Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2579–2583, October 1971

Spin-Label Electron Spin Resonance Study of the Lipid-Containing Membrane of Influenza Virus

(erythrocyte ghosts/stearate label/androstane label/membrane glycoproteins)

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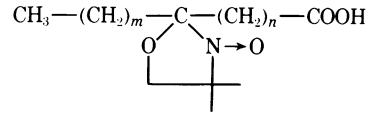
Communicated by Harden M. McConnell July 26, 1971

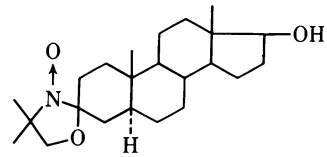
ABSTRACT **The organization of the lipid-containing membrane of influenza virus has been studied by the use of three different lipid spin labels, and the results are compared with a parallel study on human erythrocyte ghosts. The lipid phase of the viral membrane is slightly more rigid than that of the erythrocyte ghosts. The data suggest that the viral lipid is arranged in a bilayer. The glycoprotein spikes covering the viral membrane were specifically removed by proteases, and no alteration in the environment of any of the three spin labels was detected. This suggests that the spikes are not involved in determining the organization of the lipid bilayer.**

Influenza and parainfluenza viruses, like most other lipid-containing viruses, are assembled by a process of budding at the plasma membrane of the infected cell. During the budding process, there is continuity between the unit membrane of the cell and a membrane of similar appearance in the emerging virus particle (1–3). In addition, striking similarities have been found between the lipid composition of parainfluenza virus particles and that of the plasma membrane of the host cell; it has been concluded that the viral lipids are derived from the host-cell plasma membrane (4–7). On the other hand, the proteins of these viruses are limited in number and appear to be coded for entirely by the viral genome (7–11). The outer surfaces of influenza and parainfluenza viruses are covered with a layer of projections (spikes) that consist of glycoproteins, whereas the internal proteins of these viruses contain no carbohydrate (10–13).

Recently, spin-label electron spin resonance (ESR) has been widely used in the study of structural properties of membranes. The experimental spectra are sensitive to the motional degrees of freedom experienced by the probes, permitting the spectra to be interpreted in terms of the rigidity or liquidity of the environment. In this report, we compare the structure of the lipid phase of the influenza viral membrane with the erythrocyte membrane by the use of spin-label analogs of stearic acid [**1** (*12,3*)¶§, **1** (*1,14*); see Fig. 3] and androstane [**2**]. We have also investigated whether the spikes are involved in determining the lipid organization of the viral membrane.



1 (*m, n*)



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MATERIALS AND METHODS

Chemicals. Polyethylene glycol was obtained from Amend Drug and Chemical Co., Irvington, N.J. Spin labels were purchased from Synvar Associates, Palo Alto, Calif., and bovine-serum albumin (less than 0.01% fatty acid) from Sigma Chemical Co., St. Louis, Mo.

Preparation of Virus Particles and Erythrocyte Membranes. The WSN strain of influenza A_0 virus was grown in Maden bovine kidney cells, as described by Choppin (14). The line of kidney cells was grown on plastic surfaces in reinforced Eagle's medium with 10% fetal-calf serum.

Cells were inoculated with about 1 plaque-forming unit per cell, and released virus was harvested after 20–24 hr. Cell debris was removed by centrifugation at 3000 × *g* for 30 min, and 7.5 g/100 ml of polyethylene glycol no. 6000 and 7 g/100 ml NaCl was added to the supernatant (15). The mixture was stirred for 30 min at 20°C to dissolve the polyethylene glycol, and held at 4°C for 15–20 hr. The virus precipitate was pelleted by centrifugation at 1000 × *g* for 30 min, suspended in Eagle's medium, and purified in a potassium tartrate gradient (10).

Human erythrocyte membranes were prepared from fresh, heparinized human blood by the procedure of Dodge, Mitchell, and Hanahan (16).

Spin Labeling. Spin labeling was by a modification of the procedure of Hubbell and McConnell (17). The spin-label solutions were prepared as follows: 10 mg of spin label was dissolved in a small volume of chloroform in a 25-ml Erlenmeyer flask, and the chloroform was evaporated under a gentle stream of nitrogen. To the remaining thin film of spin

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¶ The numbers in brackets indicated values for *m* and *n* in structure **1**.

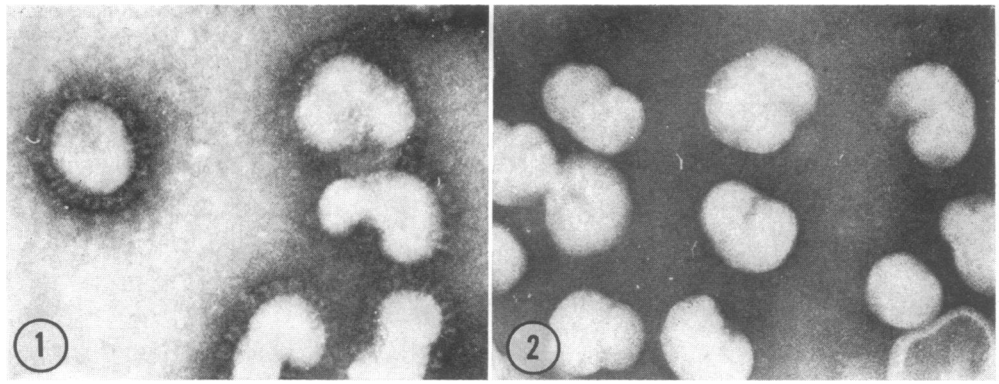

FIG. 1. Intact virus particles in a sample labeled with spin-label 2. × 100,000.
FIG. 2. Particles from the same virus preparation as in Fig. 1, after chymotrypsin treatment and purification. ×100,000.

label, 10 ml of a 5% solution of bovine-serum albumin was added. After stirring overnight at room temperature, the bovine-serum albumin–spin-label solution was passed through a Millipore filter and stored in the cold.

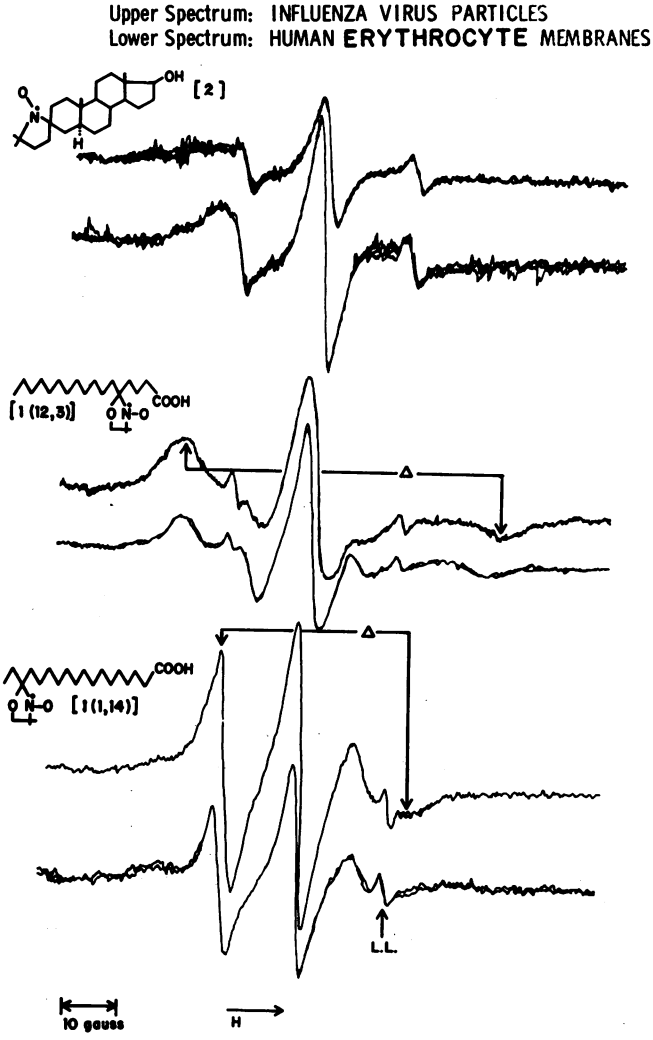

FIG. 3. Comparison of the spectra of spin-labels 1[1,14],[§] 1[12,3], and 2 incorporated into intact influenza virus particles and human erythrocyte membranes. Δ is defined as the splitting between the low- and high-magnetic-field peaks of the "broad-line" spectrum: *LL* indicates position of the high-field peak of the three "liquid lines" superimposed on the "broad line" spectrum (see text). *H* denotes the magnetic field.

Samples of 1–3 mg of purified virus were pelleted at 30,000 rpm for 30 min in a Spinco SW 50 rotor, and suspended in 2 ml of spin-label solution. For labeling with stearic-acid derivatives, the samples were left at room temperature for 4 hr. In the case of the androstane derivative, this 4-hr treatment was followed by 12–15 hr of incubation at 4°C. To remove unincorporated label, virus was pelleted, suspended in phosphate-buffered saline (pH 7.2), pelleted again, and either suspended in buffer for enzyme treatment or again rinsed with buffer and suspended in 0.5 ml of buffer for ESR measurements.

Erythrocyte membranes were labeled in a similar manner. To 1 volume of membranes (2–3 mg of protein/ml) was added 0.5 volume of buffer and 0.5 volume of spin-label solution. Samples were incubated as described for the virus, and freed of excess label by suspending in hypotonic phosphate buffer and centrifugation. This washing procedure was repeated 3 or 4 times.

ESR spectra from the supernatant solution of spin-labeled viruses or erythrocyte membranes generally showed a very small amount of mobile spin label ("liquid line"), which was negligible compared to the spectrum of the samples; this result showed that most of the excess spin label had been satisfactorily removed by the washing procedures.

Enzyme Treatment of Virus. Glycoproteins were removed from the virus particles with bromelain or chymotrypsin (10, 11). After enzyme treatment, particles were again purified in a potassium tartrate gradient. Control samples were incubated under identical conditions without enzyme and were also purified in a second gradient. After enzyme treatment of the spin-labeled virus, a second, minor band was observed in the gradient, at a lower density than the main band. This band was found by electron microscopy to consist of debris and virus fragments, and was discarded.

ESR Spectroscopy. The ESR spectra were obtained with flat quartz sample-cells in a Varian E-12 ESR spectrometer. Care was taken to avoid power saturation and overmodulation effects.

Electron Microscopy. Virus samples were negatively stained with 2% sodium phosphotungstate (pH 6.2), and examined in a Philips EM 300 microscope.

RESULTS

Figs. 1 and 2 are electron micrographs of intact and chymotrypsin-treated influenza virus particles after spin labeling with the androstane derivative. These micrographs are indistinguishable from those obtained from unlabeled particles (10, 11), and show that the labeling procedure does not alter virus morphology. Similar electron micrographs were obtained from particles treated with the other spin labels used in this study.

The ESR spectra of spin-labeled, intact virus particles are compared with those of human erythrocyte ghosts in Fig. 3. The spectra of spin-labeled intact and chymotrypsin-treated virus particles are shown in Fig. 4. These may be interpreted in a qualitative way by realizing that they are all due to the superposition of two types of spectra, a "broad line" spectrum and a "liquid line" spectrum. The "liquid line" consists of a set of three narrow lines, whose spacings do not vary significantly from probe to probe, and it arises from spin label that is undergoing rapid isotropic tumbling. Part of this spectrum arises from spin label that has not been completely removed from the suspending medium. The remainder sediments with the membrane, and may be adsorbed to its outer surface. This amount varies from preparation to preparation and, consequently, the "liquid line" has not been interpreted in terms of membrane structure. The amount of "liquid line" present in each trace may be estimated from the amplitude of the narrow line superimposed on the high-field side of the "broad line" spectrum in the traces of Figs. 3 and 4. This feature is denoted "*LL*" in Fig. 3. The "broad line" spectrum in each trace arises from spin label that is intercalated into the lipid phase of the membrane (17, 18). In general, the broadness and the splitting between the peaks of these spectra (see Fig. 3 and Table 1) increase as the hindrance to motion of the spin label is increased by its environment. A more rigorous treatment of spectral interpretation can be found elsewhere (19).

For each of the spin labels, there is a striking similarity between the spectrum obtained for the virus and that of the erythrocyte ghost. The spectral splittings are much greater for the **1**(*12,3*) spin label than for the **1**(*1,14*) label, when these labels are incorporated into either the erythrocyte membrane or the influenza virus particle (Table 1). This implies that the hindrance to motion of the spin label in the vicinity of the polar group of the molecule is considerably greater than the hindrance near the end of the hydrocarbon chain of the label (18, 19).

If the spectra from spin label **1** in Fig. 3 are carefully compared, it becomes clear that although the spectra of virus particles and erythrocyte ghosts are remarkably similar, slight differences can be detected. The splitting between the extreme high- and low-field peaks (Δ in Fig. 3 and Table 1) is greater in the virus than in the erythrocyte for both **1**(*12,3*) and **1**(*1,14*). This indicates that these spin labels are located in a more immobilizing (i.e., more "rigid") environment in the virus particles than in the erythrocyte ghosts.

TABLE 1. *Δ Values for spin-label* **1** *incorporated into influenza virus and erythrocyte membranes (see Fig. 3)*

Sample	**1**(*12,3*)	**1**(*1,14*)
Erythrocyte membrane	56.2 ± 0.8 G	31.9 ± 0.8 G
Intact influenza virus	59.3 ± 0.8	34.1 ± 0.5
"Spikeless" influenza virus	59.0 ± 0.6	33.8 ± 0.4

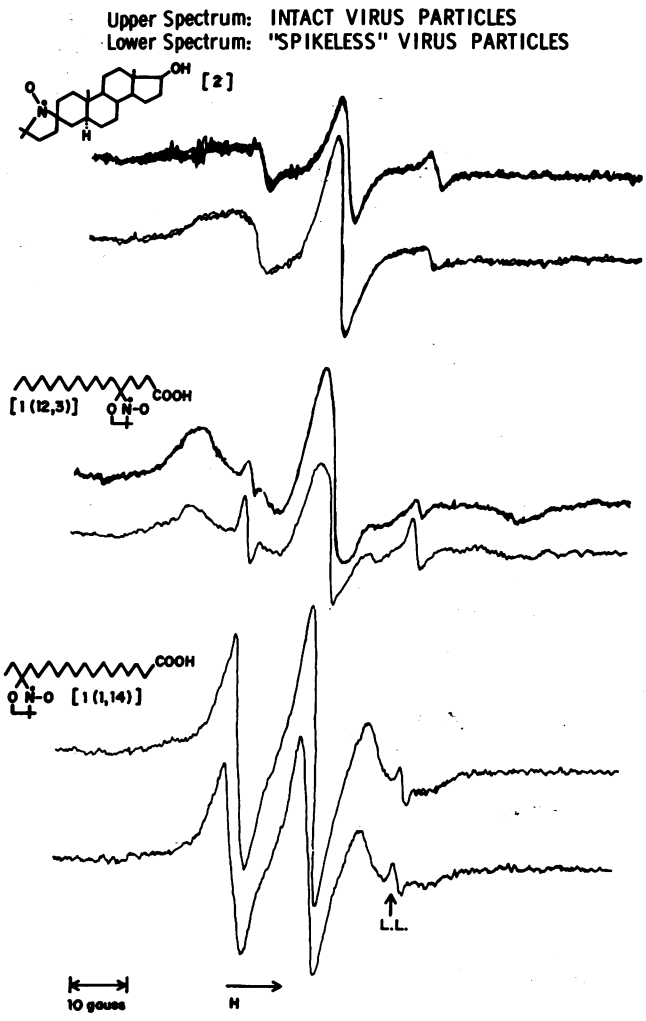

FIG. 4. Comparison of the spin-label spectra of intact virus particles and chymotrypsin-treated particles. *H* denotes the magnetic field.

To determine whether the glycoprotein spikes play a role in determining the organization of the lipid layer in the viral membrane, the ESR spectra of intact and spikeless particles were compared. The glycoproteins were removed either by treatment for 4 hr with bromelain, which removes three of the four glycoproteins (10), or by overnight chymotrypsin treatment, which removes all four glycoproteins (11). The chymotrypsin-treated particles have spectra that are indistinguishable from those obtained with intact particles (Fig. 4 and Table 1); similar results were obtained with bromelain-treated particles. In addition, particles spin-labeled after spike removal give spectra that are identical with those labeled before spike removal. Thus, the glycoproteins do not appear to be involved in determining the organization of the lipid layer.

DISCUSSION

Several physical techniques have recently been brought to bear on the study of the organization of lipids in biological

membranes. X-ray diffraction results on various biological membranes, including erythrocyte ghosts (20), have led to the conclusion that the lipids in all these membranes are arranged in a bilayer structure. Similar conclusions have been reached by Steim *et al.*, based on differential thermal calorimetry of biological membranes and of lipid dispersions prepared from the same membranes (21, 22).

By incorporating spin labels derived from cholestane and various fatty acids into synthetic multilayers, Libertini *et al.* (23) have shown that these probes are ordered in the multilayer in such a way that the hydrocarbon axis is preferentially oriented perpendicular to the plane of the multilayer. The probes were further shown to be undergoing rapid anisotropic rotation about their hydrocarbon axis. Hubbell and McConnell (18) showed similar orientation effects of similar spin labels in oriented canine erythrocytes and in the walking-leg fiber of *Homarus americanus*. These workers also showed striking increases in mobility of the nitroxide group, as it is moved away from the carboxyl end, when the fatty-acid probe is incorporated into biological membranes and phospholipid dispersions (18, 19).

We also observed these changes in mobility in human erythrocyte ghosts and in influenza virus particles. Thus, spin-label studies on synthetic bilayers, erythrocyte membranes, and influenza virus yield substantially the same conclusion: that the rotation of **1**(*12,3*) is far more hindered by its environment than is the rotation of **1**(*1,14*). This relationship suggests that the lipid of the virus is organized into a bilayer.

Tiffany and Blough have recently presented several schematic models of the influenza viral envelope in which the lipid is arranged in the form of spherical micelles having a radius of about the length of a lipid molecule (24). Such a micellar construction necessarily implies different local mass densities from those of the corresponding bilayer structure and would, therefore, be expected to give rise to relative mobilities of **1**(*1,14*) and **1**(*12,3*) that were different from those in a bilayer. The very close parallel between the behavior of the labels in erythrocyte membranes and in virus particles (Fig. 3) thus argues against the existence of lipid micelles in viral membranes.

The models of Tiffany and Blough (24) are based on a calculation that estimates that there is about twice as much lipid as is needed to form a single bilayer covering the spherical virion. We have made similar calculations using more recent data. A radius of 37 nm (370 Å) for the spikeless virion has been estimated from electron microscopy (R. Compans, unpublished). If this virion is covered by a single bilayer, and if the bilayer thickness is 56 Å (25), then, with the distance to the center of the bilayer as the radius, the bilayer area is 147×10^4 Å^2. Schulze (11) has determined the density of the spikeless particle to be 1.15 g/cm^3 in sucrose, and we have obtained a similar value in potassium tartrate. This size and density correspond to a mass of 147×10^6 daltons per particle. The intact particle contains about 1% RNA, 5–7% carbohydrate (26), 21% lipid (Klenk and Choppin, in preparation), and the balance (about 70%) is protein. Since spikes account for about 30% of the protein (10, 11), and probably about 70% of the carbohydrate (12), the lipid content of the spikeless particle is about 29%, or 43×10^6 daltons. In influenza virions grown in bovine kidney cells, there are about equimolar amounts of cholesterol (molecular weight 398) and phospholipid (molecular weight 775) (A. Scheid and R. W. Compans, in preparation), indicating that there are about 3.6×10^4 molecules of cholesterol and 3.6×10^4 molecules of phospholipid per virion. Using 35 Å^2 for the surface area occupied by a cholesterol molecule and 46.7 Å^2 for a phospholipid molecule (25), we find that the area of bilayer that can be formed by the total lipid in each virion is 148×10^4 Å^2, a value in agreement with that required to coat the virion with a single bilayer.

The minor differences previously noted in the spectra of Fig. 3 are of considerable interest. The spectra of erythrocyte membranes are identical in 8 mM phosphate buffer (pH 7.4) and in isotonic phosphate-buffer (pH 7.4), suggesting that ionic-strength changes are not responsible for these differences. Studies on the effect of cholesterol in synthetic lipid structures (27–29) have shown that cholesterol has a marked effect on the rigidity of these systems. Part or all of the observed differences may be attributable to differences in the cholesterol content between the erythrocyte ghost [about 37 mol per cent (30)] and the influenza virus particles grown in bovine kidney cells [about 50 mol per cent (A. Scheid and R. W. Compans, in preparation)], although differences in unsaturated fatty acid content may also affect the rigidity of the membrane lipid phase (31).

Complete removal of the glycoprotein spikes, which appear to represent *all* the proteins on the outer surface of the lipid layer, has no detectable effect on the lipid organization, as monitored by the three different probes. By use of an interspike spacing of 75 Å (32) and a spike radius of 20 Å (33), it can be calculated that about 25% of the particle surface is covered with spikes, if a hexagonal arrangement is assumed (32, 34, 35). The fact that such a large change in surface structure as spike removal can occur with no detectable alteration of lipid structure suggests that the spikes do not penetrate through the lipid phase. This conclusion is supported by the observations of Schulze (11), who showed that digestion by chymotrypsin completely removes all spike proteins from the particle, a result that would not be expected if there were significant penetration of the spike into the lipid bilayer.

We thank Dr. Samuel C. Silverstein for his efforts in suggesting these collaborative studies, and Mrs. Sulamita Konar and Mrs. Ann Erickson for excellent technical assistance. J. L. is an established Investigator of the American Heart Association. This research was supported by USAEC Contract AT (30-1)-3983, Public Health Service research grants CA-08748 and AI-05600, NSF Grant 6B-19797, and a grant from the Cystic Fibrosis Research Foundation.

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