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# ASYMMETRY OF THE LIPID-BILAYER OF SINDBIS VIRUS*

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The organization of the lipid bilayer of the enveloped Sindbis virus has been studied. In the model membrane which consists only of two virus specific glycoproteins and host derived lipids the latter were radioactively labelled with $^{14}$C-palmitic acid by prelabelling their BHK 21 host cell lipids. The purified virus particles were submitted to neuramidase**, bromelain and combromelain–neuraminidase treatment.

It could be demonstrated that the N-acetyl neuraminic acid residue of the total hematoside present in the virion is hydrolyzed by neuraminidase leaving the particles fully intact. Proteolysis of the spikes leads to particle aggregation yet an unchanged hematoside content. This was fully transformed into ceramidelactoside by subsequent neuraminidase treatment. The analyses of the ceramide species present in hematoside of the control particles and ceramidelactoside derived thereof by neuraminidase hydrolysis are in very close agreement.

From these experiments it is concluded that all hematoside molecules are organized in the outer half of the bilayer of the envelope.

## I. Introduction

Numerous studies have been concerned with the physical state of biological membranes and potent techniques, using fluoresence [1] and spin label probes [2] and more recently NMR-spectroscopy [3] have led to the conclusion that the lipid phase of biological membranes is "fluid" [4], a statement not surprising to lipid biochemists since it is well known that about half of all fatty acyl residues of complex phospholipids of eucaryote membranes contain unsaturated fatty acids with one or more cis-double bonds, which renders these lipids fluid at temperatures of viability of these cells. From similar experimental approachs a lateral phase separation into crystalline lipid clusters and fluid areas has been deduced [5]. Recent NMR-studies give evidence for a cooperativity in the mobility of the polar head groups and the acyl chains of the respective complex lipid and vice versa [6]. The complexity of studies on the dynamics of the membrane structure may become even greater if

* Dedicated to the honour of the late Professor Erich Baer.

** Enzymes: Neuraminidase mucopolysaccharide N-acetyl neuraminyl hydrolase (EC 3.2.1.18). Abbreviations: CE, cholesterol esters; TG, triglyceride; FFA, free fatty acids; DG, diglyceride; MG, monoglyceride; CMH, ceramidemonohexoside; CDH, ceramidedihexoside; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; SPM, sphingomyelin; hematoside, N-acetyl neuraminosyl ($\alpha$ 2–3) galactosyl ($\beta$ 1–4)-glycosylceramide; BHK 21 cells, baby hamster kidney cells.

studies currently undertaken would elaborate conclusive evidence for an asymmetry of lipid bilayer. The model membrane so far used for studies on the asymmetry of the lipid bilayer has been the erythrocyte ghost [7–16]. We have chosen the lipid bilayer of the enveloped virions vesicular stomatitis virus and Sindbis virus. We demonstrated the asymmetric arrangement of the ganglioside hematoside in vesicular stomatitis virus. It is integrated exclusively in the outer molecular layer of the envelope bilayer of the rhabdovirus vesicular stomatitis virus (VSV) [17].

In this paper we summarize the results of studies on the structure of the lipid bilayer of the Toga-virus Sindbis virus grown in BHK 21 cells. Whereas VS-virions possess a bullet shape, Sindbis virus is spherical and two glycoproteins of different degrees of glycosylation are integrated into the envelope. We present evidence that hematoside, the only ganglioside present in the lipids of the virion, and the host plasmamembrane is integrated only in the outer surface of the envelope.

## II. Experimental

[1–$^{14}$C] Palmitic acid, spec. radioact. 0.73 $\mu$Mol, has been synthesized by a $^{14}CO_2$ Grignard reaction with pentadecyl-bromide. Neuraminidase from Vibrio cholerae was purchased from Behringwerke, Marburg/Lahn (spec. activity 500 neuraminidase units/ml); bromelain from Serva, Heidelberg.

BHK 21 cells were grown in Dulbeccos medium, supplemented with 5% fetal bovine serum and 35 ng [1–$^{14}$C] palmitate/ml medium in 70 Roux flasks (250 cm$^2$). The medium was decanted from the confluent host cell monolayer and Sindbis virus adsorbed at a multiplicity of 1 pfu/cell for one hour at 37°C. 70 ml Dulbeccos medium was added to each flask and the virus isolated from the medium after 20 hr incubation at 37°C. At that time a cytopathic effect of about 70% was visible.

Virus purification was carried out essentially as described before including the following steps: sedimentation of cell debris at 600 × *g* for 15 min and 4°C in a GSA rotor (Sorvall centrifuge), half saturation of supernatant with an equal volume of saturated $(NH_4)_2SO_4$-solution, pelleting of the precipitate after 2 hr at 10000 × *g* (GSA rotor, Sorvall centrifuge), suspending the sediment in 100 ml Dulbeccos medium and sedimentation at 100 000 × *g* for 60 min at 4°C in a Ti 60 rotor (Beckman ultracentrifuge) [19, 20]. The combined pellets were homogenously suspended in 10 ml Dulbeccos medium by gentle magnetic stirring at 4°C and layered on top of three linear potassium tartrate gradients between 15 and 40% (w/v). The virus was banded in a SW 27 rotor at 23 000 rpm for 2.5 hr at 4°C and the virus bands isolated at a density of 1.1487 and 1.1881. After dialysis against PBS the purity of the virus preparations was checked by electronmicroscopy after negative staining. 5.15 × $10^6$ dpm $^{14}$C corresponding to 0.62% of the radioactivity used for the prelabelling of the host cells was recovered in the purified virus preparation. The following conditions were applied for the enzymatic treatment of the purified virions:

*A. Neuraminidase treatment*

Aliquots of the virus suspension were pelleted at 100 000 × *g* for 60 min at 4°C. The pellet was homogenized in 0.01 M Tris-acetate buffer, 10 mM in calcium acetate, pH 5.5, by magnetic stirring in the same polyallomer tube, 0.2 ml neuraminidase added and incubated for 10 min at 37°C with magnetic stirring. 5 ml ice cold PBS containing 5% fetal bovine serum were added immediately, a small sample taken for electronmicroscopy and the rest sedimented at 100 000 × *g* for 60 min. The sediment was suspended in a small volume of PBS (2 ml) for lyophilization and subsequent lipid extraction.

*B. Bromelain treatment*

The pelleted aliquot of the virus suspension was homogenized with 2 ml 0.1 M Tris-HCl buffer, 0.001 M EDTA, 0.05 M dithiothreitol pH 7.2 in a polyallomer tube and incubated with 2.6 mg bromelain for 30 min at 37°C [21]. The virions were again pelleted and processed for lipid extraction as described under neuraminidase treatment.

The *combined bromelain–neuraminidase treatment* was initiated by the proteolytic treatment. The viruses derived of their spikes were quickly sedimented at 4°C and 100 000 × *g* for 60 min and then incubated with neuraminidase under the conditions given under the respective sections. The virions were again concentrated for lipid analysis as described before.

*C. Extraction and separation of virus lipids*

The lyophilized virus fractions were extracted with chloroform/methanol 2 : 1 and 1 : 2 for 1/2 and 2 hr respectively at room temperature with magnetic stirring. A Folch distribution was carried out with the lipid extract. The upper phase was concentrated by lyophilization. This aqueous phase contained the hematoside, if present in the lipid mixture (fig. 3).

The lower organic phase was separated by silicic acid column chromatography [22] (10 g silicic acid, column 25 × 1.2 cm). Neutral lipids were eluted with 100 ml chloroform, neutral glycosphingolipids with 30 ml acetone/methanol 9 : 1 and phospholipids with 200 ml methanol. Thin-layer chromatographic separations of lipid fractions were carried out in the following solvent systems: neutral lipids: petroleum ether (30–60°C)/ether/acetic acid 70 : 30 : 1; neutral glycosphingolipids: chloroform/methanol/water 100 : 42 : 6; phospholipids: chloroform/methanol/water 65 : 25 : 4. Radioactive bands were localized by radioscanning in a Berthold radio thin-layer scanner, model LB 2722 or a Packard scanner, model 7201. The bands were visualized also in an iodine chamber. Fatty acid methyl esters liberated from individual ester and amide lipids by acid methanolysis at 80°C for 3 and 18 hr respectively were separated by gas liquid chromatography on 2 m columns, 2.5% EGS on chromosorb W (80–100) mesh at 175°C and a nitrogen gas flow of 30 ml/min.

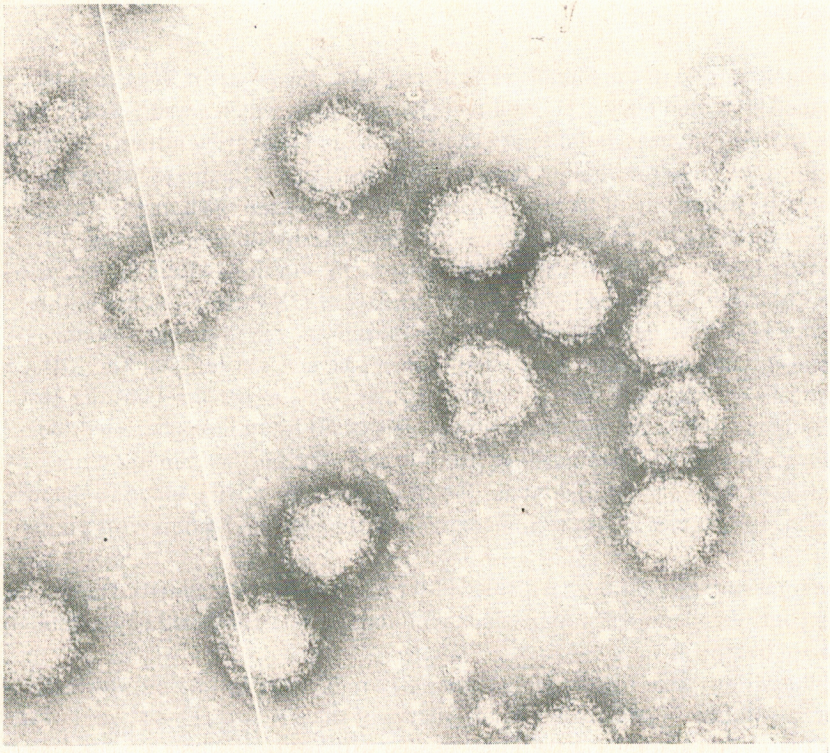
## III. Results

The nucleocapsid of the Sindbis virus belonging to the group of Toga virus is surrounded by a lipid bilayer [1] and two glycoproteins, which are directed to the outside and partially integrated or attached to the lipid bilayer by hitherto unknown interactions. Whereas the viral proteins are virus specific the lipids are derived from the host cell presumably of its plasma membrane. Due to the small number of components and the facility to label the viral lipid and glycoprotein envelope radioisotopically or with carbon 13 labelled precursors, the envelope offers the opportunity to study lipid–lipid and lipid–protein interactions. $^{13}$C-NMR studies in this laboratory [23] have demonstrated that the lipids in the envelope form a rather rigid bilayer. Our approach in the studies reported here was as follows. The BHK 21 host cells were labelled with radioactive palmitic acid as precursors of the host cell membrane lipids. The confluent monolayers of 50 Roux flasks depleted the medium up to 94% of the radioactive palmitic acid. The purified Sindbis virus suspension (10.5 mg protein) contains 0.62% of the radioactivity added to the medium. Fig. 1 resembles an electronmicrograph of the purified Sindbis virus preparation.

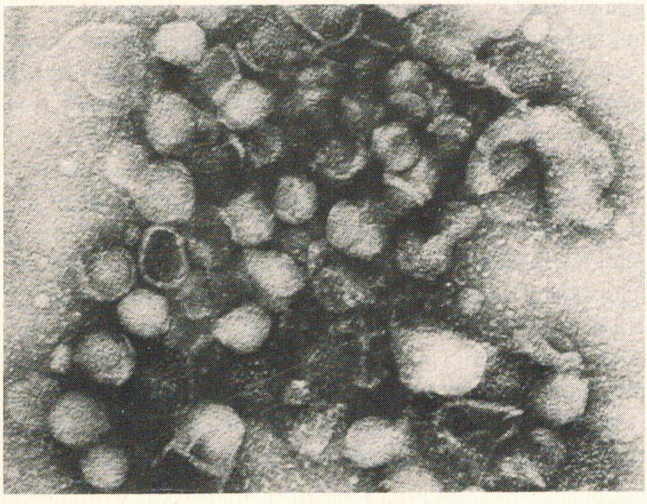
The total virus suspension was divided into four aliquots for enzymatic treatments, for the control experiment, bromelain, neuraminidase and combined neuraminidase–bromelain treatment.

Sindbis virions were treated with *neuraminidase* in order to find out the localization or orientation of hematoside in the bilayer of the envelope. One aliquot of the purified virus preparation was therefore treated with neuraminidase, a second pretreated with *bromelain* in order to digest the spikes of the virions followed by a neuraminidase treatment and the data of the complete lipid analysis of the extracts of these preparations compared with the lipid extract of the control virus (untreated) and a virus aliquot treated with bromelain alone. The results are summarized in table 1. It becomes quite clear from the data in this table that the bromelain treatment leaves the lipid bilayer composition intact. The distribution of the radioactivity in the neutral glycolipid, hematoside and phospholipid fractions is very closely comparable with those of the control virus.

If we however compare the radioactivity recovered in the hematoside fraction (which is the sum of the radioactivity of the Folch upper phase and the residual chloroform/methanol soluble hematoside recovered from the methanol fraction) and the ceramide lactoside of the control and bromelain experiment with the hematoside present in the neuraminidase, bromelain, combined neuraminidase–bromealin treated virions, the hematoside has been almost completely transformed into ceramide lactoside by neuraminidase. This is also documented in fig. 2a–d. There is a constantly small amout of ceramide lactoside present in the untreated virus envelope. The radioactive band corresponding to ceramide lactoside becomes the most prominent peak of the neutral glycosphingolipids of the neuraminidase treated Sindbis virions underlining the results of table 1.



(a)



(b)

Fig. 1. Electronmicrograph of purified Sindbis virions grown in BHK 21 cells. Negative staining with 2% phosphotungstic acid; after (a) neuraminidase, (b) bromelain–neuraminidase treatment.

Table 1
Distribution of radioactivity (% of total) in lipid classes of the envelope of Sindbis virus grown on BHK 21 cells, prelabelled with [1-$^{14}$C] palmitic acid before (control) and after neuraminidase and combined bromelain–neuraminidase treatment.

| | Control | | Neuraminidase treated | | Bromelain and neuraminidase treated | |
|---|---|---|---|---|---|---|
| | dpm × $10^5$ | % of total | dpm × $10^5$ | % of total | dpm × $10^5$ | % of total |
| Total radioactivity | 4.45 dpm × $10^3$ | 100 | 391 dpm × $10^3$ | 100 | 426 dpm × $10^3$ | 100 |
| Lipid class | | | | | | |
| Neutral lipids | 21.6 | 4.83 | 15.4 | 3.9 | 29.1 | 6.8 |
| CE | 7.8 | 1.75 | 3.2 | 0.81 | 4.3 | 1.0 |
| FFA | 3.2 | 0.71 | 3.0 | 0.76 | 9.8 | 2.3 |
| DG | 7.7 | 1.73 | 7.2 | 1.85 | 12.1 | 2.83 |
| MG | 0.82 | 0.18 | 0.5 | 1.12 | 0.6 | 0.14 |
| Ceramide | 2.05 | 0.46 | 1.5 | 0.38 | 2.3 | 0.53 |
| Phospholipids | 410 | 92.17 | 360.2 | 92 | 38.0 | 89 |
| PE | 110 | 24.72 | 103.8 | 26.5 | 116.8 | 27.4 |
| PC/PS | 180 | 40.45 | 152.8 | 39.0 | 157.8 | 37.0 |
| SPM | 120 | 27.0 | 103.6 | 26.5 | 105.2 | 24.6 |
| Neutral glycosphingolipids | 3.0 | 0.66 | 14.9 | 3.8 | 17.0 | 3.98 |
| CMH | 2.4 | 0.53 | 1.4 | 0.35 | 2.2 | 0.51 |
| CDH | 0.6 | 0.13 | 13.5 | 3.45 | 14.8 | 3.47 |
| Hematoside | 10.4 | 2.33 | 0.5 | 0.12 | 0.6 | 0.1 |

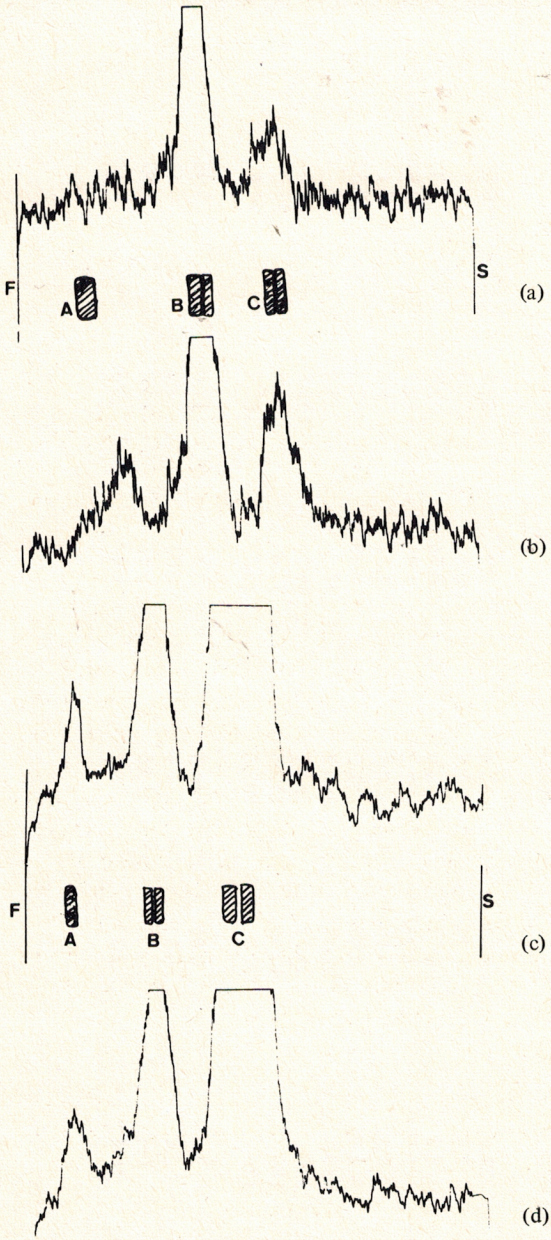



Fig. 2a–d. Radioscans of neutral glycosphingolipids of Sindbis virions grown in BHK 21 cells prelabelled with [1–$^{14}$C] palmitic acid (for details see Experimental). The neutral glycosphingolipids were separated from the total lipid extract by silicic acid chromatography [22] by elution with an acetone/methanol 9 : 1 mixture. Neutral glycosphingolipids isolated from the envelope lipids of (a) control experiment, (b) bromelain treated Sindbis virions, (c) neuraminidase and (d) bromelain-neuraminidase treated virions. Solvent system: Chloroform/methanol/water 65 : 25 : 4. The positions of the test sphingolipids are indicate below the radioscan. A = ceramide, B = ceramidemonohexoside (CMH), C = ceramidelactoside (CDH), F = solvent front, S = start.

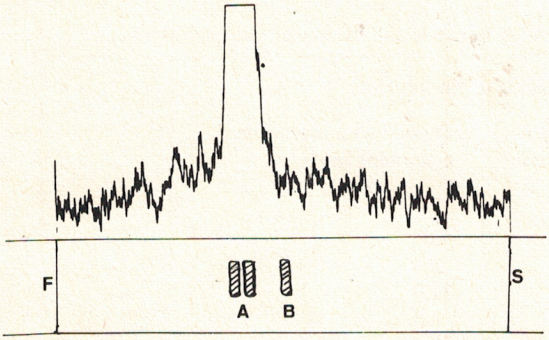



Fig. 3. Radioscan of total Folch upper phase lipids extracted from Sindbis virions grown on BHK 21 cells. The complete radioactivity is present in hematoside (substituted with N-acetyl neuraminic acid). Solvent system: n-propanol/conc. ammonia 70 : 30. A test chromatogram with N-acetyl neuraminic acid (A), N-glycolyl neuraminic acid (B), substituted ceramidelactoside is shown below the radioscan.

Bromelain cleavage of the spikes has no influence on the release of N-acetyl neuraminic acid from hematoside. The viral envelope contains only hematoside with N-acetyl neuraminic acid. Otherwise the latter would have been separated from N-glycolyl neuraminic acid containing hematoside in the solvent system used in fig. 3, which represents an example of a radioscan of the Folch upper phase obtained from the lipid extract of the control aliquot of Sindbis virus. The Folch upper phase of the lipid extract of neuraminidase and bromelain–neuraminidase treated Sindbis virions contained only extremely small amounts of hematoside as demonstrated in table 1. The concentration of radioactive hematoside is so small, that the sensitivity of radio thin-layer chromatography was insufficient to demonstrate radioactive hematoside in these neuraminidase treated virions.

The ratio of radioactivities present in ceramide lactoside and hematoside of the control virus, bromelain, neuraminidase and bromelain–neuraminidase treated Sindbis virions visualizes clearly the neuraminidase hydrolysis (table 2).

Table 2
Ratio of ceramide dihexoside/hematoside in untreated Sindbis virions and virions after neuraminidase and combined bromelain–neuraminidase treatment.

| | % of total radioactivity in lipids of virus envelope | | |
|---|---|---|---|
| | CDH | hematoside | CDH/hematoside |
| Control | 0.13 | 3.47 | 0.37 |
| Neuraminidase treated | 3.45 | 0.12 | 28.8 |
| Bromelain–neuraminidase treated | 3.47 | 0.14 | 24.8 |

Table 3
Composition (%) of fatty acids of ceramide lactoside and hematoside in Sindbis virus.

| | Fatty acids of ceramide lactoside in Sindbis virus | | |
|---|---|---|---|
| | Control experiment | Treated with neuraminidase[a] | Treated with bromelain neuraminidase[a] |
| Fatty acid | | | |
| 14 : 2 | 4.2 | 3.0 | 3.8 |
| 15 : 0 | 2.5 | 1.5 | 2.0 |
| 16 : 0 | 32.6 | 33.4 | 31.0 |
| 16 : 1 | 3.4 | 3.0 | 3.5 |
| 18 : 0 | 22.0 | 21.4 | 18.0 |
| 18 : 1 | 18.0 | 14.0 | 15.8 |
| 19 : 0 | 1.6 | — | 2.7 |
| 20 : 0 | 2.2 | 3.0 | 2.4 |
| 20 : 1 | 1.8 | 1.3 | 1.6 |
| 21 : 0 | 2.0 | 1.9 | — |
| 22 : 0 | 5.2 | 6.2 | 8.4 |
| 22 : 3 | — | 2.2 | 1.7 |
| 23 : 0 | — | — | 1.0 |
| 24 : 0 | 4.5 | 9.0 | 8.1 |

[a] Sum of fatty acids of ceramide lactoside normally present and derived from hematoside.

When the fatty acid composition of ceramide lactoside (CDH), present normally in very minute amounts in the viral envelope (see control experiments, fig. 2a) is compared with the ceramide lactoside arising from the hydrolysis of the envelope hematoside by neuraminidase it becomes apparent that ceramide lactoside and hematoside have very closely similar fatty acid substituents as shown in table 3 and also proven by the direct comparison with the hematoside fatty acid mixture directly released by acid hydrolysis from hematoside.

## IV. Discussion

The envelope of a number of viruses such as myxo-, paramyxo-, rhabdo- and toga virions represents a suitable membrane model in order to study a number of aspects such as lipid–lipid and lipid–protein interactions and the question whether the particular lipid classes are asymmetrically distributed in the two layers of the bilayer. Knowledge of the molecular architecture might also give insight into the assembly process of these simple membranes.

We have chosen the vesicular stomatitis (VS) and Sindbis virus for studies on the aforementioned aspects. The lipid components of the envelope of these viruses could be easily labelled by prelabelling the host cells, which were in this case BHK 21 cells.

Previous studies [24] have demonstrated that the lipids of the envelope are de-

rived from the plasma membrane of the host cell. The two virus specific glycoproteins (53 000 and 47 000 daltons) of Sindbis virus [25] interact with the lipid bilayer. The bilayer structure has been deduced from X-ray diffraction studies by Harrison et al. [26]. These authors concluded that the glycoproteins do not bridge through the lipid bilayer in Sindbis virus. A similar statement has been made by Landsberger et al. [27] for influenza virus. These authors claimed that the spikes are involved in determining the organization of the lipid bilayer. Garoff and Simons [28] on the other hand concluded from labelling and crosslinking experiments that the "spike"-glycoproteins of Semliki Forest virus (SFV) extend through the viral membrane and are in close contact with the nucleocapsid. Due to the discrepancy in this information chemical and physical techniques suitable to give information about possible lipid–protein interactions must be used. We have introduced $^{13}C$-NMR spectroscopy into this problem by labelling the viral envelope of VSV with $^{13}C$-enriched choline and specifically $^{13}C$-labelled fatty acids [23]. Our studies with VS-virions indicated that the N-methyl group of the choline polar head group possesses a high rotational mobility which remains unchanged after tryptic removal of the surface projections. The fatty acid chains of the phospholipids had relaxation times which indicated that the lipid bilayer is in a rather rigid arrangement. One reason certainly is the high cholesterol content of the viral envelope, another the rather high content of saturated long chain fatty acids.

Enzymatic studies have clearly demonstrated that the ganglioside hematoside in vesicular virions is integrated only into the outer surface of the envelope bilayer [16]. The results of the studies presented here give evidence that hematoside in Sindbis virus grown in the same host cell line (BHK 21) are oriented also exclusively toward the outer surface of the virus particle. Furthermore, the experiments with a combined treatment with bromelain, which completely removes the surface projections and neuraminidase point out that all neuraminic acid residues of the hematoside are exposed to the enzyme regardless of the presence or absence of the spikes. The occurrence of gangliosides is predominantly limited to the plasma membrane and [29, 30] membranes derived thereof such as lysosomal membranes. Here hematoside is the main extraneuronal ganglioside.

It is not known whether the glycosylated sphingolipids and particularly the N-acetyl neuraminic acid containing gangliosides are randomly distributed in the lipid phase of the plasma membrane, the envelope of the vesicular stomatitis virus studied earlier [23] or of Sindbis virus reported here. It is intriguing to suggest a clustering of the glycosphingolipids and gangliosides around the glycoproteins, in our subject around the spikes. Multiple hydrogen bonding between their hydrophilic moieties and the carbohydrate side chains of the spikes could easily associate with these molecules by lateral phase separation. An arrangement of gangliosides (hematoside) in the halo of the spikes is made also likely by the observation that the envelope of virions such as influenza and SV 5 virions which contain neuraminidase activity in their spike proteins [31–34] are free of hematoside when grown in host cells the plasma membranes of which are rich in this ganglioside. Neuramini-

dase containing spikes are too far distant to reach randomly distributed N-acetyl neuraminic acid residues on gangliosides. However it is not clear whether the virally coded neuraminidase acts on the host derived hematoside in the plasma membrane area before or after the virus maturation ("virus budding"). The release of the negative charges contributed by N-acetyl neuraminic acid residues present on the glycoprotein and the hematoside molecules does not lead to virus particle aggregation. This is only seen on complete proteolysis of the spikes which then leads to aggregation caused either by zwitterionic interactions of the now freely accessible polar head groups of the outer half of the envelope bilayer or/and the hydrophobic bases of the spikes residing in the envelope after proteolytic treatment.

Our previous $^{13}$C-NMR-study[23] on the organization of envelope lipids in VSV grown in BHK 21 cells has indicated that the lipid bilayer forms a rather rigid structure not very suited for a lateral lipid phase separation. It has been demonstrated by relaxation studies that the negatively charged N-acetyl neuraminic acid of hematoside integrated into phospholipid bilayers does not interact with the quarternary ammonium group of phosphatidylcholine and sphingomyelin. CPK (Corey-Pauling-Koltun) models underline the unfavorable steric arrangement of these oppositely charged groups for an ion–ion-interaction [35].

Experiments are in progress which by photochemical crosslinking aim at the elucidation of the lipid and protein interactions in the viral envelope.

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