Functional Reconstitution of the Integral Membrane Proteins of Influenza Virus into Phospholipid Liposomes†

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ABSTRACT: The integral membrane proteins of influenza virus, a hemagglutinin and a neuraminidase, have been incorporated into liposomes composed of either phosphatidylcholine or a mixture of phosphatidylcholine and phosphatidylethanolamine (2:1 w/w) using detergent dialysis. The virus spike glycoproteins for reconstitution were selectively solubilized by using cetyltrimethylammonium bromide to leave a "core particle", which lacked a lipid bilayer but possessed quaternary structure as observed by electron microscopy. The viral spike proteins were combined with exogenous phospholipid in excess sodium cholate followed by exhaustive dialysis for 150 h. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that only the viral glycoproteins were associated with all the complexes formed. The level of sodium cholate remaining after dialysis was shown to be reduced to less than 1 molecule per 80 protein molecules. Viral proteins reconstituted into dimyristoylphosphatidylcholine liposomes were shown to have retained hemagglutination, low-pH-dependent hemolysis, and neuraminidase activities and were associated with a lipid bilayer in two types of complexes with average lipid to protein mole ratios after sucrose density gradient purification of either 590:1 or 970:1. The bilayer vesicles formed were of similar sizes and were shown by negative-stain electron microscopy to be 150–300 nm in diameter with well-defined spikes on their surface. Reconstituted liposomes of dimyristoylphosphatidylcholine were found to be unstable with respect to their trapped volume and therefore were unsuitable for fusion studies, unlike complexes formed with phosphatidylcholine or a mixture of phosphatidylcholine/phosphatidylethanolamine derived from hen eggs. Liposomes of egg phospholipids [lipid to protein mole ratios of 420:1 and 236:1 for complexes composed of egg phosphatidylcholine and a mixture of egg phosphatidylcholine/egg phosphatidylethanolamine (2:1 w/w), respectively] containing viral spike proteins were not leaky with respect to their occluded volume and were induced to fuse with protein-free phospholipid vesicles by acidifying the buffer to pH 5.0, as shown by a contents mixing assay using terbium and dipicolinic acid loaded vesicles. The fusion process itself was shown to be leaky, with release of some vesicle contents to the external volume.

The lipid composition of the influenza virus has been shown to closely reflect that of the host cell from which it is derived (Kates et al., 1961). However, selective interactions between the viral membrane proteins and specific lipids of the host cell have been implied from detailed studies which have demonstrated significant differences between the acyl chain composition of the lipids of the virus and those of the host cell (Tiffany & Blough, 1969). Electron spin resonance spin-label observations on the bilayer of intact virus have also suggested that its microviscosity is significantly increased with respect to that of the host cell (Landsberger et al., 1971), even though the cholesterol content and molar ratios of lipid to protein in the two membranes are very similar. There are several processes during the infection cycle of the influenza virus where lipid–protein interactions may be of importance; in the budding of the virus from the cell surface, during membrane fusion events, and possibly in modulating the activity of the two integral membrane proteins, a hemagglutinin (HA)[1] and a neuraminidase (NA).

Virosomes, containing the viral membrane proteins and with a well-defined lipid composition, constitute a model system in which specific interactions between the lipids and proteins and the relative organization of the bilayer components may be studied. Interest in these models has increased recently since additional factors apart from the involvement of the glycoproteins have been implicated in fusion events. A regulatory role of a viral binding protein in Sendai virus during the fusion process has been postulated (Harmsen et al., 1985), and the potential of fatty acids to provide a hydrophobic interaction with target membranes has also been discussed (Skehel et al., 1982).

Previous reconstitutions of the influenza virus glycoproteins have been conducted with the detergent Triton X-100 (Oxford et al., 1981), which has a relatively low critical micelle concentration (cmc) and is therefore difficult to reduce to low levels. Octyl glucoside has also been used (Huang et al., 1979); this has a high cmc but has been shown to interfere with the assay for neuraminidase in submicromolar quantities (Warren, 1959). Residual octyl glucoside also appeared to broaden the pH range over which hemagglutinin could induce fusion and, by inference, the capacity to hemagglutinate, since membrane fusion by HA reconstituted with this detergent was observed

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-3-phosphocholine; CTAB, cetyltrimethylammonium bromide; PBS, phosphate-buffered saline; NA, neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18); HA, hemagglutinin; NANA, *N*-acetylneuraminic acid; cmc, critical micelle concentration; NP, nucleoprotein; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid disodium salt; DPA, dipicolinic acid (pyridine-2,6-dicarboxylic acid); Tris, tris(hydroxymethyl)aminomethane; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; NAU, neuraminidase unit; HAU, hemagglutinating unit; PAGE, polyacrylamide gel electrophoresis.

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in a later study (Huang et al., 1980) at neutral pH and not specifically and characteristically below the threshold of pH 5 (Skehel et al., 1982).

Presented here is the first reconstitution of the integral membrane proteins of the influenza virus involving an exchange of detergents. Selective solubilization of the proteins is achieved by using a specific concentration of cetyltrimethylammonium bromide (CTAB), and reconstitution is conducted by using sodium cholate which is readily removed by dialysis to a very low level. The reconstituted vesicles are also shown to be capable of pH-dependent fusion similar to that of the intact virus. The method now has the potential for producing complexes not only of variable lipid to protein ratios but also of defined lipid composition for use in biophysical studies. In addition, the significance of lipid–protein interactions in physiological systems may be evaluated by determining to what extent the fusion activity of the reconstituted particle of a specific lipid composition is moderated compared to the native virus.

Materials and Methods

Virus. Intact inactivated influenza virus strain A/X49 was generously provided through DUPHAR (Weesp, Holland). The virus was concentrated and purified by R. W. H. Ruigrok and P. C. J. Krygsman (Leiden, Holland), suspended in phosphate-buffered saline (PBS), pH 7.2 (0.14 M NaCl, 2.7 mM KCl, 11 mM KH_2PO_4, 28 mM Na_2HPO_4, and 0.001% sodium azide), and stored at 4 °C. Additional virus was purified as described by Mellema et al. (1981) and stored at 4 °C for no more than 24 h before reconstitution.

Protein Determination and Assays. Virus protein was determined colorimetrically following the Lowry modification of Markwell et al. (1981) using bovine serum albumin as standard.

Samples assayed for neuraminidase activity were incubated with 1.25 mg of phosphate-buffered fetuin (pH 5.9) as substrate for 30–120 min at 37 °C. The liberated *N*-acetylneuraminic acid (NANA) was then assayed by the thiobarbituric acid method of Warren (1959). The NANA chromophore was extracted into acidified *n*-butyl alcohol according to the modification of Aminoff (1961). One neuraminidase unit (NAU) is defined here as that amount which will liberate sufficient NANA under standard conditions in 30 min to give an extinction reading of 0.50 in a 1-cm cell at a wavelength of 549 nm (this is approximately equivalent to the release of 1.5 nmol of NANA/min).

Hemagglutination activity was determined by Salk's pattern technique (Salk, 1944) by adding 0.25% fresh goose red blood cells, prepared according to the method of Clark and Casals (1958), to serial 2-fold dilutions of the sample in 0.89% NaCl (isotonic saline). Control experiments using protein-free DMPC vesicles at the same lipid concentration as that of the reconstituted system were also performed. Hemolysis of goose erythrocytes was assayed spectrophotometrically at 540 nm. The 0.5-mL quantity of the highest dilution of the working dilution which completely agglutinates the standard erythrocyte suspension is defined as one hemagglutination unit (HAU).

Electrophoresis. Viral proteins were dissociated by heating at 100 °C for 2 min in the presence of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. Electrophoresis was performed under reducing conditions in 9% polyacrylamide gels at 2 mA/track for 6 h thermostated at room temperature. Protein bands were visualized either by staining with 0.25% Coomassie brilliant blue in 50% methanol and 10% acetic acid or by the silver stain method of Morrissey (1981). Glycoproteins (HA and NA) were identified by employing the technique developed by Zacharius et al. (1969) using the Schiff reagent of Fairbanks et al. (1971).

Detergents and Lipids. CTAB and sodium cholate were A.R. commercial grade (Sigma Chemical Co.). Sodium [^{14}C]cholate (Amersham, U.K.) had an activity of 52 mCi/mmol. The phospholipids 1,2-dimyristoyl-*sn*-3-phosphocholine (DMPC) (Fluka; Buchs, Switzerland), egg phosphatidylcholine, and egg phosphatidylethanolamine (Lipid Products, Surrey, U.K.) were stored in chloroform/methanol (2:1 v/v) at −20 °C until use. [$^3H_9$]DMPC, labeled in the choline methyl group, was prepared by methylation of 1,2-dimyristoyl-*sn*-3-phosphoethanolamine (Fluka) with C^3H_3I as described by Eibl (1980). All lipids were shown to be pure by thin-layer chromatography both before and after experiments.

Phosphorus Determination. Phosphate analysis was performed on samples in phosphate-free buffers according to the method of Rouser et al. (1967).

Electron Microscopy. Preparations of virus, the core particle, and the reconstituted liposomes, free of nonvolatile salts, were dried onto carbon-shadowed Formvar-coated copper grids prior to being stained with 1% phosphotungstic acid (adjusted with sodium hydroxide to pH 7.2) for 15 s. Excess fluid was then withdrawn by using filter paper. Micrographs were produced on an Phillips EM400 electron microscope.

Solubilization of Viral Membrane Components. Selective solubilization of the hemagglutinin and neuraminidase from the lipid bilayer of the virus was achieved by employing the basic recommendations of Bachmayer (1975). A solution of CTAB in PBS (pH 7.2) was added to the virus suspension, maintaining the CTAB concentration at 0.025% and the integral membrane protein to detergent ratio at 1.6:1 (w/w). The extraction (45 min, 21 °C) was allowed to proceed with continuous stirring followed by centrifugation (45 min, 4 °C, 20000*g*), and the supernatant containing the membrane proteins and lipids was removed. The pellet was then subjected to a further extraction using CTAB (45 min, 21 °C) for which it was ensured that both the concentration of CTAB (0.025%) and the membrane protein [estimated by the modified Lowry assay (see above) to give the amount of protein not extracted during the initial solubilization] to detergent ratio (1.6:1 w/w) were identical with the first extraction. Centrifugation of the preparation was then performed as for the initial extraction.

Reconstitution. (A) DMPC Complexes. Dry, solvent-free DMPC, doped with 0.24 μCi of [^{3}H]DMPC, was solubilized by a small volume (0.5 mL) of sodium cholate (including 5 μCi of [^{14}C]cholate) in PBS with vigorous mixing until a clear solution was obtained. The quantity of phospholipid dried down was calculated to produce a lipid to viral protein weight ratio of 7.85:1. To dissolve the phospholipid, a greater than 4-fold excess (w/w) of sodium cholate was employed. The solubilized preparation of viral components composed of the combined supernatants from the postextraction centrifugations was then added to the lipid–detergent mixture, and the concentration of cholate was confirmed to be above its cmc. The suspension was dialyzed (21 °C for a total of 150 h) against six changes of dialysis buffer (5 L each; 0.1 M Tris-HCl, pH 7.4, 0.5 M KCl, 1 mM EDTA, and 0.001% sodium azide). The buffer was made phosphate free to enable phosphate determinations of the lipid phosphorus present to be made later. Aliquots were taken every 24 h to assay the concentration of [^{14}C]cholate remaining and to monitor concomitant lipid loss (see Results). A lipid control, comprising sodium

Table I: Hemagglutinin (HAU) and Neuraminidase (NAU) Activities of Membrane Proteins at Various Stages during Their Reconstitution with DMPC As Described in the Text

	total protein (mg)[a]	% of original membrane protein[b]	amount of protein required to induce hemagglutination (ng)	HAU act. (units/mg of total protein)	NAU act. (units/mg of total protein)
intact virus	4.61	100	1.6	625000	15.5
isolated spikes	2.21	68	2000	500	62.0
core particle	2.30	32	300	3300	5.84
reconstituted liposomes[c]	1.25	62	360	2780	1.66
light-density band	0.351	23	400	2500	
heavy-density band	0.603	39	320	3130	

[a] Total protein as measured by a modified Lowry method (see text). (Note that hemagglutinin and neuraminidase represent a greater proportion of this amount during the reconstitution; hemagglutinin is approximately 64% of the total protein in the intact virus and 80% of the isolated spikes.) [b] Accounting for samples removed from the preparation for analysis. [c] Before fractionation on a sucrose density gradient.

cholate solubilized DMPC and free of viral bilayer components, was also subjected to dialysis.

After dialysis, the lipid–protein recombinant was centrifuged (60 min, 4 °C, 18000*g*) to sediment a well-formed pellet. This pellet was washed in dialysis buffer and then resuspended in 10% sucrose in Tris-HCl buffer (pH 7.4) and subjected to centrifugation on a 5–20% sucrose gradient (6 h, 4 °C, 38000*g*).

(*B*) *Egg–Phospholipid Complexes.* Due to the inability of DMPC vesicles to trap a stable occluded volume for extended periods, viral proteins were reconstituted, under exactly identical conditions as described, into vesicles composed either of egg phosphatidylcholine or of a mixture of egg phosphatidylcholine/egg phosphatidylethanolamine (2:1 w/w). Dialysis of the protein–lipid–detergent mixture was initially carried out by using 1 L of Tris–Tb^{3+} buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM $TbCl_3$, 50 mM sodium citrate, and 0.001% sodium azide) at room temperature for 72 h, by which time vesicles had formed, as confirmed by a spectroscopic turbidity assay. The buffer was then changed to 5 L of Tris–EDTA (10 mM Tris, 0.1 M NaCl, 1 mM EDTA, and 0.001% sodium azide, pH 7.4) and dialysis allowed to proceed for a further 24 h. EDTA was included to prevent binding of the terbium ion to the outside surface of the vesicles. Finally EDTA-free buffer (10 mM Tris, 0.1 M NaCl, and 0.001% sodium azide, pH 7.4) was used to reduce the sodium cholate level to that achieved for the DMPC system. Multilamellar vesicles, which were shown to trap no Tb^{3+}, were removed from the preparation by pelleting through low-speed centrifugation (15 min, 3000 rpm) to leave the supernatant which was used for the fusion assay.

Fusion Assay. The technique developed by Wilschut et al. (1980) for the study of mixing of aqueous vesicle contents was modified and extended to investigate the fusogenic potency of the reconstituted liposomes. Briefly, tightly sealed vesicles are formed encapsulating the terbium ion (Tb^{3+}) weakly chelated with the citrate anion. On fusion with specific receptive vesicles loaded with the dipicolinic anion (DPA), an enhancement in fluorescence intensity due to the formation of the $Tb(DPA)_3^{3-}$ complex is observed when occluded volumes mix (Barela & Sherry, 1976). The formation of this complex is very rapid and enables kinetic measurements of fusion processes to be made (Bentz et al., 1983). Formation of this complex may be inhibited by the addition of 0.1 mM EDTA.

To serve as receptors, small unilamellar vesicles of egg phosphatidylcholine/egg phosphatidylethanolamine (2:1 w/w) were formed by sonication at 4 °C, using a DAWE 7530A soniprobe equipped with a circulating water thermostat. Phospholipid (20 mg in 2 mL) was sonicated in the presence of 150 mM DPA (sodium salt), 2 mM L-histidine, and 2 mM Tes, pH 7.4. After a clearing spin to remove multilamellar fragments (105000*g*, 15 min, 4 °C), the vesicles were chromatographed on a Sephadex G-75 column (elution buffer: 0.1 M NaCl, 2 mM L-histidine, and 2 mM Tes, pH 7.4) to remove extravesicular DPA. Vesicles were kept on ice until use within the hour.

For the fusion assay, 0.8 mL of the reconstituted vesicles (50 μg of protein/mL; see above for preparation) was mixed with 0.2 mL of the sonicated small unilamellar vesicles (5 mg/mL). Fusion was induced by the addition of HCl (86 μL; 11.26 mM) to the preparation, which increased the acidity of the medium to precisely pH 5.0. Fluorescence intensities were measured on a Perkin-Elmer LS-5 luminescence spectrometer at an excitation wavelength of 276 nm and an emission wavelength of 491 nm with the sample temperature maintained at 37 °C.

Results

DMPC Complexes

Membrane Solubilization. The extract of the integral membrane proteins was achieved by using a limiting concentration of CTAB (0.025%), as recommended by Bachmayer (1975). The integral membrane protein to detergent ratio was calculated to be 1.6:1 (w/w). This represents the minimum amount of surfactant required to induce the lamellar to micellar phase transition of phospholipids of a typical biological membrane (Helenius & Simons, 1975). This level of detergent results in selective solubilization of the hemagglutinin and neuraminidase as well as disruption of the lipid bilayer, leaving a subviral core particle devoid of a lipid bilayer but possessing quaternary structure as evidenced by electron microscopy (see below and Figure 4c). Protein determinations indicated that only 35% of the membrane proteins of this particular strain of influenza were initially solubilized. A repeat extraction was performed on the pelleted particles to raise the total yield of membrane proteins to 68% of the initial level (Table I). No M protein or nucleoprotein appeared as a contaminant in the supernatant after centrifugation of the virus–detergent mixture, as seen by intentionally overexposing an SDS–PAGE gel to silver stain (not shown). The pelleted core particle showed no indication of disintegration due to the secondary extraction as seen by electron micrographs.

Current evidence suggests that on solubilization most, if not all, of the membrane proteins are originally released from the membranes as lipoprotein–detergent complexes, which become gradually but completely delipidated with time (Helenius & Simons, 1975). No attempt was made in this study to separate the lipid–detergent mixed micelles and the protein–detergent complexes, since the amount of exogenous DMPC was in large excess of the residual indigenous lipid. As found with other enzymes in comparable membrane systems, the relative enzyme activity of the solubilized neuraminidase was elevated

Table II: Lipid and Protein Compositions of Various Fractions during the Purification and Reconstitution with DMPC of Hemagglutinin and Neuraminidase

	total protein (mg)	total lipid phosphate (mg)	total lipid from [^{3}H]DMPC radioact. (mg)	av membrane lipid:protein ratio (mol/mol)
intact virus	4.61			280:1[a]
reconstitution mixture[b]	1.35		10.70	
reconstituted liposomes[c]	1.25	7.44	6.27	1920:1
light-density band	0.35	1.05	0.86	970:1
heavy-density band	0.60	1.09	0.90	590:1

[a] From Mellema et al. (1981). [b] Predialysis. [c] Postdialysis.

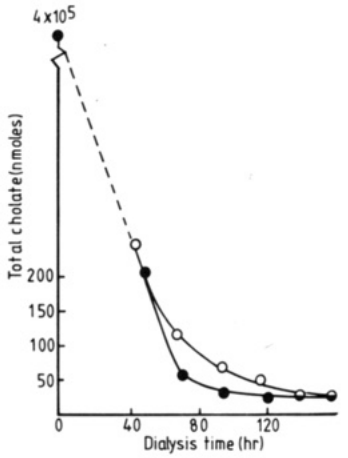

FIGURE 1: Measurement of the removal of sodium [^{14}C]cholate from the reconstitution preparation during dialysis. Initial cholate concentration was 27 mM, solubilizing 10.9 mg of DMPC and 1.35 mg of protein. Temperature, 22 °C. See text for buffer and method of cholate determination. (○) Protein–lipid reconstitution; (●) protein-free control.

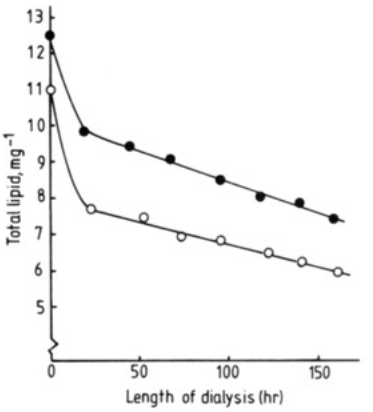

FIGURE 2: Loss of [^{3}H]DMPC from the reconstitution preparation during dialysis. Corrections have been made for the change in dialysate volume and the phospholipid content which is represented as the total lipid remaining. Other conditions as in Figure 1. (●) Protein–lipid reconstitution; (○) protein-free control.

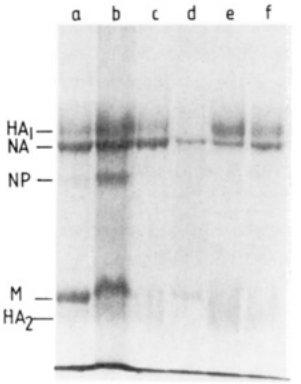

FIGURE 3: SDS–polyacrylamide (9%) gel analysis of influenza polypeptides present during the course of solubilization with CTAB (0.025%) and during reconstitution. Stain used was Coomassie blue, and running conditions are given in the text. Intact viron (a); core particle pelleted after centrifugation following the initial extraction of virus with CTAB (b); solubilized polypeptides retained in the supernatant after centrifugation following the initial extraction (c); core particle after second extraction with CTAB (d); solubilized polypeptides after second extraction with CTAB showing almost complete removal of the hemagglutinin from the original virus (e); reconstituted liposomes containing the combined protein of the two solubilizations showing that only HA and NA are present with no discernible contamination of M or core proteins (f).

with respect to the activity in the native, intact virus (Table I). It is possible that this may reflect a modulating function of the viral lipids on protein activity (Sandermann, 1986).

Reconstitution. Over 97% of the sodium cholate was shown to be removed from the reconstitution complex in the first 20 h of dialysis (Figure 1) at which time the dialysate had become cloudy and the cholate level was below its cmc (7 mM) at this temperature and salt concentration. The level of detergent remaining was determined at regular time intervals, and dialysis was terminated when the detergent to protein ratio (mol/mol) reached 1:80. The concomitant loss of lipid was also monitored (Figure 2) and showed that 40% of the initial phospholipid was lost from the reconstituted system during dialysis (Table II). The use of radiolabeled DMPC as a tracer also demonstrated that the exogenous lipid was retained and constituted the major part of the lipid component of the final liposome, since the ratio of exogenous to indigenous lipid was initially greater than 6:1.

The protein-free dialysis control and the protein-containing complex exhibited not only the same rate of lipid loss during dialysis but also similar rates of detergent removal, the final amount of residual cholate being the same in both systems (Figure 1). A negligible amount of protein (7%) was lost during the dialysis (Table II).

After formation of the liposomes by dialysis, the cloudy suspension was fractionated by centrifugation on a sucrose density gradient. A band of density 1.02 g/mL representing liposomes containing little or no protein was formed at the top of the gradient, and this contained all the remaining sodium cholate. The protein-containing liposomes were shown to be of two densities, 1.03 and 1.07 g/mL, the former being relatively poor in protein (light density band) and the other more rich (heavy density band) (Table II). On the basis of enzymatic activity determination (see below and Table I), the two populations were composed of a similar ratio of hemagglutinin to neuraminidase.

The result of gel electrophoresis of protein samples taken during the extraction procedure (Figure 3) illustrates well the degree of selectivity for protein extraction of this step. Only the membrane proteins, HA and NA, were solubilized, and on a 7.5% acrylamide gel (not shown), no P proteins were

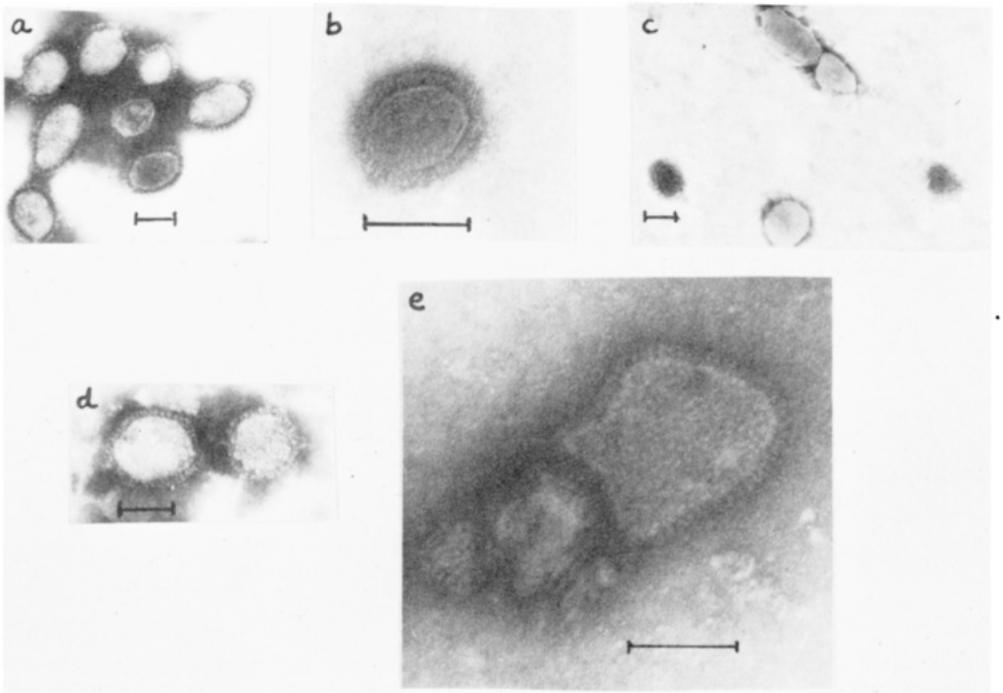

FIGURE 4: Electron micrographs of preparations negatively stained with 1% sodium phosphotungstate, pH 7.0. The bar in each micrograph represents 100 nm. Intact influenza virus at two different magnifications (a and b); the pellet obtained after the second detergent extraction showing lipid vesicles and subviral core particles (c); liposomes containing HA and NA recovered from the dialysate as detailed in the text, after sucrose density gradient centrifugation to form the heavier density (1.07 g/mL) band (d) and to form the lighter density (1.03 g/mL) band (e).

detected in the reconstituted system. A 9% acrylamide gel also demonstrated that almost all the hemagglutinin is seen to be removed from the virus after the second CTAB extraction (Figure 3a,b). Neither the nucleoprotein (NP) nor the M protein was found in the protein–lipid recombinant (Figure 3f). Reconstituted proteins therefore yield very similar gel patterns (Figure 3f) to intact virus (Figure 3a) except for the absence of P, NP, and M proteins.

Enzyme Activities. Viral proteins reconstituted into DMPC liposomes were shown to be active in terms of hemagglutination and neuraminidase potential (Table I). However, the relative activity of the enzymes (expressed in units per milligram of enzyme) was significantly reduced relative to that of the intact virus. Factors contributing to this diminution may be indicated by electron microscopic observations (Oxford et al., 1981) that in a random assembly process, proteins are not wholly accessible to substrate since they may be diametrically oriented on incorporation into a nascent bilayer, appearing as spiked protruding from both the external and internal surfaces of the liposome. Proteins may also be sequestered within the liposome, thereby further restricting the access of substrate. It is significant that the specific activity of the neuraminidase is only reduced by about a factor of 10, while the hemagglutination potential is reduced by several orders of magnitude. No estimation was made of the activity of the neuraminidase after sucrose gradient fractionation. It is well documented that the assay for sialic acid (Warren, 1959) is also sensitive to a large variety of sugars, and even after several washes, sucrose from the density gradient is still present in the liposome medium at detectable levels in the neuraminidase assay. It was found that micromolar quantities of contaminating sugar make the interpretation of the results of the Warren assay unreliable, especially since we observed that the interfering chromophore is light sensitive.

Preliminary hemolysis assays (results not shown) indicated that the reconstituted particle possesses hemolytic activity at pH 5.0, but not at pH 7.4, indicating that the hemagglutinin protein was in its native conformation in the reconstituted complex.

The apparently high hemagglutinin titer of the core particle may be ascribed to the action of detergent, residual unsolubilized hemagglutinin, and nonspecific aggregation of erythrocytes effected by hydrophobicity of exposed M protein.

Electron Microscopy. Micrographs of the intact virus (Figure 4a,b) confirm the recognized value of its spherical diameter of about 120 nm (Mellema et al., 1981). The pellet produced by centrifugation of the solubilized virus (corresponding to gel track b in Figure 3) was also examined (Figure 4c). A few intact virions were observed, as well as a number of spherical structures with an average diameter of 80 nm which lacked the classical protein spikes of the influenza virus. This diameter is consistent with that expected of a virion stripped of its lipid bilayer and membrane proteins. The fact that the particle retains its structure without the surrounding envelope substantiates the postulated role of the M protein as the principal stabilizing force during the assembly of enveloped viruses on the lipid membranes of the infected cell (Kohn, 1979). Pure lipid vesicles were also observed in this preparation, indicating, contrary to earlier reports (Bachmayer, 1975), that the CTAB disrupts and extracts a proportion of the lipid component of the viral bilayer, the vesicles presumably forming during the dialysis step necessary to produce a preparation free of nonvolatile salts for electron microscopy.

Micrographs were also taken of the reconstituted protein–lipid complexes which were shown to consist of predominantly unilamellar vesicles with an average diameter of 150 nm (Figure 4d,e). The average size of these liposomes was approximately the same in the two fractions obtained after density gradient centrifugation; however, the protein spikes were observably more compact in the high-density liposomes from the sucrose gradient. Nevertheless, even in this sample, the average distance between the spikes was significantly greater than that of the intact virus, resulting in stain penetration between the proteins. While some large vesicles (di-

ameter of 2000–5000 nm) were found in both preparations, these contained no protein and presumably had isopycnically centrifuged on the sucrose gradient with the protein–lipid recombinants by virtue of their observed multilamellar nature. It is significant that the only particles possessing any spikes were of comparable dimensions to the intact virus, and these had spikes covering their entire surface.

Lipid:Protein Ratio Analysis. The results of a modified Lowry protein analysis and phosphate assay on samples at various stages in the liposome preparation are given in Table II. The accepted value in the literature for the molar lipid to glycoprotein ratio of the native influenza virus is 280:1 (Mellema et al., 1981). The reconstituted system, due to the swamping with exogenous lipid, has a much higher value for this ratio: 970:1 in the low-density fraction and 590:1 in the high-density fraction. This is substantiated by examination of the electron micrographs of these samples (Figure 4d,e), in which the density of the protein spikes on the bilayer surface is reflected in the measured lipid to protein ratios (Table II). While the distance between the spikes of the intact influenza virus has been calculated to lie between 10.4 and 11.8 nm at the external radius of the virus (Mellema et al., 1981), measurements taken from the micrographs of Figure 4 and others yield a value for the interspike separation in the low-density fraction of 32 nm and of 22 nm in the high-density fraction. It is noted that only an estimated 68% of the integral membrane protein is solubilized during the two extraction steps (Table II), but no protein is lost during the following steps since it is virtually all (91%) accounted for in the final liposome preparation (Table II). Approximately 60% of the lipid remaining in the dialyzed mixture had formed protein-free vesicles, and these were separated from the reconstituted particles by sucrose density gradient centrifugation.

Egg–Phospholipid Complexes

The reconstituted protein-containing liposomes of egg phospholipid were characterized by analytical sucrose density gradient centrifugation (6 h, 4 °C, 50000*g*) on a small fraction of the sample. High- and low-density bands were found as for the complexes of DMPC. The average lipid to protein mole ratio for the egg phosphatidylcholine reconstitution was 420:1; for reconstitution into egg phosphatidylcholine/egg phosphatidylethanolamine (2:1 w/w), 236:1. All solubilized viral protein was shown to be complexed with phospholipid, and no lipid-free protein aggregates were found on the density gradient.

Fusion Activity. Fusion was determined by monitoring the mixing of contents of the large reconstituted liposomes containing Tb^{3+}/citrate and small sonicated vesicles containing DPA. Controls comprising protein-free hen egg phospholipid large unilamellar vesicles, formed by detergent dialysis to occlude the terbium/citrate chelation complex under conditions identical with the protein–lipid reconstitution, exhibited no leakage of contents at pH 7.4 or 5.0 in the presence of excess DPA (20 μM) at 37 °C. Likewise, the sonicated vesicles (containing DPA) showed only slow leakage of contents in the presence of excess Tb^{3+}. Consequently, when the large protein-free lipid vesicles loaded with terbium were incubated with small unilamellar vesicles entrapping DPA, no fluorescence at pH 7.4 nor after a pH drop, at pH 5.0, was observed (control in Figure 5).

The reconstituted protein–lipid vesicles showed no leakage or fusion at pH 7.4 when incubated with sonicated vesicles containing DPA, similar to the control protein-free system. However, immediately after the pH was lowered to 5.0 by the addition of HCl, a rise in the fluorescence intensity was observed for both types of reconstitutions (Figure 5). This rate of increase was calibrated by determining the maximal fluorescence possible by resolubilizing a fraction of the membrane sample to release all Tb and DPA through the addition of sodium cholate (0.5% w/v). The low-pH-induced fusion activity of the reconstituted viral proteins in egg phosphatidylcholine/egg phosphatidylethanolamine liposomes was calculated to have an initial rate equivalent to 60% of the maximum fluorescence per minute at 37 °C. Both the rate and extent of fluorescence development of the egg phosphatidylcholine reconstitution were approximately 60% that of the egg phosphatidylcholine/egg phosphatidylethanolamine reconstitution. At the end of the experiment, 20 μL of 5 mM EDTA was added by injection which immediately quenched the fluorescence (Figure 5). A parallel experiment conducted throughout in the presence of EDTA exhibited no fluorescence development, demonstrating that the fusion process is leaky and that all the fluorophore produced through vesicle contents mixing is accessible to quenching within the extravesicular medium.

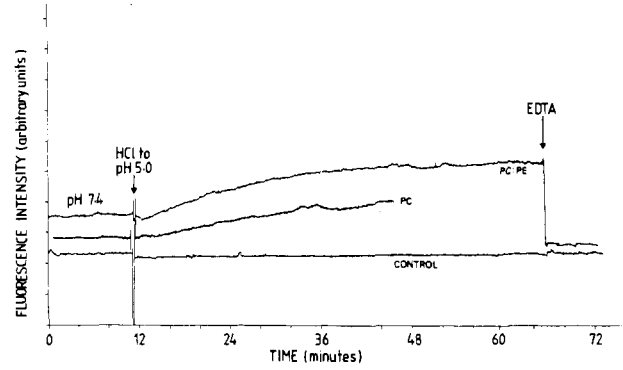

FIGURE 5: Fluorescent intensity changes recorded upon incubation of reconstituted influenza virus membrane proteins into egg PC (middle trace) and egg PC/PE (2:1 w/w) (top trace) liposomes (0.8 mL, 50 μg of protein/mL), occluding a volume containing Tb^{3+}, with small unilamellar vesicles [egg PC/PE (2:1 w/w); 0.2 mL, 5 mg/mL] containing DPA. The pH of the medium was lowered from pH 7.4 to 5.0 by the addition of HCl (see text for details). The addition of EDTA to the extravesicular space abolished the fluorescence from interacting Tb^{3+}/DPA complexes. The response of a protein-free control [large unilamellar vesicles of egg PC/egg PE (2:1 w/w); 1.0 mL, 0.2 mg/mL formed by detergent dialysis, entrapping Tb^{3+}] to the presence of the small vesicles containing DPA at pH 7.4 and 5.0 is also shown (bottom trace).

Discussion

The properties of a detergent which render it capable of biological membrane disruption may not necessarily be related to those which determine the potential efficacy of the detergent to solubilize individual membrane-associated components with retention of their biological activity. Similarly, the most suitable detergent for a defined solubilization need not be the easiest one to later remove under nondenaturing conditions, whether by dialysis or gel filtration, for example. However, in many cases, the detergent is used empirically, with little justification or rationalization for the selected use of one over another. In this study, CTAB, a cationic detergent which is nondenaturing at low concentrations, is used for protein solubilization. Sodium cholate, technically simple to remove to unperturbing levels, is then used to form protein–lipid complexes through detergent dialysis. CTAB is a type A soluble amphiphile (Helenius & Simons, 1975) and is ideally suited to protein extraction, the physical shape of the molecule conferring on it curvature-enhancing properties. Thus, the detergent appears to be able to overcome the relatively low surface curvature of the viral envelope so that particles can

be formed with sufficient curvature to close upon themselves within a small radius.

With a lipid to protein ratio of 500:1 (cf. results of Table II), the molar ratio of detergent to lipid remaining in the final reconstituted particle is calculated to be 0.003:1. This is the lowest level to which sodium cholate may be reduced during reconstitution experiments, either by dialysis or by any alternative method according to Allen et al. (1980). Here it is suggested that vesicles composed predominantly of phosphatidylcholine contain a small number of high-affinity binding sites for cholate. As previously indicated, any residual cholate remaining after the prolonged dialysis would appear to be a function of the lipid rather than the protein since the base level of cholate in the reconstituted system is of the same order of magnitude as that of the pure lipid dialysate (Figure 1). Hence, it is likely that in the preparations described here, detergent–protein interactions are weak.

The reconstituted liposomes prepared in this study have been fully characterized and direct comparisons made between the enzymatic activities of the proteins in the native, detergent-solubilized, and reconstituted environment. Of particular interest is the markedly different sensitivity of the hemagglutinin and the neuraminidase. This has been noted previously (Huang et al., 1979) where it was demonstrated that the neuraminidase appeared relatively indifferent to the nature of its lipid environment while the hemagglutinin activity of the hemagglutinin protein was significantly modulated in certain circumstances. This difference may be due, in part, to the molecular nature of the hemagglutination assay itself and the factors influencing its sensitivity, a topic which has received rather little attention. In this study, the apparent loss in activity of the HA may not be due in denaturation of the protein and consequent loss of the individual activity, but to the density of protein on the liposomal surface which is markedly lower than that of the native virus. Alternatively, it is conceivable that the cooperative action of more than one hemagglutinin trimer may be required tc initiate cell binding (Nir et al., 1986); this would also set a lower limit on interprotein separation.

Hemagglutinin and neuraminidase assays were routinely made of the viral proteins reconstituted into egg phospholipid complexes which were also used for the fusion assay (results not shown). While the neuraminidase retained the same level of activity as its counterpart reconstituted into DMPC, the hemagglutinin titer (HAU per milligram of protein) was reduced by only a factor of 4 relative to the native virus. It is possible, therefore, that there is a connection between the inability of DMPC vesicles to seal tightly, when formed by detergent dialysis, and the potential of HA to induce hemagglutination. The significance of the cohesive pressure exerted by solvating lipids on membrane proteins has already been noted by Lindstrom et al. (1980).

In the present study, vesicle fusion is demonstrated through the mixing of vesicle contents, thereby mimicking the physiological role of viral fusion. The significance of the observation that fusion activity of the reconstituted membrane involved contents mixing as well as leakiness to the previously excluded phase is unclear. It is possible that provided the influenza nucleic acid is only exposed at the appropriate time during its reproductive cycle, it is of no consequence that the fusion event in vivo, on the condition that it occurs between the lysosomal membrane and the virus membrane, is of a leaky nature. Indeed, from a mechanistic viewpoint, the fusion process is easier to explain if this is the case. The release of low molecular weight substances from target large unilamellar vesicles composed of cardiolipin has been detected as a result of intact influenza virus–liposome interaction at pH 5.0 (Stegmann et al., 1985), indicating that the fusion event in vivo may be, albeit transiently, leaky. In addition, no physiological reason for the dependence of influenza virus penetration into the cell on low pH and lysosomes is apparent; such conditions are not observed in the infection cycle of the closely related Sendai virus.

Since the lipids comprising the bilayer of the intact virus are host-derived (Patzer et al., 1979), they would present the same charge on their surface as the anionic surface plane of the host. If this is the case, then close intermembranous contact is electrostatically unfavorable. However, if the packing of viral proteins in the native virus is highly compact, then the length of the protein spike may modify and reduce such possible membrane bilayer charge interaction. In the reconstituted system, where the interprotein distance is significantly greater than that of the virion, then the charge carried by the lipids will be of significance. In this study, the zwitterionic lipids have a neutral overall charge, but since up to 10% of the lipids present may be viral in origin, then the contribution of charge repulsion may be significant. The model system devised here could now be used for an investigation of such electrostatic effects.

Registry No. CTAB, 57-09-0; DMPC, 18194-24-6; NA, 9001-67-6; sodium cholate, 361-09-1.

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Equilibrium and Dynamic Structure of Large, Unilamellar, Unsaturated Acyl Chain Phosphatidylcholine Vesicles. Higher Order Analysis of 1,6-Diphenyl-1,3,5-hexatriene and 1-[4-(Trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene Anisotropy Decay†

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ABSTRACT: Equilibrium and dynamic structural properties of minimally to highly unsaturated acyl chain, large, unilamellar phosphatidylcholine (PC) vesicles have been characterized by the dynamic fluorescence properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH). Fluorescence lifetimes and equilibrium and dynamic rotational properties of these probes were analyzed by limited-frequency phase-modulation fluorometry in egg PC, palmitoyloleoyl-PC (POPC), dioleoyl-PC (DOPC), palmitoylarachidonoyl-PC (PAPC), and palmitoyldocosahexaenoyl-PC (P-22:6-PC) vesicles over a temperature range from 5 to 37 °C. DPH equilibrium orientational distributions were derived according to a model permitting bimodal orientational distributions in which the parallel probability maximum was aligned parallel to the bilayer normal and the orthogonal probability maximum was oriented parallel to the plane of the bilayer. TMA-DPH orientational distributions were derived according to the same model except that all probability was constrained to the parallel orientation. TMA-DPH fluorescence lifetimes were much more sensitive than those of DPH to variations in acyl chain composition and temperature although the same qualitative behavior was generally observed with both probes. Greater acyl chain unsaturation and higher sample temperatures each gave rise to shorter lifetimes consistent with increased water penetrability into the bilayers. Equilibrium order of the hydrocarbon core (as probed by DPH) and of the interfacial and head group regions of the bilayers (as probed by TMA-DPH) was reduced by increasing levels of unsaturation and by higher sample temperatures. The proportion of DPH in the orthogonal orientation increased with greater unsaturation and higher temperatures, indicative of more disorder of acyl chain termini under such conditions. Both probes exhibited accelerated rates of depolarization as temperatures were increased. Rates of DPH depolarizing motion were very similar to those of TMA-DPH at any given temperature.

Model lipid vesicle systems have routinely been used to characterize the physical properties of lipid bilayers as a function of molecular composition. Lipid vesicles may be prepared with defined composition to permit the study of systems that lack the great compositional heterogeneity present in natural biological membranes (Akino & Tsuda, 1979; Benga & Holmes, 1984; Miljanich et al., 1979; Thompson & Huang, 1986). Knowledge of the dependence of lipid physical properties on compositional variation has implications in understanding the nature of lipid–protein interactions and their involvement in mediating membrane-associated functions (Applebury et al., 1974; Baldwin & Hubbell, 1984a,b; Deese et al., 1981; Devaux & Seigneuret, 1985; Jahnig et al., 1982; Litman et al., 1981; O'Brien et al., 1977; Salesse & Garnier, 1984; Stubbs & Litman, 1978; Stubbs et al., 1976). Early work in this area has concentrated on studies of saturated acyl chain phospholipid vesicles. Biological membranes, however, contain substantial and varying proportions of unsaturated acyl chains (Akino & Tsuda, 1979; Benga & Holmes, 1984; Miljanich et al., 1979; Thompson & Huang, 1986). It is

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