The Uncoating and Infectivity of the Flavivirus West Nile on Interaction with Cells: Effects of pH and Ammonium Chloride

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SUMMARY

Infectivity of the West Nile virus (WNV; Flaviviridae) was inactivated on exposure for brief periods (90 s) to pH 6.6 and below. This inactivation was not due to decreased interaction between cells and acid-treated virus. The RNA of [3H]uridine-labelled virus particles prebound to the cell surface before acidic pH treatment underwent rapid uncoating within 1 min at 37 °C at the same pH values that inactivated virus particles. The uncoating of [3H]uridine-labelled virus particles was also studied over longer time periods after synchronized internalization by P388D1 cells. At pH 7.6 uncoating occurred rapidly after a reproducible time lag of 1 min on warming to 37 °C and was essentially complete by 15 to 30 min after the start of internalization, leaving uncoated RNA in an infectious form. In contrast, at pH 6.2 viral uncoating occurred rapidly without any time lag and the uncoated RNA appeared to be far less infectious than that uncoated at pH 7.6. Ammonium chloride could almost totally inhibit both the infectivity and uncoating of virus particles on synchronized internalization into P388D1 cells, with a pH optimum of 8.0. These results suggest that the uncoating of virus particles is dependent on an acidic pH, although the location of uncoating (prelysosomal endosome or plasma membrane) decides whether the uncoated RNA will be infectious or not. Essentially the same results were obtained when infections were carried out in the presence of enhancing antibody.

INTRODUCTION

Several enveloped viruses can fuse efficiently with target membranes (including artificial liposomes and cell membranes) optimally at pH values just below neutrality (White et al., 1983) and it has been proposed that this fusion is involved in infectious penetration of virus genetic material from acidic intracellular prelysosomal compartments (endosomes), into the cytosol, where replication can then commence (Marsh, 1984). The possibility that infectious penetration of the West Nile flavivirus (WNV) nucleocapsid into the cytosol might involve an acid-catalysed fusion reaction in the endosomal compartment is investigated further in this paper. For this purpose we used the enhanced replication which results when the macrophage-like cell line P388D1 is infected in the presence of sub-neutralizing concentrations of antiviral antibody (Peiris & Porterfield, 1979, 1981). Enhancement is due to an increased interaction of non-neutralized antibody-opsonized virus particles with cell surface Fc receptors (FcR) (Peiris et al., 1981; Gollins & Porterfield, 1984).

Electron microscopic examination of the WNV entry process has shown that at neutral pH virus is taken up into P388D1 cells by receptor-mediated endocytosis, both in the absence and presence of ‘enhancing’ antibody (Gollins & Porterfield, 1985). The suggestion that WNV undergoes acid-catalysed fusion is supported by using ammonium chloride, which raises the pH of acidic, intracellular compartments. This can both inhibit viral replication (Gollins & Porterfield, 1984), and can cause a build-up of virus particles in prelysosomal (endosomal) compartments either with or without ‘enhancing’ antibody (Gollins & Porterfield, 1985). Also,
WNV fuses with artificial liposomes with an optimum of pH 6.7 and below (Gollins & Porterfield, 1986) which is possibly relevant to viral escape from an acidic intracellular compartment.

METHODS

Cells. The porcine kidney cell line PS Clone D (Madrid & Porterfield, 1969) was maintained in Leibowitz L15 medium containing 3% foetal calf serum. The mouse fibroblast cell line L929, Vero cells and P388D1 cells were all maintained as described previously (Gollins & Porterfield, 1984).

Virus. Oxford PO mice less than 1 week old were injected intracerebrally with 10⁵ p.f.u. of WNV, E101 strain, in phosphate-buffered saline (PBS) pH 7.4. After 3 days mice became ill and their brains were harvested aseptically. Each brain was assumed to weigh 0.1 g, and 10% suspensions were prepared by homogenizing infected brains in PBS with 10% foetal calf serum. During this procedure, brains were kept at 4 °C. The resulting suspension was clarified by centrifugation at 10,000 g for 1 h at 4 °C. The supernatant was distributed into 0.1 ml aliquots and immediately frozen at −70 °C.

[^3H]Uridine-labelled WNV was prepared and purified as described before (Gollins & Porterfield, 1986).

[^3S]Methionine-labelled WNV was prepared and purified as described for[^3H]uridine-labelled virus (Gollins & Porterfield, 1986), except that the labelling medium consisted of Eagle's MEM without methionine, containing 167 µCi/ml[^3S]methionine, 1.5 µg/ml actinomycin D, 0.2% bovine serum albumin (BSA), buffered with 10mM-HEPES, 10 mM-HEPES (pH 7.8).

Virus was stored at −70 °C in aliquots of Tris buffer (0.14 M-NaCl, 0.01 M-Trizma base, 0.2% BSA) at pH 8.0. Aliquots were thawed just before use.

Antibody. A rabbit hyperimmune anti-WNV (RatWNV) antiserum was prepared in a New Zealand White rabbit by giving an initial dose of a heat-killed (60 °C for 30 min) suckling mouse brain suspension of WNV, through an intravenous route, followed by several monthly intravenous injections of live virus. The rabbit was bled 2 weeks after the last boost. The IgG fraction of the antiserum was purified by Protein A-Sepharose affinity chromatography. IgG was dialysed against PBS, filter-sterilized and stored in aliquots at 4 °C for experiments carried out within 6 weeks after purification. Other aliquots were stored at −20 °C.

Assay for the uncoating of[^3H]uridine-labelled WNV on interaction with P388D1 cells. The assay used was essentially that of Helenius et al. (1982). Virus was inoculated onto monolayers of 2 × 10⁶ P388D1 cells in 35 mm diam. plastic Petri dishes (Falcon), as described in individual experiments. Medium was removed from cells which were then overlaid with 1 ml ice-cold isotonic buffer containing 0.01 M-triethanolamine, 0.01 M-acetic acid and 0.25 M-sucrose (pH 7.5) (H buffer). The cells were then scraped from plates, transferred to Eppendorf tubes at 0 °C, and pelleted for 30 s in a Beckman microfuge at 4 °C. The supernatant was aspirated and 0.5 ml of fresh H buffer with 1 mM-EDTA was added. The cells were then centrifuged for 5 min at 10,000 g in a Beckman microfuge and an aliquot of the supernatant was counted to give a figure for acid-soluble radioactivity. All radioactive counts were determined in 10 ml Unisolve-E scintillation fluid (Koch-Light).

Controls included samples to which were added 0.1 ml PBS containing no nucleases. This gave the background breakdown due to cellular nucleases. Other samples were precipitated with TCA immediately after the cells had been lysed at 0 °C, with no 30 min incubation at 37 °C. This gave an estimate of the intracellular lysosomal degradation of virus particles.

Synchronized infections. These were carried out by binding WNV particles in B medium (L15, 0.2% BSA, 15 mM-HEPES), pH 7-6 at 0 °C, to cells for 1 or 2 h, washing cells twice at 0 °C, then warming to 37 °C by addition of prewarmed medium (described in individual figure legends), and floating plates on a 37 °C water-bath.

Infectivity assay. At the end of the experiments described in Fig. 1, 3, 5, 6 and 7, cells were washed three times and incubated for 2 to 3 days with the relevant growth medium containing 1.5% CM-cellulose, and the number of viral plaques was counted after staining with naphthalene black.

RESULTS

Effects of pH on the interaction of WNV with cells

WNV particles initially at pH 8.0 and 0 °C were brought to different pH values for 90 s at 37 °C, and then brought back to pH 7.6 by dilution into pH 7.6 medium. As shown in Fig. 1, the infectivity of such viral suspensions decreased between pH 7.2 and 6.6, with 90 to 95% inactivation occurring at the latter pH.
**Flavivirus uncoating and infectivity**

Fig. 1. Effect of pH on infectivity in the absence of cells. WNV was thawed and treated for 90 s at 37 °C in A medium (L15, 0.2% BSA, 10 mM-HEPES, 10 mM-MES) at various pH values. It was then brought to pH 7.6 by dilution into B medium (see Methods) and then immediately incubated with monolayers of L929 (□), PS Clone D (△) or P388D1 (○) cells in the wells of 24-well Linbro plates for 5 h at 37 °C. At the end of this period the virus inoculum was removed and the cells were washed three times with B medium. After 3 days the numbers of viral plaques were counted. Plaques per well formed by virus pretreated at each pH are expressed as a percentage of plaques formed by virus pretreated at pH 7.6 (100%). Virus input was adjusted so that 100% represents 50 to 100 plaques/well in each case.

Fig. 2. Cell association and lysosomal degradation of virus pretreated for 90 s at various pH values. [35S]Methionine-labelled, purified WNV was pretreated for 90 s at 37 °C in A medium at various pH values, brought to pH 7.6 by dilution in B medium (pH 7.6) and then incubated with confluent monolayers of P388D1 cells in Petri dishes for 2 h at 37 °C (1.0 ml of medium/dish). At the end of this period the medium was removed and assayed for TCA-soluble radioactivity (○). The cells were scraped into 1.5 ml fresh B medium (pH 7.6) and washed twice with 1.0 ml B medium in 1.5 ml microfuge tubes. The final cell pellet was solubilized with 1% Triton X-100 and an aliquot was counted to give a figure for cell-associated radioactivity (○). Fifteen-thousand c.p.m. were added to each monolayer.

A similar drop in infectivity with pH was seen in L929, PS Clone D (which do not have FcR on their surface) and P388D1 cells (which do bear FcR) in the absence of antiviral antibody (Fig. 1). If virus was similarly pretreated at different pH values, brought back to pH 7.6, and then sub-neutralizing, purified RzWNV antiserum was added for the duration of the infection of P388D1 cells (final concentration 1 µg/ml), viral replication could be enhanced more than 20-fold over
Fig. 3. Effect on infectivity of treating cell surface-bound virus at various pH values. WNV particles were bound to P388D1 cell monolayers in 24-well Linbro plates for 1 h at 0 °C, in 0·25 ml B medium pH 7·6. Cells were then washed twice with B medium at 0 °C, 2 ml pre-warmed (37 °C) A medium at various pH values was added to monolayers to allow synchronized infection, and plates were floated on a 37 °C water-bath for 90 s. This medium was then replaced with 37 °C B medium pH 7·8 with (○) or without (△) 50 mm-ammonium chloride, and cells were incubated for a further 5 h at 37 °C. At the end of this period cells were treated as described in Fig. 1. One-hundred% represents an average of 90 plaques/well.

Fig. 4. Uncoating of WNV RNA on exposure of cell surface-bound virus particles to various pH values for 1 min. [3H]Uridine-labelled WNV was bound at 0 °C to monolayers of P388D1 cells in Petri dishes for 2 h at 0 °C in 0·5 ml B medium pH 7·6, containing 1·0 lg/ml RzWNV IgG which optimally enhanced WNV binding and replication under these conditions. The cells were washed twice and then treated for 1 min at 37 °C with A medium at various pH values. This medium was then aspirated, pre-cooled (0 °C) H buffer was added to cells and uncoated viral RNA was determined by susceptibility to digestion with RNase as described in Methods. At pH values below 6·2, approximately 25% of the viral c.p.m. eluted from cells into the medium and the results have been corrected for this so that they represent uncoated viral RNA per 2000 c.p.m. of cell-associated virus.

that of pH-treated virus in the absence of antibody. However, the major (more than 90%) drop in infectivity of virus pretreated between pH 7·2 and 6·6 was still seen in the presence of 'enhancing' antibody (not shown).

The association of [35S]methionine-labelled WNV with P388D1 cells at 37 °C increased if virus particles had been pretreated with increasingly acidic pH media (Fig. 2). Moreover, a substantial amount of cell-associated virus was endocytosed and reached lysosomes after pretreatment at all pH values, as demonstrated by TCA-soluble radioactivity (degraded virus) appearing in the medium.

In order to assess the effects of acidic pH on virus particles that had been prebound to the cell surface, the experiment described in the legend to Fig. 3 was carried out. Cell surface virus particles were inactivated by a 90 s treatment at pH values less than 7·2; less than 10% of infectivity remained at pH 6·4. No bypass of an ammonium chloride block of viral infection could be achieved using low pH treatment (Fig. 3). Very similar results were obtained for L929 or PS Clone D cells and also for antibody-enhanced infection of P388D1 cells (not shown).

When [3H]uridine-labelled WNV was inoculated onto P388D1 cells, then treated briefly with media at different pH values, it was found that at pH values below 7·0 an increase in uncoated viral RNA occurred (Fig. 4), with substantial uncoating occurring at pH 6·4 and maximum uncoating at pH 5·7. No uncoated viral RNA was found when cell-bound virus was briefly warmed at pH 7·0 and above.

The observation that the infectivity of more than 95% of virus particles bound to the plasma membrane of a variety of cells could be destroyed by a 90 s treatment of cells with pH 5·5 medium (Fig. 3) formed the basis of a sensitive assay for viral internalization into cells.
Internalized virus becomes resistant to inactivation by acid, whereas virus bound to the plasma membrane is inactivated. Accordingly, the synchronized infection of P388D1 cells was studied (both enhanced and non-enhanced) (Fig. 5). The time needed for internalization of 50% of virus in the presence of antibody in P388D1 cells was approximately 3.25 min. In the absence of antibody the figure for P388D1 cells was 6.25 min. Using a similar protocol the figure for both L929 and PS Clone D cells was also 6.25 min (not shown).

**Effects of ammonium chloride on the interaction of WNV with P388D1 cells**

The unprotonated form of ammonium chloride crosses cell membranes but the protonated form does this far less efficiently. If the uncharged form enters acidic intracellular compartments it becomes protonated, thereby raising the intravacuolar pH and inhibiting its own escape across the membrane of such vacuoles (Ohkuma & Poole, 1978, 1981).

When ammonium chloride (20 mM) was dissolved in L15 medium buffered at various pH values with 10 mM-HEPES and 10 mM-MES, infection of P388D1 cells by WNV in the presence of ‘enhancing’ antibody was inhibited (see Gollins & Porterfield, 1984) and this inhibitory effect was potentiated at alkaline pH values. Ninety-five% inhibition of WNV replication occurred at pH 8.0 and no inhibition at pH 7.2 and below (Fig. 6). Similar results to these were obtained for virus particles bound to L929 cells or P388D1 cells in the absence of ‘enhancing’ antibody (not shown).
Fig. 7. Length of time that ammonium chloride must remain in contact with cells in order to exert its inhibitory effect. A synchronized infection of P388D1 cells was established using WNV prebound for 1 h at 0 °C in the absence (○) or presence (●) of 1 μg/ml RaWNV IgG and the cells were warmed in B medium pH 8.0 containing 20 mM-ammonium chloride. At various times after warming, the medium was replaced with B medium pH 8.0 without ammonium chloride. At the end of a total incubation period of 5 h the cells were washed twice and growth medium containing CM-cellulose was added. The numbers of plaques/well are expressed as a percentage of the plaques/well obtained in the total absence of ammonium chloride (100%). Viral input was adjusted so that 100% represented 50 to 100 plaques/well with or without antibody. In some wells (■, without antibody; ■, with antibody), no ammonium chloride was added for the first 2 h after warming, then was added for a period of 5 h, before washing of the cells and addition of growth medium containing CM-cellulose.

Fig. 8. Fate of [35S]methionine- and [3H]uridine-labelled WNV after synchronized infection of P388D1 cells. [35S]Methionine- or [3H]uridine-labelled WNV was bound for 2 h to monolayers of P388D1 cells in Petri dishes, at 0 °C in B medium pH 7.6, either in the absence (a) or presence (b) of 1 μg/ml RaWNV IgG. The cells were then washed twice with cold B medium pH 7.6 and then 1 ml 37 °C B medium at pH 7.4 or pH 8.0 without ammonium chloride or pH 8.0 with 20 mM-ammonium chloride (AC) was added to each plate. The incubation was continued for 2.5 h at 37 °C, after which time TCA-precipitable and TCA-soluble [3H] c.p.m. in the medium were determined. The cells were scraped into cold B medium pH 7.6, washed twice, and the cell-associated c.p.m. were determined. The initial 35S-WNV cell-bound c.p.m. were 650 c.p.m. in the absence of antibody and 3075 c.p.m. in the presence of antibody. The initial 3H-WNV cell-bound c.p.m. were 520 c.p.m. in the absence of antibody and 2810 c.p.m. in the presence of antibody. The values expressed in the bar charts are percentages of the c.p.m. initially bound to the cells in each case.
In the above experiments, ammonium chloride was kept in contact with cells for 5 h before removal in order to exert its inhibitory effect. In later experiments (Fig. 7) it was shown that regardless of whether WNV was prebound to P388D1 cells in the absence or presence of 'enhancing' antiviral antibody, 20 mM-ammonium chloride at pH 8·0 could inhibit a synchronized infection of P388D1 cells by 85% or more, when added for 40 min after the start of internalization.

**Uncoating of WNV on interaction with P388D1 cells and its inhibition by ammonium chloride**

Initial experiments compared the interaction of [3H]uridine- and [35S]methionine-labelled WNV with P388D1 cells, using synchronized infections, after prebinding WNV particles to the cell plasma membrane in the absence or in the presence of 'enhancing' antiviral antibody (Fig. 8). TCA-precipitable radioactivity appearing in the medium (eluted virus particles), TCA-soluble radioactivity in the medium (degraded virus particles) and cell-associated radioactivity were determined on synchronized internalization at pH 8·0 and pH 7·4 in the absence of ammonium chloride or at pH 8·0 in the presence of 20 mM-ammonium chloride. Firstly, three- to fivefold more cell-associated [35S]methionine-labelled viral protein than [3H]uridine-labelled viral RNA was degraded in the presence of antibody. Without antibody the difference was less (approximately twofold), though still significant in all cases (P < 0·05). Secondly, 70 to 80% of whole virus particles eluted from cells on warming in the absence of antibody, but less than 8% in the presence of antibody.

The last observation meant that it was difficult to get enough cell-associated viral radioactivity bound to P388D1 cell monolayers to derive accurate results regarding the uncoating of the virus. Hence, in the experiments presented below, virus particles were initially bound to the surface of P388D1 cells in the presence of purified RzWNV IgG at a concentration (1 µg/ml) that had previously been shown to optimally enhance both the binding and infectivity. When monolayers of P388D1 cells were synchronously infected with WNV at pH 8·0 or 7·6, the susceptibility of viral RNA to specific degradation increased in the first few minutes of infection and was essentially complete at 15 min after warming, when approximately 27% of the initially cell-associated viral RNA had been uncoated (Fig. 9a). The presence of 20 mM-ammonium chloride at pH 8·0 reduced the uncoating by approximately 94%. At 60 min after warming, the amount of uncoated RNA present at pH 6·2 amounted to approximately 64% of that uncoated at pH 7·6 and 8·0 (Fig. 9a), i.e. 19% compared to 30% of the initially bound c.p.m.

When the degradation of [3H]-labelled RNA by the cells' own lysosomal hydrolases was examined, it was found that there was a time lag of approximately 30 min between the appearance of intracellular and extracellular TCA-soluble radioactivity (Fig. 9b).

A detailed examination of the initial 7·5 min of synchronized infection (Fig. 10) showed that at pH 6·2 a rapid initial uncoating seen within 1 min was followed by a subsequent slower increase in uncoating. At pH 7·6, however, there was a time lag of approximately 1 min before the viral uncoating started to increase, which it then did rapidly, eventually surpassing the level of uncoating seen at pH 6·2.

**DISCUSSION**

The results presented in this paper give some insight into the relationship between WNV uncoating and infection of cells using simple manipulations of pH, ammonium chloride, and an assay that directly monitors the RNA uncoating step. It is evident that brief exposure to pH values lower than pH 6·6 induces a change in virus particles, which leads to loss of virus infectivity, regardless of whether virus is treated with acid in the absence of cells, or prebound to the cell surface. Virus particles prebound to the plasma membrane of P388D1 cells were uncoated at pH values below 7, with approximately 14% of virus particles uncoated within 1 min at pH 6·4. This is probably an underestimate of the number of uncoated viral nucleocapsids as some viral RNA within uncoated nucleocapsids might be protected from digestion by RNase by the nucleocapsid structure [as was possibly occurring when viral uncoating took place through fusion with liposomes (Gollins & Porterfield, 1986)]. Secondly, it is likely that in the uncoating assay used in the present study many small membrane vesicles will be formed on mechanical
Fig. 9. Uncoating and intracellular degradation of the RNA of \( ^{3} \text{H} \)uridine-labelled WNV on synchronized infection of P388D1 cells. \( ^{3} \text{H} \)Uridine-labelled WNV was bound for 2 h at 0 °C in B medium pH 7.6, containing 1 µg/ml RzWNV IgG, to monolayers of P388D1 cells in Petri dishes. The cells were twice washed at 0 °C, then 2 ml 37 °C A medium at pH 8.0, 7.6 or 6.2 without ammonium chloride or 8.0 with 20 mM-ammonium chloride was added to cells. After various times at 37 °C, medium was removed from cells and 1 ml ice-cold H buffer was added to the monolayers. Cells were scraped from plates into the H buffer and either precipitated with TCA directly, to measure the intracellular lysosomal degradation of \( ^{3} \text{H} \)uridine-labelled RNA, or precipitated after a 30 min digestion with or without externally added RNase at 37 °C, to determine the specific intracellular uncoating of viral RNA as described in Methods. The TCA-soluble radioactivity released by cells was determined from the medium initially removed from cells. (a) Amount of uncoated viral RNA susceptible to specific degradation by externally-added RNase on synchronized internalization at pH 8.0 (O), pH 7.6 (A) or pH 6.2 (Delta) in the absence of ammonium chloride or pH 8.0 + 20 mM-ammonium chloride (Square). One-hundred% represents 440 c.p.m. (30% of the initially bound c.p.m.). For comparison, the data from Fig. 5 concerning the rate of acquisition of resistance to acid inactivation of antibody-coated extracellular virus, are also included (Circle). (b) Degradation of \( ^{3} \text{H} \)-RNA by the cells' own lysosomal hydrolases, i.e. TCA-soluble \( ^{3} \text{H} \)-RNA found intracellularly at pH 8.0 (O), pH 7.6 (Delta) or pH 6.2 (Square) in the absence of ammonium chloride or pH 8.0 + 20 mM-ammonium chloride (Square) and TCA-soluble \( ^{3} \text{H} \) radioactivity found in the extracellular medium at pH 8.0 (Circle), pH 7.6 (Triangle) or pH 6.2 (Delta) in the absence of ammonium chloride or pH 8.0 + 20 mM-ammonium chloride (Square).

Fig. 10. Uncoating of \( ^{3} \text{H} \)uridine-labelled WNV RNA on synchronized infection of P388D1 cells for 1 to 7.5 min at pH 7.6 or 6.2. A synchronized infection of P388D1 cell monolayers by \( ^{3} \text{H} \)uridine-labelled WNV was established as described in the legend to Fig. 9. Infection was initiated by adding pre-warmed medium at pH 7.6 (Circle) or pH 6.2 (Square) and monolayers were incubated for up to 7.5 min at 37 °C. After various times, cells were cooled by addition of ice-cold H buffer, and the amount of uncoated viral RNA susceptible to specific degradation by added RNase was determined as described in Methods. 1390 c.p.m. of virus were initially bound to cells.

Lysis of cells in which nucleocapsids may be trapped and thus resistant to attack by added RNase.

It is striking that both viral inactivation and uncoating occur at pH values just below pH 7.0, which indicates that the two processes might be linked. A variety of other enveloped viruses can undergo rapid fusion with the plasma membrane of cells to which they are bound at mildly acidic pH values (White et al., 1980; Matlin et al., 1981, 1982). In the light of this, and in the light of the
mildly acidic pH optimum previously described for WNV fusion with liposomes (Gollins & Porterfield, 1986), it is thus likely that the uncoating seen with WNV below pH 7.0 is due to fusion of virus particles with the P388D1 cell plasma membrane. It is possible that when virus particles encounter subneutral pH values, structural changes take place in the virus particles that enable them to fuse with target membranes. However, if these changes take place in the absence of cells, virus inactivation results (Fig. 1). Virus inactivation also results if virus particles fuse directly with the plasma membrane of cells, because this fusion is non-infectious (Fig. 3). It is thus proposed that virus particles must encounter an acidic environment when located in an intracellular vacuole, rather than at the cell surface, in order for viral membrane fusion to produce a productive infection.

The examination of the effect of ammonium chloride on viral infection made it clear that an early stage in the WNV infectious entry pathway was inhibited (Fig. 7). Furthermore, the pH dependence of this inhibition strongly suggested that the unprotonated form of the base was responsible for the inhibition of WNV infection (Fig. 6). These observations are compatible with the unprotonated form of ammonium chloride crossing the plasma membrane, accumulating in intracellular vacuoles by becoming protonated (Ohkuma & Poole, 1978, 1981), raising the pH inside these compartments and thus inhibiting an acid-dependent fusion step in viral infectious entry from such compartments. Ammonium chloride does not markedly inhibit the strong hydrolytic activity of the lysosomal compartment within the macrophage-like P388D1 cells (see below), and it is likely that any virus particles that fail to fuse with the membrane of acidic prelysosomal compartments will be rapidly degraded and inactivated once they reach the lysosomal compartment.

Fig. 8 shows that a consistently larger amount of ^35^S than ^3^H radioactivity was degraded in cellular lysosomes. This could be partially due to the relative inaccessibility of ^[3H]uridine-labelled viral RNA compared to the major (E) viral membrane glycoprotein, which is located on the outside of the viral envelope and in which most (70%) of the ^[35^S]methionine label in WNV particles resides. Additionally, some of the viral RNA became inaccessible to lysosomal enzymes by entering the cytosolic cellular compartment as a result of fusion of the WNV envelope with the membrane of an acidic vacuole. Thus, the E glycoprotein would be exposed on the inside of the vacuole and if the vacuole then fused with a primary lysosome, the E protein would be degraded.

It was shown that in a synchronized infection of P388D1 cells at pH 8.0 or 7.6, uncoating of virus particles was almost complete by 15 min after the start of internalization, and reached a maximum value of 30% of the initially bound c.p.m. (Fig. 9a). This must be regarded as a minimum estimate for the proportion of viral capsids that have had their envelope removed, because the capsid structure may protect an unknown percentage of the viral RNA from attack by nuclease. If the rate at which virus particles acquire resistance to pH 5.5 inactivation is compared with the rate of viral uncoating (Fig. 9a) it is evident that the latter must be occurring very soon after virus particles are endocytosed by P388D1 cells. Uncoating of WNV could be inhibited almost completely by 20 mM-ammonium chloride at pH 8.0 (Fig. 9a), strongly suggesting that this is the basis of its ability to inhibit WNV replication.

If the total amounts of intracellular and extracellular TCA-soluble ^3^H radioactivity are added together at 1 h after synchronized infection (Fig. 9b), it is seen that an approximately equal amount of viral RNA is lysosomally degraded in the presence and the absence of ammonium chloride. This indicates that ammonium chloride is not inhibiting lysosomal hydrolases to any major degree.

There was a lag in the virus uncoating of 1 min at pH 7.6, whereas there was no such time lag at pH 6.2. It is proposed that this is due to the time taken for virus particles to be endocytosed into cells at pH 7.6, and to encounter the acidic pH values needed for viral uncoating through fusion (i.e. pH 6.6 and below; Fig. 4). Using electron microscopy, Gollins & Porterfield (1985) showed that at neutral pH, virus particles start to appear in uncoated or partially coated endosomes after 1 to 3 min. Thus, it is from these prelysosomal compartments that the entry of viral RNA into the cytosol must first occur. Because at both pH 6.2 and pH 7.6 some ^[3H]uridine-labelled RNA was susceptible to lysosomal degradation, probably at neither pH
was the RNA of all virus particles sequestered in the cytosol where it would be protected from lysosomal hydrolases.

At 1 h after warming in a synchronized infection, the amount of RNA uncoated when the extracellular medium was at pH 6.2 was approximately 64% of that measured at pH 7.6 or 8.0. However, infectivity assays had previously shown that viral infectivity at pH 6.2 was less than 7% of that at pH 7.6 under similar conditions. Similarly, Matlin et al. (1981, 1982) were unable to infect MDCK cells with either fowl plague virus or vesicular stomatitis virus by fusion through the plasma membrane. It may be that the cytosolic environment encountered on acid-triggered fusion with the cell plasma membrane is different from that encountered on fusion with an endosomal membrane and that the cytosolic ribosomal translational machinery is not available for virus RNA released in this way.

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