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

ALTERATIONS IN MEMBRANE ChOLESTEROL AND PROTEIN CONTENT

Sanda Clejan and Robert Bittman†
From the Department of Chemistry, Queens College of The City University of New York, Flushing, New York 11367

(Received for publication, June 22, 1983)

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 biphase 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 membranes (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 transbilayer movement from the outer to inner halve (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 ≥5%; 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 [14C]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 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. [2-3H]Glycerol trioleate (2 Ci/mmol) was obtained from methanol. Hydrochloride (20 000 grade. Potassium salicylate was prepared from salicylic acid and Molecular weight cutoff, Fisher) were rinsed and boiled in distilled water prior to use in the equilibrium dialysis cells.

Methods

Growth of M. gallisepticum—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/10 ml of medium. In the experiments of endogenous phospholipid exchange, the lipids were labeled by the addition of 0.002 μCi of [14C]palmitic acid/ml 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 leakiness of M. gallisepticum cells after separation from the vesicles, the medium was supplemented with 0.25 μCi/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 a solution containing 20 mM MgCl2 and 0.005 μCi of [3H]deoxyribose (2 μCi/ml) in the presence of 20 mM MgCl2, and then washed once and resuspended in 0.20 M sucrose, 50 mM Tris buffer, pH 7.4, containing 20 mM MgCl2 (STM buffer). Cells were adapted to grow 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. After the mixture had entered the column completely, the eluate was collected at 1.0 ml of STM buffer, pH 7.4, was added. The eluate was counted in 4 ml of Liquiscint. The recovery of the uncharged acceptor vesicles in the eluate was approximately 80%, based on 3H cpm from vesicles labeled with [3H]cholesterol or [3H]glycerol trioleate as a nonexchangeable marker. Less than 6% of the 14C cpm in M. gallisepticum vesicles was lost when the absence of lysozyme in the elution buffer. An excess of acceptor vesicles was used to minimize back exchange; a 10-fold excess of vesicles (relative to cholesterol in donor membranes) was used in the exchange studies in the presence of 2% (w/v) albumin, and a 45-fold excess was used in the absence of albumin. The fraction of [14C]cholesterol exchanged at time t was calculated from the ratio of [14C]/[3H], where (14C/3H) represents the ratio of [14C]cholesterol/3H nonexchangeable marker at time zero, in the eluate obtained from membranes in the absence of acceptor vesicles. The infinity value was determined after 20–24 h.

Modification of Membrane Protein Content—To study the effect of membrane protein on the initial rate of [14C]cholesterol exchange, the exogenous protein lysozyme was incorporated into M. gallisepticum cell membranes by treating cells (1 mg of cell protein/ml) with lysozyme (1.5 mg/ml) in STM buffer at 37 °C (9). In other experiments, the endogenous membrane proteins were cross-linked with dimethyl suberimidate or 1,4-phenylenediamineimide. The cell suspension (1 mg of cell protein/ml of STM buffer) was incubated at 37 °C for 30 min with sufficient dimethyl suberimidate stock solution (10 mM in ethanol) to give a protein/dimethyl suberimidate ratio of 2:1, assuming that the average molecular weight of M. gallisepticum protein was 90,000. The final ethanol concentration did not exceed 0.5% (v/v). The reaction was terminated by dilution with 0.25 M NaCl solution, and the cells were sedimented at 12,000 × g for 20 min and resuspended in STM buffer. Protein cross-linking using 1,4-phenylenediamineimide (40 mM stock solution in ethanol) was carried out in STM buffer at 37 °C by incubating cells at protein phenylenediamineimide ratio of 2:1 for 30 min or 4:1 for 10 min. The reactions were terminated by dilution with 20 volumes of cold STM 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 washed with STM buffer. The treated cells remained intact during the 12-h incubation with vesicles, as judged by the retention of [3H]labeled components in the recovered vesicles. The extent of formation of the cross-linked proteins produced by these treatments was assessed by sodium dodecyl sulfate electrophoresis in 15% polyacrylamide gels (12). Detection was by staining with Coomassie brilliant blue. Gel densitometry of the control and cross-linked samples was performed with a Helena densitometer.

Analytical Procedures—The procedures for extraction of lipids from cell suspensions and for determination of protein, phospholipid, and cholesterol have been described elsewhere (6).

Kinetics of [14C]Cholesterol Translocation—The half-time of equilibration of [14C]cholesterol was the time required for 50% of the membrane bilayer cholesterol to equilibrate between the inner and outer leaflets of the vesicles (see subsequent section). kobs and kex are considered to represent the first order rate constants for the translocation of cholesterol from the inner to outer and from the
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, 14–16). 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 four-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 0.92 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

![Graph](image-url)
Lipid Exchange between Mycoplasma gallisepticum 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, $t_{50}^r$, 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 $t_{50}$ ($p < 0.05$) 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 ($M_r = 95,000$ and $M_r > 95,000$), 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 $k_{a2}$) and increased $t_{1/2}$ and $t_{50}$. 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 $[^{14}C]$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 chloramphenicol. There was a 73 ± 10% decrease in protein content of the outer monolayer of the membrane bilayer to vesicles when the cholesterol/phospholipid molar ratio (Table II). The rates of cholesterol translocation from one pool to the other ($k_{a2}$, $k_{b2}$) 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 $t_{50}$ was not reduced significantly ($p > 0.05$). Exogenous lysozyme, which is bound extrinsically to mycoplasma membranes (19), also did not alter $t_{50}$ (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 $[^{14}C]$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 $[^{14}C]$cholesterol exchange was 1.67 times faster in the presence of these salts relative to STM buffer alone. Furthermore, the relative rate increased by a factor of 2.0 when 5% (final concentration) dimethyl sulfoxide, which is also expected to increase the aqueous solubility of cholesterol, was added to STM 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.

Phospholipid Exchange—The kinetics of exchange of phospholipids from cells to vesicles was also measured in membranes containing different cholesterol/phospholipid molar ratios. When the membrane lipids were labeled biosynthetically with $[^{14}C]$palmitate, the incorporation of the label was almost exclusively into PG if the cells were grown without exogenous phospholipids. Exogenous polar lipids were incorporated into $M$. gallisepticum cells, as has been reported in other mycoplasmas (23, 24). The initial rate of $[^{14}C]$-labeled

### Table II

| Molar ratio of cholesterol/PL | $k_{a2}$ | $k_{b2}$ | $t_{1/2}$ | $t_{50}$ |
|------------------------------|---------|---------|----------|---------|
| $10^6 \times$ rate constant (h$^{-1}$) | h | h |
| 0.92 | 4.7 ± 0.6 | 6.5 ± 0.8 | 6.2 ± 0.3 | 2.5 ± 0.1 | 0.58 (4) |
| 0.63 | 4.9 ± 0.8 | 6.7 ± 0.9 | 6.2 ± 0.3 | 2.4 ± 0.2 | 0.69 (2) |
| 0.50 | 4.9 ± 0.9 | 7.8 ± 1.2 | 5.5 ± 0.5 | 2.1 ± 0.1 | 0.62 (3) |
| 0.36 | 6.8 ± 0.8 | 8.2 ± 1.2 | 4.6 ± 0.5 | 1.8 ± 0.2 | 0.55 (4) |
| 0.25 | 12.7 ± 1.2 | 15.0 ± 1.6 | 2.5 ± 0.1 | 1.1 ± 0.2 | 0.54 (4) |

* 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.

1 $t_{50}$ is the time required for the $[^{14}C]$cholesterol content to be reduced to 50%; it represents mostly a transfer of cholesterol.

amphenicol/ml compared with cells grown without the inhibitor. The electrophoretic pattern of the membrane proteins was not changed significantly by chloramphenicol treatment of the culture. The uptake of exogenous cholesterol was also partially inhibited by addition of more than 10 µg of chloramphenicol/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 chloramphenicol/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 chloramphenicol 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 chloramphenicol 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 ($k_{a2}$, $k_{b2}$) 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 $t_{50}$ was not reduced significantly ($p > 0.05$). Exogenous lysozyme, which is bound extrinsically to mycoplasma membranes (19), also did not alter $t_{50}$ (Table III).

### Table III

| Lipid Exchange between Mycoplasma gallisepticum and Vesicles at 37 °C | $k_{a2}$ | $k_{b2}$ | $t_{1/2}$ | $t_{50}$ |
|-----------------------------|---------|---------|----------|---------|
| Molar ratio of cholesterol/PL | $10^6 \times$ rate constant (h$^{-1}$) | h | h |
| 0.92 | 4.7 ± 0.6 | 6.5 ± 0.8 | 6.2 ± 0.3 | 2.5 ± 0.1 | 0.58 (4) |
| 0.63 | 4.9 ± 0.8 | 6.7 ± 0.9 | 6.2 ± 0.3 | 2.4 ± 0.2 | 0.69 (2) |
| 0.50 | 4.9 ± 0.9 | 7.8 ± 1.2 | 5.5 ± 0.5 | 2.1 ± 0.1 | 0.62 (3) |
| 0.36 | 6.8 ± 0.8 | 8.2 ± 1.2 | 4.6 ± 0.5 | 1.8 ± 0.2 | 0.55 (4) |
| 0.25 | 12.7 ± 1.2 | 15.0 ± 1.6 | 2.5 ± 0.1 | 1.1 ± 0.2 | 0.54 (4) |

* 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.
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 $^{14}$Ccholesterol and $^{14}$Cphospholipids 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

![Graph A](#)  
**FIG. 3.** Effect of modifying membrane protein composition and organization on kinetics of $^{14}$C cholesterol exchange between *M. gallisepticum* cells and cholesterol/PC vesicles. A, cross-linking of membrane proteins; B, partial inhibition of protein synthesis by chloramphenicol. In A, cells were treated with phenylenedimaleimide using a protein/phenylenedimaleimide molar ratio of $1:4$ or $1:2$, as described under "Methods", or with dimethyl suberimidate (C). Kinetic data obtained from cells that were not treated with a cross-linking agent are also shown (○). The cholesterol/phospholipid molar ratio was 0.25. In B, the cultures received 20 µg of chloramphenicol/ml of media and the cholesterol/phospholipid molar ratio was 0.34 (●); kinetic data obtained from control cells, cholesterol/phospholipid molar ratio of 0.36, are also shown (○).

**Table III**

| Modification               | Molar ratio of cholesterol/PL in the cell membrane | $k_{as}$ | $k_{ac}$ | $t_{1/2}$ | $t_{50}$ | $r_{max}$ |
|----------------------------|---------------------------------------------------|---------|---------|----------|---------|----------|
| Phenylendimaleimide*       | 0.25                                              | 10.4 ± 1.0 | 14.2 ± 1.5 | 2.5 ± 0.1 | 1.3 ± 0.2 | 0.55     |
| Phenylendimaleimide*       | 0.25                                              | 4.5 ± 0.9 | 12.0 ± 1.2 | 3.7 ± 0.1 | 1.5 ± 5.2 | 0.65     |
| Dimethyl suberimidate      | 0.25                                              | 11.1 ± 1.2 | 15.3 ± 1.6 | 2.6 ± 0.1 | 1.2 ± 0.1 | 0.58     |
| Chloramphenicol*           | 0.25                                              | 10.2 ± 0.7 | 12.4 ± 0.7 | 3.1 ± 0.1 | 1.4 ± 0.1 | 0.45     |
| Lysozyme*                  | 0.26                                              | 10.0 ± 0.7 | 12.0 ± 0.7 | 3.0 ± 0.1 | 1.3 ± 0.2 |

*Cells are treated with phenylenedimaleimide at 37 °C at a protein/phenylenedimaleimide molar ratio of 4 for 10 min, using a protein average $M_r = 90,000$.

*The reaction was carried out using a protein/phenylenedimaleimide molar ratio of 2 for 30 min.

*Cells were treated with dimethyl suberimidate at 37 °C at a protein/dimethyl suberimidate molar ratio of 4 for 30 min.

*Cells were grown in media containing 20 µg of chloramphenicol/ml and 10 µg of cholesterol/ml.

*Lysozyme was added to cells at a concentration of 1.5 mg/ml of cell suspension in STM buffer in the presence of 2% albumin for 1 h at 37 °C. The amount of lysozyme bound was 1.05 mg/mg of membrane protein. $f_{50}$ is the time required for one-half of the initial rapid rate to be completed.
TABLE IV

| [14C]PL exchanged | Molar ratio of cholesterol/PL | Endogenous cholesterol incorporated | Endogenous PL incorporated | Acceptor vesicles | t1/2 |
|-------------------|-------------------------------|----------------------------------|---------------------------|------------------|------|
|                    | µg/mg membrane protein         |                                  |                           |                  |      |
| Endogenous PL*     | 0.94                          | 102                              | 0                         | PG/Cholesterol   | 24.2 ± 1.2 |
| Endogenous PL*     | 0.25                          | 26                               | 0                         | PG/Cholesterol   | 14.9 ± 0.9 |
| Endogenous PL +    | 0.91                          | 106                              | 76.5                      | PG/PC/Cholesterol| 15.0 ± 1.0 |
| exogenous PC       |                               |                                  |                           |                  |      |
| Endogenous PL +    | 0.23                          | 29                               | 80.4                      | PG/PC/Cholesterol| 9.2 ± 0.5 |
| exogenous PC       |                               |                                  |                           |                  |      |

* 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 [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 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 Czokanski (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 because 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-phenylenediamine 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-phenylene-diamine 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.

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

REFERENCES

1. Bell, F. P. (1978) Prog. Lipid Res. 17, 207-243
2. Bruckdorfer, K. R., and Graham, J. M. (1976) in Biological Membranes (Chapman, D., and Wallach, D. F. H., eds) Vol. 3, pp. 103-152, J. Wiley & Sons, New York
3. Bruckdorfer, K. R., Edwards, P. A., and Green, C. (1966) Eur. J. Biochem. 4, 506-511
4. Bloj, B., and Zilversmit, D. B. (1977) Biochemistry 16, 3943-3948
5. Razin, S. (1978) Microbiol. Rev. 42, 414-470
6. Clejan, S., Bittman, R., and Rottem, S. (1978) Biochemistry 17, 4579-4583
7. Clejan, S., Bittman, R., and Rottem, S. (1981) Biochemistry 20, 2200-2204
8. Rottem, S., Shinar, D., and Bittman, R. (1981) Biochim. Biophys. Acta 649, 572-581
9. Bittman, R., Blau, I., Clejan, S., and Rottem, S. (1981) Biochemistry 20, 2425-2432
10. Razin, S., and Rottem, S. (1976) in Biochemical Analysis of Membranes (Maddy, A. H., ed) pp. 3-26, Chapman and Hall, London
11. McLean, L. R., and Phillips, M. C. (1981) Biochemistry 20, 2893-2900
12. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685
13. Bloj, B., and Zilversmit, D. B. (1976) Biochemistry 15, 1277-1283
14. Razin, S., Yashou, Y., Ne'eman, Z., and Razin, S., (1973) Biochim. Biophys. Acta 323, 495-508
15. Le Grimellec, C., and Leblanc, G. (1978) Biochim. Biophys. Acta 514, 152-163
16. Le Grimellec, C., Cardinal, J., Giocondi, M.-C., and Carriere, S. (1981) J. Bacteriol. 146, 155-162
Lipid Exchange between Mycoplasma gallisepticum and Vesicles

17. Rottem, S., Linker, C., and Wilson, T. H. (1981) *J. Bacteriol.* **145**, 1299–1304
18. Razin, S. (1979) in *The Mycoplasmas* (Barile, M. F., and Razin, S., eds) Vol. I, pp. 213–229, Academic Press, New York
19. Rottem, S., Hasin, M., and Razin, S. (1973) *Biochim. Biophys. Acta* **296**, 876–886
20. Baeker, J. M., and Dawidowicz, E. A. (1981) *Biochemistry* **20**, 3805–3810
21. Massey, J. B., Gotto, A. M., Jr., and Pownall, H. J. (1982) *J. Biol. Chem.* **257**, 5444–5448
22. Bojesen, E. (1982) *Nature (Lond.*)** **299**, 276–278
23. Razin, S., Kutner, S., Efrati, H., and Rottem, S. (1980) *Biochim. Biophys. Acta* **598**, 628–640
24. Gross, Z., Rottem, S., and Bittman, R. (1982) *Eur. J. Biochem.* **122**, 169–174
25. Rottem, S., Slutzky, G. M., and Bittman, R. (1978) *Biochemistry* **17**, 2723–2726
26. Bittman, R., and Rottem, S. (1976) *Biochem. Biophys. Res. Commun.* **71**, 318–324
27. Rottem, S., and Razin, S. (1966) *J. Bacteriol.* **92**, 714–722
28. Cirillo, V. P., and Razin, S. (1973) *J. Bacteriol.* **113**, 212–217
29. Shirvan, M. H., Rottem, S., Ne’eman, Z., and Bittman, R. (1982) *J. Bacteriol.* **149**, 1124–1128
30. Bruckdorfer, K. R., and Green, C. (1967) *Biochem. J.* **104**, 270–277
31. Quarfordt, S. H., and Hilderman, H. L. (1970) *J. Lipid Res.* **11**, 528–535
32. Bell, F. P. (1973) *Exp. Mol. Pathol.* **19**, 293–303
33. Slotte, J. P., and Lundberg, B. (1983) *Biochim. Biophys. Acta* **750**, 434–439
34. Jonas, A., and Maine, G. T. (1979) *Biochemistry* **18**, 1722–1728
35. Smith, R., and Tanford, C. (1972) *J. Mol. Biol.* **67**, 75–83
36. Haberland, M. E., and Reynolds, J. A. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2313–2316
37. Saad, H. Y., and Higuchi, W. I. (1965) *J. Pharm. Sci.* **54**, 1205–1206
38. Mohandas, N., Wyatt, J., Mel, S. F., Rossi, M. E., and Shohet, S. B. (1982) *J. Biol. Chem.* **257**, 6537–6543
39. McLean, I. R., and Phillips, M. C. (1982) *Biochemistry* **21**, 4053–4059
40. Poznansky, M. J., and Czekanski, S. (1979) *Biochem. J.* **177**, 989–991
41. Rottem, S., and Verkleij, A. J. (1982) *J. Bacteriol.* **149**, 338–345
42. Amar, A., Rottem, S., and Razin, S. (1979) *Biochim. Biophys. Acta* **562**, 457–467
43. Efrati, H., Rottem, S., and Razin, S. (1981) *Biochim. Biophys. Acta* **641**, 386–394
44. Presti, F. T., Pace, R. J., and Chan, S. I. (1982) *Biochemistry* **21**, 3831–3835
Kinetics of cholesterol and phospholipid exchange between Mycoplasma gallisepticum cells and lipid vesicles. Alterations in membrane cholesterol and protein content.
S Clejan and R Bittman

J. Biol. Chem. 1984, 259:441-448.

Access the most updated version of this article at http://www.jbc.org/content/259/1/441

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/1/441.full.html#ref-list-1