Kinetics of Cholesterol and Phospholipid Exchange between
*Mycoplasma gallisepticum* Cells and Lipid Vesicles

ALTERATIONS IN MEMBRANE CHOLESTEROL AND PROTEIN CONTENT

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The kinetics of exchange of radiolabeled cholesterol and phospholipids between intact *Mycoplasma gallisepticum* cells and unilamellar lipid vesicles were investigated over a wide range of cholesterol/phospholipid molar ratio. The change in cholesterol/phospholipid molar ratio was achieved by adapting the sterol-requiring *M. gallisepticum* to grow in cholesterol-poor media, providing cells with decreased unesterified cholesterol content. At least 90% of the cholesterol molecules in unsealed *M. gallisepticum* membranes underwent exchange at 37°C as a single kinetic pool in the presence of albumin (2%, w/v). However, we observed biphasic exchange kinetics with intact cells, indicating that cholesterol translocation from the inner to outer monolayers was rate-limiting in the exchange process. Approximately 50% of the cholesterol molecules were localized in each kinetic pool, independent of the cholesterol/phospholipid molar ratio in the cells and vesicles. A striking change in the kinetic parameters for cholesterol exchange occurred between 20 and 26 mol % cholesterol; for example, when the cholesterol/phospholipid molar ratio was decreased from 0.36 to 0.25, the half-time for equilibration of the two cholesterol pools at 37°C decreased from 4.6 ± 0.5 to 2.5 ± 0.1 h. Phospholipid exchange rates were also enhanced on decreasing the membrane cholesterol content. The ability of cholesterol to modulate its own exchange rate, as well as that of phospholipids, is suggested to arise from the sterol’s ability to regulate membrane lipid order. Extensive chemical modification of the membrane surface by cross-linking of some of the protein constituents with 1,4-phenylenediamine decreased the cholesterol exchange rate. Depletion of membrane proteins by treatment of growing cultures with chloramphenicol increased the cholesterol exchange rate, possibly because of removal of some of the protein mass that may impede lipid translocation. The observations that phospholipid exchange was one order of magnitude slower than cholesterol exchange and that dimethyl sulfoxide, potassium thiocyanate, and potassium salicylate enhanced the cholesterol exchange rate are consistent with a mechanism involving lipid exchange by diffusion through the aqueous phase.

Lipids undergo spontaneous exchange between membrane systems (reviewed in Refs. 1 and 2) and also can move between the inner and outer leaflets of the bilayer of biological and model membranes (e.g. Refs. 3 and 4). The minute prokaryotes, mycoplasmas, are well suited for studying the problem of lipid exchange. Unlike other prokaryotes, mycoplasmas (except those included in the genus *Acholeplasma*) require sterol for growth and incorporate large quantities of the sterol into their cell membrane without any structural modification of it (5). Since mycoplasmas, like other prokaryotes, cannot synthesize sterols, all of the sterol is derived from exogenous sources. Moreover, mycoplasmas lack a cell wall, assuring direct contact between the membrane surface and the external environment. In the mycoplasma cell membrane, cholesterol undergoes a relatively rapid translocation from the outer to inner halves (6, 7) and from the inner to outer halves (8) of the membrane bilayer. The movement of cholesterol into the inner half of the bilayer is impeded by increased rigidity of the phospholipid acyl chains, as in aged *Mycoplasma gallisepticum* cells (9). The rate of [3H]cholesterol exchange from cells to vesicles was enhanced by albumin, with maximal effects on the rate constants for outer-to-inner and inner-to-outer leaflet movement observed at albumin concentrations of ≥7%; however, the ability of cholesterol to undergo rapid transbilayer movement in the membrane of resting *M. gallisepticum* cells is independent of cell viability (8). We have attempted to determine what other factors may influence the rates of lipid translocation. In this report we analyze the role of cholesterol/phospholipid molar ratio of the *M. gallisepticum* cell membrane on the rates of transmembrane movement of [3H]cholesterol and [3H]phospholipid. The adapted strain of *M. gallisepticum* has been used because this organism provides an efficient method for varying the cholesterol/phospholipid molar ratio of the cell membrane over a wide range, thereby allowing study of the exchange kinetics of labeled lipids under equilibrium conditions. Depletion of the cholesterol content of the native strain was achieved by gradually decreasing the cholesterol concentration in the medium. This procedure provided cells containing cholesterol/phospholipid molar ratios that ranged from 0.25 to 0.92 in increments of about 0.15, allowing analysis of the role of cholesterol content on the kinetic parameters for cholesterol and phospholipid exchange. The cholesterol content of the membrane modulates the rates of lipid movement, probably because this sterol is a regulator of bulk membrane fluidity and affects the physical state of the membrane lipids. In the present work, we have also examined the mechanism of cholesterol exchange and the influence of some changes in membrane proteins on the exchange kinetics.

EXPERIMENTAL PROCEDURES

Materials

The sources and purities of egg phosphatidylcholine, cholesterol, bovine serum albumin (Fraction V, fatty acid poor), and oleic and...
palmitic acids have been described previously (9). [6-methyl-3H]Thymidine (30.6 Ci/mmol), [4-14C]cholesterol (53 Ci/mmol), [U-14C]alginic acid (59 Ci/mol), and [1-14C]palmitic acid (59 Ci/mol) were purchased from New England Nuclear. [7-3H(N)]Cholesterol oleate (65 Ci/mol) was kindly supplied by Dr. M. C. Phillips (Medical College of Pennsylvania). [2-14C]Glycerol trioleate (2 Ci/mol) was obtained from Pierce. 1,4-Phenylenedimaleimide, deoxyribonucleoside, chloramphenicol, and egg phosphatidylglycerol were obtained from Sigma. Lysozyme was purchased from Worthington. Chemicals were of reagent grade. Potassium salicylate was prepared from salicylic acid and potassium hydroxide. The dialysis membranes (Spectra Por 6, 50,000 molecular weight cutoff) were rinsed and boiled in distilled water prior to use in the equilibrium dialysis cells.

**Methods**

**Lipid Exchange between Mycoplasma gallisepticum and Vesicles**—Strain A5969 was grown in a modified Edward medium (10) that contained 1% albumin, palmitic and oleic acids (10 μg/ml of each), and cholesterol (2–10 μg/ml). The lipids were added as ethanolic solutions. The final concentration of ethanol did not exceed 0.5% (v/v). In the cholesterol exchange experiments, the lipids were labeled by the addition of 0.005 μCi of [14C]cholesterol/ml of medium. In the experiments of endogenous phospholipid exchange, the lipids were labeled by the addition of 0.002 μCi of [14C]palmitoylcardanol of medium. When exogenous PC (10 μg of egg PC/ml) was incorporated, the lipids were labeled with 0.005 μCi of [14C]PC/ml of medium. To monitor the recovery and leaakness of *M. gallisepticum* cells after separation from the vesicles, the medium was supplemented with 0.25 μg/ml of [6-methyl-3H]thymidine. Cultures were grown at 37°C and growth was monitored by measuring the absorbance at 640 nm. The organisms were harvested in the midexponential phase of growth by centrifugation at 12,000 × g for 10 min at 4°C. The cells were washed once in cold 0.25 M NaCl solution, and an aliquot of the cell suspension was withdrawn for lipid and protein analysis. The cells were treated at 37°C for 10 min in the absence of deoxyribonuclease (20 μg/ml) in the presence of 20 mM MgCl₂, and then washed once and resuspended in 0.40 M sucrose, 50 mM Tris buffer, pH 7.4, containing 0.1% of 2-mercaptoethanol, followed by centrifugation at 12,000 × g for 15 min. The cells were collected and sedimented at 12,000 × g for up to 2 months.

**Preparation of Unilamellar Cholesterol/Egg PC Vesicles**—Vesicles were prepared in STM buffer as described previously (8). The concentration of total lipid in the vesicles used in the [14C]cholesterol exchange studies was 15 nm, whereas in the [14C]phospholipid exchange experiments the vesicles contained 5 mM total lipid. Vesicles used in the cholesterol exchange experiments were prepared from egg PC and cholesterol, whereas those used in the studies of endogenous phospholipid exchange were prepared from egg PC and cholesterol. In the experiments with egg PC incorporated into the cell membrane, the vesicles were prepared from egg PC, egg PC, and cholesterol (PG/PC molar ratio of 1:1). The cholesterol/phospholipid molar ratio of the vesicles was identical with that in the mycoplasma cell membrane.

**Determination of [14C]Cholesterol or [14C]Phospholipid Exchange**—Cells (approximately 0.5 mg of cell protein) and vesicles (a 50- to 100-fold excess with respect to lipid concentration) were incubated in STM buffer at 37°C with gentle shaking for periods up to 14 h. Penicillin G (5000 units/ml), deoxyribonuclease (20 μg/ml), and albumin (2%) were included in the incubation medium. At defined time intervals, 0.5-ml aliquots were withdrawn, and cells were pelleted by centrifugation for 2 min at room temperature in an Eppendorf centrifuge. Duplicate samples were taken for each time point. After the supernatant was removed by aspiration, the cell pellet was washed once with STM buffer. The suspension was centrifuged again for 2 min, and the resulting cell pellet was transferred to scintillation vials containing scintillation fluid (Liquiscint, National Diagnostics). The radioactivity was determined in a Beckman LS 7500 liquid scintillation spectrometer. The fraction of [14C]-labeled lipid exchanged at time t was calculated from the ratio of (t/8g/C)/HC] or (t/8g/C)/Hg, where (t/8g/C) and (t/8g/C) represent the ratio of [14C] and [3H] in the cell pellet at time t and time 0. Spillover of [14C] cpm into the [3H] channel was typically 9%, and spillover of [3H] cpm into the [14C] channel was typically 4%. The data were corrected for spillover.

**Assay of Lipid Exchange between Isolated Membranes and Vesicles**—The time course of [14C]cholesterol exchange between isolated membranes of *M. gallisepticum* and acceptor cholesterol/PC vesicles was monitored by passage of 0.20-ml aliquots of an incubation mixture of membranes and vesicles through a column (~1 cm in length) of diethylaminoethyl-Sepharose CL-6B (Pharmacia). This procedure was adapted from McLennan and Phillips (11), who separated negatively charged vesicles from neutral vesicles. We used ion exchange chromatography to separate the neutral acceptor vesicles from the PG-rich *M. gallisepticum* membranes since isolated membranes, unlike cells, could not be recovered rapidly by centrifugation in an Eppendorf centrifuge. Cell membranes were prepared and isolated as described previously (8). Each column was treated with 0.25 mg of sonicated egg PC vesicles in 0.1 ml of 20 mM sodium phosphate buffer, pH 6.0, to improve the recovery of the acceptor vesicles. Aliquots of the incubation mixture were applied to the columns at room temperature. The elution mixture had a density of 1.058 g/ml, and a 45-fold excess was used in the absence of albumin. The fraction of cholesterol remaining in the supernatant was identical with that in the mycoplasma cell membrane. The adapted cells were more fragile, and the adapted cells were more fragile, and the adapted cells were more fragile, and the adapted cells were more fragile.

**Kinetics of [14C]Cholesterol Translocation**—The half-time of equilibration of [14C]cholesterol in the translocation experiments was determined by the method of Siebert and Schliekelman (10). The membrane bilayer was calculated using the relationship t½ = 0.693/ (kₐ + kₑ), since the pores appear to represent [14C]cholesterol in the two halves of the membrane bilayer (see subsequent section). kₐ and kₑ are considered to represent the first order rate constants for the translocation of cholesterol from the inner to outer and from the

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1 The abbreviations used are: PC, phosphatidylcholine; PG, phosphatidylglycerol; PL, phospholipid.
Lipid Exchange between Mycoplasma gallisepticum and Vesicles

RESULTS

**Growth of Native and Adapted Cells**—Various investigators have adapted mycoplasmas to grow with very low cholesterol by making serial passages of cultures to medium containing lower cholesterol concentration than that in the previous medium (6, 14, 15). To examine the role of cholesterol content in lipid translocation across the mycoplasma membrane we have grown *M. gallisepticum* at varying stages of cholesterol depletion. Fig. 1 shows growth curves of *M. gallisepticum* after serial passages into medium containing albumin, palmitic and oleic acids, and various concentrations of cholesterol. Cells grew best at high cholesterol concentrations (10 and 7 μg/ml). Cultures of the adapted strain reached the stationary phase at a lower turbidity, in accord with previous observations of poor growth of mycoplasmas in cholesterol-poor media (6, 14, 15). There was a 4-fold decrease in the cholesterol content and the cholesterol/phospholipid molar ratio of the strain adapted to 2 μg/ml compared with the native strain, which was grown with 10 μg of cholesterol/ml (Table I). The cholesterol/phospholipid molar ratio and cholesterol content of the cell membrane decreased gradually as the cells were adapted to growth with progressively decreasing cholesterol supplementation. The phospholipid content of the membrane remained the same, however (Table I), indicating that sticking of the sonicated vesicles to the mycoplasma membrane was negligible during the incubation period.

**Exchange with Lipid Vesicles**—*M. gallisepticum* was grown to midexponential phase in medium containing [14C]cholesterol and [3H]thymidine. On incubation of cells with a large excess of sonicated vesicles containing egg PC and nonradioactive cholesterol, radioactive cholesterol underwent exchange between these membrane systems. The osmotic stability of the cells was increased by using an incubation medium lacking Na+, since Na+ is permeable to the membrane of nonenergized cells (17). Moreover, *M. gallisepticum* cells are known to have a low osmotic fragility (18). The cells remained intact throughout the long period of incubation with lipid vesicles, as judged by the retention of 85–88% of [3H]thymidine-labeled components after 5 h of incubation, and 78–82% after 14 h. When cells were incubated with vesicles containing the same molar ratio of cholesterol to phospholipid as the cell membrane, there was no significant change in the cholesterol and phospholipid content of cells compared with the amounts present before incubation (Table I).

Fig. 2 shows that the incubation of [14C]cholesterol-labeled cells with an excess of vesicles resulted in a relatively rapid transfer of most of the radioactive cholesterol to the vesicles. The exchange followed biphasic kinetics. The rate and extent of exchange increased when the cholesterol/phospholipid molar ratio was decreased from 0.63 to 0.25; no significant change was found between 0.95 and 0.63 (Fig. 2). The pool sizes were found to be independent of the cholesterol/phospholipid molar ratio. Throughout the range of molar ratios we investigated, there was an approximately symmetrical distribution of lipids between the cells and vesicles.
of cholesterol between the two leaflets of the cell membrane (Table II). The half-time, \( t_{1/2} \), for equilibration of the two cholesterol pools decreased by a factor of 2.5 (from 6.2 to 2.5 h at 37 °C) when the cholesterol/phospholipid molar ratio decreased by the same factor (from 0.63 to 0.25). Similarly, the time required for 50% of the radioactive cholesterol to be exchanged, \( t_{0.5} \), decreased by a factor of 2.2 when the cholesterol to phospholipid molar ratio of the mycoplasma membrane was decreased from 0.63 to 0.25. Table II shows that significant decreases in \( t_{1/2} \) and \( t_{0.5} \) (\( p < 0.05 \)) are observed between the cholesterol/phospholipid molar ratios of 0.25 to 0.36 (20 to 26 mol % cholesterol).

**Modifications in Membrane Proteins**—Cells were treated under various conditions with dimethyl suberimidate and 1,4-phenylenedimaleimide to yield a range of cross-linked proteins. In the absence of a cross-linking agent, at least 26 individual polypeptide bands were detected in the sodium dodecyl sulfate-polyacrylamide gels ranging from about 20,000 to 80,000 Da. Rough estimates of the staining intensities of the protein spots indicated that after treatment with dimethyl suberimidate (as described under “Methods”) two bands appeared (\( M_0 = 95,000 \) and \( M_1 > 95,000 \)), and all of the bands in the range of 35,000 and 65,000 Da decreased in intensity. Treatment with 1,4-phenylenedimaleimide, under both conditions used to produce cross-linking (see “Methods”), resulted in the disappearance of three bands (22,000, 37,000, and 53,000 Da) and the appearance of two new bands at high molecular weights (120,000 and 180,000). Many of the polypeptide bands decreased considerably in intensity, especially when the cells were treated with the higher concentration of phenylenedimaleimide. However, the differences in the dye-binding capacity of proteins on the acrylamide gels makes their quantitation by Coomassie blue staining equivocal. Fig. 3A shows that the rate of cholesterol translocation across the membrane of adapted M. gallisepticum cells was relatively unaffected by the cross-linking induced by dimethyl suberimidate; treatment with phenylenedimaleimide at the higher concentration, but not the lower, decreased the cholesterol translocation rate constants (especially \( k_{s0} \)) and increased \( t_{1/2} \) and \( t_{0.5} \). Thus cholesterol movement can be impeded by extensive cross-linking of membrane proteins, depending on the conditions used.

In further efforts to learn how the kinetics of \([44C]\) cholesterol translocation are related to changes in membrane protein content, we carried out experiments with cultures taken to log phase (~22 h) in the presence of small amounts of cholephaminol. There was a 75 ± 10% decrease in protein content of the membrane of cells grown with 20 μg of cholephaminol/ml compared with cells grown without the inhibitor. The electrophoretic pattern of the membrane proteins was not changed significantly by cholephaminol treatment of the culture. The uptake of exogenous cholesterol was also partially inhibited by addition of more than 10 μg of cholephaminol/ml, probably because of growth inhibition. The decrease in protein content was more dramatic than that in cholesterol content; for example, in inhibited cells grown with 10 μg of exogenous cholesterol/ml of media in the presence of 20 μg of cholephaminol/ml there was a 4-fold decrease in the content of total membrane protein compared with a 1.4-fold decrease in the content of total membrane phospholipids and cholesterol, relative to control cells that were adapted to the same cholesterol content. Thus the addition of cholephaminol at about 20 μg/ml provided a means for increasing the lipid-to-protein ratio of the cell membrane. We compared the exchange kinetics obtained with the inhibited cells grown with 10 μg of cholesterol/ml and the corresponding kinetic parameters obtained with partially adapted control cells containing the same cholesterol/phospholipid molar ratio. Table III shows that growth in the presence of cholephaminol resulted in a 33% decrease in the half-time for cholesterol equilibration between the two pools in the cell membrane compared with the corresponding \( t_{1/2} \) value in control cells with normal membrane protein content and the same cholesterol/phospholipid molar ratio (Table II). The rates of cholesterol translocation from one pool to the other (\( k_{s0}, k_{s0} \)) increased by 50% in the inhibited cells, and the size of the outer monolayer pool decreased (Table III, Fig. 3B). However, the time course for cholesterol exchange from the outer monolayer of the membrane bilayer to vesicles was not sensitive to depletion in membrane protein content, since \( t_{0.5} \) was not reduced significantly (\( p > 0.05 \)). Exogenous lysozyme, which is bound extrinsically to mycoplasma membranes (19), also did not alter \( t_{0.5} \) (Table III).

**Dependence of Cholesterol Exchange Kinetics on Salts and Dimethyl Sulfoxide**—Lipid exchange between two vesicle systems appears to take place through the aqueous phase (e.g. Refs. 11 and 20–22). To determine if the spontaneous exchange of free cholesterol from M. gallisepticum cells to vesicles is compatible with a mechanism involving diffusion in the aqueous medium, we measured the initial rate of \([44C]\) cholesterol transfer in the presence of structure-breaking solutes such as potassium thiocyanate and potassium salicylate, and in structure-making solutes such as magnesium chloride and potassium chloride. As predicted for a mechanism that depends on the limited aqueous solubility of cholesterol, the exchange rates are enhanced in 1.0 m thiocyanate and salicylate (Fig. 4). The initial rate of \([44C]\) cholesterol exchange was 1.67 times faster in the presence of these salts relative to STIM buffer alone. Furthermore, the relative rate increased by a factor of 2.0 when 3% (final concentration) dimethyl sulfoxide, which is also expected to increase the aqueous solubility of cholesterol, was added to STIM buffer. On the other hand, the initial rate was decreased by chloride ion (Fig. 4). This probably results from a salting-out effect on the transfer of cholesterol into the aqueous phase.

### Table II

<table>
<thead>
<tr>
<th>Molar ratio of cholesterol/PL</th>
<th>( k_{s0} )</th>
<th>( k_{s0} )</th>
<th>( t_{1/2} )</th>
<th>( t_{0.5} )</th>
<th>( r_{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 10^7 \times \text{rate constant (h}^{-1}) )</td>
<td>( h )</td>
<td>( h )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.92</td>
<td>4.7 ± 0.6</td>
<td>6.5 ± 0.8</td>
<td>6.2 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>0.58 (4)</td>
</tr>
<tr>
<td>0.65</td>
<td>4.9 ± 0.8</td>
<td>6.7 ± 0.9</td>
<td>6.2 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>0.69 (2)</td>
</tr>
<tr>
<td>0.50</td>
<td>4.9 ± 0.9</td>
<td>7.8 ± 1.2</td>
<td>5.5 ± 0.5</td>
<td>2.1 ± 0.1</td>
<td>0.62 (3)</td>
</tr>
<tr>
<td>0.36</td>
<td>6.8 ± 0.8</td>
<td>8.2 ± 1.2</td>
<td>4.6 ± 0.5</td>
<td>1.8 ± 0.2</td>
<td>0.55 (4)</td>
</tr>
<tr>
<td>0.25</td>
<td>12.7 ± 1.2</td>
<td>15.0 ± 1.6</td>
<td>2.5 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.54 (4)</td>
</tr>
</tbody>
</table>

1 The rate constants, half-times, and sizes of the outer-leaflet cholesterol pool were calculated as described under "Methods." The numbers in parentheses represent the number of kinetic experiments conducted with separate cultures. The values represent the mean ± S.E.

2 \( t_{0.5} \) is the time required for the \([44C]\) cholesterol content to be reduced to 50%; it represents mostly a transfer of cholesterol.
endogenous phospholipid transfer from cells to cholesterol/PG vesicles increased when the cholesterol/phospholipid molar ratio was decreased in the cells and vesicles (Table IV). When egg PC was incorporated into the cells, there was an increase in the initial rate of transfer of $^{14}$C-labeled phospholipids from cells to vesicles prepared from egg PG, egg PC, and cholesterol. On depletion of cellular cholesterol by adaptation to growth in cholesterol-poor medium, the rate of phospholipid movement was enhanced. Thus, the exchange rates of both $^{14}$C-cholesterol and $^{14}$C-phospholipids from cells to vesicles are affected by the cholesterol content of the membrane.

**DISCUSSION**

The biphasic exchange kinetics observed with intact cells suggests that cholesterol exists in two environments in the *M. gallisepticum* cell membrane. Compartmentalization into two pools confirms our previous exchange (8, 25) and filipin-binding (9) measurements with mycoplasmas. Since exchange to acceptor vesicles involves the desorption of cholesterol molecules present only in the outer monolayer of the *M. gallisepticum* cell membrane bilayer, our results indicate that the rate of cholesterol "flip flop" from the inner to outer surface of the bilayer is comparable to or slower than that at which it undergoes exchange. Although we cannot rigorously exclude the coexistence of two immiscible cholesterol pools in the same monolayer of the bilayer, we consider the rapidly exchangeable pool to represent cholesterol molecules localized in the outer leaflet and the less accessible pool to represent cholesterol in the inner leaflet of the bilayer. This conclusion is based on the agreement between our estimates of the pool sizes determined by exchange measurements (which did not vary over a wide range of cholesterol content, Table II) and by filipin-binding measurements (9, 26). Moreover, Fig. 5
Lipid Exchange between Mycoplasma gallisepticum and Vesicles

TABLE IV

<table>
<thead>
<tr>
<th>[14C] PL exchanged</th>
<th>Molar ratio of cholesterol/PL</th>
<th>Endogenous Cholesterol incorporated</th>
<th>Endogenous PL incorporated</th>
<th>Acceptor vesicles</th>
<th>t_0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous PL*</td>
<td>0.94</td>
<td>102</td>
<td>0</td>
<td>PG/Cholesterol</td>
<td>24.2 ± 1.2</td>
</tr>
<tr>
<td>Endogenous PL*</td>
<td>0.25</td>
<td>26</td>
<td>0</td>
<td>PG/Cholesterol</td>
<td>14.9 ± 0.9</td>
</tr>
<tr>
<td>Endogenous PL + exogenous PC*</td>
<td>0.91</td>
<td>106</td>
<td>76.5</td>
<td>PG/PC/Cholesterol</td>
<td>15.0 ± 1.0</td>
</tr>
<tr>
<td>Endogenous PL + exogenous PC*</td>
<td>0.23</td>
<td>29</td>
<td>80.4</td>
<td>PG/PC/Cholesterol</td>
<td>9.2 ± 0.5</td>
</tr>
</tbody>
</table>

*In the absence of exogenous PC, PG comprised 95% of the endogenous phospholipids. Membrane lipids were labeled by growing the cells with 0.002 μCi of [1-[14C]palmitate/ml of medium.

*In the presence of exogenous egg PC (10 μg/ml), PG comprised 49% of the total phospholipids in the cell membrane. To label the membrane lipids, the cells were grown with 0.002 μCi of [1-[14C]palmitate and 0.005 μCi of egg [14C]PC/ml of medium.

shows apparent first-order kinetics of [14C]cholesterol exchange from open, unsealed M. gallisepticum membranes to acceptor vesicles. At least three-quarters of the cholesterol molecules exchanged in one kinetic pool in the absence of albumin, and more than 90% of the radiolabeled cholesterol underwent exchange as a single pool in the presence of albumin. Since membranes isolated from mycoplasma cells have been shown to be unsealed using a variety of techniques (27–29), exchange of cholesterol molecules is expected to occur from both surfaces.

Water-miscible organic solvents have been found to stimulate the rate of cholesterol exchange between lipoproteins and cells, membranes, or tissues (1, 30–32). The rate enhancements have been ascribed to disruption of hydrophobic interactions involving the lipids in the lipid-protein complexes (30). Desorption of the sterol from the membrane surface is facilitated by weakening of lipid-lipid and lipid-protein contacts; in addition, the aqueous solubility of the lipid is increased by disruption of ordered water structure. Our observations that the initial rate of cholesterol exchange is decreased by addition of chloride ion to STM buffer and enhanced by addition of several chaotropic agents (salicylate, thiocyanate, dimethyl sulfoxide) are consistent with the importance of aqueous solubility in spontaneous lipid exchange between membrane interfaces. A mechanism involving a collision complex would predict no dependence on the aqueous solubility of the lipid undergoing exchange. The rate enhancement caused by addition of albumin (Fig. 5; Refs. 8 and 33) may, therefore, arise from enhancement of the very low solubility of cholesterol in the aqueous phase. Moreover, the initial rate of phospholipid exchange from native cells to vesicles is slower than that of cholesterol by a factor of 9.7; for exchange between adapted cells and vesicles this factor is 13.5 (Table IV). Other investigators have also reported that the rate of cholesterol exchange between donor and acceptor membranes is faster than that of phospholipids by about one order of magnitude (4, 11, 34). This is expected for an aqueous diffusion mechanism, since monomeric phospholipids have even lower aqueous solubility than monomeric cholesterol (35–37). In further efforts to determine the mechanism of cholesterol exchange between membrane interfaces, we prevented direct contact between cells and vesicles by enclosing adapted M. gallisepticum cells in a dialysis membrane. The dialysis membrane allows passage of cholesterol monomers, but the rate is decreased because it represents an additional barrier (11, 20). In these experiments we observed a slow, but measurable exchange of [14C]cholesterol (5% after 25 h), providing additional support for the mechanism involving
desorption of the lipid from the cell membrane and partitioning into the aqueous solution.

In the present study we have shown that the exchange rates at 37 °C of both cholesterol and phospholipids are increased when the cholesterol/phospholipid molar ratio is decreased. Le Grimellec et al. (16) concluded from steady state changes in the fluorescence anisotropy of 1,6-diphenylhexatriene that native M. gallisepticum cells have a higher degree of membrane lipid order at 37 °C than adapted cells. Therefore, we attribute the ability of cholesterol to modulate its own exchange rate, as well as that of phospholipids, to the well known, unique ability of the sterol to increase membrane lipid order above the lipid phase transition temperature. Desorption of the lipid from the donor membranes, which is thought to be the rate-limiting step in exchange or transfer to excess acceptors (11), appears to be facilitated by decreasing cholesterol content. In accord with our data showing a dependence of lipid exchange on membrane cholesterol content, Mohandas et al. (38) found that the rate of lysophosphatidylcholine translocation decreased by 43% when the cholesterol/phospholipid molar ratio of the erythrocyte membrane was increased from 0.90 to 1.52. However, for the exchange of cholesterol between the two vesicle systems, McLean and Phillips (39) did not find a significant dependence of rate or activation energy in the cholesterol concentration range of 5 to 40 mol %.

Poznansky and Czekanski (40) reported that the initial rate of cholesterol exchange from erythrocyte ghost membranes to lipid vesicles increased by 40% when the cholesterol/phospholipid molar ratio was increased from 19 to 48 mol %, and that the activation energy was lower at 33 to 48 mol % cholesterol than below 20 mol % cholesterol. Thus further experiments are needed with membranes whose cholesterol/phospholipid molar ratio can be varied over a wide range to establish how cholesterol exchange rates are affected by cholesterol content. A wider range of cholesterol/phospholipid molar ratio could not be studied in M. gallisepticum membranes since the cells grew poorly and lysed at very low molar ratios of cholesterol to phospholipid.

Changes in the composition and organization of membrane proteins can also affect the rate of cholesterol translocation across the M. gallisepticum cell membrane. For example, extensive cross-linking of some of the protein constituents of the membrane with 1,4-phenylenedimaleimide reduced the rate constants for translocation from one monolayer to the other (Table III). In a previous study we reported that dimethyl suberimidate inhibited the rate of cholesterol movement from the outer to the inner monolayer of the membrane of adapted M. capricolum cells undergoing active cholesterol uptake (9). Chloramphenicol treatment of M. gallisepticum cultures caused a marked reduction in the unusually high protein content of the membrane. (In the absence of the inhibitor, the protein/lipid ratio by weight is about 4.0.) The reduction in membrane protein content increased the rate constants of [14C]cholesterol exchange relative to control cells with the same cholesterol/phospholipid molar ratio (Fig. 3B). Recent spin-label studies with M. gallisepticum membranes gave no evidence of a relatively immobilized lipid component on the electron spin resonance time scale (41). Since in other membranes a motionally restricted signal is characteristic of lipid interacting with protein, it appears that the membrane proteins of M. gallisepticum cause relatively little restriction of the fluidity of the lipids. Therefore, if lipid-protein contacts are not extensive in the M. gallisepticum membrane, the increases in the rate constants of cholesterol translocation on partial inhibition of protein synthesis apparently arise largely from the removal of steric bulk. A decrease in the extent of exposure of proteins on the surface of mycoplasma cell membranes has been noted in the presence of growth inhibitors (42); this effect may also contribute to facilitating the desorption step. Since sterol-requiring mycoplasmas have been postulated to possess protein receptors that are responsible for maximizing contact between the membrane and cholesterol in exogenous sources (43), the localization of proteins in the membrane bilayer is another parameter that may influence cholesterol uptake and removal. Our experiments on lysozyme incorporation and dimethyl suberimidate or 1,4-phenylenedimaleimide treatment, under conditions producing nonextensive cross-linking (Table III), suggest that a considerable latitude in protein modification can occur without a significant effect on cholesterol exchange kinetics. The inability of various protein-modifying reagents or formalin fixation to modify cholesterol exchange between low density lipoproteins and erythrocytes was considered to indicate that cholesterol exchange is more sensitive to lipid-lipid than to lipid-protein interactions (1, 32). Indeed, we observed striking changes in the kinetic parameters for cholesterol exchange between 20 and 26 mol % cholesterol (Table II). It is interesting that bilayer systems exhibit special effects with respect to molecular diffusion, transport, and enthalpy change at about 20 mol % cholesterol (for a review see Ref. 44). Our results tend to support the conclusion concerning the importance of lipid-lipid interactions in cholesterol exchange (1, 32), although we also obtained evidence that extensive modification of protein content and organization also plays a role.

Phospholipid vesicles were used in our studies in order to maintain equilibrium conditions with respect to the cholesterol/phospholipid molar ratio in the donor cells and acceptor membranes. Analysis of the parameters we found to influence lipid exchange kinetics can be extended in future experiments to the more complex situation of net transfer of free cholesterol between mycoplasma cells and serum lipoproteins which interact with the cells.

Acknowledgements—We thank Sylvia Schaffel for typing the manuscript and Bruce Robinson for preparing the art work.

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Lipid Exchange between Mycoplasma gallisepticum and Vesicles

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