Effect of Membrane Protein on Lipid Bilayer Structure: A Spin-Label Electron Spin Resonance Study of Vesicular Stomatitis Virus†

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ABSTRACT: Spin-label electron spin resonance (ESR) methods have been used to study the structure of the envelope of vesicular stomatitis virus (VSV). The data indicate that the lipid is organized in a bilayer structure. Proteolytic digestion of the glycoproteins which are the spike-like projections on the outer surface of the virus particle increases the fluidity of the lipid bilayer. Since the lipid composition of the virion reflects the composition of the host plasma membrane and the protein composition is determined by the viral genome, VSV was grown in both MDBK and BHK21-F cells to determine the effect of a change in lipid composition on the structure of the lipid bilayer of VSV. The lipid bilayer of the virion was found to be more rigid when derived from MDBK cells than from BHK21-F cells. Studies comparing spin-labeled intact cells and cell membrane fractions suggest that upon labeling the whole cell the spin label probes the plasma membrane. Comparison of spin-labeled VSV particles and their host cells indicates that the lipid bilayer of the plasma membrane is considerably more fluid than that of the virion. These results are discussed in terms of the effect of membrane-associated protein on the structure of the lipid bilayer.

Enveloped viruses which form by budding at the plasma membrane are bounded by a lipid-containing envelope, the outer surface of which is covered by glycoprotein spikes. With the inner surface of the envelope of many viruses is associated a nonglycosylated membrane (M) protein (Compans et al., 1970; Schulze, 1970; McSharry et al., 1971, 1976; Lenard and Compans, 1974). It has been shown using x-ray diffraction techniques that the lipids of Sindbis virus are arranged in a bilayer structure (Harrison et al., 1971a). Spin-label electron spin resonance (ESR) methods have provided evidence that the envelopes of influenza, SV5,[1] and Rauscher murine leukemia viruses contain lipid bilayers (Landsberger et al., 1971, 1972, 1973). Although the viral glycoproteins cover approximately 25% of the influenza virus particle surface, enzymatic digestion of the glycoprotein spikes left the rigidity of the lipid bilayer unaltered, which was interpreted to suggest that the spikes do not penetrate through the bilayer (Landsberger et al., 1971). Spin-label ESR experiments comparing influenza and parainfluenza SV5 virions grown in the same cell line have shown that differences in viral proteins are not reflected in the rigidity of the lipid bilayer (Landsberger et al., 1973). Significant differences, however, were found in the rigidity of the lipid bilayers of influenza and SV5 virions grown in two cell types differing in their lipid composition, MDBK and BHK21-F cells. It was suggested that these differences may reflect the cholesterol-to-phospholipid ratio of the virions (Landsberger et al., 1973).

We present here the results of a spin-label ESR investigation of the structure of the envelope of vesicular stomatitis virus (VSV). The virions are bullet-shaped particles. The viral glycoprotein (G protein) has an approximate molecular weight of 69 000 and forms the spikes on the outer surface of the viral envelope (Cartwright et al., 1970; McSharry et al., 1971; Wagner, 1975). Associated with the inner surface of the lipid phase is the membrane protein (M protein) having an approximate molecular weight of 29 000 (McSharry et al., 1971; Cartwright et al., 1972; Wagner, 1975). Proteolytic treatment of VSV virions produces "spikeless" particles (Cartwright et al., 1970; McSharry et al., 1971) having only a small polypeptide associated with the lipid bilayer (Mudd, 1974; Schloemer and Wagner, 1975).

Evidence is presented that suggests that the lipid of VSV is organized in a bilayer structure. Extending the results of an earlier report (Landsberger et al., 1973) that the structural rigidity of the lipid bilayers of influenza and parainfluenza SV5 virions grown in the same cell is not dependent on the viral protein composition, the fluidity of the bilayer of VSV is reported here to be different from that of either influenza and parainfluenza virions, demonstrating that the organization of the viral lipid bilayer can reflect differences in the protein composition. In contrast with earlier experiments with influenza virions (Landsberger et al., 1971), protease treatment which results in the digestion of the glycoproteins and removal of the morphological spikes from the surface of the VSV virions increases the fluidity of the lipid bilayer. Comparison of the ESR spectra of spin-labeled MDBK and BHK21-F cells with the spectra of VSV virions indicates that the lipid of the viral envelope is considerably more rigid than the lipid phase of the plasma membranes of the host cells. These results suggest (1) a possible general organizational principle involving the effect of the viral M protein on the structure of the lipid bilayer and (2) a possible role for the M protein in viral assembly.

Materials and Methods

Virus and Cells. The Indiana strain of vesicular stomatitis virus and the WSN strain of influenza virus were grown in

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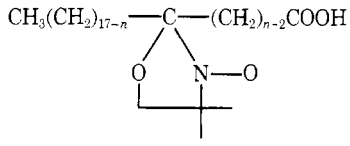
[1] Abbreviations used are: MDBK cells, Madin Darby bovine kidney cells; BHK21-F, a line of baby hamster kidney cells; VSV, vesicular stomatitis virus; SV5, simian virus 5; PBS, phosphate-buffered saline; M protein, membrane protein; G protein, glycoprotein.

MDBK or BHK21-F monolayer cultures and purified by precipitation with polyethylene glycol and equilibrium zonal centrifugation in a potassium tartrate gradient as described previously (Choppin, 1969; Choppin and Compans, 1970; Compans et al., 1970; McSharry et al., 1971). Only the results obtained from the upper of the two bands observed on the potassium tartrate gradient are reported here, as this band contained highly purified virions as shown by electron microscopy. The MDBK line of bovine kidney cells and the BHK21-F line derived from baby hamster kidney were propagated according to described procedures (Choppin, 1969; Holmes and Choppin, 1966).

Protease Treatment. Proteolytic digestion of the VSV surface glycoproteins with protease Type VI from *Streptomyces griseus* were performed as described previously (McSharry et al., 1971) or with trypsin as described by Cartwright et al. (1969). Virions were repurified in a potassium tartrate gradient. Removal of surface projections was judged to be complete by electron microscopy.

Spin Labeling of Virions. Purified virions were dialyzed overnight in phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) prior to spin labeling. Virions were labeled with nitroxide derivatives of stearic acid having the general structure



C_n: $n = 5$ or 16

where the nitroxide ring is located on the nth carbon (C_n) from the carboxyl end. Labeling was accomplished by incubating virions with a variable amount of spin-label bovine serum albumin complex adjusted to the particular virion concentration of the sample (Landsberger et al., 1971), and repurifying the virions in a potassium tartrate gradient. Some experiments were performed with Millipore-filtered spin-label bovine serum albumin complex. This has been discontinued because of the presence of Triton X-100 in Millipore filters; however, the spectra obtained with filtered and unfiltered spin-label solutions were indistinguishable. For protease-treated samples, the spin labeling was carried out prior to proteolytic digestion. Prior to ESR measurements, samples were dialyzed against PBS. In some instances, the virions were concentrated after dialysis by pelleting and resuspended in PBS.

Spin Labeling of Cells. BHK21-F and MDBK cells were labeled by washing a confluent monolayer (approximately 75 cm^2) of cells three times with PBS deficient in Ca^{2+} and Mg^{2+}. One milliliter of C_5-labeled bovine serum albumin solution was added and incubated at 37 °C for 5 min. After pouring off the spin-label solution, the cells were washed three times with PBS deficient. The cells were scraped with a rubber policeman adding a small amount of PBS and pelleted. The ESR spectra were obtained from a resuspension of the pellet in a small volume of PBS. The spectra obtained from cells labeled by this procedure are essentially the same as those obtained by similar procedures where the cells were (1) detached by scraping and then labeled, (2) detached using EDTA and then labeled, and (3) detached using trypsin and then labeled.

Cell Fractionation and Spin Labeling. Cytoplasmic membrane fractions of BHK21-F and MDBK cells were prepared in discontinuous sucrose gradients as described previously. (Caliguiri and Tamm, 1970; Compans, 1973). For spin labeling, fractions were incubated for 3 h at 20 °C with 0.5–1 volume of spin-label bovine serum albumin complex without removal of sucrose, separated from unbound label by banding in 20–60% sucrose gradients and dialyzed against PBS.

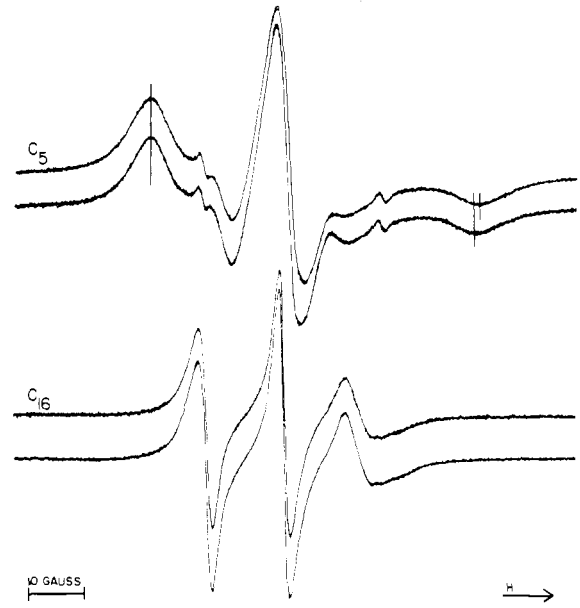


FIGURE 1: Comparison of ESR spectra of C_5- and C_{16}-labeled intact and spikeless vesicular stomatitis virus particles grown in BHK21-F cells. The upper spectrum of each pair corresponds to intact virions and the lower spectrum of each pair corresponds to "spikeless" particles after treatment with trypsin. For the C_5 pair of spectra, the left-hand vertical line aligns the position of the extrema of the low-field peaks and the right-hand vertical lines are drawn through the corresponding extrema of the high-field peaks.

ESR Spectroscopy. The spectra were recorded using a Varian E-4 or E-12 ESR spectrometer. Microwave power levels were maintained low enough to avoid sample heating and saturation effects. An aqueous ESR cell was used for all samples. Each spectrum is the superposition of multiple spectrometer retracings of the same sample.

Chemicals. Polyethylene glycol was obtained from Amend Drug and Chemical Co., Irvington, N.J. Protease Type VI, trypsin (Type III, twice crystallized), and bovine serum albumin (less than 0.01% fatty acid) were purchased from Sigma Chemical Co., St. Louis, Mo. Spin labels were purchased from Syva Corp., Palo Alto, California.

Results

In Figure 1, the ESR spectra of C_5- and C_{16}-spin-labeled intact VSV particles are shown. As discussed in detail previously, these spectra constitute the superposition of two types of spectra (Landsberger et al., 1973). The "liquid-line" spectrum consists of three sharp equally spaced lines which arise from spin label freely tumbling in the buffering medium and not associated with the viral envelope. The variable amplitude of the "liquid-line" spectrum depends in part on the concentration of virus in a given sample and the length of time elapsed between the last pelleting step and the recording of the ESR spectrum. The "broad-line" spectrum arises from spin label intercalated into the lipid phase of the viral envelope. The spectral splitting between the outermost peaks (cf. Figures 1 and 2) increases as the fluidity of spin-label environment decreases (Hubbell and McConnell, 1971; Jost et al., 1971). The splitting between the outermost peaks of the C_5-labeled particles is greater than that of the C_{16}-labeled particles. This indicates that the lipid phase is considerably more rigid in the region near the lipid glycerol backbones probed by the C_5 label

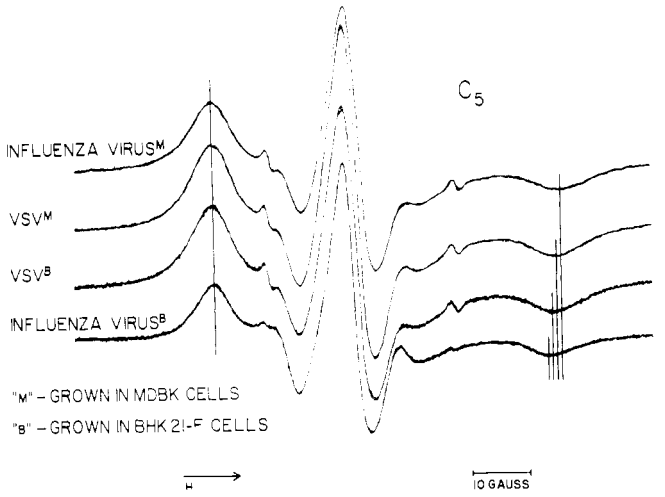


FIGURE 2: Comparison of ESR spectra of intact influenza and VSV virions both grown in BHK21-F and MDBK cells. The maxima of the extreme low-field peaks are aligned and a vertical line is drawn through them. The right-hand vertical lines are drawn through the minima of the high-field peaks showing the different spectral splittings for each of the systems.

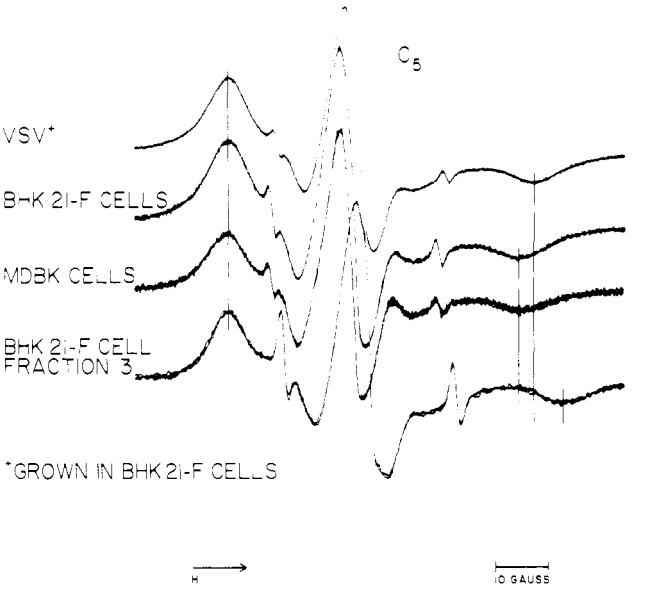


FIGURE 3: Comparison of ESR spectra of spin-labeled VSV virions grown in BHK21-F cells, BHK21-F cells, MDBK cells, and BHK21-F cell fraction (3). The cells were labeled and then removed from the monolayer by scraping (cf. Materials and Methods).

(Godici and Landsberger, 1974, 1975) than that in the deep interior of the lipid hydrocarbon phase probed by the C_{16} label (Godici and Landsberger, 1974, 1975), suggesting that the lipid is organized in a bilayer structure as discussed below.

To investigate the effect of proteolytic digestion of the glycoprotein on the organization of lipid envelope, the ESR spectra of C_5- and C_{16}-labeled intact and protease treated particles are compared in Figure 1. The splitting in the C_5 spectra between the outermost peaks of the "broad-line" spectrum (cf. the vertical lines in Figure 1) is decreased upon protease treatment. Thus proteolytic treatment increases the fluidity of the lipid phase of the VSV envelope in the region probed by the C_5 label. No changes were detected in the C_{16} spectra after similar treatment (cf. Figure 1). Since identical results were obtained using protease Type VI and crystalline trypsin, it is unlikely that the change in fluidity is attributable to lipase contamination.

To determine the effect of a change in lipid composition on the structure of the lipid membrane, the ESR spectra of C_5-labeled VSV particles grown in both BHK21-F and MDBK cells whose plasma membranes differ appreciably in lipid composition (e.g., cholesterol-to-phospholipid ratio of 0.68 and 0.75, respectively) (Klenk and Choppin, 1969, 1970a,b) are compared in Figure 2. The spectral splitting of the BHK21-F grown virions is reproducibly slightly smaller than that of MDBK grown virions suggesting that the fluidity of the lipid envelope is slightly greater for the BHK21-F grown virions.

Previously reported experiments comparing the lipid bilayers of SV5 and influenza virions have shown that a complete change in membrane associated protein has no effect on the rigidity of the bilayer (Landsberger et al., 1973). To investigate whether the fluidity of the lipid bilayer of VSV particles is also the same as that of the envelopes of the myxo- and paramyxovirions, the C_5 spectra of MDBK and BHK21-F grown influenza and VSV particles are compared in Figure 2. It can be seen that the spectral splitting for MDBK grown virions is slightly smaller for VSV than for influenza virus particles. In contrast, the spectral splitting of BHK21-F grown virions is slightly larger for VSV than for influenza virus particles. These small differences have been observed in repeated experiments. Therefore, although the rigidity of the lipid bilayer is the same for both SV5 and influenza virions, the rigidity of the VSV lipid bilayer is different from that of the two other virions. From Figure 2, the range of the structural rigidity of the lipid phase of VSV virions grown in MDBK and BHK21-F cells lies within the range defined by influenza virions grown in the same pair of cell lines. It is apparent that the change in the rigidity of the lipid bilayer of VSV particles reflects to a lesser degree a change in the lipid composition than is observed for both influenza and parainfluenza virions. The membrane associated proteins of VSV particles appear to interact with the lipid phase to produce a lipid layer whose structural rigidity is more strongly determined by the viral proteins than is the case for the influenza and parainfluenza virions.

To compare the rigidity of the lipid phase of viral envelopes with the lipid bilayer of the corresponding host plasma membrane, the ESR spectra of spin-labeled MDBK grown virions and BHK21-F and MDBK cells are shown in Figure 3. The ESR spectral splittings of C_5-labeled VSV virions are greater than those of MDBK cells or BHK21-F cells. In preliminary experiments, the spectral splittings of C_5-labeled BHK21-F and MDBK plasma membranes prepared as described by Brunette and Till (1971) with minor modifications were the same as those of the labeled intact cells, suggesting that the spin label when added to intact cells probes the lipid bilayer of the plasma membrane. To provide further evidence that the ESR signal from labeled cells arises from spin label largely intercalated into the lipid bilayer of the plasma membrane, the ESR spectra of spin labeled cytoplasmic membrane fractions were compared with those of intact cells. Cytoplasmic fractions of BHK21-F cells were separated on the basis of buoyant density on a discontinuous sucrose gradient which yields smooth and rough microsomal fractions. Although some variability was found in the ESR spectra of these fractions labeled with C_5-label, all showed a much more rigid environment than observed with the labeled intact cell; an example of the smooth cytoplasmic membrane fraction (3) is shown in Figure 3. These results indicate that labeling of the intact cell does not result in appreciable labeling of intracytoplasmic membrane components but does result in the labeling of the plasma membrane. Experiments using C_{16} label demonstrated that intact cells as

well as cytoplasmic membrane fractions possess a very fluid environment in the region probed by this label, as is characteristic of a bilayer structure.

The difference in the spectral splittings of BHK21-F grown VSV particles and the splittings of the labeled MDBK and BHK21-F cells are pronounced (cf. Figure 3). It would, therefore, seem that viral protein strongly affects the organization of the viral lipid phase. Furthermore, although the lipid composition of the plasma membranes of the two cells differ (Klenk and Choppin, 1969, 1970a,b), similar ESR spectra were detected. It would appear that the membrane associated proteins of the cells interact with the lipid such that the rigidity of the bilayer is the same. A less likely possibility is that the net rigidity of the lipid bilayers produced by the two different lipid compositions is fortuitously the same. It cannot be rigorously ruled out that the spin labels incorporated into the plasma membranes of the MDBK and BHK21-F cells are excluded from hypothetical rigid regions and occupy only the more fluid portions which are the same in both cell types. The present data would nevertheless require that these fluid regions do not exist to the same extent in the virion envelope. Thus the observations on both viral and cellular membranes indicate that membrane protein can have a strong effect on the organization of a lipid bilayer.

Discussion

The present data demonstrating the presence of a rigidity gradient in the membrane of VSV virions indicate that the viral lipid is organized in a bilayer structure (cf. references cited in Landsberger et al., 1971, 1972, 1973). This interpretation is in agreement with our previous results with influenza (Landsberger et al., 1971), SV5 (Landsberger et al., 1973), and Rauscher murine leukemia (Landsberger et al., 1972) viral envelopes. Although hexagonal phases can also exhibit rigidity gradients (Boggs and Hsia, 1973; Caron et al., 1974), this alternative seems unlikely. The virion envelope is derived from the host plasma membrane during the budding process and there is no reason to assume an hexagonal phase in either membrane system. This interpretation is supported by the results of x-ray experiments by Harrison et al. (1971a) demonstrating the presence of a lipid bilayer in Sindbis virus and in the bacteriophage PM2 (Harrison et al., 1971b). The present data demonstrate that protease treatment increases the fluidity of the lipid bilayer. These results are in contrast with earlier experiments in which it was shown that protease treatment of influenza virions did not alter the fluidity of the lipid bilayer (Landsberger et al., 1971).[2] Sefton and Gaffney (1974) have recently observed that protease digestion of the glycoproteins of Sindbis virus also increased the fluidity of the envelope.

Recent reports on the proteolytic treatment of VSV (Mudd, 1974; Schloemer and Wagner, 1975) and Semliki forest (Gahmberg et al., 1972) virions have demonstrated that enzymatic removal of the glycoprotein spikes leaves small hydrophobic polypeptide fragments associated with the lipid envelope. Treatment of influenza virions with bromelain initially results in particles containing the HA2 polypeptide (Compans et al., 1970) and on more extensive digestion small protease resistant peptides are still observed (Lenard et al., 1976). Skehel and Waterfield (1975) have also suggested from primary structure studies of influenza virus hemagglutinin that after bromelain treatment a hydrophobic peptide remains associated with the envelope. Thus it may not be possible to interpret the ESR observations on the effect of spike removal unequivocally in terms of the depth of the penetration of the glycoprotein spikes into the lipid bilayer. However, the fact that no change is observed in the spin-label ESR spectra of influenza virions upon spike removal suggests that the arrangement of the remaining hydrophobic fragments relative to the lipid bilayer is not detectably altered and that the extent to which the glycoproteins and the hydrophobic enzymatic cleavage products affect the rigidity of the lipid bilayer is the same.

There is a reproducible but only a slightly greater rigidity of the lipid bilayer for VSV grown in MDBK cells in comparison with VSV grown in BHK21-F cells. For the same change in the lipid composition, the change in the rigidity of the lipid envelope of VSV is smaller than that observed in influenza or SV5 bilayers (Landsberger et al., 1973). Specifically, the fluidity of the lipid bilayers of MDBK grown VSV particles is greater than that of MDBK grown influenza and SV5 virions, whereas the fluidity of the lipid bilayers of BHK21-F grown VSV particles is less than that of BHK21-F grown influenza and SV5 virions (Landsberger et al., 1973). This indicates that differences in membrane associated proteins can have an effect on the structure of the lipid bilayer of enveloped virions. Furthermore, this suggests that the membrane proteins of VSV particles more strongly control the rigidity of the lipid bilayer than is the case for influenza virions.

In contrast with the small differences observed in the ESR spectra of the C_5-labeled virions of several different major groups, markedly larger differences can be noted comparing the spectra of an enveloped virus with that of the corresponding host plasma membrane (cf. Figure 3). The unusual feature of all spectra of the enveloped viruses, influenza, SV5 (Landsberger et al., 1971, 1973), and VSV is that in each case the environment probed by the C_5-spin label is very rigid (i.e., more rigid than that of the plasma membrane of the host cell) and is not remarkably sensitive to changes in lipid and protein composition (cf. Figure 3 and Landsberger et al., 1973). Sefton and Gaffney (1974) have also recently reported that the lipid bilayer of Sindbis virus appears to be more rigid than the plasma membrane of the host cell. Since the lipid composition of enveloped viruses is determined largely by that of the host plasma membrane (Klenk and Choppin, 1969, 1970a,b; Choppin et al., 1971, 1972), the spin-label ESR data would argue that membrane associated viral proteins markedly increase the rigidity of the viral lipid bilayer and restrict the effect of a change in the lipid composition on the rigidity of the envelope. Furthermore, proteolytic digestion of the viral glycoprotein spikes either has no effect on the structure of the lipid bilayer (e.g., influenza virions) or results in a lipid bilayer which, although more fluid (e.g., vesicular stomatitis virions), still is markedly more rigid than the plasma membrane of the host cell. Considering these results together, it may be proposed that the M protein has a significant role in determining the relatively large rigidity of the viral lipid bilayer. If the M protein does increase the rigidity of the viral lipid bilayer, it is, by analogy with the effect of cholesterol on lipid bilayers (Darke et al., 1972; Lee et al., 1972, 1973; McConnell and McFarland, 1972; Devaux and McConnell, 1972; Saito et al., 1973; Gent and Prestegard, 1974; Godici and Landsberger, 1975), conceivable that the rate of lateral diffusion of membrane-associated components is decreased by the presence of the M protein. Because of the intimate association of the M

[2] Recently, the experiment investigating the effect of chymotrypsin treatment on the structure of the influenza envelope was repeated to obtain spectra of higher signal-to-noise than previously published (Landsberger et al., 1971). No change in the ESR spectra was observed upon protease treatment.

protein with the lipid bilayer (Choppin et al., 1972; Lenard et al., 1974; Choppin and Compans, 1975), the M protein may actually form a barrier preventing the diffusion of plasma membrane proteins into the assembly region of the virion.

Acknowledgments

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