Effects of electrolytes on virus inactivation by acidic solutions

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Abstract. Acidic pH is frequently used to inactivate viruses. We have previously shown that arginine synergizes with low pH in enhancing virus inactivation. Considering a potential application of the acid inactivation of viruses for the prevention and treatment of superficial virus infection at body surfaces and fixtures, herein we have examined the effects of various electrolytes on the acid-induced inactivation of the herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), the influenza A virus (IAV) and the poliovirus upon their incubation at 30˚C for 5 min. Eight electrolytes, i.e., phosphate, NaCl, glutamate, aspartate, pyrrolidone carboxylate, citrate, malate and acetate were tested. No detectable inactivation of the poliovirus was observed under the conditions examined, reflecting its acid-resistance. HSV-1 and HSV-2 responded similarly to the acid-treatment and electrolytes. Some electrolytes showed a stronger virus inactivation than others at a given pH and concentration. The effects of the electrolytes were virus-dependent, as IAV responded differently from HSV-1 and HSV-2 to these electrolytes, indicating that certain combinations of the electrolytes and a low pH can exert a more effective virus inactivation than other combinations and that their effects are virus-specific. These results should be useful in designing acidic solvents for the inactivation of viruses at various surfaces.

Introduction

Virus inactivation is a critical technology for both the prevention of virus infection (1,2) and the development of pharmaceutical proteins (3,4). Daily preventive measures, such as disinfection of various contaminated-surfaces that come in contact with humans, reduces the chance of acquiring infectious diseases (5-8). Such preventive measures are normally accomplished by the application of acidic solutions (2,7-10) or disinfectants (5,11,12) that inactivate the viruses present on contaminated surfaces or on human fingers and hands. Virus inactivation is also a critical process for manufacturing therapeutic proteins when produced based on mammalian-derived cell lines, which may contain infectious viruses. Unfortunately, acids and disinfectants can be severely toxic to cells and tissues or can damage pharmaceutical proteins under the conditions sufficient to inactivate viruses. These factors limit the practical applications of acidic solutions in virus inactivation (10,12-16). Alternatively, the acidic solutions alone may be insufficiently effective against certain viruses (2). Thus, the development of a safer and more effective treatment is a valuable weapon against virus infection.

We have previously shown that arginine synergizes with a low pH in virus inactivation (17-20). In other words, it is not just the acidic conditions that play a role in virus inactivation, but the presence of electrolytes, arginine in the above case, can modulate the efficacy of acidic solutions to inactivate viruses. In the present study, we have examined various electrolytes, including amino acids, organic and inorganic acids and salt, in modulating the acid-induced inactivation of four different viruses with different virion structures and replication strategies. Even a small degree of enhanced virus inactivation over the acidic solutions should provide an advantage in reducing the chance for a virus infection.

Materials and methods

Reagents. Solvents for virus inactivation were prepared by titrating each aqueous solution of organic, inorganic, and amino acids at room temperature with a 4 M aqueous sodium hydroxide solution. The pH of each solvent was adjusted to the desired values using the pH electrode 6378 and the pH meter F-54 (Horiba; Kyoto, Japan). The pH meter was manually calibrated every 2 h during solvent preparation. Citric and malic acid were purchased from Nacalai Tesque (Kyoto, Japan). Sodium chloride (NaCl), acetic acid and sodium dihydrogen phosphate dihydrate were purchased from

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Junsei Chemical Co., Ltd (Tokyo, Japan). Sodium pyrrolidone carboxylate, aspartic acid (Asp), and glutamic acid (Glu) were kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). All chemicals were of biochemical research grade.

Cells and viruses. Vero and MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum (FBS). The Herpes simplex virus type 1 strain F (HSV-1) and type 2 strain 186 (HSV-2), the influenza virus A/Aichi/68 H_3N_2 (IAV) and the poliovirus type 1 Sabin vaccine strain (PV-1) were used throughout the experiments. The viruses were propagated in Vero cells in MEM supplemented with 0.5% FBS for HSV-1, HSV-2 and PV-1 or in MDCK cells in MEM supplemented with 0.1% bovine serum albumin (BSA) and acetylated trypsin (5.3 μg/ml) for IAV and stored at -80˚C until use. The amount of virus was measured by a plaque assay on Vero or MDCK cells as described previously (21-23).

Assay for virucidal activity. All the starting materials were stored on ice prior to the virus inactivation experiments. An excess volume of solvents was mixed with the virus stock so that the pH of the electrolyte solutions would not be affected: i.e., a 190 μl aliquot of the electrolyte solutions received a 10 μl aliquot of virus preparations (approximately 10^7~10^8 plaque-forming units (PFU)/ml). This virus preparation was incubated at 30˚C for 5 min. After incubation, aliquots of these virus samples were diluted 100-fold with ice-cold Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing 0.1 % BSA for IAV or 0.5 % FBS for other viruses to quench the virus inactivation. The viruses were further diluted to yield virus counts suitable for measurements and the number of infectious virus in the treated preparation was measured by a plaque assay. Virus inactivation in PBS was insignificant and hence the amount of infectious virus in PBS was used as a control count.

Results

The basal levels of virus inactivation were assessed using 10 mM citrate, 0.15 M NaCl at a pH of 3.8-6.0. Namely, HSV-1, HSV-2, IAV and PV-1 were exposed to the low pH solutions at 30˚C for 5 min. This temperature was chosen to simulate the temperature of the surfaces or fixtures of the human body. The pH-dependence of the virus inactivation for these four viruses is shown in Fig. 1A. Both HSV-1 and HSV-2 were significantly inactivated below pH 4.6 followed by a sharp decline in relative infectivity below pH 4.0. The pH-dependence of the virus inactivation for HSV-1 and HSV-2 was similar, both with no detectable virus recovery at pH 3.8 and with a modest inactivation above pH 4.0. Such similarity may be due to their similar virion surface properties. Conversely, IAV showed a significant virus inactivation at pH 5.0 with nearly a one log reduction in relative infectivity. PV-1 showed no apparent inactivation even at pH 3.8, as expected from its acid resistance (24). Specifically, PV-1 replicates in the alimentary tract through its transmission by the fecal-oral route, which is made possible due to its resistance to acidic conditions in the stomach (24). Considering that PV-1 is a non-enveloped virus, the observed inactivation of HSV-1, HSV-2 and IAV, which are all enveloped viruses, by the acidic solutions suggests the involvement of the lipid membranes present in these viruses. It is thus, evident that the viruses respond differently to acidic pHs.

Table I. Dissociation constants of electrolytes.

Electrolyte	pK1	pK2
Phosphoric acid	2.14	7.1
NaCl	NA	NA
Glutamic acid (Glu)	2.19	4.25
Citric acid	3.12	4.76
Aspartic acid (Asp)	1.88	3.65
Malic acid	3.40	5.11
Pyrrolidone carbonic acid (PCA)	3.32	
Acetic acid	4.74	

NA, not applicable.

The variation of the virus response to low pH is plotted in Fig. 1B. At pH 3.8, all three enveloped viruses were inactivated to a level below the detection limit of the virus counts under the experimental conditions used. As stated above, no inactivation was observed for PV-1. In response to treatment at a pH 4.0 (just 0.2 pH unit higher), only one log reduction of the relative infectivity was observed for HSV-1 and HSV-2, a large difference from the observed effect of this pH on IAV. IAV was much more sensitive to low pH than HSV-1 and HSV-2. Similar results were obtained at pH 5.0, at which pH only a marginal inactivation was observed for HSV-1 and HSV-2 and a small, but significant, inactivation occurred for IAV.

We next examined the efficacy of the combination of a low pH and various electrolytes, i.e., phosphoric acid, NaCl, acetic, citric and malic acids, Glu, pyrrolidone carbonic acid (PCA) and Asp. As all these, except for NaCl, are weak electrolytes they have at least one dissociable group as shown in Table I. When these eight different acidic solutions containing 0.15 M electrolytes (or 0.095 M for Glu and Asp due to their low solubility) in the presence of 10 mM citrate were assessed on HSV-1, HSV-2, IAV and PV-1, no inactivation was observed for PV-1 by any of these combinations. Fig. 2 shows the pH-dependence of the relative infectivity for HSV-1 (Fig. 2A) and HSV-2 (Fig. 2B). These eight electrolytes were essentially grouped into three classes based on their pH dependence: circles for 0.15 M phosphate, 0.15 M NaCl and 0.095 M Glu (Group-1), triangles for 0.15 M malate, 0.15 M PCA, 0.095 M Asp and 0.15 M citrate (Group-2), and squares for acetate. In other words, the first three electrolytes (i.e., phosphate, NaCl and Glu) showed little inactivation for HSV-1 even at the pH 4.0, below which a sharp decline in the relative infectivity was observed at a level of 10^{-5} or less at pH 3.8. These electrolytes are thus weak in HSV-1 inactivation. The next four electrolytes (i.e., malate, PCA, Asp and citrate) showed a significant inactivation of HSV-1 at pH 4.2, below which a sharp decline was observed. It is evident that these four electrolytes (Group-2) have a stronger effect on HSV-1 inactivation than phosphate, NaCl and Glu (Group-1). The pH-dependence of acetate qualitatively differed from the

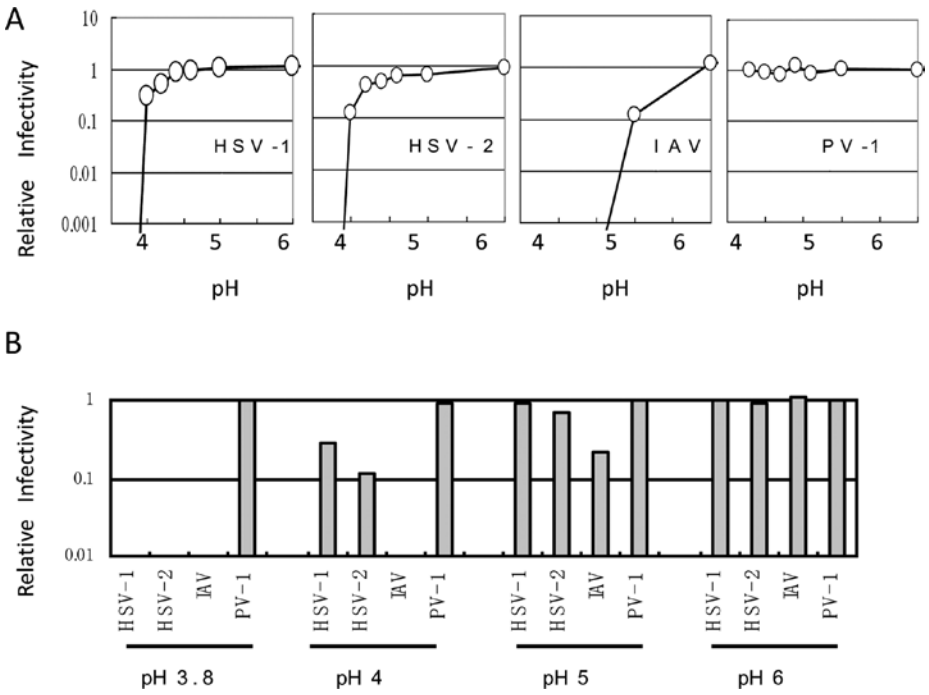

Figure 1. Inactivation of viruses at various pH values. The viruses were incubated at 30˚C for 5 min in 10 mM citrate-buffer, 0.15 M NaCl, at different pHs. The number of infectious viruses was determined by a plaque assay after incubation and was normalized to that with incubation in PBS (pH 7.4). (A) Relative infectivity of HSV-1, HSV-2, IAV and PV-1 as a function of pH. (B) Comparison of the relative infectivity of the four viruses at pHs 3.8, 4.0, 5.0 and 6.0.

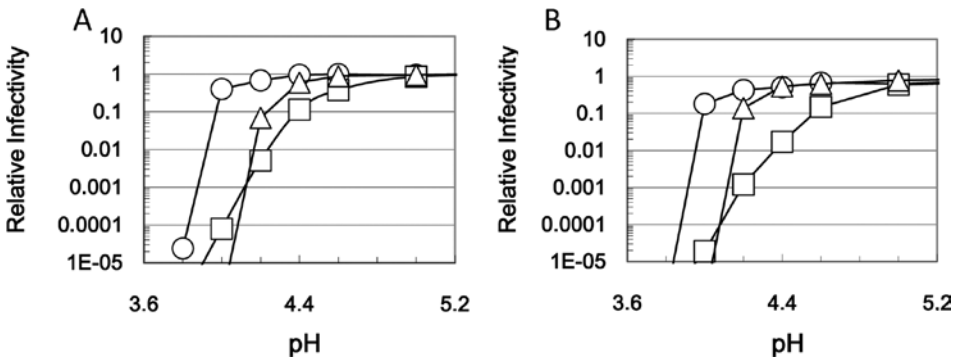

Figure 2. Inactivation of HSV-1 (A) and HSV-2 (B) in the presence of different electrolytes. The virus was incubated with the electrolytes, all containing 10 mM citrate, at the indicated pH for 5 min at 30˚C. The number of infectious viruses was determined by a plaque assay after the incubation in electrolyte and was normalized to that of incubation in PBS (pH 7.4). Circles (○) denote 0.15 M phosphate, 0.15 M NaCl and 0.095 M glutamate; triangles (△), 0.15 M citrate, 0.15 M malate, 0.15 M PCA and 0.095 M aspartate; squares (□), 0.15 M acetate.

above seven electrolytes. There was a significant inactivation already with this electrolyte at pH 4.4, at which pH the other seven electrolytes were barely effective. However, the pH-dependence was much weaker with this electrolyte, leading to a lower inactivation effectiveness at pH 4.0 than the Group-2 electrolytes (depicted by triangles). Acetate was stronger than phosphate, NaCl and Glu (Group-1) at any pH examined. A similar trend was observed with HSV-2 (Fig. 2B). Phosphate, NaCl and Glu required a lower pH than others to achieve the same level of virus inactivation. Acetate showed a lower pH-dependence, leading to a greater inactivation above pH 4.2 than other electrolytes, but a lower effectiveness at pH 4.0 than Group-2 electrolytes (triangles).

The eight electrolytes behaved differently against IAV, although they also appeared to be grouped into three classes. As shown in Fig. 3, phosphate and Glu (shown by circles) showed the weakest inactivation, similarly to their effects on HSV-1 and HSV-2. Interestingly, their effects leveled off at pH~4.6, instead of reaching the undetectable relative infectivity as the pH was further reduced. The middle group consisting of five electrolytes, i.e., NaCl, acetate, citrate, malate and PCA (triangles) showed a monotone decrease with pH in infectivity. In other words, the leveling-off of the inactivation was not observed for these five electrolytes. Their effects were intermediate at pH 5.0, but were the strongest below pH 4.4 due to the linear pH-dependent virus inactivation. Asp (diamonds) was more effective than the other seven electrolytes at a higher pH, but leveled off at pH 5.0, similarly to phosphate and Glu. It is not clear why these electrolytes (phosphate, Glu and Asp) showed a plateau in the inactivation of IAV. Such plateau is specific for IAV, as HSV-1 and HSV-2 showed no such tendency. There may be a specific interaction between

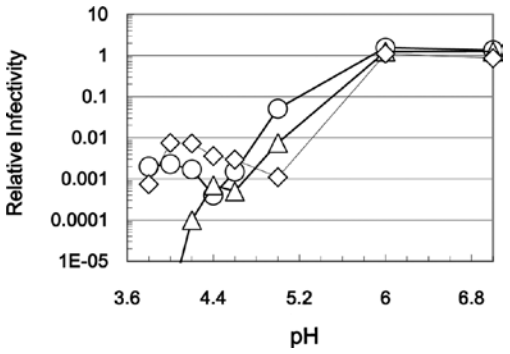

Figure 3. Inactivation of IAV in the presence of different electrolytes. IAV was incubated with the electrolytes, all containing 10 mM citrate, at the indicated pH for 5 min at 30˚C. The number of infectious viruses was determined by a plaque assay after the incubation in electrolyte and was normalized to that of incubation in PBS (pH 7.4). Circles (○) denote 0.15 M phosphate and 0.095 M glutamate; triangles (△), 0.15 M NaCl, 0.15 M acetate, 0.16 M citrate, 0.15 M malate and 0.15 M PCA; diamonds (◇), 0.095 M aspartate.

IAV and these three electrolytes (phosphate, Glu and Asp) that stabilize IAV against low pH-induced virus inactivation.

Next, the effects of the concentration of the electrolytes were examined for acetate and citrate at pH 4.2 and 4.4, pHs at which they showed moderate inactivation of HSV-1 (Fig. 4A) and HSV-2 (Fig. 4B). As shown in Fig. 4A, at pH 4.2, no increase in the virus inactivation was observed with acetate up to 0.6 M (open circles), above which a sharp increase in inactivation to the detection limit was observed. No such concentration-dependence was observed for acetate at pH 4.4 (open triangles). Citrate at 0.15 M was less effective than acetate at both pHs and became even more less effective at higher concentrations. Namely, a small, but significant, inactivation effect that was apparent at 0.15 M diminished with increasing concentration at pH 4.2 (closed circles). Essentially no virus inactivation was observed for citrate at pH 4.4 in the entire range of concentrations (0.15-0.9 M, closed triangles).

As shown in Fig. 4B, concentration-dependence for acetate was more apparent with HSV-2 (open symbols). The virus inactivation sharply increased with acetate concentration at pH 4.2, leading to undetectable virus counts at 0.6 M (open circles). Only marginal concentration-dependence was observed at pH 4.4 (open triangles). Similar to HSV-1, increased concentration abolished a small inactivation effect of citrate on HSV-2 at pH 4.2 (closed circles). Citrate was ineffective at pH 4.4 at any concentration (closed triangles). Thus, it is evident that while the effectiveness of virus inactivation for both HSV-1 and HSV-2 increases with concentration for acetate, at pH 4.2, it decreases for citrate.

We also examined the effects of NaCl and phosphate on HSV-1 and HSV-2 inactivation as a function of concentration. However, these two electrolytes showed little virus inactivation at any electrolyte concentrations, although inactivation was slightly enhanced at 0.9 M (data not shown).

IAV responded to increasing concentrations of acetate differently. Such dependence for acetate is shown in Fig. 5A (open symbols). At both pH 4.2 and 4.4, the virus inactivation at 0.3 M (open circles and triangles) was so effective that the virus recovery was below the limit of detection. At pH 5.0 (open squares), the dose-dependence curve was shifted upward, meaning that the effectiveness significantly decreased at any acetate concentrations below 0.6 M. Nevertheless, it was evident that the effects saturated at 0.6 M, above which concentration the virus recovery reached the detection limit. Interestingly, citrate also showed dose-dependent inactivation at pH 4.2 and 4.4 (closed circles and triangles). At pH 4.2, 0.3 M citrate resulted in virus inactivation to the detection limit, similarly to what was observed with acetate at pH 4.2. Virus inactivation efficacy of citrate was significantly reduced at pH 4.4 between 0.3 and 0.6 M, but reached the detection limit at 0.9 M. However, at pH 5.0 (closed squares), the concentration-dependence was opposite to the results at pH 4.2 and 4.4. The efficacy of citrate at this pH decreased with increasing concentration.

The concentration-dependence of IAV inactivation was also examined for NaCl (closed symbols) and phosphate (open symbols) (Fig. 5B). NaCl at pH 4.2 (closed circles) and 4.4 (closed triangles) was highly effective, reaching the maximum efficacy at 0.3 M. However, NaCl was totally ineffective at pH 5.0 at any concentration (closed squares). Phosphate showed completely different concentration-dependence. At any pH between 4.2-5.0, concentration increases slightly, though significantly, reduced the virus inactivation effects of this electrolyte (open circles, triangles and squares).

The comparison of the effects of NaCl, phosphate, citrate and acetate on the inactivation of IAV at pH 5.0 under the

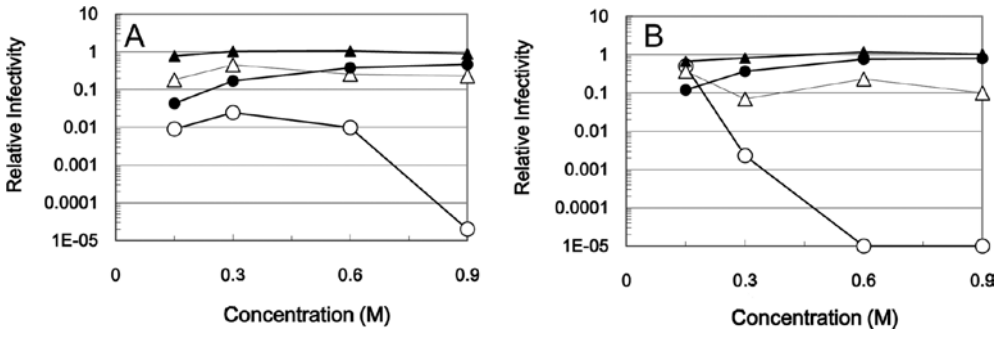

Figure 4. Effects of electrolyte-concentration on the inactivation of HSV-1 (A) and HSV-2 (B) at different pHs. The virus was incubated with the electrolytes, all containing 10 mM citrate, at the indicated pH for 5 min at 30˚C. Open symbols (○, △) denote 0.15 M acetate; closed symbols (●, ▲), 0.15 M citrate. Circles, pH 4.2; triangles, pH 4.4. The number of infectious viruses was determined by a plaque assay after the incubation in electrolyte and was normalized to that of incubation in PBS (pH 7.4).

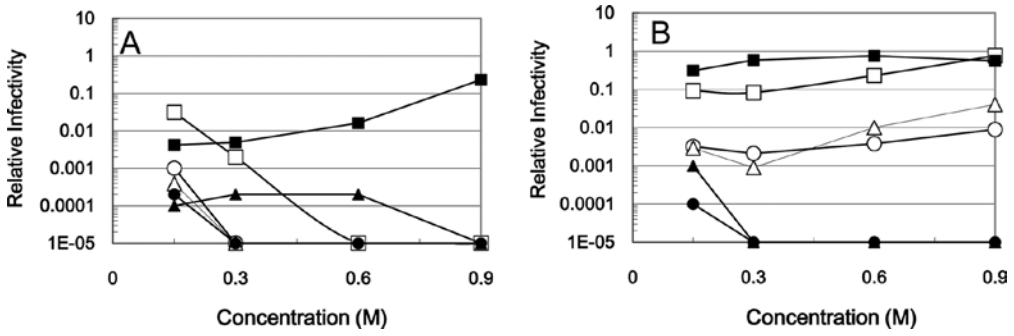

Figure 5. Effects of electrolyte-concentration on IAV inactivation at different pHs. (A), IAV incubation with 0.15 M acetate (○, △, □) or 0.15 M citrate (●, ▲, ■) at pH 4.2 (○, ●), pH 4.4 (△, ▲) or pH 5.0 (□, ■) for 5 min at 30˚C. (B), virus incubation with 0.15 M phosphate (○, △, □) or 0.15 M NaCl (●, ▲, ■) at pH 4.2 (○, ●), pH 4.4 (△, ▲) or pH 5.0 (□, ■) for 5 min at 30˚C. All the electrolyte solutions contained 10 mM citrate. The number of infectious viruses was determined by a plaque assay after the incubation in electrolyte and was normalized to that of incubation in PBS (pH 7.4).

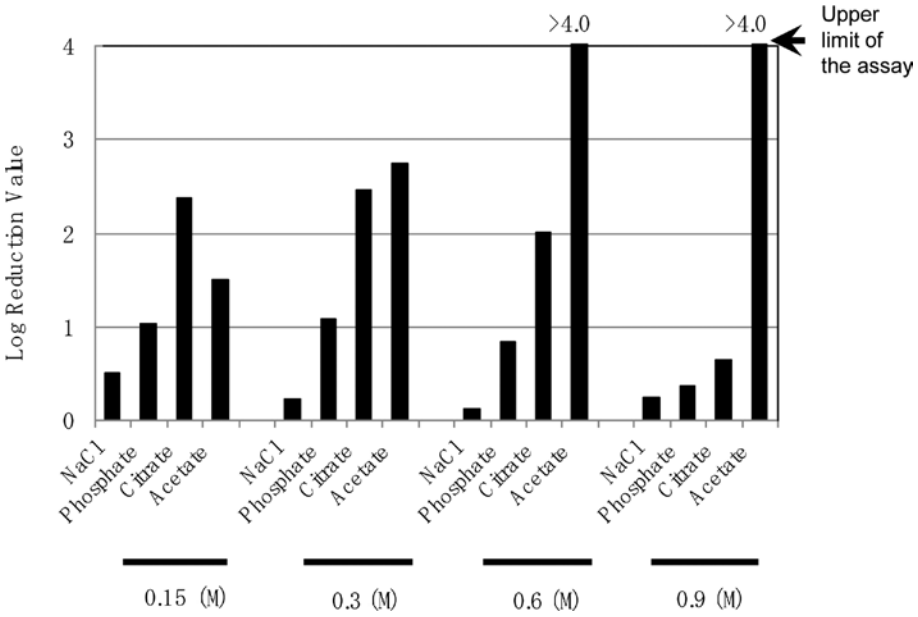

Figure 6. Comparison of the effects of the electrolytes on IAV inactivation at pH 5.0. IAV was incubated with NaCl, phosphate, citrate or acetate at 0.15 M, 0.30 M, 0.60 M or 0.90 M concentrations for 5 min at 30˚C. All the electrolyte solutions were at pH 5.0 and contained 10 mM citrate. Log reduction value (LRV) was calculated by LRV = log (virus recovery in PBS/virus recovery in the test solvent).

different electrolyte-concentrations is shown in Fig. 6. NaCl was the least effective at any concentration followed by phosphate. Interestingly, 0.15 M citrate was more effective than 0.15 M acetate. However, citrate was less effective than acetate at higher concentrations, in particular above 0.6 M, where acetate became extremely effective in inactivating IAV to the detection limit.

Discussion

The most critical information obtained from this study is that virus inactivation is a function of not only the pH but also of the type and the concentration of the electrolyte. We have previously shown that arginine synergizes with the pH conditions in inactivating HSV-1, HSV-2 and IAV (17-20). As shown in Fig. 1B, the virus inactivation was weak at pH 4.0 by acid alone. The addition of arginine concentration dependently increased the virus inactivation at this pH (17-20). We have ascribed this to the effects of arginine on protein-protein or protein-lipid interactions, as arginine has been shown to suppress protein aggregation and lipid assembly (25-38). A similar observation was made here with various electrolytes. Certain combinations of electrolytes and a low pH resulted in a greater virus inactivation than other combinations. Furthermore, the effects of the electrolytes were shown to be virus-dependent (Figs. 2 and 3). In regards to HSV-1 and HSV-2, the effects of the electrolytes were also concentration-dependent with the efficacy increasing for one electrolyte and decreasing for the other as the concentration increased (Fig. 4). Therefore, it appears essential that different electrolytes be screened for their efficacy against each virus as a function of pH and concentration. This is most clearly demonstrated with the case of IAV (Fig. 5). In addition, we have demonstrated that there is no point in using excessively low pH when combined with phosphate, Glu and Asp, whose effects leveled off at pH 4.6-5.0 (Fig. 3).

Why are the effects of the electrolytes different from each other? There are a number of reasons. First, most of the electrolytes tested are weak electrolytes with at least one dissociable group (pK), as listed in Table I. NaCl is the only strong electrolyte examined. This means that the ratio of uncharged to charged species varies with the pH value and that the pH dependence of the ratio and hence the ionic strength of the solution differs from one electrolyte to the other. However, there is no simple relation between the pK value and the effectiveness of virus inactivation. For example, the virus inactivation effectiveness of citrate diminishes with increasing concentration for HSV-1 and HSV-2, which may be ascribed to the protein stabilization effects of this electrolyte (39,40). Thus, the pK of weak electrolytes cannot be used to explain the observed differences between the electrolytes. Nevertheless, the pK may play a critical role in maintaining the pH upon mixing the electrolyte solutions with the viruses, which possess solvent-exposed charged groups on the virion surface.

How do electrolytes affect viruses? At low concentrations, ions form electrical double layers around the charged particles and screen their charges (41,42). Since the viruses are more positively charged as the pH is lowered, the ions from the electrolytes would shield their surface charges, leading to virus stabilization that may offset the inactivation effects of low pH. Such charge screening effects should depend on the ionic strength and hence the pK and also the type of the ions (e.g., the size and valency of the ions). At high concentrations, additional effects of the electrolytes should occur. One such effect is on the water structure. Cohesive force of the electrolytes on the water molecules often correlates with their stabilizing effects on macromolecular solutes (43,44) and hence may stabilize the viruses. Another effect that may occur at high electrolyte concentration is acid binding. Either the ionic or the neutral forms of the electrolytes may weakly bind to the viruses, which would alter the surface properties of the viruses and may stabilize or may destabilize the viruses. As each of these effects may depend on both the electrolytes and the viruses, it appears that screening of the electrolytes for the target virus is essential in finding the most effective combination of the electrolyte and low pH.

PV-1 exhibited resistance to both the acidic solutions and to the electrolytes tested, consistent with the reported results (24,45). Such resistance was ascribed at least in part to the lack of a lipid membrane for this virus. However, there are certain non-enveloped viruses that are sensitive to acid treatment, for example, rhinoviruses (24). The vaccine strain of PV-1 has been observed to respond to temperature-induced inactivation treatment (24). Thus, understanding why certain non-enveloped viruses are sensitive to low pH and why PV-1 is sensitive to temperature may lead to a more rational approach for acid-induced inactivation of this virus in combination with certain electrolytes or other compounds (e.g., non-toxic organic solvents).

Another factor that must be considered is the type of applications for the electrolytes. For pharmaceutical applications, the electrolytes need to be removed from the final product or to be compatible with the administration. For surface disinfection (e.g., body surfaces, food hygiene and sanitizing fixtures), the electrolyte solutions should maintain the efficacy for a prolonged period. The stability of the electrolytes at the surface may be a critical factor for such applications.

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