# Cholesterol Distribution and Movement in the *Mycoplasma gallisepticum* Cell Membrane†

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ABSTRACT: The time course and extent of transfer of [$^{14}$C]-cholesterol from resting *Mycoplasma gallisepticum* cells or membrane preparations to high-density lipoproteins were studied. More than 90% of the total cholesterol in isolated, unsealed membrane preparations was exchanged in a single kinetic process. In intact cells, however, cholesterol exists in two different environments. Cholesterol in one environment, representing approximately 50% of the total unesterified cholesterol, is readily exchanged with the cholesterol of high-density lipoproteins, with a half-time of about 4 h at 37 °C. The rate of exchange of [$^{14}$C]cholesterol from the other environment was exceedingly slow, with a half-time of about 18 days. The fraction of the total cholesterol in the readily exchangeable cholesterol pool in intact cells increased somewhat upon aging of the culture. Electron spin resonance spectra of nitroxide-labeled stearic acids incorporated into membranes of *M. gallisepticum* cells indicated increased rigidity at the late exponential phase of growth. These results suggest that cholesterol is present in approximately equal concentrations on both surfaces of the *M. gallisepticum* membrane and that in resting cells the rate of movement of cholesterol molecules from the inner to outer halves of the lipid bilayer is exceedingly slow or nonexistent.

An intriguing topic in membrane research is the determination of the localization and movement of components within the membrane (see references in review by Rothman & Lenard, 1977). Although cholesterol is an important component of membranes, little is known about its transbilayer distribution and rate of movement from one surface to the other. Transbilayer movement of cholesterol has been found to be rapid in human erythrocyte membranes (Lange et al., 1977) but very slow or nonexistent in influenza viral membranes (Lenard & Rothman, 1976). Conflicting results were obtained in two recent studies of the rate of transbilayer cholesterol movement in phosphatidylcholine vesicles (Poznansky & Lange, 1976; Bloj & Zilversmit, 1977).

Mycoplasmas, unlike other prokaryotic cells, require cholesterol for growth, making them useful tools for the study of cholesterol distribution and movement. They lack the ability to synthesize cholesterol and require an exogenous source of this sterol, which is incorporated exclusively into the cytoplasmic membrane (the only membrane that is present in the cell). In contrast to other membranous systems used for the analysis of cholesterol distribution and movement (such as erythrocytes, virions, and liposomes), mycoplasmas offer the advantage that they are capable of autonomous growth. The findings we present here show that high-density lipoprotein (HDL)[1] can be used in cholesterol exchange studies with *M. gallisepticum* cells and membrane preparations. Furthermore, the data suggest that in *M. gallisepticum* cells cholesterol is almost symmetrically distributed between the outer and inner halves of the lipid bilayer.

## Materials and Methods

*Organisms and Growth Conditions. Mycoplasma gallisepticum* (strain A5969) was grown in a modified Edward medium (Razin, 1963) containing 5% horse serum. To label cells for the cholesterol exchange experiments, 0.05 $\mu$Ci/mL of [4-$^{14}$C]cholesterol (20 Ci/mol, New England Nuclear) was added to the growth medium as an ethanolic solution. The ethanol concentration did not exceed 0.5%. To test for cell leakiness during prolonged incubation periods, 0.2 $\mu$Ci/mL of [6-*methyl*-$^{3}$H]thymidine (21.5 Ci/mmol, Nuclear Research Center, Negev, Israel) was added. The cultures were incubated at 37 °C for 16–24 h and growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with cells in the mid-exponential phase of growth ($A_{640}$ = 0.15–0.20). To test the effect of culture age on cholesterol localization, experiments were performed with cultures ranging in absorbance from 0.1 to 0.3. The cells were harvested by centrifugation at 12 000*g* for 20 min, washed once, and resuspended in a cold 0.25 M NaCl solution. The absorbance of the cell suspension was adjusted to $A_{500}$ = 9.0 (equivalent to 2.5 mg of cell protein/mL).

*Isolation of Cell Membranes.* Cell membranes were prepared from a portion of the washed cell suspension by ultrasonic treatment for two to four 30-s periods at 0 °C in an MSE ultrasonic disintegrater (60 W) operated at 1.5 A. Membrane preparations had absorbance (500 nm) values 80–90% lower than those of cell suspensions containing the equivalent membrane mass per mL. The membranes were collected by centrifugation at 34 000*g* for 30 min, washed once, and resuspended in 0.25 M NaCl solution. No loss of cholesterol was found on sonication.

*Lipoproteins.* Human low- and high-density lipoproteins prepared as previously described (Slutzky et al., 1977) were kindly provided by Professor S. Eisenberg (The Lipid Research Laboratory, Jerusalem). In some experiments LDL and HDL were iodinated with $^{125}$I (Bilheimer et al., 1972). Before use,

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[1] Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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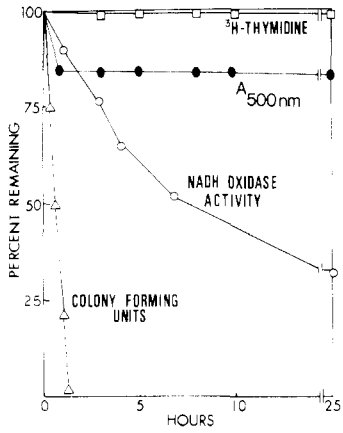


FIGURE 1: Effect of prolonged periods of incubation of *M. gallisepticum* cells with HDL on cell viability and leakiness. Cells (300 μg of protein per mL) were incubated with HDL (4.5 mg of protein per mL) at 37 °C in sucrose–phosphate buffer for various periods of time. The viability and leakiness of the cells were determined as described in Materials and Methods.

the lipoprotein preparations were dialyzed for 24 h against 200 volumes of 0.4 M sucrose solution containing 100 mM sodium phosphate buffer, pH 7.2, and 20 mM $MgCl_2$ (sucrose–phosphate buffer). The lipoprotein fraction was filtered through a 0.45-μm pore size Millipore filter and stored at 4 °C until used.

*Cholesterol Exchange Studies.* [$^{14}$C]Cholesterol-labeled *M. gallisepticum* intact cells or isolated membranes containing about 10 μg of cholesterol (250 μg of cell protein or 90 μg of membrane protein) were incubated at 37 °C for varying periods of time with HDL or LDL. The amount of HDL or LDL added up to a final volume of 1.0 mL of sucrose–phosphate buffer provided a 50–100-fold excess of unesterified cholesterol in the reaction mixture (5–10 mg of HDL protein or 1–2 mg of LDL protein). To avoid bacterial contamination, penicillin G (2000 U/mL) and sodium azide (0.02%) were added to the reaction mixture. Upon completion of the incubation period, duplicates of 50-μL samples were filtered through 0.45-μm pore size Millipore filters. The filters were washed twice with 10 mL of cold 0.25 M NaCl solution and dried. The radioactivity was assayed using a toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid. Samples containing isolated membranes were centrifuged for 30 min at 34 000*g*. The sedimented membranes were washed twice with 10 mL of cold 0.25 M NaCl solution, solubilized with 0.1 mL of 10% sodium dodecyl sulfate, and assayed for radioactivity. To determine whether the cells remained intact in the exchange reaction mixture, [$^3$H]thymidine-labeled cells were used. At varying lengths of time, the absorbance (500 nm) of the cells in the reaction mixture was determined. At each time interval samples were withdrawn and the viability of the cells in the sample was determined by the colony-count technique (Butler & Knight, 1960). The retention of [$^3$H]thymidine-labeled components in the cells was determined after filtration of the samples through a 0.45-μm filter and counting the filters as described above. NADH oxidase activity in suspensions of cell pellets and in supernatant fluid obtained after centrifugation of the samples at 12 000*g* for 20 min was determined in a reaction mixture described before (Ne'eman & Razin, 1975); to obtain complete release of the enzyme from intact cells, sodium deoxycholate was added to the reaction mixture at a final concentration of 1.25 mM.

*Analytical Methods.* Protein was determined according to Lowry et al. (1951) using bovine serum albumin as standard. Lipids were extracted from freeze-dried membranes or cells with chloroform–methanol (2:1) at 45 °C for 2 h. The concentration of cholesterol in the extracted lipids was measured by the procedure of Rudel & Morris (1973). The separation of unesterified cholesterol from esterified cholesterol was performed as previously described by Argaman & Razin (1965). Total phosphorus in the lipid fraction was determined by the method of Ames (1966).

*Electron Spin Resonance Measurements.* Membranes of *M. gallisepticum* strain A5969 were labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid and 12-ketostearic acid, hereafter called 5-doxylsterate and 14-doxylsterate (Syva, Palo Alto, Calif.), by exchange from bovine serum albumin according to Rottem et al. (1970). The freedom of motion of the spin-labeled fatty acids in the membrane preparations was assessed from the order parameter, which was calculated from spectra recorded at 37 °C as described by Gaffney (1975). The order parameter is related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the membrane.

## Results

*Mycoplasma gallisepticum* is unable to synthesize cholesterol, thus requiring it for growth (Argaman & Razin, 1965). Despite the presence of excessive amounts of esterified cholesterol in the growth medium, almost all (~95%) of the cholesterol found in *M. gallisepticum* strain A5969 was unesterified cholesterol. The cholesterol incorporated by the cells was neither esterified nor otherwise changed and the level of cholesterol in the membranes was high, as reflected by a cholesterol/phospholipid molar ratio of about 1.0. When *M. gallisepticum* cells were grown in the presence of [$^{14}$C]cholesterol, the labeled cholesterol was incorporated into the cell membrane. Since [$^{14}$C]cholesterol was present in the growth medium in almost identical quantities throughout the growth cycle, it is assumed that the specific activities of the cholesterol pools in the membrane are equal.

Our cholesterol exchange procedures require that a minimal net transfer of cholesterol occurs between *M. gallisepticum* cells and the lipoproteins, and that the permeability barrier of the cells remains intact throughout the exchange reaction. A considerable net transfer of cholesterol occurred upon a 24-h incubation of *M. gallisepticum* cells with LDL resulting in an increase in cholesterol content from 21.2 to 75.5 μg of cholesterol per mg of cell protein. Only about 15% of the increase was due to the binding of LDL to the cells [~10 μg of LDL protein bound per mg of cell protein as determined using $^{125}$I-labeled LDL (Slutzky et al., 1977)]. On the other hand, a small increase in cholesterol content (from 21.2 to 26.4 μg of cholesterol per mg of cell protein) occurred after a 24-h incubation of the cells with HDL where about 5% of the increase was due to the association of HDL with the cells (1.4 μg of HDL protein bound per mg of cell protein). There was no further increase in the cholesterol content of the cells or in the amount of HDL bound during prolonged incubation periods (up to 6 days) of *M. gallisepticum* cells with HDL. These observations exclude the possibility of fusion or cosedimentation of HDL with *M. gallisepticum* cells or membrane preparations, favoring the use of HDL but not of LDL as an appropriate cholesterol carrier in our system. Figure 1 shows that throughout a 24-h incubation period of cells in a complete reaction mixture, the absorbance of the cell suspension was almost unchanged after an initial rapid decrease. Since all of the thymidine-labeled components were retained within the cell, and no NADH oxidase was released from the cells, the permeability barrier of the cells remained essentially intact.

TABLE I: Changes in Membrane Composition and Membrane Fluidity of *M. gallisepticum* Cells upon Aging of the Culture.

| Age of culture (h) | Absorbance of culture (640 nm) | Phospholipid and cholesterol content of membranes: Lipid phosphorus (μmol (mg of membrane protein)$^{-1}$) | Cholesterol (μg (mg of membrane protein)$^{-1}$) | Cholesterol (μmol (μmol of lipid phosphorus)$^{-1}$) | Order parameter[a]: 5-Doxylstearate | Order parameter[a]: 12-Doxylstearate |
|---|---|---|---|---|---|---|
| 16 | 0.12 | 0.27 | 122.1 | 1.20 | 0.61 | 0.36 |
| 18 | 0.16 | 0.24 | 90.2 | 0.98 | 0.63 | 0.41 |
| 23 | 0.30 | 0.16 | 58.2 | 0.98 | 0.66 | 0.51 |

[a] Order parameters were calculated from spectra recorded at 37 °C.

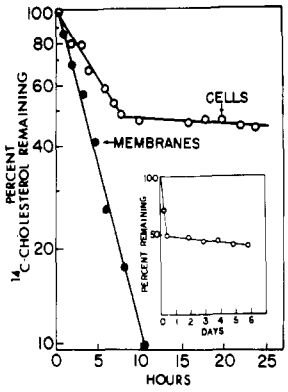


FIGURE 2: Transfer of [$^{14}$C]cholesterol from labeled *M. gallisepticum* cells or membrane preparations to HDL as a function of incubation time. Conditions were the same as in Table I. Inset: Time course of [$^{14}$C]cholesterol transfer from cells to HDL over a 6-day period.

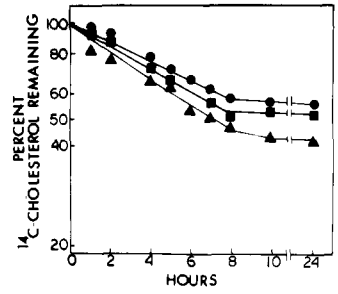


FIGURE 3: Plot of transfer of [$^{14}$C]cholesterol from *M. gallisepticum* cells harvested at various phases of growth to HDL vs. time of incubation. Cultures were harvested at $A_{640}$ values of 0.12 (●), 0.16 (■), and 0.30 (▲).

The viability of the cells showed, however, a 99% decrease within 2 h of incubation and a total loss of viability within 5 h of incubation. Although NADH oxidase activity was not found in the supernatant fluid obtained after sedimenting the cells, the level of the soluble NADH oxidase activity was reduced by 50% within 6 h of incubation. The reduced levels of the enzyme within the cells reflect the apparent intracellular inactivation of the enzyme rather than its release from the cells into the medium.

The time course for transfer of labeled cholesterol from *M. gallisepticum* cells and membranes to HDL is shown in Figure 2. About 50% of the labeled cholesterol was removed from intact cells compared with more than 90% from isolated membranes. Little additional cholesterol could be removed from intact cells over a 6-day incubation period (inset to Figure 2). During this period the cells remained intact as indicated by the unchanged absorbance of the cell suspensions and the retention of [$^3$H]thymidine-labeled components within the cells. A drop in absorbance was, however, noticed at incubation periods longer than 6 days. Since no net transfer of cholesterol between intact *M. gallisepticum* cells and HDL preparations occurred, the removal of [$^{14}$C]cholesterol from the cells can be used as a measure of the rate and extent of exchange of cholesterol between cells and HDL. This suggests that about one-half of the cholesterol in intact cells is readily exchangeable with the lipoprotein cholesterol with a half-time of about 4 h, while the other half is very slowly exchanged with a half-time of about 18 days as calculated when fitted to a single exponential. Figure 3 shows that the age of the culture affects the fraction of the cholesterol in the membranes of intact cells that exchanges in the rapid process. Thus, with cells in the early exponential phase of growth ($A_{640}$ = 0.10) only about 40% of the cholesterol was readily exchanged, whereas with cells in the late exponential phase of growth ($A_{640}$ = 0.30) almost 55% was exchanged. Table I shows the changes that take place in membranes of *M. gallisepticum* cells harvested at early ($A_{640}$ = 0.12), mid- ($A_{640}$ = 0.16), and late ($A_{640}$ = 0.30) exponential phases of growth. A marked decrease in membrane lipid-to-protein ratio occurs upon aging of the culture. However, the cholesterol to phospholipid molar ratio is almost unchanged. The variations in the lipid-to-protein ratios of membranes from cells harvested at different phases of growth had a marked effect on membrane fluidity. Membranes from cells harvested at the late exponential phase of growth showed a lower fluidity, as indicated by the decreased freedom of motion of 5-doxylstearate and 12-doxylstearate. The freedom of motion of the spin-label probe in the membrane is related to the order parameter ($S$), with higher $S$ values being associated with reduced freedom of motion of the probe (Gaffney, 1975).

## Discussion

Evidence is presented here that in intact *M. gallisepticum* cells cholesterol is present in two distinct environments. Cholesterol in one environment (approximately 50% of the total) is readily exchanged with HDL, whereas cholesterol in the other environment is exchanged with exceedingly slow rates. Since more than 90% of the cholesterol in isolated membranes was exchanged rapidly, it is likely that the environments represent the outer and inner halves of the lipid bilayer. Two pools of cholesterol have also been postulated in the membranes of influenza virions (Lenard & Rothman, 1976) and of red blood cells (Bell & Schwartz, 1971; d'Hollander & Chevallier, 1972; Cooper et al., 1972; Grunze & Deuticke, 1974; Fisher, 1976; Gottlieb, 1976; Lange et al., 1977). It was suggested that in influenza viral membranes the two cholesterol pools correspond to the two surfaces of the lipid bilayer (Lenard & Rothman, 1976); however, in the erythrocyte membrane the two pools may represent cholesterol molecules with differing accessibility to plasma free cholesterol, which may not correspond necessarily to the localization of the sterol within the bilayer (Got-

tlieb, 1976). The entire pool of exchangeable cholesterol in human erythrocytes can be removed from the membrane by incubation with plasma of lowered cholesterol content (Lange & D'Alessandro, 1977). Our observation that in unsealed membrane preparations of *M. gallisepticum* more than 90% of the cholesterol is exchanged rapidly indicates that in *M. gallisepticum* an extensive correspondence exists between accessibility of cholesterol for exchange and its localization within the bilayer. Nevertheless, approximately 10% of the total cholesterol remained unavailable for exchange from isolated membrane preparations even after a prolonged incubation period with HDL. This suggests that a small pool of cholesterol may be firmly bound or in a region of poor contact with the HDL particle. One cannot rule out the possibility that a small fraction of sealed membrane fragments may be present, even though membranes obtained from mycoplasma cells by sonication or osmotic lysis are known to be largely unsealed, based on morphological (Rottem & Razin, 1966) and transport (Cirillo & Razin, 1973) studies.

Recent studies involving manipulation of cholesterol levels in biological cells using cholesterol–phospholipid dispersions showed that the cholesterol-to-phospholipid molar ratio in the dispersion has a marked effect on the direction of cholesterol movement. Thus with cholesterol–phospholipid dispersions having a molar ratio higher than that in the cell membrane, a net accumulation of cholesterol in the cell membrane could be demonstrated, whereas depletion of cellular cholesterol could be achieved using cholesterol–phospholipid dispersions having low molar ratios (Shinitzky & Inbar, 1974; Arbogast et al., 1976). The free cholesterol to phospholipid molar ratio in HDL is much lower than that in the membrane of *M. gallisepticum* (0.2 vs. 1.0); yet no net transfer of cholesterol was observed. HDL is appropriate for the exchange process since only little net cholesterol transfer occurs and only little nonspecific binding to *M. gallisepticum* cells or membrane preparations was found. Serum lipoproteins have been found to be excellent sources of cholesterol for growth of mycoplasmas (Slutzky et al., 1977).

The increased size of the exchangeable pool observed in cells harvested at late exponential phases of growth may arise from the increase in the rigidity of the fatty acyl chains of the phospholipids, as determined by measuring the freedom of motion of fatty acid spin labels in the membrane. The increased rigidity may limit the movement of cholesterol into the inner half of the bilayer. The increased protein content of the membranes observed upon aging of cells may occur predominantly at the inner membrane surface, as shown in other mycoplasma cells (Amar et al., 1976). The movement of cholesterol to the inner half of the lipid bilayer may also be impeded by the decreased area available for lipid at that surface.

The approximate symmetrical distribution of cholesterol in the two halves of the lipid bilayer as indicated in our exchange experiments is in excellent agreement with rapid kinetic studies of filipin binding to cholesterol in intact cells and isolated membranes of *M. gallisepticum* (Bittman & Rottem, 1976). The ratio of the second-order rate constants of filipin–cholesterol association in isolated membranes relative to intact cells indicated a symmetric distribution of cholesterol in intact *M. gallisepticum* cells.

Although cholesterol taken up by mycoplasmas is first incorporated into the outer half of the lipid bilayer, only about 50% of the cholesterol in the membranes of intact *M. gallisepticum* cells is present on the outer surface. Therefore, within the growth period (18–24 h) movement of cholesterol from the outer to the inner surface occurs. The rate of this movement appears far more rapid than the rates demonstrated in influenza viral membranes and phospholipid bilayers. On the other hand, our exchange data show a very slow rate of cholesterol movement across the bilayer in resting cells. The rate of transbilayer cholesterol movement may vary with the direction; for example, the rate may be greater from the cholesterol-rich outer surface to the cholesterol-poor inner surface in growing cells than from the inner to outer surface in resting cells, where the cholesterol concentrations are more comparable. The rate may also depend on the viability of the cells.

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