A number of viruses (i.e., herpesvirus, rhinovirus, influenza virus) have been considered to be acid sensitive, since their titers decrease at pH levels of 5.0 or less. However, we recently found that influenza virus can be made acid resistant by ammonium sulfate treatment (4). At that time, we reported that acidification of influenza virus causes the formation of a precipitate as well as a loss of virus, as measured by hemagglutination (HA) activity. Addition of ammonium sulfate (10% saturated solution) causes the formation of a heavy precipitate at a neutral pH. If this precipitate is removed, no further precipitate forms upon acidification, and there is little or no loss of virus. The experiments reported here show that the so-called acid sensitivity of influenza hemagglutinin is due to virus adsorption to precipitates that form under acidic conditions rather than to virus inactivation.

The PR-8 strain of influenza virus used had been through over 150 serial passages in 10- to 11-day-old embryonated eggs. Virus stock was made by allantoic inoculation with 10 HA doses and incubation at 36 C for 48 h. The surviving embryos were chilled and allantoic fluids were harvested. The pooled fluids were stored at −90 C. The frozen samples were rapidly thawed at 37 C immediately before use and clarified at 2,000 rpm for 15 min. Influenza virus was assayed by hemagglutination. Serially diluted infectious allantoic fluids (0.5-ml volumes) were mixed with an equal volume of 0.5% thice-washed human O erythrocytes in saline, and HA patterns were scored after 2 h of incubation at 25 C.

Experiments were conducted to determine whether the allantoic precipitate that forms at pH 4.5 influences the HA titer of the virus. Influenza virus was diluted 100-fold in different materials (Table 1) and acidified. The HA titer remained the same until acidification. After acidification the titer fell markedly unless the acid precipitate (gross and colloidal) had first been removed. Virus diluted 100-fold in a suspension of the acid precipitate obtained from normal allantoic fluids and then acidified also showed a great loss of titer. These results indicate that the influenza HA titer is decreased by the precipitate that forms in allantoic fluids at pH 4.5.

When influenza virus is diluted in saline buffered with 0.05 M glycine-hydrochloride and then acidified, the effects of acidification on titer vary inversely with the dilution. Table 2 shows the results of diluting influenza virus prior to acidification. HA titers were maintained if the infectious allantoic fluids were highly diluted before acidification. Thus, under conditions not conducive to efficient collision between virus and precipitate, the virus hemagglutinin was acid resistant.

Influenza virus was treated with a number of different salts and then acidified. The experimental procedure and results are shown in Table 3. Control influenza virus fluids adjusted to pH 4.5 with HCl developed a significant precipitate in the cold. This sample failed to manifest an HA titer, indicating that the HA was either inactivated by the acidification or that the HA was adsorbed to the precipitate and was incapable of hemagglutinating red blood
cells. A massive precipitate was formed after treatment with (NH₄)₂SO₄ at neutral pH; it could be removed by centrifugation, leaving a clear supernatant fluid with virtually all the original HA activity. Upon acidification of this clear supernatant fluid, no further precipitates formed and there was no reduction in titer. Magnesium sulfate and sodium sulfate failed to produce precipitates; upon acidification, the same degree of precipitation occurred as in the control samples. Even though the acid precipitate formed, the virus was stabilized, suggesting that the virus does not adsorb to the precipitate in the presence of excess salts. The results with (NH₄)₂SO₄ indicate that the virus does not adsorb to a precipitate at a neutral pH. The adsorption of viruses to suspended particles and other solids only at acid pH levels has been reported previously (3, 4, 6).

These experiments indicate that the loss of

| Diluent | HA titers | pH 4.5 samples |
|---------|-----------|----------------|
| Physiological saline (0.15 M) | 0 800 400 | Non-acidified |
| Undiluted, normal allantoic fluid | + 800 <100 | |
| Undiluted, normal allantoic fluid treated to remove gross acid precipitate* | ± 800 <100 | |
| Undiluted, normal allantoic fluid treated to remove gross and colloidal acid precipitates* | 0 800 400 | |
| Partially purified acid precipitate* | ++ 800 <100 | |

*Influenza allantoic fluid was diluted 100-fold in the diluents indicated above and below (pH of all samples was 7.8–8.5). Samples were brought to pH 4.5 with 1 N HCl and held at 4 C for 1 h. Prior to assay, samples were adjusted to neutrality.

* Normal allantoic fluids were adjusted to pH 4.5 and the acid precipitate was allowed to form. The fluids were centrifuged (1,500 x g for 15 min) to remove the precipitate, and the supernatant fluids were adjusted to original pH (8.5) and used as a diluent for virus. The supernatant of the acidified allantoic fluid contained a colloidal precipitate which was not sedimentable at the centrifugal force used above. However, filtration of the acidified supernatant fluid through a 0.22-μm cellulose membrane removed the colloidal precipitate. The filtrates were adjusted to pH 8.5 and used as a virus diluent.

* Acid precipitate described above was sedimented from normal allantoic fluids and suspended in saline.

### Table 1. Inactivation of influenza virus hemagglutinin by the acid precipitate

| Dilution of infectious allantoic fluids | HA titers |
|---------------------------------------|-----------|
| pH 7.0 | pH 4.5 |
| Undiluted | 640 | 80 |
| 1:2.5 | 640 | 160 |
| 1:5 | 640 | 160 |
| 1:10 | 640 | 320 |
| 1:20 | 640 | 320 |
| 1:40 | 640 | 320 |
| 1:80 | 640 | 640 |
| 1:160* | 640 | 640* |

*Influenza allantoic fluid was diluted in 0.05 M glycine-hydrochloride-NaCl buffer. After acidification, samples were held at 4 C for 1 h, and then assayed.

* Represents final dilution of undiluted allantoic fluid, e.g., allantoic fluid diluted 1:160 and held for 1 h at 4 C was still considered 1:160, and the final titer of 1:640 represents a fourfold dilution therefrom.

### Table 2. Effects of dilution on acid sensitivity of influenza virus hemagglutinin

| Dilution of infectious allantoic fluids | HA titers |
|---------------------------------------|-----------|
| pH 7.0 | pH 4.5 |
| Undiluted | 640 | 80 |
| 1:2.5 | 640 | 160 |
| 1:5 | 640 | 160 |
| 1:10 | 640 | 320 |
| 1:20 | 640 | 320 |
| 1:40 | 640 | 320 |
| 1:80 | 640 | 640 |
| 1:160* | 640 | 640* |

### Table 3. Effects of salts on acid sensitivity of influenza virus

| Salt | pH of sample after salt addition | Precipitate formed after adding salts* | HA titer |
|------|---------------------------------|--------------------------------------|----------|
|      |                                 | Shaking for 15 min | 1 h, 4 C, at pH 4.5 | Non-acidified samples | pH 4.5 samples |
| None added | 8.5 | 0 | ++ | 800 | <100 |
| (NH₄)₂SO₄ | 7.4 | ++++ | 0 | 800 | 800 |
| MgSO₄ - 7 H₂O | 7.6 | 0 | ++ | 800 | 800 |
| Na₂SO₄ | 8.4 | 0 | ++ | 800 | 400 |

* Centrifuged, infectious allantoic fluid was treated with the salts indicated to make a final molarity of 0.5. Salts were added while the fluids were magnetically stirred. After addition of salts, the samples were stirred for 15 min at 25 C to allow precipitates to form; precipitates were scored as shown below. Then the samples were centrifuged to determine whether the precipitation removed virus (see non-acidified samples). The clear supernatant fluids were then acidified with HCl to attain a pH of 4.5, and the samples were held in an ice bath at 4 C for 1 h, and additional precipitates that formed were also scored. Samples were assayed without removal of the second precipitates. To make the first dilution for assay, the samples were diluted 100-fold in glycine-buffered saline at pH 7.5.

* Scoring of precipitates: ++++, massive; ++, moderate; 0, no detectable precipitate.
HA activity at low pH is due to adsorption of the virus to the precipitate that forms at low pH. Influenza can be stabilized by diluting the virus fluids to decrease the collision efficiency of the virus and precipitate, by adding a high concentration of salt which prevents the adsorption of the virus to the precipitate, or by removing the precipitate at a neutral pH with (NH₄)₂SO₄.

Attempts to recover the HA of influenza virus from precipitates with various fluids revealed that virtually 100% of the HA activity could be recovered by solubilizing the precipitate with pH 11.5 to 12.0 glycine-NaOH buffer.

It appears that reevaluation of viral sensitivity to acid is in order, to determine whether or not such sensitivity may be due to the adsorption of virus to the precipitates which form at an acid pH. Furthermore, some viruses which are acid resistant are often found to be "slightly inactivated" at pH levels from 2 to 4 (1, 2, 5). It is possible that the sensitivity of some viruses to pH 2 to 4 may be attributable to precipitates which form in tissue culture harvests at these pH levels. Fiala (personal communication) suggested that the decrease in titer of rhinoviruses after acidification may be due to induction of aggregates by the acid. However, such "aggregates" may actually be virus adsorbed on precipitates formed by the acid environment.

LITERATURE CITED
1. Brunner, K. T., and R. Ward. 1959. Differences in stability of antigen-antibody complexes formed with poliovirus and acute and convalescent phase human sera. J. Immunol. 83:405-410.
2. Pinheiro, F., and G. D. Hsiung. 1963. A study on dissociation of coxsackie B4 virus-antibody complex. Virology 20:457-463.
3. Shirobokov, V. P. 1968. Differentiation of coxsackieviruses based on the character of adsorption onto bentonite. Acta Virol. 12:185.
4. Wallis, C., A. Homma, and J. L. Melnick. 1972. Concentration and purification of influenza virus on insoluble polyelectrolytes. Appl. Microbiol. 22:740-744.
5. Wallis, C., and J. L. Melnick. 1962. Effect of organic and inorganic acids on poliovirus at 50°C. Proc. Soc. Exp. Biol. Med. 111:306-308.
6. Wallis, C., J. L. Melnick, and J. E. Fields. 1971. Concentration and purification of viruses by adsorption to and elution from insoluble polyelectrolytes. Appl. Microbiol. 21:703-709.