Complete Exchange of Viral Cholesterol†

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ABSTRACT: The exchange of the cholesterol in the membranes of two enveloped viruses, Sindbis virus and vesicular stomatitis virus, with cholesterol present in lipid vesicles and in serum was measured. Biosynthetically labeled viral cholesterol underwent spontaneous and complete transfer to both lipid vesicles and to serum. The rate with which and the extent to which this process occurred were very similar for these two viruses. During incubation with lipid vesicles in excess, half of the viral cholesterol underwent transfer in approximately 4 h and more than 90% underwent transfer in 24 h at 37 °C. Similar rates and extents of movement of viral cholesterol were observed when incubations were carried out with vesicles which contained cholesterol and phospholipid in the same molar ratio as in the virus or with egg lecithin vesicles which contained no cholesterol. When labeled cholesterol was present initially in the lipid vesicles, movement of cholesterol from the vesicles to the virus was observed. One implication of the fact that viral cholesterol undergoes extensive exchange with serum cholesterol is that cellular cholesterol is in equilibrium with that in the extracellular fluid.

Cholesterol is a major component of the surface membranes of animal cells and viruses. It has been known for years that membrane cholesterol can undergo spontaneous exchange with exogeneous cholesterol present in lipoproteins or in lipid vesicles (Hagerman & Gould, 1951; Bruckdorfer et al., 1968). This phenomenon has been used to measure both the distribution of cholesterol between the two leaflets of the membrane bilayer and the rate of movement of cholesterol from one face of the membrane to the other. Conflicting results have been obtained.

In several studies it was found that a significant portion of membrane cholesterol would not undergo exchange (Poznansky & Lange, 1976, 1978; Lenard & Rothman, 1976). This result was interpreted to mean that cholesterol is present in both leaflets of the bilayer and that unexchangeable cholesterol does not undergo exchange because it is present in the inner leaflet of the membrane and cannot undergo inside–outside transpositions. In contrast, several other studies have demonstrated the complete exchange of membrane cholesterol (Bruckdorfer et al., 1968; Bloj & Zilversmit, 1977). Since it is clear that some of the cholesterol which underwent exchange in the latter experiments was originally present in the inner leaflet of the membrane bilayer, these results argue that cholesterol can and does move readily from one leaflet of the bilayer to the other.

Lenard & Rothman (1976) have reported that no more than 50% of the cholesterol present in the membrane of influenza virus grown in bovine MDBK cells will undergo exchange with cholesterol present in sonicated lipid vesicles. We have used a similar experimental approach to determine the extent to which the cholesterol present in the membrane of Sindbis virus grown in avian cells and in the membrane of vesicular stomatitis virus (VSV), grown in mammalian cells, will undergo exchange. We find, in marked contrast to what was found with influenza virus, that the cholesterol in these two virus preparations exchanges completely.

Materials and Methods

Most experiments were performed using TNE buffer. This consisted of 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4 at 37 °C), and 1 mM EDTA.

Radioactive Virus. Sindbis virus and VSV were labeled with [^{14}C]cholesterol by growth in tissue culture cells prelabeled with [^{14}C]mevalonic acid. DL-[2-^{14}C]Mevalonic acid, DBED salt (Amersham/Searle, 22.6 mCi/mmol), was dissolved in serum or serum-containing medium by sonication, sterilized by filtration, and diluted with growth medium to a final concentration of 1.5–3 μCi/mL. Wild-type Sindbis virus was grown in primary cultures of chick embryo cells which had been labeled in Dulbecco's modified Eagle's medium supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% chick serum for 48–72 h. VSV, Indiana serotype, was grown in BHK cells which has been labeled in Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum for 72 h. Infection was at a low multiplicity, 0.05–0.1 plaque-forming units/cell. Adsorption was allowed to proceed for 60 min in a small volume. The radioactive medium was then restored to the cells. Virus was harvested after incubation for approximately 18 h at 37 °C and purified as described previously (Sefton & Keegstra, 1974; Sefton, 1976).

The incorporated ^{14}C was identified as cholesterol by chromatography on thin-layer silica gel plates (Analtech). The viruses were concentrated by centrifugation and resuspended in TNE (Sindbis virus) or water (VSV). This solution of virus (5 μL) was spotted directly on the plate. Three microliters of [^{3}H]cholesterol (G-[^{3}H], Amersham/Searle, 8.3 Ci/mmol) in $CHCl_3$ was spotted on top of Sindbis and the chromatogram was developed sequentially with isopropyl ether/acetic acid (96:4) and petroleum ether/ethyl ether/acetic acid (90:10:1). Eight microliters of chloroform/methanol (2:1) and 1 μL of [^{3}H]cholesterol ([1,2-^{3}H(N)], New England Nuclear, 54 Ci/mmol) were applied on top of the VSV, and this chromatogram was developed with ethyl ether/petroleum ether (3:1). Each plate was divided into 20 fractions and each fraction was scraped into a scintillation vial containing 10 mL of a toluene/Triton X-100/PPO scintillation fluid. Radioactivity was measured with a scintillation counter.

[^{3}H]Uridine-labeled virus was produced by growth of Sindbis virus and VSV in chick cells and BHK cells, respectively. Here infection was at a high multiplicity, approximately 10–100 plaque-forming units/cell. After adsorption, the infected cells were incubated in Eagle's medium supplemented with 2% tryptose phosphate broth, 1 or 2% calf serum, and 0.5 μg/mL of actinomycin D and containing 20

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to 50 μCi/mL of [5,6-^{3}H]uridine (New England Nuclear, 44 Ci/mmol). The virus was harvested after incubation for 8.5–15 h at 37 °C and purified as above.

Sindbis virus labeled with [^{3}H]palmitate was produced by growth of the virus on chick cells prelabeled with [^{3}H]palmitate, as has been described before (Sefton & Gaffney, 1974).

Lipids. Cholesterol and egg yolk lecithin were either obtained from Supelco or were prepared by the method of Bangham (1974; egg lecithin) and by recrystallization from methanol (cholesterol) and were stored in $CHCl_3$ at 25 mg/mL under argon at −20 °C. Thin-layer chromatography of the cholesterol on silica gel in ethyl ether/petroleum ether (3:1) revealed only a single spot. Similarly, thin-layer chromatography of the egg lecithin on silica gel in chloroform/methanol/water (65:25:4) revealed only a single spot.

Exchange Incubations

(*a*) *Transfer of Cholesterol from Virus to Vesicles.* To make lipid vesicles, cholesterol was mixed with egg lecithin in $CHCl_3$, usually at a molar ratio of 0.8, and the mixture was dried thoroughly with N_2. The lipids were then dispersed in TNE at a concentration of 2 mg/mL by vigorous mixing with a vortex mixer. The resulting suspension was sonicated for at least 45 min in an ice bath under argon with a Branson sonicator equipped with a 1-cm tip. Delipidated bovine serum albumin (Sigma) was then added to a final concentration of 100 μg/mL, and the vesicles were centrifuged for 15 min at 15000*g*. Gel filtration of vesicles doped with [^{14}C]phosphatidylcholine and [^{3}H]cholesterol revealed that at least 75% of the sonicated vesicles was included on a column of Sepharose 4B and that the molar ratio of cholesterol to egg lecithin was the same in both the large and small vesicles (0.77) and very similar to that in the initial dispersion (0.80).

Exchange incubations were done under argon or nitrogen at 37 °C in TNE containing delipidated bovine serum albumin (100 μg/mL) and, in some experiments, sodium azide (0.02%). In some experiments, a viral pellet was dissolved in the solution containing the vesicles. In others, virus in a small volume of 20–30% sucrose in TNE was added to the solution of vesicles. The concentration of the lipid in vesicles was routinely 2 mg/mL. The concentration of viral lipids was 10–50 μg/mL. At intervals, samples were taken and mixed with nonradioactive carrier virus (50 μg of viral protein) at 4 °C, and the vesicles and virus were separated by centrifugation in a sucrose gradient. Sindbis virus was sedimented for 45 min at 45000 rpm in a 15–30% w/w in TNE sucrose gradient in an SW 50.1 rotor. VSV was sedimented for 25 min at 40000 rpm in a 15–30% w/w in TNE sucrose gradient in an SW 50.1 rotor. The gradients were fractionated, and the radioactivity was determined by mixing each fraction with 1 mL of water and 10 mL of toluene/Triton X-100/PPO scintillation fluid. In preliminary experiments, Sindbis virus was separated from the vesicles by precipitation of the virus with water and collection of the precipitate by centrifugation (Sefton et al., 1973). This procedure gave essentially similar results, although a slightly greater fraction of the lipid vesicles sedimented nonspecifically with the virus in this procedure.

(*b*) *Transfer of Cholesterol from Virus to Serum.* Exchange incubations were performed essentially as were those employing lipid vesicles except that the incubation mixture contained serum (usually 10% v/v) instead of vesicles. Untreated tissue culture serum from the Colorado Serum Co. or from Gibco was used. In several experiments the serum was replaced after 24 h of incubation. This was accomplished by dilution of the virus with water and collection of the resulting viral precipitate by centrifugation. The pellet was then redissolved in fresh 10% serum.

(*c*) *Transfer of Cholesterol from Vesicles to Virus.* [1,2-^{3}H]Cholesterol (New England Nuclear) was mixed with cold, recrystallized cholesterol to a specific activity of 250 μCi/g. Cholesteryl [1-^{14}C]oleate, sp act. 50–60 μCi/mmol from New England Nuclear, was used as a nonexchangeable marker. Phosphate buffer was sodium phosphate (0.01 M), pH^{25} 7.4, sodium chloride (0.15 M), 0.02% sodium azide. Buffers were deoxygenated for all procedures by bubbling a stream of nitrogen (high purity, dry, filtered through glass wool) through the solution for 30 min. Estimates of lipid in virus samples were based on weight ratios of 100:33:11 (protein/phospholipid/cholesterol; Pfefferkorn & Hunter, 1963) and Lowry analysis for protein of samples in phosphate buffer. The protein standard was BSA.

Lipid vesicles were prepared from a mixture 25 mg of egg yolk lecithin, 10 mg of tritiated cholesterol, and 2.5–5.0 μCi of cholesterol oleate. The lipids were dissolved in chloroform/benzene, the solvent was removed with a stream of nitrogen, and lipids were further dried for 2 h at room temperature under vacuum (~1 mm). A suspension of the lipids in 4 mL of TNE buffer was sonicated and centrifuged as described above. A portion (3.5 mL) of the resulting supernatant was applied to a 1 × 40 cm column of Sepharose 2B (Bio-Rad), and fractions of approximately 0.35 mL were collected by elution with TNE. The dilution of the vesicles on the column was calculated by comparison of the radioactivity in the suspension applied to the top of the column with that in the eluted fractions.

For incubation with Sindbis virus, vesicle fractions from the lipid peak were pooled to give the required total lipid within 24 h after application to the Sepharose column. Virus was thawed (from −170 °C), precipitated by dilution from sucrose solution, and dissolved in TNE. The final incubation mixture contained 2 mg/mL of vesicle lipid and virus. Nitrogen was bubbled slowly through the reaction mixture for 5 min and it was placed in a sealed bottle in a water bath at 37 °C for 4 or 20 h. At the end of the incubation, mixtures of large volume were placed on top of 56 mL of a continuous gradient of 15–30% sucrose in phosphate buffer. After centrifugation at 23000 rpm (Beckman 25.2) for 3 h, fractions of 1.7 mL were collected. Smaller volumes were handled as described in part a above. Aliquots (50 μL) of the fractions were counted. The ^{3}H-rich virus band in the middle of the gradient was precipitated by tenfold dilution with water and centrifugation for 1 h at 11000 rpm (Beckman JA-20). The precipitate was brought to 1 mL in phosphate buffer and analyzed for radioactivity and protein. Thin-layer chromatography on silica gel of the treated and recovered virus and vesicles showed that all of the ^{3}H migrated as cholesterol and the ^{14}C as cholesteryl oleate.

Characterization of the Sindbis Virus after Incubation with Cholesterol-Containing Lipid Vesicles. The sedimentation rate of incubated virus was measured by mixing incubated virus, labeled with [^{3}H]uridine, with unincubated virus, labeled with ^{14}C-labeled amino acids and sedimentation of the mixture into a 15–30% w/w in TNE, sucrose gradient for 45 min at 45000 rpm in a SW 50.1 rotor. The radioactivity in each fraction was counted, using 10 mL of a toluene/Triton X-100/PPO scintillation fluid and 1 mL of water.

The density of the incubated virus in potassium tartrate was determined as above, except that centrifugation was for 3 h at 40000 rpm in an SW 50.1 rotor in a 10–40% potassium tartrate in water gradient. The radioactivity in each fraction was determined using Aquasol (New England Nuclear) as

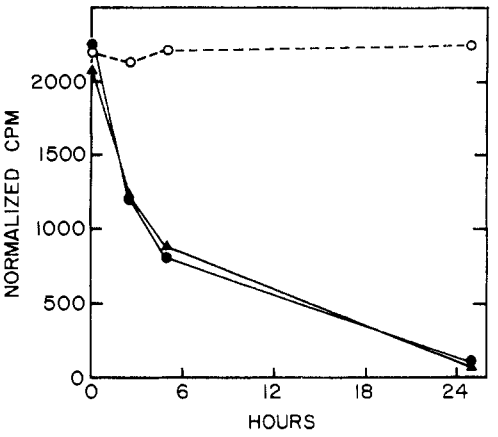

FIGURE 1: Kinetics of the transfer of radioactive cholesterol from the membrane of Sindbis virus to lipid vesicles. Sindbis virus, labeled with [^{14}C]cholesterol, was mixed virus labeled with [^{3}H]uridine and dissolved in TNE buffer containing either no lipid, sonicated cholesterol/egg lecithin vesicles (molar ratio 0.8), or sonicated egg lecithin vesicles. The amount of lipid in the vesicles (2 mg/mL) was approximately 160-fold greater than that in the virus. Incubation was at 37 °C. At intervals, samples were removed, and the virus was separated from the vesicles by sedimentation into a sucrose gradient. The data presented here are normalized to the amount of ^{3}H recovered. The actual amount of ^{14}C in each sample differed less than 20% from the normalized values presented here. (○) TNE buffer alone; (●) cholesterol/egg lecithin vesicles; and (▲) egg lecithin vesicles.

scintillation fluid. Enough water was added to clarify each sample.

The infectivity of the incubated virus was determined by plaque titration of the virus on monolayers of chick embryo cells on 50-mm plastic petri dishes. The details of the procedure have been described elsewhere (Fan & Sefton, 1978).

The behavior of the virus during gel filtration was determined by chromatography on Sepharose 2B. Virus (2 mg) was incubated with sonicated lipid vesicles containing [^{3}H]cholesterol (mole ratio of cholesterol to egg lecithin of 0.8) in a total volume of 2 mL of TNE for 21 h at 37 °C. Virus, labeled with ^{14}C-labeled amino acids, was added to the cooled mixture at the end of the incubation and the double-labeled sample was separated from the lipid vesicles on a sucrose gradient. Fractions from the virus peak (0.2 mL) were applied to a 1 × 40 cm Sepharose 2B column.

Paramagnetic Resonance Measurements. Electron paramagnetic resonance (EPR) measurements and spin-labeling of Sindbis virus were performed as described by Sefton & Gaffney (1974).

Results

Sindbis virus and VSV, containing ^{14}C-labeled cholesterol, were produced by growth of the viruses in cells preincubated for several days in [^{14}C]mevalonic acid. More than 85% of the label in Sindbis virus and more than 95% of the label in VSV comigrated with [^{3}H]cholesterol during thin-layer chromatography.

Exchange with Lipid Vesicles. We measured the exchange of the radioactive viral cholesterol during incubation of the labeled virus with a large excess of nonradioactive sonicated cholesterol/egg lecithin vesicles. The radioactive cholesterol in Sindbis virus grown in chick cells underwent transfer to the lipid vesicles (Figure 1). Within 24 h the transfer was essentially complete. The experiment presented in Figure 1 is a typical result. Repeated measurements determined that the half-time for the transfer of the radioactive cholesterol was between 3 and 5 h and that the amount of radioactive cholesterol present in the virus after incubation for 24 h was between 3 and 10% of the starting material.

Table I: Transfer of Cholesterol from Vesicles to Sindbis Virus

	expt 1	expt 2
Initial Conditions[a]		
virus cholesterol (μg/mg of viral protein)	110–140	110–140
vesicle cholesterol (μg/mg of viral protein)	740	1500
CPM cholesterol/100 CPM cholesterol oleate in vesicles[b]	83.3	39.1
Final Analysis[c]		
total radioactive cholesterol in virus fraction (CPM)[d]	1863	2438
cholesterol due to vesicle sticking (μg/mg of viral protein)	9.1	17.6
cholesterol transferred (μg/mg of viral protein)	121	101

[a] Each experiment was performed with 1 mg of viral protein. Estimates of the cholesterol content of this amount of virus are 110–140 μg (Pfefferkorn & Hunter, 1963; Hirschberg & Robbins, 1974). [b] [^{3}H]Cholesterol and cholesterol [^{14}C]oleate were used. Cholesterol oleate served as a nonexchangeable marker for vesicles sticking. [c] 117 μg of viral protein was recovered in experiment 1 and 130 μg in experiment 2. The losses of viral protein were due in large part to nonspecific sticking of virus to the sides of tubes during both incubation and centrifugation. Virus recovery was measured by chemical determination of protein at the end of the experiment. [d] Counting efficiences differed in experiments 1 and 2. Cholesterol was 122.8 CPM/μg in experiment 1 and 157.6 CPM/μg in experiment 2.

This experiment was done using sonicated lipid vesicles which contained approximately the same ratio of cholesterol to phospholipid as does the virus, 0.8. This ratio was chosen to minimize depletion of the viral cholesterol during the incubation. We have also performed this experiment using cholesterol-free egg lecithin vesicles. The transfer of radioactive cholesterol from the virus to egg lecithin vesicles occurred with the same rate and proceeded to the same extent as with vesicles which contained cholesterol (Figure 1).

We wished to know whether what we were observing was the net transfer of cholesterol from the virus to the vesicles or was, when exogeneous cholesterol was present, the exchange of radioactive viral cholesterol for nonradioactive cholesterol in the vesicles. We therefore measured the transfer of [^{3}H]cholesterol from sonicated cholesterol/egg lecithin vesicles to nonradioactive virus. Transfer of cholesterol from the vesicles to the virus occurred readily and after 20 h of incubation the amount of radioactive cholesterol present in the virus was approximately equal to the amount of viral cholesterol present at the start of the experiment (Table I). Therefore, appreciable depletion of the viral cholesterol does not occur during incubation with cholesterol-containing lipid vesicles.

To confirm that viral membranes were not altered markedly by incubation with lipid vesicles, the physical properties of the viral membrane were investigated with a fatty acid spin-label (Sefton & Gaffney, 1974). A spin-label derivative of a 20-carbon fatty acid, which is longer than the more commonly used spin-label stearate derivatives, was chosen to favor partitioning of the label into membranes rather than into water. Figure 2 shows the results of EPR measurements of virus samples, after incubation with lipid vesicles of various ratios of cholesterol to lecithin, and of EPR measurements of lipid vesicles. The observed spectra of virus which had been isolated after incubation with vesicles differed significantly from the control virus spectra only when the amount of cholesterol in

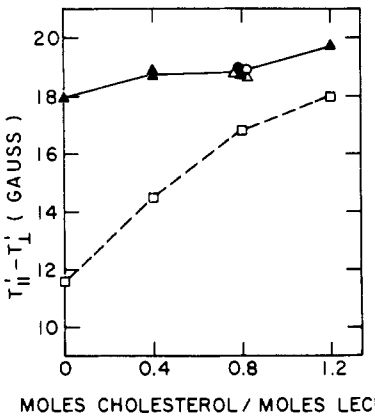

FIGURE 2: EPR spectral parameter, $T_{\parallel}'-T_{\perp}'$, of Sindbis virus which had been incubated with lipid vesicles containing varying amounts of cholesterol and of the vesicles themselves. A derivative of arachidic acid, spin-labeled at C-8 with the 4,4-dimethyl-3-oxazolidinyloxyl ring, was used. Vesicles were prepared as described in the Materials and Methods section and were fractionated on a Sepharose 2B column. [^{14}C]Cholesteryl oleate was used as a nonexchangeable marker to assay for separation of virus and vesicles. Virus was isolated after the incubation by sucrose gradient centrifugation. The ratio of cholesteryl [^{14}C]oleate to protein in the samples used for the EPR spectra was measured and demonstrated that a maximum of 3–10% of the lipid in the samples resulted from vesicles stuck to the isolated virus. EPR measurements were made at 22 °C. The parameter ($T_{\parallel}'-T_{\perp}'$) is one-half the difference of the observed outer and inner extrema and is proportional to the order parameter (Sefton & Gaffney, 1974). (▲) Virus incubated with a fivefold excess of vesicles for 4 h; (●) virus incubated with a tenfold excess of vesicles for 20 h; (△) virus incubated in buffer for 5 h; (○) virus incubated in buffer for 20 h; (□) lipid vesicles alone.

the lipid vesicles was very low (0%) or very high (cholesterol/lecithin mole ratio = 1.2). The data for the lipid vesicles are included to demonstrate that the EPR parameter is quite sensitive to the cholesterol/phospholipid ratio in membrane samples.

Our results with Sindbis virus grown in chick cells differ from those obtained with influenza virus grown in MDBK cells. It was reported that only half of the cholesterol in the influenza virion is available for exchange and this fraction undergoes exchange only after digestion of the virus with chymotrypsin (Lenard & Rothman, 1976). To determine whether complete exchange of viral cholesterol is a property unique to Sindbis virus grown in chick embryo cells, we performed similar experiments with VSV grown in hamster BHK cells. Incubation of ^{14}C-labeled VSV with sonicated cholesterol/egg lecithin vesicles resulted in the rapid and complete transfer of the radioactive viral cholesterol to the vesicles (Figure 3). The rate with which this occurred and the extent to which it proceeded were essentially the same as those observed with Sindbis virus.

To examine the ability of viral lipids other than cholesterol to undergo exchange, we incubated [^{3}H]palmitate-labeled Sindbis virus with cholesterol/egg lecithin vesicles and with pure egg lecithin vesicles. [^{3}H]Palmitate is incorporated into both phospholipids and glycolipids but not into viral protein (data not shown). We observed the transfer of less than 15% of the palmitate radioactivity under conditions where greater than 80% of viral cholesterol underwent transfer. This indicates that the viral lipids labeled by [^{3}H]palmitate undergo only very limited exchange with the lipids in the vesicles. Significant transfer of viral RNA and viral proteins to the lipid vesicles was not observed.

The fraction of cholesterol remaining in the virus in the above experiments was determined by separation of the virus from the vesicles by centrifugation. It was clear, therefore,

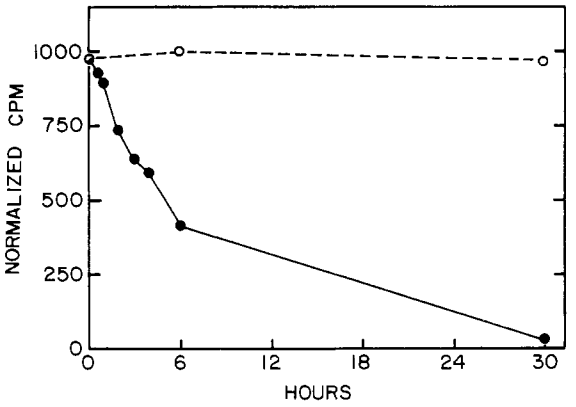

FIGURE 3: Kinetics of the transfer of radioactive cholesterol from the membrane of VSV to lipid vesicles. The experiment was performed essentially as described in the legend to Figure 1, except that the virus was not also incubated with cholesterol-free vesicles. (○) TNE buffer alone; (●) cholesterol/egg lecithin vesicles.

that, in a crude sense, the viruses were still intact at the end of the incubation. We have examined the physical integrity of Sindbis virions which have undergone incubation with sonicated cholesterol-containing lipid vesicles by velocity sucrose density gradient centrifugation, by centrifugation to equilibrium in potassium tartrate gradients, and by gel filtration on Sepharose 2B. We used for these analyses virus which had been incubated with sonicated cholesterol/egg lecithin vesicles for 17 to 21 h. Incubation for this interval results in the transfer of at least 80% of the radioactive viral cholesterol. This incubated virus preparation sedimented slightly more slowly than unincubated virus during sucrose gradient centrifugation, had a slightly reduced density in potassium tartrate, and eluted from a Sepharose 4B column as a particle slightly larger than unincubated virus (Figure 4).

The infectivity of virus which had undergone incubation with lipid vesicles was greatly reduced. Five hours of incubation, which resulted in the transfer of 50% of the radioactive viral cholesterol, caused the loss of 92% of the infectivity of the preparation. Incubation for 24 h, which resulted in transfer of 97% of the viral cholesterol, caused a complete loss of infectivity; fewer than 0.001% of the particles remained infectious. This rapid inactivation of infectious virus occurred during incubation with both cholesterol/egg lecithin vesicles and with pure egg lecithin vesicles.

Exchange with Serum. We wished to know whether the complete transfer of radioactive cholesterol which we observed during incubation with lipid vesicles would also occur with another acceptor of viral cholesterol. We therefore incubated both Sindbis virus and VSV, labeled biosynthetically with ^{14}C in cholesterol, with buffer containing various amounts of commercial tissue culture serum (Table II). Transfer of the labeled cholesterol from both viruses to the serum occurred readily. The observed rate and the extent to which this occurred under our experimental conditions were both less than those found with the lipid vesicle incubations. It seems likely that this difference is due to the fact that the capacity per unit volume of the serum to take up the viral cholesterol is less than that of the vesicles. When the serum was replaced after 24 h of incubation, and the virus incubated an additional 24 h in fresh serum, more than 90% of the viral cholesterol was transferred.

Because virus incubated for 24 h with lipid vesicles was largely noninfectious, we examined the infectivity of virus which had been incubated in 10% calf serum for 26 h. In contrast to what we found with the lipid vesicles, this virus was 20–40% as infectious as virus incubated in buffer alone.

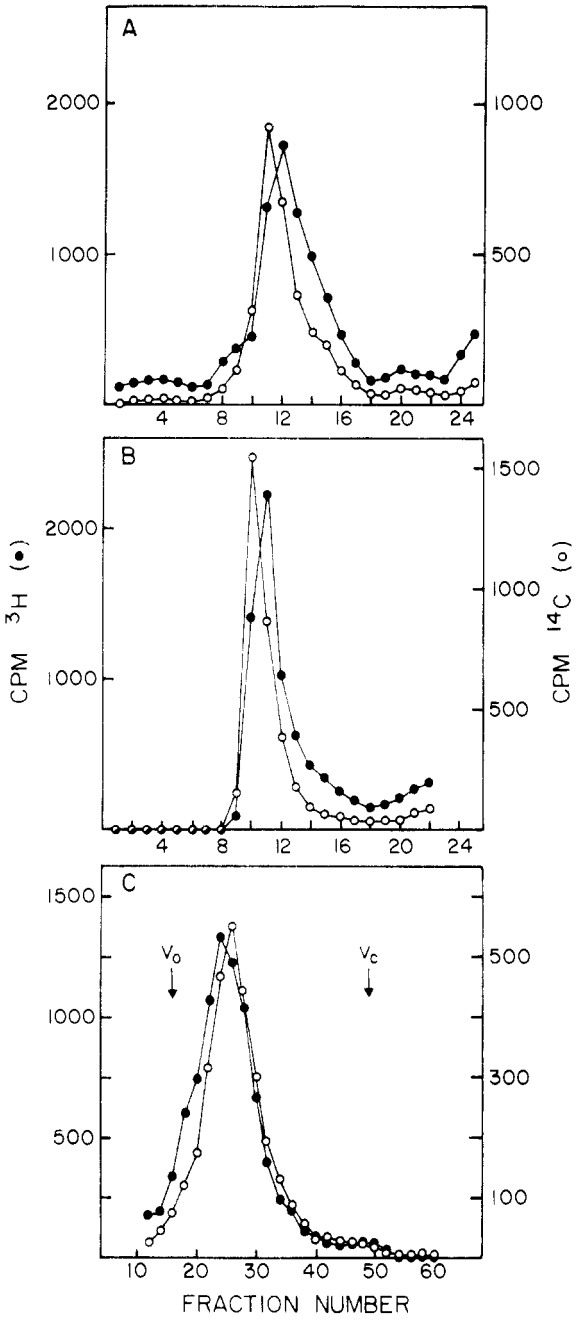

FIGURE 4: Comparison of incubated and unincubated Sindbis virus by velocity sucrose gradient centrifugation, equilibrium potassium tartrate gradient centrifugation, and gel filtration. Sindbis virus which had been incubated for 18 h at 37 °C with sonicated cholesterol/egg lecithin vesicles and was labeled with [^{3}H]uridine (A and B) or with [^{3}H]cholesterol (C) was mixed with unincubated virus labeled with ^{14}C-labeled amino acids and analyzed as described under Materials and Methods. (○) Unincubated virus; (●) incubated virus; (A) velocity sucrose gradient centrifugation; (B) equilibrium potassium tartrate centrifugation; (C) gel filtration on Sepharose 2B.

Table II: Exchange of Viral Cholesterol with Serum[a]

virus	serum	% cholesterol remaining 24 h	48 h
Sindbis	1% calf and 1% chicken	48	
Sindbis	10% calf (two experiments)	21[b]	
		17	
	10% calf, replaced at 24 h (two experiments)	42[b]	11
		35	6
	25% calf	11	
VSV	10% calf	33	

[a] Virus, labeled with [^{14}C]cholesterol, was mixed with virus labeled with [^{3}H]uridine, ^{3}H-labeled amino acids, or [^{3}H]-glucosamine and dissolved in TNE buffer containing the indicated amounts of serum, fatty-acid-free bovine serum albumin (100 μg/mL), and sodium azide (0.02%). The solution was incubated at 37 °C for the indicated periods and the virus then separated from the serum by sedimentation into a sucrose gradient. Nonspecific loss of virus was calculated from the recovery of ^{3}H in each experiment. [b] The variation in the values presented is due to differences in the amount of virus (10–50 μg of viral protein) and the volume of buffer (0.5–1.0 mL) used in the experiments. In the experiments where serum was replaced, approximately twice as much virus, per volume of serum, as in the other experiments was used. This was done to ensure good recovery of the virus after the two-step experiment.

Discussion

We have found that all the cholesterol in two different enveloped viruses will undergo transfer during incubation with sonicated lipid vesicles and with serum. From X-ray diffraction measurements, it appears that the lipids in Sindbis virus are organized in a bilayer (Harrison et al., 1971). We presume that only that cholesterol present in the outer leaflet of the viral membrane can undergo transfer. If this is so, our results indicate that either the viral cholesterol is present in both leaflets of the membrane and moves from one side of the membrane to the other at a rate which is comparable to that with which it undergoes transfer or all the cholesterol in the virion is present in the outer leaflet of the membrane. Our data do not allow us to definitively choose between these two possibilities, but we feel that the first possibility is the more reasonable. Bruckdorfer et al. (1968) and Bloj & Zilversmit (1977) have reported that all the cholesterol present in lipid dispersions will undergo exchange with red blood cells or red blood cell ghosts. Since in these studies it is certain that some of the cholesterol in the vesicles was originally present in the inner leaflet of the vesicle bilayer (Huang et al., 1974), these results can only be explained if cholesterol can move from one face of a lipid bilayer to the other. If the complete transfer which we have observed is due instead to the presence of all the viral cholesterol in the outer leaflet of the bilayer, the molar ratio of cholesterol to phospholipid in this face of the membrane would be considerably greater than 1.

Our results are different from those obtained by Lenard & Rothman (1976) who performed an apparently similar experiment with influenza virus. They have reported that none of the cholesterol in the influenza virus virion will exchange spontaneously with that in sonicated lipid vesicles. Exchange of the viral cholesterol could be observed only when the influenza virions were digested with chymotrypsin. Only one-half of the cholesterol in protease-treated virions will undergo exchange. It is not clear at this time whether the differences between our results and those of Lenard & Rothman have a technical basis or are due to a real difference between influenza virus membranes on the one hand and Sindbis virus and VSV membranes on the other.

While we feel that the most reasonable explanation of our data is that cholesterol undergoes transmembrane movement in a virus, not all investigators agree that cholesterol can move readily from one leaflet of a membrane to the other. Poznansky & Lange (1976, 1978) have argued that cholesterol will move from the inner leaflet of a lipid vesicle membrane to the outer leaflet only under conditions where net depletion of the cholesterol in the vesicle is occurring. It is not obvious, however, that depletion of the cholesterol in the vesicles occurred in the experiments of Bruckdorfer et al. (1968) and Bloj & Zilversmit (1977). In our exchange experiments with Sindbis virus, we used vesicles which contained the same molar

ratio of cholesterol to phospholipid (~0.8) as is present in virus grown in chick fibroblasts. In addition, we were able to demonstrate that significant transfer of cholesterol from the vesicles to the virus occurs during exchange incubations and that the physical properties of the viral membrane were essentially unchanged after incubation with cholesterol-containing vesicles. We therefore are confident that pronounced depletion of the viral cholesterol did not occur during our experiments.

We also observed essentially complete transfer of viral cholesterol to serum. This result makes it unlikely that the complete exchange which we observed with the lipid vesicles was a phenomenon induced by the vesicles and suggests rather that the ability to undergo exchange is an inherent property of viral cholesterol.

This result with serum has several implications. We assumed at the outset of the experiments that the specific activity of the viral cholesterol was the same in both faces of the viral membrane. However, since cholesterol in isolated virus can undergo extensive transfer to buffers containing as little as 10% serum, the viral cholesterol in our preparations must have already undergone exchange with the medium in which the virus was grown. As a result, the biosynthetically incorporated cholesterol may in fact not be evenly distributed between the two faces of the bilayer. While this uncertainty makes measurements of the initial rate of cholesterol transfer difficult to interpret, it has no effect on the interpretation of our observation that the exchange of biosynthetically incorporated cholesterol will go to completion.

One attraction of enveloped viruses as a system with which to study membrane lipids is that these viruses acquire the lipid portion of their membrane, with little selectivity, from the plasma membrane of the host cell. The viral lipid bilayer therefore closely resembles that of the host cell in lipid composition and presumably in orientation. Our observation that the viral cholesterol will undergo extensive exchange with serum raises the real possibility that the cholesterol present in the host cells of these two viruses, chick fibroblasts and hamster kidney cells, will also readily exchange with serum.

It could be argued that the exchange of cholesterol requires the presence of specific viral membrane proteins. We feel this is unlikely. We observed essentially identical rates and extents of cholesterol transfer with membranes containing either the two Sindbis virus glycoproteins or the single VSV glycoprotein. Further, there are several reports that all the cholesterol in protein-free lipid vesicles may undergo exchange. It is known that the cholesterol content of blood cells (Shinitzky & Inbar, 1974; Cooper, 1977) can be grossly altered by exchange with extracellular fluid. Our results suggest that the cholesterol content of fibroblasts and kidney cells may be similarly affected. In fact, it seems likely that the cholesterol in all cells tends toward equilibrium with that in the surrounding fluid.

We examined the integrity of Sindbis virus incubated with liposomes for prolonged periods. The physical parameters of the virus particle were largely unaffected. Its behavior during sedimentation and gel filtration was only slightly altered. The small, apparent increase in size and the decrease in density of the treated virus could be due to lipid vesicles stuck to a few of the viruses. Five to ten percent of the lipid in the reisolated virus is due to sticking. The marked adsorption of lipid vesicles to Sindbis virus observed by Mooney et al. (1975) occurs only in buffers much more acidic than those used here. Viral infectivity was, however, seriously reduced. We have no ready explanation for this. This loss of infectivity is probably not due to cholesterol exchange per se, as viral infectivity was only *moderately* reduced by an incubation with serum which resulted in the transfer of 80% of the viral cholesterol.

An asymmetric distribution of cholesterol has been postulated to explain the asymmetric electron density profile of myelin membranes (Caspar & Kirschner, 1971), although it now appears that the protein distribution accounts for most, or all, of this asymmetry (Nelander & Blaurock, 1978). Huang et al. (1974) have suggested that cholesterol may be concentrated on the inside of membrane regions that have a low radius of curvature, and Fisher (1976) has obtained some evidence for an asymmetric distribution of cholesterol in red blood cell membranes. We had hoped to determine from our experiments how the cholesterol in a viral membrane is distributed between the two leaflets of the lipid bilayer. While one interpretation of our data on Sindbis virus and VSV is that all of the cholesterol is located on the outer leaflet of the membrane, it seems more reasonable that cholesterol is present at equilibrium in both leaflets of the bilayer. However, an asymmetric distribution of cholesterol is not precluded by our results since it has been shown that cholesterol partitions differentially between lipids of varied fatty acid and head group composition (Demel et al., 1977). In addition, the obviously asymmetric protein distribution in virus membranes, the composition of the surrounding medium, and the surface tension may well influence the equilibrium distribution of cholesterol in Sindbis and VSV membranes.

Added in Proof

Results of experiments measuring the exchange of cholesterol between VSV and lipid vesicles have been reported by Patzer et al. (1978) subsequent to the acceptance of our report. These authors report an experimental approach and results essentially similar to ours.

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Thermotropic Behavior of Monoglucocerebroside–Dipalmitoylphosphatidylcholine Multilamellar Liposomes†

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ABSTRACT: The thermotropic behavior of multilamellar liposomes prepared from mixtures of glucocerebroside and dipalmitoylphosphatidylcholine has been studied by high-sensitivity scanning calorimetry. It is shown that glucocerebroside has a marked effect on the gel–liquid crystalline transition of dipalmitoylphosphatidylcholine. The pretransition seen in pure samples of dipalmitoylphosphatidylcholine is undetectable at small mole fractions of glucocerebrosides (<10%). The main transition is shifted to higher temperatures and becomes broader and less cooperative in the presence of glucocerebroside. The enthalpy change of the main transition decreases with increasing the glucocerebroside content. However, this decrease is not linear with the glucocerebroside/phospholipid mole ratio. Glucocerebroside itself does not show a separate transition in the temperature range of these studies (10–75 °C). The origin of these effects and their dependence on the glucocerebroside content suggest that the in-plane distribution of glucocerebroside molecules is affected by the physical state of the lipid bilayer and by the glucocerebroside/phospholipid mole ratio.

Glycosphingolipids are complex lipids found mostly on the outer surface of plasma membranes. These sugar-containing lipids have been shown to play a major role as surface receptors (Rothman & Lenard, 1977; Karlsson, 1977; Fishman & Brady, 1976). Physicochemical studies also indicate that these lipids increase the stability of cell surface membranes and decrease their permeability (Pascher & Sundell, 1976; Abrahamsson et al., 1977). However, little is known about the molecular organization and the interactions of these lipids with other membrane components. It is our intention to further our knowledge of the interactions of glycosphingolipids, namely, glucocerebrosides, and phospholipids as a first step in understanding the role these lipids play in biological membranes. This paper reports a calorimetric study of the gel–liquid crystalline phase transition of multilamellar liposomes prepared from various compositions of dipalmitoylphosphatidylcholine and glucocerebrosides.

Experimental Procedures

Materials. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) was synthesized by the method of Cubero Robles & Van den Berg (1969) as described by Suurkuusk et al. (1976). DPPC was dissolved in spectral grade chloroform (Fisher Scientific Co.) and stored at −20 °C until needed. Glucocerebroside (Glc-Cer), extracted from the spleen of a patient with Gaucher disease, was a gift from the Hadassah Medical School of the Hebrew University, Jerusalem. Glc-Cer was purified by silic acid chromatography and dissolved in a 2:1 ratio of spectral chloroform to methanol and kept at −20 °C until ready for use. The fatty acid composition of the glucocerebroside stock was analyzed by gas–liquid chromatographic procedures. The Glc-Cer sample was first subjected to acid hydrolysis, followed by methylation, as described by Kates (1964). These results are summarized in Table I. The sample consisted mostly of a mixture of saturated, long-chain fatty acids, principally C_{22} and C_{24} which together comprised 65% of the total.

Table I: Fatty Acid Composition of Glucocerebroside

fatty acid[a]	% of total	fatty acid[a]	% of total
$C_{16:0}$	3.90	$C_{23:0}$	13.82
$C_{18:0}$	3.22	$C_{24:0}$	31.07
$C_{20:0}$	5.25	$C_{24:1}$	6.98
$C_{22:0}$	34.62	others	1.14

[a] Designated $C_{m:n}$ where m is the number of carbon atoms and n the number of double bonds in the fatty acid side chain.

Preparation of Liposomes. Approximately 10 mg of the desired phospholipid–glucocerebroside mixture was placed in a round bottom flask and the solvent was evaporated on a rotary evaporator at 40 °C. The sample was then frozen in an ethanol bath and the mixture lyophilized for at least 4 h. The dry lipid was then heated to 55 °C and suspended in 50

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