Biochimica et Biophysica Acta, 1110 (1992) 185–192

BBAMEM 75761

Asymmetric fusion between synthetic di-n-dodecylphosphate vesicles and virus membranes

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(Received 14 April 1992)

Key words: Synthetic amphiphile; Vesicle; Sendai virus; Membrane fusion

The interaction between vesicles, prepared from the synthetic amphiphile di-n-dodecylphosphate (DDP), with Sendai virus membranes was investigated. DDP vesicles fuse in the presence of Ca^{2+} ('symmetric' fusion). However, in the absence of Ca^{2+}, DDP vesicles and Sendai virus, both displaying a high intrinsic fusion capacity with various target membranes, can also readily fuse with each other ('asymmetric' fusion). Under these conditions, fusion was found not to depend on specific viral proteins. Thus fusion occurs over a broad pH range (3.0–9.0) and is not affected by perturbation of viral protein structure. The overall interaction process was further analyzed with a mass action kinetic model. The analysis reveals that the destabilization and reorganization of the synthetic and viral bilayers are as fast as in pure phospholipid systems. Furthermore, the drastic effect of temperature on the overall reaction appears to be related to an effect of this parameter on fusion itself rather than on vesicle-virus aggregation. This could suggest that protein mobility constraints modulate the fusion reaction. The morphology of the fusion products, which consist of a single virus particle and several DDP vesicles, indicates a bilayer stabilization of the fusion product, rather than formation of tubular structures, as observed for symmetric DDP fusion products. The present results further emphasize the high susceptibility of vesicles composed of synthetic amphiphiles to engage in (protein-mediated) membrane fusion. This bears relevance to their potential application as carriers for biomolecules.

Introduction

The structural and physical properties of membranes affect many functional and dynamic processes that take place in and between membranes. Model membranes may be of particular help in revealing and understanding such properties. The extent to which such membrane models are amenable to chemical modifications or allow variation in composition, may govern the choice of a specific membrane model.

In recent work, we have employed vesicles composed of synthetic amphiphiles such as didodecylphosphate (DDP) to study fundamental aspects of the mechanism of membrane fusion. We have reported that DDP vesicles readily engage in Ca^{2+}-induced fusion, leading to the formation of large fused vesicles, which arise from tubular structures after addition of a Ca^{2+} chelating agent [1–4]. These studies have suggested the occurrence of a transition from a lamellar to a nonlamellar phase, when DDP vesicles fuse. Moreover, the mechanism proposed would be consistent with transitions (mediated by inverted micellar intermediates) reported to occur for defined phospholipid membranes as well [5].

More recently, we have shown that DDP vesicles can also participate in so called asymmetric fusion events with phospholipid vesicles and erythrocyte membranes, i.e., at conditions when symmetric fusion events cannot take place [6,7]. Fusion was triggered below the phase transition of the DDP-bilayers. The target membrane requires a fluid state, whereas Ca^{2+} was commonly essential although some fusion with target membranes was noted in the absence of the cation. Apparently, DDP bilayers possess considerable 'intrinsic' fusion capacity, likely resulting from a tendency to readily engage in nonbilayer structure formation.

These notions prompted us to investigate the susceptibility and structural consequences of DDP vesicles

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Abbreviations: DDP, di-n-dodecyl phosphate; PS, phosphatidylserine; PC, phosphatidylcholine; CL, cardiolipin; R_{18}, octadecylrhodamine B chloride; DTT, dithiothreitol; LUV, large unilamellar liposome.

toward protein-induced fusion, in particular in events involving their direct fusion with viral membranes. Enveloped viruses are known to enter cells for reproduction, via a viral spike protein-mediated membrane fusion process between virus membrane and a cellular target membrane [8].

The present work describes a study of the fusion between DDP vesicles and Sendai virus, a paramyxovirus that enters cells at neutral pH, mediated by a well-characterized spike protein, F. Taking into account the relatively high intrinsic fusion capacity of DDP vesicles, we investigated (i) whether DDP-virus fusion occurred, (ii) to what extent fusion was mediated by the viral protein and/or the amphiphilic bilayer per se, and (iii) the consequences of the fusion event with respect to the morphology of the fusion product, in light of a tubular appearance under symmetric conditions [2,3]. Known parameters commonly affecting fusion such as temperature, pH and cholesterol were also investigated.

Experimental procedures

Materials

Dialkyl phosphates were prepared as described [9]. The corresponding sodium salt was prepared by titrating alcoholic solutions of the free acid with a sodium hydroxide solution. Phosphatidylserine (PS from bovine brain) was purchased from Avanti Polar Lipids, Birmingham, AL. Cholesterol was acquired from Sigma, St. Louis, MO. Octadecylrhodamine B chloride (R_{18}) was obtained from Molecular Probes, Junction City, OR. Trypsin and trypsin inhibitor (soybean) were obtained from Boehringer (Mannheim, Germany), dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were products from Calbiochem-Behring, La Jolla, CA. Water was distilled twice in an all quartz apparatus.

Virus

The Z strain of Sendai virus was grown and purified as described elsewhere [10]. The viral membrane was labeled with R_{18} as previously reported [11]. Briefly, 10 μl of an R_{18} solution (2.2 mM in ethanol) was injected into a suspension of Sendai virus (1 mg/ml), under vigorous vortexing. After an incubation for 15 min (at room temperature) in the dark, noninserted probe was removed by chromatography on Sephadex G-75. Usually, a probe density of 2–3 mol% (with respect to total lipid) is obtained [11]. The amount of labeled virus was determined by protein measurement and the sample was stored on ice.

Vesicle preparation

PS large unilamellar vesicles (LUV) were prepared by reverse phase evaporation [10] in 5 mM NaAc, 5 mM Hepes, pH 7.4 (LS buffer) or 5 mM NaAc, 5 mM Hepes, 145 mM NaCl, pH 7.4 (HS buffer). The vesicles were sized to an average diameter of 0.1 μm by extrusion through Unipore polycarbonate filters (Bio-Rad). The lipid concentration of the resulting vesicle suspension was determined by a phosphorus determination [12].

Monitoring of membrane fusion by lipid mixing

Lipid mixing, as a measure of fusion was monitored with the R_{18} assay [11,13]. Sendai virions are labeled with R_{18} at surface densities that cause fluorescence self-quenching. Upon fusion with a non-labeled membrane relief of R_{18} self-quenching occurs, which causes an increase in fluorescence.

For calibration of the fluorescence scale, the initial residual fluorescence of the labeled virions was set to zero. 100% fluorescence was then determined after disruption of the bilayers with Triton X-100 (1%, v/v), corrected for sample dilution. Fluorescence was monitored continuously (exitation and emission wavelengths of 560 and 590 nm, respectively) on an SLM-Aminco SPF-500 C spectrophotometer, equiped with a thermostated cell holder, a magnetic stirring device and a Kipp chart recorder. Unless indicated otherwise, the measurements were performed at 37°C.

Typically 50 nmol of DDP vesicles were added to a suspension of labeled Sendai virions (5 μg protein/ml, or 1.4 μM with respect to phospholipid) in LS buffer, unless indicated otherwise. The final incubation volume was 2 ml. Initial rates of fusion were determined from the tangent at time zero of the fluorescence vs. time curve. The final extent of fusion was determined after 2 h. No further changes in fluorescence were seen between 2 and 24 h incubation at 37°C, indicating that the maximal degree of dilution was obtained after 2 h or earlier.

Kinetic analysis of virus-vesicle fusion

The procedure employed was based on a mass action kinetic model as described elsewhere [15]. The model can yield simulations and predictions of results of R_{18} fluorescence increase. The fusion process is viewed as a two step sequence: (1) second-order binding of the vesicle to the virus, followed by (2) a first-order fusion step, involving destabilization and merging of the membranes according to:

$$L_1 + V_1 \rightleftarrows A_{11} \quad (1)$$

$$A_{11} \rightarrow F_{11} \quad (2)$$

in which L denotes a DDP vesicle, V a virus particle, A the aggregated complex, and F the corresponding fusion product.

With this analysis, a possibility is provided to discriminate between the initial binding of virions to the

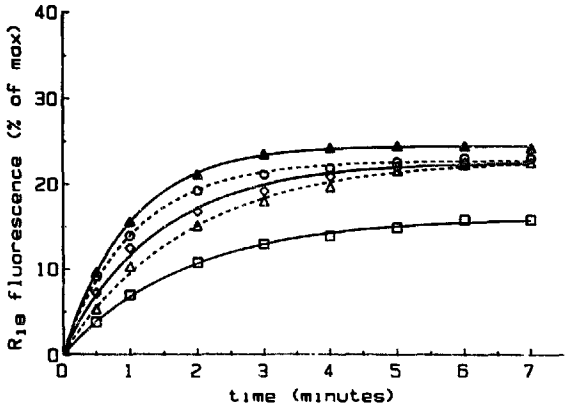

Fig. 1. Kinetics of lipid dilution occurring upon interaction of DDP vesicles with R_{18}-labeled Sendai virus (5 μg of protein/ml) at pH 7.4, as a function of DDP concentration. The experiments were as described in Experimental procedures and the incubation temperature was 37°C. DDP concentrations were: □, 6.25 μM; △, 12.5 μM; ◇, 25 μM; ○, 50 μM; ▲, 75 μM.

vesicles and the subsequent fusion reaction. Since the R_{18} fluorescence intensity varies linearly with the surface concentration of R_{18} molecules in the fusion product, the difference in size of the vesicle and the virion is explicitly taken into account. Furthermore, distinctions can be made between reversible or irreversible binding of the vesicles to the virus particles. The model can also shed light on the possible changes in adhesion (or fusion) rate, since the rate of adhesion (fusion) of vesicles to (with) virion/vesicle fusion products may differ.

The results of the model critically depend on the final extent of fusion, obtained from the R_{18} assay. From this value, the amount of active virions, at a given pH and temperature, is determined.

Calculations for lower ratios of DDP vesicles to viral particles (small excess of vesicles) tend to overestimate the experimental extents of fluorescence increase. This is due to a high degree of irreversible binding of the DDP vesicles to inactive virus. At large vesicle to virus ratios, this effect can be neglected: all active virions can fuse, because there are excess vesicles.

Electron microscopy

Electron micrographs were taken on a Philips EM400 operating at 80 kV. The samples, prepared on formvar/carbon grids exposed to pentylamine glow discharge, were negatively stained with an aqueous uranyl acetate solution (1%, v/v).

Other procedures

Sendai virus was trypsinized as described by Shimizo and Ishida [14]. The virus was incubated with trypsin (20 μg/100 μg viral protein) in 100 μl of buffer for 20 min at 37°C. The reaction was terminated by the addition of a 2-fold excess of soybean trypsin inhibitor and the mixture was kept on ice. Dithiothreitol (DTT) denaturation was performed by incubating the virus in buffer, pH 7.4, containing 5 mM of DTT in a final volume of 400 μl at 37°C. After 10 minutes, an appropriate volume of buffer was added, the mixture was transferred to a cuvette and fusion was started by injection of the DDP vesicles.

Results

Lipid mixing occurs during DDP–Sendai virus interaction

Fig. 1 shows that upon addition of various amounts of DDP vesicles to R_{18}-labeled virus particles, the fluorescence increases suggesting mixing of artificial and viral lipids. Both the initial rate and extent of probe dilution increase when the concentration of DDP vesicles increases (Fig. 2). Above 25 μM DDP, the increase in the initial rate becomes more gradual (Fig. 2) while the extent of lipid dilution tends to level off (Fig. 1). Thus in spite of a large excess of DDP surface area available for dilution, the final level of fluorescence obtained correlated with a virus fraction of approx. 25% of the total fraction that merges with the synthetic vesicles.

DDP-virus fusion, observed by electron microscopy

Lipid mixing, as described above, is consistent with the occurrence of membrane fusion between vesicle and viral membrane. To obtain further evidence, the interaction was also examined by electron microscopy. Fig. 3A shows DDP vesicles and distinct, electron dense virus particles at conditions (4°C) where fusion does not occur (see below). Incubation at 37°C shows, by contrast, spherical structures (3B and C) of greatly

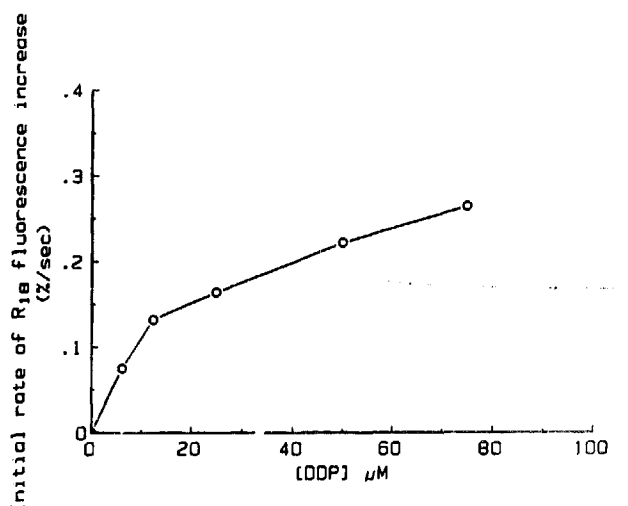

Fig. 2. Interaction of DDP vesicles with Sendai virus: initial rate of lipid dilution as a function of DDP concentration. Data were calculated from tangents drawn to curves such as in Fig. 1, at time zero. Conditions were as described in Fig. 1.

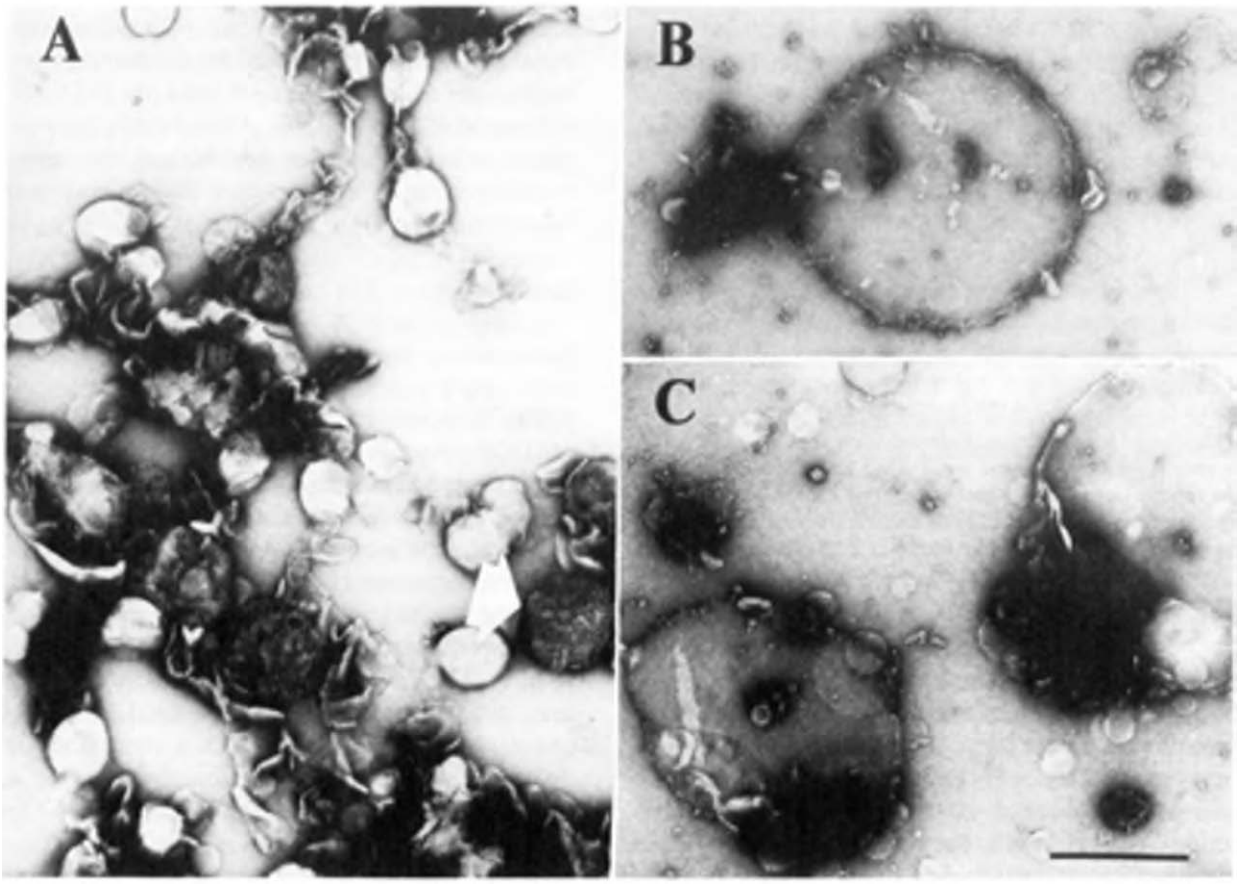

Fig. 3. Transmission electron microscopy of DDP vesicles interacting with Sendai virus. (A) 15 min, 4°C; (B and C) the sample as in A was incubated for 15 min at 37°C. Bar represents 500 nm. The arrow indicates a virus particle.

varying sizes, reflecting fusion products presumably consisting of a virus and different numbers of DDP vesicles (see below). Note that released nucleocapsid material is occasionally seen near large fused particles, suggesting some destabilization of the fusion products and/or leaky fusion. Interestingly, tubular structures, observed upon Ca^{2+}-induced fusion of DDP vesicles per se [2,3], were never seen.

Kinetic analysis of DDP-virus interaction

Approx. 25% of the total virus fraction was estimated (Fig. 1) to be involved in fusion with DDP vesicles. It should be noted that this fraction could be even less if the fusion product, consisting of a DDP vesicle and a virus, could participate in additional rounds of fusion. When adding 'fresh' virus to an incubation mixture, subsequent to the occurrence of DDP-virus fusion, no additional increase in fluorescence was seen. By contrast, a small but significant increase (approx. 5%) in the extent of fluorescence was seen when DDP vesicles were added (not shown), suggesting that fusion products may display a tendency to fuse further with vesicles, when available, but not viral membranes. Indeed kinetic analysis of the data with a mass action kinetic model (see Experimental procedures) allows a fairly accurate appreciation of the events that take place during the interaction of DDP vesicles with virus particles. As shown in Figs. 4 and 5,

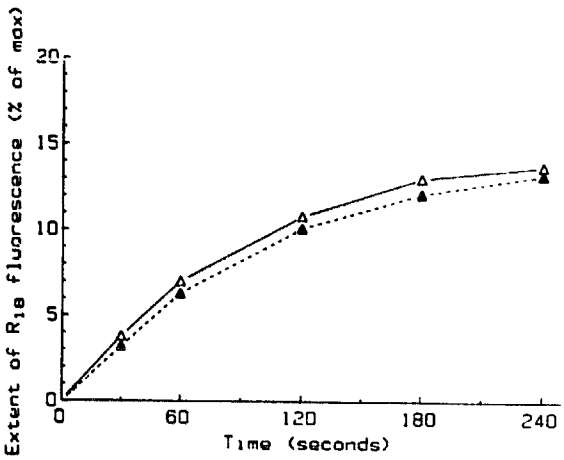

Fig. 4. Experimental values and simulation of the kinetics of lipid dilution during DDP–virus interaction. 5 μg virus was mixed with DDP vesicles (6.25 μM). The extent of lipid dilution was measured in a fluorimeter at pH 7.4 (37°C) after various time intervals (△). Alternatively, the kinetics of lipid dilution were simulated based on a mass action model as described, using $C = 4.5 \cdot 10^8\ M^{-1}\ s^{-1}$, $f = 0.12\ s^{-1}$ and $D = 0.04\ s^{-1}$ (▲).

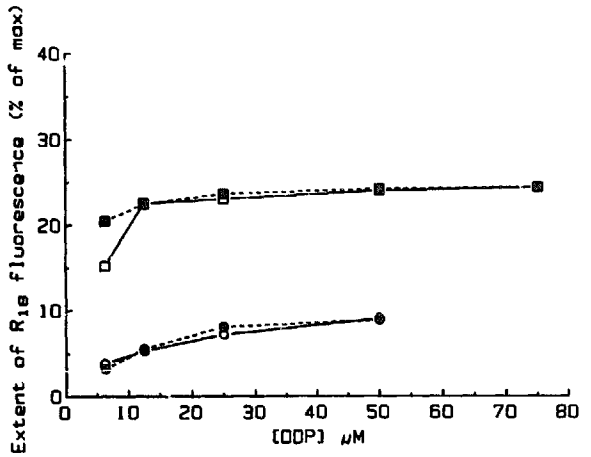

Fig. 5. Comparison of experimental and predicted values of the extents of lipid dilution occurring during DDP-virus interaction at a fixed Sendai virus concentration (5 μg/ml) and varying concentrations of DDP, as indicated. Open symbols represent experimental data obtained for the final extent of lipid dilution (□), and that obtained 30 s after initiating the incubation (○). The corresponding filled symbols represent the calculated data obtained by modelling, using the rate constants as given in Fig. 4.

very good correlations are obtained between experimental data and calculations, which are obtained when assuming that (i) the fusion products of DDP vesicles and viruses consist of any number of vesicles but only a single virus particle, and that (ii) DDP vesicles somehow prevent a fraction of the virions to fuse, involving an almost irreversible binding of these 'inactive' virus particles to the vesicles. Therefore, the conclusion is warranted that almost all vesicles are associated with virus particles, yet only a fraction fuses. With this modelling, it is also predicted that compared to fusion between an unfused DDP vesicle and a virus particle, DDP vesicles adhere to and fuse with fusion products with reduced rates. This implies that during early stages of the interaction process when excess DDP vesicles are available, fusion between DDP and (all) active virions will occur. This reaction will be followed by a slower fusion event, involving subsequent fusion of DDP vesicles with fusion products.

When the data shown in Figs. 3 and 4 are analysed in terms of active virus particles (Table I), it appears that fusion susceptibility towards DDP vesicles is comparable to that of phospholipid vesicles composed of phosphatidylserine (see Ref. 15). However, the rate constant of fusion for DDP vesicles is several fold larger than that observed for any other membrane system (cf. Refs. 15 and 16), consistent with the relatively high intrinsic fusion capacity, attributed to DDP vesicles (see Discussion). For example, at pH 7.4 the fusion rate constants for vesicles composed of cardiolipin and PS are 0.018 and 0.005 s^{-1}, respectively [15].

TABLE I

pH dependence of fusion activity; rate constants of fusion (f), aggregation (C) and dissociation (D) for fusion of Sendai virus with DDP vesicles at 37°C

Each case in this table is a summary of a comparison between calculated and experimental levels of final fluorescence for at least four ratios of DDP vesicles to viruses (cf. Fig. 1). For details see Experimental procedures and Ref. 15.

pH	% active virus	f (s^{-1})	C ($M^{-1} s^{-1}$)	D (s^{-1})
5	35	0.5	$5.0 \cdot 10^8$	0.04
7.4	24	0.1	$5.0 \cdot 10^8$	0.04

Temperature dependence

As shown in Fig. 6 significant dilution of lipid does not occur below 20°C. Above 20°C, both the rate and extent of lipid mixing increase. Interestingly, although the extent of lipid dilution increases rapidly above 20°C and levels off around 30°C, the rate increases fairly gradually in the range of 20 to 30°C. In this context it is relevant to note here that the gel-liquid crystalline transition temperature of the DDP vesicles is centered around 29°C [1]. Also, a pretransition for DDP bilayers at a temperature of about 20°C occurs. However, a threshold temperature of about 20°C for lipid mixing has also been noted with erythrocyte membranes as target membranes for Sendai virus [10,17]. With acidic phospholipid vesicles the threshold temperature for virus fusion is much less defined [10]. Kinetic analysis of the overall fusion reaction, based on the mass action model, indicates (Table II) that the virions become more prone to fusion with DDP vesicles when the temperature increases. Between 32 and 37°C, the percentage of active virus increases about 1.5-fold. Intriguingly, the simulation indicates that the steep increase in overall fusion seen above 30°C can be attributed

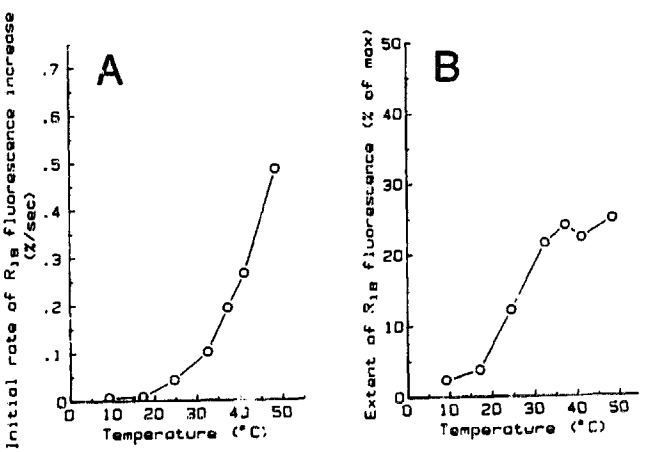

Fig. 6. Temperature dependence of DDP- Sendai virus interaction. (A) Initial rate fluorescence increase, (B) final extent of fluorescence. Rate end extent were determined as described. Virus concentration was 5 μg/ml; DDP concentration is 25 μM; pH = 7.4.

TABLE II

Rate constants for Sendai virus fusion activity with DDP vesicles as a function of temperature (pH = 7.4)

See legend to Table I for details.

Temperature (°C)	% active virus	f (s^{-1})	C ($M^{-1} s^{-1}$)	D (s^{-1})
25	15	0.07	$2 \cdot 10^8$	0.015
32	15	0.08	$4 \cdot 10^8$	0.02
37	24	0.1	$5 \cdot 10^8$	0.04
45	35	0.35	$6 \cdot 10^8$	0.35

specifically to an increase in the fusion rate constant which increases by an order of magnitude. Hence temperature affects parameters that govern the fusion reaction itself rather than factors, involved in binding of vesicles to virions.

Effect of pH

Acidic pH stimulates fusion between DDP vesicles and virus particles, as shown in Fig. 7. Both the rate and extent of lipid mixing, as a measure of fusion, increase. Relative to phospholipid vesicles composed of phosphatidylserine, DDP vesicles fuse more rapidly with the virus particles, although the extent of lipid dilution reached is quite comparable for both membrane systems. With half of the DDP substituted for cholesterol, an initial rate of lipid mixing was obtained, similar to that of PS vesicles. Yet, the extent of merging obtained with PS vesicles clearly exceeded the extent that was obtained with the cholesterol-containing DDP vesicles.

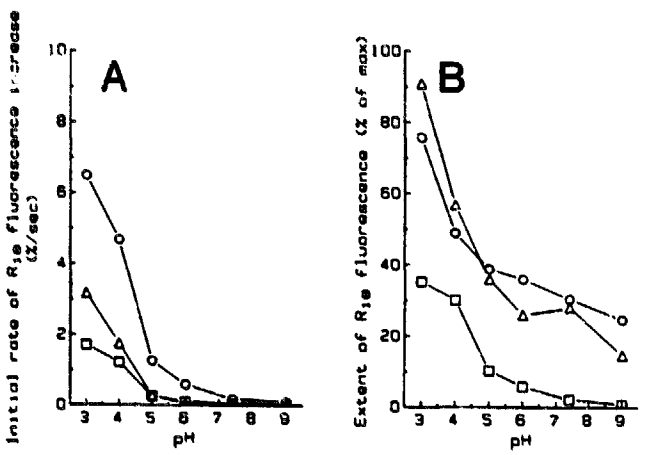

Fig. 7. Effect of pH and vesicle composition on virus fusion. Initial rates and final extent were determined as described. The incubation temperature was 37°C. The system was adjusted to the appropriate pH in a buffer consisting of 5 mM Na-acetate/5 mM Hepes/145 mM NaCl. Vesicles were as follows: ○, DDP; △, PS and □, DDP/cholesterol, 1:1. The virus concentration was 5 μg/ml; total lipid concentration was 25 μM.

Role of viral proteins in DDP-virus fusion

DDP displays a high intrinsic membrane fusion capacity. It was therefore of interest to examine how critical the viral proteins, at physiological conditions triggering the entry process into cells, were involved in fusion with DDP vesicles. Removal of the F protein by trypsinization reduced the initial rate by only 5% or less; treatment with the reducing agent dithiotreitol resulted in a similar, i.e., only marginal inhibition of fusion. The results thus indicate that the F protein as such is not critically involved while, by the same token, also protein conformational aspects are not essential for the ability of DDP vesicles to fuse with virus particles. Furthermore, when the temperature of the incubation was raised above 40°C, the rate further increased (Fig. 6A). This contrasts with analogous experiments carried out with erythrocyte membranes [18], showing that fusion decreases at such conditions, presumably due to protein denaturation.

Discussion

The results described in this work emphasize the remarkably high intrinsic fusion capacity of the synthetic amphiphile, didodecyl phosphate. Although to variable extents, bilayers prepared from the amphiphile readily merge with target membranes, irrespective of the intrinsic fusion capacity of the latter (viral membranes, this work, vs. liposomes and erythrocytes, Refs. 6 and 7). The high susceptibility to engage in intermembrane interactions may also bear relevance to the interest currently existing in the application of synthetic amphiphiles as carriers for nucleotides [19–24]. As far as the mechanism of fusion is concerned, it appears that for DDP vesicles just a protein suffices. The independence of the merging reaction of viral protein specificity is reflected by several observations. Fusion between DDP vesicles and the viral membranes occurs after trypsinization or DDT treatment. It also takes place at elevated temperatures or acidic pH. At all the various conditions, fusion of the virus with biological membranes is usually inhibited or substantially reduced (for references see Ref. 8). Even with plain liposomes, consisting of PS or CL, a more pronounced, specific protein dependence of liposome-virus fusion is observed than in the case of DDP vesicles [10]. Nevertheless, the present work suggests that (viral) proteins do play a role as fusion between DDP vesicles and the virus occurs very readily in the absence of Ca^{2+}. Interestingly, fusion of DDP vesicles with erythrocyte membranes, although proceeding slowly, can similarly occur in the absence of Ca^{2+} and seems protein dependent, although the process is facilitated in the presence of divalent cations [6]. By contrast, fusion of DDP vesicles with phospholipid vesicles requires divalent cations [7]. However, consistent with

the fusion event as seen with erythrocytes, the specificity of the conformation of the viral protein(s) is, apparently, not critical for DDP membrane merging to occur. In this context it is interesting to note that the kinetic analysis reveals that the fusion rate constant at mild acidic pH is of the same order of magnitude as that for Ca^{2+}-induced fusion of PS and CL/PC vesicles [25]. This indicates that the (required) destabilization and rearrangement of the fusing DDP and Sendai virus bilayers is as fast as those occurring in pure liposomal systems. It should also be noted in this respect that the overall bilayer structure of the fusion product is stabilized (Fig. 3). Previously, we observed that Ca^{2+}-induced fusion of DDP bilayers results in a subsequent conversion to products of a tubular nature that no longer maintain a bilayer structure [2,3].

As a function of temperature, a threshold in fusion activity was noted. Substantial lipid mixing occurred above 20°C and becomes quite distinct when the temperature further increases to 30°C, which coincides with the gel/liquid crystalline phase transition temperature of the DDP vesicles. Hence, analogous to previous observations of DDP vesicle fusion with liposomes and erythrocytes [7], DDP vesicle bilayers in the gel-like phase can also fuse with a viral membrane. Under these conditions, the fusion event is asymmetric, i.e., fusion between alike membranes (DDP-DDP, Refs. 1 and 26) does not take place. Careful analysis of the temperature dependence of DDP-viral membrane fusion revealed that in the overall merging reaction, temperature primarily affected the fusion step itself and not intermembrane adhesion (Table II). The prominent increase in fusion itself is further emphasized by the notion that as a function of temperature the dissociation constant increases by an order of magnitude. The mechanism underlying this dramatic increase is unclear. However, it should be noted that in the temperature range of interest, detectable physical changes in DDP bilayers have not been observed. Whether the facilitated merging conditions therefore reflect alterations in protein mobility, a parameter known to modulate protein-induced membrane fusion (see Ref. 8 and references therein), remains to be determined. Inclusion of cholesterol in DDP bilayers substantially reduces the fusion susceptibility. This may suggest that protein mobility constraints, presumably in conjunction with restricted protein-induced perturbation of cholesterol-containing bilayers, are relevant to the initiation of fusion. This effect of cholesterol also fits with the overall picture of a nonspecific protein-induced fusion event in the case of DDP vesicles, since the physiologically relevant fusion reaction of Sendai virus itself, has been suggested to depend on the presence of cholesterol [27–29]. Thus the non-specificity, the intrinsically high potency to merge and the very low electrostatic and hydration barrier sensed by DDP bilayers [1,2,4], as reflected by the relatively high rate constant of aggregation, appear important parameters that strongly facilitate intimate interactions of DDP with apposed non-alike bilayers. These notions will be of further relevance to develop synthetic amphiphiles into efficient systems that may serve as biomolecular carriers.

Acknowledgements

We appreciate the skillful secretarial assistance of Lies van der Wal and Rinske Kuperus. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Dutch Foundation for Scientific Research (NWO). In addition, S.N. was supported by a Visitor's grant from NWO. Further support was provided by Schonbrunn Funds from the Hebrew University of Jerusalem and by a National Institutes of Health Grant (AI-25534).

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