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Effects of Filipin on the Structure and Biological Activity of Enveloped Viruses

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The interaction of the polyene antibiotic filipin with membrane-bound cholesterol in vesicular stomatitis (VS), influenza, and Rauscher leukemia virions was studied. Exposure of virions to filipin resulted in a series of depressions and ridges in the envelope of VS virions, with a periodicity of 15 to 20 nm perpendicular to the long axis of the particle; similar morphological alterations were observed in negatively stained preparations, in thin-sectioned virions, and in protease-treated virions that lack surface glycoproteins. This morphological effect was specific for filipin, since the envelopes of VS virions that had been treated with another polyene antibiotic, amphotericin B, exhibited markedly different morphology. Morphological alterations induced by filipin in influenza and Rauscher leukemia virions differed from those seen in VS virions. The infectivity of filipin-treated VS virions was reduced up to 500-fold, whereas influenza virions were resistant to filipin treatment. Incorporation of filipin into the virions was demonstrated, and no release of either lipids or proteins from virions was detected after filipin treatment. A stoichiometry of approximately 1 mol of bound filipin per mol of cholesterol was found in both intact and protease-treated VS virions. The equilibrium dissociation constant for filipin-cholesterol interaction was approximately 74-fold larger in intact than in protease-treated VS virions. The initial rate of association of filipin with cholesterol in intact virions was slower than that in protease-treated particles. The fluidity of lipids in VS viral membranes, as probed by a stearic acid derivative spin label, was markedly reduced when either intact or protease-treated virions were treated with filipin.

The polyene antibiotic filipin is known to interact with sterols in liposomes and biological membranes, producing alterations in the lipid bilayer structure (2, 13, 24, 35, 36). Treatment of vesicular stomatitis (VS) virions with filipin resulted in modification of the permeability barrier of the viral membrane (4). In the present study, we investigated the physical and biological effects of filipin binding to virions. Equilibrium constants and initial rates of association for filipin binding to intact and protease-treated VS virions were measured. The effects of filipin on the fluidity of the lipid phase of viral envelopes and on the infectivity of virus particles are presented, and the morphological alterations induced by filipin in influenza, Rauscher leukemia, and intact and protease-treated VS virions are compared. We also present data indicating that envelope components are not released by filipin; thus, the antibiotic exerts its physical and biochemical effects solely by inducing rearrangements within the viral membrane. The findings presented indicate that the glycoproteins of the VS virions can redistribute along the membrane surface as a response to a change in the lipid matrix.

MATERIALS AND METHODS

Virus and cells. The Indiana strain of VS virus was grown and assayed in BHK-21-F monolayer cell cultures as described previously (30). The WSN strain of influenza virus was grown in MDBK cells (8), and Rauscher leukemia virus was obtained from persistently infected JLS-V9 cells (32). Viruses were purified by polyethylene glycol precipitation and by equilibrium zonal sedimentation in a 5 to 40% potassium tartrate gradient (28) and dialyzed versus a phosphate-buffered salt solution (15).

Protease treatment of virions. VS virions were treated with protease type VI from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, Mo.) as previously described (30) or with trypsin [L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated, from Worthington Biochemicals Corp., Freehold, N.J.] as described by Cartwright et al. (7). Both of these treat-

ments removed surface glycoproteins to produce spikeless particles as revealed by electron microscopy. To remove any viral aggregates in the equilibrium binding and kinetic studies, both intact and spikeless VS virions were centrifuged at 1,500 rpm in a Sorvall SS34 rotor for 20 min at 4°C and then passed through Pasteur pipettes filled with glass wool.

Spin labeling of virions. VS virions were labeled with 5- and 16-doxylstearic acid (26, 28), and unincorporated label was removed by repurification of the virions on a potassium tartrate gradient. Samples were dialyzed versus phosphate-buffered salt solution before electron spin resonance (ESR) spectra were recorded.

Chemicals. Filipin complex (lot no. 8393-DEG-11-8, The Upjohn Co., Kalamazoo, Mich.) was prepared as previously described (2). The filipin was crystallized from a slurry of chloroform as described by Whitfield et al. (38). The final concentration of dimethylformamide in solutions containing filipin was 1.5% (vol/vol), unless noted otherwise. The stearic acid spin labels were purchased from Syva Corp., Palo Alto, Calif., and were used without further purification. Bovine serum albumin (less than 0.01% fatty acid) was purchased from Sigma Chemical Co.

ESR spectroscopy. The ESR spectra were recorded at room temperature by using a Varian E-12 ESR spectrometer interfaced with a Texas Instruments 980A computer (29). A quartz aqueous sample cell was used. Virions were incubated with filipin or dimethylformamide for 15 min in the dark at room temperature before the spectra were recorded.

Measurement of stoichiometry. Appropriate volumes of VS virion suspensions and filipin solutions were added to cellulose nitrate tubes to give a constant volume of 5 ml. The virion concentration in the suspension was held constant while that of filipin was varied. After a 30-min incubation in the dark at room temperature, the virion suspensions were pelleted by centrifugation at 20,000 rpm in a Beckman SW50.1 rotor for 30 min at 23°C. Two-milliliter portions were withdrawn from the supernatants and were combined with an equal volume of methanol. The samples were allowed to stand for 30 min in the dark and then were centrifuged at 20,000 rpm for 20 min to remove salts that precipitated from the aqueous methanol solution. The concentration of free filipin in the supernatant was determined spectrophotometrically at 358 nm with the molar extinction coefficient cited previously (3). The concentration of filipin present in the supernatant above virus pellets was corrected for the small amount of filipin that sediments when the same concentration of total filipin is centrifuged without virions. Cholesterol concentration was determined by the modified Liebermann-Burchard method of Huang et al. (20).

Measurement of dissociation constants. Apparent equilibrium dissociation constants (K_D) for the binding of filipin to intact and spikeless VS virions were determined as described previously (3). Absorbance measurements were made on a Cary 14 spectrophotometer, using a 0 to 0.1 slide-wire and a 1-cm-path-length cuvette.

Measurement of initial rates of association. Changes in transmittance during the first 200 ms after mixing were recorded at 358 nm and 23°C by a stopped-flow apparatus (Durrum Instrument Corp., Palo Alto, Calif.) with a Tektronix storage oscilloscope equipped with a Polaroid camera attachment. Initial slopes of transmittance change with respect to time were analyzed from the oscillograms and converted to initial changes of absorbance, dA/dT. A slit width of 1 mm was used. The kinetic results reported here represent the analysis of at least five trials and are presented as a mean value followed by the standard deviation at the 90% confidence level.

Electron microscopy. For negative staining, virions were stained with sodium phosphotungstate (pH 7.2). Thin sections were obtained from virus pellets that had been fixed with glutaraldehyde and osmium tetroxide, embedded in an epoxy resin mixture as described previously (11), and stained with uranyl acetate and lead citrate. Specimens were examined in a Philips EM 300 microscope.

Polyacrylamide gel electrophoresis. Procedures for polyacrylamide gel electrophoresis and radioactivity determinations were described previously (5, 10).

RESULTS

Filipin-induced modification of viral envelope morphology. The morphology of control and filipin-treated virions is shown in Fig. 1. Along the periphery of the filipin-treated particles, a series of depressions and ridges occurred, with a periodicity of 15 to 20 nm; glycoprotein spikes were attached only to the ridges (Fig. 1B). A similar morphological alteration was caused by filipin on trypsin-treated virions, which lacked the surface glycoproteins (Fig. 1C), indicating that intact glycoproteins are not required for this morphological change. Striations also appeared in fixed, sectioned virions (Fig. 1D), demonstrating that similar morphological features can be observed with various preparative procedures. In addition to the intact particles, disrupted VS virions were occasionally found with 20- to 30-nm-diameter circular holes or depressions in the envelope (Fig. 1E); these particles appeared somewhat similar to filipin-treated Rauscher leukemia virions as described below. Similar structures in filipin-treated erythrocytes and lipid vesicles containing cholesterol or ergosterol have been described previously (2, 25).

The filipin-induced morphological alterations in the envelopes of influenza and Rauscher leukemia virions differed from those seen in VS virions. Influenza virions exhibited minimal alterations (not shown), whereas the envelopes of Rauscher virions were strikingly altered by filipin (Fig. 2). Circular pores, which appeared to extend completely through the membrane in some instances, were the major envelope feature observed after exposure to the polyene.

To learn whether other polyene antibiotics cause similar morphological changes, the effect

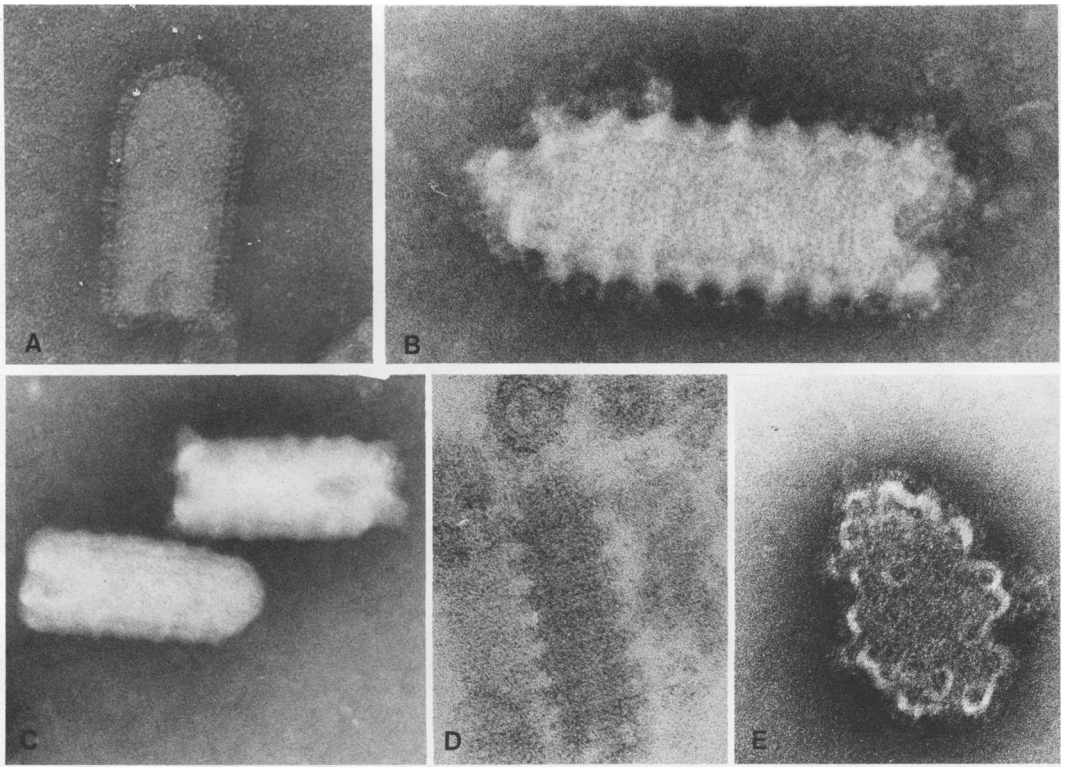


FIG. 1. *Effect of filipin on the morphology of intact and protease-treated VS virions. (A) Control particle showing projections uniformly distributed on the surface. (B) Filipin-treated particle showing depressions and ridges in the membrane and redistribution of surface projections. (C) Trypsin-treated particles that have been exposed to filipin. Despite the absence of surface spikes, depressions in the viral membrane are still detected. (D) Filipin-treated VS virion in thin section showing striated appearance. (E) Partially disrupted particle exhibiting ringlike pits or pores ~20 nm in diameter. (A, B, C, E) Negative staining with sodium phosphotungstate. Magnification: (A and D) ×150,000; (B) ×300,000; (C) ×180,000; (E) ×130,000.*

of amphotericin B on the structure of VS virions was examined. After amphotericin B treatment, the virions exhibited long tails, with numerous surface projections (Fig. 3). Therefore, amphotericin B also causes redistribution of VS viral membrane components, but in a manner different from that found with filipin. It is interesting that the effect of amphotericin B on cholesterol- and ergosterol-containing vesicles also differs from that of filipin. Amphotericin B causes disordering and swelling of the vesicles, with the formation of a homogenous electron-translucent layer in the envelope as revealed by negative-staining electron microscopy (2).

Biochemical integrity of filipin-treated virions. To determine whether filipin treatment results in the release of any protein or lipid molecules from VS virions, virions were labeled with [4,5-^{3}H]leucine and [^{14}C]glycerol. The amino acid label was incorporated into all polypeptides, whereas glycerol labeled the polypeptides as well as a major peak at the dye front (fractions 82 to 98) which included the viral lipids (Fig. 4). When labeled VS virions were treated with filipin and then analyzed by gradient centrifugation, no significant release of ^{3}H- or ^{14}C-labeled molecules was observed at the top of the gradient as compared with controls (Fig. 5). About 6% of the ^{3}H label was found at the top of the gradient in the control samples, versus 9% after filipin treatment; about 5% of the ^{14}C label in either sample was also found at the top of the gradient. Similar results were obtained with a fivefold-higher concentration of filipin. Therefore, the morphological alteration induced by filipin occurs without any extensive dissociation of components from the viral membranes.

The distribution of filipin in the gradient was determined by measurement of optical density at 338 nm, one of the absorption maxima of filipin. Most of the filipin was incorporated into the VS virus band, whereas control samples did not possess any significant absorption at this

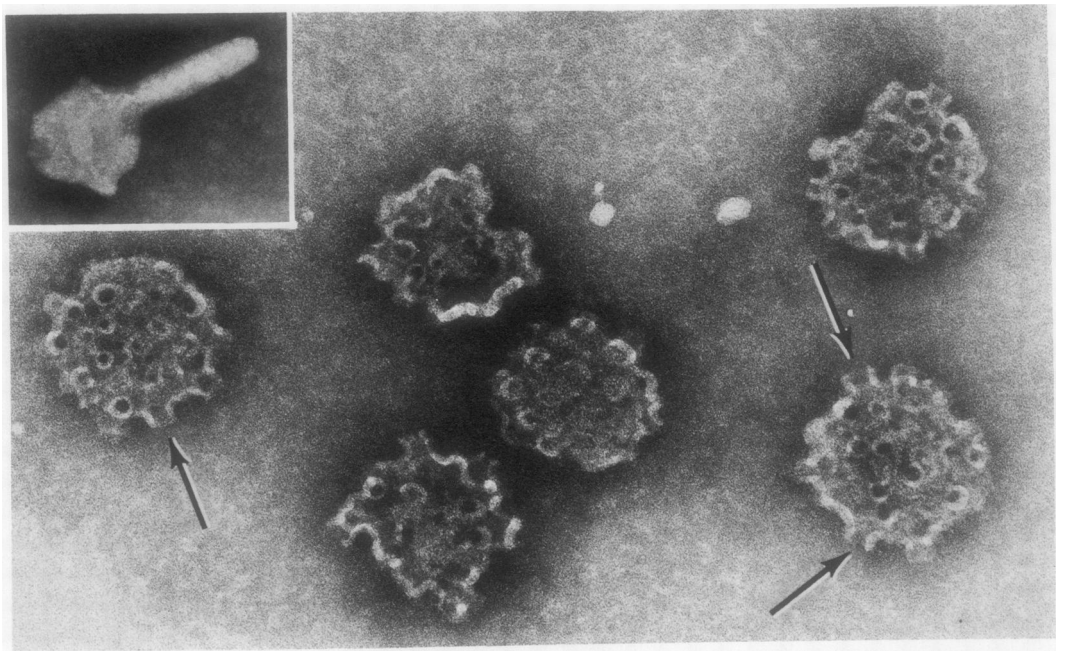


FIG. 2. *Effect of filipin on Rauscher leukemia virions. The viral membranes have become permeable to phosphotungstate and show numerous pores or pits ranging from ~10 to 25 nm in diameter, some of which appear to extend completely through the membrane (arrows). Control particles (insert) show intact envelope and typical head and tail forms in unfixed preparations. Magnification: ×165,000; insert, ×150,000.*

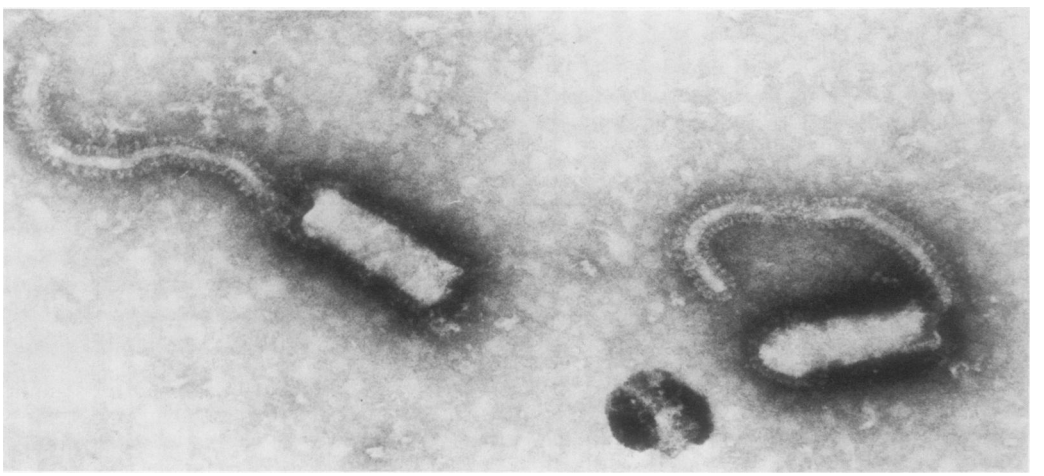


FIG. 3. *VS virions treated with amphotericin B, showing long tail-like outfoldings of the membrane. Some particles are disrupted, resulting in fragments seen in the background. Magnification: ×120,000.*

wavelength (Fig. 5A). In analogous experiments, incorporation of filipin into influenza and Rauscher leukemia virions was demonstrated.

Effect of filipin on biological activity and VS and influenza virions. The effect of filipin on the infectivity of VS and influenza virions is shown in Table 1. At a cholesterol/filipin ratio of 0.21, there was approximately a 500-fold reduction in the infectivity of VS virions. However, no appreciable reduction in infectivity of influenza virions was observed under similar conditions.

Stoichiometry of the filipin-cholesterol interaction in viral membranes. For a given cholesterol concentration, saturating concentrations of filipin gave rise to a linear region of the

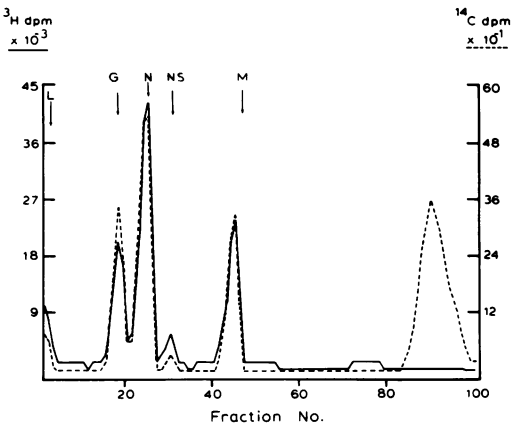
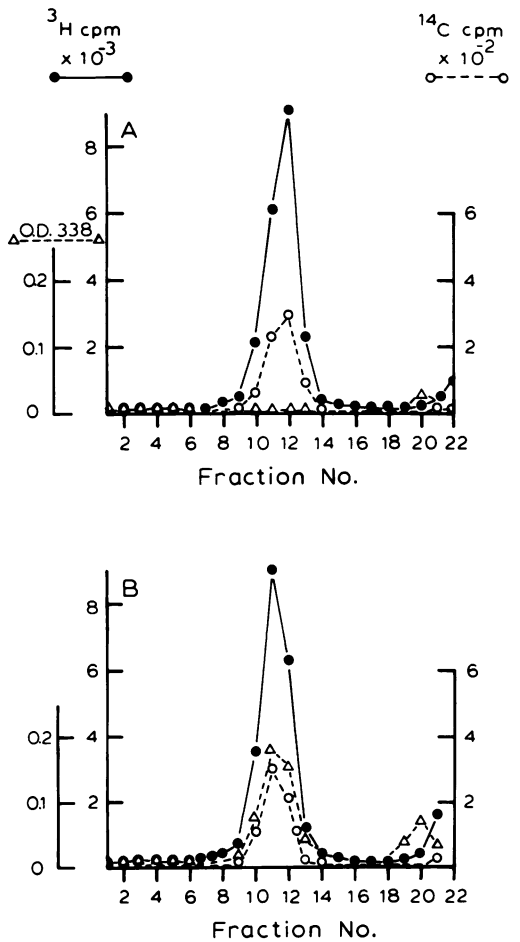


FIG. 4. *Polyacrylamide gel electrophoresis of sodium dodecyl sulfate-dissociated VS virions labeled with [4,5-^{3}H]leucine (5 μCi/ml) and [^{14}C]glycerol (0.5 μCi/ml). The designations of viral polypeptides (L, G, N, NS, M) are according to Wagner et al. (37).*



binding curve where there was a constant ratio of cholesterol to filipin (Fig. 6). For intact and spikeless VS virions, the values for the number of moles of cholesterol bound per mole of filipin were 1.25 ± 0.15 and 1.35 ± 0.33, respectively, at the 90% confidence level. Thus, the stoichiometry of the filipin-cholesterol interaction was similar for intact and spikeless VS virions, indi-

TABLE 1. *Effect of filipin on infectivity of influenza and VS virions*[a]

Virus	Molar ratio of cholesterol to filipin	PFU/ml[b]
VS		
Expt 1	Control	2.5×10^9
	1.0	3.3×10^8
	0.2	5.3×10^6
Expt 2	Control	9.6×10^9
	4.2	2.3×10^9
	1.0	1.1×10^9
	0.4	3.4×10^7
Influenza		
Expt 1	Control	6.8×10^8
	3.7	4.5×10^8
	1.8	3.0×10^8
	0.9	2.3×10^8
Expt 2	Control	7.2×10^9
	0.5	2.6×10^9

[a] Virions were incubated in the dark with filipin for approximately 30 min. Cholesterol concentrations varied between 75.3 and 233 μM. Control experiments with dimethylformamide showed no inactivation.

[b] VS virus plaque assays were carried out on 60-mm BHK-21-F cell monolayers as described previously (9), except that tryptose phosphate broth was not used in the overlay. Influenza virus plaques were as described by Choppin (8) in chicken embryo fibroblast monolayers.

FIG. 5. *Analysis of filipin-treated VS virion labeled with [^{3}H]leucine and [^{14}C]glycerol in a density gradient. To 0.3 ml of purified virus in phosphate-buffered salt solution containing ~0.5 mg of protein per ml, 1 μl of a 0.29 M solution of filipin in dimethylformamide was added, and the mixture was held at 4°C in the dark for 1 h. A similar control sample lacked filipin. The samples were layered on 4.5-ml 15 to 60% linear sucrose gradients in a solution of 0.01 M KCl, 0.0015 M $MgCl_2$, and 0.01 M Tris-hydrochloride (pH 7.4) and centrifuged for 1 h at 35,000 rpm in a Beckman SW56 rotor. Fractions of ~0.2 ml were collected, and 25 μl of each was used for radioactivity determination in a Protosol-toluene-Liquifluor mixture. To the remainder, 0.8 ml of a 1.25% solution of sodium dodecyl sulfate was added, and the optical density at 338 nm was determined on a Zeiss PMQII spectrophotometer. (A) Untreated virions; (B) filipin-treated virions.*

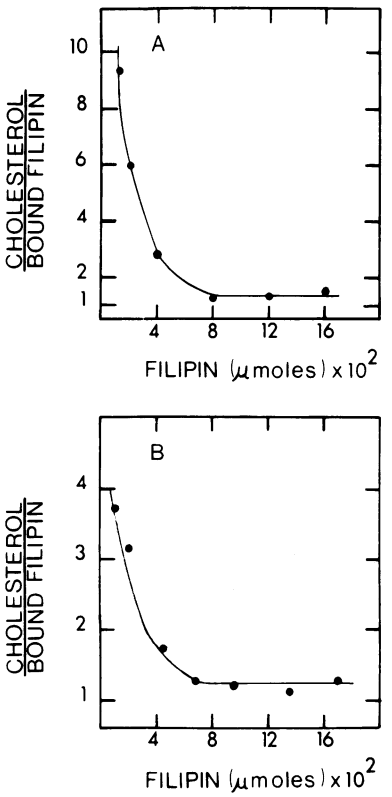


FIG. 6. *Stoichiometry of binding of filipin to intact and spikeless VS virions. To 5 ml of VS virions in phosphate-buffered salt solution, at a concentration of 2.4 and 4.8 μM viral cholesterol for intact and spikeless virions, respectively, filipin was added at concentrations that varied from 4.0 to 32.0 μM. After the samples were incubated, the virions were pelleted and the filipin concentration in the supernatant was measured. Bound filipin concentration was determined as the difference between initial and final concentrations. (A) Spikeless virions; (B) intact virions.*

cating that glycoproteins do not inhibit the binding of filipin to virions.

Equilibrium dissociation constants. The K_D values were determined by least-squares analysis of the data presented in Fig. 7. The K_D values, obtained by dividing the slope of the line by the value of the y-intercept, were 34 ± 2.0 μM and 0.46 ± 0.24 μM at the 90% confidence level for intact and spikeless VS virions, respectively. As expected, the y-intercepts were approximately 1.0, which validates the assumption used in these calculations that there is approximately a 1:1 ratio of filipin to cholesterol in the complex. The results indicate that filipin is more tightly bound to the VS viral membrane in spikeless than in intact virions.

Initial rates of association. The results of kinetic experiments are presented in Fig. 8. They indicate that the initial rate of association of filipin with cholesterol is faster in spikeless than in intact VS virions. The rates are linearly dependent on the cholesterol concentration of the viral preparation at low sterol concentrations.

ESR spectroscopy. To determine the effect of filipin binding on the structure of the viral lipid bilayer, the ESR spectra of virions spin labeled with nitroxide derivatives of stearic acid were recorded. Figure 9 shows the ESR spectrum of VS virions labeled with the C_5 spin label (where the nitroxide moiety is on the fifth carbon atom from the carboxyl end of the fatty acid; see insert in Fig. 9). The distance between vertical lines drawn through the high- and low-magnetic-field extrema reflects the motion of the spin label incorporated into the lipid bilayer (26, 27). This splitting increases as the motion of the label is restricted (21, 22). The observed increase in the splitting between the outermost spectral extrema after filipin treatment indicates that the ridigity of the region(s) probed by the label is increased. Exposure of intact influenza and spikeless VS virions to filipin appears to result in an increase in the rigidity of the probed regions of the viral bilayers (spectra not shown). In both the absence and presence of filipin, ESR spectra (not shown) of C_{16}-labeled VS virions

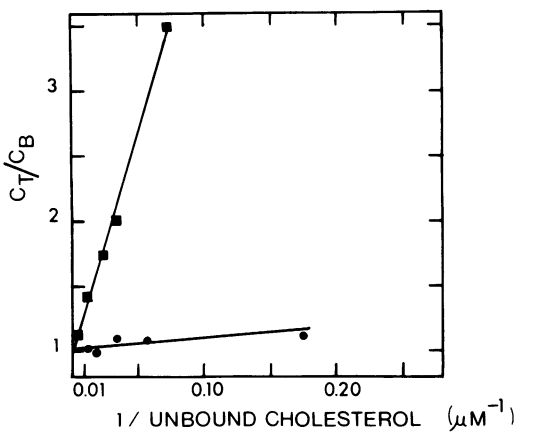


FIG. 7. *Plot of the ratio of total filipin to bound filipin (C_T/C_B) versus the reciprocal of the unbound viral cholesterol concentration in the VS virion suspension. For intact virions (■), the final filipin concentration was held constant at 4.2 μM and the cholesterol concentration was varied from 63.1 to 631 μM. For spikeless particles (●), cholesterol concentrations varied from 6.2 to 84 μM at a constant filipin concentration of 7.1 μM.*

(where the nitroxide moiety is on the 16th carbon atom from the carboxyl end of stearic acid) indicate that the deep hydrocarbon region probed by this label (18, 19) is considerably more fluid than the region near the glycerol backbone probed by the C_5 spin label.

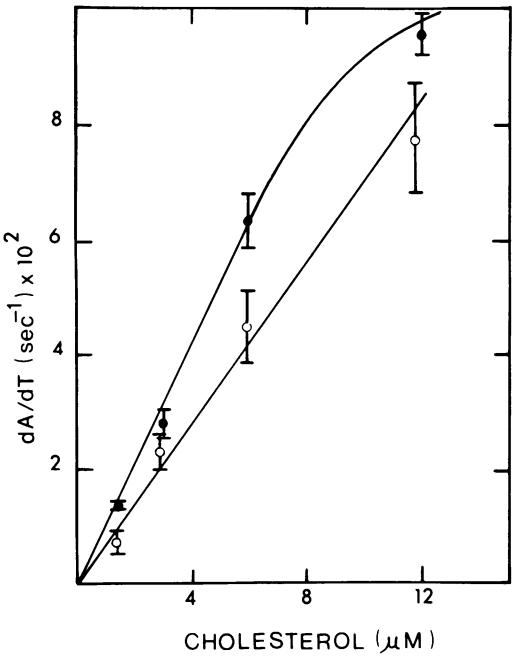


FIG. 8. *Effect of viral cholesterol concentration on the initial rate of association of filipin with intact (○) and spikeless (●) virions. The filipin concentration was 2.4 μM.*

DISCUSSION

The polyene antibiotic filipin produces profound morphological alterations in viral membranes. These alterations occur without detectable dissociation of lipids or proteins from the viral membrane and occur independently of particular preparative procedures used for electron microscopy. The fact that similar ridges and depressions were observed in intact and protease-treated VS virions indicates that these alterations do not require the presence of intact glycoprotein molecules. We cannot exclude the possibility that the small hydrophobic polypeptide fragments that remain associated with the VS viral envelope after proteolytic treatment (31, 33) may participate in the morphological alterations.

Although models for the interaction of filipin with cholesterol in biological membranes have been proposed (12, 34), none of them provides an adequate explanation of the uniform size of the 15- to 25-nm pits observed in liposomes, human erythrocytes, and ergosterol-containing *Tetrahymena* ciliary membranes, or the striations on VS viral envelopes. In the DeKruijff and Demel model (12), the cholesterol-filipin complex is envisaged as a planar aggregate lying in the hydrophobic core of the membrane parallel to the membrane surface. Models proposed for other polyene antibiotics, such as amphotericin B and nystatin, postulate a circular arrange-

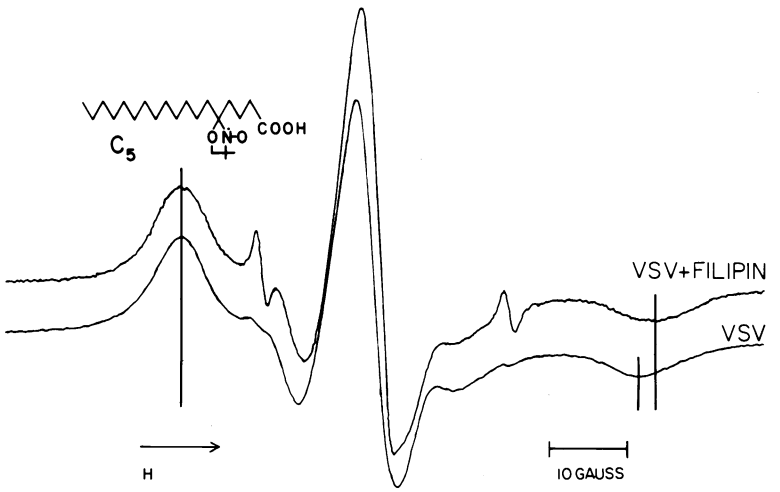


FIG. 9. *Comparison of the ESR spectra of C_5 spin-labeled untreated and filipin-treated VS virions. A vertical line has been drawn through the high- and low-magnetic-field peaks to demonstrate the greater splitting with filipin-treated virions. The cholesterol/filipin molar ratio was 0.89, and the concentration of dimethylformamide was 9.1% (vol/vol).*

ment of polyene and cholesterol molecules in the form of a pore, ca. 0.8 nm in diameter, perpendicular to the membrane surface (1, 12, 16). We propose that the pits observed in filipin-treated membranes result from the formation of a planar filipin-cholesterol complex similar to that proposed for amphotericin B and nystatin but with a diameter of 15 to 25 nm, which may be determined by the geometry of the cholesterol and filipin molecules. Because of the curvature of the bullet-shaped VS virion (70 by 175 nm), a planar complex may be unable to form in the viral membrane. As a result, the filipin-cholesterol complex may take on an elongated structure of the same diameter, forming the depressions and ridges observed in this study. Where the degree of curvature of the viral envelope is not as great, the complex would remain in the form of depressions or pits, as appear in disrupted VS virions and in the roughly spherical Rauscher leukemia virions. To determine whether the formation of either pits or striations depends on membrane geometry, further studies on filipin-induced alterations in membranes of other geometries and degrees of curvature would be valuable.

The morphological alterations of the viral membrane induced by filipin and amphotericin B are of interest in considering the mechanism that determines glycoprotein arrangement on the virion surface. A model of the structure of the VS viral envelope has been proposed, in which surface glycoproteins are regularly arranged on a hexagonal lattice of underlying membrane protein (6). Because of the stoichiometric relationship between bound filipin and membrane cholesterol and the known sterol requirement for filipin action, it is likely that the morphological alteration in the membrane results from the formation of filipin-cholesterol complexes. Therefore, if surface glycoproteins are present in a regular arrangement determined by protein-protein interactions, such interactions must be sufficiently weak to permit lateral redistribution of the glycoproteins, presumably into regions between filipin-cholesterol complexes.

Changes in viral membrane rigidity have been demonstrated to reflect changes in the lipid composition of viral membranes (26, 27). The observed increases in lipid rigidity in the membranes of intact and spikeless VS and intact influenza virions support the hypothesis that formation of cholesterol-filipin complexes occurs in the hydrophobic core of the membrane. Such complexes may laterally compress the lipids and proteins into the regions seen as ridges in VS virions. In contrast with the present results, filipin does not alter the mobility of a steroid spin label in lecithin-cholesterol planar multilayers treated with filipin (17). If it is assumed that the various stearic acid derivative spin labels have similar solubilities in the different regions of the filipin-treated viral bilayers, the observation that the region near the glycerol backbone of the viral lipids is appreciably more rigid than that of the deep hydrocarbon region for VS virions in the presence and absence of filipin suggests that the lipid regions probed by the spin labels retain a rigidity gradient characteristic of a bilayer structure (26, 27). The data of Flick and Gelerinter (17) indicate that stearic acid spin labels do not interact with filipin directly. Since no appreciable increase in spin-spin interactions is observed upon filipin treatment, the observed decrease in the motion of the C_5 spin label does not appear to reflect the formation of regions especially enriched in spin labels.

Using filipin as a probe for the state of cholesterol in the viral membrane, we measured the initial rates of association of the antibiotic with cholesterol and concluded that cholesterol is less accessible in intact than in spikeless virions, presumably because of the presence of glycoproteins on the outer surface or because the bilayer of spikeless VS particles is more fluid (26). Essentially the same stoichiometry was found for filipin binding to cholesterol in intact and spikeless VS virions. The values obtained here are similar to the published mole ratio values of 1.11 ± 0.18 reported by Katzenstein et al. (23) for liposomes and of 1.2 for *Acholeplasma laidlawii* cells (14). The fact that the difference in the equilibrium dissociation constants between spikeless and intact virions was much larger than the differences between the rates of association suggests that the rate of dissociation of filipin from the complex must be greater in intact than in spikeless virions.

The inactivation of VS viral infectivity by filipin occurs primarily at high drug concentrations, and the mechanism of inactivation remains to be established. It may be a result of viral envelope lysis, which occurs frequently at such concentrations, with only the intact particles retaining infectivity. However, we have been unable to separate populations of lysed particles by gradient centrifugation, and it is likely that filipin is incorporated essentially uniformly into all virions in a preparation. It is conceivable that inactivation of infectivity by filipin may occur because of increased viral membrane rigidity, which may have an inhibitory effect on an early event such as virus penetration. If this is the case, the observed difference in sensitivity of influenza and VS virions to inactivation by

filipin may be of significance in understanding the mechanism of virus penetration.

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