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Novel strategy with acidic arginine solution for the treatment of influenza A virus infection (Review)

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Abstract. There is already an indication of a potential worldwide spread of influenza projected for this coming autumn and winter. In this review, we propose an aqueous arginine solution as a novel agent for preventive measures and possible chemotherapy against influenza A virus infection. Influenza A virus spreads among the human population through both droplets and direct contact, and hand and mouth wash are the primary preventive measures. Upon contact, influenza A virus infects epithelial cells of the upper respiratory tracts in the initial phase of infection and spreads over the mucosal surface of the tracts, leading to varying degrees of inflammation near the site of infection. Arginine inactivates enveloped viruses, including influenza virus at an acidic pH or elevated temperature and hence may be used for preventive measures as a disinfectant and also for treatment of the infection. Because of the low cytotoxicity of arginine, virus inactivation may be performed at the site of infection in the form of a liquid or spray of an aqueous arginine solution. Acidic solvents have been used as a disinfectant and, to a limited extent, as a virus inactivation agent. Arginine may have the edge over acidic solvents due to its safety, or at least it may be used as an alternative option to acidic solvents or more specific antiviral drugs. Arginine as well as acidic solvents use a virus inactivation mechanism fundamentally different from the mechanism of antiviral chemotherapeutic drugs, i.e., through weak, but multiple, interactions with viral components. This eliminates the possibility of generating resistant viruses against arginine treatments.

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1. Introduction

The recent incident of influenza A virus pandemic has reminded us of the high risk of sporadic influenza virus infection during non-epidemic seasons. There is already indication of a potential worldwide spread of influenza projected for this coming autumn and winter. Although acquiring specific immunity through vaccination is the most effective way to prevent virus infection, daily routine preventive measures appear to be a simple, yet effective approach against influenza A virus infection. Such preventive measures are normally accomplished by the recommended procedure of hand- and mouth-wash or wearing masks. These washes, though effective, only remove viruses from the initial site of contact. It is more effective when these washes are combined with a reagent that can kill viruses. Disinfectants have been used to inactivate viruses on contaminated surfaces or human fingers and hands, but generally have severe cell and tissue toxicities and hence cannot be practically used on mucosal membranes or injured sites of the body (1-5). Less toxic acidic solvents have also been used as disinfectants and, to a limited extent, as virus inactivation agents for such sensitive surfaces (5,6).

When infected, there are several antiviral drugs against influenza A virus, including neuraminidase inhibitors, which are currently considered to be the most effective (7). There is, however, always the potential risk of generating drug resistance when using conventional antiviral drug treatments (8). Particularly in a pandemic infection, the selective pressure in nature to generate a resistant virus is much greater than the conventional epidemic mode of the infection. Therefore, an additional treatment that utilizes different mechanisms of antiviral strategy would be a valuable resource as an

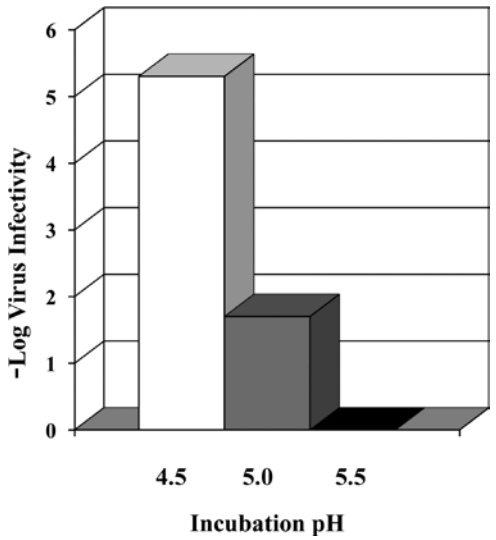

Figure 1. Virus inactivation by 0.7 M arginine as a function of pH. Influenza virus A/Aichi/68 (H_3N_2) was incubated with 0.7 M arginine solution at different pHs on ice for 60 min. The number of infectious virus was measured by the plaque assay on MDCK cells (reformatted from ref. 9).

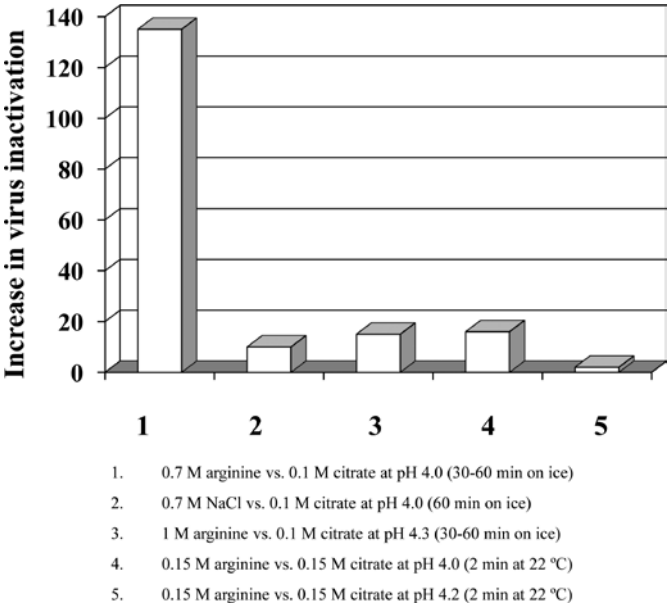

Figure 2. Relative virus inactivation of acidic arginine vs. acidic citrate. Reformatted from ref. 9 for columns 1-3. Columns 4 and 5 are unpublished observations.

alternative or addition to conventional drug treatments. Toward these goals, i.e., preventive measures and therapeutic treatments, we propose here that an aqueous arginine solution can be developed as an effective virus inactivation agent. This is based on the observations that an aqueous arginine solution inactivates influenza A virus (9,10) and exhibits antiviral activities on several enveloped viruses (11). Due to the lack of safety concerns, an aqueous arginine solution may be used, not only for hand and mouth wash as a disinfectant, but also for the inactivation of viruses at the site of infection in the form of a spray or mist. While acidic solvents provide, not only physical removal but also inactivation of viruses (5,6), arginine may have an edge over acidic solvents due to its safety, or at least may provide an alternative option to acidic solvents or antiviral drugs.

2. Conventional treatments

Preventive measures. Since no vaccine is available for new types of influenza A viruses at an outbreak of a pandemic, the only effective way to prevent the spread of infection is to reduce the number of infecting viruses to the body. As described above, recommended procedures have been hand and mouth wash to avoid the contamination of hands as well as the removal of the contaminated viruses in the nose and mouth. These procedures have been considered highly effective and recommended in particular for the recent swine flu incident. To enhance the effectiveness of washing procedures, disinfectants and acidic solvents in different formats have been developed (1-5).

Antiviral therapy. Antiviral drugs inhibit specific functions of viral proteins required for the infection and virus multiplication. Influenza virus infects cells in the upper respiratory tracts in the initial phase of infection, causing rhinitis, pharyngitis, laryngitis and simultaneously severe systemic symptoms, including fever, chill, headache and muscle pains. There are two classes of specific antiviral agents against influenza virus infections: M2 channel inhibitors (rimantadine and amantadine) and neuraminidase inhibitors (zanamivir and oseltamivir) (7). M2 channel inhibitors block dissociation (uncoating) of influenza A virus from the endosome at the very early stage of infection and consequently inhibit virus multiplication. Viral neuraminidase cleaves sialic acid of cell surface glycoproteins at the late stage of infection and, as a consequence, helps the release of progeny viruses from the infected cells and allows the spread of the virus over the surface of the respiratory epithelia. Thus, inhibition of neuramindase blocks the spread of virus in the respiratory tracts, preventing further development of the influenza symptoms. However, neuraminidase inhibitors suffer the rapid appearance of drug-resistant mutants (8). The problems of resistance are inherent to drugs that bind to a single specific site of target molecules. As discussed below, virus inactivation by arginine as well as acidic solvents use a fundamentally different mechanism through weak, multiple interactions with the viral components, i.e., no single particular target site on the viral components.

3. Virus inactivation by arginine

We have shown that an aqueous arginine solution at a low pH inactivates influenza A virus when incubated on ice for 30-60 min (9). Fig. 1 shows virus inactivation by 0.7 M arginine. The effects are extremely strong at pH 4.5, reaching a greater than 5 log reduction of virus yield. As the pH is increased, the effects rapidly decline, leading to no detectable virus inactivation at pH 5.5. These virus inactivation effects are significantly stronger than the acidic buffer alone. For example, 0.1 M citrate only leads to a 2 log reduction at pH 4.5, approximately 1000-fold less effective than 0.7 M arginine. Such a comparison is plotted in Fig. 2. Column 1

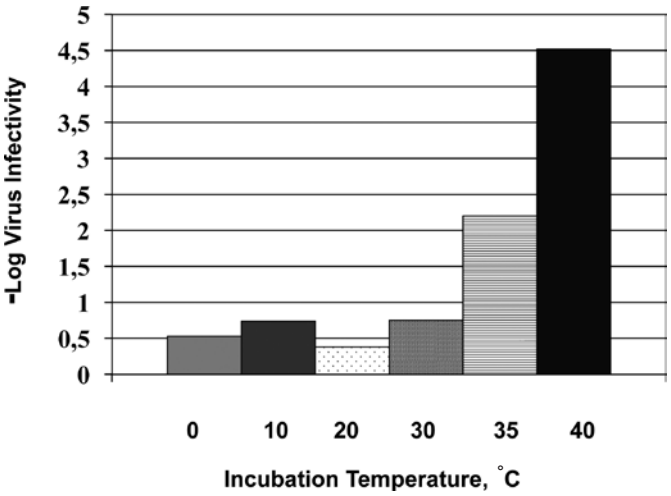

Figure 3. Virus inactivation by 0.7 M arginine at pH 4.4 as a function of temperature. HSV-2, strain 186, was incubated at indicated the temperatures for 5 min with 0.7 M arginine at pH 4.4. The number of infectious virus was measured by the plaque assay on Vero cells (reformatted from Tsujimoto *et al*, unpublished data).

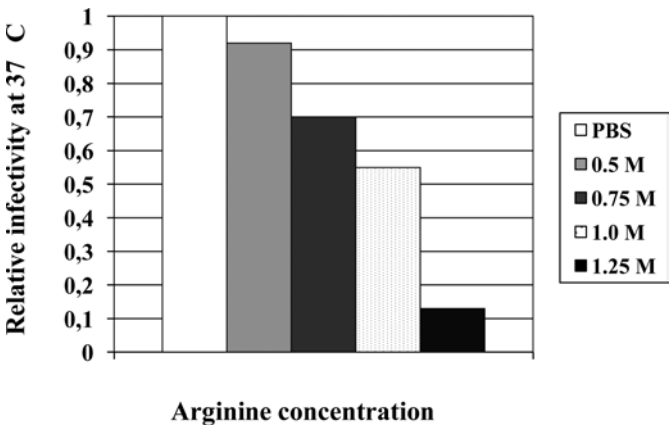

Figure 4. Virus inactivation by neutral arginine solution at 37˚C. HSV-1, strain F, was incubated with arginine at different concentrations for 5 min at 37˚C (reformatted from ref. 12).

shows that 0.7 M arginine is over 100-fold more effective than 0.1 M citrate at pH 4.0. On the other hand, 0.7 M NaCl is only 10-fold more effective than 0.1 M citrate (column 2), indicating that higher ionic strength does contribute, but cannot entirely explain, the strong virus inactivation effects of 0.7 M arginine. Consistent with this, increasing the citrate concentration to 0.7 M at pH 4.5 enhanced the effects by approximately 100-fold over 0.1 M citrate (9), which is still 10-fold less than 0.7 M arginine at the same pH. Nevertheless, it is evident that a higher citrate concentration does enhance virus inactivation at pH 4.5. The difference between arginine and citrate depends on pH, as at pH 4.3, 1 M arginine was only 15-fold more effective than 0.1 M citrate (Fig. 2, column 3).

The above experiments were conducted on ice, and the results hence may significantly differ from the effects at body or room temperature, at which disinfecting procedures or *in vivo* virus inactivation treatments take place (1-6). When the influenza virus was treated at 37˚C for 2 min with 0.7 M arginine, pH 4.0, an approximately 4 log reduction was observed, slightly less than the effect at pH 4.5, despite at a lower pH (Tsujimoto *et al*, unpublished data). This indicates the importance of incubation time. Under a identical condition, 0.1 M citrate at pH 4.0 resulted in an approximately 2 log reduction, thus still much weaker than 0.7 M arginine (4 log reduction) at the same pH. At pH 5.0, 0.7 M arginine resulted in 0.06 virus infectivity at 37˚C for a 2-min incubation, which compares with 0.02 virus infectivity on ice for 60 min, again suggesting that a longer exposure increases virus inactivation. Even at a concentration of 0.15 M, arginine is stronger than citrate (Ejima and Koyama, unpublished data). Column 4 in Fig. 2 shows an approximately 15-fold stronger effect of arginine when the influenza virus was exposed to the pH 4.0 solvent at 22˚C for 2 min. However, the difference between arginine and citrate is also dependent on pH at this condition as well. At pH 4.2, 0.15 M arginine was only 2-fold more effective than 0.15 M citrate (columns 4 and 5). We also observed that a higher citrate concentration is highly effective against influenza virus at elevated temperatures, a magnitude equal to or exceeding the level achieved by 0.7 M arginine at an identical acidic pH (Tsujimoto *et al*, unpublished data).

Virus inactivation by acidic arginine solutions has been observed with other enveloped viruses when incubated on ice for 60 min (9,10,12). When the incubation time was reduced, the effects were significantly reduced, meaning that virus inactivation is time-dependent as described above. However, a higher incubation temperature offsets the effects of a shorter incubation time. Fig. 3 shows the results of herpes simplex virus type 2 (HSV-2) when incubated only for 5 min (Tsujimoto *et al*, unpublished data). The virus inactivation effects are marginal with 0.7 M arginine at pH 4.4 when incubated on ice for 5 min. There is little change up to 30˚C, above which the virus inactivation sharply increases. Thus, even for a 5-min incubation, a higher temperature close to the body temperature can lead to extensive virus inactivation by 0.7 M arginine at pH 4.4.

A higher pH also reduces virus inactivation by arginine. At a neutral pH as well, higher temperature offsets the reduced virus-inactivating effects of arginine (12). Such temperature effects were tested using herpes simplex virus type 1 (HSV-1). Fig. 4 summarizes the effects of arginine on relative infectivity of HSV-1 at a neutral pH and a body temperature of 37˚C. Similar to the virus stability in PBS on ice, a 37˚C-exposure has no effect on virus infectivity, i.e., high temperature alone is insufficient to inactivate HSV-1 in a physiological solvent. A significant decrease in the infectivity was observed in the presence of 0.5 M arginine; approximately 10% of the virus was inactivated upon incubation at 37˚C for 5 min. Virus inactivation was enhanced with increasing concentrations of arginine, reaching approximately 10% of the surviving virus with 1.25 M arginine. It is evident that even at a neutral pH, arginine solution can inactivate HSV-1 at sufficiently high concentrations and at 37˚C. The effects are stronger at higher temperature and with longer incubation.

A similar experiment showed that influenza A virus cannot be inactivated by arginine under the conditions of 37˚C, a 5-20 min incubation and 0.5-1.25 M arginine concentration at a neutral pH (12). However, a further increase in temperature resulted in significant virus inactivation.

4. Safety of arginine

There are numerous applications of arginine, including supplementary diets, sport drinks, pharmaceutical agents, cosmetic ingredients and formulation reagents of protein and drug substances. Thus, there is no doubt about the safety of arginine administration. However, these applications are relatively used at low doses. The applications of arginine as preventive measures and topical virus inactivation require high concentrations. In other words, effective virus inactivation requires arginine at high concentrations. Our initial goal was to develop more effective virus inactivation processes for pharmaceutical proteins. One of the conventional procedures of virus inactivation has been a low pH (13), which can damage proteins (14). An idea behind the use of arginine is that it may be able to raise the pH for virus inactivation process, as arginine itself does not affect the protein stability (15). In fact, arginine at 0.3-1 M above pH 4.0 achieved virus inactivation that could be achieved by a buffer at pH 3.5 without arginine (9). An acidic arginine solution at 0.3-1 M above pH 4.0 is acceptable for virus inactivation of pharmaceutical protein solutions, but may be too toxic for any *in vivo* applications. It turns out that a low pH and high arginine concentration appear to be tolerant for certain body surfaces such as the mucosal layer of mouse genital organs and epithelial keratinocytes in rabbit eyes (Ikeda *et al*, unpublished data). The observed non-toxic nature of arginine is perhaps due to its negligible effects on proteins; arginine does not denature proteins (15). The preliminary attempts to kill viruses by topical applications for herpetic keratitis in rabbits showed that a high arginine concentration and low pH are effective and tolerant for rabbit eyes (Ikeda *et al*, unpublished data). This has opened a new window of opportunity for the use of an arginine solution for the treatment of influenza A virus infection.

5. Prospect of topical arginine applications

Preventive measures. One of the most effective preventive measures for influenza A virus infection is a hand- and mouth-wash routine. It is simply removal, not inactivation, of the contaminated virus from the primary infection site of the virus. It would be ideal if the washing or rinsing step, not only physically removes, but also kills the viruses. Along this line, an aqueous arginine solution may be used in the form of a wet towel for hand wash or spray for hand and mouth wash.

Therapeutic applications. The initial site of influenza A virus infection is the epithelial cells of the upper respiratory tracts (6). The progeny viruses are released from the infected cells to extracellular mucosal fluid on the epithelial surface by the activity of neuraminidase as described above. It might be possible to use an aqueous arginine solution in the form of spray, as has been used for acidic solvent (6) or mist that can provide a burst of concentrated arginine solution sufficient for inactivation of the viruses in the mucosal fluid, preventing the spread of progeny viruses to neighboring cells (i.e., similarly to the action of anti-neuraminidase inhibitors), although both initial arginine concentration and initial pH will change to the physiological condition with time by dilution with body fluid. This dilution will eventually abolish the virus inactivation effects of arginine. However, arginine also has a weak, but significant, antiviral effect (11). As shown in Fig. 5, arginine below 100 mM does inhibit virus growth of influenza A virus in cultured cells. This means that even after an arginine solution is diluted below the level of effective concentration and pH for virus inactivation, it can exert antiviral actions.

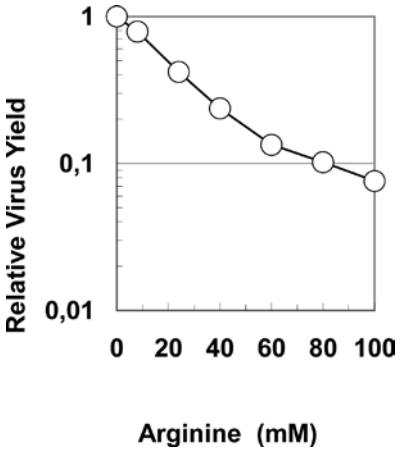

Figure 5. Antiviral activity of arginine against influenza virus. Monolayered MDCK cells were infected with influenza virus A/Aichi/68 (H_3N_2) and incubated in a medium containing the indicated concentrations of arginine. After the incubation for 14 h, the number of infectious virus in the culture medium was measured by the plaque assay (reformatted from ref. 11).

6. Advantage of virus inactivation by arginine

We demonstrated that an aqueous arginine solution can inactivate viruses, including influenza A virus, when combined with either low pH or elevated temperature or both. Although the precise mechanism of the virus inactivation by arginine has not yet been fully elucidated, one critical fact is that the virus inactivation requires a high arginine concentration, e.g., higher than 0.3 M and preferably 0.7-2 M. This requirement of high concentration makes arginine qualitatively different from currently available antiviral drugs that normally function at much lower concentrations. Such a large difference in the effective concentration inherently places a restriction on the utility of an aqueous arginine solution, namely, only a topical application for superficial infection. There is no way that arginine can be systemically or orally administered to reach an effective concentration *in vivo*.

However, such a difference in the effective concentration between conventional antiviral drugs and an aqueous arginine solution has a significant consequence in generating drug resistance. The use of antiviral drugs can quickly result in drug-resistant mutants, while the use of arginine does not. This is due to the entirely different mechanisms of their functions. First, antiviral drugs have a specific target, e.g., virus-coded enzymes to which they bind and inhibit the activity of target molecules. This leads to a mutation in the corresponding genes and loss of inhibitory activities of the drugs. Conversely, arginine has no specific viral or host cell components for its binding. Second, drug-resistant mutations in general occur when the progeny viruses are produced in the presence of suboptimal concentrations of antiviral drugs, i.e., there is a consistent

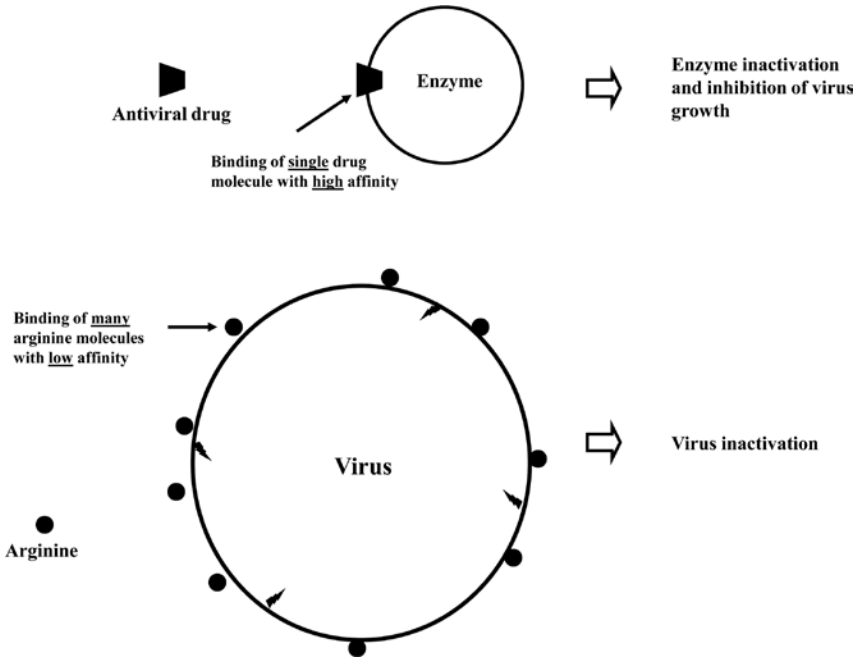

Figure 6. Mechanism of the antiviral effect and virus inactivation. The upper panel shows the binding of an antiviral drug to a target enzyme (e.g., virus-coded enzymes), leading to inactivation of the enzyme activity and inhibition of virus growth. The lower panel shows the weak interaction of arginine with the virus surface, leading to structual modification of virion and virus inactivation.

selection pressure to escape from the inhibitory effects of antiviral drugs during the course of virus multiplication in the presence of the antivirals at subeffective concentrations. On the contrary, the mode of arginine action is 'all or none'. Once the virus is killed by arginine, there is no chance to produce drug-resistant progeny virus. When the virus infects the cells even in the presence of arginine at any concentration, there is no selection pressure during virus multiplication, and hence the progeny viruses are equally sensitive to arginine. The virucidal mechanism of arginine eliminates the possibility of generating a resistant mutant against arginine treatments.

Requirement of a high concentration means that the interactions of arginine with the virus are weak, i.e., arginine interacts with the virus surface through weak interactions (16). This is schematically depicted in Fig. 6. For comparison, the upper panel shows the binding of an antiviral drug to the target enzyme that occurs with high affinity. With such high affinity, the antiviral drugs can be systemically or orally administered to reach the effective concentration. The lower panel shows the plausible mechanism of virus inactivation by arginine. Although the precise binding mechanism of arginine is still unclear, arginine does appear to bind to proteins, aromatic compounds, nucleic acids and lipids, but all with low affinity. Significant effects of arginine are not normally observed unless its concentration is higher than 0.1 M. Thus, the binding of arginine to whatever is responsible for virus inactivation is weak. Although no evidence exists, it is highly likely that multiple arginine bindings occur on the virus surface, as there are many of these arginine binding sites on the surface. Such multiple sites would result in multiple damages to the virus, leading to virus inactivation. Whether or not the damaged sites are the binding sites of arginine remains to be ascertained, but one important observation is the requirement of membrane for the mechanism of arginine effects. The non-enveloped viruses studied so far showed strong resistance to virus inactivation by arginine, implying that either membranes are involved for arginine binding and the resultant damage, or the damages occur on the membrane-protein interface. These multiple bindings and damages on the virus eliminate the possibility of generating resistant viruses against arginine treatments. Another aspect of arginine binding is that arginine binding to host proteins is most likely reversible, as expected from such low affinity. Once the arginine concentration decreases to 0.1 M, it dissociates from the proteins and becomes ineffective on any perturbation that the high arginine concentration may have caused. However, virus inactivation is not reversible, as shown by *in vitro* virus inactivation experiments. If the arginine effects were reversible, there should be enough time for the viruses to regain infectivity between the acid treatment and infection procedure under the experimental conditions used (9,10).

Currently, there are acidic solvents used as disinfectants or, to a limited extent, as virus inactivation agents (6). We showed that a high concentration of citrate is effective (9) and even more so than acidic arginine solvents against influenza virus under certain conditions (unpublished data). Clearly additional studies are required to fully understand the disadvantages and advantages of each solvent system, but the importance of such a solvent system cannot be overemphasized. Acidic arginine solutions may provide an edge over other solvent systems, or at least may be used as an alternative option to other solvent treatments due to its safety. As the spread of a new influenza virus pandemic is imminent, there is urgent need for novel treatments, and the development of an arginine solution as a disinfectant or as an *in vivo* virus inactivation agent must be given proper attention.

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