The membrane of vesicular stomatitis virus could be readily enriched with exogenous cholesterol by exposing virion suspensions to serum lipoproteins forming complexes with cholesterol or to a carrier of polyvinylpyrrolidone/bovine serum albumin mixed with cholesterol. These procedures increased the cholesterol content of the virion membrane from ~35 to ~60% of the total lipid. Endogenous cholesterol in the virion membrane was not oxidized by cholesterol oxidase, but supplementation with exogenous cholesterol to levels of 48 mol% or greater exposed the β-OH group of virion cholesterol to oxidation by the enzyme. At virion membrane levels of 54% cholesterol, 70 to 75% of total cholesterol (endogenous + exogenous) was rendered accessible to cholesterol oxidase. Similar results were obtained with a model membrane system of mixed unilamellar vesicles prepared by ultrasonication. Cholesterol present in vesicles composed wholly or predominantly of choline phospholipids became accessible to cholesterol oxidase only when the cholesterol concentration exceeded 42 to 46 mol%. When concentrations of cholesterol reached 50 mol%, cholesterol was oxidized 95% in vesicles containing egg phosphatidylcholine and 63% in vesicles containing sphingomyelin. However, even at concentrations as low as 20 mol%, cholesterol present in phosphatidyserine vesicles was readily accessible to cholesterol oxidase. Oxidizability of cholesterol in phosphatidylcholine vesicles was not influenced by phase transitions. It appears likely that the accessibility of membrane cholesterol to cholesterol oxidase is determined not only by the size but also by spacing of bulky headgroups of choline phospholipids dispersed by intercalated cholesterol.

The ratio of cholesterol/phospholipid appears to be an important determinant in regulating the properties of biological membranes (1). Although several models have been proposed to explain interaction of cholesterol with different phospholipids in model membrane systems (2–5), little information is available on lateral and transbilayer distribution of cholesterol and the effects of various phospholipids and membrane proteins. Several recent studies have shown that certain properties of biological membranes can be altered by enrichment with or depletion of cholesterol (6, 7). These effects were attributed mainly to alteration of membrane microviscosity or to differential exposure of intrinsic membrane proteins. All studies are probably of limited significance since they provide data only on average composition and not on factors which might influence heterogeneity in location of cholesterol within various membrane domains.

The role of cholesterol in the organization of biological membranes has been studied recently by several investigators who have used cholesterol oxidase (EC 1.1.3.6) as an enzymatic probe (8, 9). Inaccessibility to cholesterol oxidase has been demonstrated for the exterior membrane surface of human erythrocytes (8) and virus (9) as well as sheep erythrocytes,1 mycoplasma,2 and tissue culture cells.3 However, membrane cholesterol becomes readily susceptible to oxidation by cholesterol oxidase when phospholipase C is used to remove the polar head groups of phospholipids on the exterior layer of viral, erythrocyte, or vesicle membranes (9) or when membrane surfaces rich in aminophospholipids, such as the cytoplasmic layer of the erythrocyte membrane (8) or mixed cholesterol aminophospholipid vesicles (9), are exposed to cholesterol oxidase.

In the present study, cholesterol oxidase was used as a molecular probe to determine how supplementation with exogenous cholesterol affects the organization of the membrane of vesicular stomatitis virus (VSV). Our laboratory has extensively studied the VSV membrane, which contains 50% lipid, and only two well characterized proteins, internal M and intrinsic exterior spike glycoprotein G (10–12). Cholesterol is an important constituent of the VSV membrane, comprising 35 to 40% of total lipids (11). A parallel study was also conducted with small unilamellar mixed vesicles to determine how phospholipid content and cholesterol concentration influence accessibility to cholesterol oxidase.

**MATERIALS AND METHODS**

-Chemicals—Egg phosphatidylcholine and phosphatidylethanolamine were obtained from Makor Chemicals Ltd., Jerusalem, Israel; beef brain sphingomyelin, phosphatidylserine, and dipalmitoyl phosphatidylcholine dimyristoyl phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, MO. Cholesterol was obtained from Supelco, Inc., Bellefonte, PA. Polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA, lipid-free) were also obtained from Sigma. All

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1 Y. Barenholz, unpublished data.
2 S. Rottem, personal communication.
3 N. P. Moore, unpublished data.
4 The abbreviations used are: VSV, vesicular stomatitis virus; PVP, polyvinylpyrrolidone; BSA, bovine serum albumin; PBS, phosphate-buffered saline (NaCl/PO4, 1.5 mm KH2PO4, 2.7 mM KCl, 1 mM CaCl2, and 0.6 mM MgCl2, pH 7.5); BME medium, basal medium Eagle's medium; FCS, fetal calf serum.
lipids were found to be at least 98% pure when analyzed by thin layer chromatography on Silica Gel HR plates.

**Virus**—As previously described (9, 11, 13), the Indiana serotype of VSV was prepared by infecting confluent monolayer cultures of baby hamster kidney (BHK) 21 cells with cloned virus at a multiplicity of 0.1 plaque-forming units/cell. Homogeneous bullet-shaped virus was harvested at 18 to 20 h postinfection and was purified by differential, rate-zonal, and equilibrium centrifugation in sucrose and lactate gradients (13). Pelleted VSV was suspended in PBS at a final concentration of 2 to 5 mg/ml and was stored at −80°C.

**Preparation of Lipid Vesicles**—Small, uniform unilamellar vesicles were prepared, as previously described (14), by ultrasonication and centrifugation of various lipophilic lipids that were resuspended in 3 to 4 ml of 20 mM Tris/NaCl buffer (pH 7.6). The suspensions were sonicated under a nitrogen atmosphere at different temperatures depending on the lipids. The suspensions then were centrifuged at 100,000 × g for 90 min in the SW 50.1 rotor to pellet the residual multilamellar vesicles, leaving the supernatant unilamellar vesicles.

**Lipid Analyses**—Cholesterol concentrations were measured by the cholesterol oxidase method (15) and phospholipid concentrations were determined by the method of Mannini (16). Accessibility to oxidation of cholesterol present in intact vesicles and intact virus was determined by incubating them in the absence of detergent with cholesterol oxidase in Tris/NaCl buffer (pH 7.6). Incubation was continued until oxidation of cholesterol was complete, as determined by the reaction reaching a plateau at the desired temperature.

**RESULTS**

**Supplementation of VSV Membranes with Exogenous Cholesterol.**—Two methods were used for enrichment of cholesterol in the VSV membrane: one used serum lipoproteins present in a serum/medium mixture devised by Shinitzky (17) and the other by means of PVP beads coated with cholesterol. In the first method, 1 volume of tetrahydrofuran in 0.6% KCl (51, w/v) containing 1 mg/ml of cholesterol was added to 10 volumes of vigorously stirring BME medium containing 10% fetal calf serum (FCS); after lipoproteinization, the dried material was dispersed in specifically determined volumes of distilled water for use as cholesterol-enriching medium. In the second method, 3.6% PVP in PBS was dialyzed overnight, after which BSA was added to give a final concentration of 1% (w/v). Subsequently, a solution of cholesterol (100 μg/ml) in ethanol was added to the PVP/BSA so that the final concentration of ethanol was below 1%. This suspension then was centrifuged at 3000 × g for 10 min and the supernatant was used for enrichment of membranes with cholesterol.

Fig. 1 illustrates the rate and extent of incorporation of exogenous cholesterol into the membrane of VSV incubated with the cholesterol-supplemented preparation of BME medium/FCS or PVP/BSA over a period of 6 h. As noted, the total concentration of VSV cholesterol (endogenous + exogenous) was increased 2.5-fold by the serum mixture and 7-fold by the PVP/BSA mixture. Nothing significant can be deduced from the kinetics of these reactions except that cholesterol incorporation from PVP/BSA was linear but slower and did not exhibit any tendency to plateau by 6 h. Quite obviously, control reaction mixtures not supplemented with lipids did not alter the concentration of endogenous cholesterol present in the VSV membrane.

A series of control experiments were also performed to determine whether apparent cholesterol supplementation of the VSV membrane could be complicated by serum lipoproteins or PVP/lipid adherence to the virus surface, as was the case when phospholipid vesicles interact with VSV (19). For this purpose, VSV was incubated with BME medium/10% FCS containing high density and low density lipoproteins labeled heavily with [3H] (kindly supplied by Dr. Karen Kuehl, University of Virginia) as well as with complexes of PVP-BSA-cholesterol labeled with [3H]-BSA and [14C]-cholesterol, as a nonexchangeable marker. No significant amounts of [3H]-lipoprotein, [3H]-BSA, or [14C]-cholesterol olate could be found associated with VSV reacted with BME medium/FCS or PVP/BSA cholesterol for periods of time up to 6 h. Moreover, the interacting VSV contained no attached serum proteins or BSA as determined by polyacrylamide gel electrophoresis. It seems clear, therefore, that cholesterol supplementation of VSV membrane by these methods is not due merely to sticking of lipoprotein or PVP/BSA which formed a complex with exogenous cholesterol. Additional experimental controls were also performed to determine whether interaction with BME medium/FCS or PVP/BSA affects the morphology and biological properties of VSV. Electron microscopy of VSV enriched with cholesterol revealed no detectable alteration in virus shape, membrane integrity, or protruding glycoprotein spikes.

Table 1 summarizes the results of experiments undertaken to study the effect of cholesterol enrichment on the infectivity, membrane fluidity, and buoyant density of VSV. As noted, 2-fold or greater supplementation with BME medium/FCS or PVP/BSA had no significant effect on the infectivity of VSV as determined by accurate plaque assay. However, cholesterol enrichment resulted in a significant increase in membrane viscosity as shown by the anisotropy data obtained by fluorescence depolarization of the hydrophobic probe, 1,6-diphenyl-1,3,5-hexatriene (13).

Evidence for incorporation of cholesterol into the VSV membrane was also sought by comparing the buoyant density of BME medium/FCS/cholesterol-enriched VSV labeled with [3H]-amino-acids with that of unenriched VSV labeled with [14C]-amino-acids. As shown in Table I, enrichment with cholesterol clearly decreased the buoyant density of VSV compared with that of unenriched VSV, as determined by isopycnic centrifugation in the same 20 to 50% sucrose gradients.

Collectively, the results of these experiments strongly suggest that serum lipoproteins and PVP/albumin can promote...
The incorporation of cholesterol from an exogenous source into the membrane of VSV. This cholesterol supplementation alters the physical properties of the virus membrane without significantly perturbing its structural or biological properties.

**Accessibility of Cholesterol in VSV Membrane to Oxidation by Cholesterol Oxidase**—Cholesterol present in the membrane of intact VSV had been found to be inaccessible to oxidation by cholesterol oxidase (9, 19), presumably owing to the bulky polar head group of the predominant choline phospholipids in the outer leaflet of the bilayer (20). Kinetic analysis of cholesterol depletion and exchange reveals that ~70% of VSV membrane cholesterol resides in the outer half of the VSV membrane (21). Experiments were undertaken to determine whether increasing the cholesterol concentration of the VSV membrane would influence its accessibility to cholesterol oxidase.

To this end cholesterol in the VSV membrane was supplemented with exogenous cholesterol by interaction ofvirions with the BME medium/FCS mixture or with PVP/BSA, each containing excess cholesterol. VSV cholesterol derived only from host baby hamster kidney cells comprised 35 to 36% of the membrane lipid. VSV unenriched with exogenous cholesterol or supplemented up to final lipid concentrations of 60% were exposed to cholesterol oxidase to determine the per cent of total virion cholesterol that became accessible to cholesterol oxidase.

Fig. 2 reveals that cholesterol oxidase oxidized only negligible amounts of cholesterol present in intact virion membranes at concentrations of 46 mol % or less. However, significant amounts were oxidized when cholesterol was present at higher concentrations; at membrane levels of 54% or greater, 70 to 75% of VSV cholesterol was accessible to oxidation by the enzyme. Quite clearly, cholesterol supplementation by use of BME medium/FCS or PVP/BSA was equally effective in providing oxidizable levels of cholesterol. Kinetic analysis of the rates of exchange of radioactive endogenous and exogenous cholesterol in the membranes of cholesterol-enriched VSV revealed that the exogenously incorporated cholesterol mixed and equilibrated rapidly with the endogenous membrane cholesterol (data not shown).

These experiments clearly demonstrate that the VSV membrane loaded to a certain level with excess cholesterol becomes permeable to cholesterol oxidase, resulting in exposure of the β-OH group of the exogenous cholesterol and, to some extent, the existing endogenous cholesterol.

**Accessibility of Cholesterol in Mixed Vesicles to Oxidation by Cholesterol Oxidase**—In an attempt to gain insight into the relationship of cholesterol concentration and overall lipid composition to accessibility of membrane bilayer cholesterol to cholesterol oxidase, a parallel study was conducted using unilamellar vesicles varying in lipid composition and cholesterol content. For this purpose small, unilamellar vesicles were created by sonication of different phospholipids, singly or in combination, in the presence of varying concentrations of cholesterol (14: see also "Materials and Methods"). These mixed phospholipid-cholesterol vesicles were exposed to cholesterol oxidase and the degree of conversion to cholest-4-en-3-one was measured spectrophotically as described previously (15). The vesicle concentration of oxidizable cholesterol was determined by the cholesterol oxidase method after disruption of vesicles with tauromeoxycholate (19).

Fig. 3 compares the degree to which cholesterol oxidase oxidized cholesterol present in mixed vesicles composed of different phospholipids and containing varying concentrations of cholesterol. As noted previously (9), low concentrations of cholesterol were accessible to oxidation by cholesterol oxidase only when present in vesicles containing appreciable amounts of aminophospholipids, particularly phosphotidylserine. Vesicles composed only of choline phospholipids (Fig. 3A) or mixtures of egg phosphatidyicholine and phosphatidylethanolamine exhibited similar patterns of reactivity to cholesterol oxidase; oxidation of cholesterol in these vesicles was found to be minimal up to cholesterol levels of 42 mol %. Significant

### Table I: Comparative properties of cholesterol-enriched and unenriched (control) VSV

<table>
<thead>
<tr>
<th>Virus property</th>
<th>Control VSV (unenriched)</th>
<th>Cholesterol-enriched VSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectivity (p.f.u./ml)</td>
<td>3.4 x 10^6</td>
<td>4.6 x 10^6</td>
</tr>
<tr>
<td>Reacted with BME medium/FCS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectivity (p.f.u./ml)</td>
<td>2.5 x 10^6</td>
<td>2.1 x 10^6</td>
</tr>
<tr>
<td>Reacted with PVP/BSA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anisotropy (37°C)</td>
<td>0.211</td>
<td>0.254</td>
</tr>
<tr>
<td>Buoyant density</td>
<td>1.161 g/ml</td>
<td>1.136 g/ml</td>
</tr>
</tbody>
</table>

* Anisotropy values were calculated from the equation \( r = (A - 1)/(A + 2) \), where \( r \) is the anisotropy and \( A \) is the fluorescent intensity detected through a polarizer oriented parallel divided by the fluorescent intensity detected through a polarizer oriented perpendicular to the direction of the excitation beam (13).
of the phase transition (37°C), or above the phase transition temperature (45°C). However, when the concentration of cholesterol in vesicles was 45 mol % or higher, there was a sharp increase to almost complete oxidation of cholesterol at each of the three temperatures. Similar results were obtained when mixed cholesterol vesicles were prepared with dimyristoyl phosphatidylcholine or sphingomyelin (data not shown).

These studies indicate that the physical state of the vesicle bilayer plays no role in accessibility of cholesterol to cholesterol oxidase. The determining factor in cholesterol oxidation in vesicles containing choline phospholipids appears to be the concentration of cholesterol. Moreover, the nature of the phospholipid head group is apparently of greater significance than the fatty acyl chain.

**DISCUSSION**

The data presented here demonstrate that enrichment of VSV with cholesterol above a critical concentration exposes much of the endogenous as well as the exogenous cholesterol in the membrane to cholesterol oxidase. Enrichment with cholesterol did not affect the structural integrity or infectivity of the virus but did significantly reduce its buoyant density. Parallel studies with small unilamellar vesicles showed that, at concentrations below 42 mol %, cholesterol in intact egg phosphatidylcholine or sphingomyelin vesicles is not oxidizable by cholesterol oxidase, whereas a considerable amount is oxidized when cholesterol concentrations were above 46 mol %. Studies presented here and previously (9, 19) indicate that bulky head groups of choline phospholipids, but not amino phospholipids, appear to protect cholesterol in intact membranes from being oxidized by cholesterol oxidase.

Many recent publications have described the maximum amount of cholesterol that can be incorporated into lipid bilayers and the effect of such incorporation on the physical state of the bilayer (2–5, 22). It has been generally accepted that multilamellar vesicles composed of cholesterol and phospholipid at a molar ratio below 1:2 exhibit a complex formation in which two phases co-exist, pure phosphatidylcholine and a 1:2 complex of cholesterol and phosphatidylcholine (2–5, 23, 24). A similar model has also been proposed for cholesterol-sphingomyelin vesicles (25). Our results presented here with unilamellar phosphatidylcholine or sphingomyelin vesicles indicate that cholesterol present at molar concentrations of 42% or less is not accessible to cholesterol oxidase. It would appear that the properties of both multilamellar and unilamellar vesicles may differ, depending on their ratio of cholesterol/phospholipid in both kinds of vesicles. Indeed, such differences may be the reason for the asymmetrical distribution of cholesterol in 33% in the outer layer and a higher ratio in the inner layer of small unilamellar vesicles (26). When the cholesterol concentration in our unilamellar vesicles is 42% or greater, the cholesterol present in the outer layer may exceed the 1:2 ratio for complex formation of cholesterol and phosphatidylcholine. At high concentrations, cholesterol may form small aggregates or domains of cholesterol in the outer layer of the membrane. Cholesterol in such aggregates could well be more accessible to cholesterol oxidase than the lower concentrations associated uniformly with phospholipids. After oxidation, the outer layer cholesterol then may exchange by lateral diffusion of cholesterol in the membrane plane and between the two faces of the membrane, eventually leading to complete oxidation of cholesterol.

A number of recent reports has shown marked alterations in the properties of vesicles with concentrations of cholesterol above 42%. When the cholesterol/phospholipid molar ratio exceeds 1:2, there is a large effect on the hydrodynamic
properties of the vesicle (27). Moreover, a large increase in the $^1$H NMR line width was noted in the range of 40 to 45% cholesterol (28). The existence of a phase boundary in small unilamellar dipalmitoyl phosphatidylcholine vesicles at approximately 40% cholesterol has also been demonstrated (29). In addition, unilamellar egg phosphatidylcholine-cholesterol vesicles exhibit a critical point at which their stability is minimal at cholesterol concentrations about 42%. All these studies suggest that the properties of a lipid bilayer are markedly affected when cholesterol reaches a critical concentration of 42 to 45%, as was the case in the present study when the $\beta$-OH group of cholesterol in viral or vesicle membranes becomes accessible to cholesterol oxidase. Interestingly, this cholesterol phase is not affected by the phospholipid phase transition of the co-existing pure phospholipid domains.

The results of experiments with sphingomyelin vesicles are quite similar to those with phosphatidylcholine vesicles, except that only 60 to 65% of cholesterol in sphingomyelin vesicles is oxidized by cholesterol oxidase even at a molar ratio of 1:1. This finding appears to support the idea that cholesterol may undergo some preferential interaction with sphingomyelin compared with egg phosphatidylcholine and phosphatidylethanolamine, although the nature of this interaction remains to be determined (30, 31).

The oxidation of cholesterol in vesicles containing phosphatidylserine was found to be dependent on their phosphatidylserine content; the higher the concentration of phosphatidylserine, the lower was the cholesterol threshold required for complete oxidation of cholesterol and the greater the percentage of cholesterol exposed to the enzyme, even at low molar concentrations of cholesterol. These results suggest the possibility that the presence of cholesterol in phosphatidylcholine-phosphatidylserine vesicles may impose isothermal phase separation, providing a 1:2 cholesterol-phosphatidylcholine complex as one phase where cholesterol is not available to the enzyme, while cholesterol associated with phosphatidylserine is available to the enzyme.

All these experiments raise the question of the validity of changing cholesterol levels in membrane without investigating the organization of cholesterol before and after manipulation of cholesterol levels in the membrane. Further, as suggested here, the organization of cholesterol is dependent upon membrane lipid composition. Various membranes can have different levels of cholesterol, above which a separate cholesterol phase will appear. This could affect many of the membrane functions. Indeed, a similar idea was suggested by Shen et al. (32) for lipoproteins, although the authors feel that the particle curvature is the main factor for determining cholesterol levels at the surface.

S. Amselem and D. Lichtenberg, personal communication.

REFERENCES