

# Cell Attachment and Penetration by Influenza Virus

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Attachment and penetration of influenza virus into clone 1-5C-4 cells were quantitatively determined by the immunofluorescent cell-counting assay. Aided by centrifugal force, more than 95% of virus inocula of five representative influenza virus strains (A<sub>0</sub>/PR8, A<sub>1</sub>/Ann Arbor, A<sub>2</sub>/Japan, B/Lee, B/Great Lakes) were attached to cells at a linear rate within 10 min, in contrast to approximately 35% after stationary incubation at 35 C for 2 h. By the former procedure, a proportionality between the number of infected cells and volume of inoculum was revealed which was not evident when stationary incubation was employed. Maximal binding of virus to cells occurred at 0.2 M NaCl. The salt requirement, added to evidence of pH dependence and temperature independence, indicated that the initial virus-cell union involved electrostatic forces. Virus penetration into cells, measured by the insensitivity of virus-cell complexes to antiviral serum, was linear and complete within 15 min at 35 C for all five virus strains tested. Maximal virus penetration occurred at 0.1 to 0.2 M NaCl; the process was pH- and temperature-dependent. Both virus attachment and penetration processes were partially inhibited in the presence of diethylaminoethyl-dextran.

The early steps of interaction of animal virus and cells include the primary attachment of virus and its subsequent irreversible attachment, which is considered the first step in the penetration of virus or virus genome into the cell (26). Our knowledge of these early events in relation to influenza virus-host cell reactions (16) has been considerably enhanced by utilizing a model system, viz., erythrocyte agglutination by virus. However, this system is not in all respects an adequate model for the early interaction of virus and cells, because some steps in the host cell system may be missing or less pronounced in the erythrocyte system (26). The kinetics of the early interactions of influenza viruses in cell culture systems have not been studied extensively. A wide disparity of cell attachment and penetration rates has been reported for these myxoviruses (4, 8, 14, 20, 35, 36, 38). In part, this circumstance may reflect the diversity of both biological systems and methodology that have been employed. To characterize accurately early virus-cell interactions, a method for delineating virus attachment and penetration, a homogenous host cell system that is conducive for experimental manipulation of environmental conditions, and a quantitative procedure for assessment of influenza virus infectivity are prerequisites. In an effort to fulfill these requirements, centrifugal force was employed to delimit virus attachment

and penetration, and virus infectivity was assayed by the highly sensitive and specific immunofluorescent cell-counting technique on a uniform cellular substrate.

This report describes the attachment and penetration of cell cultures by influenza viruses and the environmental factors affecting the efficiency of these interactions. In this study, the terms employed to denote these early stages are defined as follows: "attachment" is the initial, specific union between virus and cells which may or may not be reversible. "Penetration" refers to the sequence of events starting with irreversible attachment followed by the entrance or incorporation of virus into cells as measured by the progressive insensitivity of virus-cell complexes to viral antiserum. "Adsorption" is a general term referring to the various interactions and processes involved in the initial binding of virus to cells, leading to the loss of identity of the former. Its implications of physiochemical nonspecificity limit its descriptive usefulness (26, 39).

## MATERIALS AND METHODS

**Cell cultures.** Clone 1-5C-4 derived from a variant line of Chang's conjunctival cell (49) was propagated with Eagle minimum essential medium (MEM) containing 10% fetal calf serum (FCS). Cells were maintained with MEM plus 5% FCS. For virus assay, cells were cultivated on circular cover slips (15 mm diameter) inserted in

flat-bottomed glass vials (19 by 65 mm). One milliliter of cell suspension, containing approximately  $10^6$  cells, was introduced onto each cover slip which was then incubated at 35 C for 24 h or until a complete cell monolayer was formed.

**Virus.** Strains of human influenza virus employed in this study were A<sub>0</sub>/PR8, A<sub>1</sub>/Ann Arbor/1/57, A<sub>2</sub>/Japan/62, B/Lee, and B/Great Lakes/1739/54 which were obtained from the American Type Culture Collection, Rockville, Md. Viruses were grown in the allantois of 11-day-old chicken embryonated eggs. After incubation at either 35 or 37 C for 48 h, eggs were chilled (6 C) for 5 or more h. Allantoic fluids were then harvested aseptically and individually assayed for hemagglutinin (HA). Fluids having the highest HA titers were pooled. Stock preparations of viruses were distributed in 1-ml amounts into glass vials and stored at -70 C. Each virus pool was assayed for infectivity by the immunofluorescent cell counting technique.

**Influenza antiserum conjugates and immunofluorescent staining.** Antiserum to each virus strain was prepared in rabbits by injecting intramuscularly in a 2.0-ml volume an equal quantity of virus suspension and Freund complete adjuvant. At weekly intervals thereafter, animals were twice injected intravenously with 1.5 ml of virus suspension; they were exsanguinated 10 days after the last injection. The globulin fraction of antiserum was precipitated with ammonium sulfate at 4 C and conjugated with fluorescein isothiocyanate at the rate of 0.02 mg of dye per mg of protein. Unbound dye was removed by passing conjugated globulin through a Sephadex G-50 column (Pharmacia Fine Chemicals Inc., Piscataway, N.J.). To reduce non-specific fluorescence, 5 ml of conjugated globulin was diluted with an equal volume of PBS solution and adsorbed twice with 200 mg of acetone-dried mouse liver powder and once with 100 mg of chicken embryo powder.

The direct fluorescent-antibody method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell cultures were washed three times with PBS and stained with conjugated antiserum at room temperature. Cover-slip cell monolayers were then rinsed in two changes of PBS and mounted on slides with a semi-permanent medium (31).

**Virus assay.** Virus infectivity was determined in duplicate by the immunofluorescent cell-counting technique. For this procedure, virus dilutions were prepared in PBS (0.15 M NaCl, 0.01 M phosphate), pH 7.1. Inoculum in 0.2-ml volume was introduced directly onto cover-slip cell monolayers after their transfer from glass vials to rotor chamber inserts (10). The latter were employed because they withstand the centrifugal force required to sediment virus. Rotor chamber inserts placed in a swinging-bucket SB-110 rotor were centrifuged in a model B-50 ultracentrifuge (International Equipment Co.,

Needham Heights, Mass.) at 10,000 rpm (9,838 to 16,155  $\times g$ , depending on the distance of the chamber insert in the arm of the rotor from the axis of rotation) for 12 min at 6 C. Preliminary determinations established that more than 90% of virus inoculum was sedimented under these conditions. Residual inoculum was removed after centrifugation, the cover-slip cell monolayers were placed into glass vials, and 1 ml of maintenance medium was then added to each vial. After further incubation at 35 C for 20 to 24 h, cover-slip cell monolayers were rinsed twice with PBS, fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescent staining or stored at -70 C. Fluorescence of viral antigens in fixed cell cultures was not diminished when cells were stored under these conditions for 12 weeks.

**Fluorescence microscopy and infected cell counting.** Cover-slip cell monolayers were examined with a Zeiss fluorescence microscope equipped with an FITC exciter and no. 50 and 65 barrier filters. With this optical system at a magnification of  $\times 400$ , the number of microscopic fields contained in the area of a 15-mm diameter cover slip was 1759. For each cover-slip cell monolayer, 50 microscopic fields were examined for fluorescent cells. To calculate the number of cell-infecting units (CIU) of virus per milliliter, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and the volume factor.

**Determination of virus attachment.** Attachment was measured by following the disappearance of virus from inoculum after its addition to cell monolayers. Virus inoculum at multiplicity of infection (MOI) of 0.01 in 0.2-ml volume was introduced onto cells. After designated intervals of incubation or centrifugation, residual inoculum was removed, cell cultures were immediately washed with PBS, and residual inoculum was introduced onto fresh cell monolayers to measure unattached virus. Cover-slip cell monolayers exposed to initial or residual inocula were treated in the manner described earlier for virus assay. The amount of virus that was attached to cells at a given time was expressed as a percentage of the virus input. The latter was the sum of the amounts of attached and free virus.

**Determination of virus penetration.** Virus penetration into cells was measured by the insensitivity of attached virus to antiserum. After inoculum was attached to cells by centrifugation, cell cultures were washed with PBS, overlaid at designated intervals of incubation with 0.5 ml of a prewarmed 1:15 dilution of virus antiserum, and then incubated at 35 C for 22 h. The quantity of virus that penetrated cells at a given time was expressed as a percentage of the input virus.

**Calculation of attachment and penetration constants.** The attachment and penetration rate constants (K) were calculated from the equation,  $K = 1n (V_0/V_i)/nt$ , where  $V_0$  = the input virus

concentration,  $V_t$  = unattached or unpenetrated virus concentration at time  $t$ , and  $n$  = the number of cells per cubic centimeter on a 15-mm diameter cover-slip cell culture. The latter was determined by treating cell monolayers with trypsin, resuspending the cells in a known volume, and enumerating them in a hemocytometer.

**Thermal inactivation of virus.** Equivalent concentrations of influenza virus strains were prepared with the final desired concentrations made in prewarmed (35 C) PBS. Vials containing different virus strains were held in a water bath (35 C). At designated intervals, 1.0 ml of each suspension was removed, placed in an ice bath, and subsequently assayed for viable virus. Rates of virus inactivation were calculated from the equation for a first-order reaction  $V_t/V_0 = e^{-kt}$  in which  $V_0$  is the concentration of virus at time zero,  $V_t$  is the concentration of viable virus at time  $t$ , and  $k$  is the rate of virus inactivation. Virus half-life ( $t_{1/2}$ ) was determined from the relationship  $t_{1/2} = 0.69/k$ .

## RESULTS

**Quantitative aspects of virus assay.** Before investigating the early interactions between virus and cell, the parameters of the immunofluorescent cell-counting technique employed for the assay of influenza virus infectivity were determined. The rate of appearance of fluorescent viral antigens was followed by making sequential observations of infected cell monolayers during a 24-h incubation period.

Specific nuclear fluorescence in infected cells was noted 6 h after infection, which subsequently increased in amount and intensity. Many infected cells also exhibited both nuclear and cytoplasmic fluorescence within 24 h of infection (Fig. 1). Fluorescent cells increased linearly in number within 4 to 8 h of infection; a plateau was reached at 12 h (Fig. 2). Thereafter, the number of infected cells was constant throughout the remainder of the observation period. Since the maximal number of infected cells were visualized by fluorescence staining between 12 and 24 h, infected cells were enumerated during this period.

The dose-response relationship between two-fold dilutions of virus over a range of 1.2 log units and the number of CIU of virus was linear (Fig. 3). This suggests that each fluorescent cell resulted from infection by a single infective virus particle or aggregate not divisible by dilution.

Ten determinations were made to estimate the precision of the assay by infecting cell monolayers with a standard quantity of virus inoculum and then by treating in the prescribed manner. The number of CIU of virus per ml of inoculum ranged from  $2.9 \times 10^7$  to  $3.9 \times 10^7$ , with a mean of  $3.2 \times 10^7$ , standard deviation (SD) of  $\pm 0.41 \times 10^7$ , and standard error of the mean of  $\pm 0.13 \times 10^7$ . Expressed as a percentage, the SD was 12.8% of the mean.



FIG. 1. Fluorescent influenza viral antigen in clone 1-5C-4 cells 24 h of infection.  $\times 250$ .

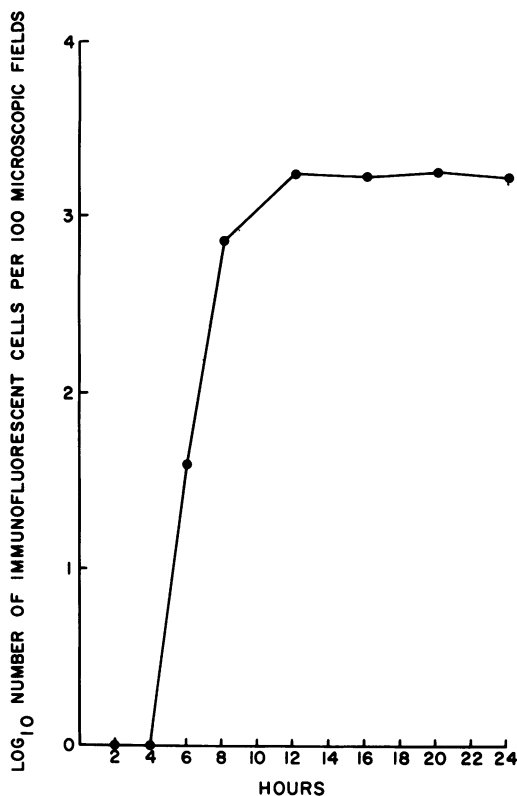


FIG. 2. Relation between the length of incubation period (35 C) on the number of influenza (PR8)-infected clone 1-5C-4 cells.

The mode of distribution of fluorescent cells on an infected cover-slip cell monolayer was determined by examining 200 random microscopic fields. Observed frequencies of fluorescent cells corresponded closely to theoretical frequencies (Fig. 4). Because there was no significant departure from the theoretical Poisson distribution ( $X^2 = 1.322$  with degree of freedom = 5), the distribution of fluorescent cells on cover-slip cultures was random.

**Virus attachment.** That the use of centrifugal force is highly efficient for promoting virus attachment onto cells has been demonstrated with several different virus-cell systems (10, 12, 25, 47). Rates of attachment of five influenza virus strains onto cell monolayers were determined during stationary incubation (35 C) and centrifugation (6 C). Each virus inoculum contained an input MOI of 0.01. Aided by centrifugal force, more than 95% of the virus inoculum of all influenza virus strains were attached within 10 min; after stationary incubation for 2 h, from 30 to 37% of virus inocula of the five strains were attached (Fig. 5). The attachment

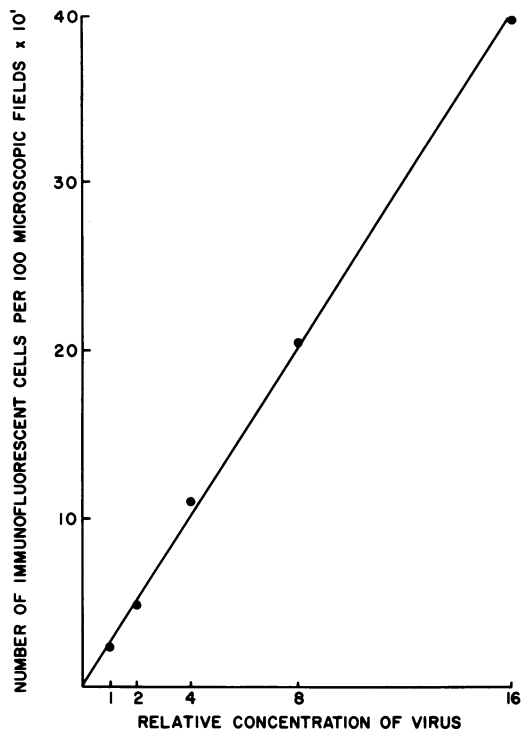


FIG. 3. Linear function between the number of immunofluorescent cells and relative concentrations of influenza (PR8) virus.

rate constants ( $k$ ) were  $3.4 \times 10^{-7}$  cm<sup>2</sup>/min with centrifugation and approximately  $2.3 \times 10^{-8}$  cm<sup>2</sup>/min with stationary incubation.

Higher estimates of the quantity of virus attached to cells by stationary incubation have been reported (20, 36, 38, 48) than that noted in the previous experiment. This may be a reflection of the procedure used to measure the free virus content of residual inoculum. To investigate this, an experiment was performed in which virus inoculum was attached to cell monolayers using stationary incubation (35 C, 2 h). Residual inoculum was then introduced onto new cell monolayers either by centrifugation or stationary incubation (35 C, 2 h). Results show that the free virus content of residual inoculum, attached by either stationary incubation or centrifugation, was 13 and 58%, respectively (Table 1). In calculating the initial quantity of virus attached to cells, it is evident that these estimates can be markedly influenced by the efficiency of the procedure employed to assess for unattached virus in residual inoculum.

That thermal inactivation may drastically reduce the viability of virus inoculum during these lengthy periods of incubation was investi-

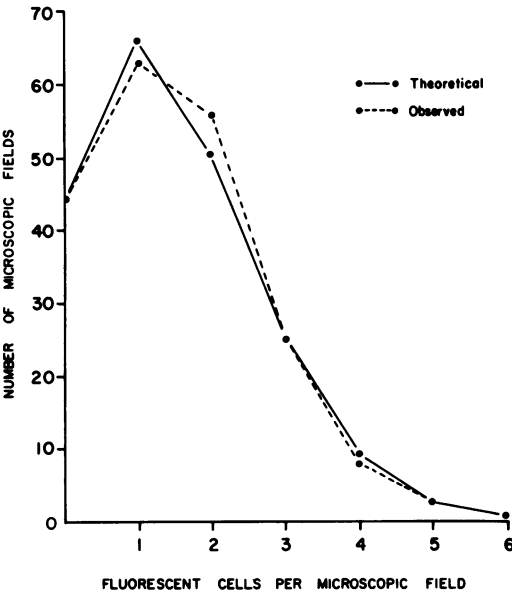


FIG. 4. Frequency distribution of fluorescent clone 1-5C-4 cells infected by influenza (PR8) virus.

gated by determining the lability of influenza viruses at 35 C. The half-life of four virus strains that were tested ranged from 55.2 to 58.4 min (Table 2). Less than 25% of the original virus inoculum was viable at 2 h. The rates of inactivation (*K*) at 35 C for the representative virus strains were comparable.

Further evidence of the efficiency of centrifugation for the attachment of influenza virus inoculum onto cell monolayers is shown in Table 3. When inoculum was centrifuged, a proportionality between the number of infected cells and volume of inoculum was revealed which was not evident when stationary incubation was employed. The inefficiency of the latter procedure has been encountered in virus plaque assays, notably, by the disproportionality between inoculum size and plaque number (1, 6).

The effect of temperature on the attachment of influenza virus to cells during centrifugation was studied by sedimenting inoculum onto cell monolayers at temperatures that ranged from 5 to 35 C. With the exception of this variable, the procedure for virus attachment and assay was similar to that described earlier. The initial binding of virus to cells was found to be independent of temperature.

The attachment response of viruses to host cells is affected by variations in the pH of the suspending medium (12, 19, 30, 40). To determine whether the influenza virus-host cell system currently under study is also affected,

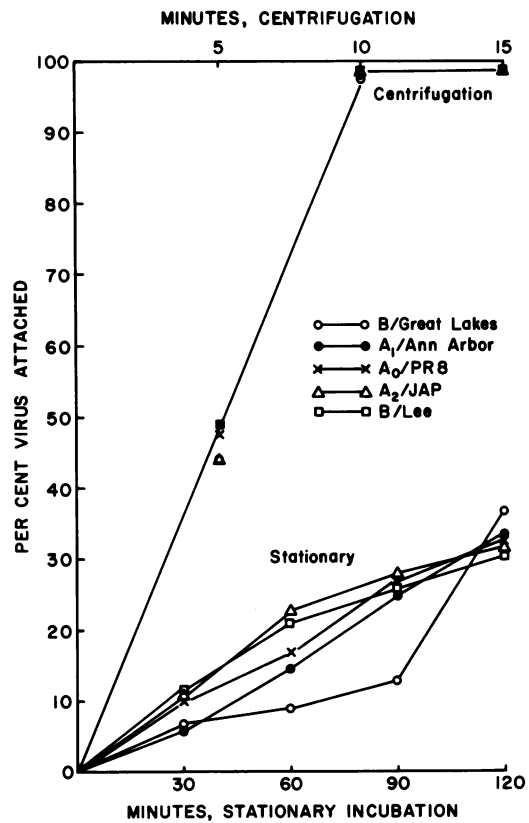


FIG. 5. Attachment of influenza viruses onto cover-slip cultures of clone 1-5C-4 cells by centrifugation ( $9,838$  to  $16,155 \times g$ , 6 C) and stationary incubation (35 C).

TABLE 1. Influence of methods for measuring residual virus on estimating the quantity of influenza (PR8) virus attached to clone 1-5C-4 cells

Method	Attachment of virus inoculum		
	Initial stationary incubation <sup>a</sup>	Residual stationary incubation	Residual centrifugation <sup>b</sup>
1	87% (40) <sup>c</sup>	13% (6)	58% (56)
2	42% (40)		

<sup>a</sup> Incubation: 35 C for 2 h.

<sup>b</sup> Centrifugation:  $16,155 \times g$ ; 12 min; 6 C.

<sup>c</sup> Infected cells per 100 microscopic fields in parentheses.

Sorensen phosphate buffer containing 0.15 M NaCl, adjusted to give pH values ranging from 5.4 to 8.0, was used as the attachment medium. Maximal binding of virus occurred near neu-

TABLE 2. Reaction rate (*K*) and half-life (*t*<sub>1/2</sub>) for inactivation at 35 C of influenza virus strains<sup>a</sup>

Virus strain	<i>K</i> (× 10 <sup>-2</sup> /min)	<i>t</i> <sub>1/2</sub> (min)
A <sub>0</sub> /PR8	1.25	55.2
A <sub>1</sub> /Ann Arbor	1.18	58.4
A <sub>2</sub> /Japan	1.25	55.2
B/Great Lakes	1.25	55.2

<sup>a</sup> Suspending medium PBS, pH 7.2.

TABLE 3. Relationship between volume of inoculum and influenza (PR8) virus-infected cells: centrifugation versus stationary incubation

Inoculum vol (ml)	Infected cells per 50 microscopic fields	
	Centrifugation <sup>a</sup>	Stationary incubation <sup>b</sup>
0.1	35	3
0.2	68	24
0.4	132	23
0.5	164	33
1.0	330	44

<sup>a</sup> Centrifugation: 16,155 × *g*; 12 min; 6 C.

<sup>b</sup> Incubation: 35 C, 2 h.

trality, indicating that the attachment of influenza virus to cells is pH dependent (Table 4). Virus attachment markedly decreased at both acidic and alkaline conditions corresponding to intervals at which ionization of carboxyl and amino groups are depressed (40).

The quantity of virus attached to cells as a function of NaCl concentration was investigated. Virus dilutions were prepared in 0.01 M phosphate buffer containing concentrations of NaCl ranging from 0.01 to 0.5 M. The maximal binding of virus to cells was achieved with 0.2 M NaCl buffer with greater or lesser salt concentrations inhibiting virus attachment (Fig. 6). Inhibition of virus attachment was more marked, however, with NaCl concentrations below 0.2 M.

**Virus penetration.** The rate of penetration of five representative strains of influenza virus into cells at 35 C was followed by determining the insensitivity of attached virus to antiviral serum at designated times. Viruses were introduced onto cell monolayers at a MOI of 0.01. The penetration of each of the five virus strains into cells was similar (Fig. 7). Penetration proceeded at a linear rate; the process was complete within 15 min. The penetration rate constant (*K*) for the influenza viruses was approximately  $2.3 \times 10^{-7}$  cm<sup>2</sup>/min.

TABLE 4. Effect of pH on attachment of influenza (PR8) virus to clone 1-5C-4 cells

Attachment <sup>a</sup> medium (pH)	Virus attached (%)	Virus titer <sup>b</sup> (CIU/ml)
5.4	21.9	$2.6 \times 10^7$
6.2	82.2	$1.0 \times 10^8$
6.8	84.3	$1.0 \times 10^8$
7.0	99.6	$1.2 \times 10^8$
7.2	99.6	$1.2 \times 10^8$
8.0	25.3	$3.1 \times 10^7$

<sup>a</sup> Sorensen phosphate buffer with 0.15 M NaCl.

<sup>b</sup> Virus input, 8,267 cell-infecting units with 0.2 ml of inoculum.

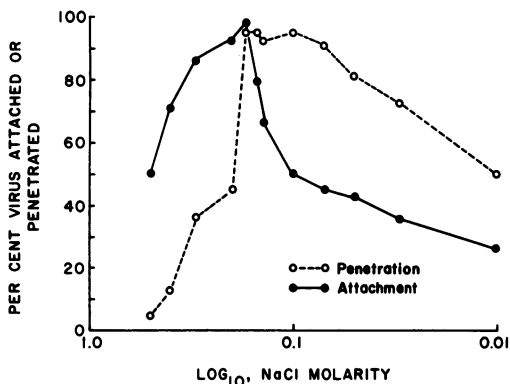


FIG. 6. Attachment and penetration of influenza (PR8) virus to clone 1-5C-4 cells in the presence of different NaCl concentrations.

The effect of different temperatures (35, 26, and 6 C) on the penetration of influenza (PR8) virus into cells was investigated. The experiment was performed in a manner similar to that of the preceding test. The results indicate that virus penetration into cells is temperature-dependent (Fig. 8). At 35 C, 50% of attached virus penetrated into cells in 7.5 min, but at 26 C penetration of an equivalent quantity of attached virus occurred in 58 min. At 6 C, only 5% of attached virus penetrated into cells within 60 min.

The influence of pH on the penetration of virus into cells was determined by adding prewarmed (35 C) Sorensen PBS, adjusted to pH values ranging from 5.4 to 8.0, immediately after virus attachment to cells. As in preceding experiments, penetration was followed by the insensitivity of attached virus to antiviral serum. Results show that the maximal penetration of virus occurred between pH 6.2 and 7.2 (Table 5). The influence of pH on virus penetration, however, appeared to be less restrictive than on virus attachment to cells (Table 4).

To ascertain the effect of NaCl concentration on virus penetration into cells, 0.01 M phosphate buffer solutions, pH 7.2, containing NaCl concentrations ranging from 0.01 to 0.5 M were added to cell monolayers immediately after virus attachment to cells. Buffer solutions were prewarmed to 35 C, the temperature condition selected for virus penetration. Maximal penetration of virus into cells occurred in 0.2 to 0.1 M NaCl solutions; the efficiency of virus penetration declined slowly at lower salt molarities (Fig. 6). Salt concentrations above 0.2 M markedly inhibited virus penetration.

**Virus attachment and penetration in the presence of DEAE-dextran.** The presence of the polycation, diethylaminoethyl (DEAE)-dextran, during the early stages of virus-cell interaction has been reported to increase the infectivity of several viruses (17, 18, 24, 32, 33, 41, 42). It has been postulated that the action may involve either increased adsorption of virus to cells or enhanced penetration. The effect of DEAE-dextran (mol wt,  $2 \times 10^6$ ; Pharmacia Inc., Uppsala, Sweden) on cell attachment and penetration by influenza (A<sub>2</sub>/Japan) virus was de-

termined as follows: virus attachment to cells was carried out in the presence of PBS containing DEAE-dextran (200 µg/ml) or PBS alone, and virus penetration was allowed to proceed in the presence of PBS. To measure the effect of the polycation on virus penetration, attachment of virus to cells was performed in PBS, and virus penetration was carried out in the presence of DEAE-dextran or PBS. Virus was attached with the aid of centrifugal force and allowed to penetrate at 35 C for 30 min. Results in Table 6 show that maximal virus attachment and penetration occurred in PBS and that the presence of DEAE-dextran was partially inhibitory to both these processes.

DISCUSSION

The immunofluorescent cell-counting technique employed for the assessment of influenza virus infectivity on the uniformly stable clone 1-5C-4 cell line proved highly amenable for quantitatively studying the early events of virus-cell interaction. Centrifugation of virus inoculum onto cell monolayers attached virus efficiently

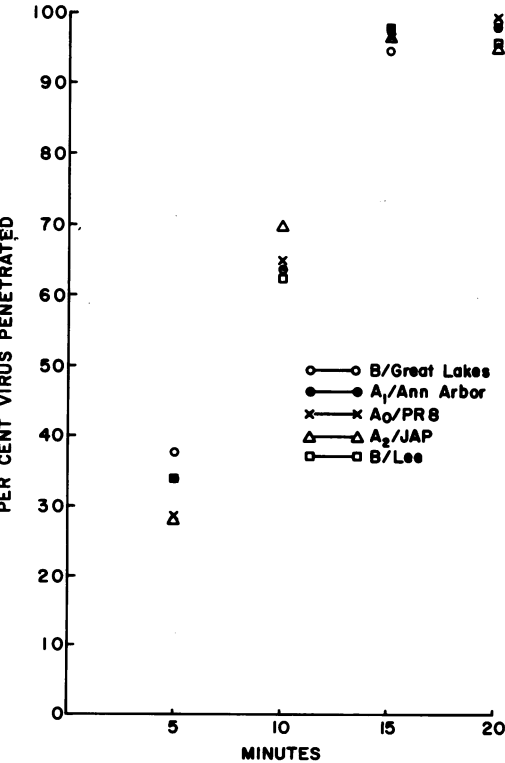


FIG. 7. Rate of penetration of influenza viruses into clone 1-5C-4 cells at 35 C as measured by insensitivity of attached viruses to antiviral serum.

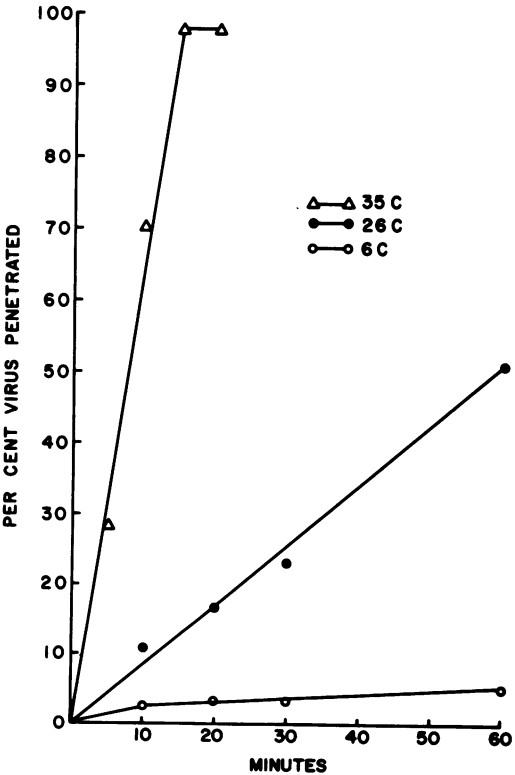


FIG. 8. Effect of temperature on the penetration of influenza (PR8) virus into clone 1-5C-4 cells as measured by insensitivity of attached virus to antiviral serum.

TABLE 5. *Effect of pH on penetration of influenza (PR8) virus into clone 1-5C-4 cells*

Penetration <sup>a</sup> medium (pH)	Virus penetrated (%)	Virus titer (CIU/ml) <sup>b</sup>
5.4	38.3	$1.4 \times 10^7$
6.2	97.2	$3.5 \times 10^7$
7.2	98.6	$3.5 \times 10^7$
8.0	46.5	$1.7 \times 10^7$

<sup>a</sup> Sorensen phosphate buffer with 0.15 M NaCl.<sup>b</sup> Virus input, 2,462 cell-infecting units with 0.2 ml of inoculum.TABLE 6. *Effect of diethylaminoethyl (DEAE)-dextran on cell attachment and penetration by influenza (A<sub>2</sub>/Japan) virus*

Medium	Virus attached <sup>a</sup> (%)	Virus penetrated <sup>b</sup> (%)
PBS <sup>c</sup>	97.4	98.0
DEAE-dextran <sup>d</sup>	40.5	22.1

<sup>a</sup> Virus input of 2,638 cell-infecting units prepared in presence of DEAE-dextran or phosphate-buffered saline (PBS) for attachment to cells; virus penetration carried out in presence of PBS.<sup>b</sup> Virus input of 3,518 cell-infecting units prepared in PBS for attachment to cells; virus penetration carried out in presence of DEAE-dextran or PBS.<sup>c</sup> At pH 7.2.<sup>d</sup> At 200  $\mu$ g per ml in PBS.

rapidly, and at a linear rate. More than 95% of virus inocula of five representative influenza virus strains were bound to cells within 10 min, in contrast to approximately 35% after stationary incubation at 35 C for 2 h. Similar results have been obtained with other viruses under comparable conditions (11-13). Our findings differed markedly from those cited by others on the kinetics of "adsorption" of influenza viruses to cells during stationary incubation. Some of the results that have been reported are as follows: 100% adsorption of A<sub>2</sub> and B viruses in 3 h (38), 75% adsorption of A/NWS virus in 30 min (36), 90% adsorption of A<sub>2</sub> virus in 4 h (4, 14), 78% adsorption of an Asian virus in 1 h (8), and 65% adsorption of A<sub>0</sub> virus in 3 h (35). An important factor that may contribute to the overall high estimate of virus attached to cells using stationary incubation is the assay of the free virus content of residual inoculum. Generally, the quantity of virus that is attached at a given time is expressed as a percentage of the virus input; the latter is the sum of attached and

free virus. As shown in Table 1, free or unattached virus measured by a relatively inefficient virus attachment procedure resulted in an estimate of the original quantity of virus attached that was high and inaccurate. After stationary incubation for 2 h, residual inoculum contained more virus than was actually attached to cells initially.

Prolonged incubation periods may also contribute to the inefficiency of virus attachment as well as to unrealistic estimates of the event. Attempts to achieve maximal adsorption of influenza virus to cells using stationary incubation (35 or 37 C) have fostered the use of incubation periods ranging from 2 to 5 h (4, 14, 20, 35, 36, 37). The half-life of four representative influenza virus strains that were tested at 35 C ranged from 55.2 to 58.4 min (Table 2). This deleterious effect on virus viability is minimized by the rapidity of virus binding to cells when augmented by centrifugal force at 6 C. That stationary incubation is relatively inefficient for attaching virus to cells is further substantiated by the theory of Brownian movement as it relates to the arrival rate of virus particles in suspension at the surface of a cell system. Virus in Brownian motion may take an average of 4 h to diffuse 0.1 mm, and several hours may elapse before half the virus particles reach the cell surface (43). Because almost synchronous attachment of virus is achieved by using centrifugal force and attached virus may be held at the cell surface without penetration at 6 C, these two events may be delineated and their rates accurately measured.

The disappearance of virus from the supernatant fluid of virus-cell mixtures introduced onto cell monolayers has been postulated to be unaffected by the temperature of the reaction mixture (1, 2, 9, 12, 19, 26, 44). The attachment of influenza virus to host cells was also independent of temperature. However, influenza virus attachment was pH-dependent; maximal binding occurred near neutrality. This suggests that chemical groups are involved in the event, most likely the amino groups of the virus and the strongly acidic phospholipid groups of the host cell (1). The pH-dependence for T phage attachment to *Escherichia coli* also showed a pH optimum indicative of carboxyl or amino groups as a prerequisite for attachment (40). The quantity of virus attached to cells as a function of NaCl concentration indicated that 0.2 M was optimal. Salt concentrations below 0.2 M markedly inhibited virus attachment. This salt requirement, added to evidence of pH dependence and temperature independence of influenza virus attachment to cells, supports



the concept that the initial union involves electrostatic forces (2, 12, 19, 26, 29, 39).

Although divalent cations, usually calcium and magnesium, have been generally found to be necessary for the electrostatic attachment of bacteriophages (46) and animal viruses to host cells (2, 23, 26), there are numerous exceptions (11-13, 21). Attachment of influenza virus to cells proved to be another exception. In the presence of 0.01 M PBS, pH 7.2, containing 0.0009 M  $\text{CaCl}_2$  and 0.0005 M  $\text{MgCl}_2$ , the quantities of virus attached did not differ significantly from that in the phosphate buffer alone. The addition of dipolarionic buffers, *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid, tris(hydroxymethyl)aminomethane, or tricine, similarly, did not enhance or adversely affect virus-cell attachment. These data, together with other parameters of virus attachment, supported by findings with the bacteriophage (28) and animal virus systems (12), suggest that the requirements for virus attachment are virus specific and not cell specific.

In general, there is a paucity of data on the rate of penetration of influenza viruses into cells. The slow penetration reported for influenza virus into clone 1-5C-4 cells (36), requiring from 3 to 4 h, differs sharply from our findings. The variable penetration times reported for other animal viruses, ranging from 20 min to several hours (3, 7, 15, 22, 45), may reflect the difficulty in isolating the penetration stage from the rest of the infective cycle (5). In this current study, the penetration of five representative strains of influenza virus into cells at 35°C proceeded at a linear rate and was complete within 15 min for all virus strains. Similar results were obtained with other animal viruses when attachment and penetration processes were delineated (11-13). In contrast, to the temperature independency of the attachment reaction, the penetration of influenza virus, as noted with other virus-cell systems (9, 12, 15, 22, 27), was temperature dependent. Fifty percent of attached virus penetrated into cells in 7.5 min at 35°C, whereas an equivalent quantity penetrated in 58 min at 26°C. Penetration was minimal at 6°C; 5% of attached virus penetrated into cells within 60 min.

The influence of pH on virus penetration, with maximal penetration occurring between pH 6.2 and 7.2, was less restrictive than on virus attachment, with maximal attachment occurring between pH 7.0 and 7.2. In contrast to the precipitous decline of virus attachment in the presence of NaCl concentrations less than 0.2 M, virus penetration declined more slowly at lower salt molarities (Fig. 6). Maximal virus

penetrated in the presence of 0.2 to 0.1 M NaCl and was minimal at salt concentrations greater than 0.2 M. Although virus penetration as it is affected by NaCl concentration cannot be presently explained, it would appear that the salt plays a role different from that postulated for virus attachment, i.e., electrostatic interaction.

The addition of DEAE-dextran during the early stages of virus-cell interaction has been shown to induce elevated plaque counts with many viruses (17, 18, 24, 32, 33, 41, 42) but exceptions have been noted (48). Either increased adsorption of virus to cells because of an electrostatic interaction (1) or enhanced penetration as a result of reversible cell damage produced by the polymer (34) has been proposed for the mode of action by the polycation. When DEAE-dextran was incorporated into influenza virus inoculum, our results showed that both cell attachment and penetration by virus was partially inhibited. That the polycation masked or competed with virus for cellular binding sites during the virus attachment step may account for this phenomenon. The inhibitory effect on virus penetration by DEAE-dextran may be related to modification or inhibition of enzymes participating in the virus penetration step, or to a breakdown of virus-cell union resulting in virus elution. In view of reports that an increase in influenza virus plaque size and number resulted when DEAE-dextran was added to agar overlay medium (14, 36, 38), it would appear that the action of the polycation in this system, as opposed to that in the present study, involves different mechanisms. That influenza virus plaque development was improved when DEAE-dextran was used to complex sulfated polysaccharides in agar (38) serves to substantiate this hypothesis. Willis et al. (48) recorded some paradoxical observations on the effect of DEAE-dextran on epizootic hemorrhagic disease virus infectivity; the polycation included in an agar overlay decreased plaque counts. However, the counts were increased when the polycation was added to the virus-cell suspension. In view of the data, generalizations on the mechanism of action relative to the favorable or adverse effect of polycations on the infectivity of intact viruses are presently difficult to formulate.

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