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Transmembrane Movement and Distribution of Cholesterol in the Membrane of Vesicular Stomatitis Virus†

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ABSTRACT: The transmembrane movement and distribution of cholesterol in the vesicular stomatitis virus membrane were studied by following the depletion of cholesterol from virions to interacting phospholipid vesicles and by exchange of radiolabeled cholesterol between virions and phospholipid–cholesterol vesicles. The kinetics of the cholesterol exchange or depletion reactions revealed the presence of two exponential rates: a rapid rate, dependent on the vesicle to virus ratio, and a slower rate, independent of the vesicle to virus ratio. The kinetics of cholesterol movement could be best interpreted by a model of the virion membrane considered as a two pool system in which ~30% of the cholesterol resides in the outer monolayer and ~70% in the inner monolayer. The half-time for equilibration of the two pools was calculated to be 4–6 h and was assumed to represent the time required for transmembrane movement of cholesterol across the bilayer. The initial rate of transfer of cholesterol from virus into vesicles increased when vesicle phospholipids contained more unsaturated and shorter chain fatty acids. Furthermore, the transfer of cholesterol appeared to occur by a collisional mechanism requiring membrane–membrane contact. Interaction with lipid vesicles did not significantly affect the integrity of the virion membrane as assessed by the relative inaccessibility of internal proteins to lactoperoxidase-catalyzed iodination and by the small loss of [^{3}H]amino acid labeled protein from the virus.

Studies of the distribution and degree of transmembrane movement of cholesterol in synthetic lipid vesicles and biological membranes have to date yielded conflicting results. Initial studies showed that synthetic liposomes (Poznansky and Lange, 1976, 1978), the erythrocyte membrane (Gottlieb, 1976), and the influenza virus membrane (Lenard and Rothman, 1976) contain cholesterol in two pools, presumably in the inner and outer monolayers; the inner monolayer cholesterol was assumed to be slowly exchangeable or nonexchangeable. In contrast, more recent evidence suggests that the cholesterol of liposomes is completely exchangeable as one pool (Bloj and Zilversmit, 1977) and the erythrocyte membrane contains two pools of cholesterol, both rapidly exchangeable (Lange et al., 1977). Clearly, more work is needed on these and other systems to establish the location and rates of transmembrane movement of cholesterol.

We undertook to study the distribution and movement of cholesterol in the membrane of vesicular stomatitis (VS)[1] virus. This virus can be purified to homogeneity in large quantities, and membrane constituents can be specifically radiolabeled during virus growth (Wagner, 1975). Since virions bud from the plasma membrane, the viral cholesterol distribution should reflect that of the host cell and other eucaryotic cell plasma membranes. Moreover, a great deal of evidence concerning the distribution of proteins and phospholipids in the viral membrane has already been obtained. Of the two membrane-associated proteins, the glycoprotein (G) has been shown to be asymmetrically oriented toward the external milieu (Schloemer and Wagner, 1975), whereas the matrix (M) protein appears to lie internal to the permeability barrier of the membrane (Moore et al., 1974). Similarly, the phospholipids and their fatty acyl chains have an asymmetric orientation with the majority of the choline phospholipids located in the external layer and the majority of amino phospholipids and polyunsaturated fatty acids located internally (Patzer et al., 1978). In previous studies it was found that >90% of the cholesterol in the VS virion membrane could be oxidized from the external surface by cholesterol oxidase after removal by phospholipase C of phospholipid head groups (Moore et al., 1977) or could be >90% depleted from the viral membrane by interaction with phospholipid vesicles (Moore et al., 1978).

In the present studies the movement of cholesterol between VS virus and lipid vesicles has been carefully examined to analyze the kinetics of the exchange process. These data have allowed us to estimate the percentage of membrane cholesterol in the inner and outer monolayers and the rates of transmembrane movement.

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[1] Abbreviations used are: VS, vesicular stomatitis; G, glycoprotein; M, matrix protein; L, large protein; N, nucleocapsid protein; BHK, baby hamster kidney; PS, phosphatidylserine; PC, phosphatidylcholine; NaDodSO$_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; DMPC, DPPC, and DOPC, dimyristoyl-, dipalmitoyl-, and dioleoylphosphatidylcholine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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Materials and Methods

Cells and Virus Growth. The Indiana serotype of vesicular stomatitis (VS)[1] virus was used to infect confluent monolayers of BHK-21 cells at a multiplicity of 0.1–1.0 plaque-forming unit/cell as described previously (Barenholz et al., 1976). Cholesterol-labeled virus was produced by using a metabolic precursor of cholesterol, DL-[5-^{3}H]mevalonic acid (1–5 Ci/mmol, New England Nuclear). It was introduced into the cell growth medium and viral infection medium at 2–3 μCi/mL for the entire cell- (~2 days) and viral-growth (~18 h) periods. This assured a homogeneous incorporation of the label throughout the viral cholesterol. Virus was also labeled with [^{3}H]leucine and purified, as previously described (Barenholz et al., 1976). Prior to use, virus preparations were stored at −80 °C.

Lipid vesicles were prepared from chromatographically pure egg phosphatidylcholine, dipalmitoyl-, dioleoyl-, and dimyristoylphosphatidylcholine, beef brain phosphatidylserine, and cholesterol; these were mixed in various proportions and dried under nitrogen. Small amounts of high specific activity labels were introduced into the lipid mixture. [^{14}C]Cholesterol oleate (50–60 μCi/μmol; New England Nuclear), trioleoyl[2-^{3}H]-glycerol (30–60 μCi/μmol; Amersham Searle), or [^{14}C]triolein were used as nonexchangeable markers (Bloj and Zilversmit, 1977; Poznansky and Lange, 1978). Mixed vesicles containing [4-^{14}C]cholesterol (57.7 mCi/mmol; Amersham Searle) were used to follow the exchange of cholesterol into VS virus. The lipids were resuspended in benzene and lyophilized overnight. The dried lipids were resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5), or 10 mM Tris (pH 7.5), and vesicles were made by high-intensity ultrasonic irradiation at 4 °C or room temperature under nitrogen, as described by Huang and Thompson (1974). The small proportion of multilamellar liposomes was removed by centrifugation at 100 000*g* for 40 min, and the region III vesicles described by Barenholz et al. (1977) were used for the exchange reactions.

Cholesterol Exchange Reaction. VS virus (150 μg, 220 nmol of cholesterol/mg of protein) was incubated with 0.81 μmol of vesicle phospholipid (containing 0.8 μmol of cholesterol) in 1.0 mL of 20 mM Tris, 100 mM NaCl (pH 7.5) at 37 °C for up to 24 h. The reaction was stopped by cooling to 4 °C, and the vesicles were separated from the virus by equilibrium centrifugation in 0–60% sucrose gradients in 10 mM Tris (pH 7.5) for 16 h in SW50.1 or SW60 Ti rotors at 40 000*g* at 4 °C. The vesicles remained at the top and the virus banded at a characteristic position near the middle of the gradient. The vesicles and virus band were collected, and the virus was pelleted in a SW50.1 or SW60 Ti rotor at 165 000*g* for 40 min at 4 °C. The virus pellet was resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5), and one aliquot was used to assay for cholesterol content and the other for scintillation counting of radioactivity. When exchange of cholesterol from the virus was being measured, the virus was labeled with [^{3}H]cholesterol and the vesicles with [^{14}C]cholesterol-oleate. When exchange into the virus was monitored, the virus was unlabeled and the vesicles were labeled with [^{14}C]cholesterol and trioleoyl[2-^{3}H]-glycerol. The cholesterol-oleate and triolein were used as nonexchangeable markers to assay the level of adherence of vesicles to virus. Since the adhering vesicles contained labeled cholesterol, their contribution had to be subtracted to obtain the actual value of labeled cholesterol remaining in the virus. The adhering vesicles were assumed to be identical to the free vesicles at the top of the gradient in their content of radioactivity; therefore, the amount of labeled cholesterol in the adhering vesicles was obtained by calculating the ratio of labeled cholesterol to nonexchangeable marker in the vesicles at the top of the gradient and multiplying it by the amount of nonexchangeable marker adhering to the virus. This value, which accounted for a maximum of 4–5% of the total, was subtracted from the total radiolabeled cholesterol in the virus pellet.

Cholesterol Depletion. VS virus (120 μg, intact or equivalent number of spikeless virions) (220 nmol of cholesterol/mg of protein) was incubated at 37 °C with 1.2 μmol of vesicle phospholipid for periods up to 24 h. Controls consisted of virus in the absence of vesicles. The depletion was terminated by banding the virions at 4 °C in a SW50.1 or SW60 Ti rotor at 40 000*g* for 16 h in continuous 0–60% sucrose equilibrium gradients, in which the free vesicles remained at the top. The virus band was further pelleted at 4 °C in a SW50.1 or SW60 Ti rotor at 165 000*g* for 40 min, and the pellet was resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5). Aliquots of the virus pellet and vesicles were used for measuring radioactivity by scintillation counting and assay of cholesterol content. To assay for vesicle adherence to virions, the nonexchangeable markers [^{14}C]cholesterol-oleate or [^{14}C]triolein were included in the vesicles in order to measure the proportion of vesicles remaining adherent to virions, as described above for the cholesterol-exchange reactions. Vesicles remaining adherent to virus accounted for a maximum of 10% of the cholesterol in the highly depleted intact virus pellets; these values were subtracted from the total cholesterol content of the pelleted virus.

Polyacrylamide Gel Electrophoresis. Virus preparations were made 1% with respect to $NaDodSO_4$, 1% in β-mercaptoethanol, and 10% with glycerol. Samples were boiled for 90 s prior to electrophoresis in 7.5% polyacrylamide gels as previously described (Moore et al., 1974). Electrophoresis was for 14–15 h at 3.0–3.5 mA/gel, and the gels were sliced into 1-mm fractions using a Mickle gel slicer. The slices were dissolved in a Nuclear Chicago Solubilizer (NCS) and counted in toluene-based liquid scintillant.

Additional Methods. Total viral protein was assayed by the method of Lowry et al. (1951), and phosphorus content was assayed as described by Bartlett (1959). Phospholipase C treatment of VS virus and assay of cholesterol content by oxidation with cholesterol oxidase were performed as described in detail elsewhere (Moore et al., 1977). Iodination of VS virus with lactoperoxidase was performed as described by Moore et al. (1974). Lipids were extracted by the technique of Bligh and Dyer (1959). VS virus was treated with trypsin (Schloemer and Wagner, 1975) prior to the cholesterol-exchange experiments and pelleted through a 20% sucrose pad at 165 000*g* in a SW60 Ti rotor at 4 °C. The pellet was resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5) or 10 mM Tris (pH 7.5) at the original concentration. Thin-layer chromatography of the virus lipids was performed as described by Mangold (1964) or by using a solvent system of chloroform/methanol (100:2, v/v) (Moore et al., 1977).

Results

Depletion of Cholesterol from the VS Virion Membrane. Cholesterol comprises ~40% of the lipid constituents of the VS virion membrane (McSharry and Wagner, 1971). Previous observations indicated that when VS virions were incubated with an excess of phosphatidylcholine vesicles the cholesterol was depleted from the virion membrane and concomitantly appeared in the vesicles (Moore et al., 1978). In an attempt to determine the distribution of cholesterol in the virion membrane and to understand its mechanism of transfer, experiments were devised to measure the rate of depletion and ex-

TABLE I: Percent Adherence of Lipid Vesicles to Intact and Spikeless VS Virions.[a]

incubat time (h)	intact virions: egg PC vesicles (0.3 mL) (+NaCl)	intact virions: egg PC/PS vesicles (1 mL) +NaCl	intact virions: egg PC/PS vesicles (1 mL) −NaCl	spikeless virions (+NaCl): vesicles (0.3 mL) egg PC	DMPC	DPPC	spikeless virions (+NaCl): egg PC/PS/cholesterol vesicles (1 mL)
0.5	1.3	1.1	0.1	0.1			0.1
1	2.0	1.4	0.1	0.2		17.7	0.2
3	4.3	1.9	0.2	0.7	13.5	14.5	0.2
6	6.3			0.9	13.4		0.3
15	3.8	3.3		0.6	19.6	18.3	0.4
17		4.0	3.1				
24	6.7	4.0	3.2	0.8	23.4	15.9	0.4

[a] Adherence of lipid vesicles to intact or spikeless VS virions was determined as the percent of [^{14}C]cholesterol-oleate (nonexchangeable vesicle marker) associated with the virions after incubation. 120 μg (protein) of intact or an equivalent amount of spikeless VS virions was incubated in the indicated volumes at 37 °C with 1.2 μmol of egg PC, egg PC/PS (4:1, mol/mol), DMPC, or DPPC vesicles containing [^{14}C]cholesterol-oleate. Virions rendered spikeless by trypsinization (equivalent to 150 μg of protein of intact virus) were also incubated at 37 °C with 0.81 μmol of egg PC/PS/cholesterol (4:1:5, mol/mol) vesicles containing [^{14}C]cholesterol-oleate. Incubation buffer consisted of 20 mM Tris and 100 mM NaCl (pH 7.5) (+NaCl) or 10 mM Tris (pH 7.5) (−NaCl). After incubation, the virions mixed with DMPC or DPPC vesicles were banded in sucrose equilibrium gradients at 37 °C; all others were banded at 4 °C, and the bands were pelleted at 4 °C. The ^{14}C dpm associated with the pellet was expressed as a percent of the total input vesicle radioactivity.

change of cholesterol from the virion membrane during reaction with lipid vesicles. Conditions were varied to compensate for differential adherence of vesicles to virions related to ionic strength and to vesicle composition and charge as well as adherence to virion surface glycoprotein. Therefore, for these experiments VS virus was incubated at 37 °C in 20 mM Tris, 100 mM NaCl (pH 7.5) or 10 mM Tris (pH 7.5) with either egg phosphatidylcholine vesicles or egg phosphatidylcholine/phosphatidylserine (4:1, mol/mol) vesicles containing [^{14}C]cholesterol-oleate as a nonexchangeable marker to measure vesicle sticking to virions. After incubation, the virus was banded in sucrose equilibrium gradients and subsequently pelleted; the free vesicles remained at the top of the gradients. Both reactants were assayed for cholesterol content and radioactivity.

Figure 1 shows semilogarithmic plots illustrating the kinetics of cholesterol depletion from the membrane of intact VS virus (Figure 1A) and VS virus stripped of glycoprotein spikes by trypsin (Figure 1B). The three experimental conditions employed with intact VS virus each resulted in two distinct exponential rates for the cholesterol-depletion process. However, the rates and extent of cholesterol depletion were greater at low ionic strength and when phosphatidylserine was included in the reacting vesicles. Greatly accelerated rates and extent of cholesterol depletion occurred when spikeless VS virions were exposed to egg phosphatidylcholine or to dioleolylphosphatidylcholine vesicles (Figure 1B).

Interacting vesicles have been found to adhere specifically to the glycoprotein spikes of VS virus, although the adherence to intact virions can be decreased when the vesicles contain a negatively charged lipid (phosphatidylserine) or when the incubation occurs at low ionic strength.

Table I illustrates the marked differences in adherence of vesicles to spikeless and intact virions. The introduction of phosphatidylserine into the vesicles decreased the adherence to intact vesicles nearly twofold compared to egg phosphatidylcholine alone, and the removal of salt from the incubation mixture caused a further decrease in adherence, especially at short incubation times. An increase in the volume of incubation with the phosphatidylserine-containing vesicles also may have caused a slight decrease in adherence. Removal of glycoprotein spikes resulted in a ca. tenfold reduction in adherence of egg PC vesicles, even in the presence of 0.1 M NaCl. Vesicles containing 40% PC, 10% PS, and 50% cholesterol were even less adherent to spikeless VS virions. Note, however, the

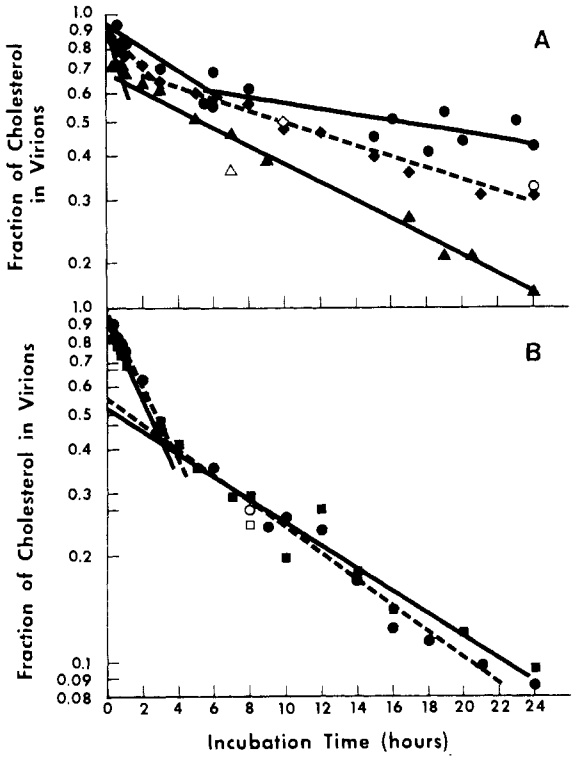

FIGURE 1: Kinetics of the depletion of cholesterol from (A) intact VS virus and (B) spikeless VS virus during incubation with phospholipid vesicles. Intact virus (120 μg of protein) or an equivalent amount of trypsinized (spikeless) virus was incubated at 37 °C with 1.2 μmol of phospholipid vesicles containing [^{14}C]cholesterol-oleate as a nonexchangeable marker. Intact virions (panel A) were incubated in 0.3 mL of 20 mM Tris, 100 mM NaCl (pH 7.5) with egg PC vesicles (●), 1 mL of the same buffer with egg PC/PS (4:1, mol/mol) vesicles (♦), or 1 mL 10 mM Tris (pH 7.5) with egg PC/PS (4:1, mol/mol) vesicles (▲). Spikeless virions (panel B) were incubated in 0.3 mL of 20 mM Tris, 100 mM NaCl (pH 7.5) with egg PC (●) vesicles or dioleoylphosphatidylcholine vesicles (■). Controls consisted of virus in the absence of vesicles. The reactions were stopped by banding the virus in sucrose equilibrium gradients at 4 °C and subsequently pelleting the virus band. The vesicles at the top of the gradient and the virus pellet were assayed for cholesterol content by the cholesterol oxidase reaction, and the cholesterol in the pellet was expressed as a percent of the total in both fractions. Open symbols depict cholesterol depleted from virions by twice the concentration of egg PC (○), egg PC/PS (◇), egg PC/PS in Tris without NaCl (△), or dioleoylphosphatidylcholine (□). The cholesterol content of the pellet was corrected for the contribution of cholesterol in adhering vesicles as described under Materials and Methods. All curves were drawn with the aid of linear-regression analysis of the data points.

marked adherence of DMPC and DPPC vesicles to spikeless VS virions.

From these results there appears to be an inverse correlation between the rates of cholesterol depletion and the amount of vesicle lipid adhering to the virus surface. An estimate of the number of vesicles adhering per virion can be calculated by assuming 4.25×10^{12} virions/mg of protein (Edward Dubovi, personal communication) and 75% recovery of the virions from the gradient and pelleting steps after incubation. The area occupied by an egg phosphatidylcholine molecule has been determined to be 71.7 Å^2 (Small, 1967); therefore, if the vesicles are homogeneous 250-Å diameter spheres, there should be 20 vesicles/virion at 5% adherence compared to only two vesicles/virion at 0.5% adherence. At the lower adherence levels (1%), there are apparently too few vesicles attached to the virions to block "free" vesicles from approaching the virus membrane, thus allowing a faster rate of depletion. This is further supported by the results with trypsinized VS virus.

Trypsin removes the glycoprotein spikes from the surface of VS virions (Schloemer and Wagner, 1975), which results in the decreased adherence of egg phosphatidylcholine vesicles illustrated in Table I. The correspondingly high rates of cholesterol depletion from these trypsinized virions are shown in Figure 1B with both egg phosphatidylcholine and dioleoylphosphatidylcholine vesicles. The adherence of dioleoylphosphatidylcholine vesicles to spikeless VS virus is nearly identical to that of the egg phosphatidylcholine (data not shown) and, similarly, the rates of depletion are almost indistinguishable. It should also be noted that the incubation volumes for experiments shown in Figure 1B are 0.3 mL, indicating that the increased rates of depletion from intact virions by reaction with phosphatidylserine-containing vesicles in Figure 1A were not a direct result of the increase in volume.

All the curves in Figure 1 are characterized by an initial rapid depletion rate followed by a second slower rate, both of which can be fitted to an exponential rate process. The second rate was independent of the vesicle to virus ratio, illustrated by the open symbols in Figure 1; these samples contained a twofold greater concentration of vesicles but caused little effect on the rate of cholesterol depletion. These data appear to preclude the possibility that the slower rate was simply due to saturation of the vesicles causing exchange of cholesterol back into the virion membrane. Moreover, considering the 36-fold excess of vesicle phospholipid to viral phospholipid in the other incubation mixtures, this possibility seems remote.

The most likely explanation for the initial rapid rate is that cholesterol is first being depleted from an accessible pool leaving a less accessible pool as the only source of cholesterol not yet depleted. The second slower rate must then reflect the rate of movement of cholesterol from the less accessible pool.

Rate of Cholesterol Depletion with Vesicle Phosphatidylcholines Containing Various Fatty Acyl Chains. As was shown above, egg phosphatidylcholine and dioleoylphosphatidylcholine exhibit the same rates of cholesterol depletion on interaction with trypsinized virus (Figure 1B) and had nearly identical levels of adherence. In contrast, when dimyristoyl- or dipalmitoylphosphatidylcholine vesicles were incubated with trypsinized VS virus under the same conditions, they showed considerably reduced rates of cholesterol depletion (Figure 2A). These reduced rates could be due to the greatly enhanced levels of adherence of these two phosphatidylcholines to trypsinized virus as illustrated in Table I or they could be due to inherent differences in the acceptance rate of these phosphatidylcholine vesicles for cholesterol.

To distinguish between these two possibilities, dimyristoylphosphatidylcholine vesicles were incubated with trypsinized virus for 3 h to allow the vesicles to adhere. The virus was then banded in sucrose equilibrium gradients, pelleted, and incubated with a second equal aliquot of either egg phosphatidylcholine or dimyristoylphosphatidylcholine vesicles for up to 6 h. The results of this experiment are shown in Figure 2B with the arrow indicating the time at which the second aliquot of vesicles was added. The egg phosphatidylcholine vesicles obviously have a faster rate of cholesterol depletion even when dimyristoylphosphatidylcholine vesicles are adherent to virions; however, the depletion rate in this experiment was slower than that during the incubation with egg phosphatidylcholine vesicles alone (Figure 1B). Identical results were obtained with dipalmitoylphosphatidylcholine vesicles (data not shown). Consequently, there does appear to be an inherently slower acceptance rate for cholesterol of phosphatidylcholine vesicles containing longer and more saturated fatty acids, although some of the differences in rates apparently are due to differences in vesicle adherence.

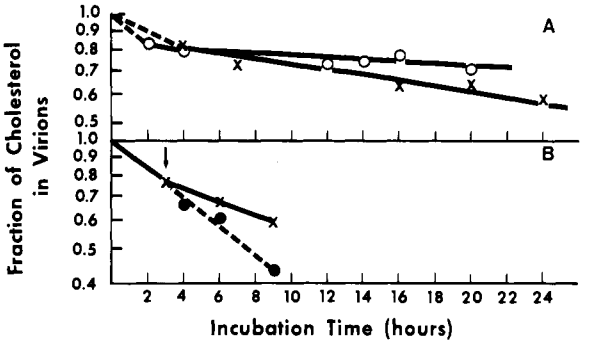

FIGURE 2: Kinetics of depletion of cholesterol from spikeless VS virions incubated with dimyristoylphosphatidylcholine (DMPC) (x) or dipalmitoylphosphatidylcholine (DPPC) (O) vesicles (panel A) and a comparison of the depletion kinetics upon reincubation with egg phosphatidylcholine (●) and dimyristoylphosphatidylcholine (x) vesicles (panel B) after initial incubation with dimyristoylphosphatidylcholine. Spikeless VS virus (equivalent to 120 μg of protein of intact virus) was incubated at 37 °C in 0.3 mL of 20 mM Tris, 100 mM NaCl (pH 7.5) with 1.2 μmol of dimyristoyl- (x) or dipalmitoylphosphatidylcholine (O) vesicles containing [^{14}C]cholesterol-oleate for the times indicated in panel A. The virus was banded at 37 °C in sucrose equilibrium gradients, and the bands were pelleted at 4 °C. The cholesterol content of the vesicles and virions was calculated as in Figure 1. In panel B, spikeless VS virions were incubated with dimyristoylphosphatidylcholine vesicles under identical conditions to panel A. The virus was banded and pelleted after a 3-h incubation and a second aliquot (↓) of 1.2 μmol of dimyristoylphosphatidylcholine (x) or egg phosphatidylcholine (●) vesicles was added to the pellet and the incubation continued. After the times indicated, the virions were again banded and pelleted and the cholesterol content was analyzed as in Figure 1.

Cholesterol Exchange between VS Virus and Lipid Vesicles. The foregoing cholesterol-depletion experiments resulted in almost complete removal of cholesterol from the virion membrane. Since this marked alteration in cholesterol content presumably perturbs the virion membrane, nondepleting experiments were devised to determine the rate of simultaneous exchange of cholesterol between VS virions and cholesterol-containing lipid vesicles. The kinetics of the exchange process was followed by radiolabeling the viral cholesterol with its metabolic precursor, [^{3}H]mevalonic acid. To ensure that the label was incorporated exclusively into cholesterol, the lipid species of the virus were isolated by TLC as described under Materials and Methods. One major peak corresponding to authentic cholesterol contained ~93% of the total radioactivity and a minor leading shoulder contained ~7% (data not shown). The minor component behaved similarly to cholesterol during the exchange experiments; i.e., it exchanged at the same rate.

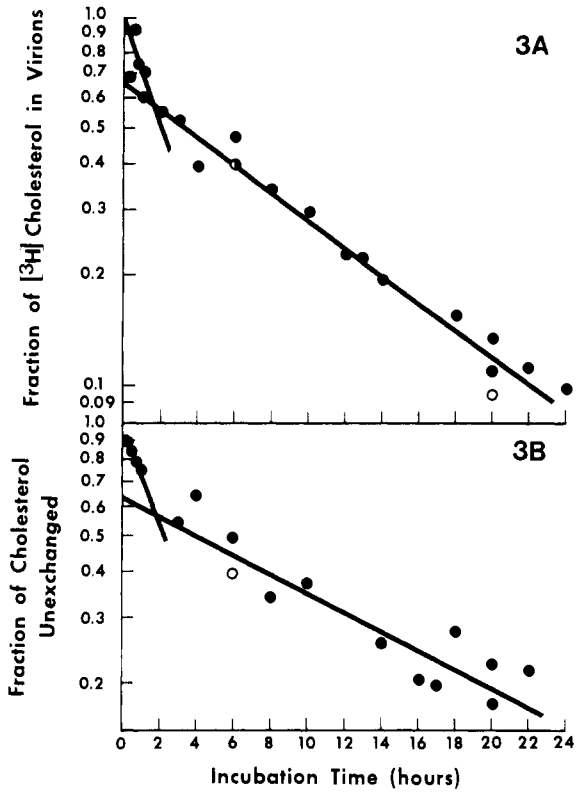

FIGURE 3: The kinetics of [^{3}H]cholesterol exchange from trypsinized VS virus into egg PC/PS/cholesterol (4:1:5, mol/mol) vesicles (panel A) and the kinetics of exchange of [^{14}C]cholesterol from egg PC/PS/cholesterol (4:1:5, mol/mol) vesicles into unlabeled trypsinized VS virus (panel B). 150 μg of spikeless VS virus was incubated at 37 °C with 0.8 μmol of vesicle phospholipid in a total volume of 1 mL of 20 mM Tris, 100 mM NaCl (pH 7.5). The reaction was terminated by banding the virions in sucrose equilibrium gradients at 4 °C. The virus band was pelleted and resuspended in 20 mM Tris, 100 mM NaCl (pH 7.5) prior to scintillation counting. The ordinate in panel A shows the fraction of total [^{3}H]cholesterol remaining in the virus pellet after incubation. The amount of cholesterol exchanged into virions from vesicles labeled with [^{14}C]cholesterol was determined from the ^{14}C dpm contained in the virus pellet multipled by the specific activity of the cholesterol in the vesicles (μg of cholesterol/^{14}C dpm). The fraction of cholesterol exchanged (unexchanged = 1 − exchanged in panel B) was then calculated by expressing the amount of cholesterol as a fraction of the known cholesterol content of the virus. Panels A and B represent semilogarithmic plots drawn using linear-regression analysis of the data points. The open circles represent samples to which twice the concentration of vesicles was added. All values were corrected for adherence to virions of vesicles which contained nonexchangeable markers, [^{14}C]cholesterol-oleate in panel A and [^{3}H]triolein in panel B.

When labeled cholesterol in vesicles was exchanged into unlabeled VS virus, this minor shoulder was not detected in the viral lipids. The minor [^{3}H]mevalonate-derived component in the virion membrane could represent an intermediate in the biosynthetic pathway of cholesterol or another minor sterol; nevertheless, it did not affect the rate of cholesterol exchange assayed by following transfer of total radioactive counts.

For the cholesterol-exchange experiments, lipid vesicles containing 50 mol % cholesterol were used to minimize cholesterol loss from the virion membrane. Throughout the 24-h period of incubation, no net loss of total cholesterol could be detected in the vesicles or virions. The vesicles also consisted of 10 mol % phosphatidylserine, and the virions were trypsinized to reduce vesicle adherence, which was again assayed by counts of nonexchangeable markers, [^{14}C]cholesterol-oleate or [^{3}H]triolein, associated with the virions. Although vesicle adherence was quite low (see Table I), the total radioactivity in the virions was corrected for the radioactive cholesterol in the adhering vesicles, which accounted for a maximum of 4–5% of the total.

Figure 3A portrays the removal of [^{3}H]cholesterol from the VS virion membrane. Again, the data can be fitted to two exponential rate processes: an initial rapid phase followed by a second slower phase. The initial rate is dependent on the vesicle to virus ratio, evidenced by a threefold decrease in the initial exchange rate with a threefold lower vesicle concentration (data not shown). The second slower phase was found to be independent of the vesicle concentration, which is illustrated by the open symbols in Figure 3A representing twice the concentration of vesicles.

If the cholesterol in the two kinetically defined pools of the viral bilayer were not radiolabeled to the same specific activity or if a small perturbation of the bilayer altered its pool sizes or integrity, the exchange results could be misleading. To ensure that this process represented a true reversible exchange of cholesterol between the two pools in the VS virion membrane, the exchange of [^{14}C]cholesterol from vesicles into unlabeled virions was determined under identical conditions. The amount of cholesterol exchanged (μg) into virions was calculated by dividing the ^{14}C dpm in the virions by the specific activity (^{14}C dpm/μg of cholesterol) of the vesicles and expressing this as a percent of the total viral cholesterol (μg). Figure 3B shows the kinetics of this exchange reaction, which is plotted as the fraction of total viral cholesterol *unexchanged* at each time point. Again, the data fit two exponential rate processes with parameters similar to those of the preceding exchange and depletion experiments.

Kinetics of Transmembrane Movement of Cholesterol. The observation that the initial rate of cholesterol exchange is dependent on the virus to vesicle ratio whereas the second rate is not suggests that the virion membrane cholesterol exists in two kinetic pools that reversibly exchange cholesterol between the pools and from only one pool to the vesicles. If it is assumed that the movement of cholesterol can be described by a two-pool model under reversible equilibrium conditions, then the following mechanism applies:

$$(\text{cholesterol})_{\text{inner}} \underset{k_i}{\overset{k_o}{\rightleftharpoons}} (\text{cholesterol})_{\text{outer}} \xrightarrow{k_v} \text{vesicles}$$

where k_o and k_i are the rate constants for equilibration of cholesterol between pools and k_v is the rate constant for its removal into vesicles. Under these conditions, the system is analogous to that of Bloj and Zilversmit (1976) and can be treated identically using the slopes and intercept in Figures 1 and 3 to calculate k_o and k_i. The half-time for cholesterol equilibration is given by $t_{1/2} = 0.693/(k_o + k_i)$, and the pool sizes can be calculated from $r_o = k_o/(k_o + k_i)$ and $r_i = 1 - r_o$, where r_o and r_i are the sizes of the outer and inner pools of cholesterol, respectively.

The calculated half-times and pool sizes for the depletion and exchange experiments are listed in Table II. The values for intact virions were only calculated from the low ionic strength conditions, since the greater adherence of the other incubations altered the kinetic rates significantly. The half-times for equilibration of cholesterol between the two pools varied between 4.3 and 6.2 h, depending on the incubation conditions. These values indicate very good agreement between the various techniques, although the calculated pool sizes are somewhat more inconsistent. The external pool size ranged from 21 to 41% of the total cholesterol, which in all cases leads to an asymmetric distribution of cholesterol.

Intactness of VS Virus during Cholesterol Depletion and Exchange. The integrity of VS virions during the cholesterol depletion or exchange experiments was determined by the loss

TABLE II: Kinetic Parameters of Cholesterol Depletion or Exchange from the VS Virion Membrane.[a]

incubation conditions	virion cholesterol: equilibration between pools – $t_{1/2}$ (h)	pool size (% total): outer (r_o)	pool size (% total): inner (r_i)
cholesterol depletion			
Egg PC/PS (−NaCl)[b]	5.2	41	59
Egg PC[c]	5.3	21	79
DOPC[c]	5.9	25	75
radiolabeled cholesterol exchange			
from VS virus[c]	4.3	31	69
into VS virus[c]	6.2	37	63

[a] The parameters were calculated using a reversible two-pool analysis of the data from Figures 1 and 3, analogous to the derivation of Bloj and Zilversmit (1976). Depletion or exchange of cholesterol from the virus membrane was characterized by equations of the general form:

$$\frac{\text{unexchanged cholesterol}}{\text{total cholesterol}} = H_1 e^{-g_1 t} + H_2 e^{-g_2 t}$$

where g_1 and g_2 are the rapid and slow exponential rate constants (see Figures 1 and 3), respectively. If the location and movement of cholesterol are described by a two-pool model under reversible equilibrium conditions, then these exponential rate constants and the intercepts in Figures 1 and 3 can be used to calculate k_o and k_i, the rates for cholesterol transposition from inner to outer and from outer to inner pools, respectively. The half-time for equilibration of the two pools is given by $t_{1/2} = 0.693/(k_o + k_i)$ and the pool sizes (r) are obtained from $r_o = k_o/(k_o + k_i)$ and $r_i = (1 - r_o)$, where r_o and r_i are the outer and inner pools, respectively. [b] Intact VS virus. [c] VS virus rendered spikeless by trypsinization.

TABLE III: Loss of Protein from VS Virus Banded on Equilibrium Gradients after Incubation with Lipid Vesicles.[a]

incubat time (h)	% radioactivity at top of gradient: spikeless virions (+NaCl) PC/PS/cholesterol (150 μg of virus)	spikeless virions (+NaCl) DOPC (120 μg of virus)	intact virions (120 μg), PC/PS (−NaCl)
1	2.7		9.7
5	4.5	9.0	10.1
14	6.8	16.1	
20	7.5	14.8	13.1
control[b]	2.4	2.2	1.2

[a] 120 μg of intact VS virus or trypsinized VS virus (equivalent to 120 or 150 μg of intact virus) labeled with [^{3}H]leucine was incubated at 37 °C in 0.3 mL of 20 mM Tris and 100 mM NaCl (pH 7.5) (+NaCl) or 1 mL 10 mM Tris (pH 7.5) (−NaCl). 0.8 μmol of egg PC/PS/cholesterol vesicles (4:1:5, mol/mol) or 1.2 μmol of DOPC or egg PC/PS (4:1, mol/mol) vesicles was included in the incubation. Each mixture was separated on a 5-mL sucrose equilibrium gradient and the top 1.5–2.0 mL was used for scintillation counting. The radioactivity at the top of the gradient was expressed as a percent of the total viral input radioactivity. [b] The controls consisted of VS virus incubated in buffer alone for 20 h.

of [^{3}H]leucine-labeled protein from virions incubated with different vesicles before banding in sucrose equilibrium gradients. The radioactivity was isolated from vesicles at the top of the gradients and was expressed as a percent of the total viral radioactivity in the incubation mixtures. The loss of radiolabeled protein for three incubation conditions is illustrated in Table III. There was a relatively significant loss of protein from intact virions incubated with phosphatidylcholine/phosphatidylserine vesicles or spikeless virions incubated with dioleoylphosphatidylcholine vesicles. This loss did not substantially increase with time under these cholesterol-depleting conditions. On the other hand, spikeless virions incubated with phosphatidylserine/phosphatidylcholine/cholesterol vesicles exhibited a twofold smaller loss of protein, possibly due to the unaltered cholesterol content of the virion membrane.

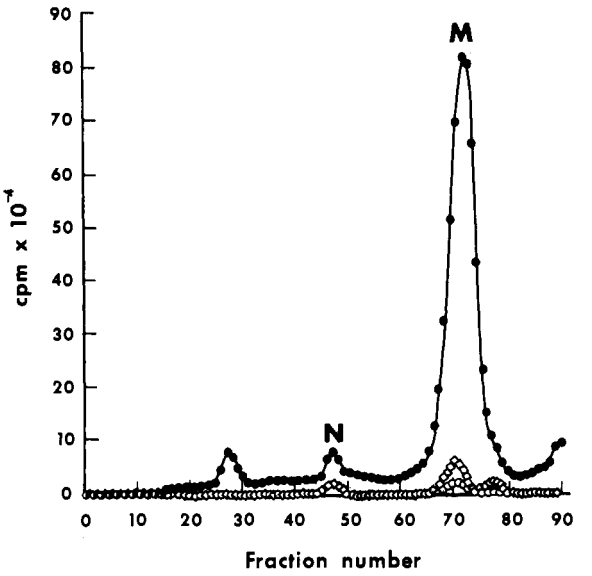

FIGURE 4: Electropherograms of lactoperoxidase-catalyzed [^{125}I]protein to illustrate the membrane integrity of spikeless VS virions following incubation with egg PC/PS/cholesterol vesicles for 6 (○) and 24 h (◇) compared with ^{125}I-labeling of Triton-disrupted VS virions (●). All VS virion samples contained 50 μg of total protein prior to trypsin treatment to remove glycoprotein spikes. Two virion suspensions were incubated with 0.095 μmol of egg phosphatidylcholine/phosphatidylserine/cholesterol (4:1:5, mol/mol/mol) vesicles for 6 or 24 h, respectively; virions were then separated from vesicles by pelleting through a 20% sucrose pad by centrifugation at 120 000g for 30 min in a SW60 Ti rotor. A control sample of VS virions not exposed to vesicles was suspended in 10 mM Tris (pH 7.5) and disrupted with 1% Triton X-100 for 1 h at room temperature. The preparations of intact VS virions exposed to vesicles and the Triton-disrupted virions were each labeled with ^{125}I by the lactoperoxidase procedure (Moore et al., 1974), and the extracted proteins were then subjected to electrophoresis in 7.5% polyacrylamide–NaDodSO$_4$ gels, as described under Materials and Methods. Under the conditions of VS virion disruption, the only tyrosine residues labeled significantly are those of the M protein and, to a very limited extent, the N protein (Moore et al., 1974).

Another method used to assay the intactness of the virion membrane after interaction with vesicles was surface labeling by lactoperoxidase-catalyzed iodination with ^{125}I. This technique can distinguish between sealed and leaky membranes rather than merely measuring complete disruption. For these studies, spikeless virions were incubated with phosphatidylserine/phosphatidylcholine/cholesterol vesicles prior to ^{125}I labeling. If the membrane remains intact, only a small percentage of the total viral M protein, which is reported to line the inner surface of the virion membrane (Wagner, 1975), should be labeled (Moore et al., 1974). The two other major internal proteins, L and N, do not label well unless fully denatured with NaDodSO$_4$ (Moore et al., 1974).

Figure 4 shows polyacrylamide gel electropherograms of lactoperoxidase iodinated proteins of Triton-disrupted VS virions compared with spikeless, but otherwise intact, VS virions after incubation with mixed PC/PS/cholesterol vesicles for 6 and 24 h, respectively. The major protein labeled is M protein with a minor amount of N protein. M protein labeling increased with time to a maximum of 60 000 cpm in the peak

fraction at 24 h. When VS virus is fully disrupted with Triton X-100 in low ionic strength buffer, the glycoprotein and membrane barrier are stripped from the virion, leaving the other proteins, including M protein, intact (Kelley et al., 1972). A polyacrylamide electropherogram of Triton X-100 disrupted virus containing an equal amount of viral proteins exposed the M protein to a maximum iodination of 800 000 cpm in the peak fraction. Thus, less than 10% of M protein was labeled even after prolonged incubation (24 h) with vesicles, indicating that the membrane of these virions remained essentially intact.

Mechanism of Cholesterol Transfer between Membranes. There would appear to be two possible mechanisms by which cholesterol can be transferred from membrane to membrane: (1) it could migrate through the aqueous phase separating the membrane systems or (2) membrane–membrane contact could be required for the transfer. There are two observations from this study which suggest that the latter mechanism is operating. The first involves the depletion of cholesterol from the intact VS virion membrane. Unless the conditions were chosen carefully, a relatively large percentage of vesicles adhered to the virus surface, thereby causing a decreased rate of depletion. These adherent vesicles are not irreversibly fused with the virion membrane and do not appear to be in direct contact with it, since they can be detached from the virus by trypsin proteolysis of glycoprotein spikes (Patzer et al., in preparation). The adherent vesicles appear to block, by steric hindrance, the transfer of cholesterol, which would be consistent with the hypothesis that cholesterol transfer occurs through membrane–membrane contact.

An alternative explanation, however, might be that the virion cholesterol partitions into the adhering vesicles, thereby reducing the concentration of cholesterol in the membrane and, consequently, in the aqueous phase. This reduced concentration of cholesterol in the aqueous phase could result in a proportionately slower rate of depletion through the aqueous phase. When the amount of lipid adhering to intact virus is determined for egg phosphatidylcholine vesicles at times greater than 6 h (Table I), a 2.2-fold increase in total phospholipid (vesicle + virion) can be calculated (data not shown). The longer incubation times correspond to the second exponential rate of depletion (Figure 1), which is ca. four times slower with egg phosphatidylcholine vesicles and intact VS virus compared to trypsinized VS virus with the same vesicles. Consequently, the dilution effect does not appear to be great enough to account for the loss in depletion rate, although the accuracy of the adherence measurements still leaves some doubt.

The second observation bearing on this problem was the change in the initial rate of [^{3}H]cholesterol exchange from the virion membrane with different concentrations of phosphatidylserine/phosphatidylcholine/cholesterol vesicles. The half-time for the initial exponential rate in Figure 3A is 2.2 h, whereas a threefold lower concentration of vesicles resulted in a half-time of 6.3 h (data not shown). Since these incubations involved a large excess of vesicle to viral lipid and the equilibrium between cholesterol in the membrane and cholesterol in the aqueous environment is vastly in favor of the membrane (Haberland and Reynolds, 1973), the rate-determining step of cholesterol transfer through the aqueous phase would be its "off" time from the virion membrane. This would require that transfer through the aqueous phase is independent of the vesicle concentration. We have already seen that transfer from the virion membrane to vesicles is directly dependent on the vesicle concentration. Therefore, it would appear that membrane–membrane contact provides the most satisfactory explanation for cholesterol transfer from VS virus to vesicles.

Discussion

Data presented here revealed that cholesterol in the VS virion membrane resides in two kinetic pools, one rapidly and one slowly exchangeable. The rapid exchange rate is dependent on the concentration of lipid vesicles as well as the degree of unsaturation and chain length of the fatty acyl residues in the phosphatidylcholine acceptor vesicles. The more unsaturated dioleoylphosphatidylcholine and egg phosphatidylcholine vesicles deplete cholesterol at much more rapid rates than do dimyristoylphosphatidylcholine vesicles or the slightly slower dipalmitoylphosphatidylcholine vesicles. These results, together with those of Bloj and Zilversmit (1977), suggest that cholesterol is not only removed more slowly but is also incorporated more slowly into phospholipid vesicles with longer chain saturated fatty acids. The slower rates in both directions cannot be explained by the observations of DeKruijff et al. (1974) that phosphatidylcholines with longer, more saturated fatty acids have a lower affinity for cholesterol. Cholesterol would be expected to be removed more rapidly from phosphatidylcholine vesicles containing long-chain, saturated fatty acids, if affinity was the only constraint.

The interpretation of two kinetic pools in protein-free lipid vesicles is straightforward in the absence of phase separations. The only physically distinct locations for two independent lipid pools must be the inner and outer halves of the bilayer. In biological membranes, however, one cannot unambiguously assign a physical location to kinetically defined pools, largely due to the presence of membrane proteins. In some cases, boundary lipid surrounding membrane proteins, rather than the transmembrane location of lipids, may cause the appearance of a less accessible pool of lipid. In VS virus, two proteins could possibly interact with membrane lipids: the glycoprotein (G) which has a hydrophobic tail fragment embedded in the membrane (Schloemer and Wagner, 1975) and the matrix (M) protein which lines the inner side of the membrane (Wagner, 1975) and may ionically interact with the membrane. There are a number of reasons why the interaction of cholesterol with membrane proteins in VS virus seems to be an unlikely explanation for the two kinetic pools. First of all, the magnitude of the slowly accessible pool would require that the majority of cholesterol in the virion membrane be immobilized as boundary lipid. Secondly, the membrane-embedded portion of G protein is apparently lost upon trypsinization (Schloemer and Wagner, 1975) without any detectable effect on the pool sizes or exchange rates. Finally, the aqueous, soluble M protein (Knipe et al., 1977) would seem to be an unlikely candidate to bind a hydrophobic cholesterol molecule, although this interaction would also result in the distribution of the less accessible pool of cholesterol on the inner side of the membrane. At present, the most plausible interpretation for two kinetic pools of cholesterol in the VS virion membrane appears to be a transmembrane distribution of cholesterol, with the less accessible pool representing the cholesterol in the inner half of the virion-membrane bilayer.

The exchange of cholesterol from membrane to membrane (e.g., virion to vesicle) can occur either directly through the aqueous medium or by a collisional mechanism through the area of membrane–membrane contact. The exchange of a model lipid molecule, pyrene, between high-density lipoproteins was found by Charlton et al. (1976) to fit more closely the movement of monomers through the aqueous phase than collision of lipoproteins. Using NMR spectroscopy to follow the exchange of cholesterol between phosphatidylcholine–cholesterol and phosphatidylcholine vesicles, however, Haran and Shporer (1977) could not distinguish between first- and

second-order reaction kinetics. Thus, the mechanism of transfer could not be conclusively determined. In a previous study with VS virus, we suggested that the majority of the cholesterol either was not transferred through the aqueous phase or was undetected by the enzyme cholesterol oxidase (Moore et al., 1978). Two observations in the present study also indicate a collisional mechanism for cholesterol exchange. First, vesicles adhering to virions can apparently slow the depletion of cholesterol from the virion membrane, and, secondly, the rate of exchange of [^{3}H]cholesterol from virions was dependent on the initial vesicle concentration, precluding migration solely through the aqueous phase. In addition, the exchange of labeled cholesterol and the depletion (mass) of cholesterol apparently occurred by the same mechanism, since they were characterized by nearly identical rate constants, in agreement with the erythrocyte membrane (Lange and D'Alessandro, 1977).

The half-time for equilibration of cholesterol between its two kinetic pools ranged between 4.3 and 6.2 h for the various incubation procedures. We have interpreted this as the half-time for the rate of transmembrane equilibration of cholesterol between monolayers of the VS virion membrane. These results differ markedly from those with influenza virus (Lenard and Rothman, 1976), in which two nearly equal pools were reported, the inner pool being only slowly exchangeable (half-time $\geq$13 days). There are no obvious experimental reasons for these different observations. Both studies used similar incubation conditions, although Lenard and Rothman included protease in their incubation mixture and no correction was mentioned for vesicles adhering to influenza virions. Neither of these factors would be expected to produce such a large discrepancy in the rate of transmembrane movement. Consequently, the different rates could be due entirely to inherent characteristics of the two virus membranes.

Smith and Green (1974) were the first to show that an analogue of cholesterol, sterophenol, underwent transbilayer movement in phospholipid vesicles with a half-time of 1–1.5 h. More recent studies showed that transmembrane movement of cholesterol occurred in lipid vesicles composed of phosphatidylcholines with various fatty acyl chains (Bloj and Zilversmit, 1977) and in erythrocyte membranes (Lange et al., 1977). The extremely fast transbilayer movement during exchange between lipid vesicles was not rate limiting, resulting in only one kinetic pool of cholesterol observed. Similarly, the rate of transmembrane movement of cholesterol in erythrocyte membranes was much more rapid (half-time $\leq$50 min) than in VS virus, although the study involved mechanical breakage of the membrane which could cause some rearrangement of the membrane constituents. Previous studies with both lipid vesicles (Poznansky and Lange, 1976, 1978) and erythrocytes (Gottlieb, 1976) led to the conclusion that no transmembrane movement occurred in either system. The discrepancies in these two systems remain to be resolved.

Although the transmembrane movement of cholesterol in VS virus does not appear to be as rapid as the reported rates in lipid vesicles and erythrocytes, the data reported here suggest two very mobile pools of cholesterol in the inner and outer monolayers of the virion membrane. Since cholesterol transposition occurs on the order of hours, equilibration of the two pools should be possible; however, an asymmetric distribution of cholesterol persists. Other membrane constituents may play a role in maintaining this asymmetry. The VS viral glycoprotein (G) on the external surface may exclude cholesterol from its proximity or the matrix (M) protein at the inner surface may select for cholesterol, although this is not consistent with our interpretation of the data. The asymmetric distribution of cholesterol in myelin membranes is presumably due to specific interactions with proteins (Caspar and Kirschner, 1971). In addition, phospholipids could play a role in maintaining the asymmetry. Demel et al. (1977) reported that the choline phospholipids have a stronger affinity for cholesterol than amino phospholipids; however, sphingomyelin and phosphatidylcholine are located in the external monolayer of the VS virus membrane (Patzer et al., 1978). An additional consideration is the location of cholesterol in mixed phospholipid–cholesterol vesicles. Above 30 mol % cholesterol, the vesicles have an asymmetric distribution with the majority of cholesterol in the internal monolayer (Huang et al., 1974; DeKruijff et al., 1976). It was postulated (DeKruijff et al., 1976) that highly curved regions, including areas around membrane proteins, may result in higher concentrations of cholesterol. Possibly, there are more highly curved regions in the inner monolayer than the outer monolayer of the VS virus membrane.

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Characteristics and Polyadenylate Content of the Actin Messenger RNA of Mouse Sarcoma-180 Ascites Cells†

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ABSTRACT: Actin is a major protein component of mouse sarcoma-180 ascites cells. It is produced in large amounts in a wheat germ cell-free system supplemented with total polysomal RNA from these cells. Adsorption of the poly(A)$^+$ RNA onto oligo(dT)-cellulose leads to the retention of the template activity for most polypeptides but leaves a substantial portion of the actin mRNA in the unadsorbed fraction. The actin mRNA that binds to oligo(dT)-cellulose contains a large proportion of chains unable to bind to Millipore filters. The other major poly(A)$^+$ mRNAs bind nearly as well to Millipore filters as to oligo(dT)-cellulose. This implies that the distribution of poly(A) sizes in the actin mRNA is atypical, with a large proportion of the chains having relatively short poly(A) segments and with many chains containing either very short segments or no poly(A) at all. The translation of actin mRNA is preferentially inhibited in the presence of excess poly(A)$^+$ RNA. Both the poly(A)-containing and poly(A)-deficient forms of actin mRNA exhibit this sensitivity to inhibition of translation. Inhibitors of polypeptide chain initiation such as poly(A) or poly(U) did not inhibit preferentially actin mRNA translation. The poly(A)$^-$ actin mRNA appears to be functional in the cell, since it is found associated with polysomes in cytoplasmic extracts. A 26-fold enrichment in the poly(A)-deficient actin mRNA was achieved by first isolating a 50S ribonucleoprotein particle from (ethylenedinitrilo)tetraacetic acid treated polysomes and subjecting the deproteinized material to oligo(dT)-cellulose fractionation, followed by zone centrifugation.

The physiological significance of the poly(A)[1] sequence at the 3′ end of eukaryotic mRNA chains remains poorly understood. This segment is present in most mRNAs characterized so far but is absent from the RNA species that code for histones (Adesnick & Darnell, 1972; Greenberg & Perry, 1972). Comparisons between the histone mRNAs and poly(A)-containing species have failed to reveal significant functional differences that might be attributed to this sequence. The histone mRNAs do not appear to be particularly unstable (Perry & Kelley, 1973) nor do they seem to have any unusual requirements for translation. Removal of the poly(A) from normally polyadenylylated species, however, leads to a marked loss of stability when the depleted species are injected into frog oocytes (Huez et al., 1974, 1975). This feature points to a role for the poly(A) in the control of mRNA stability, but it fails to account for the apparent stability of the histone mRNAs. The status of the latter species is further complicated by the finding that they can also exist as polyadenylylated components in sea urchin eggs (Levenson & Marcu, 1976; Ruderman & Pardue, 1976). The protamine mRNA of developing trout testes has also been shown to occur in both forms (Iatrou & Dixon, 1977).

The study of additional mRNA species that exist without poly(A) could conceivably reveal significant functional features specifically related to this sequence. It is suspected that a substantial portion of the mRNA of mammalian cells lacks a poly(A) segment (Milcarek et al., 1974). We observed that the polysomal RNA of certain mammalian tumor cells depleted of poly(A)-containing species can promote the synthesis of a major polypeptide of 45 000 molecular weight in wheat germ extracts (Sonenshein et al., 1976). This polypeptide has been identified as actin by several investigators (Hunter & Garrels, 1977; Kaufmann et al., 1977). In the present report we confirm the identification of this polypeptide as actin and provide evidence that it exists as polyadenylylated and non-polyadenylylated forms, both apparently functional in the intact cells. The two types of actin mRNA chains show the same characteristics of translation in vitro. It is possible that

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[1] Abbreviations used are: poly(A), polyadenylic acid; poly(U), polyuridylic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; ATP and GTP, adenosine and guanosine triphosphates; oligo(dT), oligodeoxythymidylate; DNase, deoxyribonuclease I (EC 3.1.4.5); EDTA, (ethylenedinitrilo)tetraacetic acid.

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