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^{13}C-NMR Studies of the Membrane Structure of Enveloped Virions (Vesicular Stomatitis Virus)

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Summary: The mobility of the lipids in the bilayer of the envelope of vesicular stomatitis virus has been probed over its complete space by the biosynthetic incorporation of [*N*-$^{13}CH_3$]-choline as a probe for the polar head groups and [3-^{13}C]- and [11-^{13}C]oleic acid and [16-^{13}C]-palmitic acid for the hydrophobic region of the bilayer. These precursors were effectively incorporated as established by the concomitant administration of the same precursors in radioactive form. Spin lattice relaxation time measurements (T_1) of the ^{13}C enriched segments in complete virus envelope allowed estimation of their mobility. The mobility of the polar head groups is restricted, probably due to ionic interactions with neighbouring acidic phospholipids (phosphatidylserine) and/or acidic side chains of the glycoprotein (G-protein). The rigidity of the hydrophobic part of the bilayer is due to the high cholesterol content and interaction with the immersing polypeptide chains of the G- and possibly M-protein. The rigidity is limited to a depth of about 15 Å ranging from the inner and outer surface, whereas the inner core of the bilayer is fluid. Tryptic cleavage of the hydrophilic part of the G-protein allows the lipophilic immersing polypeptide fragment to enter further the bilayer which then reduces the fluidity of the hydrocarbon chains in the core region by lipid-protein interactions.

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^{13}C-NMR-Untersuchungen über die Membranstruktur von Virushüllen (Vesicular-Stomatitis-Virus)

Zusammenfassung: Die Beweglichkeit der Lipide in der Doppelschicht des membranhaltigen Vesicular-Stomatitis-Virus (VSV) wurde über ihre gesamte Ausdehnung mit Hilfe der ^{13}C-markierten Vorstufen [*N*-$^{13}CH_3$]Cholin für die polaren Köpfe, [3-^{13}C]- und [11-^{13}C]Öl- und [16-^{13}C]Palmitinsäure für die hydrophobe Region als Sonden gemessen. Diese Vorstufen wurden in guter Ausbeute über die Wirtszelle in die Virushülle eingebaut, wie die Analyse der gleichzeitig mitverabreichten radioaktiv markierten Vorstufen bewies. Spin-Gitter-Relaxationszeit-Messungen (T_1) von ^{13}C-markierten Segmenten in der kompletten Virusmembran erlauben die Bestimmung ihrer relativen Beweglichkeiten. Die Beweglichkeit der polaren Gruppen ist eingeengt. Als Ursache erscheinen ionische Wechselwirkungen mit sauren Phospholipiden (Phosphatidylserin) und/oder sauren Seitenketten des Glykoproteins (G-Protein). Die Starrheit des hydrophoben Teils der Lipiddoppelschicht kann einmal auf den hohen Cholesteringehalt und zum anderen

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Abbreviations: VSV = Vesicular stomatitis virus; BHK 21 cell line = a baby hamster kidney cell line; pfu = plaque forming unit.

auf die Wechselwirkungen mit den eintauchenden Polypeptidketten des G-Proteins und – nicht auszuschließen – des M-Proteins zurückgeführt werden. Die Rigidität ist auf eine Tiefe von etwa 15 Å (C-11), von der inneren und äußeren Oberfläche gemessen, beschränkt, während der innere Kern der Doppelschicht flüssig ist. Tryptische Abspaltung des hydrophilen Teils des G-Proteins erlaubt dem lipophilen Fragment tiefer in den hydrophoben Teil der Doppelschicht einzudringen, wodurch die Fluidität der Kohlenwasserstoffketten in der Core-Region durch Lipid-Protein-Wechselwirkungen abnimmt.

Key words: Lipid phase, dynamics in enveloped virus; [^{13}C] precursors, incorporation; ^{13}C-NMR spectroscopy with complete virions; spin-lattice-relaxation time measurements with complete virions; virus envelope, rigidity.

The membrane of a number of enveloped animal viruses consists of an assembly of host cell derived lipids and virus specific proteins. The number of component species is relatively small and therefore the virus envelope represents a membrane of limited complexity for the study of its structural organisation[1] which implies the question for the lipid and protein interactions.

The enveloped virus used in the previous[2] and present study is the vesicular stomatitis virus (VSV). Its envelope contains one glycoprotein (G-protein) of 75000 daltons and an M-protein somehow associated with the envelope, whereas the viral polymerase (L-protein) and the nucleocapsid protein (N-protein) apparently do not interact with the envelope membrane[3,4]. The contradictory reports about the organization of the glycoprotein and the membrane protein[5-8] with the lipid bilayer point out that a broad chemical and physical methodology is required and must be applied to the study of even this simple membrane. In fact chemical labelling of the envelope proteins by enzymatic iodination[6] and pyridoxalphosphate[7], electron spin resonance (ESR)[9] and fluorescence spectroscopy[10] have all been applied to the problem. We have recently reported ^{13}C nuclear magnetic resonance studies[2] performed with whole VS viruses, in which the carbon 13 isotope has been enriched either in the choline moiety or in position 11 of the oleic acid chain of the main phospholipids of the envelope. The labelling of the envelope lipid phase has been achieved with specifically ^{13}C enriched precursors of the host cell lipids. Our general approach is as follows: The ^{13}C labelled precursors are incorporated in well defined positions of the lipid molecules of the host cell plasma membrane and therefore, after virus propagation in these cells, in the architecture of the virus membrane. The ^{13}C probe does not disturb the structures under study. The signals obtained from this probe can be detected unambigously by their known chemical shifts. More important spin-lattice-relaxation time measurements of these probes may lead to information about the mobility of the complete molecule or of its segments in the architecture of this envelope. The degree of the mobility may reflect interactions between lipids or lipids and proteins. Removal of the glycoprotein surface projections by proteolytic enzymes could shed light upon the influence of these structures on the dynamics of the lipids in the envelope. Finally liposomes produced from the viral lipids should serve as controls since their structures have been intensively studied by ^{13}C NMR spectroscopy[11].

In continuation of our previous studies we report in this paper on the incorporation studies of ^{13}C enriched precursors into the lipids of the VSV envelope. Due to their localization they permit insight into the dynamics of the whole lipid bilayer in the presence and absence of the glycoprotein. The choline moieties of lecithin and sphingomyelin, representing to a large extent the hydrophilic groups, and C-3 and C-11 of oleic acid and C-16 of palmitate of the phospholipids of the VSV envelope, representing parts of the hydrophobic regions, have been labelled with ^{13}C and their spin-lattice-relaxation times (T_1) determined. The conditions chosen in the experiments led to an efficient incorporation into the host cell and subsequently into the viral phospholipids. The results indicate that the mobility of the polar head groups of the phospholipids is not meas-

urably influenced by the glycoprotein protruding from the surface. The mobility of the major part of the oleic acid chain is markedly reduced, whereas that of the terminal methyl group of palmitic acid in the hydrophobic region is unaltered in the complete virion as compared with the liposomes prepared from the viral lipids.

The NMR data, which confirm the rigidity of the viral envelope, are interpreted in connection with the analytical data as lipid-lipid and lipid-protein interactions.

Material and Methods

^{13}C labelled compounds

[*N*-$^{13}CH_3$]choline iodide was synthesized by quaternization of *N*-dimethylethanolamine with $^{13}CH_3I$ (90% enriched) in ethanol according to Ferger and Du Vigneaud[12] in a 77% yield (based on theory). [^{13}C]methyl iodide was purchased from Amersham Buchler, Braunschweig. [3-^{13}C]Oleic, [11-^{13}C]oleic and [16-^{13}C]-palmitic acids were synthesized in this laboratory[2].

Incorporation experiments

The baby hamster kidney (BHK 21) cell line was grown in monolayer cultures in Roux flasks (250 cm^2) in Dulbecco's medium[13] containing 10% tryptose broth and 5% fetal calf serum. ^{13}C labelled choline and fatty acids were added to the medium before the cell monolayer reached confluency and ^{3}H labelled choline chloride, oleic and palmitic acids were admixed with the respective ^{13}C precursors to facilitate the quantitative and qualitative analysis. The following concentrations were applied in the labelling procedures of the BHK 21 host cells: a) 0.30 μmol [*N*-$^{13}CH_3$]choline and 1.40×10^5 dpm [*N*-$^{14}CH_3$]choline per m*l* medium; b) 35 μg (0.125 μmol) per m*l* of either [3-^{13}C]-, [11-^{13}C]oleic or [16-^{13}C]palmitic acid supplemented with [9,10-$^3H_2$]oleic acid (specif. radioactiv. 1×10^7 dpm/μmol) and [14,15-$^3H_2$]palmitic acid (specif. radioactiv. 2.7×10^7 dpm/μmol) or [1-^{14}C]-palmitic acid (specif. radioactiv. 1.6×10^6 dpm/μmol). Fatty acid synthesis de novo was inhibited by the addition of 2.85 μg desthiobiotin/m*l* of medium[14].

Cells were grown in this supplemented medium for a period of 24 h, the medium decanted and the monolayer rinsed once with phosphate buffered saline. VS virions were adsorbed (0.1 - 1 pfu/cell) for 1 h at 37 °C. 50 m*l* Dulbecco's medium supplemented with 10% tryptose and 2.5% fetal calf serum was added to each flask and incubated for 18 - 24 h. After this period of time a complete cytopathic effect was observed.

Virus purification

Virions were purified essentially as described before by ammonium sulfate precipitation and sucrose gradient (15 to 45% w/w) centrifugation[2,5,15,16]. The integrity and purity of VSV particles were controlled after negative staining (2% phosphotungstic acid in phosphate buffered saline) by electron microscopy using a Philips EM 300. The concentrated virus preparations were suspended in 2.5 m*l* of phosphate buffered saline, 1% in fetal bovine serum and 0.5 m*l* of D_2O for NMR spectroscopy.

^{13}C-NMR spectra and spin-lattice-relaxation-time measurements

Proton noise decoupled ^{13}C-NMR spectra were registered at 22.63 MHz with a Bruker WH 90 pulse spectrometer operating in the Fourier transform mode. For spin-lattice-relaxation time (T_1) measurements a 180-τ-90° pulse sequence[17] (τ = delay time between 180° and 90° pulse) was employed and T_1-values deduced by the inversion recovery technique [$M_0 - M_z = 2M_0$ exp. $(-\tau/T)$] with an estimated error below 5% in repeated experiments. A Bruker ST 100/700 variable temperature control unit with an accuracy better than ± 1 °C permitted an exact temperature setting. Sample tubes with 10 mm i.d. were used; the samples were flushed with argon and sealed before the NMR experiment. Intact and trypsinized virions were suspended in 2.5 m*l* phosphate buffered saline. containing 1% fetal bovine serum and 0.5 m*l* D_2O. Liposomes were formed in 2.5 m*l* 0.9% NaCl and 0.5 m*l* D_2O.

Not-infected controls

The monolayer of one Roux flask prelabelled with the labelled fatty acids in the manner described for the general experiments but not infected with VS virions served as a control in order to determine the radioactivity and thereby the amount of ^{13}C labelled fatty acid incorporated into each host cell lipid class.

Extraction of lipids

Lipids were extracted as described before[2]. Most of the hematoside appeared in the Folch aqueous upper phase. The rest eluted with the acetone/methanol fraction during silicic acid column chromatography (8 g SiO_2) by which neutral lipids, neutral glycosphingolipids and phospholipids were eluted with 75 m*l* chloroform, 75 m*l* acetone/methanol 9:1 and 150 m*l* methanol, respectively. These fractions were analyzed and further purified by preparative thin-layer chromatography. The following solvent systems were used in thin-layer chromatography: petroleum ether (30 - 60 °C)/ether/acetic acid 70:30:1 for neutral lipids, chloroform/methanol/water 100:42:6 for neutral glycosphingolipids and chloroform/methanol/acetic acid/water 25:15:4:2[18] for the separation of phospholipids.

Radioactive bands were collected in Pasteur pipettes plugged with chloroform washed cotton by water pump aspiration and eluted with chloroform/methanol/water 100:50:10. Spots and bands on thin-layer plates were located by a short exposure of the plates to iodine vapors in a closed chamber. Iodine evaporated from the plates on storage in a vacuum thermostat at 30 °C. Radio thin-layer chromatography was performed on a Berthold thin-layer scanner, model LB 2723, gas liquid chromatography on a Packard gas chromatograph of the eight hundred series using 2-m columns of 2.5% ethylene glycol succinate polyester (EGS on Chromosorb).

Results

Three independent but similar experiments were carried out by which VS virion lipids were enriched in their envelope lipids in the hydrophobic region with [3-^{13}C]- and [11-^{13}C]oleic acid and with [16-^{13}C]palmitic acid. Radioactive [9,10-$^{3}H_2$]-oleic and [1-^{14}C]palmitic acid were added for tracing and quantitative determination of these precursors incorporated into the complex lipids of the host cells (BHK 21 cells) and subsequently of the virions. Fatty acid synthesis de novo in the host cells was inhibited by the addition of desthiobiotin to the medium.

BHK 21 monolayer cultures were grown almost to confluency in Roux flasks, the precursors then added in order to label the host cell lipids and the monolayer infected after 24 h. Virions were harvested by standard procedures.

Quantitative aspects of incorporation of ^{13}C and radioactively labelled precursors

a) BHK 21 cells: The BHK 21 monolayer of one Roux flask was used as control for the quantitative determination of the labelled precursor fatty acids, either oleic or palmitic acid, which were later used as ^{13}C probes. The yield of radioactive fatty acids incorporated into the membrane lipids reflects the amount of ^{13}C labelled acid present in the respective lipid class since an isotope effect by the two nuclei can be neglected. After 24 h the medium was depleted of about 85% of the [^{3}H]oleic or [^{14}C]palmitic acid. About 70% of the radioactivity was recovered in the lipid extract. The total lipid extract was obtained by Folch extraction. The lower phase was separated into the neutral lipid, glycosphingolipid and phospholipid fractions by silicic acid chromatography[19]. The upper phase contained almost all of the hematoside, which amounted to 3 to 4% of the total radioactivity present in the lipid extract. The quantitative aspect of the analyses is summarized in Table 1.

Table 1. Distribution of radioactivity (% of total) in lipid fractions and in phospholipid classes of BHK 21 cells prelabelled with [3-^{13}C; 9,10-$^{3}H_2$]- and [11-^{13}C; 9,10-$^{3}H_2$]oleic acids.

Lipid fraction	Radioactiv. of the incorp. oleic acid	
	[dpm]	[% of total]
Neutral lipids (free fatty acids, glycerides, cholesterol esters)	2.56×10^7	37.5
Glycosphingolipids (hematoside, ceramide, mono- and di-glycosylceramide)	0.29×10^7	4.2
Phospholipids (phosphatidylcholine, phosphatidylserine)	3.89×10^7	58.3

The distribution of the radioactive fatty acids in the neutral and phospholipid fractions is exemplified in Fig. 1a, 1b, which illustrate the preparative thin-layer chromatographic separation of these fractions. A portion of the neutral lipid and phospholipid fractions of the BHK 21 cells was transesterified and the fatty acid methyl esters obtained for analysis. Table 2 summarizes the analytical data. These analyses proved the high level of incorporation of oleic acid into the envelope lipids.

b) Lipid analysis of VS virions: The purification procedure of VS virions by ammonium sulfate precipitation and sucrose and potassium tartrate gradient centrifugations led to preparations which appeared to be homogeneous by electron microscopy following negative staining with phosphotungstic acid. Virus preparations in the monolayers of 100 Roux flasks yielded about 70 mg of virus protein, which corresponded to about 100 - 110 mg VSV dry weight in each experiment.

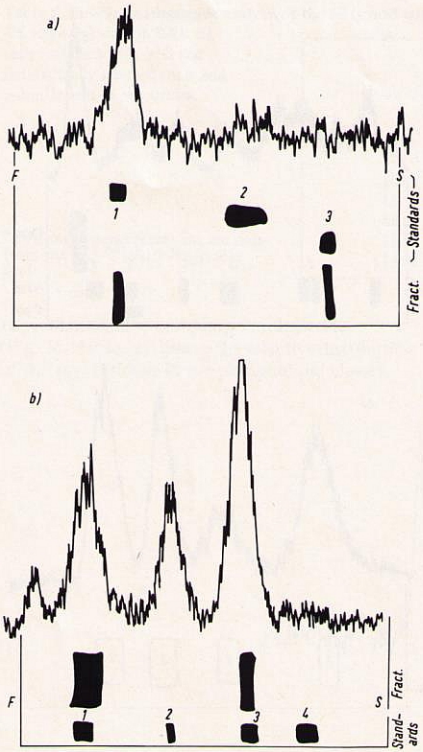

Fig. 1. Distribution of radioactivity in a) neutral lipid and b) phospholipid fraction of BHK 21 cells grown on [9,10-$^{3}H_2$;11-^{13}C]oleic acid.

a) Solvent system: petroleum ether/ether/acetic acid 70 : 30 : 1. Standards of 1 = triglyceride; 2 = free fatty acid; 3 = diglyceride.

b) Solvent system: chloroform/methanol/acetic acid/water 50 : 30 : 8 : 4. Standards of 1 = phosphatidylethanolamine; 2 = phosphatidylserine; 3 = phosphatidylcholine; 4 = sphingomyelin; S = start, F = front.

30 mg of crude lipids were extracted after the NMR experiments had been performed. The purified VS virions contained 4.5 to 5% of the radioactive precursors used in the different experiments for prelabelling the host cells.

Table 2. Distribution of the main fatty acids in the neutral and phospholipid fatty acid fractions of BHK 21 cells after supplementing the medium with [^{3}H]oleic acid.

Fatty acid	Phospholipids	Neutral lipids
14:0	–	4.4
16:0	27.2	18.0
16:1	5.2	5.4
18:0	17.1	6.9
18:1	50.5	65.3

The analytical procedures were those described for the lipid separation and analysis of the BHK 21 cells. Table 3 compares the data of the distribution of the radioactive precursors in the neutral and phospholipid fractions of VS virions grown on [3-^{13}C; 9,10-$^{3}H_2$]- and [11-^{13}C; 9,10-$^{3}H_2$]-oleic acid and [16-^{13}C; 1-^{14}C]palmitic acid.

The radioactive components of the neutral lipid fraction were free fatty acid (50.8%), diglyceride (42.4%) and triglyceride (6.8%) in the palmitic acid experiment (Fig. 2a) and 61.9% free fatty acid, 21.4% diglyceride and 16.7% triglyceride in the oleic acid experiments. Hematoside was recovered to its largest extent in the Folch upper phase, the rest of it residing in the lower phase was eluted with acetone/methanol (1 : 9 v/v) during silicic acid chromatography together with

Table 3. Distribution of radioactive precursors in lipid classes of the envelope of VS virions grown on [3-^{13}C; 9,10-$^{3}H_2$]- and [11-^{13}C; 9,10-$^{3}H_2$]oleic and [16-^{13}C; 1-^{14}C]palmitic acid.

Lipid fraction	Radioactive precursor	
	Oleic acids	Palmitic acid
	[% of total radioactivity]	
Neutral lipids	20.1	18.5
Glycolipids[a] (hematoside)	2.3	13.1[b]
Phospholipids	74.5	68.4

[a] The predominating component was hematoside, with traces of mono- and diglycosylceramide.

[b] This fraction contained traces of phosphatidylcholine, -serine and -ethanolamine, which eluted with acetone/methanol 9 : 1 on silicic acid chromatography. The main component was again hematoside (see Fig. 2b).

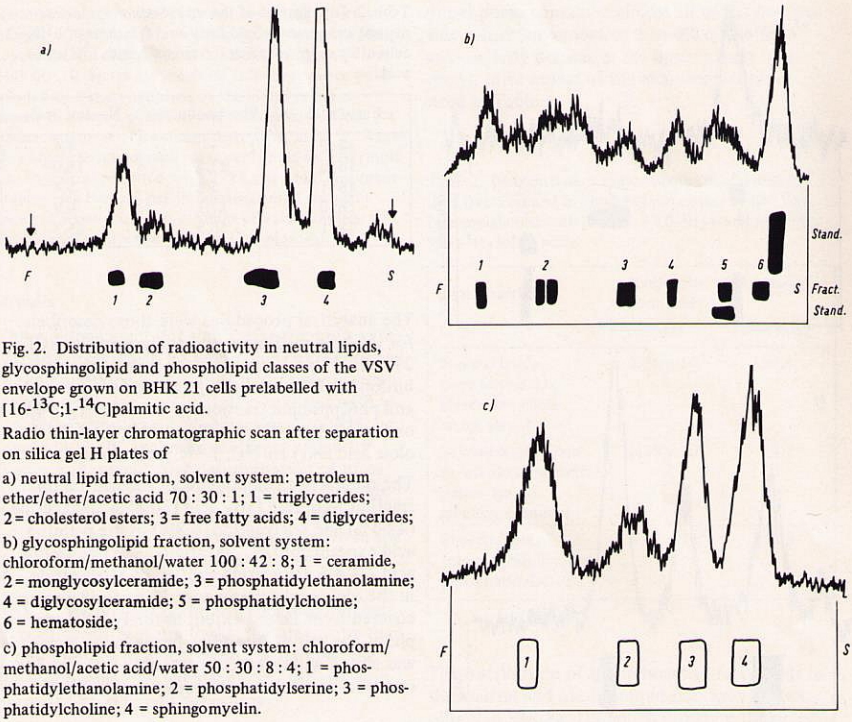

Fig. 2. Distribution of radioactivity in neutral lipids, glycosphingolipid and phospholipid classes of the VSV envelope grown on BHK 21 cells prelabelled with [16-^{13}C;1-^{14}C]palmitic acid.

Radio thin-layer chromatographic scan after separation on silica gel H plates of

a) neutral lipid fraction, solvent system: petroleum ether/ether/acetic acid 70 : 30 : 1; 1 = triglycerides; 2 = cholesterol esters; 3 = free fatty acids; 4 = diglycerides;

b) glycosphingolipid fraction, solvent system: chloroform/methanol/water 100 : 42 : 8; 1 = ceramide, 2 = monglycosylceramide; 3 = phosphatidylethanolamine; 4 = diglycosylceramide; 5 = phosphatidylcholine; 6 = hematoside;

c) phospholipid fraction, solvent system: chloroform/methanol/acetic acid/water 50 : 30 : 8 : 4; 1 = phosphatidylethanolamine; 2 = phosphatidylserine; 3 = phosphatidylcholine; 4 = sphingomyelin.

ceramide, mono- and diglycosylceramide (Fig. 2b). This fraction was contaminated with traces of phosphatidylcholine and phosphatidylethanolamine. About 8% of the radioactivity was lost on mild alkaline hydrolysis. Mainly four phospholipid classes were heavily labelled, regardless of the radioactive precursor fatty acid used: phosphatidylcholine, sphingomyelin, phospha-

Table 4. Distribution of radioactivity in phospholipid classes in the envelope of VS virions grown in a medium supplemented with [11-^{13}C; 9,10-$^{3}H_2$]oleic and [16-^{13}C; 1-^{14}C]palmitic acid as precursors.

Lipid fraction	Radioactive precursor	
	[11-^{13}C; 9,10-$^{3}H_2$]-Oleic acid	[16-^{13}C; 1-^{14}C]-Palmitic acid
	[% of total radioactivity]	
Sphingomyelin	19.2 (22.7)[a]	42.6
Phosphatidylcholine	38.4 (28.4)	35.2
Phosphatidylserine	6.2 (4.8)	18.5
Phosphatidyl-ethanolamine	36.2 (44.1)	3.7

[a] Values in parentheses are the data from the [3-^{13}C; 9,10-$^{3}H_2$]-oleic acid experiment.

Table 5. Gas chromatographic analysis of the fatty acid composition of phospholipids isolated from the envelope of VS virions grown on BHK 21 cells enriched with ^{13}C and radioactively labelled oleic and palmitic acid as precursors.

Fatty acid	Radioactive precursor	
	[11-^{13}C; 9,10-$^{3}H_2$]-Oleic acid	[16-^{13}C; 1-^{14}C]-Palmitic acid
	[% of total radioactivity]	
14:0	2.2	–
16:0	27.1 (40.5)[a]	54.0
16:1	3.2 (2.0)	5.0
18:0	9.1 (9.5)	14.8
18:1	49.4 (45.0)	26.2
18:2	9.0 (3.0)	–

[a] Values in parentheses are the data from the [3-^{13}C; 9,10-$^{3}H_2$]oleic acid.

tidylethanolamine and phosphatidylserine (Fig. 2c). However, due to the selective distribution of the acyl residues in the phospholipid classes depending on their structure (chain length and unsaturation) differences in the labelling pattern were observed. They are summarized in Table 4.

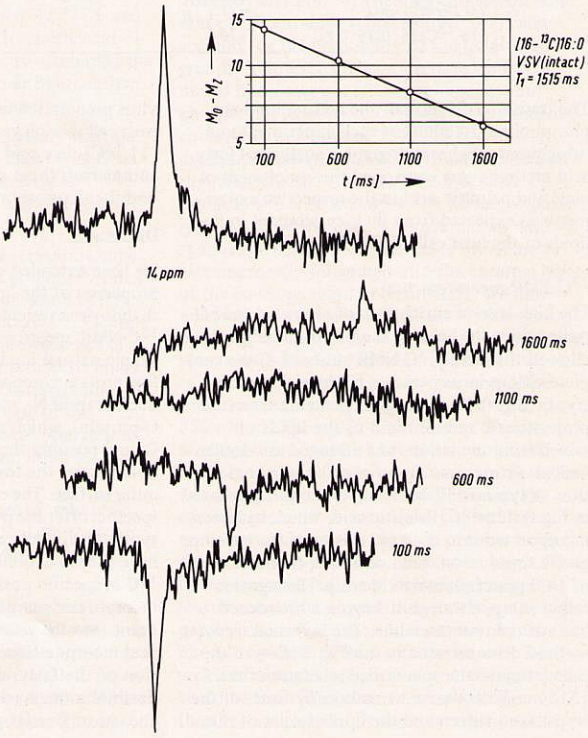

Fig. 3. Determination of spin-lattice-relaxation time (T_1) of [16-^{13}C]palmitic acid containing lipids in the envelope of complete VS virions.
Virions were suspended in phosphate buffered saline 1% fetal bovine serum at a concentration of 20 - 30 mg virus protein/ml. The broad band between δ = 24 and 31 ppm corresponds to resonances of the natural abundance of ^{13}C nuclei of the hydrocarbon chains of the lipid.

Table 6. Spin-lattice-relaxation-times (T_1) of specifically ^{13}C-labelled oleic, palmitic acid and choline incorporated into the phospholipids of the envelope of complete and spikeless vesicular stomatitis virus and liposomes formed from total envelope lipids.

For comparison T_1 values are added of phosphatidylcholine (PC) and sphingomyelin (SPM) substituted with fatty acids of different saturation.

The figures added to each T_1 time represent the error introduced by the graphical evaluation.

^{13}C Labelled precursor	Spin-lattice-relaxation times (T_1 in ms) at 37 °C		
	Complete VSV	Liposomes	Trypsinized VSV
[3-^{13}C]Oleic acid	127 ± 10	157 ± 20	120 ± 15
[11-^{13}C]Oleic acid	169 ± 38	225 ± 15	–
[16-^{13}C]Palmitic acid	1489 ± 70	1626 ± 80	1348 ± 75
[*N*-$^{13}CH_3$]Choline	238 ± 18	213 ± 21	165 ± 22
Relaxation times of labelled model substances in liposomes			
[*N*-$^{13}CH_3$]18:2/18:2-PC	470 ± 7		
[*N*-$^{13}CH_3$]16:0/16:0-PC	300 ± 20		
[*N*-$^{13}CH_3$]18:0/18:0-PC	260 ± 10		
[*N*-$^{13}CH_3$]-SPM	341 ± 6		
[16-^{13}C]16:0/16:0-PC	1470		

The transesterification of the neutral lipid and phospholipid fractions of each experiment and subsequent gas chromatographic analysis of fatty acid methyl esters confirmed the enrichment of oleic and palmitic acid in the respective experiments as expected from their enrichment in the lipids of the host cells, Table 5.

^{13}C-NMR-measurements

The high level of enrichment of oleic acid and palmitic acid in the lipids of the VS virion preparations allowed the direct ^{13}C-NMR study of a) the complete virions in suspension, b) the virions after tryptic digestion of the glycoprotein surface projections ("spikes") and c) the lipids obtained from the virions and arranged in lamellar vesicular structures (liposomes) by ultrasonication. A typical ^{13}C-NMR experiment is presented in Fig. 3. [16-^{13}C]Palmitic acid, which had been incorporated into the lipid classes of the envelope gave a single resonance line with a chemical shift of 14.0 ppm (relative to Me_4Si). The signal is rather sharp, although sitting on a broadened basis of the resonance line. The inversion recovery method demonstrated in the Fig. 3 allowed the calculation of the spin-lattice-relaxation time T_1 = 1515 ms. With the same method T_1 times of the trypsinized virions and the lipid vesicles of this virus preparation and those propagated in BHK 21 host cell monolayers enriched with [3-^{13}C]- and [11-^{13}C]oleic acid were determined. Table 6 summarizes these spin-lattice-relaxation times and their corresponding correlation times (τ_c).

Discussion

We have extended our studies of the physical properties of the lipid phase of the enveloped rhabdovirus vesicular stomatitis virus (VSV) by ^{13}C-NMR spectroscopy. Its envelope represents a simple natural model membrane because of the few protein components (viral polymerase L-, nucleocapsid N-, membrane M- and glycoprotein G-protein), which are virus specific and only the G- and possibly the M-protein interact with a lipid bilayer the former protruding from the outer surface. The envelope lipids, which are host specific, offer the possibility of being labelled biosynthetically with appropriate precursors. We have chosen choline and fatty acids labelled with ^{13}C in specific positions which were effectively incorporated via the host cell (BHK 21) membrane into the virus envelope lipids. The fatty acid incorporation was enhanced by the inhibition of the fatty acid synthesis de novo by desthiobiotin. As demonstrated under Results the use of free fatty acids is as effective as that of

fatty acid-tween esters[14], as shown earlier in this laboratory[2,20]. This biosynthetic incorporation of our ^{13}C labelled precursors for biophysical studies or of fatty acids with photosensitive groups for chemical studies of lipid-lipid and lipid-protein interactions is much superior to the secondary addition of spin labelled fatty acids[9], fluorescent probes like 12-anthroyl stearic acid [10] to the purified viruses or the attempt to incorporate spin labelled phosphatidylcholine vesicles by fusion with the virus envelope[22].

As reported in previous publications ^{13}C-NMR spectroscopy in conjunction with spin-lattice-relaxation time (T_1) measurements give insight into the dynamics of the lipid molecules. The enrichment of the ^{13}C isotope in particular segments of the lipid molecules, into polar head groups via [N-$^{13}CH_3$]choline and along the fatty acyl chains by the incorporation of [3-^{13}C]- and [11-^{13}C]oleic acid and [16-^{13}C]palmitic acid opens up the possibility of directly estimating the relative mobilities of the particular hydrocarbon segments in the complete virus envelope.

Because the T_1 times correlate directly with the trans-gauche isomerization and kink formations which this respective segment undergoes[11,23-26] conclusions can be drawn from any changes of this parameter.

We carried out these spin-lattice-relaxation time measurements with suspensions of whole virions and compared these with those measured in liposomes prepared by ultrasonication[27] from the extracted envelope lipids and liposomes of other model phospholipids or mixtures of lipids. Changes of T_1 times allow us to estimate interactions qualitatively and in the case of our ^{13}C labelled precursors to localize these interactions exactly. The mode of interaction, however, cannot be specified.

Since spin-lattice-relaxation times of only protonated C atoms are measured contributions by other relaxation mechanisms than dipol-dipol relaxation can be excluded[11].

It is striking that the *polar head groups* of the phosphatidylcholine molecules have a rather low T_1 value. The quaternary ammonium group has a restricted mobility both in the complete virion and the liposomes of the envelope lipids (238 ms and 213 ms). The mobility is further reduced after tryptic release of the glycoprotein (G-protein) (165 ms).

Reduced rotational mobility of the N-CH_3 groups in phosphatidylcholine and sphingomyelin can be caused a) by highly saturated hydrocarbon chains of the acyl residues, e.g. the N-CH_3 group of choline in dipalmitoyl- and distearoyllecithins have T_1 times of 300 ± 20 ms and 260 ± 10 ms, respectively, due to the close packing of the acyl chains and cooperativity of the polar head groups and therefore reduced availability of space. In contrast dilinoloyllecithin has highly mobile N-CH_3 groups in its choline residue (470 ± 7 ms). Since half of the fatty acyl residues of the phospholipids of the VSV envelope are oleic and palmitoleic acids one would expect an intermediate T_1 time but not this short time below that of distearoyllecithin. b) The high cholesterol content of the envelope (cholesterol/P 0.7) cannot account for the immobility of the polar head groups. In previous relaxation time studies of mixed liposomes containing sphingomyelin/phosphatidylcholine/phosphatidylethanolamine/cholesterol in a ratio of 1:4:1:3 and sphingomyelin/phosphatidylcholine/-ethanolamine/-serine/cholesterol 1:1:1:1:3 we measured T_1 times of 480 ± 16 ms and 430 ± 37 ms, respectively. c) Ionic interactions with neuraminic acid residues of hematoside molecules which are asymmetrically integrated into the external layer of the envelope seem to be unlikely. We have previously demonstrated that the choline group of phosphatidylcholine in hematoside containing liposomes before and after complete hydrolysis of N-acetylneuraminic acid by neuraminidase preserves its mobility unchanged (340 and 350 ms). d) The rather high content of phosphatidylserine could lead to the immobilization of the positively charged quaternary trimethylammonium group by ionic interactions with the carboxy group of serine. Also a contribution of ionic interactions with negatively charged side chains of the G-protein cannot be excluded. This however would require phosphatidylcholine-cluster formation around the immersing G-polypeptide chain. The ionic interactions of acidic phospholipids with the zwitterionic polar head group of phosphatidylcholine and sphingomyelin could be facilitated after enzymatic release of the G-protein which for steric interactions of

their carbohydrate side chains could hold the outer bilayer in a more expanded state and cause the further reduced T_1 time of the N-CH_3 groups of phosphatidylcholine and sphingomyelin to 165 ± 22 ms. e) A closer packing and thereby reduced rotational freedom could also be induced by enhanced interactions of the phospholipid and sphingolipid hydrocarbon chains clustered around the hydrophobic part of the G-protein.

The rigidity of the lipid phase of VSV envelope is most strikingly documented by the low T_1 values of the ^{13}C nuclei within the alkyl chain of the incorporated oleic acid. Although about 50% of the acyl residues of all phospholipids are oleic acid, the ^{13}C enriched carbon atoms 3 and 11 are slightly more immobile in the complete and trypsinized virus than in liposomes. The interaction with cholesterol certainly exerts a condensing effect. In 18:0/[11-^{13}C]18:1-phosphatidylcholine vesicles C-11 exhibits a T_1 time of 278 ms but in cholesterol containing vesicles one of 210 ms. An additional contribution by interactions with hydrophobic polypeptide regions immersing into the lipid bilayer might also be considered.

The T_1 times of [16-^{13}C]palmitic acid incorporated into the virus envelope require some interpretation. This terminal CH_3 group located in the center of liposomal bilayers has a high mobility. Pure dipalmitoyl vesicles possess a high degree of order. Its T_1 is 1470 ms, but that of 18:0/[16-^{13}C]16:0-phosphatidylcholine with less order due to the unequal chain lengths is 1840 ms. Mixed vesicles also enhance the disorder and lead to increasing spin-lattice-relaxation (T_1) times. The mobility of the terminal CH_3 group is slightly reduced in the complete virion but more pronounced in the trypsinized virus particles (Table 6), when compared with liposomes formed from the viral lipid. We interpret this change in mobility in the following way: After tryptic digestion of the G-protein the residual lipophilic polypeptide which has a molecular weight of 5200[8] after thermolysin or of 4700 to 11000[28] after trypsin or pronase treatment, may further immerse into the hydrocarbon environment leading to stronger interactions in the central region of the bilayer thereby reducing the T_1 time of the [16-^{13}C]palmitic acid residue. Brown et al.[29] stated that the G-protein penetrates the bilayer and contacts the nucleocapsid. However a stronger alteration of the mobility parameters of [16-^{13}C]-palmitate containing phospholipids in the complete VS particle would be expected.

We suggest that the glycoprotein might be integrated into the outer half of the bilayer in which interactions between phospholipids and cholesterol cause similar immobilizing effects to those of the hydrophobic polypeptide of the G-protein. Our ^{13}C-NMR data regarding the fluidity (rigidity) changes of the viral membrane of complete VSV and trypsinized virus particles are contradictory to the ESR data on the Sindbis virus envelope of Sefton and Gaffney[22]. The data of these authors are based on the fusion of spin labelled phospholipid vesicles with the virus. However, any proof of such a fusion is lacking and therefore so is the certainty of the interaction in the structure being probed. This uncertainty and drawback is inherent in many spin label-addition experiments. The approach chosen here used the biosynthetic pathway which places the ^{13}C labelled precursor in the genuine membrane with an exact localization of the probe. Also no perturbating structures are attempted to be integrated.

Combinations of different physical, chemical and enzymatic methods must be further used to gain insight into the molecular array of the virus envelope which represents a suitable membrane model for studies of lipid-protein interactions.

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Literature

1 Choppin, P. W., Klenk, H. D., Compans, R. W. & Caliguri, L. A. (1971) *Perspect. Virol.* **7**, 127 - 158.
2 Stoffel, W. & Bister, K. (1975) *Biochemistry* **14**, 2841 - 2847.
3 Walter, G. & Mudd, J. A. (1973) *Virology* **52**, 574 - 577.
4 Lenard, J. & Compans, R. W. (1974) *Biochim. Biophys. Acta* **344**, 51 - 94.
5 Cartwright, B., Smale, C. J. & Brown, F. (1969) *J. Gen. Virol.* **5**, 1 - 10.
6 Moore, N. F., Kelley, J. M. & Wagner, R. R. (1974) *Virology* **61**, 292 - 296.
7 Eger, R., Compans, R. W. & Rifkin, D. B. (1975) *Virology* **66**, 610 - 615.
8 Schloemer, R. H. & Wagner, R. R. (1975) *J. Virol.* **16**, 237 - 249.

9 Landsberger, F. R., Compans, R. W. & Choppin, P. W. (1973) *Biochemistry* **12**, 4498 - 4502.
10 Lenard, J., Wong, C. Y. & Compans, R. W. (1974) *Biochim. Biophys. Acta* **332**, 341 - 349.
11 Stoffel, W., Tunggal, B. D., Zierenberg, O., Schreiber, E. & Binczek, E. (1974) *this J.* **355**, 1367 - 1380.
12 Ferger, M. F. & Du Vigneaud, V. (1950) *J. Biol. Chem.* **185**, 53 - 57.
13 Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167 - 182.
14 Williams, R. E., Wisniesky, B. J., Rittenhouse, H. G. & Fox, C. F. (1974) *Biochemistry* **13**, 1969 - 1977.
15 Klenk, H. D. & Choppin, P. W. (1969) *Virology* **38**, 255 - 268.
16 McSharry, J. & Bluzinger, R. (1970) *Virology* **40**, 745 - 746.
17 Freeman, R. & Hill, D. D. W. (1971) *J. Chem. Phys.* **54**, 3367 - 3377.
18 Skipski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374 - 378.
19 Vance, D. E. & Sweeley, C. C. (1967) *J. Lipid. Res.* **8**, 621 - 630.
20 Stoffel, W. & Scheid, A. (1967) *this. J.* **348**, 205 - 226.
21 Landsberger, F. R., Compans, R. W., Paxten, J. & Lenard, J. (1972) *J. Supramol. Struct.* **1**, 50 - 54.
22 Sefton, B. M. & Gaffney, B. J. (1974) *J. Mol. Biol.* **90**, 343 - 358.
23 Solomon, I. (1955) *Phys. Rev.* **99**, 559 - 565.
24 Allerhand, A., Doddrell, D. U., Komoroski, R. (1971) *J. Chem. Phys.* **55**, 189 - 198.
25 Metcalfe, J. C. (1972) *Chem. Phys. Lipids* **8**, 333 - 340.
26 Levine, Y. K., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1972) *Biochemistry* **11**, 1416 - 1421.
27 Huang, C. (1969) *Biochemistry* **8**, 344 - 351.
28 Mudd, J. A. (1974) *Virology* **62**, 573 - 577.
29 Brown, F., Smale, C. J. & Horzinek, M. C. (1974) *J. Gen. Virol.* **22**, 455 - 458.

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