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Dynamic properties of Newcastle Disease Virus envelope and their relations with viral hemagglutinin-neuraminidase membrane glycoprotein

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Abstract

The lipid composition of Newcastle Disease Virus (NDV) Clone-30 strain shows a low lipid/protein ratio, a high cholesterol/phospholipid molar ratio, and major phospholipids being qualitatively different to other NDV strains. The major fatty acyl constituents are palmitic, stearic, oleic, and linoleic acids; cerebrosides, sulfatides and two kinds of gangliosides are also found in the NDV membrane. It is reported for the first time in NDV that phospholipid classes are asymmetrically distributed over the two leaflets of the membrane: $60 \pm 4.5\%$ of the phosphatidylcholine and $70 \pm 5.0\%$ of the sphingomyelin are in the outer monolayer. Intact viral membranes and reconstituted NDV envelopes showed similar dynamic properties. Hemagglutinin-neuraminidase (HN) and fusion (F) proteins of NDV membrane affect the lipid thermotropic behaviour in reconstituted proteoliposomes made up of a single class of phospholipids. It is shown that the lipid composition is more important than the bulk membrane fluidity/order for both sialidase (neuraminidase) and hemagglutinating HN activities. Sialidase and hemagglutinating activities requires the presence of definite phospholipids (phosphatidylethanolamine) in its environment. © 1997 Elsevier Science B.V.

Keywords: Viral membrane lipid; Lipid asymmetry; Sialidase activity; Hemagglutinating activity

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TNBS, 2,4,6-trinitrobenzene sulfonic acid; NDV, Newcastle Disease Virus; HN, hemagglutinin-neuraminidase glycoprotein; F, fusion glycoprotein; M, matrix protein; *S*, order parameter; r_s, steady-state anisotropy; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; APL, asolectin phospholipids; PBS, phosphate-buffered saline; PMSF, phenyl methyl sulfonil fluoride; Enzymes, acylneuraminyl hydrolase (sialidase = neuraminidase) (EC 3.2.1.18)

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1. Introduction

Newcastle Disease Virus (NDV) is an avian enveloped single-stranded RNA virus belonging to the family of Paramyxoviridae. The virion consists of a membrane derived from the plasma membrane of infected host cells that encloses a helical nucleocapsid. The nucleocapsid protein (NP), phosphoprotein (P) and a large protein (L) are components of the ribonucleocapside together with a single-stranded RNA. The membrane contains a nonglycosylated membrane M-protein located underneath the lipid bilayer, and two transmembrane glycoproteins: hemagglutin-neuraminidase (HN) and fusion F-protein [1]. These glycoproteins are associated with the viral membrane projecting from the external surface of the lipid envelope.

Enveloped viruses provide a suitable model system for studying membrane structure and function and although it is well established that viral membrane proteins play an important role in the viral life cycle, very little is known about viral lipids role, composition and dynamics. In the present study, we turned our attention to the lipid composition of NDV Clone-30 strain membrane and to some of its physical properties. Detailed information on the lipid composition and organization of the NDV envelope shed light on important processes such as the early events occurring in NDV infection. An asymmetric distribution of the different phospholipid classes over the two membrane monolayers has been observed in a variety of biological membranes, including viral envelopes [2–4]. In this study we report on the asymmetric distribution of the viral phospholipids between the two membrane leaflets. Membrane fluidity was also studied by measuring the movement of hydrophobic DPH fluorescent probe by means of fluorescence polarization spectroscopy. Initially, steady-state fluorescence anisotropy data were interpreted exclusively in terms of microviscosity. However, it has been shown [5] that these data contain not only information on the dynamics but also on the statics (order) of the membrane. In order to investigate some lipid–protein interactions, we prepared reconstituted envelopes from NDV viral membrane and vesicles made from protein-free viral lipids or from commercial lipids plus viral glycoproteins. We studied some of the order and dynamic properties of these systems by measuring the fluorescence polarization of the DPH incorporated into these liposomes and into intact NDV.

Additionally we have checked the effect of lipid composition on the biological activities of HN glycoprotein. The HN protein has two well-established activities: hemagglutinating activity (responsible for NDV interaction with cellular receptors) and sialidase activity, responsible for viral spreading [6]. The role of HN protein in viral fusion has been discussed and in this sense different authors consider that this protein has three different activities: hemagglutinating, sialidase and fusion promotion [7,8].

Reconstitution of functional HN into liposomes could provide a useful model for studying membrane protein functions and interactions between different membrane constituents. Reconstituted viral envelopes have been obtained from different myxoviruses and paramyxoviruses such as influenza [9], Sendai virus [10], Semliki Forest [11] and stomatitis vesicular virus [12]. Among the reconstitution methods used for membrane proteins, the detergent removal procedure is one of those most commonly described. In the present paper we describe two different reconstitution procedures from NDV membrane components: (1) reconstituted envelopes from detergent-solubilized membranes and (2) virosomes made from glycoprotein/exogenous phospholipid/detergent mixtures. The protein/phospholipid ratio and classes of proteins present in the reconstituted vesicles, the size of the vesicles, sialidase and hemagglutinating activity and reconstitution efficiency were analysed.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine from egg yolk, Phosphatidylethanolamine from egg, L-α-Phosphatidylserine from brain, Phosphatidylinositol from bovine liver and Sphingomyelin from brain were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The probe 1,6-Diphenyl-1,3,5-Hexatriene (DPH) was obtained from Aldrich (Milwaukee, WI, USA) and from Molecular Probes, Inc. (Junction City, OR, USA), and picryl sulfonic acid (TNBS) from Serva Feinbiochemica (Heidelberg, Germany).

The enzyme Phospholipase C from *Clostridium perfringens* (EC 3.1.4.3.), soybean asolectin (L-α-Phosphatidylcholine type II from soybean), chicken erythrocytes, Triton X-100 and 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bio-beads SM2 (50–20 mesh) and PAGE reagents were acquired from Bio-Rad (Richmond, CA, USA).

2.2. Virus growth and purification

The lentogenic 'Clone-30' strain of NDV was used. The virus was grown in the allantoic cavity of 11-day-old Specific Pathogen-Free chick embryos and purified as previously described [13].

2.3. Extraction and analysis of lipids

Total lipids from NDV were routinely extracted by the methods of Bligh and Dyer [14] and Rose and Oklander [15], the latter method being used when only very small samples were available. The different classes of lipids were analysed by TLC on Silica Gel 60 plates from Merck (Germany) according to González-Ros et al. [16], by the one-dimensional procedure for neutral lipid analysis or the two-dimensional procedure for phospholipid analysis. The latter procedure completely separated all the major phospholipid species of the virus except phosphatidylserine and phosphatidylinositol.

The glycolipid composition of the three phases (chloroformic, methanolic-aqueous, and aqueous), which were obtained by the procedure of Bligh and Dyer [14], was analysed by one-dimensional TLC with the solvent system chloroform/methanol/water (50:45:10, v/v/v) with 0.02% $CaCl_2$. The plate was developed with the resorcinol reagent as described by Svennerholm [17], where gangliosides showed a purple colour, or with the orcinol reagent for the detection of cerebrosides (25 ml of orcinol reagent was made up with 25 mg of orcinol in a volume of a mixture of 35 ml of H_2SO_4 and 12 ml of water).

2.4. Fatty acid analysis

Fatty acid analysis was performed by GLC. Fatty acid methyl esters were prepared by the method of Metcalfe et al. [18] by hydrolysing and methylating the phospholipid fraction (total lipids from NDV membrane or scraped phospholipid spots separated by TLC) in the presence of 1 ml of BF_3-methanol (Supelco Inc., Bellefonte, PA, USA), at 100°C for 3 min under a N_2 atmosphere. The fatty acid methyl esters were extracted with isooctane and then analysed in a Konik 3000 gas chromatograph with a FID hydrogen flame detector and a fused silica capillary column (30 m × 0.53 mm) from Supelco Inc. (Bellefonte, PA, USA). Appropriate fatty acid standards from Supelco were also used. The area under each peak was calculated using a Konik SP 4290 (Spectra-Physics) integrator.

2.5. Phospholipase C treatment

The hydrolysis of viral phospholipid by this enzyme was performed according to Patzer et al. [3] and Roelofsen and Op den Kamp [19]. Phospholipase C was dissolved in 20 mM Tris-HCl, 100 mM NaCl (pH 7.4), and stored at 4°C. Virions were suspended in the same buffer rendered 1.3 mM with respect to $CaCl_2$ at a concentration of 1 mg/ml and treated with 0.2 unit/ml of lipase for 2, 3 or 4 h. The reaction was stopped by the addition of 3.75 volumes of chloroform/methanol (1:2, v/v) and 1 volume of 24 mM EDTA at a concentration of 4 mM and lipids were extracted as described by Bligh and Dyer [14].

2.6. TNBS labelling

A 9 mM aqueous solution of TNBS was prepared in 5% $NaHCO_3$ and stored at 4°C in the dark until use. Virions were suspended in 100 mM $NaHCO_3$, 50 mM KCl (pH 8.6) at a total protein concentration of 0.5 mg/ml, and TNBS was added at a final concentration of 3.25 mM of the probe, according to Patzer et al. [3]. The reaction was stopped by the addition of 1/5 volumes of 1 M HCl. Viral lipids were extracted using chloroform/methanol (2:1, v/v) and analysed by TLC running the first dimension in a solvent system of chloroform/methanol/water (65:25:4, v/v/v).

2.7. Preparation of lipid vesicles

Total lipids from NDV membrane were extracted by the method of Bligh and Dyer [14]. The organic solvents were evaporated with N_2 and then placed under vacuum for 3 h to eliminate any residual

solvent. Dried lipids were resuspended in PBS (pH 7.4) at a lipid concentration of 5 mg/ml. After 30 min at room temperature under a N_2 atmosphere, samples were subjected to high-intensity ultrasonic irradiation for 5 min under a stream of N_2 at 4–10°C using a Branson B-30 sonicator. Suspensions were then centrifuged at $100\,000 \times g$ for 60 min in order to pellet large liposomes. The supernatant was used as the source of lipids vesicles.

2.8. Reconstitution of NDV envelopes

Purified virions were solubilized at a total protein concentration of 5 mg/ml with 2% (v/v) Triton X-100 and at high ionic strength (1 M KCl), as described by Scheid et al. [20] and Scheid and Choppin [21]. Viral envelope-solubilized proteins were isolated from ribonucleocapsids and intact viruses by pelleting the latter at $200\,000 \times g$ for 90 min. M protein was eliminated after dialysis against 10 mM Tris-HCl, 150 mM NaCl, 0.05 mM PMSF (pH 7.4) and precipitation by centrifugation at $12\,000 \times g$ for 30 min. The detergent present in the supernatant was removed by direct addition of SM-2 Bio-beads to solubilized envelopes without M protein, according to Vainstein et al. [10], with some modifications. The polymer was previously washed with methanol as reported by Holloway [22] and 40 mg of SM-2 Bio-beads per each 6 μl of Triton X-100 (the Bio-beads/Triton X-100 ratio was 7:1, w/w) was added. After gentle shaking at room temperature for 3 h, a second similar portion of SM-2 Bio-beads was added concomitantly with a volume of the buffer. After an additional incubation period of 12 h, the liquid phase was collected by a polymer-proof syringe. The solution was centrifuged at $12\,000 \times g$ for 30 min in order to eliminate high molecular weight aggregates and SM-2 Bio-bead remains. The supernatant was later centrifuged at $100\,000 \times g$ for 90 min and the pellet, containing the reconstituted viral envelopes, was resuspended in 100–500 μl of 10 mM Tris-HCl, 150 mM NaCl, 0.05 mM PMSF (pH 7.4) buffer or PBS (pH 7.4).

2.9. Reconstitution of vesicles containing viral glycoproteins HN and F

Membrane vesicles, containing HN and F glycoproteins and a single class of phospholipid, were prepared as follows: commercial phospholipids were suspended in an appropriate volume of 10 mM Tris-HCl, 150 mM NaCl, 0.05 mM PMSF (pH 7.4) buffer containing 2% (v/v) Triton X-100, to achieve a lipid concentration of 2 mg/ml. Samples were kept at room temperature for 30 min to allow lipid solubilization and then mixed with an aliquot of solubilized envelopes in the same buffer. The lipid/protein ratio was 0.5 (w/w). This solution was shaken thoroughly and maintained at room temperature for 2 h. Then, Triton X-100 was removed with Bio-beads as described above.

2.10. Sialidase (neuraminidase) assay

Enzyme activity (EC 3.2.1.18) was determined by the fluorimetric procedure [23], essentially as described by Cabezas et al. [24], using 2́-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid as substrate at 37°C. One enzyme unit (U) was defined as the amount of enzyme activity that gave 1 μmol of product in a minute. Specific activity was expressed as enzyme units per mg (U/mg).

2.11. Hemagglutinating activity

Hemagglutination assays were performed according to Barret and Inglis [25] using 2% chicken erythrocytes at pH 7.4 in PBS. Serial 2-fold dilutions of samples in PBS were set up in 96-well microtiter plates. An equal volume of 2% red blood cells was added to each well. The plate was gently shaken to mix the contents of each well and incubated for 60 min at room temperature. Sedimentation of the cells was observed in each well. Hemagglutinating activity (HAU) was defined as the smallest concentration of virus able to inhibit the sedimentation of red blood cells; the hemagglutination titre was expressed as HAU/ml or HAU/mg.

2.12. Fluorescence measurements

Fluorescence labelling of NDV viral membranes with DPH and steady-state fluorescence anisotropy measurements were done according to Shinitzky and Barenholz [26]. All experiments were performed on a Hitachi F-4010 fluorescence spectrophotometer with polarisers in the excitation and the emission beams.

The DPH probe was dissolved at a concentration of 10 mM in tetrahydrofuran and stored at −80°C in the dark. When used, the probe was diluted to 0.25 mM in tetrahydrofuran and 1 μl of this solution was added to a 4-ml aliquot of virus or membrane samples in PBS. The solution was mixed thoroughly for 10 s and then incubated for 90 min at the desired temperature. After excitation of samples at 360 nm, the emission light at 430 nm was simultaneously analysed through a polarizer oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of polarization of the excitation beam. The correcting factor (G) for the polarization inherent to the instrument was measured using vertically polarized excitation light. All $I_{\parallel}$ and $I_{\perp}$ values were corrected for light scattering by subtracting the values of the measurable intensities in the absence of DPH. Steady-state fluorescence anisotropy (r_s) was calculated using the relationship:

$$r_s = (I_{\parallel} - I_{\perp} G)/(I_{\parallel} + 2I_{\perp} G)$$

The r_s values were means of 3 measurements taken in the same sample.

The temperature dependence of the *S* lipid order parameter of DPH was calculated from the r_s data, according to the relationship proposed by Van der Meer et al. [27]:

$$S^2 = \left[r_o r_s^2/\left(r_o r_s + (r_o - r_s)^2/m\right)\right]/ro$$

where r_o is the maximal fluorescence anisotropy value in the absence of any rotational motion of the fluorophore (0.362 for DPH) and m = 1.71.

Data were fitted by non-linear regression with the SIMFIT version 3.86 computer package (for simulation, curve-fitting and statistical analysis, developed by Dr. W.G. Bardsley, University of Manchester, UK). To visualize the inflection points, which are indicative of phase transitions in membranes, curves as a function of temperature were smoothed by the cubic splines method and their first order derivatives were calculated using the same computer package.

2.13. Determination of Triton X-100

The amount of Triton X-100 in the samples was determined as previously described [28].

2.14. SDS polyacrylamide gel electrophoresis

This was carried out under reducing conditions, employing the method of Fairbanks et al. [29] with a slight modification: the 0.75-mm thick polyacrylamide slab gel consisted of a continuous 5–25% (w/v) gradient gel. 1 μg of protein was applied to each lane. Gels were silver-stained according to Merril et al. [30]. Prior to SDS-polyacrylamide gel electrophoresis, Triton X-100, when present, was removed from the samples by butanol precipitation, essentially as described by Scheid and Choppin [21].

2.15. Sucrose density gradient centrifugation

After reconstitution, the incorporation of proteins into membranes was monitored by discontinuous flotation gradients [31]. 500 μl of the samples were thoroughly mixed with 1500 μl of sucrose (50% w/v) in 10 mM Tris-HCl, 150 mM NaCl, 0.05 mM PMSF (pH 7.4) buffer in 12 ml centrifuge tubes. 2 ml each of 40%, 30%, 20%, and 10% sucrose (w/v) were layered, followed by 2 ml of 10 mM Tris-HCl, 150 mM NaCl, 0.05 mM PMSF (pH 7.4). The samples were centrifuged at $220\,000 \times g$ for 24 h in a Beckman SW 40Ti rotor. Fractions of 0.5 ml were collected from the top to the bottom and were analysed for lipid and protein contents.

2.16. Electron microscopy

The reconstituted vesicles were examined by electron microscopy. Briefly, the samples were fixed with glutaraldehyde and embedded in an EPON-812 resin and contrasted with uranyl acetate and lead nitrate. Samples were then examined under a Zeiss 900 transmission electron microscope.

2.17. Additional methods

Phosphorus determination was carried out as described by Rouser et al. [32]; cholesterol was determined using the procedures of Zlatkis et al. [33] and Courchaine et al. [34]. Protein concentrations were determined according to the method of Markwell et al. [35] using BSA as standard.

3. Results

3.1. Lipid composition of NDV membrane

Table 1 shows the overall lipid (a) and phospholipid (b) composition present in NDV 'Clone-30'.

The aminophospholipids present in the viral membranes represented a large proportion of total phospholipids (46%).

Most of the glycolipids from NDV membranes were found in the more polar phases (methanolic-aqueous and aqueous) obtained in the lipid extraction by the procedure of Bligh and Dyer [14] (data not shown). The glycolipid composition of these phases was analysed by TLC. Galactosyl or glucosylceramides, cerebrosides with a long carbohydrate chain (with a lower R_f than galactosyl or glucosylceramides), sulfatides, and the gangliosides GD_{1b} and GT_{1b} were also detected.

3.2. Fatty acid composition of NDV

Table 2a shows the overall fatty acid composition of NDV viral membrane phospholipids and also the fatty acids present in three of the major viral phospholipids separated by TLC. Basically, four major fatty acids were the most abundant phospholipids: palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2). These four fatty acids represented more than 70% of the total NDV viral fatty acids and were distributed among the individual phospholipids in different proportions: 70% in PE; 64% in PC, and 84% in PS + PI. The fatty acid constituents of PE closely resembled the general pattern of total phospholipid fatty acids. PC had the highest percentage of palmitic and lowest percentage of stearic acid. The most remarkable difference in the fatty acid pattern of the PS + PI fraction was seen in the high percentage of stearic and the low proportion of palmitic acid. This fraction had the highest degree of saturation while PE had the highest degree of unsaturation, although in all the cases the saturation/unsaturation ratio was close to 1 (Table 2b).

3.3. Distribution of major phospholipids within the two layers of NDV viral membrane

The localization of PC and SM was accomplished by treating intact NDV with phospholipase C. The hydrolysis of PC with phospholipase C (data not shown) reached a plateau at 2 h, and of SM at 3 h. In both cases, incubation for 4 h did not increase the percentage of hydrolysis, supporting the notion that the enzyme did not compromise the membrane integrity of the viral particles. Phospholipase C treatment of total NDV octyl glucoside-solubilized lipids showed that $96 \pm 4\%$ of total viral PC and $92 \pm 5\%$ of total SM were hydrolysed. These data were used for correcting the percentages of hydrolysed phospholipids. According to these values, the choline-phospholipids PC and SM are preferably externally oriented, $60 \pm 5\%$ and $70 \pm 5\%$, respectively, being located at the external leaflet.

The distribution of aminophospholipids, PE and PS, was analysed using the non-penetrating reagent TNBS. Labelling reached a plateau for both lipids, at 60 min for PE and 90 min for PS (data not shown). The lack of additional labelling after 120 min of reaction showed that TNBS did not penetrate the membrane of intact NDV virions. The percentages of total PE and PS labelled with TNBS were $47 \pm 3\%$ and $54 \pm 3\%$, respectively, which means that both aminophospholipids are distributed about equally between the inner and outer layers of the viral membrane. These data were calculated considering the rate of labelling of completely detergent-solubilized aminophospholipids. All (100%) of the PE was labelled in solution together with a $98 \pm 1\%$ of the PS + PI fraction.

Table 1
Lipid composition of NDV Clone-30 membrane

(a) Overall lipid composition *	
Total lipids	$17.4 \pm 0.8\%$
Phospholipids/total lipids (w/w)	0.6 ± 0.1
Total phospholipids	$10.2 \pm 0.6\%$
Cholesterol/total lipids (w/w)	0.24 ± 0.01
Total cholesterol	$4.2 \pm 0.5\%$
Cholesterol/phospholipids (w/w)	0.51 ± 0.04
Cholesterol/phospholipids (molar ratio)	1.02 ± 0.08
(b) Phospholipid composition **	
Phospholipid	**Percent composition**
Phosphatidylethanolamine	26.8 ± 0.9
Sphingomyelin	23.0 ± 0.8
Phosphatidylcholine	22.7 ± 0.9
Phosphatidylserine + phosphatidylinositol	15.5 ± 0.6
Lysophosphatidylethanolamine	4.2 ± 0.3
Lysophosphatidylcholine	3.5 ± 0.8
Phosphatidic acid	2.8 ± 0.2
Phosphatidylglycerol	2.1 ± 0.2

* ± S.E. of 5 separate determinations.
** ± S.E. of 20 separate determinations.

Table 2
Fatty acid composition of NDV Clone-30 membrane

(a) Distribution in percentages of major fatty acids in total lipids and main phospholipids of NDV membrane				
Fatty acids	Total lipids [a]	PE [b]	PC [c]	PS + PI [b]
Saturated				
15:0	3.5 ± 0.5	1.6 ± 0.7	0.8 ± 0.3	0.34 ± 0.23
16:0	19.9 ± 1.4	14.2 ± 0.7	29.0 ± 2.3	12.0 ± 0.8
17:0	3.6 ± 0.5	3.2 ± 1.2	0.13 ± 0.06	0.77 ± 0.16
18:0	17.5 ± 0.4	16.8 ± 2.1	8.0 ± 0.5	38.1 ± 1.3
20:0	1.04 ± 0.03	0.6 ± 0.1	0.07 ± 0.05	1.83 ± 0.17
Monoenoic				
16:1	1.0 ± 0.1	0.27 ± 0.08	0.34 ± 0.13	N.D.
18:1	22.0 ± 0.4	27.6 ± 2.0	15.3 ± 1.7	23.4 ± 1.0
22:1	1.00 ± 0.15	0.33 ± 0.14	1.06 ± 0.41	1.20 ± 0.27
Polyenoic				
18:2	11.7 ± 0.3	11.8 ± 0.7	12.1 ± 0.9	10.1 ± 0.6
20:3	6.5 ± 0.2	6.96 ± 0.83	2.11 ± 1.04	N.D.
21:5 n-3	1.29 ± 0.07	1.34 ± 0.35	2.26 ± 1.04	N.D.
22:6 + 24:0	5.33 ± 1.18	6.3 ± 1.1	4.2 ± 1.0	2.1 ± 0.9
Total	94.4	90.8	75.4	89.8
(b) Percentages of saturation and unsaturation of total and individual species of phospholipids in NDV virus [d]				
Saturated	52.1 ± 0.8	43.3 ± 07	44.7 ± 2.5	55.9 ± 0.6
Monoenoic	24.4 ± 0.5	28.3 ± 1.9	17.8 ± 1.3	26.8 ± 0.9
Polyenoic	27.1 ± 1.5	30.9 ± 2.8	29.8 ± 1.9	18.6 ± 1.9
Saturated/unsaturated [e]	1.02 ± 0.03	0.74 ± 0.03	0.96 ± 0.07	1.3 ± 0.03

N.D. Not detected.
[a] ± S.E. of 3 separate determinations.
[b] ± S.E. of 5 separate determinations.
[c] ± S.E. of 6 separate determinations.
[d] Data calculated from (a).
[e] Ratio calculated from percentages of saturated fatty acids to percentages of unsaturated fatty acids.

3.4. Dynamic properties of intact NDV membrane, reconstituted envelopes and liposomes

Fluorescence depolarization of the hydrophobic probe DPH was used to study some of the physical characteristics of these NDV systems. All experiments were performed with a ratio of lipid molecules to DPH probe of 280, in the case of intact virus, or 450 in liposome and reconstituted envelope samples. In both cases, the concentration of DPH lay in the linear part of the probe concentration vs total fluorescence intensity curve (data not shown).

The rate of DPH penetration into the hydrophobic region of the viral membrane and liposomes, calculated as the time in minutes required for 50% (t_{50}) of the maximal total fluorescence intensity to be reached,

Table 3
Anisotropy (r_s) and S parameters of intact Clone-30 NDV, reconstituted envelopes and liposomes at three temperatures [*]

	10°C		25°C		37°C	
	r_s	*S*	r_s	*S*	r_s	*S*
NDV	0.32 ± 0.004	0.94 ± 0.006	0.28 ± 0.006	0.86 ± 0.007	0.25 ± 0.003	0.80 ± 0.004
Reconstituted envelops	0.31 ± 0.002	0.92 ± 0.002	0.28 ± 0.008	0.86 ± 0.011	0.24 ± 0.002	0.77 ± 0.002
Liposomes	0.26 ± 0.002	0.83 ± 0.003	0.22 ± 0.001	0.72 ± 0.016	0.19 ± 0.001	0.66 ± 0.002

[*] Data are expressed as means ± S.E. of 3 separate experiments.

was the same (0.55 ± 0.1 and 0.52 ± 0.1 min respectively). This could mean that the membrane surface proteins do not affect probe incorporation. However, the rate of incorporation into reconstituted envelopes was lower, 0.77 ± 0.02 min. When the envelopes were reconstituted with M protein (by not including the dialysis step in the reconstitution procedure described in Section 2), t_{50} showed an intermediate value (1.18 ± 0.3) between those of intact NDV and reconstituted envelopes.

Table 3 shows the effect of temperature on the r_s values and *S* parameters for intact virions, reconstituted envelopes and liposomes. The reconstituted envelopes showed thermotropic behaviour similar to that of intact NDV, which is consistent with the similarities in the lipid and intrinsic protein compositions of both systems (data not shown). However, from our data differences are seen in the absolute anisotropy and *S* parameter values, at all three temperatures studied, between viral membranes and lipid vesicles prepared from viral membrane. In the latter, these parameters had lower values.

The temperature-dependent changes in r_s values for the DPH-labelled membranes are shown in Fig. 1. $I_{\parallel}$ and $I_{\perp}$ were measured each approximately every 5°C after 10 min for temperature stabilization. For all temperatures studied anisotropy was lower in liposomes than in intact virus and reconstituted envelopes. In the latter two systems, r_s had similar values, although they were slightly lower in reconstituted envelopes at low temperatures and slightly higher at high temperatures. The plots of r_s vs temperature (Fig. 1) pointed to a continuous decrease, with no observed discontinuities. Fig. 2 shows the temperature dependence of the structural order parameter of DPH in the three types of samples investigated. Computer-smoothed first derivatives vs temperature were plotted in order to detect breaks in the temperature profiles of *S* [36]. The coefficient R^2 had values higher than 0.99, as deduced from the transformed data fitted with the SIMFIT computer package. Reconstituted envelopes and intact NDV membranes had the same break point at 38.5 ± 0.5°C; in the case of liposomal membranes the inflection in the derivative plot was 1 ± 0.5°C higher.

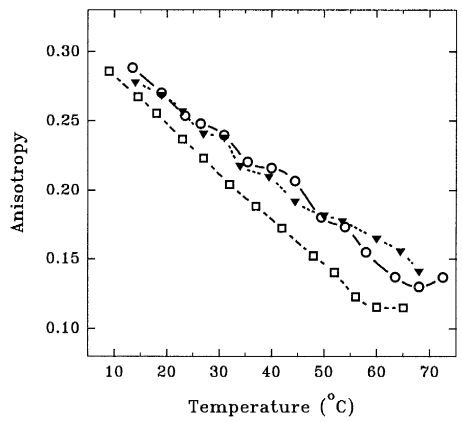

Fig. 1. Temperature dependence of steady-state fluorescence anisotropy in DPH-labelled systems derived from NDV: ○, intact NDV; ▼, reconstituted envelopes; □, liposomes. Data are from a single representative experiment; each point represents the average of three determinations.

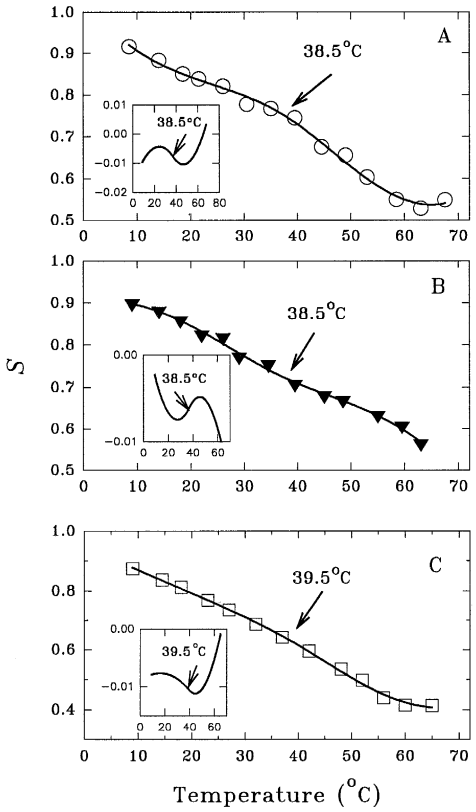

Fig. 2. Temperature dependence of the order parameter of DPH (S) in three systems derived from NDV membrane: A (○), intact NDV; B (▼), reconstituted envelopes; C (□), liposomes made from NDV lipids. Insertions: the corresponding first derivatives were calculated, smoothed and plotted vs temperature. Inflection points are indicated by arrows. Data are from a single representative experiment; each point represents the average of three determinations.

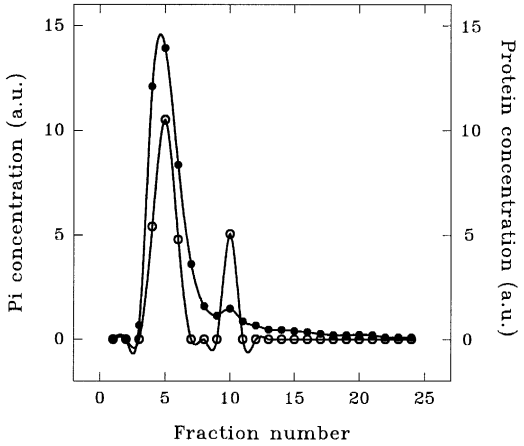

Fig. 3. Sucrose flotation ultracentrifugation gradient of PC virosomes. Vesicles were constituted by HN and F glycoproteins and PC as exogenous phospholipid. The gradient ranged from 0% sucrose on the top to 50% of sucrose on the bottom. Fractions were extracted from the top (no. 1) to the bottom (no. 24). On left axis, phospholipid concentration (●) in arbitrary units (a.u.); on right axis, protein concentration (○) in arbitrary units (a.u.).

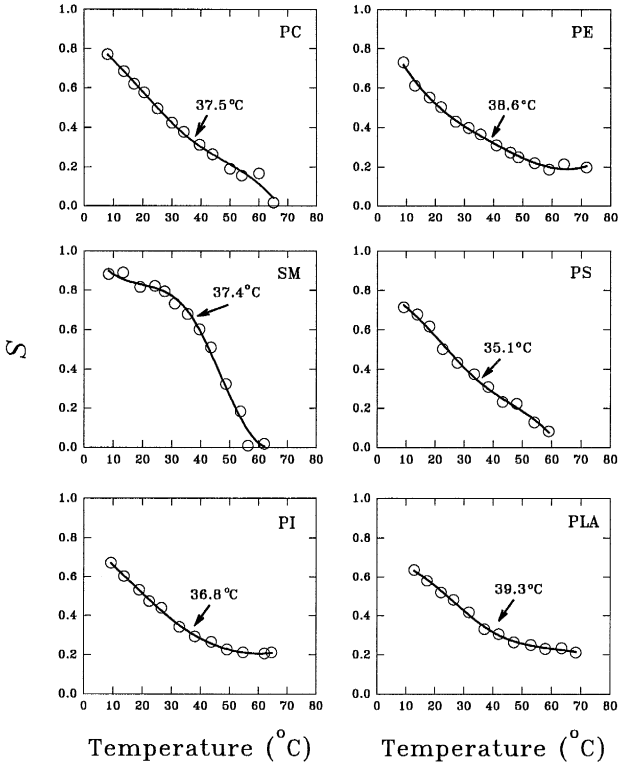

Fig. 4. Temperature dependence of the order parameter of DPH (S) in reconstituted vesicles with the HN and F proteins, and with the lipids PC, PE, SM, PS, PI and the complex mixture of asolectine (PLA). PLA composition is 59.5% PE, 10.9% PC, 8% PS + PI, 10% PA, 4% DPG, 3.1% SM; 9 mg/ml of cholesterol. Inflection points are indicated by arrows. Data are from a single representative experiment; each point represents the average of three determinations.

We also studied the effect of the two intrinsic viral glycoproteins, HN and F, on the thermotropic behaviour of lipid vesicles composed of a single phospholipid species. After reconstitution, vesicles were analyzed by discontinuous flotation sucrose gradients (0–50%, w/w) as detailed in Section 2. The positions of the lipids and proteins were determined in the aliquots collected from the gradient (Fig. 3). Reconstituted envelopes appear as a turbid band in the top half of the gradient tube. The major protein peak (80.5% of the total proteins in the gradient) and the major lipid peak (87% of total lipids) coincided, meaning that the procedure was affording proteoliposomes. Reconstituted vesicles were also analyzed by SDS-PAGE (data not shown) being F protein the major protein component in the reconstituted proteoliposomes. The proportions of M protein varied within 1–15% range of total proteins, as determined by

Table 4
Sialidase and hemagglutining activities of HN protein in virosomes constituted with a single molecular species of phospholipid and HN and F proteins. Both activities are expressed taking the activities of the reconstituted envelopes as 100%

Phospholipid	Sialidase activity (mU/mg)	% of sialidase activity (HAU/mg)	Hemagglutinating activity	% of hemagglutining activity
Control	1450	100	55650	100
PC	1387	96	24151	43
PE	2818	194	49231	89
SM	511	35	11852	21
PS	98	7	6154	11
PI	0	0	8421	15
APL	2068	143	51200	92

Table 5
Anisotropy, r_s, and structural order parameter, S, of control envelopes and virosomes reconstituted with different lipid environments

	Control [1]	PC [2]	PE [2]	SM [2]	PS [2]	PI [2]	APL [2]
r_s	0.234 ± 0.003	0.086 ± 0.0001	0.090 ± 0.007	0.156 ± 0.003	0.075 ± 0.001	0.084 ± 0.003	0.098 ± 0.0005
S	0.761 ± 0.006	0.311 ± 0.0004	0.33 ± 0.02	0.548 ± 0.01	0.272 ± 0.004	0.309 ± 0.008	0.356 ± 0.002

[1] ± S.E. of 6 separate determinations.
[2] ± S.E. of 3 separate determinations.

densitometry. The lipid percentage was found to be higher than 83% of the total phospholipids in the vesicle. We studied six different proteoliposomes, five with a single lipid species (PC, PE, SM, PS and PI) and the sixth with the complex mixture of asolectin phospholipids (the composition of asolectin vesicles is specified in the legend to Fig. 4). The percentage of the lipid was found to be higher than 83% of the total phospholipids in the vesicle. As in intact NDV and reconstituted envelopes, the discontinuities in the plot of *S* vs temperature were calculated from the corresponding slopes of smoothed first derivatives. These temperatures are shown in Fig. 4. For all types of liposomes, except for SM vesicles, as mentioned, the break points were higher than the transition temperature described in the literature for liposomes without proteins. For liposomal membranes obtained with negatively charged lipids, PS and PI, the breaks observed in the derivative plot of *S* occurred at 35.1 ± 0.5°C and 36.8 ± 1.0°C respectively. For SM vesicles, the temperature was 37.4 ± 0.5°C, similar to the described transition temperature. For PE membranes, the break was at 38.6 ± 0.3°C and for asolectin vesicles, the discontinuity temperature lay at 39.3 ± 0.2°C.

3.5. Influence of the lipid environment on the biological activities of HN glycoprotein

The lipid environment of HN and F protein was modified with pure phospholipids (as detailed in Sections 2 and 3.4), investigating the sialidase and hemagglutinating activities of HN protein in the vesicles constituted with different phospholipids (Table 4). The activities of virosomes containing different phospholipids were compared with those of reconstituted envelopes composed of the two viral glycoproteins and total lipids from NDV membrane as controls. In one set of experiments, different concentrations of exogenous PC added to the solubilized envelopes were assayed. Almost the same sialidase and hemagglutinating activities were obtained in the reconstituted vesicles with different PC/protein ratios (w/w) (data not shown). The phospholipid/protein ratios (w/w) were always measured in the virosomes. No correlation between these values and the biological activities of HN was found (data not shown). The results obtained showed that PE and asolectin phospholipids led to an increase in sialidase activity (194% and 143% as compared with controls); in PC virosomes this activity was similar to that of the controls, whereas SM, PS and PI caused a strong inhibition of sialidase activity; about 65%, 93% and 100%, respectively (Table 4).

The results for hemagglutinating activity were different from those of the sialidase activity: PE and asolectin phospholipid did not significantly influence hemagglutinating activity (89% and 92% of control activity). However, in PC, SM, PI and PS vesicles activity appeared to be inhibited, with 43%, 21%, 15% and 11% of the activity detected in control envelopes (Table 4).

On investigating some of the fluidity/package parameters (Table 5), a reduction in the steady-state fluorescence anisotropy and the structural lipid order parameter (*S*) were detected in all virosomes constituted with exogenous phospholipids and viral glycoproteins as compared to the parameters from reconstituted control envelopes. The highest values were observed in SM vesicles and the lowest ones in PS vesicles. Nevertheless, the enrichment of the HN lipid environment with all the phospholipids assayed had different effects on the biological activities of the protein (Tables 4 and 5).

4. Discussion

The importance of viral lipid composition in the infectivity process has only begun to be understood.

Treatment of NDV or other enveloped viruses with several lipophilic drugs, such as butylate hydroxytoluene has been shown to significantly block viral infectivity [37]. It seems that the lipid composition of RNA-viruses is critical and derives from sequestration of specific lipids from the host cell surface membrane lipids [38,39]. Similar to other enveloped RNA viruses [3,40,41], NDV has a low lipid/protein (w/w) ratio (0.21) and a high molar cholesterol/phospholipid ratio (1.02).

Cholesterol is the major neutral lipid present in NDV membrane. A high cholesterol/phospholipid ratio has been shown to be essential for the infectivity of many enveloped viruses (see, for example, Ref. [42]). The high ratio in the NDV Clone-30 membrane could be necessary for the fusion process between the host cell and viral membranes, since in Sendai virus (a very related paramyxovirus) it has been shown [43] that cholesterol is necessary for the fusion process.

Our analysis of NDV membrane lipids by two-dimensional TLC showed that PE, PC, SM and PS + PI (Table 1b) are the major phospholipids present in NDV membrane. Roughly, the phospholipid class pattern seems to be similar to those of Semliki Forest and SV5 viruses [44], vesicular stomatitis virus [45], Sendai virus [46] and HIV [39]. However, if we compare in detail this pattern with those of different NDV strains important differences can be seen. These differences are particularly significant between the phospholipid class distribution in NDV Clone-30 strain and that of the NDV Blacksburg strain described by Blough and Lawson [47], which to our knowledge is the main bibliographic reference for NDV lipids. Although PE is in both strains the major phospholipid, noticeable differences are in the proportions of PE, PS + PI and PA higher in Blacksburg than in Clone-30 and in the percentages of SM and PC lower in the strain studied by Blough and Lawson [47] than in Clone-30. Since both strains, Blacksburg and Clone-30, were grown in the allantoic cavity of chick embryos, such differences must be strain-specific. Results presented here differ from those of Sakaguchi et al. [48] and Toyoda et al. [49]. These authors compared the sequences of HN and F proteins in different NDV strains and divided them in three different lineages. The strain studied by Blough and Lawson [47] and LaSota strain [50], from which our Clone 30 derived, belong to the same lineage, with very close nucleotide and amino acid sequences on F and HN proteins. Therefore, according to the data presented here, both strains present more differences in the lipids selected from those of the host membrane. The few differences found in [48,49] may be enough for controlling the lipid composition of the viral envelope and/or the sequestration of lipids may be also influenced by the M adjacent membrane protein. For this reason, lipid analysis of the viral membrane could be a useful tool for strain identification, in addition to other methods.

Glycolipids play an important role in cell surface phenomena [6] but their function in viruses is still unknown. The presence of gangliosides in the NDV Clone-30 membrane is surprising, taking into account the existence of a neuraminidase (sialidase) activity associated to the HN protein. It is widely accepted that this sialic acid-cleaving activity has a very important role in the release of newly formed virions from the cell surface after viral budding [1], thus avoiding viral aggregation. Klenk and Choppin [6] detected trace amounts of gangliosides in only one sample of BHK-grown viruses after checking their presence in several samples of viruses grown in four different types of cells. Our analysis of Clone-30 NDV lipids by TLC shows that galactosyl and/or glucosyl ceramides, cerebrosides with a long carbohydrate chain, sulfatides and the gangliosides GD_{1b} and GT_{1b} are present in all the samples of NDV Clone-30 membrane (data not shown). The neutral glycolipids with long carbohydrate chains could be products of the HN sialidase activity coming from gangliosides such as GD_{1b} or GT_{1b}. These compounds have similar carbohydrate chains with neuraminic acid in different positions. The presence of the above-mentioned sialic acid-containing glycolipids could be due to the failure of the HN glycoprotein sialidase activity to cleave all sialic residues present in newly synthesised virions. However, other possibilities could be that complete removal of such residues might be not necessary to avoid viral aggregation, or that some sialic acid residues would not be accessible to cleavage. It is also possible that uncleaved sialic residues in glycolipids could be involved in virus-cell recognition events or have other unknown roles.

We report, to our knowledge for the first time, the asymmetric distribution of the different phospholipid

classes over the two monolayers of NDV membrane. From comparison of phospholipid distribution in erythrocyte [51] and in NDV Clone-30 membranes (this work) we have found that the most noticeable difference in the lipid topology was in the distribution of aminophospholipids. It was shown that in both systems choline-containing phospholipids are predominantly at the outer layer, although for NDV more PC and SM are found in the inner leaflet. Similar findings have been reported for influenza virus [2] and stomatitis vesicular virus [52]. In Semliki Forest virus, however, the membrane topology is different; SM lies predominantly at the inner layer [4]. Aminophospholipids are found at the outer layer in erythrocytes although in our NDV strain they are symmetrically distributed, as detected by TNBS-labelling of their amino-groups. These results are similar to those described for influenza virus [2] and to those on PE distribution in stomatitis vesicular virus [3].

The function of transbilayer distribution in membrane fusion processes is a matter of current investigation. According to Devaux [53], transmembrane asymmetry gives each membrane a vectorial character that may be important for membrane–membrane contacts. Only the layer that contains aminophospholipids is fusion-competent. Erythrocytes with asymmetric distribution of PS (concentrated in the inner monolayer) do not exhibit spontaneous cell fusion, which would be observed if the aminophospholipids were symmetrically distributed [54]. Farooqui et al. [55] have found that PC and/or PS are necessarily located at the outer leaflet for calcium-induced fusion with bovine erythrocytes. The high percentage of PE and PS in the external monolayer found by us in the NDV membrane could be essential for the fusion of the virus with host cell membranes. Moreover, this distribution may be necessary for the activating effect that PE exerts on the biological activities of HN protein, as mentioned above.

The outer localization of PS in the membrane could be related to the increase in membrane surface potential of cellular membrane systems [56]. The increase in the amount of anionic phospholipids, such as PS, in the NDV outer membrane leaflet could facilitate cation-viral membrane interactions. Indeed, we have previously shown [57] that Ca^{2+} elicits an activating effect on NDV sialidase at pH's of 6.6 and 6.8. This effect may be due to the interaction between the cation and the external PC molecules. The symmetric distribution of PS could also help calcium-induced membrane fusion [58].

Reconstitution of biological membranes is a very good tool for studying the structure and function of different membrane constituents. The characterization of reconstituted and liposome systems was considered as the first step in our investigation. Using fluorescence polarization of DPH, we have characterized the differences in the apparent fluidity of lipid bilayers in several systems obtained from NDV Clone-30 membranes: intact virus, reconstituted envelopes and liposomes. Evaluation of membrane fluidity is often achieved by measuring the motion of hydrophobic fluorescent probes using fluorescence polarization spectroscopy, the most widely used probe being DPH [26]. Initially, steady-state fluorescence anysotropy data were associated to membrane fluidity. However, it was later shown that these data also contain information on the statics of the membranes. Structural parameters such as the lipid order parameter describe molecular order, whereas motion parameters like membrane fluidity describes molecular dynamics [5]. The lipid order parameter (S), determining the degree to which fluorophore movement in the membrane is restricted by the molecular packing of surrounding lipids [27], was calculated from r_s data. This technique has been extensively used to study the dynamic properties of the viral membranes: stomatis vesicular virus [3], togavirus [59], rhabdovirus [60], influenza virus and NDV [61]. The close correspondence between the anisotropy (r_s) and the order parameter S for the intact NDV membrane (Table 3) and reconstituted envelopes suggests that in both systems the DPH may have a very similar environment, which is unaffected by the reconstitution process and support the validity of this method. The values of these parameters suggest that the viral envelope is relatively rigid. Factors such as the cholesterol/phospholipid ratio [62], the degree of unsaturation of the phospholipid and sphingolipid acyl chain [63] or proteins could affect membrane fluidity and packing. We observed differences in the r_s and S parameters between intact NDV and liposomes derived from total viral lipids. The values for both parameters are lower in liposomes, being up to 24% lower in the anisotropy values (0.07 r_s units at 37°C) or 17.5% lower in the order parameter values

(0.14 *S* units at 37°C). These data indicate that the NDV membrane proteins HN and F could have a rigidizing and immobilizing effect on the membrane since the lipid compositions of intact NDV and liposomes composed of lipids from the viral envelope are identical and proteins are absent from the latter.

Mixed lipid systems such as those found in cell and viral membranes do not show a well-defined transition from gel to the liquid-crystalline state. However, by plotting the order parameter *S* and its first derivative as a function of temperature, changes in membrane lipid physical properties might be discernible. Break points defined from the inflections or discontinous values in slope, indicative of phase transitions, can be seen. The temperatures for these break points are only slightly affected by the presence of proteins, in the three systems derived from NDV membrane (intact viruses, reconstituted envelopes and liposomes) (Fig. 2). Similar break points were detected in reconstituted systems with HN and F proteins and a single class of phospholipids (Fig. 4). The inflection temperature in the slope lies within the 35–39.5°C range. These break points may be indicative of some kind of structural reorientation within the lipid bilayer. They would represent temperatures at which sub-populations of molecules in the membrane begin the transition from an ordered to a disordered arrangement, representing relatively modest structural transitions and not the classical transition from gel to the liquid-crystalline state [64]. In liposomes with a single phospholipid species plus the two viral glycoproteins, except for SM liposomes, the presence of viral components broadens the profile of 'anisotropy vs temperature' and prevents the appearance of a phase transition (Fig. 4). The break points are found at higher temperatures than those described in the literature. The inflections in the first derivative plots would be defined into the liquid-crystalline state of liposomes, meaning less dramatic transitions than the main transition from gel to liquid-crystalline phase, elicited by the proteins incorporated into lipid vesicles according to our hypothesis.

The 'temperature dependence of anisotropy' curves in intact NDV and reconstituted envelopes (Fig. 1) proved to be very similar, with lower anisotropy values for reconstituted envelopes at temperatures under the 'break temperature' (Fig. 2). These results suggest an effect of M protein on membrane dynamics: this protein (present in intact NDV) would exert a rigidifying effect at low temperatures and a fluidizing effect at high temperatures. Our hypothesis is supported by similar results described by Pal et al. [45] for the M protein of stomatitis vesicular virus. Despite this, Neitchev and Dumanova [65] have described an opposite effect of M protein in proteoliposomes reconstituted with lipids, HN and M protein from Russeff and La Sota strains of NDV. These discrepancies could lie in the different strain studied by Neitchev and Dumanova [65] and the Clone-30 strain studied by us. Moreover, those authors used only artificial reconstituted systems with a different detergent (octyl glucoside), which could have different physical properties.

As can be seen in Tables 4 and 5, the effects of PE and asolectin lipids on sialidase activity of HN protein are noteworthy. We suggest that there is an activating effect of PE on the sialidase and hemagglutinating activities of NDV. Since PE is the major viral phospholipid (27% of total phospholipids, Table 1) and since it represents 60% of phospholipid in asolectin vesicles this could represent a direct effect on HN protein. The activation caused by PE on different membrane-bound proteins has been observed by other authors [66]. PE is a lipid with a head group smaller than that of PC and frequently adopts the hexagonal H_{II} phase. The function of this aminophospholipid in biological membranes may be to control the nature of the hydration of the membrane surface [67]. This parameter is important in processes such as membrane fusion or protein–membrane surface interactions. The role of PE in viral fusion may be dual: destabilization of the viral membrane and activation of HN functions. Here, we report the activating effect of this phospholipid on sialidase and hemagglutinating activities although PE could also enhance the proposed HN fusion promotion activity [7,8]. The N-terminal region of HN proteins in different paramyxoviruses seems to play a major role in the virus type specificity of the fusion promotion activity of these proteins [68–70]. Such HN interacting domain with F protein is determined by the transmembrane anchor and the region at the membrane-proximal end of the ectodomain, the putative HN stalk [68]. In this sense, these regions are very close to the membrane for being affected by its characteristics. The activating effect of PE in the two

HA biological activities reported here could be also exerted in the fusion promotion activity. Lipids in the viral membrane may modulate in some extension the communication needed for efficient fusion by interacting with the transmembrane domain of HN protein.

In conclusion, our data imply that sialidase activity requires the presence of definite phospholipids (PE > APL > PC) in its environment; hemagglutinating activity also requires the presence of APL > PE. Nevertheless, regulation of the biological activities of integral glycoproteins of the NDV envelope may be a complex process requiring further detailed investigations.

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