^{13}C Nuclear Magnetic Resonance Studies on the Lipid Organization in Enveloped Virions (Vesicular Stomatitis Virus)†

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ABSTRACT: ^{13}C nuclear magnetic resonance (NMR) studies are described regarding the lipid organization in the envelope of the vesicular stomatitis virion. The fatty acid chains (oleic acid) and the choline moiety of the 3-*sn*-phosphatidylcholine and sphingomyelin have been labeled specifically with ^{13}C by growing the virions in prelabeled host cells (BHK 21 cells). The results suggest that ^{13}C NMR spectroscopy is a very feasible method for the study of natural membranes provided the isotope is highly enriched in specific positions and incorporated biochemically. Spin-lattice relaxation (T_1) measurements of particular C atoms have been carried out with whole virions, with virions deprived of their surface projections by trypsinization but unaltered in their shape and size, and with liposomes prepared from the total lipid mixture of the envelope in order to get insight into the molecular structure of this model membrane. The mobility of the central part of 11-^{13}C-labeled oleic acid incorporated into the ester and amide lipids and the choline group of 3-*sn*-phosphatidylcholine and sphingomyelin is very restricted as indicated by their short T_1 times. It is concluded from the data presented here that the high cholesterol content (cholesterol/P: 0.7) of the envelope lipid phase is responsible for the rather rigidly packed envelope structure. The mode and extent of the interactions between lipids and glycoprotein surface projections are subjects for further study.

Enveloped viruses characterized by their lipid and protein containing limiting membrane structure, which surrounds the nucleocapsid, represent a model system of moderate complexity for the study of the structure of a membrane. Whereas the envelope proteins are virus specific their lipids are host cell derived, in many cases originating from the plasma membrane (Klenk and Choppin, 1969, 1970, 1971; Choppin et al., 1971).

The obvious advantage enveloped viruses offer is that they can be isolated in high purity for analytical studies and biophysical and biochemical investigations aiming at the lipid-protein interactions since the number of proteins associated with the envelope and coded by the virus genome is very small. The lipid composition and thereby the physical properties of the envelope can be manipulated via the host cell by growing the same virus on different cell lines with different membrane composition or growing the host cell in a medium supplemented with particular fatty acids. Their influence on the physical properties of the lipid in which they are incorporated is predictable. We have given detailed information on the fatty acid incorporation and distribution in membrane phospholipids of HeLa cell cultures, the growth medium of which had been supplemented with particularly labeled polyenoic fatty acids (Stoffel and Scheid, 1967). Also the lipid composition of SV-5 viruses has been altered by the addition of fatty acids into the growth medium of MDBK cells (Klenk and Choppin, 1969).

Two physical methods, namely electron spin resonance (ESR) (Landsberger et al., 1971, 1972, 1973) and fluorescence spectroscopy (Lenard et al., 1974), have recently been applied to elucidate fundamental questions regarding (a) the arrangement of the different lipids in the envelope of the virion, (b) the interactions of the envelope proteins with the lipids, and finally (c) the possible insertion into the lipid phase of the envelope.

A useful development and extension of the physical methods for the study of lipid-lipid and lipid-protein interactions, of the dynamics of lipid phases, and the structure-function relationship of lipids is ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR).

Recently we have studied the lipid-lipid interaction in artificial bilayer membranes (liposomes) consisting of single phospholipid species specifically enriched in their hydrophilic and hydrophobic moieties and of mixed lipids (phospholipids, sphingomyelin, cholesterol, cholesterol esters, and gangliosides) (Stoffel et al., 1974a) and the lipid-apo-high-density lipoprotein interactions by ^{13}C NMR (Stoffel et al., 1974a-c). The results of these studies encouraged us to study even more complex structures.

In the present paper we want to present data which demonstrate that ^{13}C NMR spectroscopy is a valuable tool for the study of the lipid arrangement in membranes of enveloped viruses of which we have chosen the vesicular stomatitis virion[1] (VSV) as an example. Furthermore, it opens up the possibility of studying lipid-polypeptide interactions of a membrane considerably simpler than most membranes of mammalian origin.

The approach which we have chosen is the following one. The host cell membrane lipids were labeled with an 80-90% ^{13}C-enriched fatty acid ([11-^{13}C]oleic acid) and with [*Me*-^{13}C]choline and then VS virions were grown in these cells. The virions enveloped with the host cell labeled lipids were purified and then directly used for NMR spectroscopy. This approach avoids the use of perturbating probes such as the

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[1] Abbreviations used are: VSV, vesicular stomatitis virion; PBS, phosphate buffered saline; PFU, plaque-forming unit; Tos-PheCH$_2$Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

bulky spin labels or even bulkier fluorescence groups. The discrepancies of spin label ESR experiments and deuterium labeled lipids in view of the perturbation effect by the spin label groups have been discussed in recent papers (Seelig and Niederberger, 1974; Seelig and Seelig, 1974a,b). More importantly this approach excludes uncertainties regarding the localization of the probe, which is added to a membrane or virus preparation and expected to be integrated in the desired hydrophobic or hydrophilic area. ^{13}C-Labeled probes incorporated in the way described here guarantee that the signal comes from C atoms of lipids in the proper natural arrangement of the membrane or envelope. We added the respective radioactively labeled fatty acid and choline to the ^{13}C-labeled probe in order to facilitate the quantitative analysis of the envelope–lipid mixture.

Materials and Methods

^{13}C-Labeled Compounds. [11-^{13}C]Oleic acid has been synthesized in this laboratory (Stoffel et al., 1972). [*Me*-^{13}C]Choline was obtained by quaternization of dimethylaminoethanol with [^{13}C]methyl iodide (Ferger and du Vigneaud, 1950). [*Me*-^{14}C]Choline was purchased from Amersham-Buchler, Braunschweig. The BHK 21 baby hamster kidney cell line was grown in monolayer cultures in Roux flasks (250 cm^2) in Dulbecco's medium, containing 10% tryptose broth and 10% fetal calf serum. Cells were grown to confluency. The Indiana serotype of VSV, kindly provided by Professor Dr. H. J. Eggers (Institute of Virology, University of Cologne), was propagated by inoculation (1 plaque-forming unit (PFU)/cell) of prelabeled monolayers.

Labeling of 3-*sn*-phosphatidylcholine and sphingomyelin of VS virions with [*Me*-^{13}C;^{14}C]choline was done by the following method. BHK 21 cells (38th passage) were grown in 80 Roux flasks. [*Me*-^{13}C]Choline (250 mg; 2 mmol) and 42 μCi of [*Me*-^{14}C]choline were added to 5600 ml of choline-free Dulbecco medium supplemented with 10% tryptose broth and 5% fetal bovine serum (70 ml/flask). Radioactivity (12.3%) was removed from the medium, which was decanted after this period of labeling. The cell monolayer was rinsed three times with phosphate buffered saline (PBS). VS virions were adsorbed (about 1 PFU per cell) for 1 hr at 37°. The choline-free medium (50 ml) was added to the monolayer per flask and incubated for 18 hr. After this period of time a complete cytopathic effect was achieved.

[11-^{13}C;1-^{14}C]Oleic acid was incorporated into the envelope lipids by the following method. BHK 21 cells (30th passage) in Roux flasks were allowed to attach to the glass surface for 24 hr in Dulbecco medium, 10% in tryptose broth and 5% in fetal bovine serum. Then the medium was decanted and the cells were rinsed with PBS. Serum-free Dulbecco medium (70 ml) devoid of biotin but with 200 μg of desthiobiotin, 10% tryptose, and 4.0 mg of [11-^{13}C;1-^{14}C]oleic acid (1.28×10^5 dpm) per flask was added. After 24 hr the cells of one flask were harvested, their lipids were extracted, and the total radioactivity was determined. About 70% of the administered labeled oleic acid was incorporated into the cell lipids. Cells were infected as described for the choline experiment, except that the Dulbecco's medium was supplemented with fetal bovine serum.

Virus Purification. Virions were purified essentially as described before (Cartwright, 1969; Klenk and Choppin, 1969). The virus preparation was dialyzed against phosphate buffered saline (PBS) at 4° for 70 hr and centrifuged for 2 hr at 40,000 rpm in a 60 Ti rotor, Beckman. The pellet was homogenized in 2.5 ml of PBS (1% fetal bovine serum) and 0.5 ml of D_2O by gentle magnetic stirring. After the completion of the NMR experiments the integrity of the VSV particles was controlled by electron microscopy. The intact VS virions were then lyophilized and extracted with chloroform–methanol (2:1 and 1:2). Vesicles were prepared from the total lipid extract in saline. The liposome suspension was used for NMR spectroscopy. Following the spin-lattice relaxation studies with the vesicles composed of the virion lipids, the distribution of radioactivity in 3-*sn*-phosphatidylcholine and sphingomyelin was determined by preparative radio thin-layer chromatography of the lipid extract (solvent system: chloroform–methanol–water, 65:25:4). The radioactive bands were separately recovered from the thin-layer plates (Goldrick and Hirsch, 1963) and eluted with chloroform–methanol (2:1 and 1:2) and chloroform–methanol–water (100:50:10). The surface projections (spikes) of virions were removed by treatment with trypsin. After the NMR measurement the virus suspension, which had been protected against bacterial contamination by the addition of 0.01% NaN_3, was dialyzed against 0.04 *M* sodium phosphate buffer (pH 7.6). The sample was concentrated by centrifugation (90 min at 40,000 rpm). The pellet was suspended homogeneously in 7.5 ml of the same buffer by a short 2-sec sonication (80 W) burst with a microtip of a Branson sonifier. Tos-PheCH_2Cl[1] treated trypsin (1 mg/ml, 2 U/mg, Merck, Darmstadt) was added and the suspension incubated for 35 min at 37° with stirring. The sample was immediately cooled in an ice bath and layered on top of a 15–40% dipotassium tartrate gradient without delay and centrifuged in an SW 27 rotor at 27,000 rpm for 3 hr at 4°. The spikeless virions formed a sharp band with a density of 1.1525 g cm^{-3}. No surface projections were detected by negative staining in electron microscopy. The gradient fractions containing the VS virions were again collected, dialyzed against PBS, pelleted, and homogeneously suspended in PBS containing 30% D_2O.

^{13}C NMR Spectroscopy. Proton noise decoupled ^{13}C NMR spectra were obtained at 22.63 MHz with a Bruker WH-90 pulse spectrometer operating in the Fourier transform mode. A 180°–*t*–90° pulse sequence (Freeman and Hill, 1971) was employed for spin–lattice relaxation time (T_1) measurements, where *t* is the delay time between the 180 and 90° pulses. The T_1 values were derived from the inversion recovery technique according to $M_0 - M_z = 2M_0 \exp(-t/T)$.[2] An internal deuterium field-frequency lock was used. The free induction decay signals were obtained with a 20 μsec pulse. Sample temperature was controlled by a Bruker ST 100/700 variable temperature controller with an accuracy better than ±1°. Sample tubes of 10 mm o.d. were used, which were constricted to about 1 mm i.d. below the liquid surface in order to minimize the liquid interchange, which might affect the T_1 value due to the contribution of rapidly relaxing nuclei in the vapor phase (Aksnes et al., 1968). Cell samples were flushed with purified argon gas.

Liposomes were formed from the total lipid extract of virions by ultrasonication in saline (Huang, 1969).

Results

Enrichment of [11-^{13}C]Oleic Acid in Complex Lipids of the Vesicular Stomatitis Virion Envelope. The BHK 21 host cells were first labeled optimally with radioactive oleic

[2] The estimated error in repeated T_1 determinations was below 5%; in repeated experiments it was between 5 and 12%.

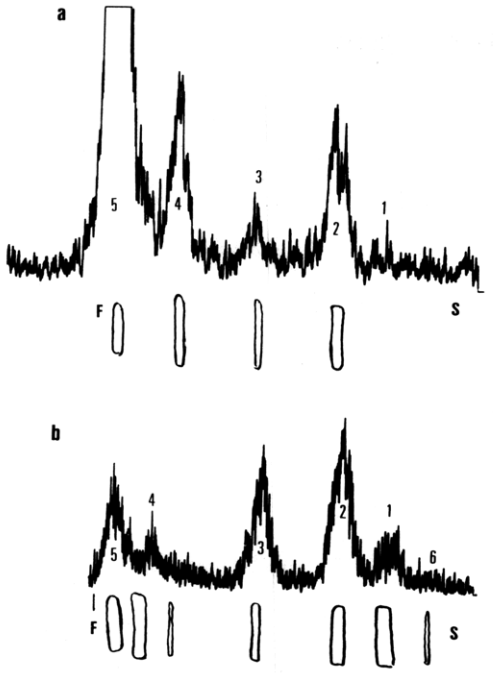

FIGURE 1: Radio thin-layer chromatogram of total lipid extract of (a) BHK 21 cells (control); (b) VS virions. BHK 21 cells were grown in a [1-^{14}C;11-^{13}C]oleic acid containing medium; solvent system, chloroform–methanol–water (65:25:4); (1) sphingomyelin; (2) 3-*sn*-phosphatidylcholine; (3) 3-*sn*-phosphatidylethanolamine; (4) cardiolipin; (5) triglycerides and free fatty acids; (6) hematoside.

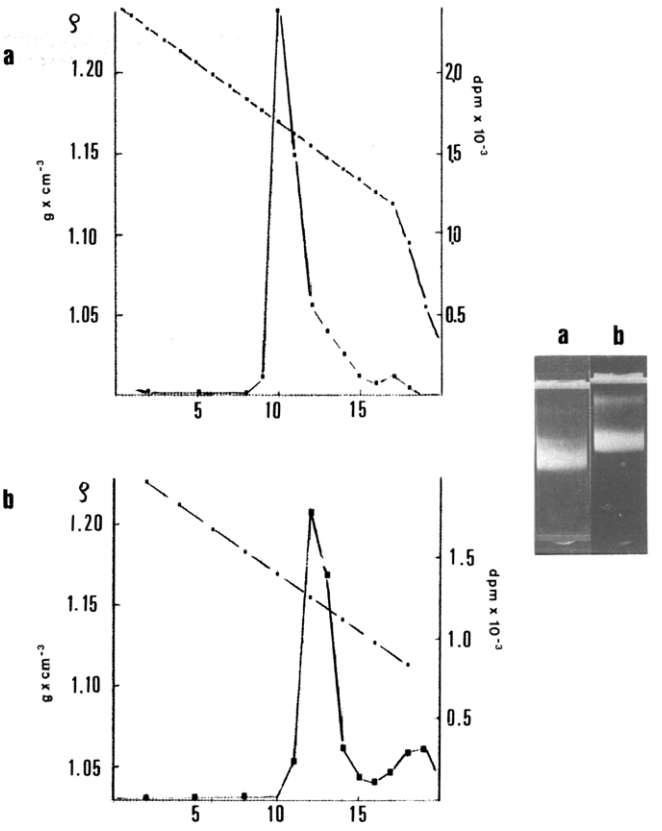

FIGURE 2: Purification of VS virions by gradient centrifugation (potassium tartrate, 15–40% w/v): (a) before; (b) after trypsin treatment.

acid. BHK 21 cells were grown for 24 hr in serum containing medium followed by serum-free medium, to which desthiobiotin (2.85 μg/ml) had been added in order to stop de novo fatty acid synthesis. Cell growth was promoted by the addition of oleic acid (70 μg/ml) in ethanol solution. On the basis of optimizing experiments with [1-^{14}C]oleic acid it was calculated that 70% of the radioactivity was recovered in the ester lipids of the BHK 21 cells (Figure 1a). Transesterification of the total phospholipids yielded 65% 18:1, 14.5% 18:0, 3.0% 16:1, 14.0% 16:0, and 3.5% 14:0 fatty acids. VS virions grown in these radioactively labeled cells were purified by gradient centrifugations and their purity controlled by electron microscopy after negative staining with phosphotungstic acid. Virus envelope lipids were obtained by Folch extraction of the thoroughly dialyzed and lyophilized virus band. Their analysis is given in Figure 1b.

Of the radioactivity added to the growth medium 0.5% was recovered in the total lipid extract. Silicic acid column chromatography separated the total lipids into a neutral lipid fraction (28.3% of total radioactivity), a glycolipid fraction (1.7%), and a phospholipid fraction (70%). The glycolipid fraction consisted only of hematoside, whereas in the phospholipid fraction 15% of the radioactivity was present in sphingomyelin, 50% in 3-*sn*-phosphatidylcholine, and 35% in phosphatidylethanolamine. Preparative thin-layer separation of the phospholipids yielded pure radioactive compounds, which were transesterified for radio gas–liquid chromatography. In all cases the only labeled fatty acid was oleic acid.

In the pilot ^{13}C experiment BHK 21 cells were cultured in 80 Roux bottles under the conditions elaborated in the screening experiments, but now the serum-free medium had been enriched with 50 μg/ml of [11-^{13}C]oleic acid and for better tracing of the labeled lipids with 3.5 μg/ml of [1-^{14}C]oleic acid. The confluent cell layer was infected with VS virions (1 PFU/cell) adsorbed over a period of 1 hr at 37°. Then fatty acid free medium containing 5% fetal bovine serum was added. All cells showed a massive cytophathic effect after 12 to 18 hr (rounding and detachment of cells). Virus was isolated and then purified by two gradient centrifugations, the first in 15–45% sucrose–phosphate buffer, the second in a 15–40% potassium tartrate gradient as described before (Klenk and Choppin, 1969). One main virus band with a density of 1.1777 (Figure 2) yielded 66.5 mg of VS virion protein after dialysis. The concentrated sample (see Materials and Methods section) was used immediately for ^{13}C NMR spectroscopy. The suspension was made 0.01% in sodium azide.

The total lipids of the enveloped virions contained 7.5 mg (26.6 μmol) of labeled oleic acid as calculated from the radioactive [1-^{14}C]oleic acid incorporated into the virus preparation. Gas–liquid chromatography of the fatty acid methyl esters indicated that 70% oleic acid was present in the total mixture.

The resonance line of the C-11 appears at 28.27 ppm related to tetramethylsilane (Me_4Si). T_1 time was measured to 169 msec.

Figure 3 represents spin–lattice relaxation time (T_1) determination of [11-^{13}C]oleic acid incorporated in the lipids of VSV envelope. Also, the polar head groups of phosphatidylcholine and sphingomyelin were enriched with the ^{13}C probe by supplementing the chemically defined medium with [*Me*-^{13}C]choline hydrochloride.

Optimal conditions for the labeling of the host cell phospholipids were determined by exploratory experiments, using [*Me*-^{14}C]choline. BHK 21 cells incorporated 5.3, 12.7, and 18% of the choline added to the medium within 45, 69, and 72 hr, respectively. The label was present exclu-

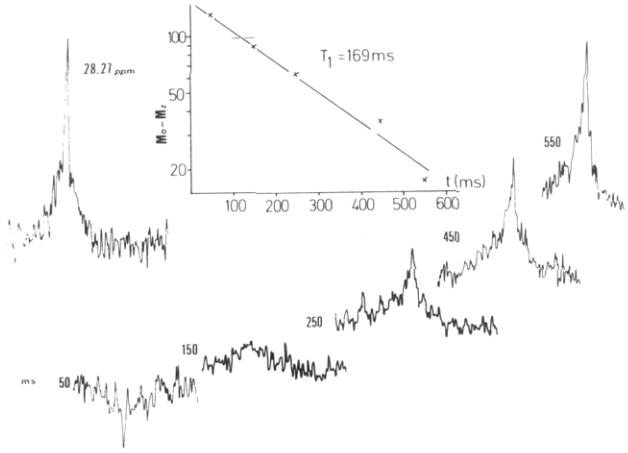

FIGURE 3: Determination of spin–lattice relaxation time (T_1) of [11-^{13}C]oleic acid containing phospholipids of VS virions; aqueous suspension; 37°; 22 mg of virus protein/ml.

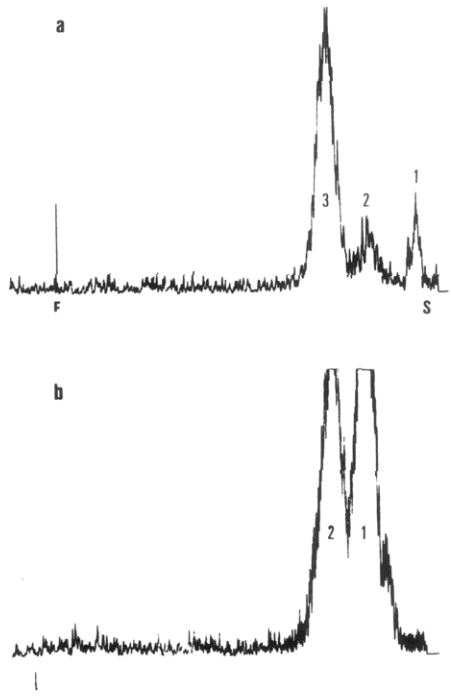

FIGURE 4: Radio thin-layer chromatography of total lipid extract of (a) BHK 21 cells; (b) VS virions grown in these cells. BHK 21 cells were grown in a [*Me*-^{13}C;^{14}C]choline containing medium; solvent system, chloroform–methanol–water (65:25:4); (a) (1) phosphorylcholine; (2) sphingomyelin; (3) 3-*sn*-phosphatidylcholine; (b) (1) sphingomyelin; (2) 3-*sn*-phosphatidylcholine.

sively in 3-*sn*-phosphatidylcholine (94%), sphingomyelin (4%), and phosphorylcholine (2%) (Figure 4a). The radioactivity was distributed in the VS virions grown in these cells only in 3-*sn*-phosphatidylcholine (57%) and sphingomyelin (43%) (Figure 4b). Both phospholipids had the same specific radioactivities. On the basis of these results the BHK 21 cells were grown for 72 hr in a medium containing [*Me*-^{13}C]choline (0.33 μmol/ml) and [*Me*-^{14}C]choline before being infected with VS virions at a multiplicity of 0.1–1 PFU/cell. Virions were harvested and purified as outlined before and described under Materials and Methods. From the radioactivity present in the lipid extract of an aliquot of the virus preparation it could be calculated that 0.63% of the choline was present in the choline-containing phospholipids, which is equivalent to 1.62 mg (11.6 μmol).

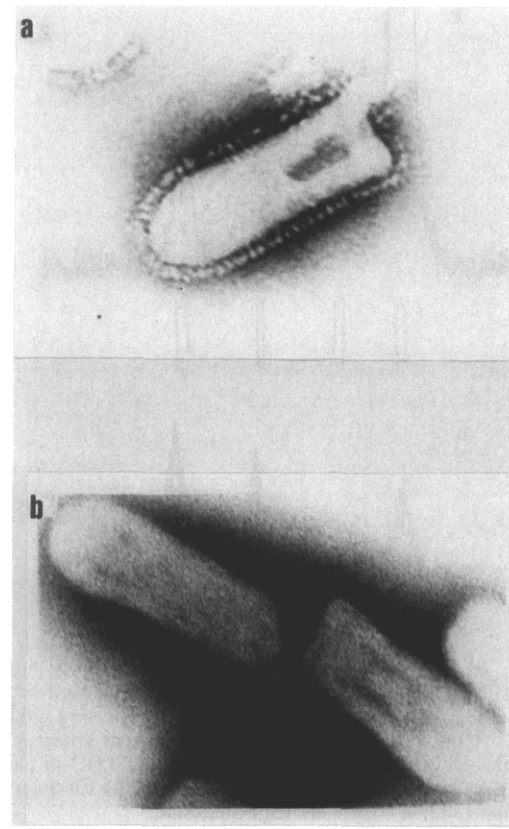

FIGURE 5: Electron microscopy of VS virions: (a) before; (b) after removal of surface projections with trypsin; magnification: 185,000×.

The VS virions were intact as demonstrated by electron microscopic controls (Figure 5a). The sharp virus bands from the tartrate gradient were dialyzed against phosphate-buffered saline, concentrated, and prepared for the ^{13}C NMR measurements as described before.

The ^{13}C NMR spectrum of the VS virion suspension showed a strong signal characteristic for the enriched choline *N*-methyl group with the resonance band at 54.0 ppm. T_1 time was determined at 37° to be 224 msec and in a second experiment it was 252 msec (Figure 6).

The ^{31}P NMR spectrum showed a very broad signal expanding over 800 Hz. This could correspond to a T_2 time of 0.4 msec (Feigenson and Chan, 1974).

In order to study the influence of the surface projections on the lipid organization in the virus envelope another purified VS virion preparation labeled again in the choline moiety of 3-*sn*-phosphatidylcholine and sphingomyelin of the envelope was treated with trypsin under conditions safe to guarantee the complete removal of the surface glycoprotein projections (Cartwright, 1969). Electron microscopy of the trypsin-treated virions confirmed that all spikes had been removed (Figure 5b). These virions banded in the continuous tartrate gradient at a density of 1.1525 (Figure 2b).

Spin–lattice relaxation measurements in the manner described above had the result documented in Figure 7. The spin–lattice relaxation time of the *Me*-^{13}C group of choline in 3-*sn*-phosphatidylcholine and sphingomyelin was considerably reduced to 185 and 162 msec in two independent experiments.

The total lipids (32 mg) of the trypsinized VS virions were extracted and used for the formation of liposomes by ultrasonication (Huang, 1969) and subsequent T_1 time determination of the [*Me*-^{13}C]choline group of 3-*sn*-phospha-

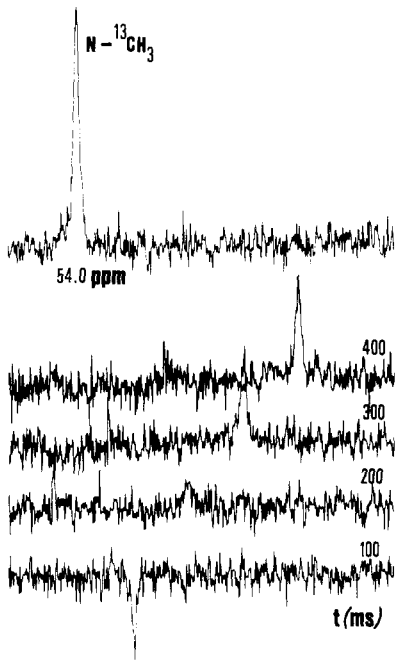

FIGURE 6: Determinations of spin-lattice relaxation time (T_1) of the *N*-methyl group of choline-containing phospholipids of VS virions labeled with [*Me*-^{13}C]choline (aqueous suspension; 37°; 20–30 mg of virus protein/ml).

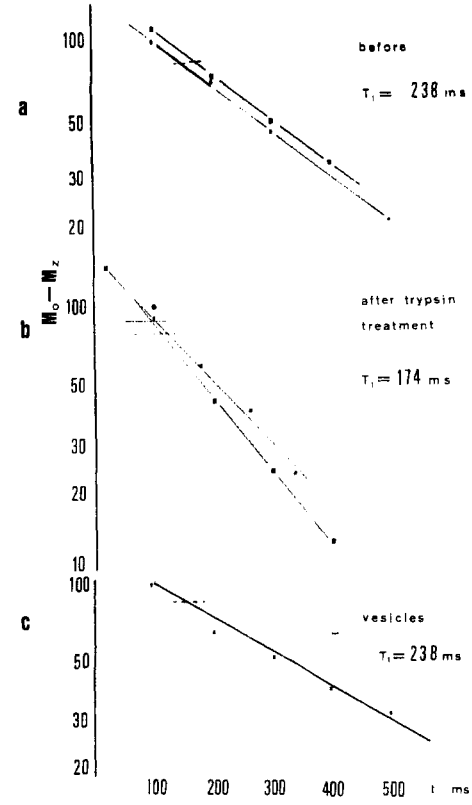

FIGURE 7: Graphical representation of determination of T_1 times: (a) complete virions; (b) trypsinized VS virions; (c) liposomes from VS virion lipids. T_1 values are averaged values of two determinations.

tidylcholine and sphingomyelin. A T_1 of 238 msecs identical with that of the lipids in the envelope of the native virus particle, was found.

On the basis of our own lipid analysis which is in satisfactory agreement with previous reports (McSharry and Wagner, 1971) mixed liposomes containing 3-*sn*-phosphatidylcholine, 3-*sn*-phosphatidylethanolamine, sphingomyelin, 3-*sn*-phosphatidylserine, and cholesterol in a ratio of 1:1:1:1:3 were prepared for comparison. The phospholipids consisted of the dilinoloyl species. In 3-*sn*-phosphatidylserine-free liposomes the [*Me*-^{13}C]choline T_1 time was about 469 msec, which decreased to T_1 = 418 msec after admixture of the serine-containing phospholipid.

Discussion

Among the numerous chemical and physical methods applied in membrane research only those which use probes with the biologically most suitable properties are valuable. The optimal requirements would be: (1) that the chemical structure of the probe does not affect the molecular organization of the macromolecular structure under study, either by chemical or by physical perturbation; (2) it should be possible to introduce the probe in defined positions of a biological membrane covalently integrated either at the lipid site or the protein components; (3) the probe must be present there at concentrations allowing accurate measurements.

The ^{13}C NMR technique and the labeling of the lipids of biological membranes with ^{13}C in specific positions of the hydrophobic and hydrophilic parts of the phospho- and sphingolipids are suitable approaches to the study of lipid–lipid and lipid–protein interactions (Stoffel et al., 1974a–c). The experiments described in this communication prove that it is possible to use the ^{13}C NMR spectroscopy also for a biological membrane and the interactions of its component.

We have chosen the simple model membrane of an enveloped RNA virus, namely vesicular stomatitis virus (VSV). All the evidence suggests that one glycoprotein which represents the surface projections interacts with a lipid bilayer. Apparently during maturation the nucleocapsid is wrapped into a bilayer of lipids derived from the host cell.

The primary objective in these studies was to find out whether it is possible to incorporate ^{13}C-labeled precursors by a biochemical approach into the hydrocarbon chain of the complex ester and amide lipids and the head group of the two choline-containing phospholipids 3-*sn*-phosphatidylcholine and sphingomyelin. If this was possible, then the problem of lipid–lipid and lipid–protein, particularly lipid–glycoprotein (spike), interaction in the envelope was approached since the relaxation times of the ^{13}C-enriched carbon atoms of both regions of the bilayer should allow insight into their mobilities and therefore interactions.

We labeled the host cell with [11-^{13}C]oleic acid as a probe of the hydrocarbon region of the phospho- and sphingolipids which comprise about 50% of the viral envelope lipids.

^{13}C was also enriched biochemically in the polar head groups by growing the host cells in [*Me*-^{13}C]choline, thus labeling the choline-containing lipids 3-*sn*-phosphatidylcholine and sphingomyelin. This approach then facilitated not only obtaining the NMR signals of the labeled C atoms but also measuring their spin-lattice relaxation times (T_1). Based on our earlier studies on the metabolism of polyenoic acids in tissue culture (Stoffel and Scheid, 1967) we could demonstrate now that free oleic acid added to the medium (2% serum) is incorporated more than 60% into the phospho- and sphingolipids of BHK 21 cells and even more into the complex lipids of the viral envelope.

Recently it has been described that the fatty acid compo-

sition of animal cells grown in chemically defined medium can be manipulated by the addition of fatty acid sorbitan esters to the medium with simultaneous suppression of de novo fatty acid synthesis with desthiobiotin (Williams et al., 1974).

The spin–lattice relaxation time (T_1) of [11-^{13}C]oleic acid incorporated into the phospholipids of the envelope to about 60% of all acyl groups is 169 msec. This value differs considerably from the T_1 of, e.g., 1-stearoyl-2-[11-^{13}C]oleoyl-3-*sn*-glycerophosphorylcholine in chloroform solution (1435 msec) or in liposomes of the single species lecithin (278 msec).

We have described the influence of cholesterol on the spin–lattice relaxation time of the hydrocarbon chain C atoms of this and other lecithin species in liposomes (Stoffel et al., 1974c). Increasing ratios of cholesterol/phospholipid led to a reduced T_1 of C-11 of oleic acid of lecithins, indicating an increased immobilization.

The restriction in the mobility of the central part of the hydrocarbon chain of the virus membrane can be caused by this interaction of phospholipid hydrocarbon chain and cholesterol since the cholesterol/phospholipid ratio in the VSV envelope is very high (0.7). Another restriction in mobility could be imposed on the hydrocarbon chains by their interaction with sites of the glycoproteins (spikes) embedded in or penetrating the lipid bilayer. The question how the glycoproteins are integrated or interact with the other envelope components has not yet been settled. Also the [*Me*-^{13}C]choline group of viral envelope 3-*sn*-phosphatidylcholine and sphingomyelin exhibited a low spin–lattice relaxation time (T_1 = 238 msec). This T_1 value is considerably reduced to 174 msec after the glycoprotein surface projections have been tryptically digested. Therefore, we assume that the spikes with their polyhexoside side chains which are rendered negatively charged due to terminal neuraminic acid residues do not reduce the mobility of the choline residues of phosphatidylcholine and sphingomyelin. The high cholesterol content in the envelope lipids not only leads to a restricted mobility of the alkane chains but also of the polar head group. When vesicles consisting of the [*Me*-^{13}C]choline-labeled 3-*sn*-phosphatidylcholine, sphingomyelin, 3-*sn*-phosphatidylethanolamine, and cholesterol in a ratio of 1:1:1:3, imitating the natural lipid composition of the viral envelope, are used for the determination of the spin–lattice relaxation time of their choline polar head group a T_1 of 460 msec was measured as reported in a previous paper. The only differences of these vesicles to those produced from the envelope lipids are the presence of 1 part of 3-*sn*-phosphatidylserine and the fatty acid substitution. The envelope lipids contain 25% palmitic, 20% stearic, and 34% oleic acid as main constituents and they are therefore much more saturated. Therefore, 3-*sn*-phosphatidylserine-containing liposomes with the aforementioned composition and 1 part of phosphatidylserine in addition were subjected to ^{13}C NMR spectroscopy. The [*Me*-^{13}C]choline group had a reduced T_1 time (418 msec). Therefore, the difference in the fatty acid composition in conjunction with the cholesterol must be responsible for the low mobility of the alkane chain and the choline head groups of the viral phospholipids. All the evidence deduced from our experiments points out that the lipid phase of the viral envelope is in a very rigid state mainly due to lipid–lipid interactions. If further studies will prove that the surface projections are anchored in the lipid phase (bilayer) this rigid lipid arrangement should prohibit a lateral diffusion of the glycoproteins. Our studies do not rule out that part of the glycoprotein basis might enter or penetrate the lipid bilayer of the envelope.

Concurrent studies on the possible asymmetric distribution of these lipids in the viral envelope and further studies of the interaction between lipids and the spike glycoprotein and the membrane protein bordering the inner lipid layer will contribute to our understanding of the structure of a simple model membrane as that present in an enveloped virus.

We have demonstrated the usefulness of the ^{13}C NMR technique in these structural studies by a suitable biochemical approach to the specific labeling of the membrane lipid phase. The method lends itself to more sophisticated studies by further refinements.

Acknowledgment

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Nonspecific Acid Phosphatase from *Schizosaccharomyces pombe.* Purification and Physical Chemical Properties†

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ABSTRACT: Repressible nonspecific acid phosphatase from *Schizosaccharomyces pombe* was purified to apparent homogeneity, as ascertained from ultracentrifugal, electrophoretic, and chromatographic data. The native protein has a molecular weight of 383,000 as determined by sucrose density gradient centrifugation and 381,000 as determined by gel filtration. The native protein can be dissociated in the presence of 8 *M* urea–1% sodium dodecyl sulfate into subunits possessing an approximate molecular weight of 104,000. Neutral sugars account for about 66% of the total molecular weight and contribute to the high solubility and some of the other physical properties of this enzyme. Purified enzyme preparations have a K_m for 4-nitrophenyl phosphate of 0.17 m*M* and a broad substrate specificity, but do not show diesterase activity. Phosphate and sulfate are competitive inhibitors. The enzyme is inactivated at neutral and alkaline pH and at relatively low temperatures. Mannose and galactose were found as the main components of the carbohydrate moiety; glucosamine was present in lower amounts. The amino acid analysis revealed a high content of aspartate, threonine, and serine; no sulfhydryl group could be detected. P_i is released in stoichiometric amount (1 mol per enzyme monomer) on protein digestion.

The regulatory mechanisms of phosphatase formation have not yet been clearly elucidated in eukaryotic microorganisms. The available data on the regulation of the synthesis of the nonspecific acid phosphatase of *Saccharomyces cerevisiae* (Shurr and Yagil, 1971; Toh-e et al., 1973) and nonspecific alkaline phosphatase of *Neurospora crassa* (Lehman et al., 1973; Toh-e and Ishikawa, 1971) suggest a more complex regulatory mechanism with respect to that of the corresponding alkaline phosphatase in *Escherichia coli,* which has been extensively studied by Garen and his colleagues (Garen and Echols, 1962a,b). Our previous work on the nonspecific acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) in *Schizosaccharomyces pombe* has been concerned with preliminary characterization both in intact cells and in cell-free extract and investigations on the factors affecting the enzymatic activity of the cells (Dibenedetto, 1972). Our results suggested that, besides the repression by inorganic phosphate, additional factors influence the levels and changes of phosphatase activity. Evidence was then obtained indicating that unimpeded formation of mannan is a necessary condition for the formation of the acid phosphatase. To investigate further the genetic control of the formation of the acid phosphatase in *S. pombe* it was essential to obtain pure preparations and to investigate the chemical and physical features of the enzyme molecule. The present article describes the purification procedure and the physical and chemical properties of the pure enzyme.

Materials and Methods

Urea was recrystallized from hot methanol, norleucine was obtained from Mann Research Laboratories, and dansyl-Cl[1] was from Calbiochem. Diisopropyl fluorophosphate treated carboxypeptidase A was supplied by Worthington. Twice distilled constant boiling HCl was used for hydrolyses. Bovine serum albumin (from Calbiochem), β-galactosidase (from Sigma), and catalase (from Boehringer) were used without further purification. Phosphorylase was kindly supplied by the laboratory of Dr. E. Fischer and arginine decarboxylase by Dr. E. Boeker. Bio-Gel A-5m, 200–400 mesh, was obtained from Bio-Rad and washed with buffer before using. Dextrans T-150, T-250, and T-500 were purchased from Pharmacia, Fine Chemicals.

Activity Assay. The nonspecific acid phosphatase was assayed as described before (Dibenedetto, 1972) by a modification of the method of Torriani (1960) and by determination of phosphorus using the method of Fiske and Subba-Row (1925) with minor modification. One unit of acid phosphatase activity represents the amount of enzyme catalyzing the release of 1 μmol of 4-nitrophenol/min at 30°,

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[1] Abbreviation used is: dansyl, 8-dimethylamino-1-naphthalenesulfonate.