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Fusion of Sendai virus with negatively charged liposomes as studied by pyrene-labelled phospholipid liposomes

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Sendai virus particles fuse with negatively charged liposomes but not with vesicles made of zwitterionic phospholipids. The liposome-virus fusion process was studied by dilution of the concentration-dependent excimer-forming fluorophore 2-pyrenyldodecanoylphosphatidylcholine contained in the liposomes by the viral lipids. The data were analyzed in the framework of a mass action kinetic model. This provided analytical solutions for the final levels of probe dilution and numerical solutions for the kinetics of the overall fusion process, in terms of rate constants for the liposome-virus adhesion, deadhesion and fusion. This analysis led to the following conclusions: (1) At neutral pH and 37°C, only 15% of the virus particles can fuse with the phospholipid vesicles, although all the virions may aggregate with the liposomes. The rate constants for aggregation, fusion and deadhesion are of the orders of magnitude of 10^7 $M^{-1} \cdot s^{-1}$, 10^{-3} s^{-1} and 10^{-2} s^{-1}, respectively. The fraction of active virus increases with temperature. (2) At acidic pH, both the fraction of 'fusable' virus and the rate of fusion increase markedly. The optimal pH for fusion is 3–4, where most of the virus particles are active. At higher pH values, an increasing fraction of the virus particles become inactive, probably due to ionization of viral glycoproteins, whereas at pH values below 3.0 the fusion is markedly reduced, most likely due to protonation of the negatively charged vesicles. (3) While only 15% of the virions fuse with the liposomes at pH 7.4 and 37°C, all the liposomes lose their content (Amselem, S., Loyter, A. Lichtenberg, D. and Barenholz, Y. (1985) Biochim. Biophys. Acta 820, 1–10). We therefore propose that release of entrapped solutes is due to liposome-virus aggregation, and not to fusion. Both trypsinization and heat inactivation of the virus particles inhibit not only the fusion process but also the release of carboxyfluorescein. This demonstrates the obligatory role of viral membrane proteins in liposome-virus aggregation. (4) Reconstituted vesicles made of the viral lipid and the hemagglutinin/neuraminidase (HN) glycoprotein fuse with negatively charged liposomes similar to the intact virions. This suggests that the fusion of virions with negatively charged vesicles, unlike the fusion of the virus with biological membranes, requires only the HN and not the fusion glycoprotein.

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Abbreviations: PC, phosphatidylcholine; Pyr-PC, 2-pyrenyldodecanoylphosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; V, The total concentration of viral lipid; F glycoprotein, Sendai virus envelope fusion glycoprotein; HN glycoprotein, Sendai virus envelope hemagglutinin/neuraminidase glycoprotein.

Introduction

Fusion of membranes is an event of high significance in biology of eukaryotic cells [1]. Therefore, much effort has been devoted to gain in-depth understanding of the mechanistic aspects of various fusion processes of both biological membranes and model membranes. It is accepted that several steps are common to most fusion processes. These include the obligatory contact between the membranes involved, which should then be followed by dehydration of the fusing membranes and perturbation in their lateral organization [2,3]. Such perturbation may result from formation of highly curved regions [4] or formation of non-lamellar domains [5].

Of special interest is the fusion of virus particles with eukaryotic cells. It is clear today that enveloped virions introduce their nucleocapsid into host cells by two different pathways, both involving fusion processes. Those are (1) the fusion at low pH of virions such as Toga and Rhabdoviruses with endosomal membranes of the host cell, following endocytosis of the intact virus and (2) the pH-independent fusion between virions such as Paramyxoviruses and host cell plasma membranes. Both processes are believed to require receptor molecules in the host cell membrane, for virus-cell recognition [6,7]. Recently, however, several groups, including ours, have shown that such receptors are not obligatory for fusion of Sendai virus with negatively charged liposomes [8], or cholesterol-containing phosphatidylcholine vesicles [25,26]. Both Sendai virus and proteoliposomes reconstituted of viral lipids and both the F and HN Sendai virus glycoproteins fuse with liposomes made of PS, PG, or PI but not with vesicles made of pure PC. This could be demonstrated by mixing of contents and of the membrane lipids of the liposomes and virosomes or intact virions. Fusion is also accompanied by release of the liposome content, but the relationship between fusion and lysis is not yet clear.

The prime goal of the present work is to gain insight into the mechanism of these processes through further information on the consequent mixing of lipids. This is done by investigation of the dilution of 2-pyrenyldodecanoylphosphatidylcholine (Pyr-PC) present in the lipid vesicles [9,10]. These data are analyzed in the framework of a mass action kinetic model, which provides analytical solutions for the final levels of probe-dilution due to fusion and numerical solutions for the kinetics of vesicle-virus adhesion and fusion. Although these studies do not resolve the intriguing question of the relevance of vesicle-virus fusion to virus-cell fusion processes and to Sendai virus infectivity, some light is shed on the factors governing the fusion of Sendai virus with negatively charged vesicles.

Experimental procedures

Materials

Phosphatidylcholine (PC) was obtained from egg yolk, as described elsewhere [11]. Phosphatidylserine (PS) was prepared from egg PC by transesterification using partially purified phospholipase D from Savoy cabbage [12]. The PS obtained was purified by silicic acid chromatography and was better than 99% pure. Egg phosphatidylglycerol (PG), bovine brain sphingomyelin and cholesterol were obtained from Sigma Chemical Co., St. Louis, MO. Pyrenyldodecanoylphosphatidylcholine (Pyr-PC) was prepared from egg lyso-PC and pyrenedodecanoic acid [13]. A single spot was obtained by thin-layer chromatography ($CHCl_3/MeOH/H_2O = 65:25:4$) and showed the same R_F as pure egg PC. 6-Carboxyfluorescein was obtained from Eastman-Kodak (Rochester, NY) and purified as described elsewhere [14]. Trypsin (Type III) and pronase were from Sigma. Triton X-100 was from Koch-Light Laboratories (Colnbrook, U.K.).

Cells and virus. Human blood, type O, was obtained from the blood bank of Hadassah Hospital, Jerusalem. Blood aged 4–8 weeks was used. The blood was washed five times with Solution A (160 mM NaCl/20 mM Tricine, pH 7.4) and finally suspended in Solution A to give a concentration of 2.0% (v/v).

Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutinin titer and its hemolytic activity were determined as previously described [8].

Most virions were roughly spherical and their diameter was 100–300 nm [27,30].

Methods

Preparation of Sendai virus reconstituted vesicles

containing the viral hemagglutinin / neuraminidase (HN) glycoprotein. Intact, pelleted Sendai virions (10 mg) were solubilized by 0.5 ml of a 4.0% (w/v) solution of Triton X-100 [15]. The HN glycoprotein was isolated from the solubilizate as described before [16] and HN-containing vesicles were prepared by removal of the detergent from a mixture of HN and viral lipids, using SM-2 Bio-Beads as previously described for the reconstituted Sendai virus envelope [15].

Preparation of liposomes labelled with pyrenyldodecanoylphosphatidylcholine. Small unilamellar vesicles (SUV) were prepared by ultrasonic irradiation, as previously described [17]. For the preparation of SUV containing pyrenylphosphatidylcholine, the lipids were first co-dissolved in chloroform, then dried and finally resuspended in the buffer solution and sonicated at 4°C under nitrogen. Residual metallic particles from the sonicator probe were removed by a 10 min centrifugation of the sonicated dispersion at $5000 \times g$. The resultant vesicle dispersion contained vesicles of diameters of 20–40 nm, as detected by electron microscopy of negatively stained vesicles using Philips EM 300 electron microscope.

Fluorescence measurements. Carboxyfluorescein solution was excited at 490 nm and fluorescence emission was detected at 520 nm [14]. Complete release (100%) of carboxyfluorescein from all the liposomes was obtained by the addition of Triton X-100 to a final concentration of 0.1% (w/v) [8]. All routine measurements were performed at 37°C. All fluorescence experiments were performed using either a Perkin-Elmer LS-5 or MPF-44 spectrofluorimeter. The pyrene-labeled PC in vesicles was excited at 340 nm and the spectra were recorded at wavelengths from 360 to 530 nm [9,10].

Phospholipids and protein determination. Phospholipids were assayed by the Barlett method [18] and protein was determined by the method of Lowry et al. [19].

Theoretical analysis of kinetics and final levels of vesicle-virus fusion. In this work we have studied the interaction between negatively charged liposomes and Sendai virus particles by following the reduction of excimer formation of liposomal Pyr-PC observed upon mixing the liposomes with the virions. We suggest (see Discussion) that this reduction of excimer formation is a result of probe dilution caused by mixing of liposomal and viral lipids as a consequence of liposome-virus fusion.

The fusion process is viewed as a sequence of two steps: (1) adherence of liposomes to the virus particles (2) the fusion process itself, involving merging of the membranes [2,20,21]. Detachment of adhered vesicles from the surface of virus particles may occur and is explicitly taken into account. The extent and kinetics of fusion were analyzed in the context of the model recently developed and applied for the fusion of influenza virus with cardiolipin liposomes at low pH [3].

Recent results on the fusion of influenza virus with cardiolipin vesicles [3,22] indicate that the fusion products consist of a single virus and any number of liposomes. As will be shown in this study, our results suggest that the fusion of negatively charged liposomes with Sendai virus yields similar products. Hence, we will consider the equations pertaining to this situation. First consider the case where all virus particles are active. In this case, all liposomes would eventually fuse with virions but not all the virus particles fuse with liposomes.

Assuming that the probe, initially contained in liposomes, is diluted by the lipid of the virus particles with which the liposomes fuse, the probe dilution, D_p, is given by

$$D_p = (V_F + L)/L = V_F/L + 1 \quad (1)$$

where L is the total concentration of the liposomal lipid and V_F is the lipid concentration of the fused virions.

The maximal dilution of the probe is obtained when all the virus particles fuse with all the liposomes, that is, when V_F is equal to the total concentration of the viral lipid, V. The maximal probe dilution D_L is therefore given by

$$D_L = (V + L)/L = V/L + 1 \quad (2)$$

The fraction of the virus particles which fuse with liposomes of course depends on the molar concentration ratio of liposomes and virus particles [3]. This ratio is given by (R^2L/V), where R is the ratio between the average radii of virus particles and liposomes. The concentration of viral lipid in the fused virus particles is therefore given by:

$$V_F = V(1 - \exp - LR^2/V), \quad (3)$$

However, some of the virus particles may be inactive, i.e., incapable of fusing with liposomes at a given pH and temperature.

Let α denote the fraction of inactive virus particles. We have considered two cases. (1) The adhesion of liposomes to virus particles is reversible; consequently, at equilibrium all the liposomes fuse with the active virus particles. In analogy to Eqn. 3, the concentration of viral lipid in fused virions is

$$V_F = (1-\alpha)V(1-\exp - LR^2/(V(1-\alpha))) \quad (4)$$

Now only a concentration L_F fuses with virions ($L_F < L$) and

$$D_P - 1 = V_F/L_F = (1-\alpha)\frac{V}{L}(1-\exp - LR^2/(V(1-\alpha))) \quad (5)$$

(2) The inactive virions bind liposomes irreversibly. In this case the concentration of liposomal lipid in fused form is $L_F = (1-\alpha)\ L$. The concentration of viral lipid in either fused or adhered form, V^*, is

$$V^* = V(1-\exp - LR^2/V), \quad (6)$$

and

$$V_F = (1-\alpha)V(1-\exp - LR^2/V) \quad (7)$$

The expression for D_p is

$$D_p = \alpha + (1-\alpha)(V_F + L(1-\alpha))/(L(1-\alpha)) \quad (8)$$

in which the first term arises from a dilution factor of unity for unfused liposomes. Rearrangement of Eqn. 8 and the use of Eqn. 7 gives

$$D_p - 1 = \alpha + \frac{V_F + L(1-\alpha)}{L} - 1 = V_F/L$$

$$= (1-\alpha)\frac{V}{L}(1-\exp - LR^2/V) \quad (9)$$

The analysis of the kinetics of the process of aggregation-fusion followed the numerical solution of the non-linear differential equations given by Nir et al. [3]. In analyzing the data we first determined the fraction α of inactive virus particles from the final D_p values. Then the parameters C, f, and D, i.e., the rate constants of adhesion, fusion and deadhesion, respectively, were determined from the kinetics of the aggregation-fusion process for suspensions of several L and V (and D_L) values.

Results

Mixing of viral and liposomal lipids at neutral pH

The ratio (E/M) between the intensity of excimer emission (E; measured at 460 nm) and that of the monomer (M; measured at 376 nm) of Pyr-PC in PS liposomes was a linear function ($r^2 = 0.997$) of the molar fraction of Pyr-PC in PS vesicles given by $E/M = 0.05 \cdot$ (mol% Pyr-PC). Thus, assuming that dilution of the probe by the viral lipid, due to fusion, is similar to its dilution in the PS liposomes, the extent of dilution of the probe can be estimated from the consequent reduction of E/M.

Incubation of PS vesicles containing 10 mol% Pyr-PC with a large excess of PC vesicles at pH 7.4 and 37°C resulted in only a slight decrease of E/M (Fig. 1, ×). On the other hand, incubation of the Pyr-PC containing PS vesicles with Sendai virus under the same conditions resulted in a time-dependent decrease in E/M (Fig. 1, ●), indicating dilution of Pyr-PC by virus components. The presence of an excess of unlabelled PC vesicles in the mixture of the labelled PS liposomes and virus particles resulted in a time-dependent decrease in E/M (Fig. 1, ▲) which was practically identical to that observed in a mixture containing only the virus and the labeled PS liposomes. Thus, the PC vesicles do not serve as acceptors for the Pyr-PC. In all the experiments described in Fig. 1, an apparent equilibration of the E/M value was approached in 120 min. At this point, doubling the virus concentration had no effect on the E/M (Fig. 1; ○), but addition of unlabelled PS liposomes caused a time-dependent decrease of E/M (Fig. 1, ■). Addition of labelled PS liposomes (Fig. 1, ⊗) resulted in an increase of the overall E/M, which was then followed by a time-dependent decrease of this ratio to a final E/M, which was then followed by a time-dependent decrease of this ratio to a final E/M ratio higher than that observed prior to the second addition of the

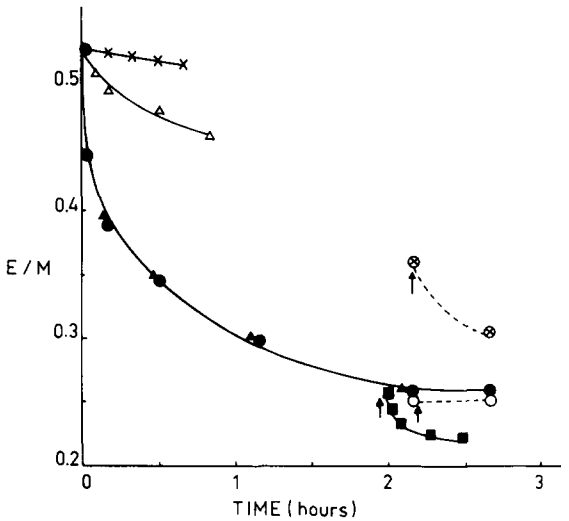

Fig. 1. Time dependence of E/M of Pyr-PC following mixing of PS liposomes (3 μM) containing 10 mol% Pyr-PC with PC liposomes (120 μM; ×) or with Sendai virus particles (27 μM viral lipid in all the other experiments). The symbols ● and ▲ represent experiments in the absence and presence of PC vesicles (40 μM PC), respectively. In the experiment described by the symbol △, the virus was trypsinized (by 10 μg of trypsin per 150 μg viral protein) prior to being mixed with the Pyr-PC-containing PS liposomes. Following a 2 h incubation of the mixture of virus (27 μM viral lipid) and labelled PS liposome (3 μM PS), aliquots of 2 ml of the mixture were mixed with additional amounts of labelled (3 μM; ⊗) or unlabelled (15 μM; ■) PS liposomes, or with Sendai virus particles (27 μM viral lipid; ○). All the experiments were carried out at 37°C and pH 7.4.

labelled PS liposomes.

Similar to our previous results [8], the fusion process was partially inhibited by both trypsinization of the virus (Fig. 1, △) or thermal inactivation by pre-incubation of the virus for 20 min at 70°C (not shown), suggesting involvement of a viral protein in the process.

Virus-induced dilution of the probe depended on both the concentration of the viral lipid (V) and the liposomal lipid (L). The apparent equilibrium value of E/M of mixtures with a constant liposome concentration decreased monotonically with increasing concentrations of virus particles, whereas in a medium containing a constant number of virus particles, the E/M increased with increasing the liposome concentration. The data of the experiments on the dependence of probe dilution on the virus and liposome concentrations are presented in Fig. 2 in terms of the dependence of the probe dilution factor ($D_p - 1 = [(E/M)_o - (E/M)\text{eq.}]/(E/M)\text{eq.}$) on the maximal possible dilution of the liposomal lipid ($D_L - 1$; Eqn. 2).

It is clear that the dilution of the probe is a monotonically increasing function of ($D_L - 1$), as it is dependent on the ratio of virus and liposome concentrations and independent on any of these concentrations per se. Yet, the rate of lipid dilution depended on the concentrations of the PS liposomes and/or the virions, as shown in Fig. 3. This figure also presents the analysis of this dependence in terms of the various steps involved in the fusion process, as described in the discussion.

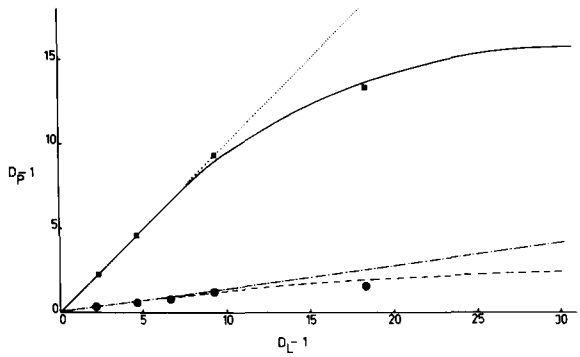

Fig. 2. Calculated and experimental dependencies of the probe dilution factor ($D_p - 1$) on the maximal lipid dilution factor ($D_L - 1$). All the lines present theoretical dependencies: both the dotted and solid lines assume that all the virus particles are capable of fusing with liposomes; the dotted line assumes that each liposome can fuse with any number of virus particles, whereas the solid line is for fusion of any number of liposomes with one virus particle. Both the broken and dashed-dotted lines present the theoretical dependencies obtained on the assumptions that 14% of the virions can fuse, each with any number of liposomes ($\alpha = 0.86$). The broken line is based on the assumption of irreversible binding of liposomes to the inactive virus particles (Eqn. 5), whereas the dashed-dotted line is obtained if it is assumed that all the liposomes can fuse with the (14%) active virions (Eqn. 9). The data points are from experiments in which the value of E/M, of mixtures of Pyr-PC-labelled PS liposomes and Sendai virus particles was measured 30 min after mixing of the two components. In one series of experiments the concentration of PS was kept constant (3 μM) and the viral liquid was varied over the range of 1–60 μM. In another series, the concentration of viral particles was kept constant (39 μM viral lipid) and the PS concentration varied from 0.5 to 12 μM. ■, experimental values at pH 3.6; ●, experimental values obtained at pH 7.4. All the measurements were carried out at 37°C.

Effect of pH

The pH dependence of the apparent equilibrium fluorophore dilution is described in Fig. 4. Fig. 4A presents the data of a control experiment carried out with labelled PS liposomes in the absence of virus particles. As shown in this figure, E/M is pH independent over the range of pH values of 3.0–7.4, while at lower pH values a decrease in the pH of the medium is accompanied by a decrease of E/M. This decrease in E/M, in the absence of virions, was reversible, as readjusting the pH to levels higher than 3.5 yields increase of E/M to its original value. At all pH values, E/M was time independent at 37°C, at least for 1 h. At pH > 3.5, E/M was also independent of the presence of a 5-fold excess of unlabelled PS liposomes, indicating that acidification of the liposomal dispersion does not induce vesicle-vesicle fusion. In the presence of excess of virus particles ($D_L = 10.2$), E/M decreased with time and after 30 min of incubation of the mixture it reached an apparent equilibrium level which depended upon the pH of the incubation medium (Fig. 4B). This virus-induced decrease of E/M is expressed in Fig. 4C in terms of the dilution factor D_p as a function of pH. Noticeably, the dilution was maximal over the pH range of 3.0 to 4.0 and decayed to much lower values for higher or lower pH values. In addition, the rate of probe dilution was also maximal over the range of pH 3.0–4.0. Thus, while 3 min of incubation of PS liposomes (3 μM PS) with Sendai virus (27 μM viral lipids) at pH 4.0 resulted in a 3-fold decrease in E/M, similar incubation at pH 7.4 resulted in merely 10% decrease of E/M (Fig. 5). 2 h later, the E/M value in the latter mixture was still higher than a half of its original value, but acidification of the mixture resulted in a rapid decrease of E/M (Fig. 5) to a level similar to that obtained upon equilibration at pH 3.6.

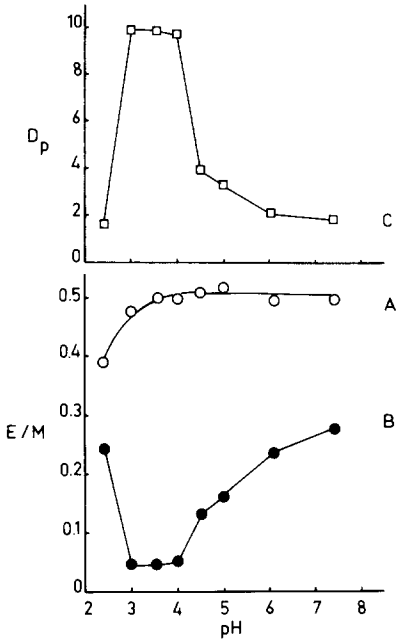

Fig. 4. Effect of pH on the E/M ratio of Pyr-PC (10 mol% in 3 μM PS liposomes) after 30 min of incubation at 37°C in the absence (A) and presence (B) of Sendai virus particles (27 μM viral lipid). The probe dilution factor D_p, obtained by dividing each point in A by its respective point in B, is presented in C as a function of the pH.

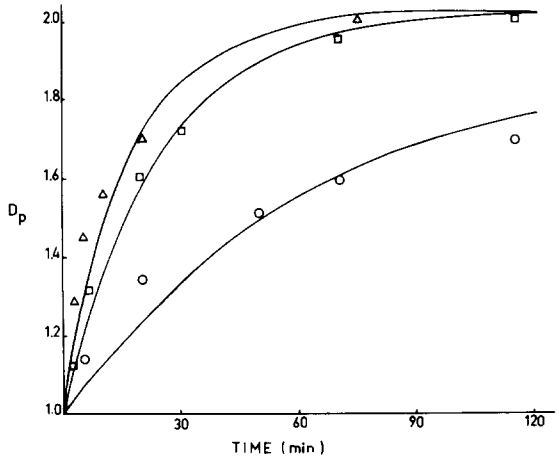

Fig. 3. Time dependence of the probe dilution factor, D_p, at 37°C and pH 7.4 for $D_L = 8.3$. Liposomal lipid concentrations were: 1.13 (○), 4.5 (□) and 9 μM (△). The curves were computed by the use of the procedure described in Ref. 3 with the parameters: $\alpha = 0.86$; $C = 2 \cdot 10^7\ M^{-1} \cdot s^{-1}$, $f = 0.002\ s^{-1}$, $D = 0.03\ s^{-1}$.

The acid-induced instantaneous activation of the virus is reversible. This conclusion is based on the finding that preincubation of the virus by itself at pH 3.6 did not result in virus activation when the pH was adjusted to pH 7.4 and liposomes were subsequently added (Table I). None-

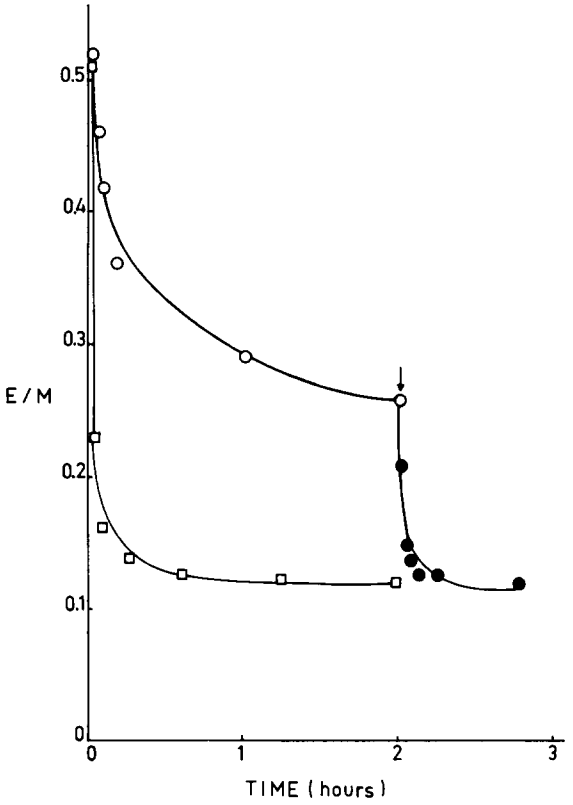

Fig. 5. Time-dependent decrease of E/M at neutral and acidic pH values. Sendai virus particles (27 μM viral lipid) were incubated at 37°C with PS liposomes (3 μM, labelled with 10% Pyr-PC), at pH 7.4 (○) and pH 4.0 (□) and the decrease with time of E/M was measured. After 2 h of incubation, the mixture incubated at pH 7.4 was acidified to pH 4.0 (see arrow) and the consequent decrease of E/M followed with time (●).

theless, a 1 h preincubation of the virions at pH 3.6 (but not at 7.4) resulted in a decrease of virus-induced probe dilution, both at pH 3.6 and 7.4. This inactivation of the virus may be due to irreversible denaturation of (a) viral protein(s) caused by the relatively long exposure to low pH. In any event, it may be concluded that the pH of the incubation medium plays an important role in determining the fraction of virus particles involved in diluting the probe contained in the PS liposomes.

Effect of temperature, lipid composition and calcium ions

As expected [13], the ratio of E/M in PS liposomes, in the absence of virus, is temperature

TABLE I

EFFECT OF PREINCUBATION OF SENDAI VIRIONS AT pH 3.6 AND 7.4 ON THEIR FUSION WITH PS LIPOSOMES

Sendai virus particles were incubated at pH 3.6 or 7.4 for 20 min, centrifuged at 100000 × g for 1 h and resuspended in Solution A. PS liposomes (3 μM) containing 10 mol% Pyr-PC were then incubated with the preincubated virions (27.5 μM viral lipid) for 30 min at pH 3.6 or 7.4. D_p is the probe dilution factor, as measured from the decrease of E/M after 30 min of incubation of the liposomes with the pretreated virus at pH 3.6 or 7.4.

Preincubation pH	D_p	
	pH = 3.6	pH = 7.4
3.6	4.3	1.14
7.4	10.2	1.82

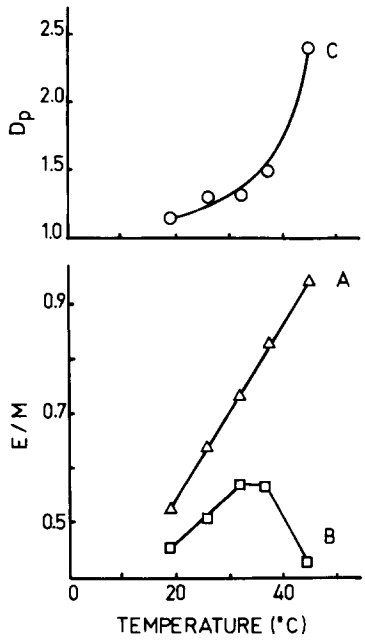

Fig. 6. Dependence of the E/M ratio of Pyr-PC (10 mol% in 10 μM PS liposomes) after 20 min of incubation at pH 7.4 in the absence (A) and presence (B) of Sendai virus (27 μM viral lipid) on the temperature of incubation. The probe dilution factor, D_p, obtained by dividing each point in A by its respective point in B, is presented in C as a function of the incubation temperature.

dependent (Fig. 6A). The E/M ratio obtained after 20 min of incubation of the liposomes with an excess of virions ($D_L = 3.8$) is presented in Fig. 6B. Evidently, the two temperature dependencies differ markedly. The ratio of E/M values after 20 min of incubation in the absence and presence of the virus is the probe dilution (D_p). This ratio is described in Fig. 6C as a function of temperature. Clearly, the probe dilution is much higher at 45°C than at 37°C. At temperatures lower than 37°C, the dilution is significantly reduced and at 20°C the dilution is very slight.

Negatively charged liposomes made of PG gave similar results to those obtained with PS liposomes both in terms of the rate and final level of dilution. As an example, 30 min incubation of virus particles with PG vesicles at 37°C and pH 7.4 at $D_L = 15$ resulted in $D_p = 1.75$, as compared to a $D_p = 1.54$ obtained with PS liposomes under the same conditions. In contrast, Pyr-PC contained in liposomes made of zwitterionic phospholipids was not diluted by the viral proteins, as evident from experiments carried out under the same conditions with PC ($D_p = 1.05$) and sphingomyelin ($D_p = 1.02$). Ca^{2+} did not affect the mixing of Pyr-PC (not shown) in spite of inhibiting the leakage of carboxyfluorescein [8].

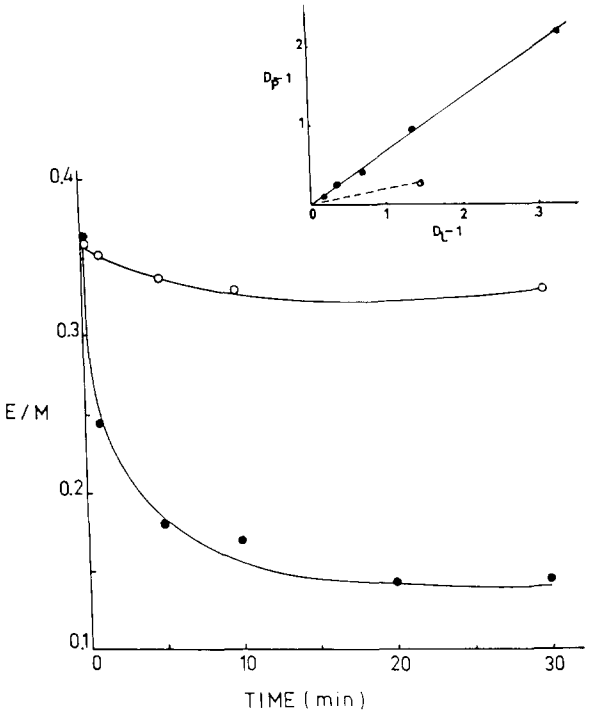

Fig. 7. Time-course of the E/M ratio of Pyr-PC in liposomes upon incubation with HN-glycoprotein reconstituted Sendai virus envelopes (HN-RSVE). PS (●) and PC (○) liposomes (5 μM, 10 mol% Pyr-PC) were incubated at 37°C and pH 4.0 with HN-RSVE (22 μg/ml viral protein) and the E/M ratio was measured with time. The inset presents the dependence of the probe dilution factor ($D_p - 1$) on the maximal lipid dilution factor ($D_L - 1$) for the Pyr-PC-containing PS liposomes, which were incubated at either pH 7.4 (○) or 4.0 (●) with varying concentrations of HN-RSVE. The ratio (D_p) between the initial E/M and the E/M value measured after 30 min is plotted as a function of the ratio ($D_L - 1$) between the viral and liposomal lipid concentrations.

Comparison between HN glycoprotein-reconstituted Sendai virus envelopes (HN-RSVE) and intact virions

Fig. 7 presents the time-dependent decrease of E/M observed upon mixing of Pyr-PC-containing PS liposomes ($L = 5.0$ μM) with reconstituted membranes containing 32.0 μM viral phospholipid and the glycoprotein HN (22.0 μg/ml) at pH 4.0. It is obvious from this figure that the fluorescent probe was diluted significantly only when it was included in PS liposomes (●), but not in PC liposomes (○), similar to the results with the intact virus. The dependence of ($D_p - 1$) on ($D_L - 1$) both at pH 7.0, and pH 4.0 (inset to Fig. 7) is also very similar to that obtained with the intact virus.

Discussion

This work assays in detail the mixing of viral and liposomal lipids by the use of the concentration-dependent excimer-forming probe Pyr-PC, under a variety of experimental conditions. The use of this technique for studies of the processes leading to mixing of lipids [9,10] is limited by the possibility that the linear dependence of E/M observed in PS liposomes may be different in the presence of other lipids (and proteins) in the membrane. Furthermore, as in all cases in which an external probe is used in monitoring a process of interest, the possible effect of the probe on the process has to be considered. In fact, the virus-induced release of carboxyfluorescein entrapped in PS liposomes was not affected by the presence of

10 mol% Pyr-PC in the liposomal membranes (not shown). This does not necessarily mean that further steps, that lead to mixing of lipids, are not altered. However, the close agreement of the experimental results with predictions based on the assumptions that the lipids of the virus particles behave as if they were merely a 'lipid diluting matrix' and that the probe reflects mixing of the lipids, justify these assumptions as a basis for approximations of the extent of mixing of virus and liposome membranous lipids.

Two distinctly different mechanisms could have led to the observed mixing of lipids. One possibility is that negatively charged vesicles can fuse with either intact virions or RSVE. This, of course, would lead to mixing of the probe by the lipid of those virus particles with which the liposomes fused. Alternatively, dilution of the probe could have resulted from exchange of lipids between membranes. In general, lipid exchange could either occur through the aqueous phase or else be mediated by collision of the liposomes with the virions. In our system, lipid exchange through the solution can not have a significant contribution to the overall probe dilution. This conclusion is based on the finding that over the time scale of our experiments, PC vesicles do not serve as an acceptor for the Pyr-PC contained in PS vesicles, neither in the absence (Fig. 1, ×) nor in the presence of virus particles (Fig. 1, ▲). Thus, not only that spontaneous lipid exchange through the aqueous medium can not account for the probe dilution, but that any virus-induced lipid exchange which might have occurred through the solution can also be ruled out. On the other hand, we cannot exclude the possibility that protein-induced lipid exchange occurs between vesicles and virions in liposome-virus aggregates and contributes to the probe dilution. In our previous work [8], we have shown that mixing of aqueous contents of reconstituted virus and liposomes accompanies the mixing of viral and liposomal lipids. Recently, we have also demonstrated by electron microscopy that the interaction between PS liposomes and HN-RSVE yields large virosomes [34]. While these results indicate that fusion occurs in the liposome-virus mixtures, we have insufficient evidence to attribute the entire lipid-mixing studied in this work to the accompanying fusion. (As a matter of fact, even the analysis of the rate of mixing of lipids through fusion would have been essentially unaltered if it were assumed that virus-induced contact-exchange of lipids is also involved in the observed probe dilution.) Nonetheless, we believe that the data available on the interaction of Sendai virus with negatively charged vesicles, when taken as a whole, can best be interpreted in terms of a virus-vesicle fusion mechanism. Therefore, we have chosen to analyze the lipid mixing studied in this work in terms of such fusion mechanism, under all experimental conditions.

Theoretically if any liposome could have fused with any number of virus particles or if virus particles could have fused amongst themselves, the final probe dilution D_p, observed when apparent equilibrium is approached, should have been equal to D_L (dotted line in Fig. 2). On the other hand, if any virus could have fused with any number of liposomes, one would have expected that $D_p = D_L$ only for low values of D_L. At higher D_L values, the number of virus particles is so large that some of those particles will have no liposomes to fuse with and consequently the dilution of the probe cannot involve all the viral lipid. Thus for high D_L values, D_p will be smaller than D_L. The exact characteristics of the dependence of D_p on D_L, for mixtures of equally active virus particles and a homogeneous population of PS liposomes, depend on the ratio (R) of sizes of the virus particles and liposomes (Eqn. 1). For vesicles whose radius equals one fifth of that of the virus, dilution of the probe would have been expected to follow the solid line in Fig. 2. For any other value of R within the range of 4–8, this solid line would have been only slightly different.

The dependence of the equilibrium value of $D_p - 1$ on $D_L - 1$ obtained experimentally at pH 3.6 (squares in Fig. 2) fits the dependence described by Eqn. 4 for $\alpha = 0$ (solid line in Fig. 2) very well. This supports the conclusion that at pH 3.6 all the virus particles are active. It also supports (indirectly) our estimate of the ratio of virus and liposome radii, R. We therefore suggest that at pH 7.4 only a fraction of the virus particles can fuse with the PS liposomes. The experimental points obtained at pH 7.4 fit very well the values calculated on the basis of the assumption that only 14% of the virus particles can fuse with PS

liposomes ($\alpha = 0.86$). The broken line in Fig. 2 was calculated on the basis of the assumption of irreversible binding of PS liposomes to the inactive virus particles (Eqn. 9). This possibility would mean that at equilibrium only 14% of the liposomes fused with the active virus particles, while 86% of the liposomes are bound to the latent virus particles with which they do not fuse. On the time scale of our experiments, deadhesion of liposomes from the virus surface may be very slow such that, apparently, only those liposomes which were bound to the surface of active virions fused with them. An alternative possibility is that deadhesion is relatively rapid, which implies that all the liposomes fused with the (14%) active virus particles. The dashed-dotted line in Fig. 2 presents calculations based on Eqn. 5, which formulates this assumption. If this possibility is valid then at equilibrium two populations co-exist. Those are 'virosomes' made of active virus particles which contain in their membranes all the PS of all the liposomes, and latent virions. As described above, the difference between D_p values given by Eqns. 5 and 9 is significant only at a high ratio between viral and liposomal lipid concentration, i.e., at large D_L values. Thus, at $D_L = 10$ the difference can be ignored and even when $D_L = 20$, the values predicted by Eqn. 9 are merely 10% smaller than those given by Eqn. 5. By inspection of the experimental points in Fig. 2 it is impossible to differentiate between the two theoretical curves.

Nonetheless, the possibility of an apparent irreversible binding seems more likely, since acidification of the equilibrated mixture resulted in a rapid but time-dependent decrease of E/M (Fig. 5) to a level similar to that predicted by the solid line in Fig. 2; thus, the liposomes bound to the latent virus particles after 120 min of incubation fuse with them when they are activated by acidification. Such pH-induced reduction of E/M could not have been expected if prior to acidification all the liposomes would have already fused with the active virus particles. The decrease of E/M to the value predicted with the assumption that dilution of the probe by the viral lipids is similar to its dilution in PS liposomes supports this latter assumption very strongly.

Another alternative to explain the relative limited lipid dilution at pH 7.4 is that virus particles which have fused with PS liposomes at this pH become inactive. This possibility can be ruled out on the basis of the reduction of E/M observed upon addition of unlabelled PS liposomes to an equilibrated mixture of virus and labelled liposomes (Fig. 1, ■). This conclusion is further supported by the experiment presented by the symbol ⊗ in Fig. 1. In this experiment, addition of labelled PS liposomes to an equilibrated virus-liposome mixture resulted in an initial increase in the overall E/M ratio due to the non-diluted probe. However, this was followed by a reduction of E/M to a value which could have been predicted for the new D_L value. The rate of this post-equilibration probe dilution process is of the same order of magnitude as the initial probe dilution. This, of course, indicates that those virus particles that have fused with PS liposomes are not very different in terms of their fusion capacity from virus particles that did not fuse with PS liposomes.

Thus, we conclude that the final level of dilution, in terms of $D_p - 1$ (derived from the initial value of E/M and the value of this parameter after 2–3 h of incubation) is best described by Eqn. 5. Theoretically, the final levels of $(D_p - 1)$ depend on the ratio between the concentrations of virus particles and liposomes (i.e. on $D_L - 1$) and are independent of the actual concentrations. In addition, $D_p - 1$ depends on the fraction of inactive virus particles (α), which can therefore be calculated for known values of D_L and D_p. In fact, for both PG and PS at pH 7.4 and 37°C, our calculations predicted the final D_p values fairly well for the whole range of D_L values from 3 to 20 and for a one order of magnitude variation in the absolute concentration, if and only if a value of $\alpha = 0.86$–0.90 was used. Hence, only 10–14% of the virus particles were capable of fusing with vesicles made of either of these two acidic phospholipids.

It is of interest to note that although only a minority of the liposomes fused with virus particles, all the carboxyfluorescein entrapped within all the liposomes leaked out of the liposomes under similar conditions [8]. Thus, it appears that aggregation of the liposomes with Sendai virus is sufficient to cause destabilization of the liposomal membrane to the extent that all the carboxyfluorescein can leak out of the liposomes. Thus,

trypsinization of the Sendai virus which inhibits the dilution of liposomal Pyr-PC, while avoiding the leakage of carboxyfluorescein, probably interferes with virus-liposome aggregation.

Inclusion of either cholesterol in the liposomes or $CaCl_2$ in the medium has no effect on the lipid dilution, in contrast to the reduction of carboxyfluorescein leakage from PS liposomes previously reported [8]. These findings further support our previous suggestion that neither cholesterol nor Ca^{2+} reduce the rate of virus-liposomes fusion and that their effect on the leakage of carboxyfluorescein is due to sealing of the liposomal membranes, thus making the overall liposome-virus interaction less leaky. In view of the results of the present work we conclude that this membrane sealing prevents the leakage of carboxyfluorescein caused by vesicle-virus aggregation.

Decreasing the pH from pH 7.0 to pH 4.0 results in activation of most of the virus particles (Figs. 2, 4 and 5). Protonation of the negatively charged carboxyl and/or phosphate groups of the PS liposomes occurs at pH values below 4.0 [24]. This protonation probably causes the decrease of E/M observed in the absence of virions at low pH (Fig. 4A). It may also be responsible for the decrease in fusion at pH values below 3.0, where the liposomes are not negatively charged. (In agreement with our previous work [8], liposomes made of zwitterionic phospholipids which have a larger hydration than those made of negatively charged lipids [32], do not fuse with the virus). However, protonation of the PS cannot explain the decrease of vesicle-virus fusion observed upon increasing the pH to values above 4.0 (Fig. 4C) as there is no evidence for any ionization step of the PS within the range of pH 4.0 to 7.4. We therefore suggest that the latter effect is caused by ionization of the viral glycoproteins, which is known to be broad and occurs within this range of pH values [23]. This conclusion accords with the finding that only protonated (positively charged) polyhistidine, but not the neutral form of this polypeptide, induces fusion of negatively charged vesicles [33].

As stated above, the experimental values of D_p at low pH are in good agreement (Fig. 2) with the values predicted by Eqn. 1 (i.e., Eqn. 9 with $\alpha = 0$). However, at pH 4.0 fusion is very extensive and consequently the final levels of E/M are very low. Measurements of the excimer fluorescence intensity under these conditions is therefore not very accurate. The measured intensity of the excimer may yield overestimated values of the actual excimer intensity, thus leading to an overestimation of E/M and an underestimation of D_p. Nonetheless, in all our experiments at acidic pH, the measured value of D_p yielded calculated values of $\alpha < 0.3$, which means that more than 70% of the virus particles are active. The strong dependencies of α on the pH and temperature most likely reflect conformational changes of viral glycoproteins but the details of these dependencies have yet to be investigated. Similar acid-induced activation of virus particles has been observed for influenza and vesicular stomatitis virions and explained in terms of pH-induced conformational changes [28,29].

Our theoretical analysis results in determination of three kinetic parameters, which characterize the overall fusion process at any given pH and temperature. Those are the rate constants of adhesion (C) fusion (f) and deadhesion (D). A fair simulation of all our kinetic results for the fusion of Sendai virus with either PS or PG liposomes at pH 7.4 and 37°C was found for $C = (0.5\text{–}2) \cdot 10^7\ M^{-1} \cdot s^{-1}$, $f = (0.2\text{–}2) \cdot 10^{-2}\ s^{-1}$ and $D = (1\text{–}5) \cdot 10^{-2}\ s^{-1}$. It is of interest to note that f values of 0.84 min^{-1} (0.014 s^{-1}; [35]) and 0.02–0.07 s^{-1} [36] were found for the fusion of Sendai virus with red blood cells or ghosts, respectively. These values are of the same order of magnitude found here. However, the exact determination of the appropriate set of parameters required many more kinetic measurements. Most serious, the values of the parameters C and f which give the best simulations are strongly dependent on that of D. The effect of deadhesion on the overall fusion process in our system at pH 7.4 is complex. As $\alpha > 0.85$, an increase in D decreases the calculated initial D_p values, while increasing the calculated D_p values at later stages. The latter effect can be explained by deadsorbtion of the vesicles initially bound to inactive virus particles. Subsequent adhesion and fusion of these vesicles to active virus particles results in a consequent increase of D_p, which explains the increase in the overall rate of fusion due to an increase in D. The most reliable determination of D was

obtained from experiments performed at a given D_L value but with different absolute concentrations. Calculations were capable of simulating the experimentally observed increase in D_p values with lipid concentrations only for D values in excess of 0.01 s^{-1}. This value of D is sufficiently high to explain the concentration dependence of the overall rate of fusion while still being low enough to be consistent with the post-equilibration acidification-induced increase of D_p. Despite the significant effect of deadhesion processes on the kinetics of aggregation and fusion, it is to be noted that a finite fraction of liposomes remain bound to inactive virus particles after an apparent equilibrium is approached.

The results previously obtained for the fusion of influenza virus with large cardiolipin vesicles [3,22] were qualitatively similar to those of this study. In the former case all the virus particles were active at pH 5.0 and at pH 6.0, α had a value of 0.5–0.75, while at neutral pH very little fusion was detected [22]. The value of f found for the fusion of influenza virus particles with cardiolipin vesicles at pH 6.0 was one order of magnitude above the f value found in our system at pH 4.0 and two orders of magnitude above the f value at neutral pH. Contradistinctively, the rate constant of adhesion, C, in our system is one order of magnitude below that of influenza virus fusing with cardiolipin vesicles and the value of D is similar to that found in the former case at pH 6.0. These differences reflect different interactions between the viral glycoproteins and target membrane phospholipids, but the details of these differences have yet to be studied.

In conclusion, this communication strongly supports our previous hypothesis that virus-induced leakage of carboxyfluorescein, entrapped in negatively charged liposomes accompanies a process of virus-liposome fusion. However, fusion is not always accompanied by leakage of entrapped solutes such as in the cases of calcium-containing media or cholesterol-containing liposomes. Furthermore, it is aggregation, not fusion, which causes the release of solutes entrapped in the liposomes: while only 15% of the Sendai virions can fuse with negatively charged liposomes at neutral pH, all the virions interact with liposomes, making their membranes leaky to entrapped solutes. Acidification of the incubation medium renders activity to the latent virions, which results in a complete mixing of viral and liposomal lipids. These findings support our previous assumption [8] that fusion of Sendai virus with negatively charged phospholipid vesicles is different from the fusion involved in viral infectivity. Maximal fusion of Sendai virus with biological membranes occurs at pH 7–8 with very little fusion at pH values below pH 6.0 [31]. Since maximal fusion with negatively charged vesicles occurs at pH 4.0, it is likely that the two fusion processes are quite different. Furthermore, proteoliposomes containing HN glycoprotein fuse with liposomes made of negatively charged phospholipids but not with biological membranes, which fuse with reconstituted virus envelopes only if they contain the F glycoprotein as well. For negatively charged, less hydrated liposomes the latter glycoprotein is not required.

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