Osmotic Swelling Allows Fusion of Sendai Virions with Membranes of Desialized Erythrocytes and Chromaffin Granules†

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ABSTRACT: Sendai virus particles are able to fuse with Pronase-neuraminidase-treated human erythrocyte membranes as well as with vesicles obtained from chromaffin granules of bovine medulla. Fusion is inferred either from electron microscopic studies or from the observation that incubation of fluorescently labeled (bearing octadecyl Rhodamine B chloride) virions, with right-side-out erythrocyte vesicles (ROV) or with chromaffin granule membrane vesicles (CGMV), resulted in fluorescence dequenching. Fusion of Sendai virions with virus receptor depleted ROV was observed only under hypotonic conditions. Fusion with virus receptor depleted ROV required the presence of the two viral envelope glycoproteins, namely, the HN and F polypeptides. A 3-fold increase in the degree of fluorescence dequenching (virus-membrane fusion) was also obtained upon incubation of Sendai virions with CGMV in medium of low osmotic strength. This increase was not observed with inactivated, unfusogenic Sendai virions. The results of the present work demonstrate that, under hypotonic conditions, fusion between Sendai virions and biological membranes does not require the presence of specific receptors. Such fusion is characterized by the same features as fusion with and infection by Sendai virions of living cultured cells.

Infection of animal cells by enveloped virions is mediated by a virus-membrane fusion step, occurring either with the plasma membrane or with membranes of intracellular organelles such as those of the endosomes (White et al., 1983). Virus-membrane fusion is absolutely dependent on the presence of specific membrane components which can serve as virus receptors (Choppin & Scheid, 1980).

Enveloped virions have been shown also to fuse with phospholipid vesicles (White et al., 1983; Citovsky & Loyter, 1985; Kawasaki et al., 1983). Recently, using fluorescent dequenching methods, we have observed that Sendai virions can fuse with liposomes composed of neutral lipids and lacking any virus receptors [Citovsky & Loyter, 1985; Citovsky et al., 1985; also see Hsu et al. (1983)]. Such virus-liposome fusion occurred only if cholesterol molecules were present within the liposome bilayer. Similar observations have been reported for other enveloped viruses such as Semliki Forest virus (White & Helenius, 1980). These results may indicate that no specific receptors are needed for fusion between enveloped virions and membranes possessing exposed lipids. However, since in biological membranes the lipid bilayer is unsusceptible to interaction with external ligands (Laster et al., 1972; Gazitt et al., 1976), it is probably also available to viral envelope glycoproteins. Indeed, it has been suggested before (Citovsky & Loyter, 1985; Citovsky et al., 1986b) that binding of Sendai virions to their membrane receptors, namely, sialoglycolipids, promotes unmasking of the membrane lipid bilayer and consequently allows its interaction with the viral envelope glycoproteins. Support for this view was obtained from recent experiments showing that reconstituted Sendai virus envelopes (RSVE)[1] are able to fuse with human erythrocyte membranes which were depleted of their virus receptors by treatment with Pronase and neuraminidase. Such fusion occurred only if the membrane phospholipids were exposed to the viral glycoproteins by osmotic stress (Citovsky & Loyter, 1985).

These observations led to the hypothesis (Loyter & Citovsky, 1987) that animal enveloped virus particles, such as Sendai virions, will fuse with any biological membrane under the following conditions: (i) cholesterol molecules should be present within the recipient membrane, and (ii) membrane lipids should be exposed and susceptible to interaction with the viral glycoproteins. In order to substantiate this hypothesis further, we have studied in the present work the interaction between intact Sendai virions and virus receptor depleted erythrocyte membranes or membranes of intracellular organelles, i.e., chromaffin granules. Receptors for Sendai virus have been removed from the erythrocyte membranes by treatment with neuraminidase and Pronase. It has been well established (Laster et al., 1979; Wolf et al., 1980; Nussbaum et al., 1984) that Sendai virions neither bind to nor fuse with such treated erythrocytes. Intracellular organelles can also serve as a convenient model system for biological membranes which naturally never encounter or fuse with enveloped animal virions. Using the fluorescence dequenching method and electron microscopy techniques, we have shown in this study that intact Sendai virions are indeed able to fuse with both membrane preparations. A relatively high degree of virus-membrane fusion was observed only under hypotonic conditions.

Experimental Procedures

Chemicals. Phosphatidylcholine (PC, type V-E from egg yolk), cholesterol (Chol), phenylmethanesulfonyl fluoride (PMSF), trypsin, Pronase, and dithiothreitol (DTT) were all

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[1] Abbreviations: CF-6, carboxyfluorescein; CGMV, chromaffin granule membrane vesicle(s); $CGMV_{GA}$, glutaraldehyde-treated CGMV; Chol, cholesterol; DTT, dithiothreitol; LUV, large unilamellar vesicle(s); PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; R_{18}, octadecyl Rhodamine B chloride; ROV, right-side-out vesicle(s); ROV_{GA}, glutaraldehyde-treated ROV; RNP, ribonucleoprotein; RSVE, reconstituted Sendai virus envelope(s); SUV, small unilamellar vesicle(s); Tris, tris(hydroxymethyl)aminomethane.

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purchased from Sigma (USA). Octadecyl Rhodamine B chloride (R_{18}) was obtained from Molecular Probes (USA). Neuraminidase (*Vibrio cholerae*, 1 unit/mL) was purchased from Boehringwerke (FRG), and 6-carboxyfluorescein (CF-6) was from Eastman Kodak (USA).

Virus. Sendai virus was isolated from the allantoic fluid of fertilized chicken eggs, and its hemagglutinating units and hemolytic activity were determined as previously described (Peretz et al., 1974). Reconstituted Sendai virus envelopes (RSVE) or enveloped vesicles bearing the viral hemagglutinin/neuraminidase (HN) and/or fusion (F) glycoproteins were obtained, and their purity was verified as described before (Chejanovsky et al., 1986).

Cells. Human blood, type O, was freshly drawn from a healthy donor. The blood was washed 5 times with solution Na (160 mM NaCl/7 mM Tris-HCl, pH 7.2) and finally suspended in the same buffer, to give 2–3% (v/v).

Preparation of Right-Side-Out Vesicles (ROV) from Human Erythrocyte Membranes. Sealed ROV were prepared from freshly drawn human blood (type O, RH^-) and purified as described (Steck & Kant, 1974; Citovsky & Loyter, 1985), with the following modifications: After the incubation period at low ionic strength, carboxyfluorescein (CF) (80 mM in 120 mM NaCl final concentration), sucrose (330 mM final concentration), or NaCl (160 mM final concentration) was added to obtain ROV loaded with CF or with isoosmotic concentrations of sucrose or salt, respectively (Citovsky & Loyter, 1985). The suspension was incubated for an additional 10 min at 4 °C. All subsequent steps such as addition of $MgSO_4$ (0.1 mM), centrifugation (10000*g* for 30 min at 4 °C), and passage through a G27 needle were performed as previously described (Steck & Kant, 1974; Citovsky & Loyter, 1985), except that CF (80 mM in 120 mM NaCl), sucrose (330 mM), or NaCl (160 mM) was present throughout the entire procedure.

Pronase- and/or neuraminidase-treated ROV were obtained from erythrocyte membranes treated with Pronase (0.5 mg/mL, 30 min at 37 °C) and/or neuraminidase [30 milliunits for 1 mL of 40%, (v/v) blood, 1 h at 37 °C], respectively (Laster et al., 1979; Lalazar et al., 1977).

Chromaffin Granule Membrane Vesicles (CGMV). Chromaffin granules were isolated from bovine adrenal glands, essentially as described by Kirshner (1962). Membrane vesicles were obtained by osmotic shock, sealed by centrifugation (10000*g*, 30 min at 4 °C) in solution K (300 mM KCl/8 mM Tris-HCl, pH 7.2) (Schuldiner et al., 1978), and used immediately.

Anti-Sendai Virus Antibodies. Anti-Sendai virus antibodies were raised in rabbits, as previously described (Volsky et al., 1979). The IgG fraction of the immune serum was purified by ammonium sulfate precipitation, and the antibody titer was determined by its ability to inhibit viral hemagglutinating and hemolytic activities, as described before (Volsky et al., 1979).

Preparation of Fluorescent Sendai Virions or Fluorescent Reconstituted Viral Envelopes. Sendai virions, RSVE, or enveloped vesicles containing the HN and/or F glycoproteins were labeled with R_{18} as described earlier (Hoekstra et al., 1984; Citovsky et al., 1985). Briefly, 7 μL of a 6 mg/mL ethanolic solution of R_{18} was rapidly injected into 700 μL of solution Na, containing 1.7 mg of viral proteins. After incubation for 15 min at room temperature in the dark, the various preparations were washed in 20 volumes of the appropriate buffer (100000*g*, 30 min at 4 °C) and resuspended in the same buffer to give a final protein concentration of 0.2 mg/mL. Under such conditions, R_{18} surface density in viral membranes was 3 mol % of total phospholipids, and its decrease was shown to be proportional to the fluorescence dequenching (Hoekstra et al., 1984; Citovsky et al., 1985).

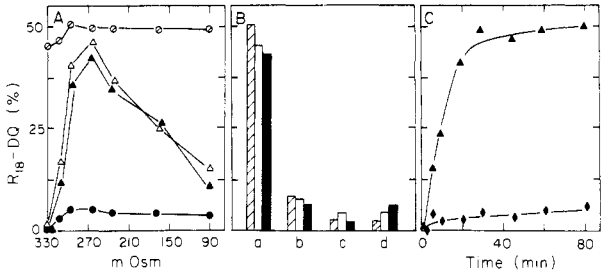

FIGURE 1: Interaction of Sendai virions with virus receptor depleted ROV. (A) Effect of osmolarity: R_{18}-labeled Sendai virions (2 μg) were incubated for 10 min at room temperature in solution Na or sucrose (330 mM) with 100 μg of ROV preparations, containing NaCl or sucrose, respectively (see Experimental Procedures). Then the osmolarity of the incubation medium was adjusted to the indicated values by addition of double-distilled water. The reaction mixture (0.4 mL final volume) was incubated for 45 min at 37 °C, after which the degree of fluorescence dequenching (R_{18}-DQ) was estimated as described under Experimental Procedures. (∅) Intact ROV; (●) neuraminidase-treated ROV; (△) neuraminidase–Pronase-treated ROV (all in solution Na); (▲) neuraminidase–Pronase-treated ROV in sucrose (330 mM). (B) Effect of inhibitors of the viral fusogenic activity: Sendai virions were rendered unfusogenic by incubation of 30 min at 37 °C with either trypsin (60 μg/mg of viral protein) (Asano et al., 1983), 3 mM DTT (Ozawa et al., 1979), or 7 mM PMSF (Israel et al., 1983) and then washed in 20 volumes of solution Na (100000*g*, 30 min at 4 °C). R_{18}-labeled intact (a), trypsinized (b), DTT-treated (c), or PMSF-treated (d) Sendai virions (2 μg) were incubated at 270 mOsm with 100 μg of various ROV preparations, exactly as described in (A). At the end of the incubation period, the degree of fluorescence dequenching was determined as described under Experimental Procedures. (⊠) Intact ROV; (□) neuraminidase–Pronase-treated ROV (all in solution Na); (■) neuraminidase–Pronase-treated ROV in sucrose (330 mM). (C) Kinetic studies: R_{18}-labeled Sendai virions (2 μg) were incubated for the indicated periods of time with 100 μg of neuraminidase–Pronase-treated ROV in sucrose, either at 270 mOsm (▲) or at 330 mOsm (♦). All other experimental conditions were as described in (A).

Fluorescence Measurements. Fluorescent Sendai virions or reconstituted viral envelopes were incubated with recipient membrane vesicles, in a final volume of 0.4 mL, under conditions as indicated for each experiment. After incubation, the degree of fluorescence was estimated (excitation at 560 nm and emission at 590 nm). The fluorescence degree in the presence of 0.1% of Triton X-100 was considered to represent an infinite dilution of R_{18} molecules, i.e., 100% dequenching. All fluorescence measurements were carried out with a Perkin-Elmer MPF-4 spectrofluorometer, with narrow excitation slits to reduce light scattering.

Protein and Lipid Determinations. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. Lipid concentrations were estimated by the method of Stewart (1980), using phosphatidylcholine (PC) as a standard.

Results

Fusion of Intact Sendai Virions with Virus Receptor Depleted Human Erythrocyte Membranes. The results in Figure 1 confirm and further extend our previous observations (Citovsky & Loyter, 1985) showing that incubation of fluorescently (R_{18}) labeled Sendai virions with ROV of erythrocyte membranes results in fluorescence dequenching. On the other hand, no increase in fluorescence (fluorescence dequenching) was observed when inactive, unfusogenic, namely, DTT-, PMSF-, and trypsin-treated, virions (Citovsky & Loyter, 1985) were incubated with ROV, thus supporting the view that the fluorescence dequenching observed indeed reflects a process of virus–membrane fusion (Figure 1B). This may also be

Table I: Interaction of Sendai Virions and Reconstituted Viral Envelopes with Virus Receptor Depleted ROV: Requirement for the Two Viral Envelope Glycoproteins

		R_{18}-DQ (%)		binding of Sendai virions (% of added)		virus-induced agglutination	
expt	system	330 mOsm	270 mOsm	330 mOsm	270 mOsm	330 mOsm	270 mOsm
1[a]	Sendai virions + ROV	50	55	64	63	+	+
	Sendai virions + ROV_{GA}	5	6	ND[c]	ND	ND	ND
	Sendai virions + neuraminidase–Pronase-treated ROV	4	46	5	5	-	-
	Sendai virions + neuraminidase–Pronase-treated ROV_{GA}	4	6	ND	ND	ND	ND
2[b]	RSVE	0	2				
	F vesicles	2	8				
	HN vesicles	2	4				
	F–HN vesicles	4	44				

[a] Experiment 1: Intact and neuraminidase–Pronase-treated ROV (600 μg) were incubated for 20 min at 37 °C with 0.1% glutaraldehyde, in final volumes of 0.2 mL of solution Na (Maeda et al., 1977a; Chejanovsky & Loyter, 1985). After incubation, the glutaraldehyde-treated ROV (ROV_{GA}) were washed 3 times with solution Na. For induction of fluorescence dequenching (R_{18}-DQ), R_{18}-labeled Sendai virions (2 μg) were incubated with the various ROV preparations (100 μg) in solution Na, at either 330 or 270 mOsm. All other experimental conditions were as described in Figure 1. Binding of Sendai virions to ROV was determined by the use of fluorescently labeled Sendai virions. Mixtures of R_{18}-labeled virions (5 μg) and ROV (100 μg) were incubated at room temperature, as described in Figure 1. Following adjustment of the osmolarity of the medium by addition of distilled water, the suspension was further incubated for 45 min at 4 °C. At the end of the incubation period, unbound virus was separated from bound virus by centrifugation through 0.3 M sucrose cushions, as described before (Chejanovsky et al., 1984). The amount of virus associated with ROV was estimated by determination of the fluorescence intensity of the pellets. Virus-induced agglutination of ROV was followed by phase microscopy observations (Peretz et al., 1974). [b] Experiment 2: Reconstituted Sendai virus envelopes (RSVE) or enveloped vesicles bearing the purified viral F (F vesicles), HN (HN vesicles), or both F and HN (F–HN vesicles, 1:1 w/w) glycoproteins were prepared and labeled with R_{18}, as described under Experimental Procedures and before (Chejanovsky et al., 1986). Fluorescent viral envelopes or enveloped vesicles (2 μg) were incubated with neuraminidase–Pronase-treated ROV (100 μg) under the same conditions as described in experiment 1 for intact virions. [c] ND, not determined.

inferred from the results summarized in Table I, experiment 1, which shows that an increase in the degree of fluorescence dequenching was observed upon incubation of R_{18}-labeled Sendai virions with untreated, but not with 0.1% glutaraldehyde-treated, ROV. It has been shown previously that such glutaraldehyde-treated erythrocyte membranes allow lipid–lipid exchange processes but are resistant to virus–membrane fusion (Maeda et al., 1977a).

No increase in the degree of fluorescence was obtained upon incubation of untreated fusogenic virions and ROV from which the virus receptors were removed by treatment with neuraminidase (Figure 1A). However, treatment of ROV with both neuraminidase and Pronase did render the ROV susceptible to fusion with Sendai virions, although this occurred only under hypotonic conditions (Figure 1A and Table I, experiment 1). As can be seen (Figure 1A), a gradual increase in the degree of fluorescence was obtained when the incubation was performed in a medium composed of decreasing amounts of salt. Maximum fluorescence dequenching was observed in a medium of about 270 mOsm, while in a medium of lower osmolarity a decrease in the degree of fluorescence was noted. This may be due to a considerable disruption of the erythrocyte vesicles in media of such low osmolarity (see also Figure 2). The results in Figure 1B also show that no significant increase in the degree of fluorescence was observed under hypotonic conditions, following incubation of unfusogenic virions, namely, DTT-, PMSF-, and trypsin-treated virions, with the neuraminidase–Pronase-treated ROV. Similarly, no increase in the degree of fluorescence dequenching was observed upon incubation of Sendai virions with glutaraldehyde–neuraminidase–Pronase-treated ROV (Table I, experiment 1), again emphasizing the view that the fluorescence dequenching observed is due to virus–membrane fusion. The results in Table I, experiment 1, also show that Sendai, virions neither attach to nor agglutinate neuraminidase–Pronase-treated ROV, as opposed to their ability to bind to and agglutinate untreated ROV.

From the results in Figure 1, it should also be inferred that the phenomenon observed is not due to a decrease in ionic strength. The same degree of fluorescence dequenching was obtained when sucrose was used instead of NaCl during the preparation of ROV as well as to maintain the osmolarity of the incubation medium. Maximum fluorescence dequenching was observed in medium adjusted to 270 mOsm, either by NaCl or by sucrose (Figure 1A,B). Kinetic studies revealed

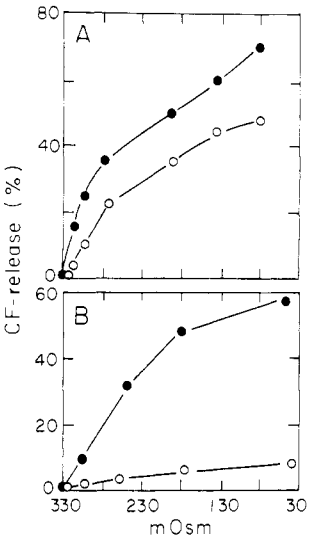

FIGURE 2: Hypoosmotic lysis of ROV (A) and lipid vesicles (B). Intact and neuraminidase–Pronase-treated ROV, loaded with CF, were obtained as described under Experimental Procedures. Liposomes composed of phosphatidylcholine and cholesterol (1:0.5 PC:Chol molar ratio) were prepared as follows: (a) Small unilamellar vesicles (SUV) were prepared by sonication of the lipid mixture in 7 mM Tris, pH 7.2, containing 80 mM CF and 120 mM NaCl (Papahadjopoulos & Miller, 1967); (b) large unilamellar vesicles (LUV) were prepared from the octyl glucoside (5% in the above sonication buffer) solution of the lipids, following removal of the detergent by the direct addition of SM-2 Bio-beads, as described before (Citovsky & Loyter, 1985; Citovsky et al., 1986a,b). Loaded vesicles were separated from free CF as previously described (Citovsky & Loyter, 1985; Citovsky et al., 1986b). To induce hypoosmotic lysis, CF-loaded vesicle preparations (5 μg) were incubated for 30 min at 37 °C in solution Na (0.4 mL final volume), adjusted to the indicated osmolarity with double-distilled water. Before and after the incubation, CF fluorescence (excitation at 490 nm and emission at 520 nm) was measured, and the extent of CF release was determined as described before (Citovsky et al., 1986b). (A) (○) Intact ROV; (●) neuraminidase–Pronase-treated ROV. (b) (●) LUV; (○) SUV.

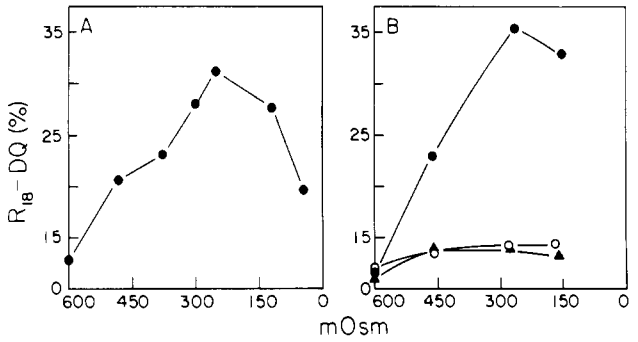

FIGURE 3: Interaction of Sendai virions with CGMV: effect of osmolarity. R_{18}-labeled intact (●), trypsinized (▲), or DTT-treated (○) Sendai virions (2 μg) were incubated for 10 min at room temperature with 400 μg of CGMV in solution K, after which the osmolarity was adjusted to the indicated values by addition of double-distilled water. The reaction mixture (0.4 mL final volume) was incubated for 45 min at 37 °C. Following incubation, the degree of fluorescence dequenching (R_{18}-DQ) was estimated (see Experimental Procedures). (A) Effect of osmolarity. (B) Effect of trypsin and DTT. Trypsinized and DTT-treated Sendai virions were prepared as described in Figure 1 and were labeled with R_{18} as described under Experimental Procedures.

that maximum fluorescence dequenching was observed following 20–30 min of incubation at 37 °C under hypotonic conditions (Figure 1C).

Infection of cells and fusogenic activity of the Sendai virus envelopes require the presence of two viral envelope polypeptides, i.e., the HN and F glycoproteins (Poste & Pasternak; 1978; Fukami et al., 1980). It was therefore of interest to study whether fusion with virus receptor depleted ROV showed the same characteristics. Incubation of reconstituted vesicles, bearing either the viral HN or the F glycoproteins, with neuraminidase–Pronase-treated ROV did not result in any increase in the degree of fluorescence (Table I, experiment 2). Such increase was observed only upon incubation with RSVE or with coreconstituted, hybrid vesicles bearing the two viral glycoproteins within the same membrane (Table I, experiment 2). As mentioned above, vesicles bearing the individual glycoproteins are unfusogenic. Thus, it appears that fusion with receptor-depleted ROV, observed under hypotonic conditions, reflects the viral fusogenic activity needed for infection and fusion with cultured cells (Poste & Pasternak, 1978; Fukami et al., 1980).

From the results in Figure 2A, it is clear that untreated and neuraminidase–Pronase-treated ROV are impermeable and osmotically sensitive. As can be seen (Figure 2A), no release of carboxyfluorescein (CF) was observed following incubation of the loaded ROV under isotonic conditions, while up to 50–70% of the CF was released under hypotonic conditions. Similar results were obtained following incubation of CF-loaded, large unilamellar liposomes (LUV), composed of phosphatidylcholine and cholesterol, in a medium of low ionic strength (Figure 2B). No release of CF was observed from small unilamellar vesicles (SUV) upon incubation at either isotonic or hypotonic conditions (Figure 2B). SUV are known to be osmotically insensitive (Portis et al., 1979).

Fusion of Sendai Virions with Chromaffin Granule Membrane Vesicles (CGMV): Fluorescence Dequenching Studies. The results described above (Figure 1) clearly demonstrate that, under hypotonic conditions, no specific receptors are required to allow fusion between Sendai virions and biological membranes. It was therefore of interest to study whether Sendai virions would interact and fuse also with membranes of intracellular organelles. The results in Figure 3A,B show that incubation of R_{18}-labeled Sendai virions, under isotonic

Table II: Fusion of Fluorescently Labeled Sendai Virions with CGMV: Effect of Neuraminidase Treatment

expt	fluorescent Sendai virions incubated with	R_{18}-DQ (%) for incubation at 600 mOsm	R_{18}-DQ (%) for incubation at 240 mOsm
1[a]	CGMV	12	31
	$CGMV_{neur\ 600}$	13	29
	$CGMV_{neur\ 240}$	15	33
2[b]	CGMV	11	31
	$CGMV_{GA}$	9	8

[a] Experiment 1: CGMV (2 mg) were incubated for 90 min at 37 °C with 30 milliunits of neuraminidase in 1 mL of a solution containing 4 mM sodium acetate (pH 5.6), 7 mM $CaCl_2$ and 0.3 M KCl (600 mOsm) ($CGMV_{neur\ 600}$), or 0.12 M KCl (240 mOsm) ($CGMV_{neur\ 240}$). At the end of the incubation period, the membranes were washed with 30 volumes of solution K (20000*g*, 30 min at 4 °C) and resuspended in the same buffer. The fluorescence dequenching (R_{18}-DQ) measurements were performed at 600 and 240 mOsm, using 400 μg of membrane protein, as described in Figure 3 and under Experimental Procedures. [b] Experiment 2: For treatment with glutaraldehyde, 1.2 mg of protein of CGMV was incubated for 20 min at 37 °C with 0.1% glutaraldehyde, in a final volume of 0.4 mL of solution K (Maeda et al., 1977a; Chejanovsky & Loyter, 1985). At the end of the incubation period, the treated CGMV ($CGMV_{GA}$) were washed 3 times with solution K. All other experimental conditions were as described in experiment 1.

conditions (CGMV have been sealed in a solution containing 300 mM KCl, namely, a solution of 600 mOsm, which therefore is an isotonic medium for these organelles), with CGMV resulted in a certain low degree (10–13%) of fluorescence dequenching. A significant increase in the fluorescence degree was observed, following incubation of CGMV with Sendai virions in medium containing decreasing concentrations of salt (Figure 3). Maximum fluorescence dequenching was observed at 240 mOsm, while at lower values of osmolarity the extent of fluorescence dequenching decreased, probably due to breakage of the chromaffin granules at these salt concentrations. A relatively low degree of fluorescence was obtained upon incubation of CGMV with nonfusogenic, namely, trypsinized or DTT-treated (Citovsky & Loyter, 1985), Sendai virions (Figure 3B). These results indicate that the fluorescence dequenching observed is due to fusion between the virus envelopes and membranes of CGMV.

Fusion of Sendai virions with CGMV cannot be mediated by virus receptors such as GD_{1a}, GT_{1b}, and GQ_{1b} gangliosides (Markwell et al., 1981), because chromaffin granule membranes are known to lack such sialoglycolipids (Ekerdt et al., 1981; Abbs & Phillips, 1980). This view is further confirmed by the results in Table II. The same degree of fluorescence dequenching was observed with untreated or neuraminidase-treated CGMV (Table II, experiment 1). Fusion with Sendai virions was observed even with CGMV that were treated with neuraminidase under hypotonic conditions. It is conceivable that under such conditions, all the membrane sialic acid residues should be available to hydrolysis by neuraminidase. No increase in the degree of fluorescence dequenching was observed following incubation of Sendai virus with glutaraldehyde-treated CGMV (Table II, experiment 2). On the basis of previous experiments (Maeda et al., 1977a) with erythrocyte membranes, it is likely that such treated CGMV are susceptible to lipid–lipid exchange but not to virus–membrane fusion processes.

Support for the view that the fluorescence dequenching observed under hypotonic conditions indeed reflects specific interaction or fusion between the viral envelopes and the CGMV was obtained by the use of anti-Sendai virus antibodies. Such antibodies can be used as a tool to detect nonviral components which are associated or fused with Sendai virus

Table III: Precipitation of CGMV by Anti-Sendai Virus Antibody

virus	^{125}I-ANPA precipitated by anti-Sendai virus antibody at 240 mOsm (hypotonic conditions) (% of total added)	600 mOsm (isotonic conditions) (% of total added)	hemolysis (% of total)
SV	33	5	92
trypsinized SV	7	5	5
DTT-treated SV	4	1	8
PMSF-treated SV	5	2	4
no virus	2	1	1

[a] ^{125}I-4-Amino-3-nitrophenyl azide (^{125}I-ANPA) was synthesized, and the membranes of chromaffin granules (0.4 mg/mL) were labeled with ^{125}I-ANPA as previously described (Gabizon et al., 1982). Trypsinized and DTT- and PMSF-treated Sendai virions (SV) were obtained as described in Figure 1, except that the preparations were performed in solution K. Both untreated and treated Sendai virions (15 μg) were incubated for 45 min at 37 °C with 2 μg of ^{125}I-ANPA-labeled CGMV in 0.2 mL of solution K, adjusted to the indicated final osmolarity, as described in Figure 3. At the end of the incubation period, 30 μg of rabbit anti-Sendai virus antibodies (titer 1:256) was added. Following an additional 30 min of incubation at 37 °C, the system was diluted with 1 mL of cold solution K and centrifuged (1000*g*, 2 min), and the amount of radioactive material in the supernatants and the pellets was determined. Under the same conditions, 80% of untreated or treated Sendai virions could be precipitated by the antibody. For induction of hemolysis, 5 μg of untreated or treated Sendai virions was incubated for 15 min at 37 °C with 250 μL of 2–3% human erythrocytes in solution Na, and the degree of hemolysis was estimated as described before (Peretz et al., 1974).

envelopes. We have previously shown (Volsky et al., 1979) that the human erythrocyte band 3 polypeptide can be precipitated by anti-Sendai virus antibody after being inserted into virus envelopes during their reconstitution. Similarly, ^{14}C-labeled phospholipid vesicles can be precipitated by anti-Sendai virus antibodies, following fusion of Sendai virus envelopes with the radiolabeled liposomes (Citovsky & Loyter, 1985).

The results in Table III show that, when the mixture of Sendai virions and CGMV was incubated at 37 °C under hypotonic conditions (240 mOsm), about 33% of the radiolabeled CGMV were precipitated by anti-Sendai virus antibodies. On the other hand, anti-Sendai virus antibodies failed to precipitate radiolabeled chromaffin granule membranes which were incubated with intact Sendai virions under isotonic conditions (600 mOsm). Furthermore, anti-Sendai virus antibodies precipitated only radiolabeled membranes which were incubated with fusogenic–hemolytic Sendai virions. Practically no or very little precipitation of radiolabeled material was observed following incubation of CGMV with virions which were treated with trypsin, PMSF, or DTT (Table III). Treatment with trypsin or PMSF specifically inactivates the viral fusogenic activity, without affecting its binding ability (Israel et al., 1979; Asano et al., 1983). Conversely, treatment with DTT affects both the viral fusogenic and cell binding activities (Ozawa et al., 1979). This is confirmed by the results in Table III, which show that trypsinized or DTT- or PMSF-treated Sendai virions failed to hemolyse human erythrocytes. It has been well established that virus-induced hemolysis reflects a process of virus–membrane fusion (Maeda et al., 1977b). The failure of anti-Sendai virus antibodies to precipitate CGMV incubated with trypsin- and PMSF-treated virions strongly suggests that the virus–CGMV interaction is not mediated by specific binding of virions to membrane receptors (via the viral HN glycoprotein).

The degree of fluorescence dequenching was highly dependent upon the amount of CGMV present in the incubation mixture (Figure 4A). Maximum fluorescence dequenching

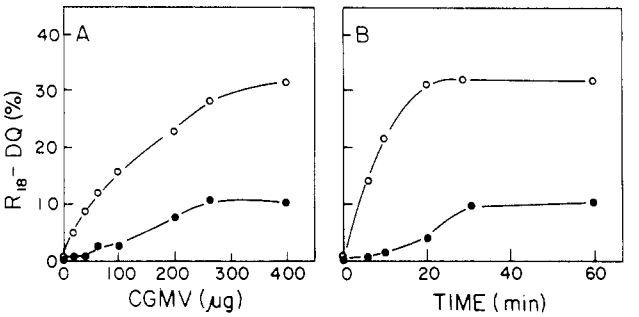

FIGURE 4: Fusion of Sendai virions with CGMV: effect of membrane concentration and time of incubation. R_{18}-labeled Sendai virions (2 μg) were incubated at 37 °C in solution K, at a final osmolarity of 600 (●) or 240 mOsm (○), for 30 min with the indicated amounts of CGMV protein (A) or with 400 μg of CGMV for the indicated periods of time (B). All other experimental conditions were as in Figure 3. After the incubation period, the degree of fluorescence dequenching was estimated as described under Experimental Procedures.

was observed upon incubation of 2 μg of Sendai virions with 400 μg of membrane vesicles. In accordance with the results shown in Figure 3, the results in Figure 4A also demonstrate that a significantly lower degree of fluorescence dequenching was observed under isotonic conditions, as compared to that obtained under hypotonic conditions (Figure 4A). Kinetic studies revealed that maximum fluorescence dequenching was reached within 20 min of incubation at 37 °C (Figure 4B), indicating that the fusion between Sendai virus envelopes and CGMV is a relatively slow process.

Experiments to study the pH dependence of the fusion process revealed that maximum fluorescence dequenching (37%) was observed between pH 6.0 and 9.0 (Figure 5A), a pH range in which the viral fusion factor is maximally activated (Chejanovsky & Loyter, 1985). Low degrees of fluorescence dequenching were obtained when Sendai virions were incubated under isotonic conditions with CGMV, even at optimal pH values, i.e., between pH 6.0 and 9.0 (Figure 5A).

Fusion of Sendai virions with biological membranes is highly dependent on the temperature of incubation (Chejanovsky & Loyter, 1985). This is also demonstrated by the results summarized in Figure 5B. Maximum fluorescence dequenching was observed when Sendai virions and CGMV were incubated at 37 °C under hypotonic conditions. Very little fluorescence dequenching was observed at temperatures below 20 °C or above 45 °C (Figure 5). The decrease in the extent of virus–membrane fusion at temperatures higher than 45 °C is probably due to thermal inactivation of the viral fusion protein (Chejanovsky & Loyter, 1985).

Fusion of Sendai Virions with CGMV: Electron Microscopy Studies. Electron microscopy studies (Figure 6) revealed that incubation of Sendai virus particles with CGMV at 37 °C in a medium of low osmolarity (240 mOsm) resulted in the formation of large membrane vesicles. It is conceivable that these large vesicles are the result of either virus–CGMV or virus-induced CGMV fusion, or both. No such large membrane vesicles were detected following incubation of inactive, unfusogenic virus particles and CGMV (Figure 6A). Similar results, namely, crowded fields containing a mixture of intact virus particles and CGMV, were also observed following incubation of fusogenic, intact virus particles and CGMV at 4 °C or at zero time of incubation at 37 °C (not shown).

Virus particles in close contact with (Figure 6B) or probably in the process of fusion (Figure 6C) with CGMV were ob-

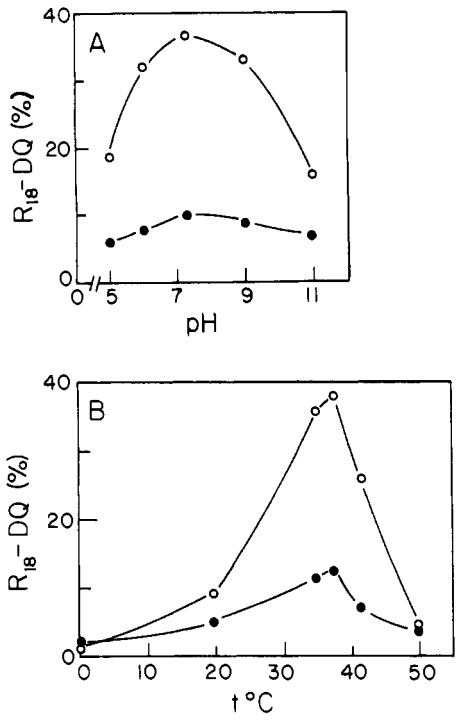

FIGURE 5: Effect of the incubation temperature and the pH of the medium on the interaction between Sendai virions and CGMV. (A) Effect of pH: R_{18}-labeled Sendai virions (2 μg) were incubated for 45 min with 400 μg of CGMV, in a medium of final 600 (●) or 240 mOsm (○) osmolarity of KCl buffered with 7 mM glycine hydrochloride (pH 5–6), Tris-HCl (pH 7.2), and glycine–NaOH (pH 9–11). All other experimental conditions were as in Figure 3. (B) Effect of temperature: R_{18}-labeled Sendai virions (2 μg) were incubated with 400 μg of CGMV in solution K, with a final osmolarity of 600 (●) or 240 mOsm (○), for 45 min at the indicated temperatures. All other experimental conditions were as described in Figure 3.

served in preparations containing a mixture of virus particles incubated at 37 °C for 3 min only. Examination of such systems after 10 min (Figure 6D) or 20 min (Figure 6E,F) of incubation at 37 °C revealed the formation of large membrane vesicles, some of them containing threads of viral ribonucleoprotein (RNP) (small arrows, Figure 6D,E) and bearing viral spikes on part of their surfaces (large arrows, Figure 6D). Unlike intact viral particles (average diameter of 180 nm) which contain tightly packed threads of RNP (Figure 6A), the large vesicles (average diameter 640 nm) show the viral RNP as diffusely localized threads (Figure 6D–F). This may result from fusion of intact Sendai virions and CGMV, a process which should lead to introduction of the viral RNP into the fused vesicles.

Fusion of Sendai Virions with Lipid Vesicles: Effect of Salt. The possibility exists that the fusion observed, under hypotonic conditions, between Sendai virions and virus receptor depleted membranes is due to activation of the viral fusion protein and not to osmotic swelling of the recipient membrane vesicles. The interaction between Sendai virions and liposomes lacking virus receptors can be used as an experimental system to study this possibility. The results in Table IV, experiment 1, confirm previous observations showing that Sendai virions are able to fuse with liposomes composed of phosphatidylcholine and cholesterol but lacking virus receptors [see also Citovsky and Loyter (1985) and Citovsky et al. (1985)]. Fusion was observed with small unilamellar vesicles (SUV) as well as with large unilamellar vesicles (LUV), although, as expected, the degree of fluorescence dequenching observed was significantly higher when LUV were used. No fusion (fluorescence dequenching) was observed following incubation

Table IV: Fusion of Sendai Virions with Small and Large Unilamellar Lipid Vesicles: Effect of Osmolarity

		R_{18}-DQ (%)			
		SV		SV_{DTT}	
expt	osmolarity (mOsm)	SUV	LUV	SUV	LUV
1[a]	330	20	45	8	7
	290	18	46	7	9
	230	18	45	7	7
	130	17	48	7	7

expt	osmolarity (mOsm)	R_{18}-DQ (%) for liposomes from ROV lipids	
2[b]	330	41	5
	270	39	7
	130	39	7

[a] Experiment 1: LUV and SUV (1:0.5 PC:Chol molar ratio) were prepared as described in Figure 2, except that CF was omitted and all preparations were performed in solution Na. To induce fluorescence dequenching, R_{18}-labeled Sendai virions (SV, 2 μg) were incubated for 30 min at 37 °C with 200 μg of liposomes in solution Na, adjusted to the indicated osmolarity values, as described in Figure 1 for solution K. The degree of fluorescence dequenching (R_{18}-DQ) was determined as described under Experimental Procedures. DTT-treated Sendai virions (SV_{DTT}) were obtained as described in Figure 1. [b] Experiment 2: ROV lipids were isolated by the method of Folch et al. (1957). Briefly, 1 volume of ROV (4 mg/mL) was added to 20 volumes of chloroform/methanol (2:1) and incubated for 15 min at room temperature with occasional stirring. The insoluble material was sedimented (2000*g*, 15 min), and $^1/_5$th volume of water was then added to the clear supernatant with vigorous stirring. Subsequent to separation of two phases, the upper phase was removed, and the lower one was washed with the same volume of water and dried under a flow of nitrogen. Liposomes were prepared from the dried ROV lipids as described in experiment 1 for the preparation of PC/Chol LUV. All other experimental conditions were as in experiment 1.

of DTT-treated virions with the phospholipid vesicles. Moreover, the results in Table IV, experiment 1, show that no change in the extent of virus–liposome fusion was observed following incubation of the virus with the liposomes in media of decreasing osmolarity. From these results, it should be inferred that no activation of the viral fusion protein occurred in a medium of low osmolarity.

Essentially the same results were obtained with liposomes prepared from lipids extracted from ROV (Table IV, experiment 2). This clearly demonstrates that the results obtained in experiment 1 are not due to the specific lipid composition of the liposomes.

Discussion

Sialic acid residues of membrane sialoglycoproteins and sialoglycolipids have been shown to serve as specific membrane receptors for Sendai virions (Suzuki et al., 1983; Markwell et al., 1981). In the present work, sialic acid residues have been removed from the erythrocyte membranes by extensive treatment with neuraminidase and Pronase. Membranes of CGMV are also known to carry sialic acid containing components. However, it is not yet clear whether these are merely sialoglycolipids or also sialoglycoproteins (Ekerdt et al., 1981; Abbs & Phillips, 1980). A detailed analysis revealed the presence of GM but not of GD, GT, or GQ classes of gangliosides. As mentioned above, only the latter sialoglycolipids have been shown to serve as receptors for Sendai virions (Markwell et al., 1981).

The results of the present work clearly show that fusion of Sendai virus with biological membranes does not necessitate the presence of specific virus receptors. This is inferred from our observations showing that, under hypotonic conditions, the incubation of fluorescently labeled Sensai virions either with

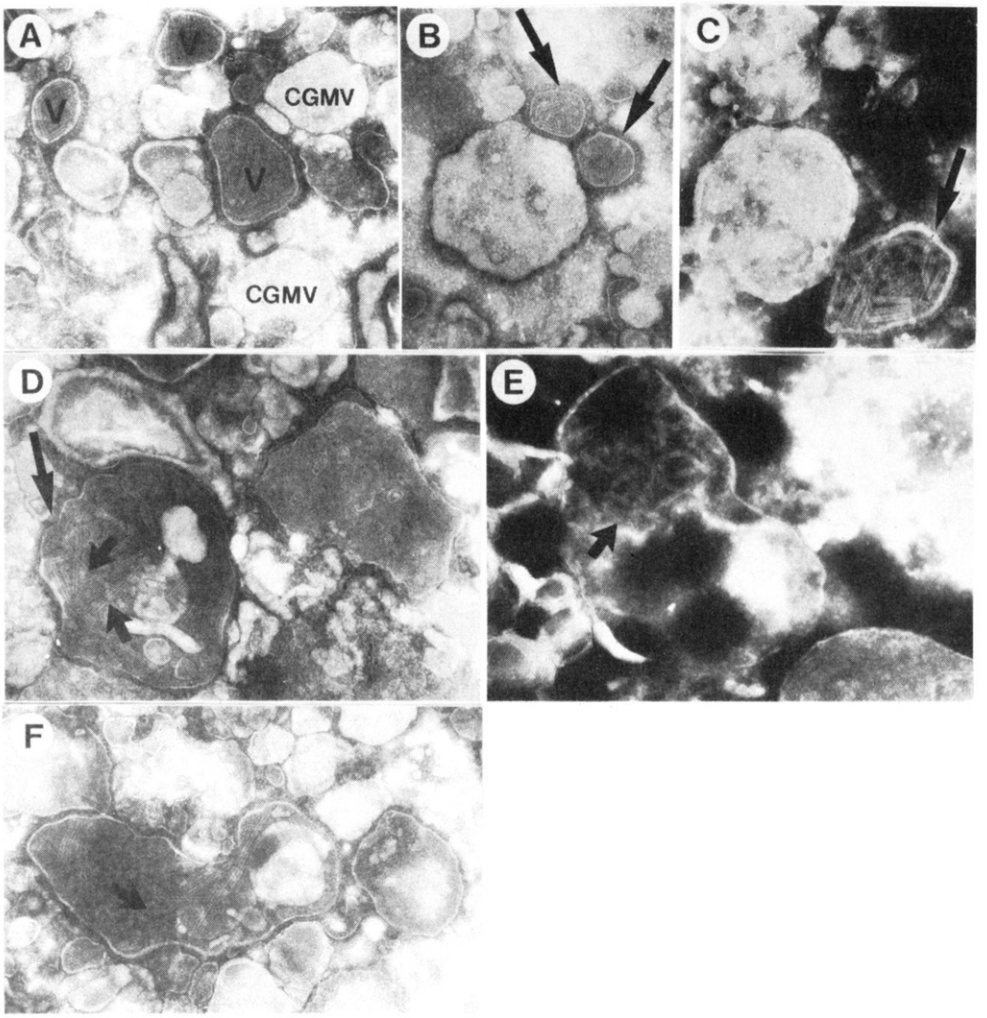

FIGURE 6: Fusion between intact Sendai virions and CGMV: electron microscopic studies. Sendai virions (100 μg) were incubated at 37 °C with a suspension of CGMV (500 μg), in a final volume of 0.4 mL of solution K, adjusted to 240 mOsm, as described under Experimental Procedures. After 3 (B, C), 10 (D), and 20 min (E, F) of incubation, samples were withdrawn and stained with 2% phosphotungstic acid for electron microscopic observations, as described before (Vainstein et al., 1984). In these experiments, relatively high amounts of virions were used in order to increase the probability of finding virus–CGMV fusion events. Also, trypsinized Sendai virions (V) (obtained as described in Figure 1B) were incubated for 30 min with CGMV (A). Note the tightly packed RNP threads (short arrows) and the viral glycoproteins (spikes) (long arrows) within and on the virus envelopes, respectively. Magnification 33800×.

neuraminidase–pronase-treated human erythrocyte membranes (ROV) or with CGMV resulted in fluorescence dequenching. Preliminary experiments in our laboratory (unpublished results) also showed that incubation of fluorescently labeled influenza virions with virus receptor depleted ROV, under hypotonic conditions, resulted in fluorescence dequenching. However, this occurred only after incubation at pH 5.0–5.2, a pH range that causes activation of the influenza virus' fusion protein (Sato et al., 1983).

The view that the increase in fluorescence dequenching observed in a medium of low osmolarity indeed reflects a process of virus–membrane fusion is supported by the results showing that such an increase was not observed with inactivated, unfusogenic Sendai virions. Moreover, no increase in fluorescence dequenching was observed upon incubation with glutaraldehyde-treated membranes, strongly suggesting that the results observed with untreated membranes are due to virus–membrane fusion and not to lipid exchange or lipid transfer processes (Maeda et al., 1977a). This conclusion is also supported by the results obtained with membrane vesicles bearing the individual viral glycoproteins. Fluorescence dequenching was observed only upon incubation of ROV (and CGMV, not shown) with membrane vesicles bearing the viral HN and F glycoproteins within the same membrane. If this increase in fluorescence was due to a collision-mediated transfer of the fluorescent probe, it is reasonable to assume that the same process will also occur with membrane vesicles bearing either the F or the HN glycoprotein. The results of the present work clearly show that no fluorescence dequenching was observed upon incubation with HN or F membrane vesicles. In this regard, fusion under hypotonic conditions with virus receptor depleted ROV shows the same features as fusion with liposomes, intact erythrocytes, or living culture cells (Gitman et al., 1984; Nussbaum et al., 1984; Citovsky & Loyter, 1985).

The requirement for the viral HN glycoprotein, in addition to the F polypeptide, for fusion with ROV (and CGMV, not shown) is understandable on the basis of previous experiments in our laboratory (Gitman et al., 1984; Nussbaum et al., 1984; Citovsky et al., 1986a,b) as well as in others (Ozawa et al., 1983). These experiments clearly showed that, in addition to its function as the viral binding protein, the HN glycoprotein also plays an active role in the membrane fusion step itself and its presence is required even for fusion with liposomes lacking virus receptors (Citovsky et al., 1985). Virus–CGMV fusion as well as virus-induced fusion of CGMV can also be inferred from electron microscopic studies.

It is possible that under the conditions used, namely, a

medium of low osmolarity, the fusion protein (F glycoprotein) of Sendai virus is highly activated, thus allowing fusion with virus receptor depleted membranes. The results showing that virus–ROV fusion was promoted in media which were made hypotonic by low concentrations of sucrose eliminate the possibility of a specific effect of the salt in the process. In addition, the observation that virus–liposome fusion was independent of the osmolarity of the medium further supports the view that it is the structure of the recipient membrane which is affected by the decreased osmolarity and not the biological activity of the virus. It is conceivable that incubation of the ROV or the CGMV in a medium of low osmolarity induces osmotic swelling of the membrane vesicles, leading to exposure of the membrane lipid bilayer, thereby allowing its interaction with the viral envelope glycoproteins. Indeed, our results clearly show that the ROV used in this work are osmotically sensitive. The findings (White & Helenius, 1980; Hsu et al., 1983; White et al., 1983; Citovsky & Loyter, 1985) that enveloped virions, including Sendai virus, fuse extensively with liposomes lacking virus receptors clearly demonstrate that the eventual target of the virus is the lipid bilayer of the recipient cell plasma membrane. Since in liposomes the lipid bilayer is exposed and available to interaction with the viral glycoproteins, manipulation with the medium's salt concentration should not affect the degree of fusion between liposomes and Sendai virus. On the other hand, lipid bialyers of biological membranes are masked and can be exposed to external ligands by osmotic swelling (Laster et al., 1972; Gazitt et al., 1976). Our suggestion that the membrane lipid bilayer must be unmasked so as to allow its fusion with envelopes of animal viruses is strengthened by our experiments showing that only neuraminidase–Pronase-treated ROV were susceptible to fusion with Sendai virions. It is conceivable that a large part of external glycoproteins of the cell plasma membrane must be removed by treatment with proteolytic enzymes in order to allow exposure of the lipid bilayer even in a hypotonic medium. It appears that treatment with Pronase is not required to allow fusion of Sendai virus with CGMV under hypotonic conditions. This may indicate that osmotic swelling of CGMV is sufficient for the exposure of its membrane phospholipids, suggesting that in these organelles the phospholipid bilayer is not as masked as in the erythrocyte membranes. In this respect, osmotically swollen CGMV behave similarly to proteoliposomes whose phospholipid bilayer is readily susceptible to fusion with Sendai virus (Citovsky & Loyter, 1985; Citovsky et al., 1986a,b).

Since, under isotonic conditions, Sendai virions fuse readily with biological membranes bearing virus receptors (see Figure 1), it may be speculated that the interaction between the virions and their membrane receptors induces exposure of the membrane lipid bilayer. Indeed, it has been well established that interaction of Sendai virions with membrane receptors at 37 °C promotes an increase in the membrane permeability and, consequently, osmotic swelling of the recipient cells (Bashford et al., 1985; Citovsky et al., 1986b). This should lead to exposure of membrane phospholipids to the viral envelope glycoproteins. It is therefore possible that membrane receptors for enveloped virions play an active role in the entire process of virus penetration and do not merely serve as passive binding entities.

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Fluorescence Lifetime Distributions of 1,6-Diphenyl-1,3,5-hexatriene in Phospholipid Vesicles†

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ABSTRACT: The fluorescence emission properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) in 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine and 1,2-dimyristoyl-3-*sn*-phosphatidylcholine multilamellar vesicles have been measured by using multifrequency phase fluorometry. The fluorescence decay of DPH in the phospholipid vesicles has been analyzed by assuming either that the decay is made up of a discrete sum of exponential components or that the decay is made up of one or more continuous distributions of lifetime components. The fit of the decay curve using exponentials required at least two terms, and the reduced χ^2 was relatively large. The fit using a continuous distribution of lifetime values used two continuous components. Several symmetric distribution functions were used: uniform, Gaussian, and Lorentzian. The distribution function that best described the decay was the Lorentzian. The full width at half-maximum of the Lorentzian distribution was about 0.6 ns at temperatures below the phase transition temperature. At the phospholipid phase transition and at higher temperatures, the distribution became quite narrow, with a width of about 0.1 ns. It is proposed that the lifetime distribution is generated by a continuum of different environments of the DPH molecule characterized by different dielectric constants. Below the transition temperature in the gel phase, the dielectric constant gradient along the membrane normal determines the distribution of decay rates. Above the transition, in the liquid-crystalline phase, the translational and rotational mobility of the DPH molecule increases, and the DPH experiences an average environment during the excited-state lifetime. Consequently, the distribution becomes narrower. The physical interpretation of the continuous distribution of lifetime values is based on the heterogeneity of the molecular environments of the DPH molecule, and this better describes the observed decay than the use of a discrete number of exponential components.

During the past several years, there has been an increasing interest in the study of the physical properties of lipid bilayers. A variety of spectroscopic techniques have been employed for the investigation of the structure and dynamic properties of synthetic and natural membranes. In particular, fluorescence techniques have been used to investigate lateral and rotational diffusion in lipid bilayers to quantitate the amount of gel and liquid-crystalline phases. Fluorescence studies generally use probes embedded in the membrane. Also, parinaric acid isomers have been used (Sklar et al., 1975, 1977a,b; Wolber & Hudson, 1981; Parasassi et al., 1984). 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] and its derivatives are perhaps the most commonly used probes to investigate the physical-structural properties of membranes at the molecular level (Shinitzky & Barenholtz, 1975; Lentz et al., 1976a,b; Chen et al., 1977; Cranney et al., 1983). DPH is a very sensitive probe, and it has a large partition coefficient for the lipid with respect to the solvent. DPH locates in the membrane interior,

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[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,2,5-hexatriene; DPH-PC, 2-[3-(diphenylhexatrienyl)-propanoyl]-3-palmitoyl-L-α-phosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene.

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