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SYMMETRICAL DISTRIBUTION AND RAPID TRANSBILAYER MOVEMENT OF CHOLESTEROL IN *MYCOPLASMA GALLISEPTICUM* MEMBRANES

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The exchange of cholesterol between [^{14}C]cholesterol-labeled *Mycoplasma gallisepticum* cells and an excess of sonicated egg phosphatidylcholine/cholesterol vesicles (molar ratio of 0.9) was measured. More than 90% of the radioactive cholesterol underwent transfer from intact cells to the vesicles. The kinetics of the transfer was biphasic. About 50% of the radioactive cholesterol was exchanged with a half-time of about 4 h. The residual was exchanged at a slower rate with a half-time of about 9 h at 37°C. Bovine serum albumin had a pronounced effect in enhancing both the fast and slow rates of cholesterol exchange, but did not affect the pool sizes significantly. The half-time for equilibration of the two pools in the presence of 2% albumin, calculated using a reversible two-pool method of analysis, was 6.2 h. The effect of albumin was also obtained with isolated membrane preparations and with cells treated with growth inhibitors, suggesting that this effect is independent of albumin preservation of cell viability. The rate enhancement of albumin was concentration dependent with maximal effects observed with ≥2%, where the rates of exchange of both the rapidly and slowly exchanging pools were twice as fast. The mechanism by which albumin may affect the exchange rates is discussed.

Introduction

Mycoplasma species require cholesterol for growth, making them a good system for studying the localization and transbilayer movement of this sterol in the cell membrane. It is well established that cholesterol in biological membranes is capable of spontaneous exchange with cholesterol present in other membranous systems (e.g. Refs. 1–8). Kinetic analyses of such intermembrane exchange measurements revealed that many membranes contain cholesterol in two pools, interpreted to be located in the inner and outer leaflets of the membrane bilayer [1,5,6,7]. Recent evidence, however, indicates that in some membranes cholesterol is almost completely exchangeable as one kinetic pool suggesting that transbilayer movement of cholesterol is faster than the intermembrane exchange process [3,4,9,10]. The transbilayer distribution of cholesterol in membranes of *Mycoplasma gallisepticum* was investigated using filipin as a probe [11]. The initial rate of filipin-cholesterol association in intact cells and unsealed isolated membranes was taken as a measure of cholesterol distribution in the membrane. These measurements indicated a symmetrical distribution of cholesterol in the two halves of the *M. gallisepticum* membrane. The major obstacle in utilizing filipin is membrane perturbations caused by this probe. Therefore, the results obtained by fast kinetic studies with filipin were confirmed by exchange studies in which cholesterol was exchanged from *M. gallisepticum* membranes to high-density lipoprotein (HDL) particles [7]. These studies

Abbreviations: sucrose-Tris-$MgCl_2$ buffer, 0.4 M sucrose/50 mM Tris-HCl/20 mM $MgCl_2$ buffer, pH 7.8; HDL, high density lipoprotein; PC, phosphatidylcholine.

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showed that in intact *M. gallisepticum* cells 50% of the total unesterified cholesterol is readily exchanged with HDL-cholesterol, supporting the symmetrical distribution of the molecule between the two leaflets of the cell membrane. However, the rate of translocation from one leaflet to the other was exceedingly slow or nonexistent. The slow translocation rate was unexpected since in mycoplasmas cholesterol is taken up from an exogenous source into the outer leaflet, and is then translocated inward. Furthermore, filipin binding experiments with another mycoplasma, *Mycoplasma capricolum,* showed that translocation of cholesterol from one side of the membrane to the other is very rapid but was dependent on cell viability and growth [12,13]. It was therefore suggested that the slow translocation rates observed in the HDL-exchange experiments were due to the fast death rate of *M. gallisepticum* cells in the HDL-exchange system [12]. The present study describes results obtained in exchange studies with unilamellar phosphatidylcholine/cholesterol lipid vesicles where cell viability could be preserved. It was found that *M. gallisepticum* cholesterol underwent almost complete transfer to lipid vesicles independent of cell viability.

Materials and Methods

Organism and growth conditions. Mycoplasma gallisepticum (strain A5969) was grown in a modified Edward medium [14] containing 4% horse serum. To label cell phospholipids, 0.01 μCi/ml of [^{3}H]palmitic acid (57.9 Ci/mol, The Radiochemical Centre, Amersham, U.K.) was added to the growth medium. To test for cell leakiness during the incubation period, 0.25 μCi/ml of [*6-methyl*-^{3}H]thymidine (35.2 Ci/mol, Nuclear Research Center, Negev, Israel) was added. To label cells for the cholesterol exchange experiments, 0.005 μCi/ml of [4-^{14}C]cholesterol (54 Ci/mol, New England Nuclear, Boston, MA) was added to the growth medium as an ethanolic solution. The cultures were incubated at 37°C. Most experiments were performed with cells in mid-exponential phase of growth (A_{640} = 0.16–0.20). The cells were harvested by centrifugation at 12000 × *g* for 15 min, washed once and resuspended in 0.4 M sucrose solution containing 50 mM Tris-HCl (pH 7.8) and 20 mM $MgCl_2$ (referred to as sucrose-Tris-$MgCl_2$ buffer).

Isolation of cell membranes. Cell membranes were prepared by ultrasonic treatment of a washed cell suspension containing 0.125 mg cell protein per ml for 3 min at 0°C in a W-350 Heat Systems sonicator operated at 50% duty cycle at 160 watts. The membranes were harvested by centrifugation at 30000 × *g* for 30 min and resuspended in the sucrose-Tris-$MgCl_2$ buffer.

Preparation of lipid vesicles. Vesicles were prepared by pipetting 2 ml of egg phosphatidylcholine (PC) solution (15 mg/ml) in chloroform (Makor Chemicals, Jerusalem, Israel) and 1 ml of cholesterol solution (13.5 mg/ml in chloroform, Sigma, St. Louis, MO) into 20-ml test tubes. The solvent was evaporated under nitrogen. The dried lipids were then dispersed in 10 ml of sucrose-Tris-$MgCl_2$ solution by ultrasonic irradiation for 60 min at 0°C under nitrogen in a W-350 Heat Systems sonicator operated at 50% duty cycle with a large probe at 160 watts. The lipid dispersions were then centrifuged at 30000 × *g* for 40 min. The sediment containing large liposomes was discarded. In some experiments the supernatant fluid was applied to a 1 × 40 cm column of Sepharose-4B (Pharmacia, Uppsala) and eluted with sucrose-Tris-$MgCl_2$. Fractions of approx. 1 ml were collected and analyzed for lipids. Fractions from the lipid peak were pooled to give the required vesicle preparations. These preparations were kept at 4°C and used within 2 days. The homogeneity of the vesicle preparations was determined by electron microscopy. The vesicles were negatively stained with 1% phosphotungstic acid and analyzed in a Philips-EM 400 electron microscope operated at 80 kV. The vesicle preparations used were found to contain mainly the following distribution of diameters: 400 Å (50%), 500 Å (16%), and 600 Å (20%).

Albumin binding to lipid vesicles. To determine binding of albumin (bovine serum, Fraction V, Sigma) to the vesicle preparations, equal volumes of a vesicle suspension and albumin (2% final concentration) in sucrose-Tris-$MgCl_2$ buffer were mixed and incubated at 37°C for 3 h. The vesicle-bound albumin was separated from the unbound soluble albumin by chromatography on a Sephadex G-150 column (50 × 2.5 cm). The column was eluted with sucrose-Tris-$MgCl_2$ buffer. Fractions (5 ml) were collected and analyzed for protein and phospholipids. An almost complete separation of the vesicles from albumin was

obtained, with 99.5% of the albumin being eluted in fractions 20–28 (unboud albumin) and 0.5% of the albumin eluted with the vesicles in fractions 9–16 (bound albumin).

Cholesterol exchange studies. [^{14}C]Cholesterol-labeled *M. gallisepticum* intact cells or isolated membranes (containing about 0.4 mg of cell protein or 0.08 mg of membrane protein) were incubated at 37°C for varying periods of time with egg PC/cholesterol vesicles (containing 3 mg/ml of PC and 1.35 mg/ml of cholesterol). The vesicles added provided about a 100-fold excess of unesterified cholesterol in the reaction mixture. To avoid bacterial contamination penicillin G (5000 U/ml) was added to the reaction mixture. To keep the cells equally dispersed deoxyribonuclease (Sigma, 20 μg/ml) was added to the medium. Upon completion of the incubation period duplicates of 0.5 ml were withdrawn. Incubation mixtures containing intact cells were centrifuged in a Beckman Microfuge B for 2 min. Incubation mixtures containing isolated membranes were centrifuged in a Sorvall RC-2B at 30 000 × *g* for 20 min. The cell membrane pellets were washed once in 0.25 M NaCl solution and solubilized in 0.1 ml of 1% sodium dodecyl sulfate. The solubilized material was applied to GF/C fiberglass filters (Tamar, Jerusalem, Israel). The filters were air-dried and assayed for radioactivity in a Packard Tricarb liquid scintillator using toluene scintillation liquor. In some experiments the washed pellets of cells or membranes were analyzed for protein and lipids. To determine whether the cells remained intact in the exchange reaction mixture absorbance (at 500 nm) and the retention of [^{3}H]-thymidine-labeled components were determined as described before [7].

Albumin was added to the exchange mixture in some experiments. To determine the extent of recovery of the lipid vesicles from *M. gallisepticum* cells, vesicles containing phosphatidyl [*N-methyl*-^{14}C]-choline (50 Ci/mol, New England Nuclear) were incubated with nonradioactive cells. Vesicles were separated from the cells as described above. The recovery of the vesicles in the supernatant was 98–99%, regardless of the presence or absence of albumin. In some experiments the viability of the cells throughout the exchange reaction was determined using the colony-count technique [15].

Analytical methods. Protein was determined by the method of Lowry et al. [16]. Lipids were extracted from membranes or cell preparations according to Bligh and Dyer [17]. Total membrane lipids were chromatographed on silica gel HR plates as described before [18]. The lipid spots were scraped from the plates into scintillation vials containing 3 ml of toluene scintillation liquor and radioactivity was determined. Total phosphorus in the lipid spots and in the total lipid fraction was determined by the method of Ames [19] after treatment of the sample with an ethanolic solution of $Mg(NO_3)_2$. The concentration of cholesterol in the total lipid extract was measured as described before [18].

Electron spin resonance measurements. Membranes were labeled with *N*-oxyl-4′, 4′-dimethyloxazolidine derivatives of 5-ketostearic and 16-ketostearic acid, hereafter called 5-doxylstearate and 16-doxylstearate (Syva, Palo Alto, CA), as described before [20]. The freedom of motion of the spin labels in the membranes was assessed from the order and motion parameters. The order parameter (S) is related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the membrane, and was calculated according to Gaffney [21]. Low values of order parameter are associated with higher freedom of motion of membrane lipids. The motion parameter (τ_0) was calculated according to Henry and Keith [22].

Results

Mycoplasma gallisepticum cells containing ^{14}C-labeled cholesterol were obtained by growth in a medium containing [^{14}C]cholesterol. These cells are unable to synthesize cholesterol [23] and therefore incorporate it from the growth medium. The cholesterol incorporated by the cells was neither esterified nor otherwise changed and the level of cholesterol was high, as reflected by a cholesterol/phospholipid molar ratio of 0.9–1.0.

Exchange with lipid vesicles

We measured the exchange of the radioactive mycoplasmal cholesterol during incubation of the mycoplasma cells with a large excess of nonradioactive sonicated cholesterol/egg PC vesicles. The lipid vesicles were unilamellar vesicles obtained by gel filtration after sonication. They contained approxi-

mately the same ratio of cholesterol to phospholipid as does the mycoplasma cell, 0.9. This ratio was chosen to minimize depletion of mycoplasma cholesterol during the incubation. When [^{3}H]-labeled palmitate was incorporated into lipids of *M. gallisepticum*, and the cells were incubated with lipid vesicles in surcrose-Tris-$MgCl_2$ buffer for up to 8 h at 37°C, no transfer of radioactivity to the vesicles was observed. Similarly, when unlabeled *M. gallisepticum* cells were incubated with vesicles containing [^{14}]PC, no transfer of radioactivity into the cells was found. As shown in Table I, the cholesterol content as well as the overall chemical composition of *M. gallisepticum* membranes were not affected upon prolonged incubations in the exchange system. However, the radioactive cholesterol in the mycoplasma preparations underwent transfer to the lipid vesicles. The transfer was completed after 16–24 h of incubation. During this period, cells incubated in the sucrose-Tris-$MgCl_2$ buffer with or without 2% albumin remained intact. This was indicated by the essentially unchanged absorbance of the cell suspensions at 500 nm and the retention of [^{3}H]thymidine-labeled components within the cells (Fig. 1). A drop in absorbance and in [^{3}H]thymidine-labeled components was, however, noticed when the cells were incubated in media containing 100 mM sodium phosphate buffer (pH 7.8) instead of the Tris-HCl buffer. The osmotic fragility in the presence of sodium phosphate apparently

TABLE I

CHEMICAL COMPOSITION OF *M. GALLISEPTICUM* MEMBRANES OBTAINED FROM CELLS INCUBATED IN THE EXCHANGE SYSTEM

The cells were incubated with the lipid vesicles for up to 8 h. The isolation of membranes and the chemical analyses were performed as described in Materials and Methods. Protein, lipid phosphorus and cholesterol are expressed per 10^4 cpm of [^{3}H]thymidine-labeled material.

Time (h)	Protein (μg/10^4 cpm)	Lipid (μmol/10^4 cpm)	Cholesterol (μmol/10^4 cpm)	[^{14}C] Cholesterol (cpm/ 10^4 cpm)
0	135	0.16	0.15	4 830
2	117	0.15	0.14	4 100
4	111	0.16	0.15	3 750
8	112	0.17	0.15	3 080

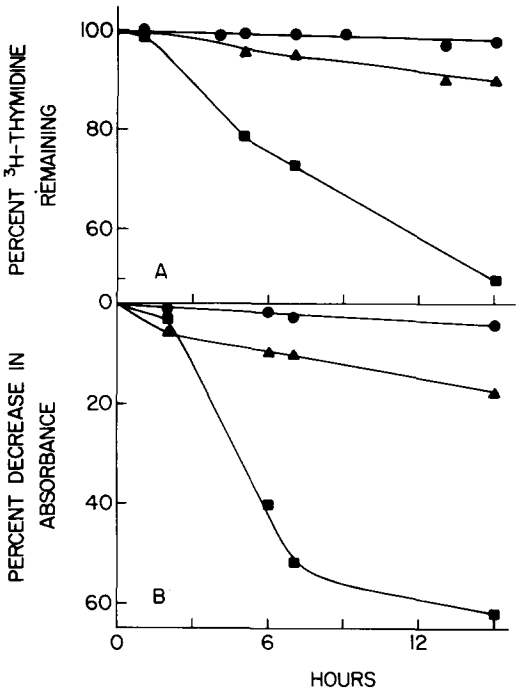

Fig. 1. The effect of prolonged incubation periods in various buffers on the leakiness of *M. gallisepticum* cells. Cells were grown in medium containing [^{3}H]thymidine and were incubated at 37°C in various solutions for up to 15 h. The leakiness of the cells was determined as described in Materials and Methods by following the release of ^{3}H-labeled materials from the cells (A) and by determing the absorbance of the cell suspension at 500 nm (B). ■———■, 0.4 M sucrose solution containing 100 mM sodium phosphate buffer (pH 7.8) and 20 mM $MgCl_2$; ▲———▲, 0.4 M sucrose solution containing 50 mM Tris (pH 7.8) and 20 mM $MgCl_2$; ●———●, sucrose-Tris-$MgCl_2$ solution containing 2% albumin.

results from the high permeability to Na^+ of non-energized *M. gallisepticum* cells [24]. The viability of *M. gallisepticum* cells during the exchange period was strictly dependent on the presence of albumin in the exchange mixture (Fig. 2). Without albumin the viability of the cells in sucrose-Tris-$MgCl_2$ buffer containing lipid vesicles showed a 4 log decrease with 2 h of incubation and a total loss of viability within 7 h. With 2% albumin, however, viability was fully preserved for up to 8 h.

Kinetics of cholesterol exchange

The time course for the exchange of labeled cholesterol from *M. gallisepticum* cells to the lipid vesicle

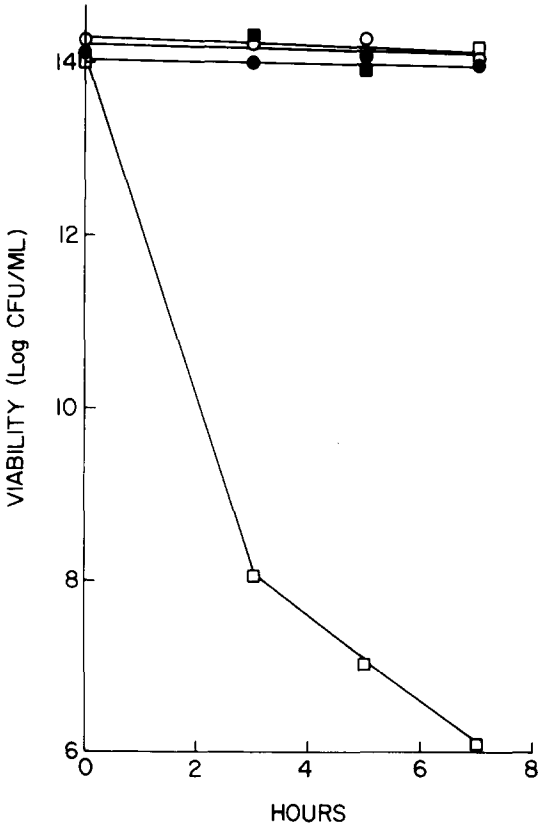

Fig. 2. Viability of *M. gallisepticum* cells in the exchange mixture. Open symbols: exchange mixture without albumin. Closed symbols: exchange mixture with 2% albumin. □ and ■, a complete exchange mixture containing PC/cholesterol vesicles; ○ and ●, incubation in sucrose-Tris-$MgCl_2$ buffer without vesicles.

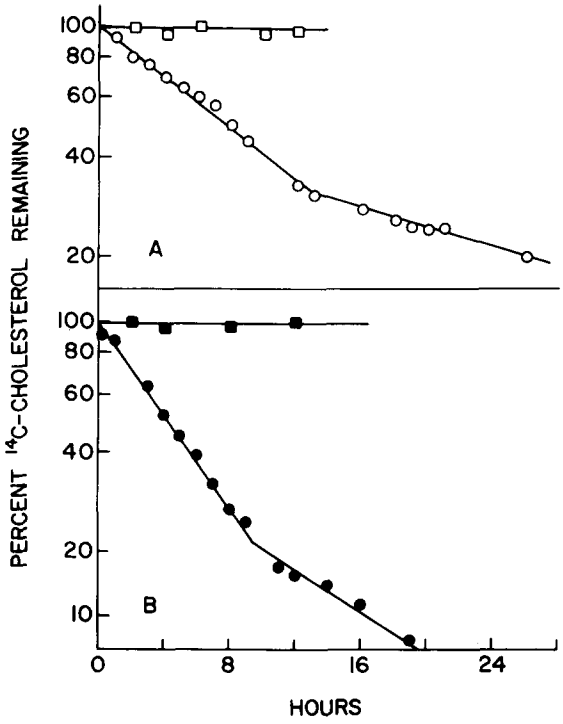

Fig. 3. Exchange of [^{14}C]cholesterol between *M. gallisepticum* cells and PC/cholesterol vesicles. The exchange was performed at 37°C in sucrose-Tris-$MgCl_2$ buffer, pH 7.8, for up to 24 h as described in Materials and Methods in the absence (A) or presence (B) of 2% albumin. ○ and ●, complete exchange mixture; □ and ■, incubation in sucrose-Tris-$MgCl_2$ buffer without vesicles. The data presented are the average values of at least 10 separate experiments in which the rapidly exchanging kinetic pool was measured, and of at least three experiments in which the second kinetic pool was measured. The correlation coefficients of the semilogarithmic plots of the rapid and slow phases are 0.988 and 0.956, respectively.

preparation is shown in Fig. 3. The exchange in sucrose-Tris-$MgCl_2$ buffer not containing albumin is shown in Fig. 3A. Ninety percent of the labeled cholesterol was removed from intact cells. The kinetics of the exchange was biphasic, with one cholesterol pool being exchanged at a fast rate and a second pool being exchanged at a slower rate. The exchange of cholesterol between lipid vesicles and isolated membranes can also be fitted to essentially the same two exponential rate processes as observed with the intact cells. The membrane fragments were apparently unsealed, as indicated by their extremely slow swelling rates in isosmotic solutions of glycerol or erythritol and by negatively stained electron microscopy which revealed vesicles containing holes (Shinar, D., unpublished data).

Effect of albumin on exchange rates

In the presence of 2% albumin both the fast and slow rates were enhanced by a factor of about 2 (Fig. 3B). The effect of albumin on the fast exchanging cholesterol pool was also observed with intact *M. gallisepticum* cells treated with growth inhibitors (Table II) or with isolated membrane fragments obtained by sonication. The time course of the slowly exchanging cholesterol pool in iodoacetate- or gramicidin-treated

TABLE II

THE EFFECT OF INHIBITORS ON THE VIABILITY AND RATE OF [^{14}C]CHOLESTEROL EXCHANGE IN *M. GALLISEPTICUM* CELLS

The exchange was performed in the system described in Materials and Methods in the presence of gramicidin D and *N*-ethylmaleimide (both products of Sigma) and iodoacetate (BDH Chemicals, Poole, U.K.). Cell viability was determined after 5 h of incubation. $t_{1/2}$ is the time required for one-half of the initial rapid rate to be completed based on a symmetrical cholesterol distribution. c.f.u., colony-forming unit.

Inhibitor	Albumin (2%)	Viability (c.f.u./ml)	$t_{1/2}$ of exchange (h)
No inhibitor	–	$1.1 \cdot 10^6$	4.1
	+	$2.4 \cdot 10^{13}$	2.1
Gramicidin D (5 μM)	+	$1.2 \cdot 10^7$	2.2
Iodoacetate (10 mM)	+	$<10^6$	2.3
N-Ethylmaleimide (10 mM)	+	$<10^6$	2.0

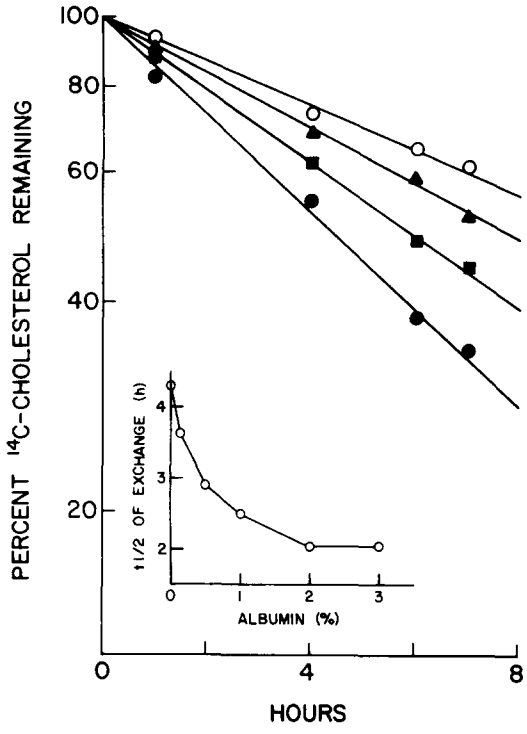

Fig. 4. The effect of albumin on the initial rate of exchange of [^{14}C]cholesterol from *M. gallisepticum* cells into egg PC/cholesterol vesicles. The exchange was performed at 37°C in sucrose-Tris-$MgCl_2$ buffer for the times indicated in a reaction mixture without albumin (○——○), and with albumin at the following concentrations: ▲——▲, 0.125%; ■——■, 1.0%; and ●——●, 2.0%. Inset: Plot of the half-time of removal of labeled cholesterol from the cells into the vesicles vs. albumin concentration. The half-time of exchange was calculated as described in Table II.

cells was also enhanced by albumin (data not shown). The kinetic parameters and pool sizes for exchange of [^{14}C]cholesterol between *M. gallisepticum* cells and lipid vesicles were calculated as described by Bloj and Zilversmit [25] in an analogous system. The half-time, $t_{1/2}$, for equilibration of the two cholesterol pools was calculated using the relationship $t_{1/2} = 0.693/(k_{ab} + k_{ba})$, where k_{ab} and k_{ba} are the rate constants for outer-to-inner monolayer and inner-to-outer monolayer movement, respectively. In the absence of albumin, the values of k_{ab} and k_{ba} at 37°C were 0.025 and 0.026 h^{-1}, respectively, and the half-time for cholesterol equilibration was 13.6 h^{-1}. In the presence of 2% albumin, the values of k_{ab} and k_{ba} were 0.054 and 0.058 h^{-1}, respectively, and $t_{1/2}$ was 6.2 h^{-1}. The fraction of cholesterol in the outer pool, which is calculated from the ratio $k_{ba}/(k_{ab} + k_{ba})$, was found to be 0.51 in the presence and absence of albumin.

The enhanced rate of cholesterol exchange induced by albumin was concentration dependent (Fig. 4), reaching maximal values at concentrations ≥2%. The enhancement by albumin was not affected by incubating the albumin solution at 56°C for 30 min, filtering the albumin through a column of active charcoal, or dialyzing the albumin solution against deionized water. Other albumin preparations (fraction V, Armour Laboratories, Sussex, England, and fatty acid poor, Calbiochem, La Jolla, CA) gave virtually identical results. No enhancement was obtained, however, when albumin was replaced by 2% human γ-globulin solution (Sigma). Likewise, it was not obtained when either *M. gallisepticum* cells or lipid vesicle preparations were pretreated with 2% albumin for 1–8 h at 37°C, followed by the removal of free albumin by centrifugation or chromatography on a G-150 Sephadex column, respectively.

TABLE III

THE FREEDOM OF MOTION OF SPIN-LABELED FATTY ACIDS IN *M. GALLISPETICUM* CELL MEMBRANE

The cells were incubated for 8 h at 37°C in the exchange reaction mixture with or without 2% albumin. The isolation and spin labeling of membranes and calculations of ESR parameters were as described in Materials and Methods.

Incubation	5-Doxylstearate		16-Doxylstearate	
	Order parameter (*S*)		Motion parameter (ns)	
	15°C	35°C	15°C	35°C
With albumin	0.697 ± 0.001	0.630 ± 0.007	5.40 ± 0.26	3.90 ± 0.18
Without albumin	0.675 ± 0.003	0.615 ± 0.007	4.90 ± 0.45	3.80 ± 0.26

To analyze the possibility that albumin in the reaction mixture may have induced alterations in chemical composition and/or physical properties of *M. gallisepticum* membranes, we determined the composition of the membranes and the freedom of motion of spin-labeled fatty acids incorporated into the membranes. Chemical analyses revealed no differences between membrane preparations incubated for 8 h in the exchange system with or without 2% albumin with respect to total lipid and protein content, and the relative amounts of cholesterol, various phospholipids and neutral lipids. The only difference was the free fatty acid content of the membranes, which was about 2% of the total lipid in membranes incubated without albumin and practically zero in membranes incubated with albumin. The freedom of motion of spin-labeled membranes incubated with albumin was very similar to that of membranes incubated in sucrose-Tris-$MgCl_2$ buffer without albumin, as indicated by the order parameter of 5-doxylstearate and motion parameter of 16-doxylstearate incorporated into the membranes (Table III).

Discussion

This investigation was performed with *M. gallisepticum* cells which are known to have a low osmotic fragility and to remain intact throughout long incubation periods. The osmotic stability of the cells was increased in a medium not containing sodium ions, since Na^+ was shown to permeate freely into non-energized cells [24]. Under our experimental conditions no net transfer of cholesterol or phospholipids was detected between the cells and the lipid vesicles and the cholesterol/phospholipid molar ratio remained unchanged.

Our studies of spontaneous cholesterol transfer from cells or membrane preparations to sonicated lipid vesicles show that cholesterol in the membrane of *M. gallisepticum* cells is present in two kinetic pools. The rapidly exchangeable pool represents removal of cholesterol from the outer leaflet, since we presume that only the cholesterol molecules localized in the outer leaflet of the bilayer can undergo transfer. The less accessible pool represents cholesterol in the inner leaflet of the *M gallisepticum* bilayer. The two pools had been found to be of essentially equal size (Fig. 3), i.e. chlosterol in *M. gallisepticum* membranes is distributed symmetrically between the two leaflets of the bilayer. A symmetrical distribution was also found in previous studies using a filipin binding approach [11] and exchange of cholesterol with HDL [7]. The time course for transfer of labeled-cholesterol from the outer leaflet of the *M. gallisepticum* membrane to lipid vesicles was the same as to HDL ($t_{1/2}$ = 4 h at 37°C). The cholesterol in the less accessible pool (inner leaflet) was almost completely exchangeable indicating that transbilayer equilibration of cholesterol between the two leaflets of the *M. gallisepticum* bilayer occurs at a relatively fast rate ($t_{1/2}$ = 13.6 h at 37°C (Fig. 3A). Such a mobile equilibration of the two pools may explain how a symmetric distribution of cholesterol is achieved in *M. gallisepticum* cells, where cholesterol is initially incorporated from an exogenous source into the outer leaflet of the membrane. It is interesting to

note that cholesterol in the less accessible pool was exchanged very slowly with HDL even after a prolonged incubation period [7]. Differences between HDL and lipid vesicles in size and composition (phospholipid-cholesterol molar ratio, lipid-protein ratio, presence of cholesterol esters, etc.) are apparent. Furthermore, the viability of *M. gallisepticum* cells was dramatically decreased within 1 h of incubation with HDL (Fig. 1 of Ref. 7). The extent to which these differences inhibited the rate of cholesterol equilibration between the two leaflets is yet unknown.

Our observation that the time course of exchange consisted of two phases even with unsealed *M. gallisepticum* membrane vesicles may be explained by the small size of the holes present in the membrane preparations (approx. 200 Å in diameter, Shinar, D., unpublished data). Thus, it is likely that the relatively large lipid vesicles (average diameter of 400 Å) are unable to penetrate and interact with the inner leaflet of the membrane. In contrast, isolated membranes incubated with HDL particles (average diameter of 70–100 Å) showed a single exchange rate process [7] apparently because HDL is capable of direct contact with the inner as well as the outer leaflets.

The enhancement in the rates of the rapidly and slowly exchanging cholesterol pools upon the addition of albumin to the incubation medium is striking. In other studies of the kinetics of cholesterol exchange, albumin was added to the incubation medium to increase the stability of cells or to improve the recovery of vesicles after prolonged incubations, probably by reducing vesible adherence to cell membranes [1,3,8–10]. The extent and initial rate of cholesterol transfer were found by Bloj and Zilversmit [3] and Nakagawa et al. [10] to be unaffected by the presence of albumin at concentrations of 5 mg/ml or 110 μg/ml, respectively. We found that albumin caused practically no changes in chemical composition and physical properties of *M. gallisepticum* membranes and did not affect the extent of recovery of lipid vesicles from the exchange mixture. However, albumin preserved cell viability throughout the long incubation periods with the lipid vesicles. Preservation of viability cannot account for the enhancement of cholesterol exchange rate, since the albumin effect was observed with isolated membrane preparations as well as with cells treated with agents that caused a marked decrease in cell viability (Table II). Rapid spontaneous cholesterol exchange was also observed in lipid vesicles [3,4,9,10]. This observation supports our conclusion that relatively rapid cholesterol transbilayer movement in a resting cell system, under conditions in which no net transfer of cholesterol or other lipids takes place, is independent of cell viability.

Although the mechanism by which albumin enhances the rate of cholesterol transfer to vesicles is not yet known, its effect is probably on the rate determining step in an equilibrium reaction. The reversibility of this effect rules out a long-lived non-equilibrium state. Cholesterol may be transferred from one membrane to another by either a collision mechanism [26], or as recently suggested, by diffusing through an aqueous phase [27]. If cholesterol is transferred by the collision mechanism, albumin, or small amounts of impurities may enhance either collision frequency or the interactions between *M. gallisepticum* cells and lipid vesicles. If cholesterol exchanges by diffusing into an aqueous phase, the enhanced rate observed with albumin would be due to a change in solubiluty or dispersibility of cholesterol in the aqueous exchange mixture.

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