Effect of Phospholipase C and Cholesterol Oxidase on Membrane Integrity, Microviscosity, and Infectivity of Vesicular Stomatitis Virus†

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ABSTRACT: Exposure of intact vesicular stomatitis (VS) virus or intact mixed-lipid vesicles to phospholipase-free cholesterol oxidase resulted in <5% oxidation of membrane cholesterol compared with >95% oxidation of cholesterol in detergent-disrupted virus or liposomes. Phospholipase C hydrolyzed ~55% of phospholipids in intact VS virion membranes and ~90% of phospholipids in small, single-walled lipid vesicles. Prior or simultaneous exposure of VS virions or liposomes to phospholipase C resulted in >90% oxidation by cholesterol oxidase of membrane cholesterol to cholest-4-en-3-one. Phospholipase A_2 did not expose VS virion cholesterol to oxidation by cholesterol oxidase. Treatment with phospholipase C and cholesterol oxidase did not greatly alter the integrity of VS virion membrane, as determined by electron microscopy and protein electropherograms; only minimal amounts of oxidized cholesterol were released from virions exposed to both enzymes. Exposure to cholesterol oxidase alone resulted in only minor alterations in VS viral infectivity and membrane microviscosity monitored by fluorescence depolarization. Phospholipase C alone also had only a minimal effect on VS viral infectivity but markedly decreased the virion membrane apparent microviscosity. Treatment of VS virions with both phospholipase C and cholesterol oxidase caused a considerable decline in infectivity without further significant reduction in virion membrane microviscosity. Loss of VS viral infectivity appears to be due to the action of cholesterol oxidase on cholesterol exposed by the action of phospholipase C on phospholipid head groups in the outer surface of the viral membrane bilayer. This effect of cholesterol oxidase can be attributed to its oxidation of cholesterol in the viral membranes, or to production of hydrogen peroxide, or both.

The membrane of vesicular stomatitis (VS) virus contains only two proteins, a glycoprotein (G) and matrix (M) protein, both of which are specified by the viral genome (Wagner, 1975). The lipids of the VS viral membrane are derived from the host cell, but the relative proportions of cholesterol and aminophospholipids and the sphingomyelin to lecithin ratio are greater in the viral membrane (McSharry and Wagner, 1971; Moore et al., unpublished data). As indicated by surface labeling (Moore et al., 1974; Eger et al., 1975), protease susceptibility (Schloemer and Wagner, 1975; Bishop et al., 1975), and Triton solubilization (Kelley et al., 1972; Emerson and Wagner, 1973), the G protein is superficially located in the viral membrane and protrudes externally, whereas the M protein lies deep within or under the viral membrane. While evidence is available on the location of proteins and lipid composition of VS viral membrane, only recently have data been obtained on the asymmetric distribution of phospholipids (Fong et al., 1976), similar to that shown for influenza virus membrane (Tsai and Lenard, 1975). Fluorescence depolarization studies reveal no evidence for protein–lipid interaction in the most hydrophobic region of the VS viral membrane (Barenholz et al., 1976) but ^{31}P nuclear magnetic resonance spectroscopy indicates some influence of viral membrane proteins on rotational mobility of phospholipid head groups (Moore et al., 1977).

The purpose of the present study was twofold: (1) to locate cholesterol in the VS viral membrane as determined by its accessibility to cholesterol oxidase; and (2) to relate enzymatic alteration of lipids to virus membrane integrity and microviscosity as well as to infectivity of VS virus.

Materials and Methods

Cells and Virus. Baby hamster kidney (BHK-21)[1] cells were grown at 37 °C in BHK-21 medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum as previously described (Barenholz et al., 1976). The Indiana serotype of vesicular stomatitis (VS_{Ind}) virus was used to infect confluent monolayers of BHK-21 cells at a multiplicity of 0.1–1.0 plaque-forming units (pfu)/cell resulting in the formation of bullet-shaped (B) virions. Labeled virus was produced by adding 1 μCi/mL (specific activity of 57 Ci/atom) [^{14}C]amino acid hydrolysate or 5 μCi/mL [^{3}H]leucine (specific activity of 60 Ci/mmol) into the virus growth medium containing BHK-21 medium with 10% tryptose phosphate broth. VS_{Ind} virus was purified, as previously described, by sequential differential, velocity, and equilibrium centrifugation (Barenholz et al., 1976). Purified virus was stored in phosphate-buffered saline (PBS), pH 7.5, or 100 mM NaCl–20 mM Tris, pH 7.5, at −80 °C. Plaque assays of virus inoculums, purified virus, and virus treated by various procedures were

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[1] Abbreviations used are: BHK, baby hamster kidney cells; VS_{Ind}, Indiana serotype of vesicular stomatitis; B, bullet shaped; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; DPH, 1,6-diphenyl-1,3,5-hexatriene; *F*, fluorescence intensity; TC, taurodeoxycholate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPM, sphingomyelin; PS, phosphatidylserine; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

performed by plating on monolayer cultures of L-929 cells, as previously described (Wagner et al., 1963).

Protein Determination. Protein concentrations were measured by the method of Lowry et al. (1951) using crystalline bovine plasma albumin as the standard.

Phosphorus Determinations. Total phospholipid phosphorus was determined in the lipid extract by the method of Bartlett (1959).

Polyacrylamide Gel Electrophoresis. Virus preparations were made 1% with respect to sodium dodecyl sulfate, 1% with β-mercaptoethanol, and 10% with glycerol. Samples were boiled at 100 °C for 90 s prior to electrophoresis in 7.5% polyacrylamide gels in phosphate buffer as previously described (Wagner et al., 1970). Electrophoresis was for 14–15 h at 3.5–4 mA/gel, and gels with radioactive viral proteins were sliced into 1-mm fractions and counted by scintillation spectroscopy.

Iodination with ^{125}I. Virus preparations were iodinated using the "penetrating" oxidant (Chloramine-T) procedure as previously described (Moore et al., 1974).

Fluorescence Measurements. Fluorescence measurements, using 1,6-diphenyl-1,3,5-hexatriene (DPH), were made on a modified Perkin Elmer MPF3 spectrofluorimeter (Barenholz et al., 1976; Lentz et al., 1976) operating in the ratio mode. One microliter of a 0.3–0.5 mM solution of DPH was added to a 4-mL aliquot of virus. The final lipid to DPH ratio was always in excess of 300:1, and equivalent ratios of lipid to fluorescence probe were used in all preparations (Barenholz et al., 1976). The sample-chamber temperature was controlled by means of a Lauda MK-2 thermoregulated bath, and the temperature used for penetration of the probe was 39–40 °C. A Yellow Springs Instrument thermistor probe was connected to a digital ohmmeter to accurately measure the sample temperature (±0.1 °C).

Fluorescence depolarization measurements (Shinitzky and Barenholz, 1974) were based on the rotational motion of the DPH probe and characterized by the anisotropy (r) equation

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured, respectively, parallel ($\parallel$) to and perpendicular ($\perp$) to the plane of polarization of the excitation beam. Both $I_{\parallel}$ and $I_{\perp}$ were corrected for sample scattering (see Barenholz et al., 1976), which was normally less than 10% of the contribution to the $I_{\parallel}$ intensity (to which scattering makes the major contribution). Total fluorescence intensity (F) was calculated from the equation

$$F = I_{\parallel} + 2I_{\perp} = (I_{\parallel}/I_{\perp} + 2)I_{\perp} \quad (2)$$

DPH does not fluoresce in an aqueous environment and its uptake into the virus membrane was observed as an increase in F, which can then be calculated as a percentage of the maximum fluorescence intensity. The DPH labeling procedures had neither an effect on virus infectivity nor an effect on the virus membrane integrity, as determined by electron microscopy (Barenholz et al., 1976).

Microviscosity values were calculated as previously described (Barenholz et al., 1976) over a range of temperatures and the data were plotted as Arrhenius plots of the natural log of the apparent microviscosity (in poise) as a function of $1/T$ (where T is the absolute temperature). All calculations and plotting were done with the aid of a CDC computer.

Determination of Cholesterol in Membranes. The procedure used for the determination of cholesterol in biological membranes (Barenholz, Y., Moore, N. F., Patzer, E. J., and Wagner, R. R., in preparation) was based on a modification of a procedure developed for the determination of cholesterol in serum (Allain et al., 1974), using the enzyme cholesterol oxidase (supplied by Supelco Inc., Bellefonte, Pa.). In short, cholesterol oxidase converts cholesterol to cholest-4-en-3-one, which absorbs light at 240 nm in aqueous solutions and 235 nm in heptane; hydrogen peroxide is the second product of the reaction. Standard curves were established by solubilizing various concentrations of cholesterol in 30 μL of 10% taurodeoxycholate (TC) (sodium salt, Sigma Chemical Co.), diluting the solution to 1 mL with PBS, and adding 10 μL of cholesterol oxidase (2.5 units/mL). The reaction mixtures were incubated at 37 °C for 3 h, since kinetic studies showed that complete oxidation of 5–50 μg of cholesterol occurred under the above conditions within this time period. Each cholesterol concentration was run in triplicate and blanks consisted of all the reagents except enzyme. The molar extinction is identical to the one obtained for cholest-4-en-3-one. Identical calibration curves were obtained when cholesterol in 2-propanol was dispersed directly into buffer (final concentration of 2-propanol 1%) in the absence or presence of detergent.

Although the standard solutions could have been read directly at 240 nm, biological membranes contain protein and VS virus contains protein and RNA both of which would contribute to the absorbance in the ultraviolet spectrum. In order to reduce the background, the modified Dole procedure (Dole, 1956) was used to terminate the enzymatic reaction and to separate the neutral lipids from the nonlipid and polar lipid molecules. Two milliliters of Dole reagent (400 mL of 2-propanol, 100 mL of heptane, 10 mL of H_2O) was added to the 1-mL reaction mixture, followed by 1 mL of heptane (Eastman, glass distilled). To the blank at each dilution was added 10 μL of cholesterol oxidase immediately prior to extraction. The mixtures were extracted by vortexing, and centrifuged at 2000 rpm for 10 min in a refrigerated (4 °C) International centrifuge. The upper phase (heptane) was removed and read at 235 nm in a Gilford spectrophotometer. For reaction of cholesterol oxidase with intact VS virus and liposomes essentially the same conditions were used. Variations in procedure, particularly with reference to the absence of detergent, are described under Results.

Preparation of Lipid Vesicles. Chromatographically pure egg phosphatidylcholine, beef brain sphingomyelin, egg phosphatidylethanolamine, beef brain phosphatidylserine, and cholesterol were mixed in the desired molar ratios. Organic solvents were removed by flash evaporation, and the lipids were resuspended in a small volume of spectral-grade benzene and colyophilized as described by Huang and Thompson (1974). The dried lipids were resuspended in PBS (pH 7.2) or 20 mM Tris, 100 mM NaCl, pH 7.5, and vesicles were made by high-intensity ultrasonic irradiation of the lipids for 5–10 min under nitrogen, at 4–10 °C using a Heat Systems W-350 Sonifier (Barenholz et al., 1976; Lentz et al., 1976). The small proportion of large multilamellar liposomes present were removed by centrifugation at 100 000g for 60 min.

Phospholipase C Treatment of VS Virus. Purified VS virus (1 mg/mL) in 100 mM NaCl–20 mM Tris (pH 7.5) was incubated with phospholipase C (*Clostridium welchii* type I, purchased from Sigma and used without further purification) at a concentration of 0.2 unit/mL in 1.3 mM $CaCl_2$ (Coleman et al., 1970). This mixture was incubated at 37 °C for the times reported under Results. The reaction was stopped by cooling to 4 °C and by pelleting the reacted virus mixture through a pad of 20% sucrose or glycerol at 85 000g for 90 min, or extracting the lipids using 2 volumes of chloroform–methanol

(1:1) (Bligh and Dyer, 1959). After pelleting, the virus was resuspended in 100 mM NaCl, 20 mM Tris (pH 7.5) for reacting with cholesterol oxidase. Following the lipid extractions, the aqueous and organic phases were separated and the amount of phosphorus present in both phases was determined (Bartlett, 1959).

Phospholipase A_2 Treatment of VS Virus. VS virus suspended at 0.1–0.2 mg/mL in 20 mM Tris, 100 mM NaCl (pH 7.5) and made 10 mM with respect to $CaCl_2$ was reacted with 1 unit/mL phospholipase A_2 (Bee Venom, Sigma Chemical Co.) and used without further purification (Zwaal et al., 1975). The reaction was incubated at 37 °C for up to 5 h and terminated by the addition of 1.25 mM EDTA and/or by cooling to 4 °C and pelleting through a 20% sucrose pad at 85 000*g* for 90 min. The pelleted virus was resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5) and reacted with cholesterol oxidase, or diluted further for plaque assays.

Electron Microscopy. Intact VS_{Ind} virus or virus which had been treated with cholesterol oxidase, phospholipase A or C, and both cholesterol oxidase and phospholipase C was examined by electron microscopy as previously described (Barenholz et al., 1976). Virus preparations (0.5 mg/mL protein) were mixed with equal volumes of 1% glutaraldehyde and incubated at 4 °C for 20 min. The samples were allowed to spread onto formvar carbon-coated grids for 2 min, and 2% phosphotungstic acid was added to the grids for 30 s. A Siemens Elmiskop 1A electron microscope was used to examine the grids.

Results

Baseline Studies on the Specificity and Activity of Cholesterol Oxidase and Phospholipases on Intact and Detergent-Disrupted Liposomes and VS Virion Membranes. The effect of cholesterol oxidase was tested on sonicated vesicles composed of cholesterol–PC (1:3) or cholesterol–SPM (1:3), either intact or disrupted with 0.3% sodium taurodeoxycholate. In the absence of detergent, cholesterol oxidase oxidized less than 5% of the cholesterol in intact mixed PC vesicles and 2% of the cholesterol in mixed SPM vesicles following incubation for 9 h at 37 °C. The enzyme rapidly oxidized virtually 100% of the cholesterol in both types of detergent-disrupted lipid vesicles. Thin-layer chromatography in a solvent system of chloroform–methanol (100:2) of organic phase material extracted with 2 volumes of chloroform–methanol (1:1, v/v) revealed that cholesterol oxidase converted all the cholesterol in detergent-disrupted mixed vesicles to cholest-4-en-3-one.

Parallel experiments using cholesterol oxidase on intact VS virus and virus from which the glycoprotein spikes had been removed by trypsin yielded similar results. For both virus preparations less than 5% of the cholesterol was oxidized in a 3-h incubation period under standard cholesterol assay conditions (with a range of enzyme concentrations) in the absence of detergent. Similar results were obtained using higher concentrations of cholesterol oxidase.

A previous preparation of cholesterol oxidase (also obtained from Supelco) was found to oxidize approximately 90% of the cholesterol in cholesterol–PC and cholesterol–SPM vesicles as well as in VS virus membranes even in the absence of detergent. When the reaction products of cholesterol oxidase on these cholesterol–PC or cholesterol–SPM vesicles were analyzed on a TLC system of chloroform–methanol–water (170:2.5:0.2), it was discovered that diglyceride from PC or ceramide from SPM was also produced during the reaction but no lysolecithin or other degradation products. Similar results were also found when the virus was exposed to this cholesterol oxidase preparation, suggesting contaminating phospholipase C activity in the original enzyme preparation. From these results and the fact that the present cholesterol oxidase enzyme preparation that we are using has no phospholipase C activity and cannot oxidize cholesterol in intact membranes, a requirement was suggested for phospholipase C to expose cholesterol in intact membranes to the action of cholesterol oxidase. Furthermore, when phospholipase C (*Clostridium welchii*) was added to VS virus for 3 h at 37 °C and the virus was separated from the enzyme by pelleting through a 20% glycerol pad, or both phospholipase C and cholesterol oxidase are added simultaneously and incubated with VS virus for 3 h at 37 °C, greater than 95% of the virus membrane cholesterol was oxidized. This substantiates the need for phospholipase C activity to allow action of cholesterol oxidase on intact virus or vesicle membranes. The specificity of our preparation of phospholipase C was determined by the failure to hydrolyze PS in VS viral membrane or in mixed-lipid vesicles of PC–PS and SPM–PS.

To further our understanding of the interaction of cholesterol oxidase with cholesterol in the intact viral membrane, phospholipase A_2 was added prior to cholesterol oxidase, as described under Materials and Methods. It was found that phospholipase A_2 did not make cholesterol available to oxidation by cholesterol oxidase even though greater than 50% of the phospholipids in the viral membrane was converted to lysophosphlipids (Patzer, E. J., Moore, N. F., Barenholz, Y., and Wagner, R. R., unpublished data). Neither lysophospholipids nor free fatty acids (the products of phospholipase A_2) up to a concentration of 1 mM are inhibitors of cholesterol oxidase.

Phospholipase C Requirement for Cholesterol Oxidase Activity on Intact VS Viral Membrane. The finding that contaminating phospholipase C makes cholesterol in vesicle and viral membranes susceptible to oxidation by cholesterol oxidase led us to a further investigation of this phenomenon. To this end, we designed kinetic experiments to determine the extent to which phospholipase C exposes cholesterol to oxidation in otherwise intact VS viral membranes.

VS virus was treated for different periods of time with phospholipase C and then freed of residual enzyme by pelleting through a sucrose or glycerol pad at 4 °C. Using this procedure, the virus pellet was free of phospholipase activity, all of which remained in the supernatant. Resuspended virus was then assayed for lipid composition or infectivity, or incubated with cholesterol oxidase (0.5 unit/mg of virus protein) for 3 h at 37 °C; the amount of cholesterol converted to cholest-4-en-3-one was determined as outlined under Materials and Methods. Total oxidizable cholesterol in each sample was determined by the action of cholesterol oxidase on virus membrane solubilized in taurodeoxycholate.

Figure 1A illustrates the kinetics of the reaction of phospholipase C with intact VS virus expressed as a percentage of the phosphate released with time. Figure 1B shows the extent to which the action of phospholipase C is necessary before cholesterol oxidase can react with cholesterol in an otherwise intact VS viral membrane. As indicated, at least 40% of the phospholipid head groups must be removed before 96% of viral cholesterol can be oxidized. Shorter exposure to phospholipase C results in less hydrolysis of phospholipid head groups, and in correspondingly less cholesterol oxidase-catalyzed conversion of cholesterol to cholest-4-en-3-one.

Structural Integrity of VS Virion Membrane after Treatment with Phospholipase C and Cholesterol Oxidase. Experiments were designed to determine whether oxidized cholesterol, diglyceride, and ceramide remained associated with the VS virion and to what extent the integrity of its membrane

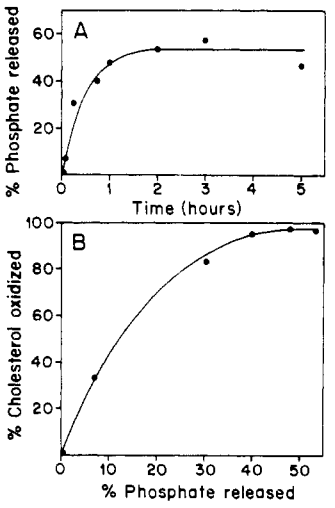

FIGURE 1: Kinetics of hydrolysis of head groups from membrane of VS virus exposed to phospholipase C (panel A) and the relationship of head groups released to exposure of virion membrane cholesterol to the action of cholesterol oxidase (panel B). VS_{Ind} virus (2 mg/mL) was reacted with phospholipase C (0.25 unit/mg of protein) at 37 °C for the periods indicated in panel A. Phosphate released into the aqueous phase after extraction with chloroform/methanol (1:1) is expressed as the percentage of total lipid in the aqueous and organic phases at each time point. Following exposure to phospholipase C at each time point, the virus was separated from the enzyme by pelleting through a 20% glycerol pad. Cholesterol oxidase (0.5 unit/mg of viral protein) was added to aliquots of the resuspended virus in the presence or absence of taurodeoxycholate. After incubation for 3 h at 37 °C, the amount of cholesterol oxidized to cholest-4-en-3-one was determined by absorbance at 235 nm as described in Materials and Methods. The percentage of cholesterol oxidized in each virus sample not exposed to detergent was calculated on the basis of total cholesterol in the corresponding virus sample disrupted with detergent. Plotted in panel B is the percentage of cholesterol oxidized by cholesterol oxidase action on intact VS virions as a function of percentage of virion membrane phosphate released by the action of phospholipase C. Less than 4% of membrane cholesterol was oxidized in virions not treated with phospholipase C or detergent.

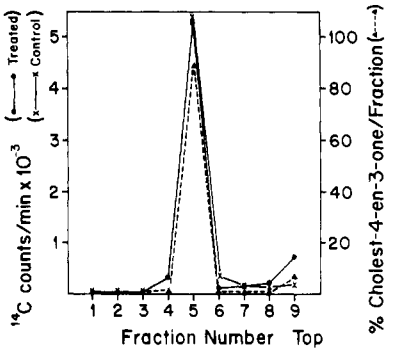

FIGURE 2: Association of cholest-4-en-3-one and ^{14}C-labeled protein with VS virions treated with phospholipase C and cholesterol oxidase prior to equilibrium centrifugation. VS_{Ind} virus (2 mg/mL) labeled with ^{14}C-labeled amino acids was reacted with phospholipase C (0.25 unit/mg of protein) and cholesterol oxidase (0.5 unit/mg of protein) for 3 h at 37 °C. The enzyme-treated virus was then centrifuged to equilibrium (16 h at 70 000g) in a 5-mL gradient of 0–40% potassium tartrate:0–20% glycerol. Fractions of 0.5 mL each were assayed for cholest-4-en-3-one and radioactivity. Also depicted are ^{14}C counts/min in control VS virions not treated with enzymes and centrifuged in a parallel gradient (X—X).

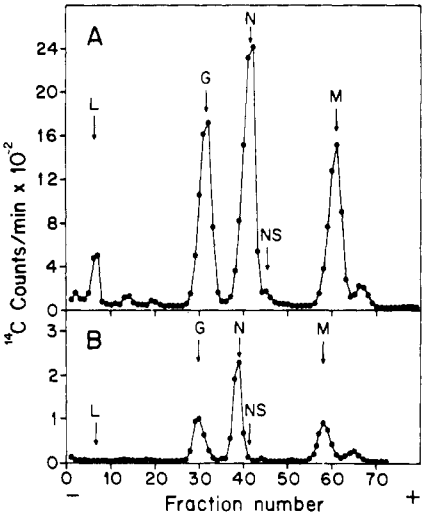

FIGURE 3: Polypeptide electropherograms of VS virions labeled with ^{14}C-labeled amino acids, treated with phospholipase C and cholesterol oxidase, and then fractionated by equilibrium centrifugation (16 h at 70 000g) in the 0–40% tartrate:0–20% glycerol gradient shown in Figure 2. Panel A shows the protein profiles of enzyme-treated VS virions collected from the light-scattering band of fraction 5. Panel B shows the protein profiles of material collected from the top of the gradient (fraction 9). Each fraction was dialyzed against PBS to remove tartrate, dissociated in 1% $NaDodSO_4$, and 1% 2-mercaptoethanol, and electrophoresed on 7.5% polyacrylamide gels, as described in Materials and Methods. Arrows mark the positions of the known VS viral structural proteins. Not shown is a coelectrophoresed protein electropherogram of control VS virions not treated with phospholipase C and cholesterol oxidase; control VS virus showed the identical protein pattern as that depicted in panel A, except for absence of the unidentified, minor ^{14}C peak which migrates just in front of protein M.

was compromised by the action of phospholipase C and cholesterol oxidase.

VS virus labeled with ^{14}C-labeled amino acids was treated for 3 h at 37 °C with phospholipase C and cholesterol oxidase and banded by equilibrium centrifugation in a 0–40% tartrate gradient. Comparison was made by equilibrium centrifugation of ^{14}C-labeled VS virus not exposed to enzymes.

Figure 2 demonstrates that 90% of oxidized cholesterol remained associated with most of the ^{14}C-labeled VS virus banded at density equilibrium. Similarly, the diglyceride and ceramide remained associated with the virus band (data not shown). A small amount of cholest-4-en-3-one was found at the top of the gradient along with some of the ^{14}C-labeled protein. This experiment suggests that the virus remains relatively intact after enzyme treatment but not completely so. To examine further the degree to which VS virus integrity was compromised, the protein compositions of the major equilibrium band and of the dissociated radioactivity at the top of the gradient shown in Figure 2 were analyzed by polyacrylamide gel electrophoresis (Figure 3).

Figure 3A shows the protein electropherogram of VS virus treated with phospholipase C and cholesterol oxidase followed by equilibrium centrifugation and dialysis to remove the tartrate. The electrophoretic profile was identical to that of control virus not exposed to enzymes (data not shown) except for the appearance of a minor peak of ^{14}C-labeled protein (<1.5% of the total protein), presumably a degradation product, which migrates slightly faster than the smallest (M) protein of VS virus. The electropherogram of ^{14}C-labeled protein recovered from the top of the tartrate–glycerol gradient reveals the presence of all three major VS viral proteins plus small amounts (less than 3% of the total protein) of the same rapidly migrating degradation product found associated with banded VS virions (Figure 3B).

These data show that treatment of VS virions with phospholipase C and cholesterol oxidase results in complete dissolution of a small proportion of the virions plus a minor degree of viral protein degradation. No dissolution of virions or protein degradation was observed when VS virions were treated with

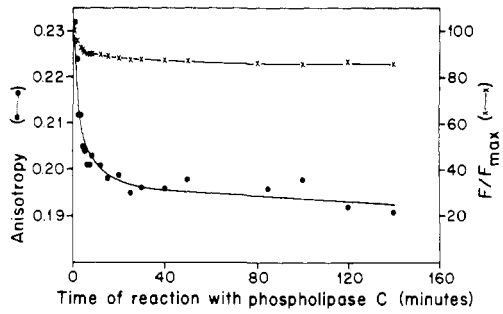

FIGURE 4: Effect of phospholipase C digestion on the anisotropy of the hydrophobic region of the membrane of VS virus. Virus (200 μg in 4 mL) was interacted with DPH at a probe to membrane lipid ratio of 1:300. When the probe had completely penetrated the membrane (i.e., no further increase in total fluorescence intensity), phospholipase C (preheated at 37 °C) was added and incubation was continued at 37 °C. Simultaneous readings of $I_{\parallel}$ and $I_{\perp}$ were recorded over the period of time indicated on the abscissa. Corrections were made for slight reductions in scattering due to digestion of the virus membrane by running in parallel a preparation of control virus (i.e., no DPH added) containing phospholipase C. A correction was also made for the slight decrease in total fluorescent intensity due to the small dilution caused by the initial addition of phospholipase C. Total fluorescence intensity (F/F_{max}) is indicated on the right ordinate and is expressed as a percentage of the initial total fluorescence intensity at time zero (corrected for dilution). Anisotropy is indicated by the left ordinate.

TABLE I: Effect of Phospholipase C and Cholesterol Oxidase on the Pattern of G and M Protein Labeling of VS Virus with ^{125}I and Chloramine-T.[a]

Treatment	G/M protein ratio of counts/min
Control (no enzyme treatment)	4.3
Phospholipase C	2.5
Cholesterol oxidase (free of phospholipase C)	3.8
Cholesterol oxidase + phospholipase C	1.2

[a] VS_{Ind} virus was reacted with cholesterol oxidase, phospholipase C, neither enzyme, or both enzymes. Virus was then pelleted and resuspended in PBS (pH 7.5). Fifty micrograms of protein from each sample was iodinated with 100 μCi of ^{125}I and 7 μg of chloramine-T. Twenty-eight micrograms of sodium metabisulfate was added to each reaction mixture after iodination for 1 min. The dialyzed samples were disrupted in 1% $NaDodSO_4$, 1% 2-mercaptoethanol and electrophoresed on 7.5% polyacrylamide gels for 16 h at 3.5 mA/gel. The polyacrylamide gels were sliced into 1-mm fractions using a Mickle gel slicer and each slice was incubated in 0.5 mL of Nuclear-Chicago solubilizer-H_2O (9:1) for 2 h at 50 °C. Ten milliliters of toluene-based scintillation fluid was added to each sample and radioactivity was determined in a Beckman LS-230 liquid scintillation counter. The total radioactivity for the G and M protein peaks was calculated and their ratios determined.

phospholipase C alone or cholesterol oxidase alone (data not shown). VS virion degradation is possibly due to a protease impurity in the cholesterol oxidase, although none was detected by direct protease assay (see Materials and Methods).

When examined by negative-stain electron microscopy, VS virions treated with cholesterol oxidase were indistinguishable from untreated VS virions. VS virions treated with both phospholipase C and cholesterol oxidase, or with phospholipase C alone, contained somewhat more broken virions, but most retained their bullet shape and glycoprotein spikes.

To gain more information on the integrity of the viral membrane after exposure to lipases, VS virions were iodinated with Chloramine-T. As we have shown previously (Moore et al., 1974), the G protein tyrosines of intact VS virions are almost 100% labeled with ^{125}I, whereas under similar controlled conditions the tyrosine residues of M protein are less extensively labeled with ^{125}I (~25%). Therefore, we compared the Chloramine-T-catalyzed iodination of G and M proteins in intact control VS virions (not exposed to enzymes) with VS virions treated with phospholipase C alone, cholesterol oxidase alone and both enzymes. Table I shows that the iodination of M protein was increased by a factor of 1.72 when VS virions were pretreated with phospholipase C alone but not significantly increased by exposure to cholesterol oxidase alone. In contrast, M protein iodination was increased 3.6 times when VS virions were previously treated with phospholipase C and then cholesterol oxidase, or with both enzymes together. Therefore, it appears that the integrity of VS virion membrane is quite significantly compromised by the combined action of phospholipase C and cholesterol oxidase.

Effect of Phospholipase C and Cholesterol Oxidase on Microviscosity of the Hydrophobic Region of the VS Virion Membrane. Previous fluorescence depolarization studies have shown that the membranes of VS virus and another rhabdovirus are more rigid than the plasma membranes of their host cells (Barenholz et al., 1976; Moore et al., 1976). It seemed important to determine the degree to which phospholipid and cholesterol alterations produced by the action of phospholipase C and cholesterol oxidase influence the microviscosity (fluidity) of the VS virion membrane.

Figure 4 depicts a plot of the change in anisotropy with time of VS virion membrane prelabeled with DPH due to action of phospholipase C at 37 °C; these data indicate a marked reduction in anisotropy and, therefore, microviscosity, particularly during the first 30 min, when phospholipase C activity was most marked. When total fluorescence intensity at each time (corrected for loss of scattering for the $I_{\parallel}$ and $I_{\perp}$ values) was calculated as a percentage of initial total fluorescence intensity (corrected for initial dilution due to the addition of phospholipase C), the values were found to be somewhat reduced. This can be explained by the reduction in the $I_{\parallel}/I_{\perp}$ ratio which is mainly due to a change in $I_{\parallel}$, causing the reduction in F (see eq 2); no quenching or loss of DPH from the membrane occurred. Similar experiments with phospholipase C mixed with cholesterol oxidase also showed similar drops in anisotropy of the hydrophobic regions of the viral bilayer, while treatment of VS virus with cholesterol oxidase alone, as expected, caused almost no decreases in the anisotropy values.

To eliminate the possibility that these enzymes were affecting the DPH probe directly, VS virus was reacted with various combinations of these enzymes for 3 h and pelleted through a sucrose pad to separate virus from the enzymes, as previously described. The resuspended virus samples were then incubated with DPH at 40 °C with complete penetration as evidenced by the total fluorescent intensity reaching a plateau. Then the samples were cooled through a temperature range and anisotropy and microviscosity were measured as described in Materials and Methods. Table II shows a summarized evaluation of the Arrhenius plots for VS virus treated with cholesterol oxidase, phospholipase C, and phospholipase C + cholesterol oxidase. Microviscosity values are shown at four temperatures for each of the systems. The microviscosity values for the phospholipase C treated virus are considerably lower at all temperatures than the values for the untreated virus or for virus treated with cholesterol oxidase alone. The cholesterol oxidase treated virus exhibits microviscosity values at the four temperatures shown which are only slightly lower (less than 8% difference in the values and in the slope of the Arrhenius plot) than the values for untreated virus. Cholesterol oxidase had little additional effect on the microviscosity of the VS virus membrane (Table II) when added with phospholipase C, al-

TABLE II: Membrane Microviscosity of VS_{Ind} Virus Treated with Cholesterol Oxidase, Phospholipase C, or Phospholipase C followed by Cholesterol Oxidase Determined at Four Temperatures Based on Data Calculated from Arrhenius Plots.[a]

Treatment	Microviscosity (poise) 10 °C	20 °C	25 °C	37 °C	ΔE (kcal/mol)	Intercept (poise × 10^5)	Correlation coefficient
Untreated	11.69	7.96	6.29	4.05	6.55 ± 0.08	−9.24 ± 0.13	0.9963
Cholesterol oxidase[b]	9.80	7.35	5.95	3.90	6.1 ± 0.07	−8.5 ± 0.12	0.9964
Phospholipase C[c]	5.90	4.39	3.63	2.17	6.5 ± 0.13	−9.8 ± 0.20	0.9894
Phospholipase C–cholesterol oxidase[d]	7.50	5.02	4.00	2.10	8.1 ± 0.12	−12.33 ± 0.30	0.9951

[a] The energy of activation, intercepts, and correlation coefficients were calculated from the Arrhenius plots of natural logarithm of microviscosity against $1/T$ (T = absolute temperature). [b] VS_{Ind} virus was treated with cholesterol oxidase free of phospholipase C. [c] VS_{Ind} virus was treated with phospholipase C for 3 h. [d] VS_{Ind} virus was digested with phospholipase C for 3 h in the presence of cholesterol oxidase.

though the slope of the Arrhenius plot increased by 20%.

Effect of Phospholipases and Cholesterol Oxidase on Infectivity of VS Virus. Invasion of host cells by VS virus appears to take place as a result of fusion of virus and cell membranes leading to entry of the infectious viral nucleocapsid into the cell cytoplasm (Heine and Schnaitman, 1971). It was of interest to determine, therefore, whether VS viral infectivity is affected by chemical and physical alterations in virion membrane integrity caused by various lipases. To this end VS viral infectivity, as measured by plaque assay, was determined following exposure to phospholipases C and A_2 and cholesterol oxidase, singly or in combination, as well as by the secondary effects of products generated by enzyme action.

Table III summarizes the data on losses of infectivity resulting from exposure of VS virus to lipases and their products. As previously noted by Cartwright et al. (1969), phospholipase C alone caused only minimal loss of VS viral infectivity. Nor is the infectivity of Semliki Forest virus significantly reduced by phospholipase C (Friedman and Pastan, 1969). Cholesterol oxidase likewise had little effect. In contrast, reaction of VS virus with phospholipase C followed by cholesterol oxidase, or simultaneous incubation with both enzymes, resulted in reduction of plaque titer of 10^{-4} to 10^{-5}. These data indicate that phospholipase C removal of 55% of polar head groups results in only comparatively small reduction in viral infectivity. However, subsequent oxidation of cholesterol causes a substantial decrease in infectivity.

The effect on viral infectivity of cholesterol oxidase may be explained by either the direct alteration of cholesterol, or by the H_2O_2 generated during the enzymatic reaction, or both. At first it did not appear to be caused by the production of H_2O_2 for the following reasons: (a) the levels of H_2O_2 were found to be invariably less than 0.001% even in the most concentrated cholesterol–cholesterol oxidase reaction mixtures; (b) moreover, removal of H_2O_2 by extensive dialysis of cholesterol oxidase treated VS virus during the incubation with the two enzymes did not reverse this loss of infectivity when compared with enzyme-treated undialyzed virus; (c) the addition of catalase to the reaction mixture containing virus, phospholipase C, and cholesterol oxidase resulted in only slightly reduced loss in infectivity (however, catalase alone caused a slight increase in VS viral infectivity); and (d) the reducing agent, 2-mercaptoethanol equimolar to cholesterol in the viral membrane, had a very limited effect on restoring infectivity to oxidized virus. The potential effect of H_2O_2 cannot be ruled out unequivocally because H_2O_2 at a concentration of 0.0001% (equimolar to viral cholesterol in the infectivity studies) decreased the infectivity of VS virus 2–7-fold; when the H_2O_2 was added at a 10-fold higher concentration of 0.001%, the VS virus infectivity dropped 10^5-fold (data not shown). The minimal reduction in infectivity caused when H_2O_2 (0.0001%) plus cholesterol oxidase were both incubated with VS virus may be attributable to contaminating catalase in the enzyme preparation.

TABLE III: Effect of Enzymes, Singly or in Combination, and Enzyme Products on Infectivity of Vesicular Stomatitis Virus.[a]

Treatment of virus	Fractional reduction of plaque titer (experimental/control)[b] Experiment 1	Experiment 2
Phospholipase C[c]	0.60	0.16
Phospholipase A_2[d]	2.1×10^{-3}	
Phospholipase A_2[d] + EDTA (2.5 mM)	1	
EDTA alone (2.5 mM)	1	
Cholesterol oxidase[e]	0.82	0.74
Phospholipase C[c] + cholesterol oxidase[e]	$>4 \times 10^{-5}$	7×10^{-5}
Phospholipase A_2[d] + cholesterol oxidase	2×10^{-3}	2.1×10^{-3}
H_2O_2[f] + cholesterol oxidase[e]	0.46	0.13
Lysophosphatidylcholine[g]	1	

[a] Each enzyme, combination of two enzymes, or other chemical was incubated at 37 °C in parallel with an equivalent virus control incubated in buffer without enzyme or added chemical. After the incubation period, the virus samples were pelleted through a 20% glycerol or sucrose pad at 100 000g for 90 min. The pellets were gently resuspended and plaque assays were performed by plating resuspended virus on monolayers of L cells. [b] Plaque titers of enzyme-treated VS virus were calculated from duplicate platings and recorded as the fractional level of five simultaneously assayed virus controls of the following titers (PFU/mL): 4.5×10^9, 7.3×10^9, 2.7×10^9, 8.6×10^9, and 2.0×10^9. [c] Phospholipase C = 0.2 units/mg of virus protein for 3 h. [d] Phospholipase A_2 = 6.7 units/mg of virus protein for 1–5 h. This experiment was repeated four times with similar results regardless of duration of incubation. [e] Cholesterol oxidase = 0.25 unit/mg of virus protein for 3 h. [f] H_2O_2 added in three equivalent aliquots at hourly intervals to produce a final concentration of 0.0001%, the amount of H_2O_2 calculated to be produced by cholesterol oxidase (~93% of cholesterol oxidized). [g] Lysophatidylcholine was added in two aliquots to produce 0.02×10^{-9} M, the amount of lyso-PC calculated to be produced by phospholipase A_2 (~50% of reacted phospholipids).

When phospholipase A_2 (bee venom) was added to VS virus under the conditions described in Table III, there was a 2–3 log loss of infectivity as compared with the control. The infectivity decreased up to an hour and then reached a plateau which did not change up to 5 h incubation time (data not shown). Since bee venom phospholipases, unless completely purified, contain melittin (Shipolini et al., 1971), a protein which strongly interacts with phospholipids, it was decided to determine whether there were any effects on viral infectivity due to melittin. This was accomplished by adding 2.5 mM EDTA to the reaction mixture. Since the enzyme requires calcium for activity, any loss in infectivity should be due to the

melittin. As seen in Table III, neither EDTA alone nor EDTA in the presence of phospholipase A_2 caused any decrease in infectivity.

Lysophosphatidylcholine is one of the products of the phospholipase A reaction. Although having high solubility in aqueous solution as micelles, lysophosphatidylcholine remains in the viral membrane in a manner similar to that observed for human red blood cell membranes treated by various phospholipases A_2 (Zwaal et al., 1973, 1975). Since lysophospholipids promote lysis of cells when added to erythrocytes (Cho and Proulx, 1971), it was of interest to determine whether the viral lysophospholipids might partition into the host cell membrane causing an alteration that results in a decrease in cell susceptibility to virus infectivity. This would represent an entirely different mechanism of action as compared with the lysophospholipids that are formed in situ in the viral membrane and exert their effect internally in the viral membrane. As seen in Table III, if lysophosphatidylcholine is added externally to the virus sample in two aliquots as described in the legend, no decrease in infectivity was observed. This suggests that lysophospholipids in the aqueous environment have little effect on the cell or virus membrane and that the viral lysophospholipids formed in situ have a much more deleterious effect.

To ensure that none of the infectivity results due to the enzymes were caused by contaminating proteases, each of them was checked using the protease assay described by Tomarelli et al. (1949). No detectable proteases were found in any of the enzymes at concentrations greater than that used for virus treatment.

Discussion

The free β-hydroxyl group of the cholesterol in intact phospholipid bilayers was found to be essentially inaccessible to the enzyme cholesterol oxidase in both VS virion membranes and mixed lipid vesicles in various molar ratios of cholesterol to phospholipids. Two procedures served to expose the β-OH group to cholesterol oxidase. In one, the membrane was disrupted by detergent which formed mixed micelles similar to those described by Small (1971). In our system the mixed micelles contained 1 molecule of cholesterol to 2000 molecules of sodium taurodeoxycholate. In the second, removal of the phospholipid polar head groups by phospholipase C also exposes the hydroxyl group of cholesterol to cholesterol oxidase either by concerted or sequential action of the two enzymes. Phospholipase C present as a contaminant in some preparations of cholesterol oxidase and the *Cl. welchii* enzyme both were active on PC, PE, and SPM (but not PS) as substrates present in liposomes and in VS viral membranes.

VS viral and other biological membranes differ from membranes of phospholipid vesicles in their reactivity to phospholipase C. Hydrolysis of phospholipids in liposomes went almost to completion, whereas hydrolysis in virion membrane did not exceed 55% of total phospholipids. VS virions exposed to phospholipase C remain relatively intact in shape and infectivity; moreover, neither proteins nor lipids located in the inner leaflet of the viral bilayer become accessible to chemical labeling by iodination or by trinitrobenzenesulfonic acid (Patzer et al., manuscript in preparation).

Phospholipase C removal of virion or vesicle phospholipid head groups produced a drastic reduction in apparent microviscosity as measured by the fluorophore DPH, presumably related to marked differences in the physical properties of the phospholipid substrates and their products of hydrolysis, the diglycerides and ceramides. Similar observations have been made with erythrocyte membranes treated with phospholipase C (Glaser et al., 1970). Cholesterol oxidase alone had little effect and did not significantly augment the effect on apparent membrane microviscosity of phospholipase C, except for a 20% increase in the slope representing the flow energy of activation. This latter effect of cholesterol oxidase may indicate less interaction of oxidized cholesterol than reduced cholesterol with membrane neutral lipids, hydrolyzed phospholipid products, and residual unhydrolyzed phospholipids.

The inaccessibility of intact VS virion membrane to the action of cholesterol oxidase can be explained in three ways: (i) the hydroxyl group of cholesterol is tightly bound to the ionogenic head group of the phospholipids and therefore cannot interact with the cholesterol oxidase unless the head group is removed by phospholipase C; (ii) the cholesterol oxidase is inhibited by the presence of ionogenic charged groups; and (iii) the cholesterol hydroxyl group is located at the interface of the lipid bilayer in the plane of the glycerol backbone of the phospholipids (Huang, 1976) where the cholesterol oxidase cannot penetrate. The first two explanations seem unlikely because mixed micelles of bile salts with cholesterol and phospholipids are charged but the cholesterol is still an excellent substrate for the enzyme. The third explanation, location of cholesterol in the plane of the glycerol backbone, seems most probable and is further supported by the fact that the cholesterol of the VS viral membrane previously treated with phospholipase A_2 did not serve as a substrate for cholesterol oxidase. Although about 50% of viral phospholipids were hydrolyzed by phospholipase A_2, the lysophospholipids and fatty acids remain in the viral membrane, thus keeping the cholesterol inaccessible to cholesterol oxidase.

Accessibility of VS virion cholesterol to oxidation by cholesterol oxidase is proportional to the degree to which phospholipase C degrades polar head groups. Maximal accessibility of cholesterol was noted at a level of ~40% phospholipid degradation (see Figure 1). Four hypotheses come to mind to explain the phospholipase C induced exposure of all the VS virion membrane cholesterol to oxidation: (i) complete degradation of the virion membrane by the enzymes; (ii) membrane cholesterol is asymmetrically distributed in the outer leaflet of the virion bilayer; (iii) symmetrically arranged cholesterol in the inner layer can flip-flop across the bilayer and exchange with outer layer oxidized cholesterol; and (iv) cholesterol oxidase can penetrate to the inner membrane layer of the virion exposed to phospholipase C.

Among these alternatives, the third hypothesis seemed to be the most difficult to exclude. There was evidence against complete enzymatic degradation of virion membrane. Even if cholesterol oxidase could penetrate through the bilayer, for which there is no evidence, the cholesterol in the inner part of the bilayer would not be exposed to the enzyme due to the presence of the phospholipid head groups in the inner layer. Also, it is difficult to visualize all the cholesterol being present only in the outer layer of the virion membrane since cholesterol represents at least 40% of the virion bilayer lipids (McSharry and Wagner, 1971) and at least half of the phospholipids are in the outer layer (Patzer et al., to be published). Therefore, the most logical hypothesis would seem to be flip-flop of symmetrically distributed cholesterol by rapid exchange of oxidized cholesterol in the outer layer resulting in exchange of unreacted cholesterol in the inner layer. The failure of Lenard and Rothman (1976) to detect flip-flop of unoxidized cholesterol in the membrane of influenza virus may be due to inability of completely reduced cholesterol to undergo redistribution across the lipid bilayer.

Since exposure of VS virus to phospholipase C alone resulted in a marked decrease in membrane microviscosity and cholesterol oxidase did not further reduce the apparent micro-

viscosity of the virion membrane caused by phospholipase C, viral membrane fluidity appears to be due, at least in some measure, to the phospholipid head groups. These data also suggest that removal of the phospholipid head groups can effect lipid–lipid interaction in the hydrophobic and interface region of the lipid bilayer, as well as the polar region. Decreased interaction of cholesterol with phospholipid head groups may explain the decrease in microviscosity caused by phospholipase C.

It seemed possible at one time that the greater viscosity of VS viral membrane promotes infectivity of VS virus by fusion of the VS virion membrane with the less rigid host cell membrane (Barenholz et al., 1976). This hypothesis now seems untenable based on the evidence that phospholipase C, which greatly reduces virus membrane rigidity, only slightly affects the infectivity of the virus. Exposure of VS virus to cholesterol oxidase alone, as expected, did not significantly influence VS viral infectivity. However, simultaneous or sequential treatment with phospholipase C and cholesterol oxidase markedly reduced infectivity by an as yet unknown mechanism. Although it is possible that the sharp decline in infectivity is caused by oxidation of cholesterol, further proof is required by experiments in which cholest-4-en-3-one is directly inserted into the budding virus membrane or by exchange of VS virion cholesterol for cholest-4-en-3-one in reacting liposomes. One cannot rule out H_2O_2 formed by the oxidation of cholesterol as being responsible for the marked loss in infectivity of VS virions after treatment with phospholipase C and cholesterol oxidase. However, removal of H_2O_2 either by dialysis or by catalase did not reverse the effect of cholesterol oxidase. Although loss of infectivity resulted from direct exposure of VS virions to H_2O_2, at least tenfold larger amounts of exogenous H_2O_2 were required to reduce infectivity than those amounts of H_2O_2 presumed to be generated enzymatically.

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