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THE STRUCTURE OF VESICULAR STOMATITIS VIRUS MEMBRANE

A PHOSPHORUS NUCLEAR MAGNETIC RESONANCE APPROACH

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Summary

The proton decoupled 40.48 M Hz ^{31}P NMR spectrum of intact and unperturbed membrane-enclosed vesicular stomatitis virus (serotype Indiana) exhibited two distinct maxima. These can be resolved into a narrow, symmetric line and a broad asymmetric line. The ^{31}P NMR spectrum of a multilamellar (unsonicated) preparation of the extracted viral lipids exhibited a line shape similar to that of the intact virus. A sonicated vesicle preparation of the extracted viral lipids exhibited a narrow symmetric line. The narrow component in the intact virus spectrum may be attributed to small membrane fragments. Phospholipase C digestion of the intact virus resulted in substantial reduction in intensity of both components which suggests that much of the contribution to both peaks is due to phosphate in the phospholipid polar head groups.

The phospholipid phosphates in both sonicated and unsonicated preparations of the extracted viral lipids exhibited substantially longer relaxation times than did those in the intact virus. The short relaxation time emanating from the intact virus preparation is caused by immobilization of the phospholipid head groups which could be due to lipid-protein interactions. Trypsin treatment of vesicular stomatitis virions, which results in complete removal of the exterior hydrophilic segment of the membrane glycoprotein, increased the ^{31}P relaxation time to a value similar to that observed in the protein-free total lipid extracts; this finding provides supporting evidence for the role of virus glycoprotein in shortened relaxation times. A reversible temperature-dependent change in apparent line width and absence of an effect of cholesterol on the ^{31}P phospholipid spectrum were also demonstrated.

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Abbreviation: VS virus, vesicular stomatitis virus.

Introduction

Vesicular stomatitis virus (serotype Indiana) (VS_{Ind} virus) is a membrane-enclosed, bullet-shaped rhabdovirus which contains five structural proteins [1—4]. Two of these proteins, the glycoprotein (G) and matrix (M) protein, have been shown to be associated with the virus lipid bilayer [5—9]. The remaining three proteins, the major nucleocapsid (N) protein and the minor large (L) protein and non-structural (NS) protein are associated with the RNA of the virus to form a nucleocapsid structure which can be isolated from the virus by various treatments [10—12].

VS virus (serotypes New Jersey and Indiana) has been shown by McSharry and Wagner [13] to have a bilayer membrane composed of lipids derived from the host cell but not identical in composition to the lipids of the plasma membrane from which the virus buds. It has also been demonstrated, using fluorescence depolarization analysis of the lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene, that the virus membrane has a higher microviscosity than that of the host cell plasma membrane [14—16]. Little difference was observed in a comparison of the microviscosities of the hydrophobic region of the intact membrane of VS virus and small vesicles made from the extracted virus lipids [14—16]. Protease treatment of VS_{Ind} virus also produced only small changes in the microviscosity as measured by fluorescence depolarization [14—16].

While general information is available on the location of protein, lipid, and carbohydrate in the membrane of VS virus, little information is available on how membrane proteins interact with the lipid moieties. Stoffel and Bister [17] have suggested from results of ^{13}C nuclear magnetic resonance studies of [^{13}C] choline-labelled VS virus that the mobility of the choline group on 3-*sn*-phosphatidylcholine and sphingomyelin was restricted. They suggested that this may be attributable to the high cholesterol content of the VS virus membrane. Phosphorus nuclear magnetic resonance has been used for examining the behavior of the polar head group in multilamellar lipid membranes [18,19], sonicated vesicles [18,20—23], and various native membrane systems including those of *Escherichia coli* [24], sarcoplasmic reticulum [25], and *Acholeplasma laidlawii* [26].

The purpose of this work was to examine the behavior of the phosphate moieties of the lipids of intact VS virus using ^{31}P NMR without any perturbing probes. ^{31}P spectra were obtained of intact VS virus and compared to spectra of phospholipase C-treated virus, trypsin-treated virus, and large multilamellar structures and sonicated lipid dispersions made from the extracted virus lipids. Using these systems we attempted to differentiate between components of the spectra attributable to RNA, lipid-lipid interaction and protein-lipid interaction.

Materials and Methods

Cells, virus growth and purification. BHK-21 cells were grown at 37° C in BHK-21 medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum as previously described [14]. BHK-21 cells diluted 1 to 10 in 75 cm^2 Falcon plastic bottles reached confluency in 48 h. The Indiana serotype of VS virus (VS_{Ind} virus) was used to infect confluent monolayer of BHK-21

cells at a multiplicity of infection of 0.1—1.0 plaque-forming unit (p.f.u.)/cell resulting in the formation of bullet-shaped (B) virions. The virus was grown in monolayers of BHK-21 cells at 37°C for 16—18 h, and the supernatant fluids were then harvested. VS_{Ind} virus was purified as previously described by sequential differential, velocity and equilibrium centrifugation [14].

Preparation of VS_{Ind} virus for nuclear magnetic resonance spectroscopy. Purified VS_{Ind} virus (~2 mg/ml) was dialyzed at 4°C against three changes of 100 volumes of 20 mM Tris, 100 mM NaCl (pH 7.5) for 16 h. Purified virus was concentrated by pelleting at 65 000 × *g* for 90 min and resuspended at a concentration of 50 mg/ml (protein) in 100 mM NaCl, 20 mM Tris (pH 7.5). Alternatively, purified VS_{Ind} virus in 20 mM Tris, 100 mM NaCl (pH 7.5) was made 1 mM with EDTA and pelleted at 80 000 × *g* for 90 min. The virus was resuspended at a concentration of 50 mg/ml (protein) in 20 mM Tris containing 100 mM NaCl and 1 mM EDTA.

Lipid extraction, multilamellar lipid preparation and sonicated vesicle formation. 10 ml of VS_{Ind} virus (5 mg/ml protein) was extracted with 37.5 ml chloroform/methanol (1 : 2, v/v) [27] under N_2 for a minimum of 16 h at 4°C. Protein was removed by pelleting at 2000 × *g* for 10 min at 4°C; 12.5 ml chloroform and 12.5 ml water was added to the supernatant which was then further extracted by vortex mixing. The lower organic phase was removed and dried under a stream of N_2. The extracted lipids were then dissolved in 2—3 ml of spectral grade benzene, frozen and dried in vacuo. The dried lipids were resuspended, under argon, in 1 ml of 20 mM Tris, 100 mM NaCl (pH 7.5) by vortexing for 15—20 min. Liposomes were made by high intensity ultrasonic irradiation of the lipids under nitrogen for 25—30 min using Heat Systems W-350 Sonifier. Multilamellar lipid preparations and sonicated small vesicles were made in the presence and absence of 1 mM EDTA in the 20 mM Tris, 100 mM NaCl buffer.

Phospholipase C digestion of VS_{Ind} virus. 15 ml of VS_{Ind} virus (total protein = 47 mg) in 20 mM Tris, 100 mM NaCl was mixed with 5 ml of 6.6 mM $CaCl_2$ and 5.0 ml of phospholipase C (*Clostridium welchii* enzyme Type I purchased from Sigma Chemical Co., St. Louis, Mo.) at a concentration of 1 unit/ml in 20 mM Tris, 100 mM NaCl. Digestion was performed at 37°C for 4.5 h under which conditions the reaction was completed and ~55% of the total lipid phosphate was removed (Patzer, E.J., Moore, N.F. and Wagner, R.R., unpublished). The phospholipase C-digested virus was then pelleted through a 2 ml pad of 20% sucrose at 65 000 × *g* for 90 min. After centrifugation the pellet of digested virus was carefully drained and resuspended in 1 ml of 20 mM Tris, 100 mM NaCl (pH 7.5) in preparation for nuclear magnetic resonance spectroscopy.

Nuclear magnetic resonance measurements. ^{31}P NMR spectra were obtained in 10-mm tubes under N_2 using a JEOL-PS100/EC100 Fourier transform spectrometer at 40.48 MHz and 23°C [22]. 4096 data points were obtained in the frequency domain with a spectral width of 5 KHz, using a JEOL 5 KHz RF crystal filter and continuous proton noise decoupling. Most spectra were obtained with successive 90° pulses (20 μs) 4—5 T_1 apart. T_1 measurements were made with the 180°-τ-90° pulse sequence, using 6—8 data points and data were analyzed by a least squares procedure. Spectra of whole virus required up

to 9 h accumulation time. Spectra were calculated using exponential filters large enough to obtain maximum sensitivity without lineshape distortion.

Dodecyl sulfate gel electrophoresis. Purified VS_{Ind} virus was made 1% in sodium dodecyl sulfate, 1% in 2-mercaptoethanol, 10% in glycerol and the sample was heated at 100°C for 90 s. Samples were electrophoresed in 7.5% polyacrylamide gels in phosphate buffer as previously described [28]. Polyacrylamide gels containing radioactive viral proteins were sliced into 1-mm fractions using a Mickle gel slicer and each slice was incubated in 0.5 ml of Nuclear-Chicago solubilizer/water (9 : 1, v/v) for 2 h at 50°C. 10 ml of toluene-based scintillation fluid was added to each sample and radioactivity was determined in a Beckman LS-230 liquid scintillation counter.

Protein determinations. Protein was determined using the procedure of Lowry et al. [29] with crystalline bovine plasma albumin as the standard.

Cholesterol determinations. Cholesterol was quantitated using the enzyme cholesterol oxidase, by a modification of the procedure developed by Allain et al. [30].

Phosphorus determinations. Phosphorus was determined as described by Bartlett [31].

Electron microscopy. Purified virus preparations at a concentration of 0.25—0.50 mg/ml (protein) were mixed with an equal volume of 1% glutaraldehyde and incubated on ice for 20 min. The samples were adsorbed from droplets (2 min adsorption) onto Formvar carbon-coated grids and 2% phosphotungstic acid was added to the grid for 30 s. Electron microscopy was performed using a Siemens Elmiskop 1A at an initial magnification of 62 000.

Results

Structure, purity and composition of VS_{Ind} virus

Fig. 1 is an electron micrograph demonstrating the unusual bullet-shaped structure of VS virus. The viral particles are 170—183 nm in length and 60—76 nm wide [32,33]. The glycoprotein spikes extend out from the surface to a distance of approx. 10 nm. As seen by electron microscopy the viral particles exhibited a high degree of intactness and there was no evidence of contamination by cellular membranes. No defective (truncated) particles [34] were apparent in any of the preparations. Polyacrylamide gel analysis of viral proteins containing ^{14}C-labeled amino acid showed no evidence of cellular protein contamination (Fig. 2). The three major structural proteins (G, N and M) and the two minor components (L and NS) are all apparent.

VS_{Ind} virus was found to have a cholesterol to phospholipid molar ratio of 0.72 which agrees closely with published values [13,17]. The phospholipid composition of the VS virus using two different thin-layer chromatography systems [36,37] and phosphate analysis [31] of the iodine-stained phospholipids in the silica gel is as follows: 23.9% phosphatidylcholine, 17.9% phosphatidylserine, 30.9% phosphatidylethanolamine, 23.9% sphingomyelin, 1.9% phosphatidylinositol (Patzer, E.J., Moore, N.F. and Wagner, R.R., unpublished). Although this is the phospholipid composition of VS_{Ind} virus grown on BHK-21 cells, it agrees fairly closely with published values for the same serotype of VS virus grown on L-929 cells [13,35].

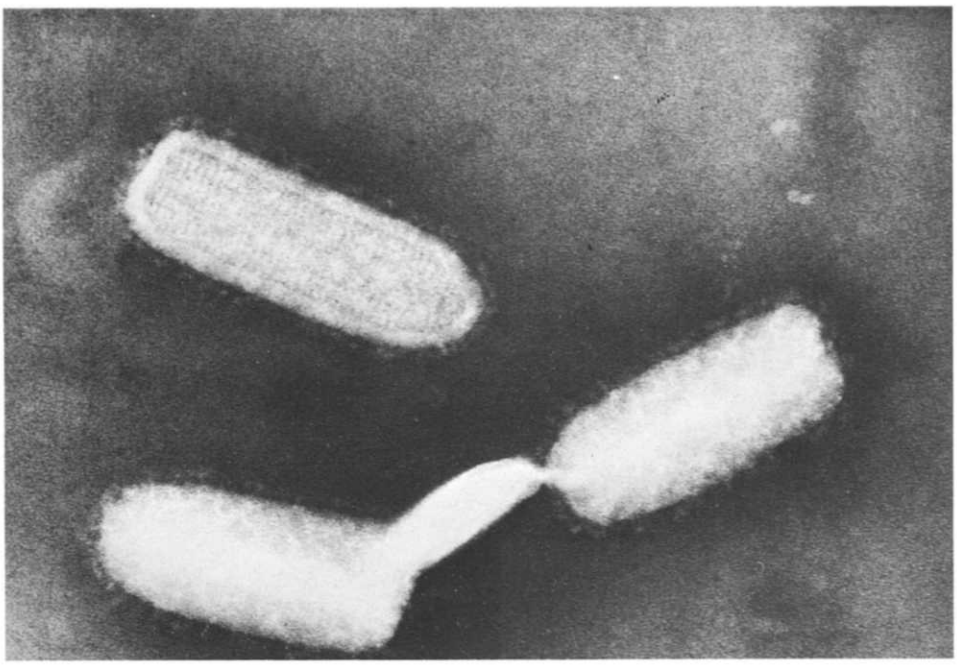

Fig. 1. Electron micrograph of pure VS_{Ind} virus. Virus particles were fixed with 0.5% glutaraldehyde for 20 min at 4°C prior to spreading onto Formvar carbon-coated grids. The grids were stained with 2% phosphotungstic acid for 30 s. Initial magnification was × 62 000.

The electron microscopy, cholesterol and phospholipid analyses and protein content of the purified virus indicates that our virus preparations are structurally intact and have a similar composition to those described by other workers. In addition the infectivity of the pure virus, as assayed by plaque assay on monolayer cultures of L-929 cells [38] was equivalent to previously described values.

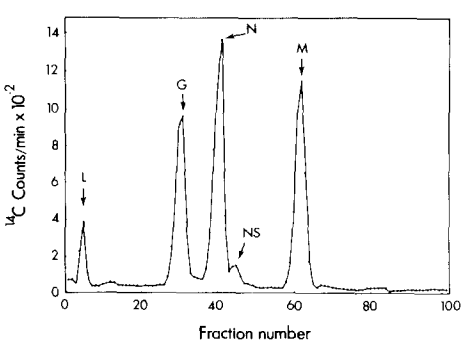

Fig. 2. Dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins of pure VS_{Ind} virus labeled with ^{14}C-labeled amino acid. The virus was grown on confluent BHK-21 cells and purified as described in Materials and Methods. 1 μCi/ml (57 Ci/atom) of ^{14}C-labeled amino acid hydrolysate was introduced into the virus growth medium.

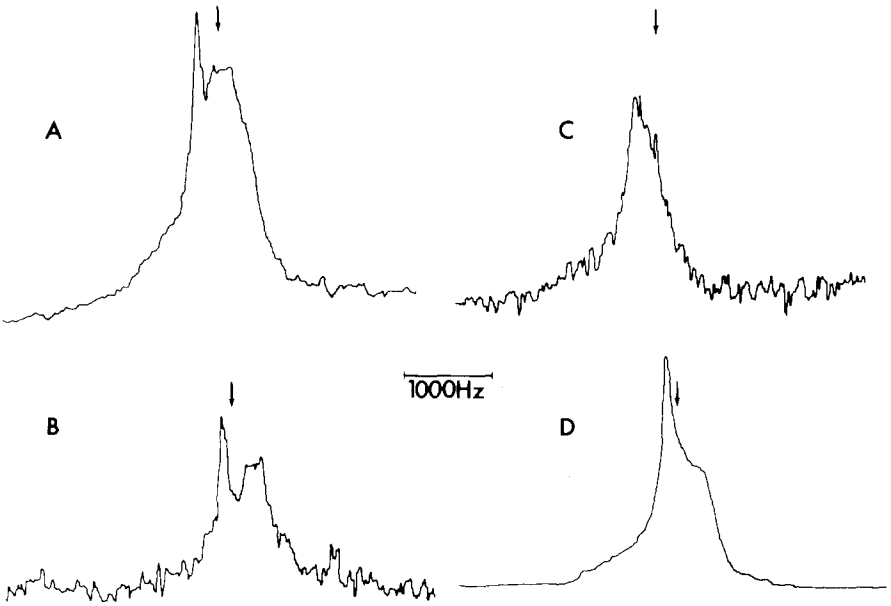

Fig. 3. Proton noise decoupled ^{31}P NMR spectra of VS virus and total lipid extracts. A, intact virus at 23°C; B, unsonicated total lipid extract at 23°C; C, intact virus at 40°C; D, unsonicated dispersion of phospholipids which mimic the virus composition, but with no cholesterol, at 23°C. The arrow refers to the resonance position of egg phosphatidylcholine unilamellar vesicles.

^{31}P NMR spectra of intact VS_{Ind} virus, lipid dispersions and sonicated vesicles made from the extracted virus lipids

The proton decoupled spectrum obtained with intact VS_{Ind} virus is shown in Fig. 3A. Two maxima are observed; a small, narrow symmetric line and a broad

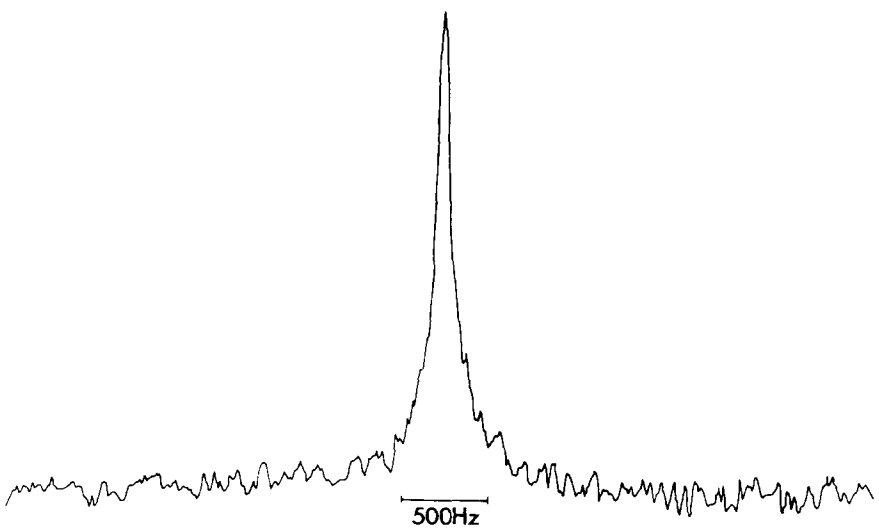

Fig. 4. Proton noise decoupled ^{31}P NMR spectra of sonicated vesicles prepared from total lipid extract of VS virus at 23°C.

asymmetric line. Previous studies with ^{31}P NMR have indicated that the narrow component can be ascribed to small membrane fragments [24]. A similar conclusion appears to be valid here as a sonicated preparation of the extracted virus lipid exhibited a similar lineshape and chemical shift to the narrow resonance in the whole virus spectrum (Fig. 4). The intensity of the narrow component (less than 10% of the total intensity in most cases) varied from sample to sample, also suggesting it was due to membrane fragments formed during suspension of the virus for NMR analysis. Further support for this assignment comes from partially relaxed spectra of the native virus which indicated that the spin lattice relaxation time of the narrow component was significantly longer than the major broad component (the small contribution of the narrow peak to the total intensity prevented an accurate measurement of its T_1 relaxation time). The longer T_1 also demonstrates that this peak is not due to incomplete proton decoupling since the broad asymmetric peak with a shorter T_1 is decoupled effectively. (This latter statement is based on a comparison between proton decoupled and undecoupled spectra of the virus.)

The unsonicated dispersion of the total lipid extract gave a ^{31}P spectrum (Fig. 3B) which was similar to the whole virus. This spectrum also contained a substantial narrow component which arose from vortex mixing of the sample to suspend the lipids and is due to small liposomes [19]. The lineshape of this spectrum is such that the chemical shift anisotropy cannot be accurately evaluated but a rough estimate of 30—40 ppm was determined for both the intact virus and the unsonicated lipid extract. In contrast for unsonicated egg phosphatidylcholine we obtained a value of 48 ppm, similar in magnitude and lineshape to a dispersion of dipalmitoyl phosphatidylcholine at a similar field strength [39].

Origin of the ^{31}P signal

In VS virus there are potentially two major contributors to the ^{31}P spectrum; the membrane phospholipids and the RNA genome. The ^{31}P spectrum of the virus is nearly indistinguishable from the spectrum of the total viral lipid extract in multilayered liposomes, and has a shape characteristic of axial symmetry around the phosphate which is typical for aqueous phospholipid dispersions. Phosphate analyses of the extracted virus lipids and viral RNA indicated that the RNA contributes about 20% of the total phosphorus of the system (the small amount of phosphoprotein [40,41] is included in the RNA phosphorus value). Since the shape of the ^{31}P spectrum of RNA phosphates is likely different from phospholipid phosphates and 80% of the total phosphorus in the system is in the phospholipids, the phospholipids must dominate the observed ^{31}P spectrum, and the properties measured are observed because those phospholipids reside in the viral membrane.

This conclusion is supported by the results of the phospholipase C digestion of the virus. 55% of the total phosphate of the viral phospholipids is removed by phospholipase C, and the resulting ^{31}P NMR spectrum exhibited reduced intensity in all portions of the spectrum.

Other possible contributions to the spectrum of intact virus from nucleotides or larger phosphate-containing molecules trapped within the virus structure are unlikely. Extensive dialysis probably eliminated the vast majority of small

trapped molecules and no narrow resonances characteristic of these small molecules were observed. There was no evidence of larger phosphate-containing molecules from the polyacrylamide gel data.

Spin lattice relaxation times of the ^{31}P resonances

The spin lattice relaxation time (T_1) of the ^{31}P resonances due to whole virus, trypsin-treated virus, dispersed total lipid extracts and sonicated lipid extract are shown in Table I. In spectra containing asymmetric lines the T_1 values are given for the broad component which accounted for the majority of the resonance intensity. It is obvious from Table I that the unsonicated and sonicated extracts and trypsin-treated virus exhibit substantially longer ^{31}P relaxation times at 23° C than the intact virus.

Apparent linewidths of the ^{31}P signals were also compared. Increasing the temperature to 40° C, reduces the linewidth of the intact virus by about 40% (Fig. 3C) and that of unsonicated extract by only 10%. Increasing the temperature of the intact virus from 23 to 40° C, increases the T_1 relaxation time by several-fold (Table I).

Effect of cholesterol on the ^{31}P NMR signal

Stoffel and Bister [17] suggested that the high content of cholesterol in the VS viral membrane may provide some restriction to the mobility of the choline group on 3-*sn*-phosphatidylcholine and sphingomyelin. We were interested to investigate if the cholesterol present in the lipid bilayer of intact VS virus or the extracted viral lipids would influence the ^{31}P spectrum. While the obvious approach is to remove cholesterol from the virus bilayer by incubation with phosphatidylcholine liposomes the degree of sticking (attachment and/or fusion) of the added liposomes does not make this a practically feasible technique. However, we approached the question of the effect of cholesterol on the ^{31}P spectrum by creating a phospholipid dispersion which mimicked the lipid composition of the virus membrane. Chromatographically pure phospholipids were also mixed in the presence of cholesterol to give a cholesterol to phospholipid molar ratio of 0.72. The two unsonicated dispersions gave spectra which were very similar in shape, although the sample containing cholesterol more closely resembled the unsonicated total virus lipid extract. The chemical shift anisotropy was estimated at 38 ppm in the cholesterol-free sample, which has a more distinctive downfield component (Fig. 3D) than any of the cholesterol-

TABLE I

^{31}P NMR SPIN LATTICE RELAXATION TIMES (T_1) OF INTACT VIRUS AND LIPID DISPERSIONS

System	T_1 * (s)
Intact virus	0.1
Intact virus at 40° C	0.9
Trypsin-treated virus	0.6
Unsonicated virus extract	0.7
Sonicated lipid vesicles	0.8

* To ± 20% at 23° C unless otherwise stated.

containing samples. Fig. 3D also contains a larger component due to smaller liposomes than does the total lipid extract (Fig. 3B).

Discussion

The T_1 relaxation time difference between the total lipid extract, whether it be sonicated vesicles or unsonicated multilayers, and the phospholipid present in the intact virus is striking. This T_1 difference is all the more interesting when compared to the apparent absence of a significant difference in microviscosity between the intact virus and protein-free virus lipid vesicles as measured by fluorescence depolarization analysis of 1,6-diphenyl-1,3,5-hexatriene [14—16]. Apparently, the head groups of the phospholipids are experiencing a significant change in their environment not felt by the phospholipid fatty acid chains, at least as measured by the fluorescence depolarization analysis of a probe in the hydrophobic regions of the membrane. A shortening of T_1 is indicative of immobilization of the head group in the virus, relative to its environment in the total lipid extract. Since the head group is affected and not the chains, the source of the immobilization apparently is located in the exterior segment of the membrane. The most likely difference in the immediate lipid environment of the lipid virus membrane compared to that of the total virus lipid extract is the presence of proteins. Therefore, it may well be that the restriction of head group movement detected by ^{31}P NMR T_1 relaxation time is due to phospholipid head group-protein interactions. If such phospholipid-protein interactions are taking place in the virus, a prime candidate for the protein would be the glycoprotein (G protein) forming the spikes on the exterior of the virus. Two recent studies have shown that a hydrophobic piece of about 50 amino acids is left associated with the membrane after proteolytic cleavage of G protein [43,44].

To test this hypothesis we examined the spectra of VS virus treated with trypsin which digests off the hydrophilic portion of the exteriorized glycoprotein but otherwise leaves the virus intact [44]. Electron micrographs of trypsinized virus revealed virions devoid of spikes (compare with Fig. 1). The ^{31}P T_1 relaxation time of trypsin-treated virus was much longer than that for the native virions and nearly indistinguishable from the T_1 of extracted virus lipid dispersions. Therefore, removal of the hydrophilic portion, and possibly the hydrophobic segment of G [44], results in an increased mobility of the phosphate groups. The most simple conclusion is that the G protein is interacting with the bulk of the phospholipid phosphates detected. Although these results provide evidence for a principal role of G protein in immobilizing polar head groups, a role for the matrix (M) protein and the nucleocapsid is not ruled out.

The marked temperature dependence of the T_1 relaxation time of the intact virus may also be accounted for by the proposed protein-phospholipid head group interactions. Several other possible explanations for the strong temperature dependence can be considered unlikely. First of all, a simple thermally induced increase in phospholipid rotational rate would not be sufficient to explain the large changes observed over such a small temperature range. T_1 of pure phospholipid dispersions increases by approximately one-fourth with a

similar temperature increase. Secondly, an increase in the virus rotation rate with temperature is inadequate to account for the increase in T_1, which is insensitive to rotation of particles as large as the virus. For example, in phospholipid systems, the ^{31}P T_1 is nearly identical for both small vesicles and large multilamellar liposomes. Thirdly, using fluorescence depolarization no phase transitions are detectable in the virus or virus lipids from 4 to 40°C [14–16]. In pure phospholipid systems the ^{31}P T_1 is relatively insensitive to phase transitions. Furthermore, the unsonicated total lipid extract does not show as strong a temperature dependence for line narrowing as the whole virus. Although the strong temperature dependence of the relaxation times in the intact virus is consistent with a cooperative effect, a simple lipid phase transition does not appear to be adequate to account for the results.

The remaining possibility to account for the strong temperature dependence of the relaxation times found only in the intact virus is an alteration in the lipid-protein interaction. If the association between the head groups and the G protein suggested by the T_1 results were weakened by raising the temperature, a linewidth and T_1 change might be observed, due to release of the phospholipids from the immobilization imposed upon them by the protein. Independent evidence is at present lacking which would corroborate this suggestion. Other probe studies might be useful in this regard.

The chemical shift anisotropy in the virus is less than that which has been reported for other membrane systems [45]. The spectrum of the phospholipids without cholesterol demonstrated that the relatively small anisotropy is a property of the phospholipids alone, and is not due to cholesterol, as has been observed in other systems [19], nor is it due to the presence of proteins.

These results demonstrate the usefulness of ^{31}P relaxation times for the study of intact membrane systems, providing a new way to probe effects on the phospholipid head groups without perturbing the system being studied.

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