cells, we took advantage of our earlier observation that in the presence of the ionophore monensin or the ATPase inhibitor N,N'-dicyclohexylcarbodiimide (DCCD) infection is strongly inhibited at neutral pH (Madshus *et al.*, 1984). Monensin dissipates H^+ gradients across membranes by electroneutral exchange of H^+ for monovalent cations, while DCCD inhibits the proton-translocating ATPase (Yamashiro *et al.*, 1983; Stone *et al.*, 1983). In both cases the cells are unable to acidify their endosomes. The protection by monensin and DCCD is overcome when cells with bound virus are exposed to low pH (Madshus *et al.*, 1984).

The effect of monensin and DCCD on the entry of poliovirus is shown in Figure 1. In this experiment we used light-sensitive virus which had been grown in the presence of neutral red (Mandel, 1967). As long as the RNA genome of such a virus is contained within the intact viral capsid it is inactivated upon exposure to visible light. However, once the virus capsid is opened and the RNA genome is released, e.g., into the cytosol, the dye diffuses away from the viral RNA, which thereby becomes insensitive. The fraction of the virus that becomes light resistant under the conditions used is therefore a measure of virus entry (Mandel, 1967). It follows that it is possible to interrupt the continuous uptake of virus after defined periods of time by exposing the cells to visible light, a procedure that will inactivate virus that has not yet entered the cells. The entry can therefore be studied while the cells are exposed to conditions and drugs which they can only tolerate for short periods of time (Madshus *et al.*, 1984).

The data in Figure 1A and C confirm that the inhibition of cellular protein synthesis that occurs in virus-infected cells is reduced in the presence of monensin or DCCD and that this effect is counteracted by low pH in the medium. At pH 5.5 the virus entered in the presence of monensin to the same extent as at pH 7.5 in the absence of the inophore. At pH below 5.5, monensin is toxic to HeLa S_3 cells, probably due to acidification of the cytosol. We also tested in detail the ability of low pH to overcome the protection by DCCD, which is well tolerated even at pH 4.5. It is clear that the optimal sensitization is reached at pH 5.5. As shown in Figure 1C and D, the effect of different pH values on the entry of virus was essentially the same in cells treated with monensin and DCCD. In both cases half maximal entry occurred at ~pH 6.1.

The sensitization does not occur as rapidly in the case of poliovirus as in the case of diphtheria toxin, where exposure to low pH for a few seconds is sufficient for maximal effect (Sandvig and Olsnes, 1981). Thus maximal sensitization to the virus was only obtained after 10 – 20 min at 37°C, when the virus had been pre-bound to the cells on ice.

When cells with bound virus are incubated at 37°C, the particles are modified. Of the altered particles, the A-particles lack one of the structural proteins, VP4, whereas the B-particles represent empty capsids (Crowell and Philipson, 1971; Lonberg-Holm and Korant, 1972; Lonberg-Holm *et al.*, 1975). A number of different conditions which inhibit virus entry, such as monensin treatment, were found also to inhibit this modification (Madshus *et al.* 1984). In the presence of monensin, modification occurred to a much larger extent at pH 5.5, than at pH 7.5 (Figure 2A and C). At pH 6.1, in the presence of monensin (Figure 2B), modifica-

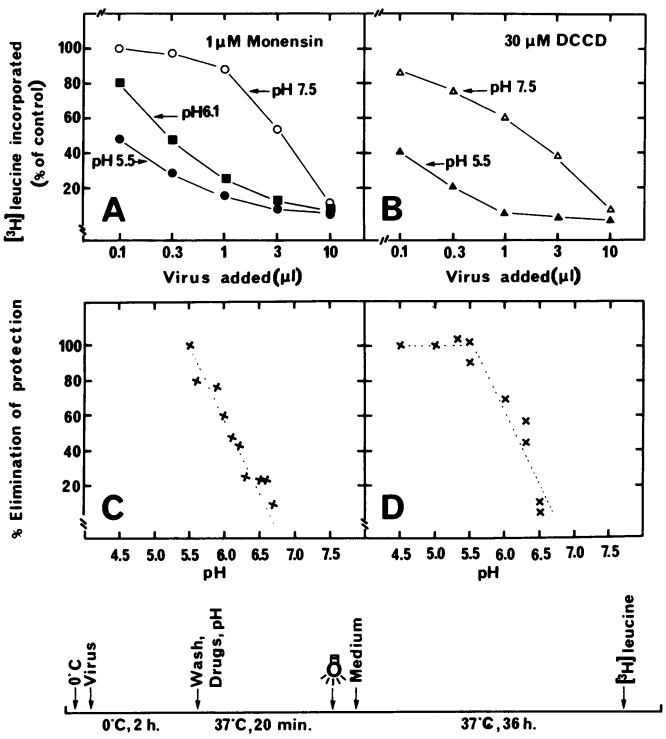

Fig. 1. Ability of low pH to counteract the protective effect of monensin and DCCD. In **A** and **B** increasing amounts of light-sensitive virus were added to HeLa S_3 cells in 24-well microtiter plates. The virus was allowed to bind for 2 h on ice. The cells were then washed, and Hepes-medium at 37°C containing 1 μM monensin or 30 μM DCCD with pH as indicated, was added. After 20 min, the plates were exposed to light, and growth medium with 10% fetal calf serum was added. The cells were incubated at 37°C for 36 h, and then the incorporation of [^{3}H]leucine during 30 min was measured. **C** and **D** show the ability of medium adjusted to different pH to eliminate the protective effect of 1 μM monensin (**C**) and 30 μM DCCD (**D**). The data are expressed as (ID_{50} control/ID_{50} test) x 100, where 'ID_{50} control' is the amount of virus that reduced protein synthesis to half at pH 7.5 in the absence of drugs, and 'ID_{50} test' the amount of virus required to obtain the same reduction in the presence of 1 μM monensin or 30 μM DCCD at the pH indicated.

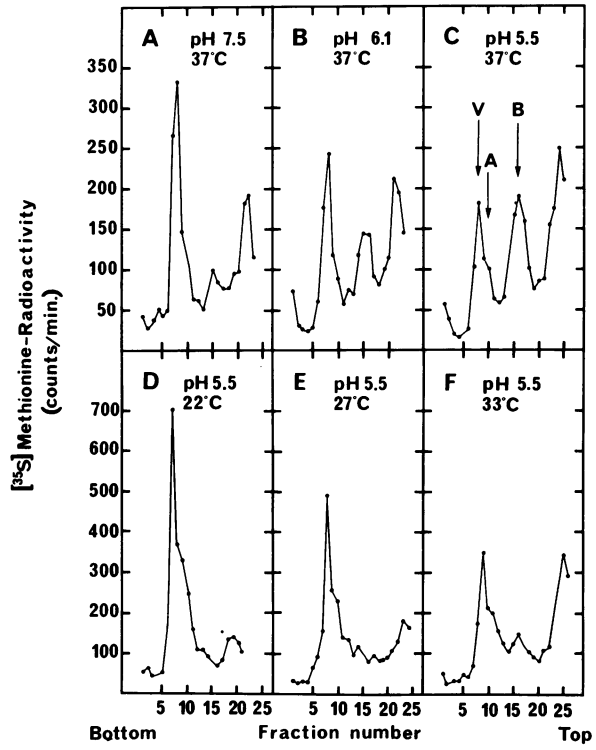

Fig. 2. Effect of pH and temperature on the ability of cells to induce alteration of poliovirus in the presence of monensin. [^{35}S]Methionine-labeled virus was added to HeLa S_3 cells and allowed to bind at 0°C for 2 h. Then the cells were washed, and Hepes-medium, with 1 μM monensin and pH and temperature as indicated, was added. The cells were incubated for 40 min. Then the pH in each well was adjusted to 7.5, and the cells were dissolved with 0.5% Triton X-100. The nuclei were removed by centrifugation in an Eppendorf centrifuge for 3 min. 0.2% SDS was added to the supernatants which were then layered on top of 15–30% sucrose gradients and centrifuged in a Beckman SW 50.1 rotor at 234 000 *g* for 50 min. Fractions were collected dropwise, and the radioactivity in 180 μl of each fraction was measured.

tion took place to an intermediate extent, indicating that it is promoted by the low pH to roughly the same extent as the infection (Figure 1A). The results suggest that virus modification is somehow linked to the entry mechanism.

Induction of amphiphilic properties in poliovirus at low pH

Diphtheria toxin requires low pH for entry (Draper and Simon, 1980; Sandvig and Olsnes, 1980). Low pH exposes a hydrophobic region in the toxin which then apparently inserts itself into the membrane (Sandvig and Olsnes, 1981; Kagan *et al.*, 1981; Donovan *et al.*, 1982). To test if hydrophobic regions are exposed at low pH also in poliovirus, we measured the ability of virus exposed to Triton X-114 at different pH values to enter the detergent phase. The non-ionic detergent Triton X-114 is soluble in water at 0°C, but after incubation at temperatures above 20°C, two phases are formed. It has been shown by Bordier (1981) that after this phase separation, hydrophilic proteins are present in the aqueous phase, while amphiphilic proteins are found in the detergent phase.

We first tested the ability of [^{35}S]methionione-labeled poliovirus to enter the detergent phase at different pH values. As shown in Figure 3, at neutral pH most of the radioactivity remained in the water-phase, whereas at pH values below 5, the amount of labeled virus present in the Triton X-114 phase increased abruptly, and at pH 3 most of the radioactivity was found in the detergent phase. This indicates that the virus exposed hydrophobic domains at this low pH.

The transfer of the virus from the aqueous to the detergent phase was half-maximal at ~pH 4, whereas the half-maximal entry of virus into cells occurred at pH 6.1 (Figure 1C and D). The reason for this discrepancy could be that binding of the virus to cell surface receptors somehow facilitates the exposure of hydrophobic regions in the virus at low pH. To test this, radioactively labeled virus was bound to formaldehyde-fixed cells and then extracted with Triton X-114 at different pH values. As shown in Figure 3, the transition from the water phase to the detergent phase now occurred at less acidic pH, and it was half maximal at ~pH 6.5, which is close to the value found for virus entry into cells in Figure 1. It should be noted, however, that in this case some virus was found in the detergent phase even at neutral pH, whereas very little virus entered the cells at neutral pH under the conditions in Figure 1.

To test the possibility that lipids extracted from the cells could alter the pH dependence of virus transfer into the Triton X-114 phase, we first extracted fixed cells with the detergent and then measured the ability of added [^{35}S]-methionine-labeled virus to enter the detergent treated in this

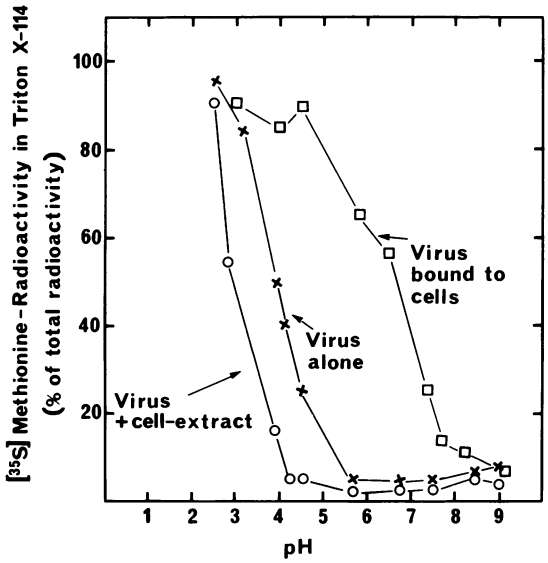

Fig. 3. Effect of pH on the ability of poliovirus to bind Triton X-114. A mixture of medium (300 μl) and Triton X-114 (100 μl of a stock solution, prepared as described in Materials and methods), adjusted to the pH values indicated on the abscissa, was incubated for 15 min at 4°C with [^{35}S]-methionine-labeled virus, as indicated. The samples were then heated to 37°C and incubated for 15 min to induce phase separation, and they were subsequently centrifuged for 2 min in an Eppendorf centrifuge 3200. The radioactivity in the two phases was measured. The amount of radioactivity in Triton X-114, as a percentage of the total radioactivity in each sample, is given at the ordinate. (x), virus alone; (□), virus pre-bound to formaldehyde-fixed cells and then extracted with the detergent; (○), virus added to Triton X-114 which had been pre-treated for 15 min with formaldehyde-fixed cells.

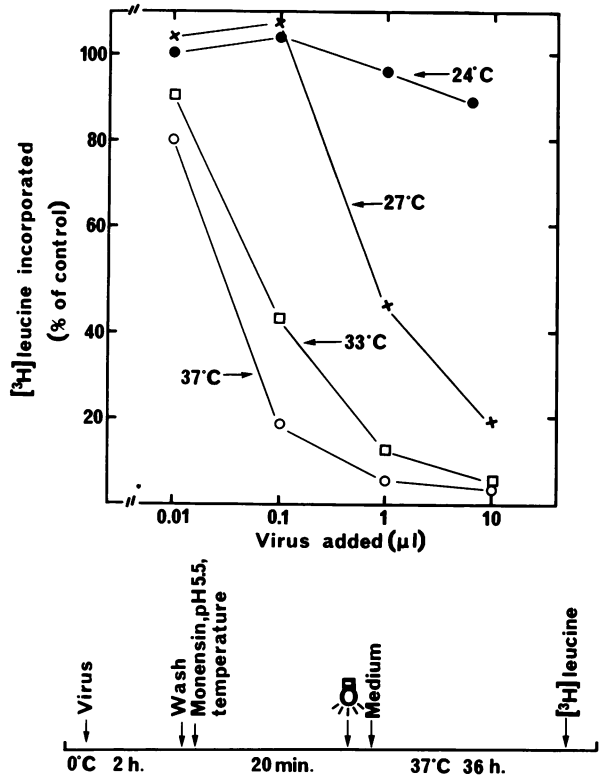

Fig. 4. Temperature requirement for entry of poliovirus at low pH in the presence of monensin. Increasing amounts of light-sensitive virus were added to HeLa S_3 cells in 24-well microtiter plates. The virus was allowed to bind in the cold for 2 h. The cells were then washed, Hepes-medium, pH 5.5, containing 1 μM monensin and adjusted to different temperatures as indicated, was added, and the cells were incubated for 20 min. The plates were then exposed to light, and growth medium, pH 7.4, was added. The cells were incubated at 37°C for 36 h, and then the incorporation of [^{3}H]leucine was measured. The exposure to virus was at: (○), 37°C; (□), 33°C; (x), 27°C; (●), 24°C.

way. As shown in Figure 3, after this treatment it was necessary to reduce the pH even more than with pure Triton X-114 to permit the virus to enter the detergent phase. Clearly therefore, lipids extracted from the fixed cells cannot be the reason for the finding that virus bound to cells enters the detergent phase at a less acidic pH than the free virus. Together, the data indicate that after binding to cell surface receptors the virus exposes hydrophobic regions at higher pH than the free virus.

The pH-induced transfer of virus from the aqueous to the detergent phase was fully reversible in the absence of cells. In the presence of cells it was found to be reversible when virus had been bound at 0°C. Thus, virus which had entered the detergent phase at low pH, returned to the aqueous phase when the pH was increased.

Effect of temperature on the entry of virus at low pH

To further characterize the entry process induced by low pH in monensin-treated cells, we tested the effect of temperature on this process. As shown in Figure 4, at 24°C, very little infection occurred at pH 5.5, while at 33°C the entry at low pH was almost as efficient as at 37°C.

The ability of the monensin-treated cells to modify the virus showed a similar temperature dependence as that found for infection. Thus at 22°C very little alteration took place at pH 5.5 (Figure 2D), while most of the virus was modified at 33°C (Figure 2F). At 27°C the virus was altered to an intermediate extent (Figure 2E).

Altogether the data indicate that low pH as well as physiological temperature are required both for alteration of the virus particles and for efficient infection. When the temperature is reduced, there does not appear to be an abrupt inhibition of infection. This is in accordance with previous findings (Mandel, 1967).

Effect of weak acids on the entry of poliovirus

A pH gradient across the membrane appears to be required for entry of diphtheria toxin (K. Sandvig and S. Olsnes, in preparation). When cells are incubated with sodium acetate, a certain fraction of the acetate is present as acetic acid, and in this form it is able to penetrate the membrane. Once it enters into the cell, the acid dissociates and acidifies the cytosol (Rogers *et al.*, 1983a, 1983b). To test if a pH gradient is required for the entry of poliovirus, cells were incubated with sodium acetate at pH 5.5 for 30 min to acidify the cytosol. Light-sensitive virus was then added, and the incubation was continued for a further 30 min at 37°C. Then virus that had not entered the cytoplasm, was inactivated by light exposure and the cells were incubated overnight. As shown in Figure 5, virus entry did not occur in the sodium acetate-treated sample while, in the control sample containing no sodium acetate, infection occurred. Clearly, exposure of cell-bound virus to low pH is not sufficient for entry. The data therefore indicate that a certain pH gradient must exist across the membrane for virus entry to occur. If the cells were pre-incubated with 0.14 M sodium acetate, pH 7.0, no protection was obtained. This indicates that sodium acetate as such does not interfere with the entry, but that acidification of the cytosol is the reason for the protective effect.

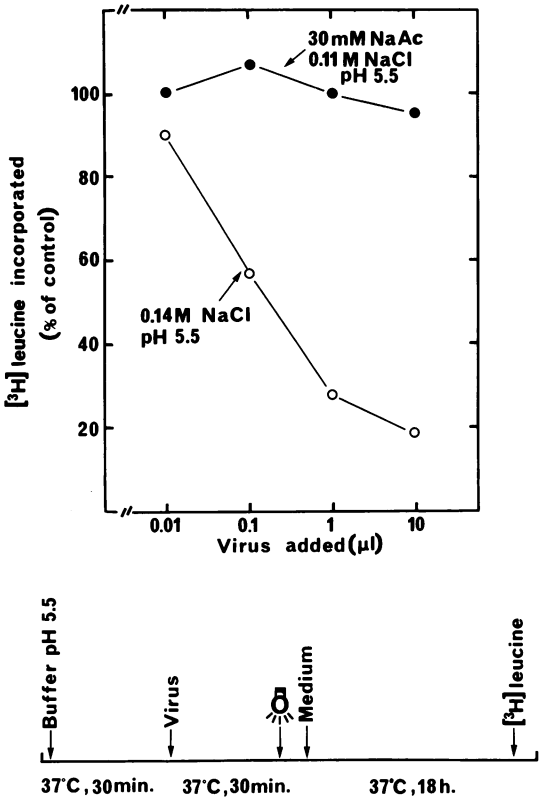

Fig. 5. Ability of acetic acid to inhibit infection induced by low pH. HeLa S_3 cells in 24-well microtiter plates were pre-incubated at 37°C for 30 min with 10 mM sodium phosphate, pH 5.5, containing 5 mM KCl and either 0.14 M NaCl or 0.11 M NaCl and 30 mM sodium acetate. Then increasing amounts of light-sensitive virus were added and the cells were incubated at 37°C for 30 min. Subsequently the plates were exposed to light, growth medium was added and the cells were incubated at 37°C for 18 h. The ability of the cells to incorporate [^{3}H]leucine was then measured.

Table I. Ability of fixed cells to bind [^{35}S]methionine-labeled poliovirus

Fixation method	Virus bound c.p.m.
None	7502
1% formaldehyde	9194
1% paraformaldehyde	8723

HeLa S_3 cells growing in 24-well microtiter plates were fixed with aldehydes, as described in Materials and methods, and washed three times with buffer. Then the cells were chilled to 0°C and [^{35}S]methionine-labeled poliovirus (~100 000 c.p.m., 5 x 10^4 virus/c.p.m.) was added and allowed to bind for 2 h. Unbound virus was removed by washing the cells three times in 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl, the cells were dissolved in 0.2 ml of 0.1 M KOH and the cell-associated radioactivity was measured.

When cells were treated with sodium acetate, virus binding and alteration was not inhibited (data not shown). The data therefore suggest that although low pH is sufficient to induce alteration of cell-bound virus, a pH gradient across the membrane is necessary for transfer of the virus genome across the membrane.

Ability of fixed cells to bind and alter virus

The results above, as well as earlier data (Madshus *et al.*, 1984; Sandvig *et al.*, 1984b), indicate that alteration of virus to form A-particles is a necessary, but not sufficient, process for virus entry. Since at low pH alteration occurs in ATP-depleted cells (data not shown), we decided to test if it could take place even in dead cells. To study this, it was first necessary to find conditions to kill the cells while retaining their ability to bind the virus. As shown in Table I, cells treated with formaldehyde and paraformaldehyde bound approximately the same amount of virus as living cells. The fixed cells were permeable to Trypan blue, indicating that they were fully permeabilized. Furthermore, they were unable to incorporate [^{3}H]leucine into acid-precipitable material, indicating that they were metabolically inactive.

The data in Figure 6C show that virus bound to paraformaldehyde-fixed cells was indeed altered upon exposure to pH 4.5 at 37°C. Exposure to pH 4.5 at 0°C, or to pH 7.5 at 37°C, did not efficiently alter the virus (Figure 6A, B). Furthermore, exposure of virus to pH 4.5 at 37°C in the absence of cells did not result in alteration (Figure 6D). It therefore appears that efficient alteration of virus occurs only when it is bound to cells and exposed to low pH at physiological temperature.

Discussion

We have presented data indicating that poliovirus exposes hydrophobic regions at low pH, and that virus which is bound to cells is altered to A-particles at physiological temperature. During this process it may inject its RNA into the cytosol if a suitable pH gradient exists across the membrane. The present data indicate that the virus entry, as well as the alteration, occur at maximal rate at pH 5.5 and below. The entry appears to be half-maximal at pH 6.1, and even at pH 6.7 some entry occurs. It therefore appears that the requirement for low pH is not as strict as in the case of Semliki Forest virus which enters only at pH below 6.2 (Kiellian *et al.*, 1984). Even at low pH the virus does not enter efficiently, unless the temperature is 33–37°C, and the same temperature requirement was found for the virus alteration. The same pH and temperature requirements as for virus entry were found for alteration with paraformaldehyde-fixed cells.

It is important to stress that our measurements of virus entry are based on the transition of virus from light-sensitivity to resistance. As discussed earlier (Madshus *et al.*, 1984), we cannot exclude the possibility that the virus becomes light resistant before the RNA genome is released into the cytosol; it is not known if the light-sensitivity persists in altered particles. Our data only provide information on the entry process as long as the RNA is light sensitive. This does not necessarily comprise the whole process of RNA entry into the cytosol. The fact that virus alteration occurs in acetate-treated cells, while infection is inhibited, shows that alteration as such is not sufficient for entry of the virus genome to occur. The same conclusion was reached in experiments with DMSO-treated cells which efficiently altered the virus without being infected (Sandvig *et al.*, 1984b).

The experiments with Triton X-114 indicate that upon exposure of virus to low pH the hydrophobic domains, present in the capsid proteins, are transiently exposed. In Figure 7 we propose the existence of an amphiphilic intermediate particle in the infection pathway. It is possible that if a suitable membrane comes in close contact with this particle the lipids may interact with the hydrophobic domains of the virus and induce irreversible alteration of the virus (A-particles). Such altered virus is able to insert itself into liposomes, indicating that hydrophobic domains are indeed exposed (Lonberg-

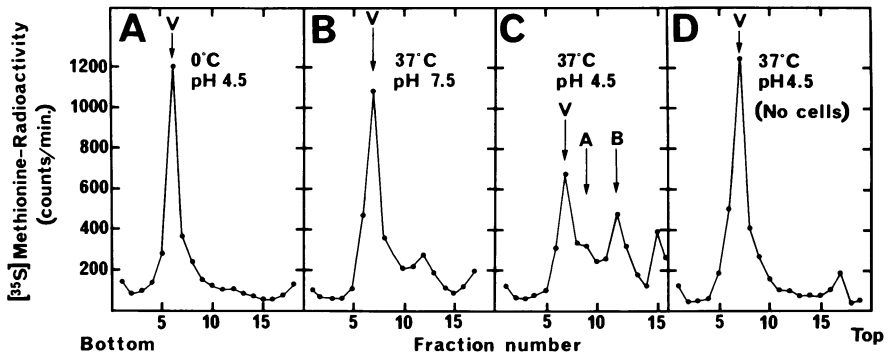

Fig. 6. Temperature and pH requirement for virus alteration by paraformaldehyde-fixed cells. The cells in (**A,B,C**) were fixed in 1% paraformaldehyde, as described in Materials and methods. [^{35}S]Methionine-labeled virus was added and allowed to bind for 2 h, and then the cells were washed to remove unbound virus. In (**A**) the cells were incubated with Hepes-medium, pH 4.5, for 40 min at 0°C, whereas in **B** and **C** the incubation was carried out in Hepes-medium, pH 7.5 and 4.5, at 37°C. In **D** virus alone was incubated at 37°C for 40 min in medium at pH 4.5. Subsequently, 0.5% Triton X-100 and 0.2% SDS were added to each well and, after 10 min at room temperature, the extracted material was transferred to Eppendorf centrifuge tubes and centrifuged for 3 min. The pellet was discarded and the supernatant was analyzed by sucrose gradient centrifugation as in Figure 2.

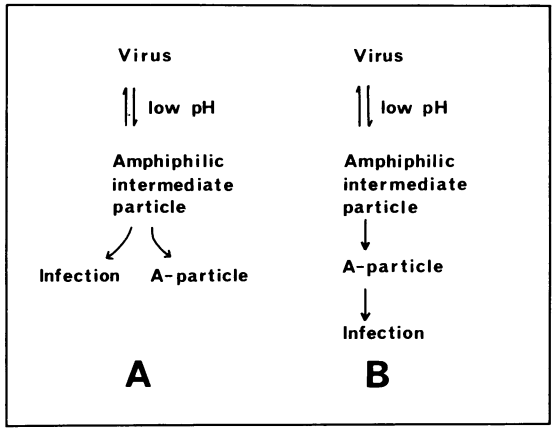

Fig. 7. Hypothetical schemes of poliovirus entry.

Holm *et al.*, 1976). In some rare instances the pH-induced interaction of the virus with the membrane could result in complete integration of the capsid proteins into the lipid bilayer and release of the RNA genome at the cytosolic side. The A-particles could either be the result of abortive alteration, where the insertion of the virus capsid into the membrane had not succeeded (Figure 7A), or they could represent a necessary intermediate stage in the entry process (Figure 7B).

It is not clear why a pH gradient across the membrane appears to be required for entry. Possibly, assymetric pH may modify the virus and the membrane enabling the virus to enter the membrane on the one side and the RNA to leave the membrane on the other side. However, it is also conceivable that the pH gradient plays a more direct role as a driving force for the entry of the RNA genome. In this connection it is interesting that a proton gradient may be the driving force for the entry of dipeptides into renal brush border cells (Ganapathy and Leibach, 1983).

Materials and methods

Virus

A strain of poliovirus type I (Brunende) was propagated in HeLa S_3 cells as described earlier (Madshus *et al.*, 1984). Light-sensitive virus was prepared in cells growing in medium containing 10 μg/ml neutral red (Madshus *et al.*, 1984). The light-sensitive virus preparation contained 1.4 x 10^5 p.f.u./μl. [^{35}S]-Methionine-labeled virus was prepared as described earlier (Madshus *et al.*, 1984).

Cell cultures

HeLa S_3 cells were maintained as monolayer cultures in minimal essential medium (Gibco, Glasgow, UK) with 10% fetal calf serum (Sandvig and Olsnes, 1981). The same medium, containing 20 mM Hepes, pH 7.5, instead of sodium bicarbonate (Hepes-medium), was used when indicated.

Measurement of virus-induced inhibition of protein synthesis

HeLa S_3 cells growing in disposable trays with 24 wells (5 x 10^4 cells/well in 1 ml medium) were treated with virus, as described in the legend to the Figures and, in most cases, the plates were incubated for 18–36 h at 37°C to amplify the infection as described (Madshus *et al.*, 1984). The experiments were terminated by measuring the incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble material during 1 h as described earlier (Sandvig and Olsnes, 1982).

In those cases when light-sensitive, neutral red-containing virus was used, the cells were exposed to the virus in the dark. After the indicated period of time, the cells were exposed for 5 min to the light from a 60 W bulb at a distance of 15 cm. This treatment inactivated >99.9% of the virus that had not penetrated into the cells. The cells were then incubated overnight and their ability to incorporate [^{3}H]leucine was measured. The control values (no virus present) varied between 5000 and 20 000 c.p.m. in different experiments.

Virus binding

Cells were pre-incubated as indicated and then chilled to 0°C and [^{35}S]-methionine-labeled virus (~100 000 c.p.m.) was added to each well. The cells were kept at 0°C for 2 h and then washed three times with medium to remove unbound virus. The cells were finally dissolved in 0.2 ml of 0.1 M KOH and the bound radioactivity was measured.

Aldehyde fixation of cells

To test virus alteration in metabolically inactive cells, cells in 24-well microtiter plates were first washed three times in 10 mM sodium phosphate, pH 7.4, 140 mM NaCl, to remove serum proteins. 1% paraformaldehyde or 1% formaldehyde in the same buffer was added, and the cells were fixed for 30 min at 4°C. Then the cells were treated for 5 min with 50 mM sodium borohydride in 10 mM sodium phosphate, pH 7.4, 140 mM NaCl, and subsequently washed twice with buffer without sodium borohydride and once with Hepes-medium.

Sucrose gradient centrifugation

HeLa S_3 monolayer cultures in 24-well disposable trays (10^5 cells/well) were incubated in a moisture chamber with [^{35}S]methionine-labeled virus (~10^5 c.p.m./well in 20–30 μl). After exposure to the virus for 2 h at 0°C, the cells were washed to remove unbound virus. Medium (100 μl per well) was added, and the incubation was continued as described. Triton X-100 was added to a final concentration of 0.5% (w/v) to dissolve the cells, and the nuclei were removed by centrifugation for 3 min in an Eppendorf centrifuge. To the supernatant, SDS was added to a final concentration of 0.2% (w/v), and the mixture was layered on top of sucrose gradients (15–30% w/v) in 0.14 M NaCl, 2 mM EDTA, 10 mM sodium phosphate, pH 7.4. The gradients were centrifuged for 50 min at 234 000 *g* in a Beckman SW 50.1 rotor, fractions were collected, and the radioactivity was measured as described (Madshus *et al.*, 1984).

Chemicals

Neutral red was obtained from G.T. Gurr, Ltd., London. Monensin, DCCD, 2-deoxyglucose and NaN_3 were obtained from Sigma Chemical Co., St. Louis, MO. Formaldehyde and paraformaldehyde were obtained from E. Merck, AG, Darmstadt, FRG.

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland. To remove material that remained in the water phase at 37°C, a stock solution of detergent was prepared by dissolving 40 g Triton X-114 in 1 litre of 20 mM sodium phosphate, pH 7.5, containing 0.14 M NaCl, by stirring overnight at 4°C and then incubating this solution at 37°C until two phases appeared. The upper phase which consisted of buffer, was discarded, and new buffer was added. This procedure was repeated three times. Finally this pre-treated Triton X-114 was made up to 200 ml by addition of 20 mM sodium phosphate, pH 7.5, containing 0.14 M NaCl.

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