Depletion and Exchange of Cholesterol from the Membrane of Vesicular Stomatitis Virus by Interaction with Serum Lipoproteins or Poly(vinylpyrrolidone) Complexed with Bovine Serum Albumin†

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ABSTRACT: Cholesterol was depleted from the membrane of vesicular stomatitis virus by exposing virion suspensions to serum lipoproteins enriched with phospholipids. Unlike the reaction of virions with phospholipid vesicles, nonspecific adherence of lipoproteins and exogenous lipids to the envelope of the virus was found to be minimal. The extent of cholesterol depletion was dependent upon the type of phospholipid complexed with interacting lipoprotein; sphingomyelin and dipalmitoyllecithin were found to be highly effective depleters of cholesterol compared to egg phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine. Similar depletion of cholesterol from the virion membrane was also observed when vesicular stomatitis virus was exposed to a complex of poly(vinylpyrrolidone) and bovine serum albumin coated with egg phosphatidylcholine or dioleoylphosphatidylcholine. Cholesterol depletion was found to alter the morphology but not the membrane integrity of the virus. Directly correlated with depletion of cholesterol was a substantial loss in the anisotropy of the viral membrane as determined by fluorescence depolarization of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene. Interaction with poly(vinylpyrrolidone) complexed with albumin, phosphatidylcholine, and cholesterol resulted in exchange of cholesterol from the virion membrane which followed biphasic kinetics with a rapid and a slow phase; these data indicate that 75–85% of viral membrane cholesterol is present in the outer monolayer, and 15–25% is located in the inner monolayer. Depletion of cholesterol from the virion membrane resulted in a significant drop in the infectivity of the virus as measured by plating efficiency on L-cell monolayers. Such an effect was not observed when virion cholesterol was exchanged without net reduction in the concentration of viral membrane cholesterol. Part of the loss in infectivity following depletion of cholesterol could be restored by reincorporation of cholesterol in the membrane, thus demonstrating that membrane cholesterol partly contributes to the infectivity of vesicular stomatitis virus.

Vesicular stomatitis virus (VSV)[1] is a negative-strand RNA virus consisting of a lipoprotein membrane and a nucleoprotein core (Wagner, 1975). The envelope of the virus contains two proteins, G (glycoprotein) and M (matrix protein), both of which are specified by the viral genome. The intrinsic glycoprotein forms the spikes which protrude from the outer surface of the membrane whereas the matrix protein lines the inner surface of the viral membrane (Dubovi & Wagner, 1977). Although virion lipids are derived from the plasma membrane of the host cell, the virion membrane contains relatively higher proportions of cholesterol and aminophospholipids (McSharry & Wagner, 1971; Patzer et al., 1978a). Because of the simplicity of the system and the ability to obtain highly purified virus, VSV has been used extensively as a model system for studying lipid organization in biological membranes (Patzer et al., 1979).

Although in recent years considerable literature has accumulated on the structural aspects of the viral membranes, relatively few studies have been directed toward understanding the role of the membrane in viral invasion of cells. The concept of viral membrane fusion during infection has been supported by studying the interaction of enveloped viruses with multilamellar vesicles (Haywood, 1975; Mooney et al., 1975). Correlative experiments in our laboratory demonstrated that interaction of VSV with small unilamellar vesicles depletes cholesterol from the viral membrane; this resulted in a significant drop in the infectivity of the virus (Moore et al., 1978) as did the oxidation of virus cholesterol by cholesterol oxidase after pretreatment of the virus with phospholipase C (Moore et al., 1977; Patzer et al., 1978c).

Cholesterol present in many biological membranes has been shown to exchange quite readily with serum lipoproteins as well as with lipid vesicles (Jackson et al., 1976; Bruckdorfer & Graham, 1976; Bell, 1976). Although exchange reactions of this type have been utilized in many instances for calculating the distribution and transbilayer movement of cholesterol, results in most cases appear to be contradictory (Poznansky & Lange, 1976; Gottlieb, 1976; Lenard & Rothman, 1976; Bloj & Zilversmit, 1977; Lange et al., 1977; Nakagawa et al., 1979; Backer & Dawidowicz, 1979; Sefton & Gaffney, 1979). Kinetic analyses of cholesterol depletion and exchange from the VSV membrane into lipid vesicles performed in our laboratory have clearly demonstrated that VSV cholesterol is present in two distinct pools, in which we now know less than 30% resides in the inner monolayer and more than 70% in the outer monolayer (Patzer et al., 1978b); an error in the original calculations resulted in presentation in the reverse order of cholesterol bilayer distribution.

Most cholesterol depletion/exchange studies done with lipid vesicles, however, suffer from a serious artifact: a considerable number of residual vesicles remain firmly adherent to the surface of the virus (or cells) following interaction with the vesicles (Moore et al., 1978; Patzer et al., 1978b). Such nonspecific adherence of lipid vesicles to VSV may affect the

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[1] Abbreviations used: VSV, vesicular stomatitis virus; G, glycoprotein; M, matrix protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPM, sphingomyelin; DPL, dipalmitoyllecithin; BSA, bovine serum albumin; PVP, poly(vinylpyrrolidone); BHK, baby hamster kidney; BME, basal medium Eagle; pfu, plaque-forming units; PBS, phosphate-buffered saline; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCS, fetal calf serum; THF, tetrahydrofuran; HDL, high-density lipoproteins; LDL, low-density lipoproteins; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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kinetic parameters of cholesterol exchange or depletion which are used to derive the $t_{1/2}$ values for time of cholesterol transposition as well as the value of cholesterol distribution, both of which are calculated from these kinetic parameters (Bloj & Zilversmit, 1976). Nonspecific adherence of vesicles can be reduced, but not eliminated, by removing the glycoprotein spikes of the virus envelope with trypsin (Patzer et al., 1978b; Shaw et al., 1979), but this results in drastic loss in infectivity (Bishop et al., 1975). Although certain experiments can be performed with spikeless virions, others can not, and the problem of residual adherent lipids always poses an unknown factor.

In this article, we describe two different methods for studying the dynamics of depletion, exchange, and transbilayer movement of cholesterol in the membrane of intact infectious VSV. The minimal amount of nonspecific adherence of exogenous lipids to virions under these conditions has also allowed us to examine with greater confidence the possible biological role of cholesterol in infectivity of the virus.

Materials and Methods

Reagents. Egg PC and egg PE were obtained from the Makor Chemical Co., Jerusalem, Israel; beef brain PS and SPM were provided by the Sigma Chemical Co., St. Louis, MO. Cholesterol was obtained from Supelco, Inc., Bellefonte, PA, and was used without further purification. All lipids were found to be pure as tested by thin-layer chromatography heavily loaded with 500 μg. Cholesterol oxidase (EC 1.1.3.6) was a product of Supelco, Inc. and was found to be free of contaminating phospholipase C (Moore et al., 1977). Sigma Chemical Co. provided BSA and poly(vinylpyrrolidone) (PVP), a water-soluble polymer characterized by its unusual complexity and colloidal properties [see *Kirk-Othmer Encycl. Chem. Technol.* (1970)]. In these studies, we used PVP VK-30, which has an average molecular weight of 40 000 and minimal effect on viscosity at the concentrations used. Concentrations of cholesterol, phospholipids, and proteins were determined as described elsewhere (Barenholz et al., 1978).

Cells and Virus. Baby hamster kidney (BHK) cells were grown at 37 °C in BME supplemented with 10% tryptose phosphate broth, 10% calf serum, and antibiotics, as described elsewhere (Barenholz et al., 1976). Plaque-purified Indiana serotype VSV was used to infect BHK-21 cells at a multiplicity of 0.1 pfu/cell. Bullet-shaped virions were harvested 21 h after infection and were subsequently purified by differential, rate zonal, and equilibrium centrifugation in sucrose and tartrate gradients (McSharry & Wagner, 1971). Purified VSV was stored in PBS at a concentration of 2–5 mg/mL at −80 °C until further use. Infectivity of all virus preparations was performed by plaque assay on monolayers of L-929 cells (Wagner et al., 1963).

Cholesterol-labeled VSV was produced as follows: DL-[5-^{3}H]mevalonic acid lactone (New England Nuclear, specific activity 2.0 Ci/mmol) was converted to the sodium salt by treating with 0.1 N NaOH at 50 °C. The cholesterol in uninfected BHK cells was prelabeled by adding 2–3 μCi of [^{3}H]mevalonate to the medium during 3 days of cell proliferation. VSV was grown in these prelabeled cells in the presence of 3 μCi of [^{3}H]mevalonate. Lipid analysis of purified virus revealed that at least 95% of the radioactivity in the virus was associated with the cholesterol fraction. Under the above conditions, the viral cholesterol reached a specific activity of 2.79×10^6 dpm/mol.

Electron Microscopy. Virus treated under various conditions and complexes of PVP–BSA–PC were examined by negative staining in a Siemens 1A electron microscope (Moore et al., 1977). A drop of each sample in PBS was placed on a Formvar carbon-coated grid for 30 s, and the excess liquid was blotted off before addition of 1–2% phosphotungstic acid for 15 s. After the excess phosphotungstic acid was removed, the grids were examined by electron microscopy.

Fluorescence Measurements. Membrane dynamics were studied by fluorescence depolarization of the lipophilic fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) by using a modified Perkin-Elmer MPF-3 spectrofluorometer exactly as described by Barenholz et al. (1976). Fluorescence anisotropy (r) was determined as described by Shinitzky & Barenholz (1978).

Depletion of Cholesterol from VSV Membrane. Two methods were used to deplete cholesterol from the VSV membrane. In one, serum lipoproteins present in a serum–medium mixture were enriched with various phospholipids as described by Shinitzky (1978) for serum enriched with egg PC. One volume of different phospholipids (2–3 mg/mL) in a 5:1 mixture of tetrahydrofuran (THF) and 0.6% KCl (v/v) was added to 10 volumes of vigorously stirred BME containing 10% fetal calf serum (FCS). The solution after freezing was lyophilized to complete dryness; this cholesterol-depleting medium was prepared just before use by dissolving the lyophilized material in appropriate volumes of sterile distilled water. Control medium, devoid of added phospholipids, consisted of 1 volume of THF mixed with 10 volumes of BME–FCS and was treated as described above.

The second method for depleting cholesterol from VSV used PVP complexed with BSA and coated with phosphatidylcholine (PVP–BSA–PC complex) as described by Shinitzky et al. (1979). The complex was prepared as follows: 3.5% PVP in PBS was dialyzed overnight, and BSA was added to provide a PVP–BSA complex at a concentration of 1%. Egg PC or dioleoylphosphatidylcholine (DOPC) (200–300 μg/mL) in ethanol was introduced into the stirred solution in such a way that the concentration of ethanol in the solution was less than 1%. The same results were obtained if ethanol was completely removed by dialysis. The solution was centrifuged for 10 min at 3000g, and the supernatant was used as a cholesterol-depleting medium. PVP complexed with BSA without lipid constituted the control reaction mixture.

For cholesterol depletion, purified VSV was incubated with BME–FCS–phospholipid or PVP–BSA–PC complexes for different time intervals, and the reaction was terminated by cooling in ice at 4 °C. The VSV thus treated was banded by centrifuging in a 5–40% sucrose gradient at 45 000g for 60 min; this band of VSV appeared to be somewhat broader compared to that of the control VSV. The virus band was collected, and the VSV was pelleted by centrifuging through a glycerol pad at 82 500g for 90 min. The pellet was resuspended in PBS, and aliquots were taken to determine concentrations of protein (Lowry et al., 1951) and cholesterol (Moore et al., 1978). Control reactions consisted of identical incubation of VSV with mixtures of PVP–BSA or BME–FCS devoid of added phospholipids in which virus was repurified as described above.

Cholesterol Exchange Reaction. PVP–albumin complexed with PC and cholesterol in a 1:1 mole ratio was used to determine the cholesterol exchange reaction from the VSV membrane. The complex of PVP–albumin with the lipids was prepared as described above except that the PVP–albumin, egg PC, and cholesterol were complexed in a 1:1 mole ratio (0.267 mM of each).

[^{3}H]Cholesterol-labeled VSV was incubated with cholesterol exchange medium for different time intervals. The reaction

was terminated by cooling the solution at 4 °C, and VSV was banded by centrifuging at 50000*g* in 5–40% sucrose gradients for 60 min in the SW50.1 Ti rotor. The VSV band was collected and then pelleted by centrifuging at 189000*g* for 45 min. The pellet was resuspended in PBS, and aliquots were taken for protein assay (Lowry et al., 1951) and radioactivity measurement.

Repletion of Cholesterol into Cholesterol-Depleted Viral Membrane. Cholesterol-enriching PVP–BSA solution was prepared by adding cholesterol in ethanol to a complexed PVP–BSA mixture so that the concentration of cholesterol in the solution was 260 nmol/mL and the content of ethanol was less than 1%. The solution was centrifuged at 3000*g* for 10 min, and the supernatant was used for cholesterol enrichment. Cholesterol-depleted or undepleted VSV equivalent to 100–150 μg of protein was incubated with 1.5 mL of cholesterol-enriching medium for 10 h. The VSV was banded by centrifuging in a 5–40% sucrose gradient at 50000*g* for 60 min. The band was collected, and the VSV was pelleted by centrifuging at 84000*g* for 90 min through a glycerol pad. The pellet was resuspended in PBS, and aliquots were taken for protein assay and for infectivity by plaque assay (Wagner et al., 1963).

Analysis of the Kinetics of Cholesterol Depletion and Exchange. The method of Bloj & Zilversmit (1976) was adapted to calculate the kinetics of depletion or exchange of radioactive cholesterol (cholesterol*) from the membrane of VSV into the interacting menstruum, the lipid concentration of which could be adjusted to provide conditions for either net depletion of VSV cholesterol or exchange of virion cholesterol without net depletion. This two-pool model, under conditions of reversible equilibrium, can be described as follows:

$$(\text{cholesterol*})_a \underset{k_{ab}}{\overset{k_{ba}}{\rightleftharpoons}} (\text{cholesterol*})_b \xrightarrow{k_o} \text{cholesterol acceptor} \quad (1)$$

where a represents one monolayer of the VSV membrane, b represents the other monolayer, k_{ba} and k_{ab} are the rates of movement of cholesterol between the two leaflets of the bilayer, and k_o is the rate at which cholesterol moves from the VSV bilayer to the interacting acceptor.

The kinetics of cholesterol depletion or exchange can be described by

$$\frac{\text{unexchanged viral cholesterol*}}{\text{total cholesterol*}} = H_1 e^{-g_1 t} + H_2 e^{-g_2 t} \quad (2)$$

where t is time and g_1 and g_2 are the rapid and slow exponential rate constants, which are obtained by plotting the natural log of viral cholesterol* concentration as a function of time (see Figures 4 and 5). These two rate constants (g_1 and g_2) as well as the intercept (c) with the y axis of the line having the slope g_2 are used to calculate k_{ab}, k_{ba}, and k_o. Pool a is assumed to be cholesterol in the inner monolayer, and pool b is assumed to be cholesterol in the outer monolayer of the viral membrane. The size of the two VSV cholesterol pools can be calculated from

$$r_a = \frac{k_{ab}}{k_{ab} + k_{ba}} \quad (3)$$

$$r_b = 1 - r_a \quad (4)$$

The half-life time ($t_{1/2}$) of equilibration of cholesterol between the two pools is calculated as follows:

$$t_{1/2} = \ln 2/(k_{ab} + k_{ba}) \quad (5)$$

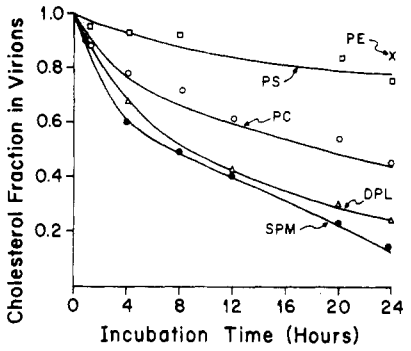

FIGURE 1: Depletion of cholesterol from the membrane of VSV by interaction with BME–FCS enriched with one of the following phospholipids: PC (○), PE (x), PS (□), SPM (●), or DPL (△). Purified VSV (containing 200 μg of protein, 60 nmol of phospholipid, and 340 nmol of cholesterol) was incubated in individual tubes at 37 °C with 2 mL of BME–FCS to which had been added the different phospholipids (of which 79% was the enriched phospholipid and 21% the original phospholipids present in the serum) and 0.06 mol of cholesterol. The mole ratio of cholesterol to phospholipid in the enriched BME–FCS complex was 0.063, lower than the cholesterol/PC ratio in the virus; the amount of phospholipids in the enriched BME–FCS was 31.7 times that in the virus. BME–FCS not enriched with phospholipids served as controls. The reaction was terminated at various times after incubation by cooling in an ice–water bath. The virions were separated from the serum lipoproteins by density centrifugation, and the cholesterol concentration was measured as described under Materials and Methods.

Results

Lipid Composition of Fetal Calf Serum (FCS). The concentrations of cholesterol and phospholipids in the FCS used in these experiments were determined as described under Materials and Methods. The FCS contained 0.29 mM cholesterol and 1 mM total phospholipids; the cholesterol to phospholipid mole ratio was therefore 0.29. Sphingomyelin accounted for about 30% of the total phospholipids; the remainder was mainly phosphatidylcholine. After enrichment of the serum with one of the phospholipids (described in Figure 1), the concentration of the phospholipids was increased to 4.8 mM, and, therefore, the cholesterol to phospholipid mole ratio was reduced to about 0.06. In most experiments, the serum was diluted 5-fold with BME, the final concentration of cholesterol was 0.06 mM, and that of the phospholipids was 0.95 mM.

Lipoprotein Depletion of Cholesterol from the VS Virion Membranes by Phospholipid-Enriched FCS Lipoproteins. Previous attempts to study depletion of cholesterol from the membrane of VSV with phospholipid vesicles resulted in considerable nonspecific adherence of the lipids to G spikes of the virus (Moore et al., 1978; Patzer et al., 1978b). We describe here some alternative ways to deplete cholesterol from membranes of intact virus where the adherence of the exogenous lipids could be reduced to minimal levels. To this end, we used fetal calf serum lipoproteins as a carrier of phospholipids for depleting cholesterol from the virion membrane, as described under Materials and Methods. Nonspecific adherence of serum lipids and protein to virions following incubation with a phospholipid-enriched serum was determined with ^{125}I-labeled serum proteins, ^{125}I-labeled HDL, or ^{125}I-labeled LDL (kindly supplied by Dr. Karen Kuehl). No radioactivity could be detected in repurified virus following prolonged incubation (>24 h) with medium containing ^{125}I-labeled serum proteins or ^{125}I-labeled lipoproteins. This was also confirmed by electron microscopic examination, which revealed perfectly normal virus with clear, protruding glycoprotein spikes even after prolonged exposure to lipoproteins.

Table I: Kinetic Parameters of Chlolesterol Depletion from VS Virions Using BME–FCS Enriched with Various Phospholipids[a]

serum-enriched phospholipid	fraction depleted after 24 h	t_{50}[b] (h)	rate constants (h^{-1})			$t_{1/2}$ (h)	% pool sizes[c]	
			k_o	k_{ba}	k_{ab}		pool a	pool b
control	0				0			
SPM	0.86	7.6	0.08	0.08	0.01	7.35	15.1	84.9
DPPC	0.76	8.3	0.07	0.064	0.01	9.36	13.6	86.6
egg PC	0.56	17.4	0.04	0.045	0.02	11.66	24.0	76.0
PS[d]	0.23	66.35						
PE[d]	0.15							

[a] See text and legend for Figure 1 for experimental details. [b] t_{50} = the time in which the level of cholesterol is reduced to 50%; it represents mostly a transfer of cholesterol. [c] Expected value of cholesterol level in the virions at equilibrium is 7% of its original value. [d] Kinetic parameters cannot be calculated since only a single pool was observed during 24 h.

Moreover, gel electrophoretic patterns of the viral proteins did not reveal the presence of any exogenous proteins associated with VSV preparations incubated with ^{125}I-labeled lipoprotein–phospholipid complexes.

Figure 1 and Table I illustrate the extent and the rate of depletion of cholesterol from the membrane of VSV following incubation with BME–FCS enriched with different phospholipids. These results suggest the possibility of preferential interaction of various lipids with cholesterol, which may influence the kinetics of depletion. It is evident that the amount and rate of cholesterol depletion were strongly influenced by the kind of phospholipid used in the reaction mixture; there was no change in VSV cholesterol level with control BME–FCS not enriched with phospholipid (data not shown). SPM served as the best acceptor for virion cholesterol, closely followed by DPL, whereas egg PC was a less efficient cholesterol depletor and PS and PE were relatively ineffective. Reaction of VS virions with BME/FCS resulted in only 20% exchange of phospholipids in 24 h as measured by loss from virions of [^{3}H]palmitate-labeled PC and SPM (data not shown).

A logarithmic plot of these data revealed two-phase kinetics for depletion of virion cholesterol by BME–FCS enriched with SPM, DPL, or egg PC but only a single, slow exponential rate for depletion of virion cholesterol by PS and PE (data not shown). The comparative efficiencies for virion cholesterol depletion by each phospholipid were determined according to the formula used by Bloj & Zilversmit (1976) as described under Materials and Methods where k_o = cholesterol movement to the enriching phospholipid acceptor, k_{ba} = rate of cholesterol movement from its small pool to its large pool, k_{ab} = rate of cholesterol movement from its large pool to its small pool, and $t_{1/2}$ = the half-time for cholesterol equilibration between the two pools as well as the distribution of cholesterol between these two pools. Table I summarizes the data calculated from the experimental results of virion cholesterol depletion shown in Figure 1. Since identical amounts of each phospholipid were used with BME–FCS for virion cholesterol depletion, the k_o values represent the capacity of each phospholipid to serve as a cholesterol acceptor. It is clear from these data that virion cholesterol is asymmetrically distributed between the two membrane pools regardless of whether SPM, DPL, or egg PC was used in the depletion mixture; no calculations for cholesterol distribution could be made for PS and PE. Also shown in Table I is the comparative efficiency of each phospholipid in fractional cholesterol depleted in 24 h and the t_{50} reduction in virion cholesterol in rank order SPM > DPL > egg PC > PS > PE. These data also permit calculation of two pool sizes for virion membrane cholesterol, in which the large pool (b) contains 76–85% and the small pool (a) contains 15–24% of the cholesterol. Depletion of viral cholesterol also affected the buoyant density of the virions, which was increased from a control level of 1.167 g/mL after incubation with BME/FCS to 1.176 g/mL after incubation with BME/FCS/SPM, which resulted in >80% depletion of cholesterol; this increase in buoyant density was due to the decrease in the lipid-to-protein ratio from 0.303 to 0.248 g/g (data not shown).

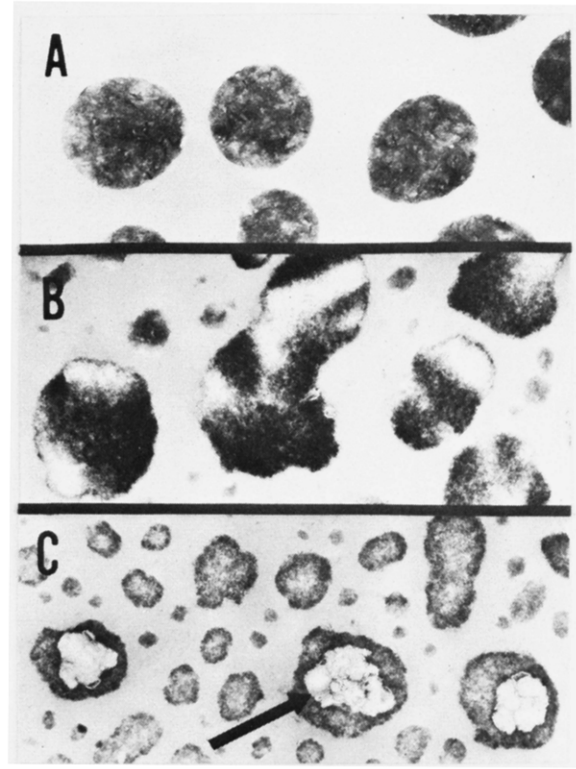

FIGURE 2: Negative-stain electron microscopy of beads consisting of (A) PVP alone, (B) PVP–BSA complex, and (C) PVP–BSA–PC complex. Suspensions of 3–5% PVP in PBS were either left untreated or mixed with 1% lipid-free BSA. Egg PC in ethanol was added to a portion of the clear suspension of complexed PVP–BSA to provide a final concentration of 300 μg/mL PC and <1% ethanol. Each suspension was further clarified by centrifugation at 3000*g* for 10 min. A drop of each supernatant suspension was examined with a Siemens 1A electron microscope (×31 500). The arrow indicates a multilamellar vesicle.

Cholesterol Depletion by PVP–BSA–PC Complexes. In an attempt to increase the extent of cholesterol depletion, a new method involving a PVP–BSA "complex" coated with egg PC as a lipid carrier was used. Unfortunately, SPM, DPPC, PS, or PE did not exhibit any significant capacity to form an active complex with PVP–albumin since they precipitated when added to PVP–BSA mixtures. However, egg PC or DOPC complexed quite well. The reason for the different behavior of the various phospholipids is not yet clear and requires further investigation.

Figure 2 illustrates the types of complexes formed when PVP interacts with BSA which, in turn, is coated with PC. Electron

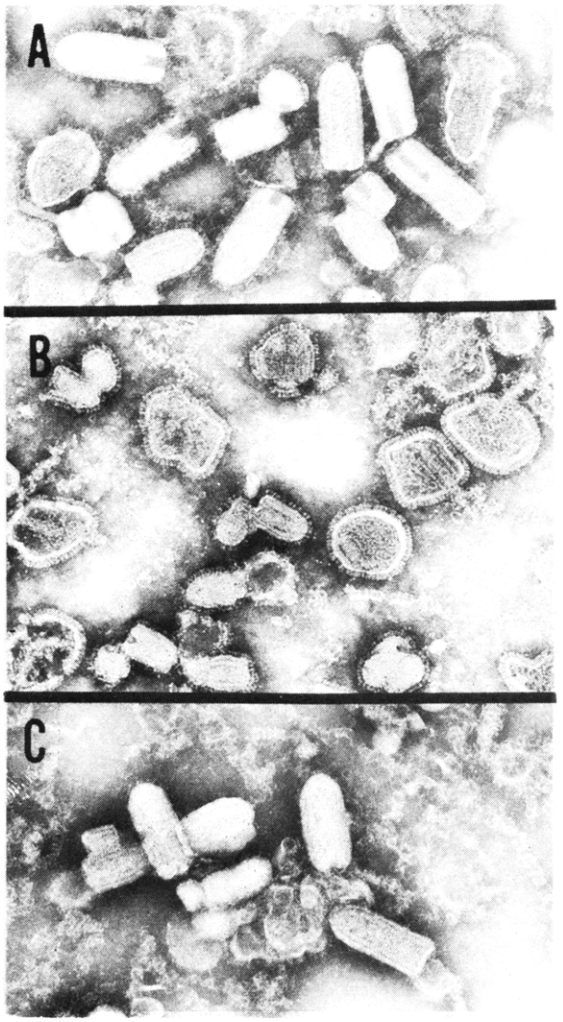

FIGURE 3: Negative-stain electron microscopy of VSV after interaction with (A) PVP–BSA without lipid (control), (B) PVP–BSA–PC complex (cholesterol depleted), and (C) PVP–BSA–PC–cholesterol (cholesterol exchanged). As described under Materials and Methods, PVP was complexed with BSA alone, with BSA and egg PC (250 μg/mL), or with BSA and 300 μg of egg PC + cholesterol (1:1 molar ratio). Purified VSV (200 μg) was incubated for 18 h at 37 °C with 2 mL of each PVP/BSA complex, after which the reaction was terminated by cooling in an ice–water bath. The virions were then separated from the beads by density centrifugation and examined by electron microscopy as described in the legend for Figure 2 (×81 000).

micrographs of negatively stained PVP dispersions show symmetrical bead particles upon interaction with BSA (Figure 2B); these beads became smaller and less symmetrical when a lipid was added (Figure 2C). It is not known if the beads observed in the micrographs are an artifact of these preparations for electron microscopy since large particles were not expected to form for this PVP in suspension. The lipid was found to complex with PVP–BSA, either in the form of multilamellar vesicles or in a dispersed form (Figure 2C). Moreover, no lipid vesicles were detected free in suspensions unattached to PVP–BSA complexes. The possibility of electron microscopy artifacts is also suggested by the fact that this complex of PVP–BSA–PC was not sedimentable when centrifuged for 90 min at 189 000g in the SW50.1 rotor, and it could pass through a filter of pore size 0.22-μm diameter (FG LPO Millipore Teflon filters).

Experiments were undertaken to determine whether the protein or lipid components in the PVP–BSA complex showed any nonspecific adherence to the virus following incubation. For this purpose, ^{125}I-labeled BSA and [^{14}C]cholesterol oleate

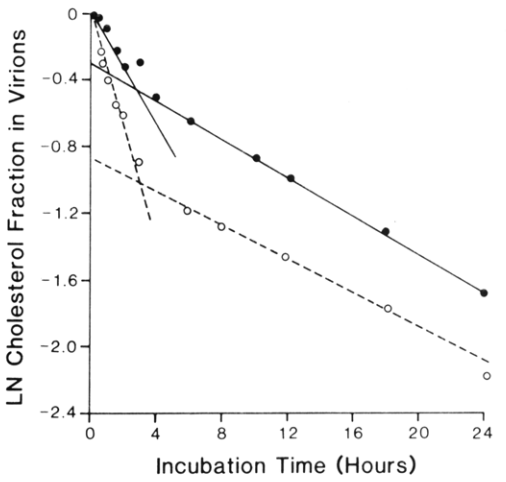

FIGURE 4: Depletion of cholesterol from the VSV membrane by interacting virions with PVP–BSA–egg PC complex (●—●) or exchange of cholesterol from the membrane of VSV by interacting the virions with PVP–BSA–PC–cholesterol complex (O---O). In the cholesterol depletion reaction, purified VS virions (750 μg of protein, 225 nmol of phospholipid, and 150 nmol of cholesterol) were incubated with a PVP–BSA–PC complex (400 nmol of egg PC) at 37 °C for varying time intervals. The total amount of phospholipids in the PVP–BSA–PC complex was 7.1 times that in the virions. For the cholesterol exchange reaction, [^{3}H]cholesterol-labeled VSV (150 μg of protein, 45 nmol of phospholipids, and 30 nmol of cholesterol ^{3}H labeled with 2.79 × 10^6 dpm/μmol) was incubated at 37 °C in individual tubes containing 2 mL of PVP–BSA–PC–cholesterol complex (1:1 molar ratio of PC/cholesterol, 25 nmol/mL of each). The depletion and exchange reactions were both terminated by cooling at 4 °C, and the virions separated by density centrifugation were assayed for protein concentration (Lowry et al., 1951). In the exchange reaction, cholesterol in the virions was assayed after disruption with 0.3% taurodeoxycholate by the method of Moore et al. (1977). In the exchange reaction, radioactive cholesterol in virions was assayed by scintillation spectrometry. In each case, the data are plotted as the natural log of the cholesterol fraction remaining in the virions as a function of time. See Materials and Methods for the method of calculating g_1 and g_2 constants.

were used as nonexchangeable markers. While no ^{125}I-labeled BSA was found in association with the virus following incubation for long periods of time, some radioactivity (~2%) was detected when the virus was incubated for 16 h with [^{14}C]-cholesterol oleate present in the PVP–BSA–lipid complex (but this also may be due to minimal transfer).

Figure 3 shows representative electron microscopic pictures of VSV incubated with PVP–BSA without lipid (Figure 3A, control), VSV incubated with PVP–BSA–PC complex (Figure 3B), and VSV incubated in the cholesterol-exchange reaction with PVP–BSA–PC–cholesterol (Figure 3C). The virus depleted of cholesterol by interaction with the PVP–BSA–PC complex obviously underwent alteration in shape from the typical bulletlike structure to round particles whereas control and cholesterol-exchanged VSV retained their classical bullet shape. However, clearly visible on the surface of cholesterol-depleted, round VSV particles are glycoprotein spikes free of lipids and PVP beads (Figure 3B). In fact, the spikes are more clearly visible in round VSV particles, unlike the obscured spikes on VSV after interaction with lipid vesicles [see Figure 2 in Moore et al. (1978)].

Figure 4 shows the kinetics of depletion of cholesterol from the viral membrane after interaction with the PVP–BSA–egg PC complex. Similar results were obtained by using PVP–BSA–DOPC complexes (data not shown). Based on the kinetic analysis devised by Bloj & Zilversmit (1976), this depletion pattern reveals the presence of two different pools of cholesterol in the VS virion (see Materials and Methods). By

this means, it is possible to determine the kinetic parameters of the process as well as the distribution of viral cholesterol between its two pools. All kinetic parameters are given in Table II. It is clear that the virus lost 81.8% of its original cholesterol after incubation for 24 h with PVP–albumin–egg PC. Since the ratio of phospholipids in this cholesterol-depleting complex to that of the viral phospholipids is 7.1, and assuming a simple nonpreferential cholesterol distribution, it is very close to the theoretical value of 14% of the original cholesterol remaining with the virions. The data suggest that cholesterol is distributed in two pools, the large one containing about 82% of the total virion cholesterol. This result thus supports the observation of Patzer et al. (1978), who also observed similar biphasic kinetics for depletion of cholesterol from the VSV membrane by interaction with phospholipid vesicles.

This biphasic reaction is apparently not due to increasing membrane permeability. It had been clearly demonstrated earlier that the membrane of intact VSV is not permeable to nucleoside triphosphates; the nucleocapsid transcriptase of the virus exhibits enzymatic activity only when the virion membrane is totally disrupted with detergents (Emerson & Wagner, 1972; Hunt & Wagner, 1975). To test the membrane integrity of VSV distorted to a round shape following interaction of virions with complexed PVP–BSA–PC, we performed transcription reactions to determine whether the membrane of the round virions had been made permeable to nucelosides following depletion of cholesterol with PVP–BSA–PC (see Figure 3B). No transcriptional activity could be detected, although the same rounded VSV particles contained a fully active transcriptase, demonstrated when the virions were subsequently disrupted by Triton X-100 (data not shown).

Exchange of Cholesterol between VSV and a PVP–BSA–PC–Cholesterol Complex. The foregoing cholesterol depletion experiments resulted in >80% removal of cholesterol from the membrane of virions but also altered the morphology of the virus. To provide further information on the membrane distribution and transbilayer movement of cholesterol, a somewhat less perturbing method was devised to measure the exchange of cholesterol between virions and the cholesterol of a PVP–BSA–PC–cholesterol complex. For this purpose, VSV was labeled with [^{3}H]cholesterol by growing the host BHK cells and the virus in the presence of [^{3}H]mevalonic acid (98% pure); >93% of the ^{3}H radioactivity was present in virion cholesterol as determined by thin-layer chromatography. Cholesterol in combination with PC at a molar ratio of 1:1 was complexed with PVP–BSA as described under Materials and Methods. The appearance of this complex, when examined by electron microscopy, was very similar to that of the PVP–BSA–PC complex (see Figure 2). The morphology of the virus was found to be unaffected following interaction of VSV with the PVP–BSA–PC–cholesterol complex (see Figure 3C).

Figure 4 portrays the kinetics of exchange of [^{3}H]cholesterol from the virus following interaction with the PVP–BSA–PC–cholesterol complex. As was the case with cholesterol depletion kinetics, the cholesterol exchange data analyzed as described under Materials and Methods were consistent with virion cholesterol distributed in two membrane pools, the larger one containing 88% of the cholesterol (3–4 h). The kinetic parameters are given in Table II. Limited phospholipid was found to be exchanged from virus undergoing 85% cholesterol exchange by interaction with PVP–BSA–PC–cholesterol; only 20% of virion PC and SPM labeled with [^{3}H]palmitate was transferred by the acceptor during a 24-h reaction period.

Table II: Kinetic Parameters of Cholesterol Depletion or Cholesterol Exchange from VS Virions Using PVP–BSA Complexed with Lipids[a]

	fraction of [^{3}H]cholesterol removed from VSV in 24 h	t_{50}[b] (h)	rate constants (h^{-1})			$t_{1/2}$[d] (h)	% pool sizes		ratio of phospholipid in acceptor to phospholipid in virus	expected value of exchange at equilibrium (%)
			k_o	k_{ba}	k_{ab}		pool a	pool b		
PVP-BSA (control)	0–0.02									
PVP–BSA–egg PC	0.82	4.3	0.11	0.08	0.03	6.28	22.8	77.2	7.1	86.0
PVP–BSA–egg PC–cholesterol	0.88	20	0.26	0.06	0.01	9.02	16.3	83.7	11.5	93.5
PVP–BSA–egg PC–cholesterol with virions enriched with cholesterol	0.84	6.3	0.17	0.09	0.05	4.96	35.6	64.4		
PC–cholesterol small unilamellar vesicles[c]	0.91	3.5	0.26	10.11	0.01	4.07	32.5	67.5	17.8	91.0

[a] See text and Figures 4 and 5 for details. [b] t_{50} = the time at which the VSV level of [^{3}H]cholesterol or [^{3}H]- and [^{14}C]cholesterol in the cholesterol-enriched virions was reduced by 50%. This represents a net cholesterol transfer in the depletion experiment and exchange of radioactive cholesterol in the virus with the nonradioactive cholesterol of the PVP–BSA–PC–cholesterol complex. [c] Data from Patzer et al. (1978b). [d] $t_{1/2}$ = the half-lifetime of equilibration of the labeled cholesterol between its two pools.

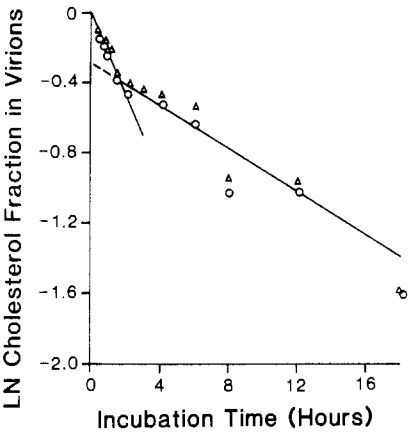

FIGURE 5: Exchange of endogenous [^{3}H]cholesterol (Δ) and exoogenous [^{14}C]cholesterol (O) from the membrane of cholesterol-enriched VSV by interaction with PVP–BSA–PC–cholesterol. Purified [^{3}H]cholesterol-labeled VSV (2.79×10^6 dpm/μmol, containing 1.2 mg of viral proteins, 0.4 μmol of viral phospholipids, and 0.27 μmol of viral cholesterol) was incubated in 15 mL of BME–FCS enriched with [^{14}C]cholesterol. The enriching medium contained 0.58 μmol/mL cholesterol (2.34×10^6 dpm/μmol). The cholesterol to phospholipid mole ratio was 2.9, and the total cholesterol in the medium was 8.7 μmol. The enrichment reaction was stopped after 3 h by cooling to 4 °C; the virions were then separated from the serum lipoproteins by centifugation at 45 000*g* for 60 min in a continuous gradient of 5–40% sucrose. The virions banded near the middle of the gradient had a cholesterol concentration of 245 nmol/mg protein compared with 200 nmol/mg protein in unenriched virions; therefore, the cholesterol to phospholipids mole ratio increased from 0.67 in the untreated virions to 0.816 in the enriched virions. A 150-μg sample of this doubly labeled virus (45 nmol of phospholipids and 30 nmol of cholesterol) was incubated with 2 mL of PVP–BSA–egg PC–cholesterol complex (egg PC–cholesterol mole ratio of 1:1.5), and the exchange of the endogenous [^{3}H]cholesterol and exogenous [^{14}C]-cholesterol was measured as described in the legend to Figure 4.

We have also attempted to exchange cholesterol between the VSV membrane and serum lipoprotein by using BME–FCS–PC–cholesterol as an acceptor. Results were similar to those with the PVP–BSA–egg PC–cholesterol complex (data not shown).

Finally, we also studied the cholesterol exchange reaction with cholesterol-enriched VS virions, the membranes of which had been endogenously labeled with [^{3}H]cholesterol and enriched with [^{14}C]cholesterol by interaction with BME–FCS–[^{14}C]cholesterol. This pretreatment of VSV increased its molar ratio of cholesterol to phospholipid from 0.67 in the untreated virus to 0.816 in the cholesterol-enriched virus. These doubly labeled, cholesterol-enriched virions were incubated with the PVP–BSA–PC–cholesterol complex, and the rate of exchange of both [^{3}H]cholesterol and [^{14}C]cholesterol was measured over a period of 24 h.

Figure 5 illustrates the rate at which both [^{3}H]cholesterol and [^{14}C]cholesterol are exchanged from the VSV membrane; the kinetic parameters of the cholesterol exchange reaction are summarized in Table II. Clearly, both the endogenous [^{3}H]cholesterol and the exogenous [^{14}C]cholesterol are exchanged from virions at the same rate throughout the duration of the reaction. These data indicate that the endogenous and exogenous cholesterols are randomly mixed and distributed between both cholesterol pools of the VSV membrane bilayer. Apparently, even exogenous cholesterol in the enriched virions is still asymmetrically distributed with ~65% in the large pool and 35% in the small pool.

Effect of Cholesterol Depletion on Microviscosity of the VSV Membrane. The ratio of cholesterol to phospholipid in biological membranes appears to determine many important properties, including the fluidity of the membrane (Cooper,

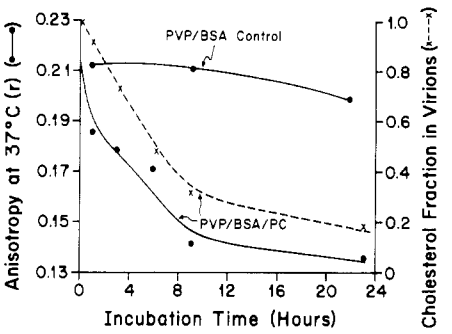

FIGURE 6: Correlation between depletion of VSV cholesterol and membrane microviscosity determined by depolarization of the hydrophobic fluorescent probe diphenylhexatriene. VSV was depleted of cholesterol by interaction for 23 h with PVP–BSA–PC complex as described in the legend for Figure 4. Virions collected at each interval were purified and analyzed for cholesterol concentration by the cholesterol oxidase method after disruption of membranes with 0.3% taurodeoxycholate. As described under Materials and Methods and in Barenholz et al. (1976), DPH was incorporated into the membranes of cholesterol-depleted virions as well as undepleted control virions exposed to PVP–BSA in the absence of PC, and anisotropy was measured at 37 °C.

1977). The role of cholesterol in the microviscosity of the VSV membrane had been examined by Moore et al. (1978), who compared fluorescence depolarization of DPH inserted into native virus and virus depleted of cholesterol following interaction with PC vesicles. However, in those experiments, the authors faced the problem of discriminating between the signals coming from the DPH probe inserted into the viral membrane and the signals from DPH which partitioned into residual adherent vesicles. The problem of lipid adherence was not completely obviated when this study was conducted with spikeless virions.

Therefore, experiments were undertaken here to determine whether the depletion of cholesterol from the viral membrane by the nonadherent PVP–BSA–PC complex could be correlated with changes in apparent microviscosity of the viral membrane. For this purpose, the fluorophore DPH (Shinitzky & Barenholz, 1978) was inserted into the lipid bilayer of the virion, as described elsewhere (Barenholz et al., 1976) and briefly under Materials and Methods. After equilibration at optimal fluorescence intensity, anisotropy was measured at 37 °C.

Figures 6 illustrates that a very good correlation could be established between the cholesterol content of VSV and the anisotropy of the membrane. The initial rapid bphase of depletion was accompanied by a sharp drop in anisotropy, which appeared to level off in parallel to the onset of the slow phase of depletion of the remaining viral cholesterol. The drop in anisotropy from 0.213 in the untreated virus to 0.136 in the virus after 23 h of cholesterol depletion is coincident with a drop of the apparent microviscosity from 3.43 to 1.44. Also, as noted, no real difference in anisotropy was observed in control VSV incubated for 22 h with PVP–BSA devoid of PC.

Effect of Cholesterol Depletion on Infectivity of the Virus. In previous studies, it was reported that the interaction of VSV with lipid vesicles led to a significant drop in the infectivity of the virus; this was attributed partly to the depletion of cholesterol from the membrane but was also due to the adherence of lipid vesicles to glycoprotein spikes of the virions (Moore et al., 1978). It was of interest, therefore, to determine whether depletion of cholesterol from VSV by the nonadherent PVP–BSA–PC complex affects the infectivity of the virus. For this purpose, VSV was incubated with PVP–BSA, PVP–BSA–cholesterol, PVP–BSA–PC–cholesterol, and PVP–

Table III: Comparative Infectivity of VS Virus after Cholesterol Depletion, Exchange, Enrichment, and Repletion after Depletion of Cholesterol in Virions Reacted for 16 h with PVP-BSA Complexes[a]

expt no.	treatment	lipid composition of PVP-BSA reaction mixture	16-h ratio of chol/protein (nmol/mg)	VS virus infectivity	
				pfu/mL	% of control
1	control	no lipid	186	3.75×10^9	
	exchange	0.266 mM PC + 0.266 mM chol	168	3.25×10^9	87
	depletion	0.4 mM PC	30	3.40×10^8	9
2	control	no lipid	181	2.50×10^9	
	enrichment	0.266 mM chol	460	2.05×10^9	82
	depletion	0.4 mM PC	25	5.02×10^7	2
3	control	no lipid		2.10×10^8	
	depletion	0.4 mM PC		3.50×10^6	1.7
	depletion followed by repletion	0.4 mM PC + 0.52 mM chol		1.50×10^7	7.1
4	control	no lipid	201	3.50×10^8	
	depletion	0.4 mM PC	25	1.25×10^6	0.3
	depletion followed by repletion	0.4 mM PC + 0.52 mM chol	323	1.20×10^7	3.4

[a] For depletion of cholesterol, purified VSV (700 μg of protein, 210 nmol of phospholipid, and 140 nmol of cholesterol) was incubated with 4 mL of PVP-BSA-PC complex (0.4 mM PC) for 16 h at 37 °C. For exchange of cholesterol, purified VSV (200 μg of protein, 60 nmol of phospholipids, and 40 nmol of cholesterol) was incubated with 2 mL of PVP-BSA-PC-cholesterol complex (1:1 molar ratio of PC cholesterol, 0.266 nmol of each) for 16 h at 37 °C. For cholesterol enrichment, the same amount of VSV was incubated with 2 mL of PVP-BSA-cholesterol complex (0.266 mM cholesterol). Controls consisted of VSV incubated with PVP-BSA alone at 37 °C for 16 h. Cholesterol-enriching PVP-BSA solution was prepared by adding cholesterol in ethanol to PVP-BSA mixtures so that the concentration of cholesterol in the solution was 0.260 mM and the content of ethanol was less than 1%. The solution was centrifuged at 3000*g* for 10 min, and the supernatant was used for cholesterol enrichment. A 2-mL aliquot of the latter was used for the repletion experiment. Each reaction was terminated by cooling the solution at 4 °C, and the virions were separated from the PVP beads and lipids by centrifuging at 50 000*g* for 60 min. The PVP-BSA-lipid complex banded at the top of the gradient while the virions banded near the middle of the tube. The visible virus band was collected and resuspended in PBS and centrifuged at 82 000*g* for 90 min. The pellet was resuspended in PBS, and aliquots were analyzed for protein (Lowry et al., 1951) and for infectivity by plaque assay (Wagner et al., 1963). In experiments 3 and 4, VSV depleted of cholesterol for 10 h was repleted with cholesterol by incubation for 10 h with PVP-BSA-cholesterol and compared with appropriate controls.

BSA–PC for 16 h at 37 °C; the virus thus treated was repurified by density gradient centrifugation and assayed for infectivity by plating on L-cell monolayers.

Plaque assay data presented in Table III demonstrate that incubation of VSV with PVP–BSA–cholesterol and PVP–BSA–PC–cholesterol had little effect on the infectivity of virus undergoing cholesterol exchange but no net loss in VSV membrane cholesterol. However, when cholesterol was depleted to an extent of ~70% following incubation with the PVP–BSA–PC complex, infectivity was found to decrease significantly compared to that in the control group treated similarly with PVP–BSA alone. In two separate experiments, the infectivity of cholesterol-depleted VSV declined by 91% and 98%.

For further establishment of the role of membrane cholesterol in the infectivity of the virus, attempts were made to reincorporate cholesterol back into the depleted VSV membrane. In this experiment, VSV that had been 70% depleted of cholesterol by interaction with PVP–BSA–PC complexes was divided into two equal portions; one aliquot was treated with PVP–BSA–cholesterol complex for 10 h at 37 °C while the other was similarly incubated with PVP–BSA alone. Another control group consisted of virus incubated with PVP–BSA alone throughout the two periods of "depletion" and "repletion". As shown by experiments 3 and 4 in Table III, infectivity of cholesterol-depleted virus could be restored significantly (4–11-fold) following reincorporation of cholesterol into the depleted virions. However, it was not possible to restore the infectivity to the original control level. The morphology of virus repleted with cholesterol resembled that of depleted virus (see Figure 3B) with a predominance of round-shaped structures and some broken particles.

Discussion

Although a number of studies have established the fact that cholesterol can affect the packing of acyl chains of membrane phospholipids and that cholesterol plays a key role in determining membrane fluidity (Chapman, 1973), very little is known about the distribution of cholesterol in biological membranes and its interaction with phospholipids. This hiatus in knowledge is mainly due to the relative chemical nonreactivity of the cholesterol molecule, thus precluding the experimental approaches which have made possible the elucidation of the distribution of phospholipids in both natural and model membranes. In recent years, attempts have been made to answer such questions by studying the kinetics of depletion or exchange of cholesterol from one membrane to another membrane or other acceptors. This technique is best represented by the approach suggested by Bloj & Zilversmit (1976) for PC exchange since it described a quantitative approach to the problem while most other approaches are mainly descriptive. Using these techniques, it has been demonstrated that cholesterol in synthetic liposomes, erythrocyte membrane, and influenza viral membrane is apparently present in two kinetic pools, presumably the inner and outer monolayers; the inner monolayer cholesterol was assumed to be slowly exchangeable or nonexchangeable (Poznansky & Lange, 1976; Gottlieb, 1976; Lenard & Rothman, 1976). In sharp contrast to these observations, it has been demonstrated that cholesterol in liposomes is completely exchangeable as one pool (Bloj & Zilversmit, 1977) whereas erythrocyte and VSV membranes contain two pools of cholesterol exchangeable at different rates (Lange et al., 1977; Patzer et al., 1978b). These previous results from our laboratory on the asymmetrical distribution of cholesterol in the VSV membrane had been erroneously reported as ~30% present in the outer monolayer (Patzer et al., 1978b); recalculation of these data reveals ~70% cholesterol in the outer monolayer and ~30% in the inner layer. Moreover, these data also suggested that the two VSV cholesterol pools are mobile, and the transmembrane movement of cholesterol is on the order of 4–6 h (Patzer et al., 1978b).

Our study on the depletion of cholesterol by lipoproteins in serum enriched with phospholipids showed that beef brain SPM and DPL were more effective in depleting cholesterol from the membrane of VSV than were egg PC, egg PE, or beef brain PS. Only with FCS enriched with SPM, which gave

the fastest depletion rate, was equilibration of cholesterol reached by 24 h. It is of interest that serum enriched with aminophospholipids is a very inefficient cholesterol depletor, and the time to reach 50% depletion (t_{50}) with them is 10-fold slower than with SPM. Similar results had been previously obtained for depletion of cholesterol from cells by using a dilipidated serum reconstituted with various phospholipids (Burns & Rothblatt, 1969). This marked effect on depletion of cholesterol by SPM and DPPC suggests the possibility that these phospholipids may have preferential affinity for cholesterol compared to that of egg PC or PE. Recent studies with differential scanning calorimetry have led to the suggestion that, in mixtures of phospholipids which show phase separation, cholesterol interacts preferentially with certain phospholipids (DeKruijff et al., 1974; Demel et al., 1977; Van Dijck et al., 1976). One such study showed that cholesterol has a preferential affinity for SPM in a mixture of SPM and egg PC where SPM was the phospholipid with the higher melting point. By measuring the uptake of cholesterol by vesicles from erythrocyte ghosts, it has been shown recently that the affinity of cholesterol for DPPC is the same as that of SPM, both above or below the phase transition. This affinity was always more than that of egg PC (Lange et al., 1979). The significance of such preferential interactions in bilayers between various phospholipids and cholesterol remains to be determined.

On the basis of both cholesterol-depletion and -exchange reactions with various acceptors, the kinetic analysis suggested by Bloj & Zilversmit (1976) allows one to conclude that the cholesterol in VSV membranes is present in two pools. The larger pool (pool b, see Tables I and II), which is apparently located in the outer monolayer of the viral membrane, contains 75–85% of the total VSV cholesterol; the smaller pool (pool a), which presumably resides in the inner monolayer, contains 15–25% of the total viral cholesterol. The half-time for equilibration of cholesterol between these two pools ($t_{1/2}$) is ~9 h for exchange conditions and 6–11 h under conditions of cholesterol depletion, depending on the system used for cholesterol depletion. Since ~55% of the VSV phospholipids are present in the outer monolayer (Patzer et al., 1978a) and ~80% of the cholesterol (which represents ~40 mol % of the membrane lipids) is also present in the outer monolayer of the membrane, the mole ratio of cholesterol to phospholipids is 1.0 in the outer monolayer but only 0.3 in the inner monolayer. Upon enrichment to a cholesterol-to-phospholipid mole ratio of 0.82 (from 0.67 in untreated virus), the asymmetric distributions of cholesterol are reduced to 65% in the outer monolayer and 35% in the inner monolayer; most of the added cholesterol, although not necessarily the same exogenous molecules, seems to wind up in the inner monolayer.

A number of biological properties of a virus may be affected following depletion of cholesterol from its envelope. One such important property is the infectivity of the virus. The results presented here demonstrate that the depletion of cholesterol from the membrane affects the infectivity of the virus to a significant extent. Indeed, no such effect was noted when VSV membrane cholesterol was either exchanged or enriched. Similar loss in infectivity was also observed when vesicles were interacted with virus to deplete cholesterol from the membrane (Moore et al., 1978); however, part of the loss in infectivity in those studies could be attributed to vesicles adhering to the glycoprotein spikes. In the present study, interaction of VSV with the PVP–BSA–PC complex resulted in alteration of the morphology of the virus, although the glycoprotein spikes remained free of any adhering beads or lipids. However, the envelope was found to be impermeable to nucleosides as is the case with normal bullet-shaped virus. The most convincing evidence regarding the influence of membrane cholesterol on the infectivity of the virus was the fact that a significant portion of the infectivity lost following depletion of membrane cholesterol could be restored by reincorporation of cholesterol into the membrane.

Initiation of the infection by VSV can be divided into two phases. The glycoprotein spikes play a key role in promoting attachment of virus to cell surface. Whether penetration of the virus into the cell occurs by virus membrane fusion at the cell surface or by phagocytosis has been the subject of controversy. However, in all likelihood, both phagocytosis and fusion of viral membrane with the surface or internalized plasma membrane take place, eventually leading to release of nucleocapsids and productive infection. The results presented here suggest the possibility that the penetration of the virus and release of its nucleocapsid by fusion with the cell membrane may be a direct function of membrane cholesterol content. Recent studies have demonstrated that vertical displacement of membrane proteins is affected by the microviscosity of the membrane (Borochov & Shinitzky, 1976; Borochov et al., 1979; Shinitzky & Rivnay, 1977). Also, cholesterol seems to have a dramatic effect on lateral organization of lipids and proteins in the plain of membranes (Rintoul et al., 1979; Cherry et al., 1980). Therefore, depletion of cholesterol from the VSV membrane may affect the lateral distribution of glycoprotein (as well as its exposure), which in turn may affect the attachment of virus to cell surfaces. In this context, it should be noted that interaction of filipin with cholesterol in VSV membrane resulted in marked morphological alteration accompanied by a dramatic loss in the infectivity of virus (Majuk et al., 1977). A clear answer to this problem may be achieved by adopting more biological ways to produce virions with a low level of membrane cholesterol. Indeed, another approach we are taking is to grow VSV in mutant cell lines defective in cholesterol synthesis; these data indicate that a low cholesterol level in the membrane of such viruses results in appreciable loss of infectivity (Pal et al., 1980).

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