

## Transbilayer Distribution of Sterols in Mycoplasma Membranes: A Review

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The polyene antibiotic, filipin, binds to 3 $\beta$ -hydroxysterols. The initial rate of filipin-sterol association, monitored in a stopped-flow spectrophotometer, was first order in each reacting partner. The ratio of rate constants in intact mycoplasma cells relative to isolated, unsealed membranes provides an estimate of sterol distribution in the membrane bilayer. Cholesterol is distributed symmetrically in the bilayer of *M. gallisepticum* cells from the early exponential phase. However, in the *M. capricolum* membrane two-thirds of the unesterified cholesterol is localized in the outer leaflet; alkyl-sterols are distributed predominantly in the external monolayer. Cholesterol is translocated rapidly in the bilayer of *M. capricolum* cells. Exogenous phospholipids incorporated into the membrane had no effect on the cholesterol distribution in *M. capricolum*.

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### INTRODUCTION

Cholesterol is a major component of many biological membranes. This sterol is known to modulate the fluidity of the plasma membrane over a wide temperature range by interfering with the cooperative interactions of phospholipids [1], thereby preventing formation of gel-phase domains at the physiological temperature. It also affects some of the cell's biochemical and physiological properties [2]. Although cholesterol has been shown to undergo exchange readily with serum lipoproteins and lipid vesicles [3-5], until recent years relatively few studies have been directed to analysis of cholesterol bilayer distribution and kinetics of cholesterol movement between the two halves of the bilayer. Mycoplasma cells offer several advantages for the study of the distribution and transbilayer movement of sterols between the two halves of the bilayer. Among the features that make these cells especially attractive for such studies are their lack of a cell wall, absence of internal membranes, requirement of cholesterol for growth, high capacity for incorporating cholesterol into the cell membrane, and inability of the strains we have used to synthesize sterols or modify sterol structure. For these studies we have chosen *M. gallisepticum* (because of its high osmotic stability) and *M. capricolum* (because it accepts various sterols into its membrane, grows well in the presence of exogenous lipids, and is readily adapted to grow with very low cholesterol).

In this paper we review our recent studies using the polyene antibiotic filipin to

probe the transbilayer distribution of sterols in membranes that can be prepared in both intact and unsealed states. For filipin to interact with sterols incorporated into membranes the minimum sterol structural requirements are an unesterified  $3\beta$ -hydroxyl group and a planar nucleus (see references in [6]). For strong interaction, an apolar side chain at C-17 of the sterol is also needed. These structural requirements for maximal filipin-sterol interaction are the same as those for phosphatidylcholine (PC)-sterol interaction [7], suggesting that the bilayer configuration is important for the action of filipin on membranes. The stoichiometry of the complex formed between filipin and unesterified cholesterol in solution [8], in liposomes [9], and in virions [10] is approximately 1 mole of filipin per mole of cholesterol. Conceptual models for aggregates of the filipin-cholesterol complex have been proposed [11–13].

Since filipin does not react rapidly with cholesterol esters, phospholipids, or proteins, we have used a stopped-flow apparatus to measure the initial rates of filipin binding to unesterified cholesterol in intact and unsealed membrane preparations. From these measurements we have determined the relative amounts of cholesterol on the outside and inside of the membrane, which presumably correspond to the amounts in the two halves of the bilayer. The short reaction times obtained with the stopped-flow technique also minimize filipin-induced membrane disruption; high cholesterol/filipin molar ratios and low temperature also minimize the disruptive effects of filipin. Stopped flow data are available, describing the kinetics of filipin-cholesterol association in model membranes [14–16], virions [10], erythrocytes [17], and lipoproteins [18].

The specificity with which filipin binds to sterols has led to other applications of this antibiotic in membrane research. Since filipin interferes with hormone expression, it has been used in the study of membrane receptors [19,20]. In addition, filipin is a useful tool for disrupting membranes containing  $3\beta$ -hydroxysterols without solubilizing membrane-bound enzymes [21]. Filipin may also become an effective cytochemical probe to distinguish between membrane regions of high and low sterol density. The interaction of filipin with cholesterol in fixed and unfixed cells and membranes [21,22] results in filipin-cholesterol complexes which are visualized in freeze-fracture preparations by their unique geometry (see references in [22]). Negatively stained preparations show a dramatic rearrangement of certain membrane components after filipin treatment, e.g., aggregation of glycoproteins in lipid-enveloped virions [10]. In order to interpret the distribution of the complexes in freeze-fracture preparations in terms of sterol localization, it is obviously necessary to minimize or control the degree of filipin-induced membrane perturbation. Fortunately, it was recently found that filipin-induced damage appears to be minimized when cells are treated with filipin after glutaraldehyde fixation [22]. Freeze-fracture examination of the distribution of membrane sterol-filipin complexes have been conducted in unfixed preparations of retinal rod outer segments [23], ergosterol-containing *Tetrahymena* membranes [24], and sperm and epithelial tissues [13]. The evaluation of freeze-fracture cytochemistry as a reliable indication of sterol distribution in membranes awaits further development of this electron microscope technique for visualizing filipin-cholesterol interactions.

## MATERIALS AND METHODS

### *Kinetics of Filipin Association with Sterols*

The initial rates of filipin binding to unesterified sterols were measured at 10°C by stopped-flow spectrophotometry at 360 nm. Equal volumes of filipin solution and

mycoplasma suspensions (either intact cells or isolated, unsealed membranes) were mixed rapidly in a Durrum-Dionex stopped-flow spectrophotometer. To minimize cell shearing, the pressure used to initiate mixing was reduced with an air-actuator assembly. The filipin "complex" was purified as described previously [15]. The final concentration of dimethylformamide was 0.3 percent (by volume). The filipin concentration was determined spectrophotometrically [15]. The initial rate of absorbance change per second,  $dA/dt$ , was calculated from the increase in the transmittance of filipin upon binding to cells or membranes. Transmittance changes were photographed using a Tektronix storage oscilloscope and a Polaroid camera.

#### *Growth of M. gallisepticum and M. capricolum and Isolation of Membranes*

*M. gallisepticum* (strain A5969) and *M. capricolum* (California kid) were grown in a modified Edward medium [25] as described previously [16]. The procedures for the adaptation of *M. capricolum* to grow in a cholesterol-poor medium and for isolation of membranes from aliquots of cell suspensions were also described previously [26]. *M. capricolum* was grown in the presence of various sterols or exogenous lipids as described in [27]. The extent of cell lysis was monitored using methods described in [26].

### RESULTS AND DISCUSSION

Figure 1 shows that the initial rates of filipin-cholesterol association in intact *M. gallisepticum* cells are significantly lower than those in isolated membranes [28]. Membranes obtained from mycoplasma cells by sonication (or by osmotic lysis of osmotically fragile cells) are largely unsealed, based on morphological [29] and transport [30] studies. Table 1 presents the second-order rate constants,  $k_{\text{cells}}$  and  $k_{\text{membranes}}$ , calculated from the initial rates of filipin binding to cholesterol in intact cells and isolated membranes of *M. capricolum*. The ratio of these rates constants,  $k_{\text{cells}}/k_{\text{membranes}}$ , provides an estimate of the transbilayer distribution of cholesterol

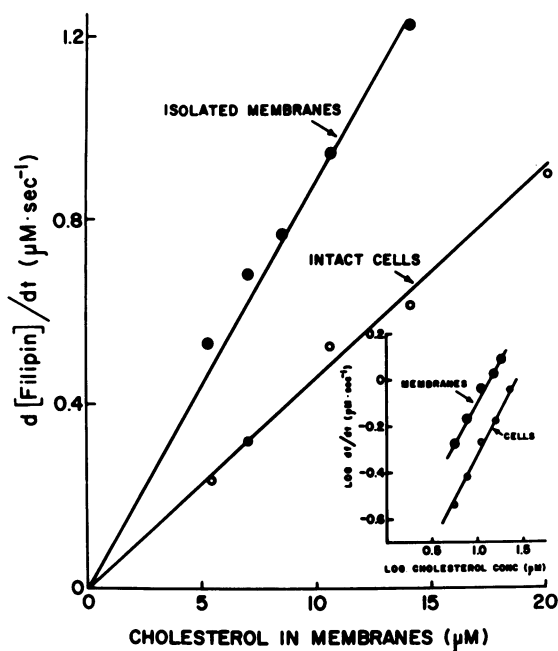


FIG. 1. Initial rate of disappearance of free filipin at 10°C on binding to varying concentrations of unesterified cholesterol in intact *M. gallisepticum* cells (○) and unsealed isolated membranes (●). *Insert*: A plot of the logarithm of the initial rate vs. the logarithm of cholesterol concentration, showing that the binding reaction is first order with respect to cholesterol in both cells and membranes. The indicated cholesterol concentrations represent the final concentration of unesterified cholesterol after mixing of equal volumes of antibiotic with cells or membranes in the stopped-flow spectrophotometer in 0.4 M sucrose containing 10 mM sodium phosphate (pH 7.2) and 20 mM MgCl<sub>2</sub> [28].

TABLE 1  
Second-Order Rate Constants for Filipin Binding to Free Cholesterol in Intact Cells  
and Isolated Membranes of *M. gallisepticum* and *M. capricolum*

Organism	Cells	k (M <sup>-1</sup> sec <sup>-1</sup> )	
		Membranes	Cell/membrane rate ratio
<i>M. gallisepticum</i>	7.8 · 10 <sup>3</sup> ± 0.1 · 10 <sup>3</sup>	15.3 · 10 <sup>3</sup> ± 0.4 · 10 <sup>3</sup>	0.51
<i>M. capricolum</i>	14.3 · 10 <sup>3</sup> ± 0.4 · 10 <sup>3</sup>	21.7 · 10 <sup>3</sup> ± 0.6 · 10 <sup>3</sup>	0.66

Data were taken from [28].

between the inner and outer halves of the mycoplasma membrane. The ratio with *M. gallisepticum* is 0.5 in cells from the early exponential phase, indicating a symmetrical distribution of cholesterol in the two leaflets of the membrane [16,28]; in stationary-phase cells the ratio is 0.6, which indicates that on aging of the culture cholesterol becomes enriched in the outer half of the bilayer [16]. Since spin-label studies indicate an increased rigidity of the phospholipid fatty acyl chains of *M. gallisepticum* membranes on aging, the movement of cholesterol into the inner half of the bilayer may be impeded by the increased rigidity of the hydrocarbon chains. With *M. capricolum* the ratio of  $k_{\text{cells}}/k_{\text{membranes}}$  is 0.66, indicating that about two-thirds of the unesterified cholesterol is localized in the outer half of the lipid bilayer [28]; this distribution remained constant throughout the growth cycle of *M. capricolum* [16]. When the polar lipid content of the *M. capricolum* membrane was enhanced by growing the cells in the presence of exogenous phospholipids, the ratio of the rate constants remained the same, i.e., approximately 65 percent of the unesterified cholesterol was present in the outer leaflet [16]. These kinetic studies show that cholesterol, which first must be incorporated from the growth medium into the outer leaflet of the membrane, is distributed in both halves of the bilayer of cells grown to the early logarithmic phase.

The localization of cholesterol can also be determined by exchange studies under conditions where no net transfer occurs. The results obtained by fast kinetic studies with filipin were confirmed by exchange studies in which cholesterol was exchanged from the *M. gallisepticum* cell membrane to high-density lipoprotein particles [31] or to unilamellar PC/cholesterol lipid vesicles [32]. Two cholesterol pools were detected. The rapidly exchangeable pool was interpreted to represent cholesterol molecules in the outer leaflet of the bilayer, whereas the less accessible pool was assumed to represent cholesterol in the inner leaflet. The fraction of total unesterified cholesterol in the outer pool was 0.51. A mobile equilibration of the two pools ( $t_{1/2} = 6.2$  h at 37°C in the presence of 2 percent albumin) may explain how a symmetrical distribution of cholesterol is achieved in *M. gallisepticum* cells [32].

Evidence that rapid translocation of cholesterol from the outer to inner surface of the membrane takes place was obtained using the filipin-binding approach with growing *M. capricolum* cells [26]. A cholesterol-depleted adapted strain of *M. capricolum* in the early logarithmic phase of growth was transferred to a cholesterol-rich medium containing fetal-calf serum [26] or fatty acids and albumin [27]. This resulted in an approximately sixfold enhancement in the unesterified cholesterol content of the membrane within four hours of incubation. The value of  $k_{\text{cells}}/k_{\text{membranes}}$  was essentially invariant after only one to two hours of incubation (Table 2). (The lower value of  $k_{\text{cells}}/k_{\text{membranes}}$  in the adapted strain than in the native strain presumably reflects a difference in cholesterol distribution arising from differences

TABLE 2  
Second-Order Rate Constants for Association of Filipin and Free Cholesterol in Intact Cells and Isolated Membranes of the *M. capricolum* Adapted Strain Transferred to a Cholesterol-Rich Medium<sup>a</sup>

Time of incubation in 10% FCS (h)	A <sub>640</sub>	Free cholesterol (μg/mg of membr protein)	k <sub>cells</sub> <sup>b</sup> (M <sup>-1</sup> s <sup>-1</sup> )	k <sub>membr</sub> <sup>b</sup> (M <sup>-1</sup> s <sup>-1</sup> )	k <sub>cells</sub> /k <sub>membr</sub> <sup>c</sup>
0	0.08	33	5.0 × 10 <sup>4</sup>	7.1 × 10 <sup>4</sup>	0.73 ± 0.08 (14)
1	0.16	44	4.3 × 10 <sup>4</sup>	8.3 × 10 <sup>4</sup>	0.53 ± 0.04 (9)
2	0.24	92	4.0 × 10 <sup>4</sup>	8.8 × 10 <sup>4</sup>	0.45 ± 0.06 (16)
4	0.26	127	4.4 × 10 <sup>4</sup>	9.1 × 10 <sup>4</sup>	0.45 ± 0.07 (16)

<sup>a</sup>The initial rates of filipin-free cholesterol association were measured at 10°C at various cholesterol concentrations in intact cells and isolated membranes obtained from at least nine different cell cultures, each incubated with medium containing 10 percent FCS for the indicated period of time. The number of cell cultures investigated is indicated in parentheses in the last column.

<sup>b</sup>A representative example of second-order rate constants analyzed from one culture.

<sup>c</sup>Average ratio of second-order rate constants. Error limits of the kinetic data are standard errors of the mean. Data were taken from [26].

in membrane composition and physiology of the organism upon adaptation.) After more than one hour of incubation in cholesterol-rich medium, about 55 percent of the total unesterified cholesterol was present in the inner half of the bilayer. When cell growth at 37°C was inhibited by addition of ionophores (valinomycin, gramicidin, nonactin) or chloramphenicol to the adapted cells, cholesterol ac-

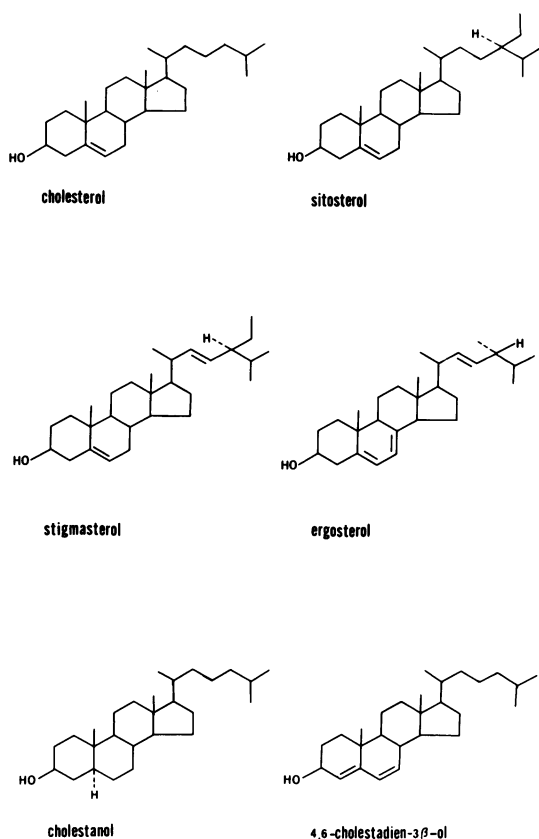


FIG. 2. Structures of sterols used to support growth of *M. capricolum*. Solutions of sterols (10 μg/ml) and palmitic and oleic acids (10 μg/ml of each) in ethanol were mixed and added together to the growth medium. See [27].

cumulation into the cell membrane was partially inhibited and cholesterol was localized predominantly in the outer half of the bilayer [26].

The sterol specificity of *M. capricolum* is relatively broad [33–35]. Furthermore, filipin binds to various sterols [7]. We have, therefore, used the stopped-flow kinetic technique to measure the transbilayer localization of a variety of sterols in the membrane of *M. capricolum* [27]. Figure 2 shows the structures of the sterols that were used to support growth of *M. capricolum*. Growth curves showed that the introduction of an alkyl substituent at C-24 (ergosterol,  $\beta$ -sitosterol, and stigmasterol), unsaturation at C-22 (ergosterol and stigmasterol) or at C-4 and C-6 (4, 6-cholestadien-3 $\beta$ -ol), or reduction of the  $\Delta^5$ -bond ( $\beta$ -cholestanol) did not markedly affect the rate of the logarithmic phase of growth after an initial lag phase. The value of  $k_{\text{cells}}/k_{\text{membranes}}$  was 0.66 with cholesterol,  $\beta$ -cholestanol, and 4, 6-cholestadien-3 $\beta$ -ol, i.e., 66 percent of the sterol that reacted with filipin is present in the outer leaflet of the membrane. Each of these sterols has the cholesterol side chain. In contrast, approximately 85–90 percent of the ergosterol,  $\beta$ -sitosterol, or stigmasterol is present in the outer leaflet [27]. Thus, the nature of the alkyl side chain of the sterol appears to affect the extent of sterol translocation from the outer to the inner surface of the *M. capricolum* bilayer. Alkylation at C-24 and unsaturation at  $\Delta^{22}$  increase the bulk of the side chain and may thereby cause steric interference in the movement of the sterol molecule across the lipid bilayer.

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