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Fusion activity and inactivation of influenza virus: kinetics of low pH-induced fusion with cultured cells

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The kinetics of fusion of influenza virus (A/PR/8/34) with human promyelocytic leukaemia (HL-60), human T lymphocytic leukaemia (CEM) and murine lymphoma (S49) cells were investigated. Fusion was demonstrated by electron microscopy, and monitored by fluorescence dequenching of octadecylrhodamine incorporated in the virus membrane. Rapid fusion was induced upon mild acidification of the medium. At pH 5, all virus particles were capable of fusing with the cells. The initial rate and the extent of fusion were maximal between pH 4·9 and 5·2 and declined sharply below and above this range. The rate constants of adhesion of influenza virus to cells or erythrocyte ghosts were large, indicating a diffusion-controlled process. The rate constants of fusion of the virus with cells were smaller than those found previously for fusion with various liposomes. Although preincubation of the virus at acidic pH in the absence of target membranes almost completely inactivated the virus in its ability to fuse with erythrocyte ghosts, it reduced the extent of fusion with cultured cells by only 20 to 40%. Kinetic analysis of fusion revealed a mode of inactivation of the virus bound to erythrocyte ghosts or suspension cells, below pH 5·4, different from that of the virus preincubated at low pH without target membranes.

Introduction

Certain lipid-enveloped viruses, such as influenza, Semliki Forest and vesicular stomatitis viruses, infect their host cells by fusing with the endosome membrane after endocytosis of the virion and acidification of the endosome lumen (Matlin *et al.*, 1981; Yoshimura *et al.*, 1982; White *et al.*, 1983; Asano & Asano, 1984; Ohnishi, 1988; Hoekstra & Kok, 1989; Marsh & Helenius, 1989). The kinetics of fusion of viruses with liposomes, erythrocyte ghosts and cultured cells provide insights into the mechanisms of interaction of viruses with target membrane components and of the fusion process. The development of fluorescence assays for the fusion of lipid-enveloped viruses with their target membranes has greatly facilitated the study of the kinetics and mechanisms of fusion (Hoekstra *et al.*, 1984, 1985; Düzgüneş & Bentz, 1988; Loyter *et al.*, 1988).

In one of these assays, octadecylrhodamine (R-18) is initially incorporated in the viral membrane at a self-quenching surface concentration; fusion with a target membrane results in the relief of self-quenching upon dilution of the probe, due to rapid lateral diffusion (Hoekstra *et al.*, 1984, 1985). This assay has been used previously to monitor the fusion of several types of viruses with cultured cells (Blumenthal *et al.*, 1987; Miller & Hutt-Fletcher, 1988; Sinangil *et al.*, 1988). Investigations of the fusion of influenza virus with erythrocyte ghosts as a biological target membrane (Stegmann *et al.*, 1986) indicated that the pH dependence of fusion was similar to that of the cell–cell fusion activity of the virus (White *et al.*, 1981). These studies, and others on the haemolytic activity of the virus, also revealed a phenomenon of 'inactivation,' i.e. a reduction of the fusion activity of the virus, when first incubated at low pH in the absence of target membranes (Junankar & Cherry, 1986; Sato *et al.*, 1983; Stegmann *et al.*, 1986, 1987*b*). Significant inactivation was not observed, however, when the target membranes were cardiolipin (CL) liposomes (Stegmann *et al.*, 1986).

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We investigated the kinetics and extent of low pH-induced fusion of influenza virus with the plasma membrane of several types of living cells, using the R-18 assay, to obtain insights into the mechanisms of the fusion of the virus with cellular membranes. The interaction of the virus with the cells was also observed by electron microscopy. Our studies indicated that human promyelocytic leukaemia (HL-60), the human T lymphoblastic leukaemia (CEM) and murine lymphoma (S49) cell lines used as target cells do not internalize the virus to a significant extent during the relatively short times of preincubation before the reduction of the pH of the medium. Experiments were designed to yield the pH and temperature dependence of the rate constants of the actual fusion process, as well as estimates for the rate constant of adhesion (Nir *et al.*, 1983, 1986*a, b, c*). Comparison of the final extents of fusion, as well as the rate constants of adhesion and fusion, in virus–cell systems with those of virus–liposome systems can elucidate the role of various membrane components in the fusion process. By combining experimental studies with a theoretical analysis we have also provided details of the process of low pH inactivation of influenza virus.

Methods

Cells. HL-60 and CEM cells were maintained in RPMI 1640 medium containing 25 mM-HEPES buffer, supplemented with 10% foetal bovine serum (FBS), and S49 cells were grown in the same medium supplemented with 10% heat-inactivated horse serum. The cells were free of *Mycoplasma* contamination and were grown under a 3% CO_2 atmosphere at 37 °C up to a density of about 10^6/ml. The cells were harvested by centrifugation in a Beckman TJ-6 cell centrifuge at 1000 r.p.m. for 10 min, washed twice in Dulbecco's PBS, once in phenol red-free RPMI 1640 containing 25 mM-HEPES buffer, resuspended in the latter buffer at a stock cell density of 10^8/ml, and kept on ice in polypropylene centrifuge tubes until use. The percentage of viable cells, determined by trypan blue exclusion, was routinely between 95 and 98%, except for the S49 cells which had a viability of about 82%.

Erythrocyte ghosts were prepared by first isolating the red blood cell fraction from whole blood by centrifugation on Ficoll-Hypaque, washing in PBS, lysing the cells in ice-cold 5 mM-EDTA, 10 mM-TES buffer pH 7·4, and washing repeatedly in this medium until a colourless sediment was obtained. The ghosts were then suspended in PBS containing 5 mM-$MgCl_2$, at 37 °C, allowed to equilibrate for 1 h, and pelleted and resuspended in RPMI medium without phenol red.

Virus labelling. The A/PR/8/34 (H1N1) strain of influenza virus was purified by discontinuous sucrose density gradient centrifugation after harvesting from the allantoic cavity of embryonated eggs. The X-87 strain of influenza virus, inactivated with β-propiolactone for use as a vaccine preparation, was obtained from Dr Jan Wilschut (University of Groningen, The Netherlands). Viruses were labelled with R-18 (Molecular Probes) by mixing 20 µl of a 1·8 µmol/ml ethanolic solution of the fluorophore with 1 ml of virus (2 mg protein) and incubating for 0·5 to 1 h at room temperature (20 °C). The final ethanol concentration was 1 to 2%. This concentration of added probe corresponds to 5 mole % of total viral lipid; the lipid concentration was determined by phosphate analysis after Bligh-Dyer extraction of the lipids (1 mg viral protein corresponded to 0·362 µmol lipid). The mixture was then placed on a discontinuous sucrose gradient (60 to 20% sucrose, buffered to pH 7·5 with 10 mM-TES), and centrifuged for 3 h at 45000 r.p.m. in a Beckman SW50.1 rotor at 4 °C, to remove free probe molecules. The protein concentration of the virus band was determined by the Lowry assay. Labelled virus was chromatographed on Sephadex G-75 (Pharmacia) with TES-buffered saline to remove unincorporated probe molecules.

Fluorescence measurements. Labelled virus was pre-incubated with cells at neutral pH and the pH of the incubation medium (RPMI 1640 with 25 mM-HEPES buffer) was reduced by adding acetate buffer. The pH was measured at the end of each experiment. Fusion was monitored as the increase of R-18 fluorescence due to dequenching of the probe upon lateral diffusion and dilution into the target membrane, as a result of the fusion of the two membranes (Hoekstra *et al.*, 1984, 1985). The maximal (100%) fluorescence (F_{max}) was determined by lysing the virus and cell membranes with 1 mM (final concentration) octaethyleneglycol dodecyl ether ($C_{12}E_8$; Calbiochem) or $C_{13}E_9$ (a gift of Dr Barry Lentz), and waiting for the fluorescence to equilibrate. Fluorescence measurements were performed in a Spex Fluorolog 2 fluorometer, using the front-face configuration in the emission channel [excitation: 550 or 560 nm; emission: 590 or 600 nm (using 5 and 2·5 mm slits in both monochromators]. A high-pass filter (50% transmission at 590 nm; Schott Glass OG590, Melles-Griot) was placed between the cuvette and the emission monochromator. The absence of any appreciable contribution to fluorescence from light-scattering was apparent in experiments where the extent of fusion was about 100%, and the addition of detergent (and hence the dissolution of the cells) did not reduce the fluorescence intensity.

Analysis of the kinetics and extent of fusion. The analysis of fusion yields the percentage of virions capable of fusing with the plasma membrane and the number of virions that can fuse per single cell (Nir *et al.*, 1986*b*), provided that the cellular membranes are in sufficient excess over those of the virions. The number of virions per µg protein was estimated from the average size of the virus particles, the expected amount of lipid in a spherical particle of this diameter, and the lipid to protein ratio of the virus preparation. The overall fusion process was analysed as a sequence of kinetically coupled reactions (Nir *et al.*, 1983): (i) adhesion of the virus to the cell, with a rate constant C (M^{-1} s^{-1}), (ii) dissociation of the virus from the cell surface, with a rate constant D (s^{-1}) and (iii) the fusion reaction *per se*, with a rate constant f (s^{-1}). The analysis presumes that the fraction, B, of bound virus, following 10 to 20 min of preincubation with cells has been determined, and that during 1 min following the reduction of pH the fraction of bound virus does not change significantly. B was determined by incubating identical amounts of virus and cells as in the fusion experiments, sedimenting the cells by centrifugation, and determining the fluorescence in the supernatant and pellet following detergent lysis. Since the bound virions occupy a small fraction of the cellular surface area, the increase in fluorescence during the first 5 to 10 s after reducing the pH may reflect a first-order process given by (Nir *et al.*, 1990):

$$I(t) = [1 - \exp(-ft)]B \quad (1)$$

in which $I(t)$ is the normalized fluorescence increase. The value of f can then be determined from equation (1). Although influenza virus can be inactivated by exposure to low pH in the absence of target membranes (Sato *et al.*, 1983; Stegmann *et al.*, 1986, 1989), virus inactivation can be neglected during the first few seconds. The values of $I(t)$ at later times may be affected by virus inactivation, as discussed in the next section.

Analysis of low pH-induced inactivation. Low pH inactivation of influenza virus may be quantified by an inactivation rate constant, γ,

which reduces the value of $f(t)$ according to either one of the following (Nir *et al.*, 1988, 1990):

$$f(t) = f(0)\exp(-\gamma t) \quad (2)$$

or

$$f(t) = f(0)/(1+\gamma t)^2 \quad (3)$$

A reduction of $f(t)$ according to equation (2) would arise from first-order processes affecting the haemagglutinin (HA) units. These processes could be the binding of the exposed hydrophobic segment of HA (Skehel *et al.*, 1982) to the viral membrane, or other conformational changes not involving clustering of HA molecules. A decay of $f(t)$ according to equation (3) would result if the number of active units of HA is reduced due to clustering in the viral membrane, as proposed by Junankar & Cherry (1986). If $f(t)$ is decaying according to equation (2), then (Nir *et al.*, 1990; Nir, 1991):

$$I(t) = \{1 - \exp[f(\exp(-\gamma t) - 1)/\gamma]\}B. \quad (4)$$

The analysis involves the determination of f from $I(t)$ values at the first 5 to 10 s, and then determination of γ from $I(t)$ at later times, e.g. up to 1 min.

Electron microscopy. The suspensions of virus and cells were fixed at room temperature with 1% paraformaldehyde, 3% glutaraldehyde and 1% acrolein buffered to pH 7·4 with 0·1 M-cacodylate buffer. The fixed suspension was washed with 0·1 M-cacodylate buffer containing 7% sucrose, fixed for 2 h at 4 °C in acetate–Veronal-buffered 1% OsO_4 (pH 7·4) with 5% sucrose, quickly dehydrated in graded ethanol solutions and embedded in Epon. Sections were cut on a Porter-Blum MT-2 microtome, stained with alkaline lead and 5% uranyl acetate, and examined in a JEOL 100CX electron microscope at 80 kV.

Results

Fusion of influenza virus with cultured suspension cells and erythrocyte ghosts

The time course of R-18 fluorescence during incubation of influenza virus (A/PR/8/34 strain) with HL-60, CEM or S49 cells is shown in Fig. 1. No detectable increase in the fluorescence was observed upon incubation of HL-60 or CEM cells with the virus at neutral pH for the first 10 min of incubation; the fluorescence increased slightly in the case of S49 cells. When the pH was lowered to 5, the fluorescence intensity increased rapidly, indicating the fusion of the viral membrane with the cell plasma membrane. Similar results were obtained with erythrocyte ghosts as target membranes, the fluorescence intensity reaching 100% within 2·5 min of the pH being lowered (Fig. 2*a*).

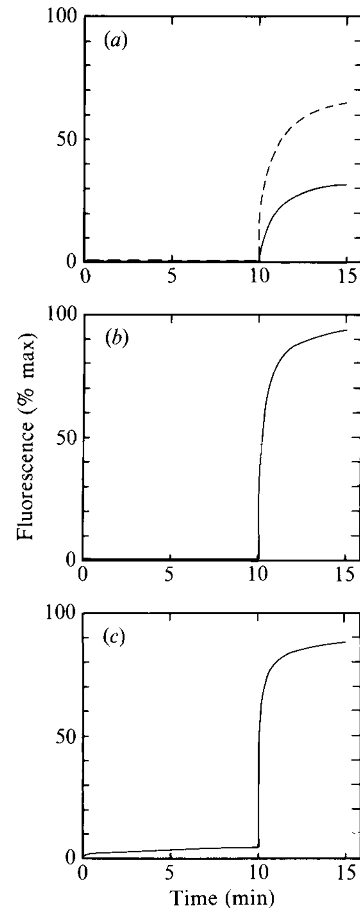

Fig. 1. The time course of R-18 fluorescence during incubation of 5 µg viral protein/ml influenza virus (A/PR/8/34 strain) with 2 × 10^7/ml (*a*) HL-60, (*b*) CEM and (*c*) S49 cells. Virus was incubated with cells at pH 7·6 at 37 °C for 10 min, and the pH lowered to 5. The dashed line in (*a*) shows the fusion kinetics obtained with 2 µg/ml virus. The value of fluorescence is given as the percent of maximum, and is defined by $F(\%) = 100\,[I(t) - I_0]/[I(\infty) - I_0]$, where I_0 is the fluorescence intensity at the beginning of the preincubation and $I(\infty)$ is the fluorescence at infinite dilution of the probe, achieved by the addition of detergent. The fluorescence scale is defined similarly in subsequent figures.

Fig. 3 shows influenza virus adhering to HL-60 and CEM cells after a 10 min incubation at neutral pH. Under these conditions no endocytosis or fusion was observed, and neither was a significant increase of R-18 fluorescence. When the pH was lowered to 5 for 0·5 to 1 min, several virus particles were observed with their membranes in continuity with the plasma membrane of the cells (Fig. 4), occasionally near a coated pit (Fig. 4*d*). The spike glycoproteins of the virus appeared amorphous at low pH. Membrane evaginations were also observed at the plasma membrane following fusion.

Binding of influenza virus to cultured cells and erythrocyte ghosts

When the PR/8 strain (2 µg/ml) was incubated with 2 × 10^7 HL-60 cells/ml at pH 7·5, about 60% of the virions were bound at 37 °C. Fifty percent of the X-87 strain bound at 37 °C, and 80% bound at 0 °C, the lower binding at 37 °C indicating a certain degree of reversibility of binding, whereas at 0 °C the dissociation rate constant D, is expected to be significantly reduced (Nir *et*

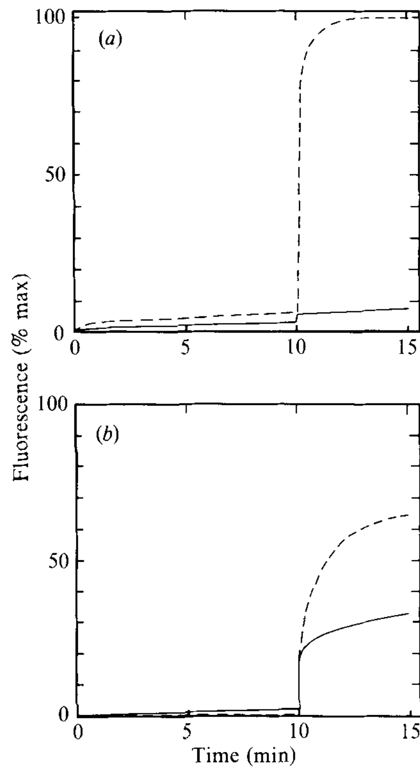

Fig. 2. The effect of preincubation of influenza virus (PR/8 strain, 2 μg protein/ml) at low pH on its fusion with (*a*) erythrocyte ghosts (2×10^8/ml), or (*b*) HL-60 cells (2×10^7/ml). Virus alone was incubated at pH 5 and 37 °C for 20 min, and incubated with erythrocyte ghosts or cells at pH 7·6 for 10 min (solid curves). The pH was then reduced to pH 5 at 10 min. The dashed curves are the controls where the virus was not preincubated at low pH.

al., 1983). The binding data can provide an estimate for the forward (adhesion) rate constant C. The concentration of free virus $V(t)$ is reduced initially according to:

$$V(t) = V_0 \exp(-CG_0t) \quad (5)$$

in which V_0 and G_0 are initial molar concentrations of the virus and cells, respectively, and t is the time of incubation (Nir *et al.*, 1986*b*). Equation (5) assumes that virus dissociation from the cell surface can be ignored. Thus, we will employ equation (5) in the form of an inequality, since less binding must occur than according to equation (5), as a result of dissociation:

$$V(t)/V_0 > \exp(-CG_0t), \quad (6)$$

or

$$C > \ln[V_0/V(t)]/(G_0t). \quad (7)$$

By taking $V_0/V(t) = 2$, $t = 1200$ s, and $G_0 = 3{\cdot}3 \times 10^{-14}$ (i.e. 2×10^7 cells/ml), we find that for the X-87 strain adhering to HL-60 cells, $C > 1{\cdot}7 \times 10^{10}\ \mathrm{M^{-1}s^{-1}}$. For the PR/8 strain adhering to HL-60 cells, $C > 3 \times 10^{10}\ \mathrm{M^{-1}s^{-1}}$, and to erythrocyte ghosts $C > 1{\cdot}2 \times 10^{10}\ \mathrm{M^{-1}s^{-1}}$. This result is remarkable, since the upper limit on C values in diffusion-controlled processes (Smoluchowski, 1917) at 37 °C is $5 \times 10^9\ \mathrm{M^{-1}s^{-1}}$ in the case of aggregation of spherical particles of equal sizes. C values can be larger for spherical particles of unequal sizes (Berg & von Hippel, 1985). When the radius of a cultured cell is larger than that of influenza virus by more than 100-fold, computation reveals that the value of C can exceed $5 \times 10^9\ \mathrm{M^{-1}s^{-1}}$ by more than 25-fold in the diffusion-controlled case. In the case of one batch of PR/8 and HL-60 cells, the increase in R-18 fluorescence was relatively more rapid (Fig. 5*b*). An analysis of these results yielded $C \geqslant 3 \times 10^{11}\ \mathrm{M^{-1}s^{-1}}$. In this case, the upper value limit on C has indeed been reached.

Fusion rate constants

The fusion (f) and inactivation (γ) rate constants were determined according to equation (4) (Table 1). Examples of the fit of equation (4) to the data are shown in Fig. 6. The values of f and γ listed in Table 1 vary from batch to batch. Hence it was essential to use the same batch of virus and cells when investigating the effect of pH or temperature. The fusion rate constants obtained with the PR/8 strain were several-fold larger than those with the X-87 strain, but the same trend was observed in both cases. The highest f values found for virus–cell systems, $f = 0{\cdot}1\ \mathrm{s^{-1}}$, correspond to the lowest f values found in virus–liposome fusion.

The pH dependence of fusion

The initial rate of fusion with HL-60 cells displayed a maximum in the pH range 4·7 to 5·0, whereas the extent of fusion within 5 min was maximal in the range 4·9 to 5·2 (Fig. 5*a*). A similar optimum was observed in the rate and extent of fusion of the X-87 strain with HL-60 cells, but the extent of fusion did not exceed 8% F_{max} at pH 4·8.

The fusion rate constant was sensitive to the pH, increasing six-fold with a change in pH from 5·38 to 5·01, and 26-fold with a change from 5·8 (Table 1). The rate of inactivation was dramatically affected. The extent of fusion was higher at pH 4·74, compared to that at 5·01, up to 1 min after lowering of the pH; but after 5 min the extents of fusion were 46·6% for pH 5·01 and 30·5% for pH 4·74. The analysis explains this difference by a relatively small increase in f and γ values in the case of the lower pH.

Effect of temperature, the time of pre-incubation and cell concentration

Reduction of the incubation temperature to 15 or 25 °C greatly reduced the initial rate and extent of fusion.

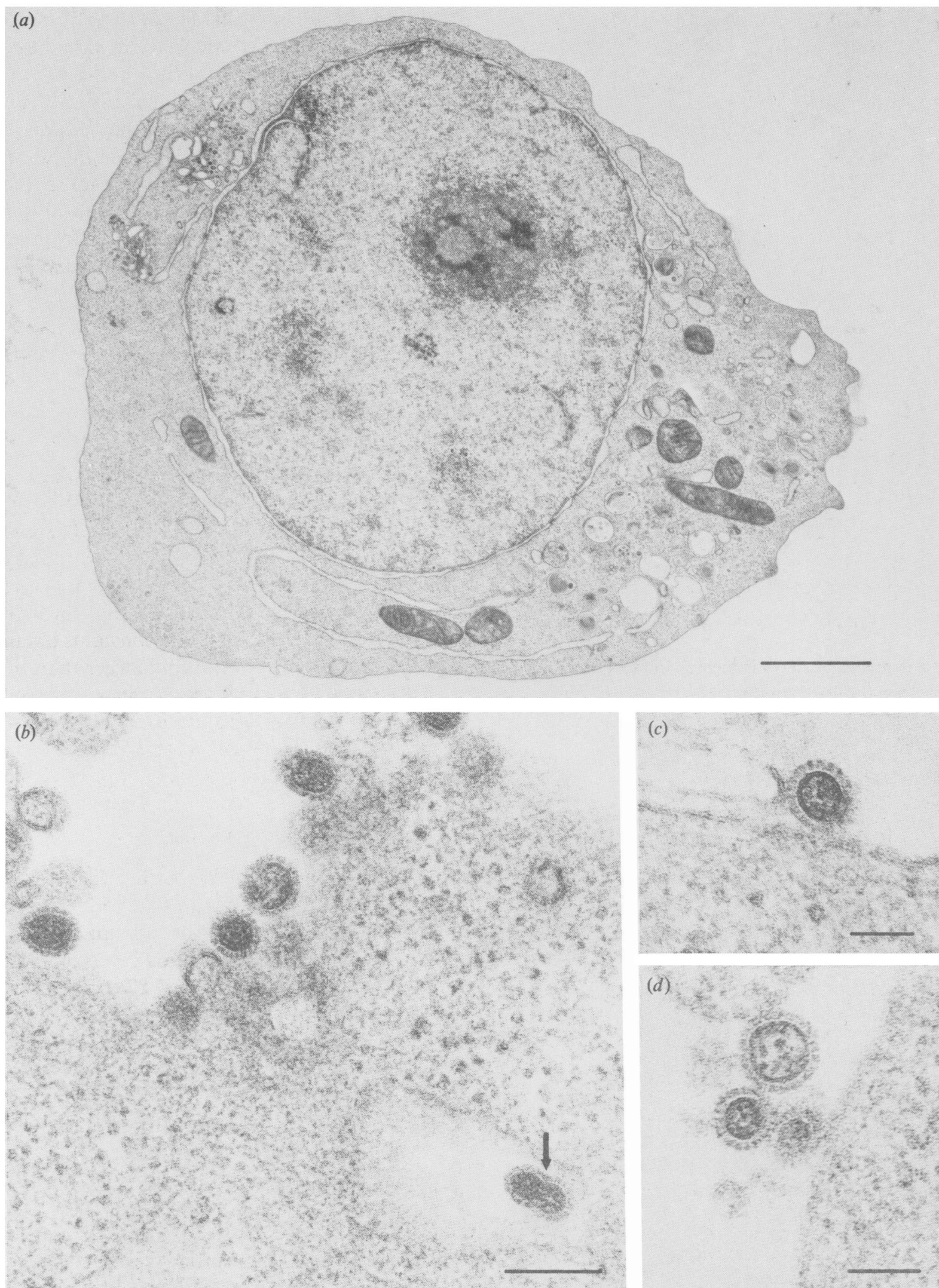

Fig. 3. Electron micrographs of the PR/8 strain of influenza virus adhering to the cell surface at neutral pH, following a 10 min incubation at neutral pH at 37 °C. (*a*) A CEM cell; (*b*) A section through the periphery of an HL-60-cell. The viruses are clustered at the cell surface and one (arrowed) appears as a twin within a cell surface invagination. (*c* and *d*) The viral HA is well fixed and prominent. Viruses remain at the surface of both CEM and HL-60 cells. Bar markers represent: (*a*) 2 μm; (*b*) 200 nm; (*c*) 100 nm; (*d*) 100 nm.

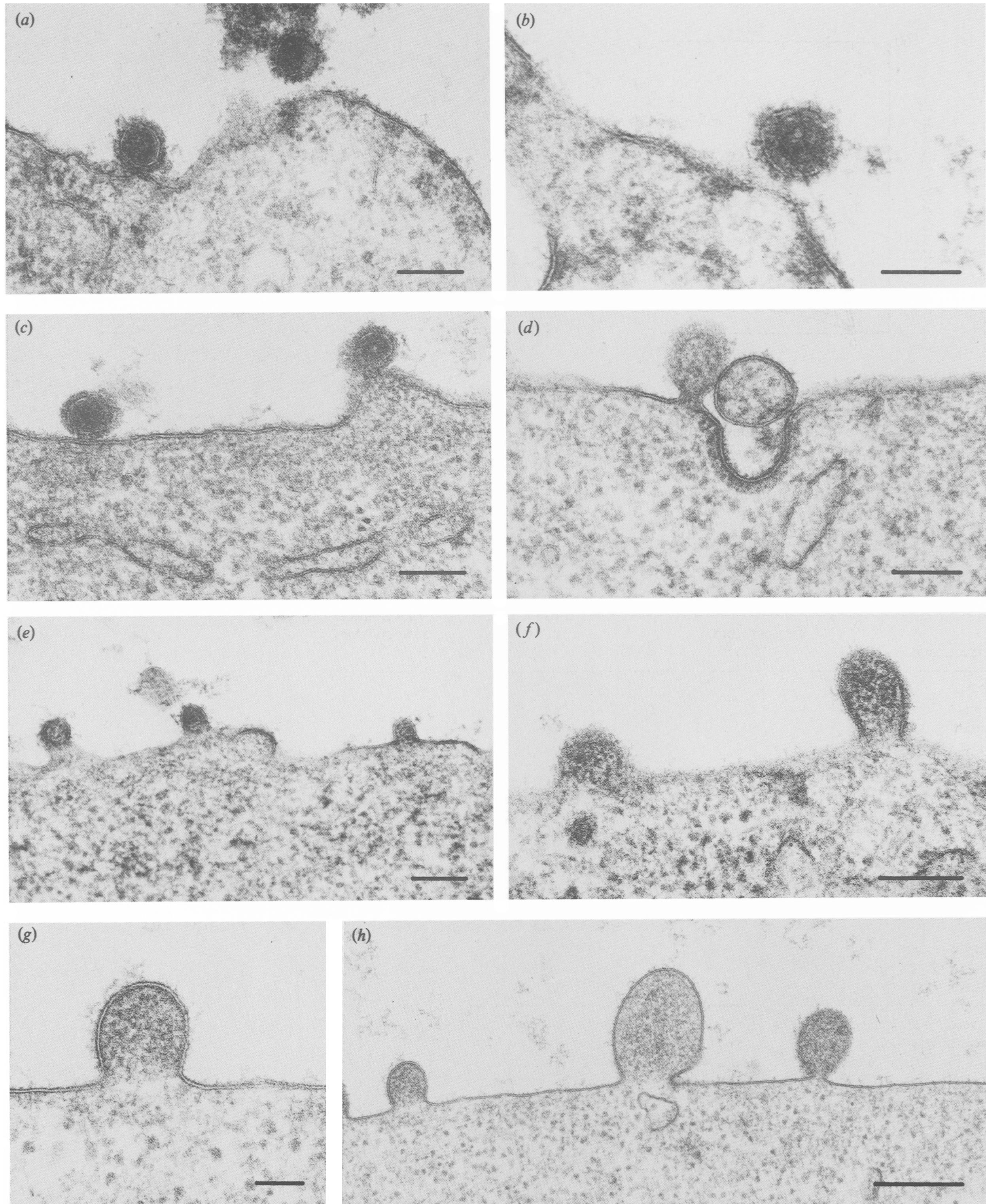

Fig. 4. Electron micrographs of the PR/8 strain of influenza virus incubated with CEM or HL-60 cells, 30 s after lowering the pH to 5. (*a*, *b* and *c*). At pH 5, the spike proteins of the virus are partially collapsed and less distinct than at neutral pH. Fusion of the virus with HL-60 cells occurs rapidly at low pH. (*d*) Fusion of a virus adjacent to a coated pit on the surface of a CEM cell. (*e*) Lower magnification image of several completed fusions at the surface of a CEM cell. (*f*, *g* and *h*). Following fusion of influenza virus with HL-60 and CEM cells, membrane evaginations seem to mark the sites of previous viral fusion, enlarging to several times the diameter of the initial virus particle. It is not known whether viral proteins remain in these structures. Bar markers represent: (*a*) 100 nm; (*b*) 100 nm; (*c*) 100 nm; (*d*) 100 nm; (*e*) 200 nm; (*f*) 200 nm; (*g*) 100 nm; (*h*) 500 nm.

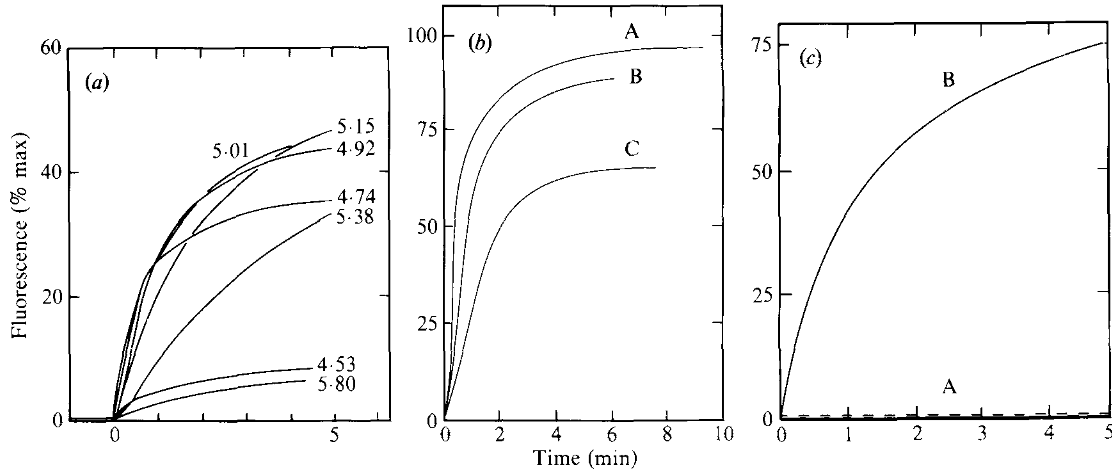

Fig. 5. The effect of pH (*a*), time of preincubation and of cell concentration (*b*) and proteinase K (*c*) on the kinetics of fusion of 2 μg/ml influenza virus (PR/8 strain) with 2 × 10^7/ml HL-60 cells at 37 °C. (*a*) After a preincubation of the virus with the cells for 10 min, the pH was lowered to the indicated values. (*b*) The pH was lowered to 5 either 1 min (curve A) or immediately (curve B) after the virus and cells were mixed at pH 7·3; curve C was similar to curve B, except that the cell concentration was 1 × 10^7/ml. (*c*) Eight μg of virus was incubated with 4·8 μg of proteinase K in 100 μl PBS at 37 °C for 15 min at pH 5 and neutralized (curve A), or at pH 7·4 for 10 min (curve B). Four μg of this virus was then incubated with 4 × 10^7 cells in 2 ml at pH 7·4 For 1 min, and the pH was lowered to 5 at time zero.

Table 1. *Dependence of rate constants of fusion and inactivation of influenza virus on the target membrane, pH and temperature*

Target membrane	Temperature (°C)	pH	Fusion rate constant f (s^{-1})	Inactivation rate constant γ (s^{-1})
S49 cells*	37	5	0·005–0·05	ND†
CEM cells*	37	5	0·005–0·05	ND
HL-60 cells*	37	5	0·01–0·1	0·01–0·03
Erythrocyte ghosts*	37	5	0·03–0·1	0·015–0·02
Liposomes‡	37	5	0·1–0·05	0·04–0·05
CL liposomes‡	37	5	1	0
HL-60§	37	4·53	0·0025	0·015
HL-60	37	4·74	0·02	0·02
HL-60	37	5·01	0·018	0·018
HL-60	37	5·38	0·003	0
HL-60	37	5·8	0·0007	0
HL-60	25	5	0·004	0·01
HL-60	15	5	0·002	0·001
Erythrocyte ghosts	37	5	0·1	0·015
Erythrocyte ghosts plus 'inactivated' virus‖	37	5	0·0008	0·015

* In these cases a range of values is given, as obtained for several batches of cells and several batches of the two virus strains X-87 and PR/8. The lower f values were generally obtained for the X-87 strain. The values of f and γ were calculated according to equation (4), as described in the text. The values of B used for suspension cells and erythrocyte ghosts were 0·5 and 0·93, respectively.

† ND, not determined.

‡ The values for a variety of liposome compositions are taken from Nir *et al.* (1988) and Stegmann *et al.* (1989), and were obtained with the X-47 strain.

§ The experiments with HL-60 cells and erythrocyte ghosts listed below were performed with the PR/8 strain.

‖ Influenza virus was incubated alone at pH 5 and 37 °C for 20 min before addition to erythrocyte ghosts.

The fusion rate constant was decreased by 4·5-fold at 25 °C, and nine-fold at 15 °C (Table 1). These observations are consistent with the requirement of the mobility, e.g. the rotational diffusion, of HA to mediate membrane fusion, as revealed by studies on influenza virus fusion with erythrocyte ghost membranes (Junankar & Cherry, 1986).

The time of preincubation of the virus with HL-60

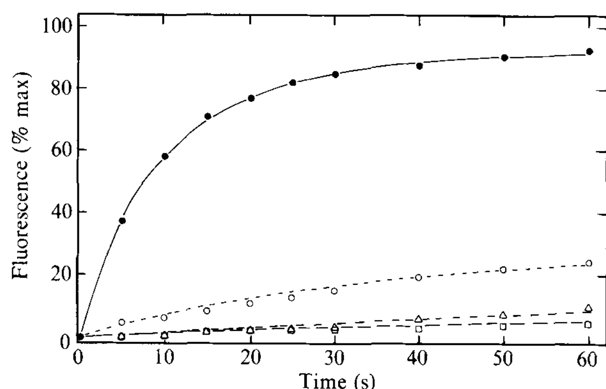

Fig. 6. Kinetics of fusion of influenza virus with HL-60 cells and erythrocyte ghosts. The PR/8 strain of influenza virus (2 μg viral protein/ml) was incubated at pH 7·6 and 37 °C with 2×10^7/ml HL-60 cells or with 2×10^8/ml erythrocyte ghosts. After 10 min, the pH was lowered. The experimental points are: fusion with ghosts at pH 5 (●); fusion with HL-60 cells at pH 5·01 (○); 5·38 (△) or 4·53 (□). The dashed curves are calculated according to equation (4) with $f = 0{\cdot}1\ s^{-1}$ and $\gamma = 0{\cdot}015\ s^{-1}$ (ghosts); $f = 0{\cdot}017\ s^{-1}$ and $\gamma = 0{\cdot}018\ s^{-1}$ (HL-60; pH 5·01); $f = 0{\cdot}003\ s^{-1}$ and $\gamma = 0$ (HL-60; pH 5·38); $f = 0{\cdot}0025\ s^{-1}$ and $\gamma = 0{\cdot}015\ s^{-1}$ (HL-60; pH 4·53).

cells affected the initial rate and extent of low pH-induced fusion. Fusion was significantly higher following a 1 min preincubation at neutral pH (Fig. 5*b*, curve A), than if the pH was lowered immediately after the addition of the virus (curve B), particularly at the initial stages. Fig. 5*b* demonstrates also that the initial rate and extent of fusion were higher for 2×10^7 cells/ml than for half this concentration, under conditions where the pH was lowered immediately upon addition of the virus. This observation indicates the effect of the kinetics of adhesion of the virus to the cells on the overall rate of fusion.

Fusion or probe exchange?

Several experiments indicated that the increase in R-18 fluorescence was mainly due to fusion, rather than molecular exchange or transfer of the probe. (i) No significant increase in fluorescence was observed at neutral pH despite extensive virus–cell binding (Fig. 1). (ii) If exchange contributes significantly to probe dilution, then the addition of membranes into which the probe can dilute should result in an enhanced fluorescence increase. When a 50-fold excess of unlabelled virus was added to HL-60 cells in the presence of labelled virus, only 30% of the added labelled virus was bound, and lowering the pH resulted in a lower extent of fusion, compared to controls (Table 2). Hence, at least two-thirds of the fluorescence increase is due to actual fusion. (iii) There was no increase in fluorescence when the labelled virus was incubated for 30 min with a 10-fold excess of unlabelled virus in the absence of cells. (iv) Incubation of the labelled virus with 10% FBS (in which lipoproteins can extract labile lipids) at pH 7·4 or pH 5 did not result in the transfer or exchange of the probe (data not shown). (v) When influenza virus was pretreated with proteinase K, the low-pH-induced increase in fluorescence was completely abolished, although a significant fraction (44%) of the added virus was bound to the cells (Fig. 5). Since the proportion of the untreated virus that was bound to cells was about 70%, the virus retained 63% of its binding capacity following proteinase K treatment. Thus, the probe does not transfer to the plasma membrane from bound virus even at low pH.

Table 2. *The effect of excess unlabelled virus on the fusion of R-18-labelled influenza virus (PR/8) with HL-60 cells*

Conditions of fusion	Extent of fusion after 4 min pH 5 [F_{max} (%)]	Bound virus [labelled virus (%)]*
Labelled virus only†	65·7	70
With 20-fold excess of unlabelled virus‡	20·6	ND§
With 50-fold excess of unlabelled virus‡	11·5	30

* The percentage of added labelled virus that was bound after 15 min at pH 7·4.

† Four μg of labelled virus was preincubated with 4×10^7 cells in 2 ml for 5 min at pH 7·4 and 37 °C. The pH was then lowered to 5 at time zero. The fluorescence scale was set as in Fig. 1.

‡ Same as in †, except that a 20- or 50-fold excess of unlabelled virus (80 or 200 μg) was also present.

§ ND, Not determined.

Number of fusion sites

The experiments with excess unlabelled virus (Table 2) indicate that, although at virus/cell ratios of about 100 all the virus will eventually fuse, only a fraction of the bound virus can fuse when the number of virions bound per cell exceeds 1000. Thirty percent of the added labelled virus bound to the cells and 10% of this virus fused. When the concentration of virus incubated with HL-60 cells was reduced from 5 to 2 μg/ml, the initial rate and extent of fusion were greater (dashed line, Fig. 1*a*). This observation supports the hypothesis that the fusion sites for influenza virus on HL-60 cells may be saturated in the case of the higher concentration. Thus, it appears that the number of sites on the cell where the virus can fuse is lower than those available for binding.

Kinetics of inactivation of influenza virus during fusion at low pH

Previous studies have shown that the haemolytic or fusion activity of influenza virus can be irreversibly

inhibited when incubated at low pH in the absence of target membranes (Junankar & Cherry, 1986; Sato *et al.*, 1983; Stegmann *et al.*, 1986; Puri *et al.*, 1990). Electron micrographs of inactivated virions indicated the absence of normally distinguishable HA spikes (Fig. 4 and Ruigrok *et al.*, 1986). The fuzzy layer on the surface may result from the deformation and/or clustering of the spikes. The kinetics of fusion at pH 5 were adequately simulated by equation (4) for up to several minutes, whereas setting γ to 0 could simulate only the first 15 s of fusion of the prebound virus. Irrespective of the cell and virus type employed, the range of γ values was just between 0·01 to 0·03 s^{-1}.

Inactivation of influenza virus pre-incubated at low pH without target membranes

We examined whether the low pH 'inactivation' reported for influenza virus fusing with erythrocyte ghosts or certain liposomes is observed for fusion with the plasma membrane of living cultured cells. We first determined whether the fusion of the PR/8 strain with erythrocyte ghosts is inhibited by low pH pretreatment.

Fig. 2*a* shows that pretreatment of influenza virus alone at pH 5 for 20 min at 37 °C reduced dramatically the fusion activity of the virus at low pH (following a 10 min incubation with the ghost membranes at neutral pH). Similar results were also obtained with phosphatidylserine (PS) liposomes as target membranes (data not shown). In contrast, when low pH pretreated virus was incubated with HL-60 cells at neutral pH for 10 min and the pH was then dropped to 5, rapid fusion was observed (Fig. 2*b*). The extent of fusion after 5 min at low pH was, however, reduced from about 56% F_{max} for mock pretreated virus to 33% F_{max}. With CEM cells as targets, low pH pretreatment resulted in the reduction of the extent of fusion at 5 min, from about 70% to 55% F_{max}.

Discussion

In this study we have established experimental and theoretical procedures for studying the fusion of influenza virus with suspension cells. We have found exceptionally high rate constants of adhesion of influenza virus to cells. *C* values found earlier for adhesion to CL liposomes were in the range of $0·5 \times 10^9$ to 1×10^9 $M^{-1}s^{-1}$ at pH 5, and 4×10^8 $M^{-1}s^{-1}$ at pH 6 (Nir *et al.*, 1986*a*), values close to what can be achieved in diffusion-controlled processes unaffected by repulsive forces. We conclude that the adhesion of influenza virus to cells (and liposomes) is practically unaffected by repulsive potential barriers, and is mainly controlled by diffusion. The larger *C* values found for cells may be attributed partly to the large size of the cells relative to the virions, and to cell surface receptors for the virus. Future studies on the differential removal of these proteins by proteases, and of the oligosaccharides by endoglycosidases will elucidate the influence of these structures on viral binding and fusion. Nussbaum & Loyter (1987) observed an immediate increase in R-18 fluorescence upon incubation of influenza virus with S49 cells at either pH 7·4 or pH 5, the former being attributed to fusion from within endosomes, since it could be inhibited partially by methylamine. In contrast, our results reveal no significant fusion at neutral pH. The observation (Nussbaum & Loyter, 1987) that fusion proceeded without a lag phase, which would have been required for endocytosis and endosome acidification, is puzzling. Stegmann *et al.* (1987*a*) found that fusion with adherent cell lines proceeded after a lag phase of 10 to 15 min.

An unexpected result obtained in our study is that 'low pH inactivation' of influenza virus is not as pronounced with cellular target membranes as with erythrocyte ghost membranes. It is clear that a residual fusion activity of the virus is retained even after 20 min of 'inactivation' at low pH. This can be reconciled by assuming that a certain degree of reversibility exists, or alternatively that the 'inactive' conformationally altered HA molecules retain some fusion activity towards suspension cells. One group of target membranes towards which inactivation of the virus is observed includes erythrocyte ghosts and most liposome types, such as PS, PC/PE (phosphatidylcholine/phosphatidylethanolamine) and PS/PC/PE (Nir *et al.*, 1988; Stegmann *et al.*, 1989). The other group includes CL liposomes and cells, towards which limited inactivation is seen. Erythrocyte ghosts are usually considered to be 'model' biological membranes; but our results indicate a clear difference between these membranes and cellular membranes as targets for influenza virus.

Our analysis indicates a different type of viral inactivation observed in the case of virus already bound to the surface of erythrocyte ghosts or suspension cells (Table 1), and not appearing at pH 5·38 and above. It exists even at lower temperatures (25 or 15 °C), although at a reduced level. It is possible that this mode of inactivation is due to interactions between viral glycoproteins and cellular components. The values of γ found for suspension cells are similar to those found for erythrocyte ghosts and are about 1·5 to threefold lower than those found for most liposomes. Whether the kinetics of decay of γ are described better by equation (2) or equation (3) could not be resolved by our analysis. The former represents a decay independent of the surface concentration of HA molecules, whereas the latter describes a clustering process. Equation (3) would imply a reduced inactivation for a lower surface concentration

of HA molecules. Investigating the inactivation of reconstituted virions with defined protein/lipid ratios may resolve this issue.

We have proposed earlier that the process of conformational change of HA induced by low pH is necessary for fusion with target membranes (Düzgüneş & Gambale, 1988; Düzgüneş *et al.*, 1991*b*), and not merely the static low pH conformation. Puri *et al.* (1990) have proposed that fusion occurs during the transition from a 'relaxed' conformation at low pH to an 'active' conformation of HA under conditions where the molecule is associated with the target membrane, and that such transitions in the absence of the target membrane lead to inactivation. The observation that fusion occurs within a narrow pH range may be attributed to the requirement for the exquisite control by pH of this transient conformation via the proper protonation of various amino acids in HA.

At pH 5 all the virus particles are capable of fusing with cells and erythrocyte ghosts, provided that the number of virions per cell is not excessive (several hundred). Stegmann *et al.* (1986, 1987*b*) also found 100% fusion activity of the X-47 strain towards erythrocyte ghosts and CL or PS liposomes, but only 20 to 45% of the virions fused with PC/PE liposomes with or without disialoganglioside G_{D1a} (Stegmann *et al.*, 1985, 1989; Nir *et al.*, 1988). Sendai virus also showed complete fusion activity towards erythrocyte ghosts (Hoekstra & Klappe, 1986; Nir *et al.*, 1986*b*) and cells (Pedroso de Lima *et al.*, 1991), but had incomplete fusion activity towards several liposomes, including PS and phosphatidylglycerol (Amselem *et al.*, 1986; Nir *et al.*, 1986*c*). It remains to be investigated which components in biological membranes mediate the complete fusion activity of Sendai or influenza viruses.

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