**Distribution and Movement of Sterols with Different Side Chain Structures between the Two Leaflets of the Membrane Bilayer of Mycoplasma Cells**

(Received for publication, June 22, 1983)

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Mycoplasma gallisepticum was adapted to grow with $\Delta^8$-sterols modified in the aliphatic side chain, and stopped-flow kinetic measurements of filipin association were made to estimate the sterol distribution between the two leaflets of the membrane. Cholesterol derivatives with unsaturated side chains (desmosterol, cis- and trans-22-dehydrocholesterol, and cholesta-5,22E,24-trien-3β-ol) or an alkyl substituent ($\Delta$-sitosterol) were predominantly (86–94%) localized in the outer leaflet of the bilayer. However, cholesterol, 20-isocholesterol, and sterols with side chains of varying lengths (in the 20(R)-n-alkylpregn-5-en-3β-ol series where the alkyl group ranged from ethyl to undecyl) were distributed nearly symmetrically between the two halves of the bilayer. Kinetic measurements of $\beta$-$[^{14}\text{C}]$sitosterol and $[^{14}\text{C}]$desmosterol exchange between M. gallisepticum cells and an excess of sonicated sterol/phosphatidylcholine vesicles confirmed the filipin-binding studies. More than 90% of these radiolabeled sterols underwent exchange at 37°C with unlabeled vesicles in a period of 12–14 h in the presence of 2% (w/v) albumin. $\beta$-$[^{14}\text{C}]$Sitosterol exchange was characterized by biphasic exchange kinetics, indicative of two pools of sitosterol molecules in the cell membrane. Only a single kinetic pool was detected for $[^{14}\text{C}]$desmosterol exchange. Stopped flow measurements of filipin binding to $\beta$-sitosterol and stigmasterol also revealed an asymmetrical localization of these sterols in membranes of growing Mycoplasma. cell membrane. When an early exponential culture of $\beta$-sitosterol- or stigmasterol-adapted M. capricolum was transferred to a sterol-rich medium at 37°C, approximately three-quarters of the $\beta$-sitosterol or stigmasterol was localized in the outer leaflet after growth was continued for 6 h; in contrast, cholesterol was distributed symmetrically after about 1 h. The asymmetric localization of sterols with alkylated or unsaturated side chains suggests that growth-supporting sterols need not be translocated extensively into the inner leaflet of the bilayers of M. gallisepticum and M. capricolum.
chain raises questions about the roles of lipid-lipid and lipid-protein interactions in sterol translocation across mycoplasma membranes.

EXPERIMENTAL PROCEDURES

Materials

Egg phosphatidylcholine, cholesterol, desmosterol, bovine serum albumin (Fraction V, fatty acid poor), oleic and palmitic acids, and deoxyribonuclease were obtained from Sigma. β-Sitosterol (referred to as sitosterol below) and stigmasterol were from Steraloids (Wilton, NH). Cholesterol, sitosterol, and stigmasterol in dodecane were recrystallized several times from ethanol. Desmosterol was purified by preparative thin layer chromatography using Silica Gel H plates (Analytech, Newark, DE) developed with diethyl ether/petroleum ether (3:1, v/v). The sterols and PC12 migrated as single spots on Silica Gel G thin layer chromatography plates as previously described (6) when visualized by sulfuric acid spray and charring. The unesterified side chain derivatives of cholesterol, cis- and trans-22-dehydrocholesterol and cholesta-5,22E,24-trien-3β-ol, were generously supplied by Dr. Dr. Henry Kircher, University of Arizona, and characterized as described elsewhere (11-13). 20-Isocholesterol, which was also a gift from Dr. Kircher, was free of traces of the stereoisomer, as judged by gas-liquid chromatography on a 5% OV-101 column and by argentation thin layer chromatography of the sterol acetate precursor. The series of cholesterol analogs in which the length of the side chain varies and the terminal methyl branch is absent (20R)−n-alkylpregn-5-en-3β-ol) was generously supplied by Dr. Toshiyuki Akiyama, University of Tokyo. These analogs, which are referred to by the total number of carbon atoms they contain (14, 15), were synthesized, characterized, and shown to be pure by Morisaki et al. (16). [6-methyl-3H]Thymidine (30.6 Ci/mmol) was obtained from New England Nuclear. [4-14C]Sitosterol (98 Ci/mmol) and [2B-27C]desmosterol (53 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Labeled sterols were analyzed for radiochemical purity by counting zones scraped from thin layer plates (silica gel, Analtech); the radiochemical purity of all of the sterols was ≥98%.

Methods

Growth of Mycoplasma Cells and Adaptation to Various Sterols—M. gallisepticum strain A5969 and M. capricolum (California kid) were grown in a modified Edward medium (17) containing 10 Fg of thymidine-labeled components was retained in the cell pellet after 12–14 h of incubation with the lipid vesicles, indicating that the cells containing sitosterol or desmosterol remained largely intact. Aliquots were withdrawn in duplicates at various times, cells were separated from vesicles, and the fraction of 14C-sterol exchanged was calculated from the 14C/13C counts as described previously (18). The half-time for equilibration of [14C]sitosterol between the two kinetic pools we observed was calculated using the relationship $t_{1/2} = 0.693/(k_{eq} + k_{emembranes})$, where $k_{eq}$ and $k_{emembranes}$ are the first order rate constants for [14C]sitosterol translocation from the inner to outer monolayers (slower exchanging pool) and from the outer to inner monolayers (faster exchanging pool), respectively. These rate constants were calculated from the slopes of the fast and slow phases, $g_{f}$ and $g_{s}$, respectively, in semilogarithmic plots of the percentage of 14C-sterol exchanged versus time (6). The half-times for exchange of [14C]sitosterol and [14C]desmosterol from the rapidly exchangeable pool, $t_{50}$, were calculated using the relationship $t_{50} = 0.693/g_{f}$.

Filipin Binding Kinetics—Initial rates of filipin binding to sterols in mycoplasma cells and membranes were measured at 360 nm as described previously (7, 8). The initial reaction rate was first order with respect to sterol and filipin. Second order rate constants for filipin association with sterol in intact cells and isolated membranes, designated as $k_{cell}$ and $k_{membranes}$, were calculated as described elsewhere (7). All rate measurements were made in STM buffer at 10°C. The air pressure used to initiate mixing of equal volumes of filipin solution with mycoplasma suspensions was reduced to 30 p.s.i. to minimize cell shearing. At least six measurements of the initial rates of filipin binding were made at each sterol concentration.

Analytical Procedures—Lipids were extracted from cells and isolated membranes and lipid phosphorus and sterol content were determined using procedures described before (5). The wavelengths used for estimating sterol concentration were as follows: 542 nm for cholesterol, sitosterol, and stigmasterol, 555 nm for desmosterol; and 550 nm for the other sterols.

Growth of M. gallisepticum with Sterols Differing in Side Chain Structure—One of the attractive features about using mycoplasmas as a system with which to investigate the relationship between sterol structure and transbilayer distribution, in addition to their well known inability to synthesize sterols, is their ability to incorporate sterols from an exogenous supply without structural modification. We adapted M. gallisepticum to grow with various Δ9-sterols with identical steroid nuclear structures but different aliphatic side chains (see Fig. 1 for structures). At 10 μg/ml, sitosterol supported growth nearly as well as cholesterol; good growth was also obtained with cholesterol, 5,22E,24-trien-3β-ol, 22-dehydrocholesterol, and desmosterol (Fig. 2A). However, cells grew less well with trans-22-dehydrocholesterol, although it has an extended side chain resembling that of cholesterol, and with 20-isocholesterol, which differs from cholesterol only with respect to the configuration at C-20. In addition, the cell yields were low with trans-22-dehydrocholesterol and 20-isocholesterol. Lower amounts of these sterols were incorporated into the cell membrane compared with sitosterol and desmosterol (which differ from cholesterol in having a 24α-ethyl group and a Δ9-bond, respectively), cholesterol, and the sterols bearing unsaturation at C-22 and C-24 (Table 1). Sterols with short side chains also supported growth. For example, C26 was especially effective (Fig. 2B), although the cell membranes contained less sterol than cells grown on...
cholesterol (Table I). Substantial growth was found with C28; the cholesterol analogs C22 and C24 supported growth less well, and cell death was apparent after about 25 h. With C32, there was a marked reduction in the maximum absorbance of the culture and substantial cell death after 23 h (Fig. 2B); with C30, the cell yield was poor, although the cultures reached maximum absorbance values comparable to those grown with C22 and C24 and the sterol contents of the membranes were similar. Thus, various sterols can approach the effectiveness of cholesterol as a growth supporter of \( M. \) \( gallisepticum \) (sitosterol, desmosterol, cis-22-dehydrocholesterol, and C26), and others promote substantial growth (cholesta-5,22E,24-trien-3\( \beta \)-ol, C22, C24, C28). However, poor growth-promoting efficiencies were found with sterols having greatly extended side chains (as in C32 and trans-22-dehydrocholesterol) and with 20-isocholesterol.

\[ ^{14}C \]Sitosterol and \( ^{14}C \)Desmosterol Exchange between \( M. \) \( gallisepticum \) Cells and Lipid Vesicles—On incubation with an excess of sonicated vesicles in STM buffer, cells remained intact (see "Methods") and the sterol/phospholipid molar ratio was maintained constant. More than 90% of the labeled sterol was removed from the cells during a 12-14-h period of incubation in the presence of 2% albumin (Fig. 3). Since the kinetic data for \( ^{14}C \)sitosterol exchange (Fig. 3A) can be fitted by the sum of two exponentials, it is concluded that \( ^{14}C \) sitosterol is distributed between two pools in the cell membrane. Thus semilogarithmic plots of the percentage of \( ^{14}C \) sitosterol remaining in \( M. \) \( gallisepticum \) cells versus time are biphasic (Fig. 3A). By extending the straight line of the slower process of the semieponential plot to time zero, we found that the faster phase represents approximately 76% of the total \( ^{14}C \)sitosterol undergoing exchange with nonlabeled sitosterol in vesicles. \( ^{14}C \)Cholesterol also gave biphasic kinetics of exchange between cells and vesicles (10, 18), and it was presumed that the rapidly and slowly exchangeable pools represent sterol molecules initially in the outer monolayer and inner monolayer of the bilayer, respectively. The size of the faster exchangeable pool of \( ^{14}C \)sitosterol molecules, \( T_{\text{fast exchangeable}} \), and the half-time of equilibration of \( ^{14}C \)sitosterol between the two pools, \( t_{1/2} \), were calculated from the fast and slow exponential constants, \( g_1 \) and \( g_2 \), respectively (19). Table II lists these parameters and also shows the corresponding values obtained for \( ^{14}C \)cholesterol exchange (18). The fraction of \( ^{14}C \)sitosterol molecules in the rapidly exchangeable pool is considerably greater than that of \( ^{14}C \)cholesterol (0.76 and 0.58, respectively). A comparison of the slopes of the two phases obtained for exchange with sitosterol and cholesterol (Fig. 3A compared with Fig. 2 of Ref. 18) shows that the more accessible pool underwent exchange faster with cholesterol than sitosterol (see \( g \), and \( t_{1/2} \) values in Table II). However, the slower exchange process, which is thought to represent sterol movement from the inner to outer leaflet, occurred at the same rate. Table II also shows that \( t_{1/2} \) is lower for the equilibration of sitosterol between the two pools than for equilibration of cholesterol. This unexpected result arises because the half-time is a function of the sum of the rate constants \( k_{\text{fast exchangeable}} \) and \( k_{\text{slow exchangeable}} \), and the rate constants are calculated from \( g_1 \), \( g_2 \), and the pool size (19). (The pool size is determined from the extension of the straight line of the slower process to the y axis of Fig. 3A.) Fig. 3B shows that only a single kinetic pool was detected for \( ^{14}C \)desmosterol exchange. This is in contrast to the semilogarithmic plots of \( ^{14}C \)sitosterol and \( ^{14}C \)cholesterol exchange between cells and vesicles, which are characterized by a rapid and a slow rate. Bovine serum albumin (2\%, w/v) stimulated the rate of \( ^{14}C \)desmosterol exchange by a factor of 1.9, as found in our previous study of \( ^{14}C \)cholesterol exchange between \( M. \) \( gallisepticum \) cells and lipid vesicles (10).

Rates of Filipin Binding to Sterols in Cells and Membranes—In several recent studies we have used stopped flow measure-
ments of filipin-sterol association to probe the transbilayer distribution of cholesterol (8, 20) and other free sterols (6) in membranes that can be prepared in both sealed and unsealed states. An example of the initial, linear increase in transmittance arising from filipin binding is shown for sitosterol-containing cells (Fig. 4A). The initial rate of absorbance change, $\frac{dA}{dt}$, with cells and membranes is first order in sterol (Fig. 4B) and filipin (7) concentrations. The extent of cell membrane disruption induced by filipin binding is minimized by the use of initial velocity measurements, high sterol/filipin molar ratios, and low temperature. Fig. 4D shows that the rates of binding of filipin to isolated mycoplasma membranes (which are open; see Ref. 10) are faster than those with the corresponding intact cells. Table III presents the second order rate constants for filipin binding to various $\Delta^2$-sterols in intact cells ($k_{cell}$) and isolated membranes ($k_{membranes}$). High ratios of $k_{cell}/k_{membranes}$ (0.86–0.94) indicate that sitosterol and the cholesterol derivatives with unsaturated side chains, desmosterol, cholesta-5,22E,24-trien-3$\beta$-ol, and cis- and trans-22-dehydrocholesterol, accumulate in the outer half of the bilayer. However, cholesterol, 20-prisorcholesterol, and the cholesterol analogs with short and long side chains are nearly symmetrically distributed between the two leaflets, with 52–60% of the sterol in the outer half of the bilayer.

In a previous report we used second order rate constants of filipin-cholesterol association in cells and membranes to show that cholesterol moves rapidly from the outer to inner half of the lipid bilayer of growing *M. capricolum* (5). The results of stopped flow kinetic measurements of the filipin-sitosterol and filipin-stigmasterol association in *M. capricolum* cells and membranes are given in Table IV. To test the relative abilities of cholesterol, sitosterol, and stigmasterol to undergo rapid translocation across the *M. capricolum* cell membrane, we adapted this organism to grow with 1.25 $\mu$g of each sterol/ml. When a culture of the adapted strain was transferred to a sterol-rich medium (10 $\mu$g/ml), the growth rate was enhanced; the sterol content of the membranes was increased by more than 3-fold for sitosterol and stigmasterol within 6 h of growth, and more than 4-fold for cholesterol within 4 h (Table IV). When samples were withdrawn at various time intervals after the cultures had been supplemented with sterol, stopped flow studies with the intact cells and isolated membranes revealed that sitosterol and stigmasterol were predominantly localized in the outer leaflet of the membrane. For example, after 4 h of growth stimulation and sterol incorporation, approximately three-fourths of the sitosterol or stigmasterol was estimated to be in the outer half of the bilayer. Cholesterol, however, was distributed about equally between the two halves of the bilayer after only 1 h of incubation. Furthermore, the amount of sitosterol or stigmastanol incorporated was lower than that of cholesterol. These experiments show that the rates of sitosterol and stigmastanol transbilayer movement in growing *M. capricolum* cells are inhibited relative to the

### Table I

<table>
<thead>
<tr>
<th>Sterol used for growth</th>
<th>$A_{452}$</th>
<th>Membrane protein $^{a}$</th>
<th>Sterol content $^{b}$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mg $^{a}$</td>
<td>$\mu$g/mg</td>
</tr>
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<td>Sitosterol</td>
<td>0.135</td>
<td>6.2</td>
<td>96</td>
</tr>
<tr>
<td>Desmosterol</td>
<td>0.130</td>
<td>5.8</td>
<td>85</td>
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<td>Cholesta-5,22E,24-trien-3$\beta$-ol</td>
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<td>107</td>
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<tr>
<td>cis-22-Dehydrocholesterol</td>
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<td>6.0</td>
<td>107</td>
</tr>
<tr>
<td>trans-22-Dehydrocholesterol</td>
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<td>70</td>
</tr>
<tr>
<td>Cholesterol</td>
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<td>124</td>
</tr>
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<td>20-Isoscholesterol</td>
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<td>C22</td>
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</tr>
<tr>
<td>C24</td>
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<td>79</td>
</tr>
<tr>
<td>C26</td>
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<td>6.4</td>
<td>67</td>
</tr>
<tr>
<td>C28</td>
<td>0.115</td>
<td>5.8</td>
<td>79</td>
</tr>
<tr>
<td>C30</td>
<td>0.100</td>
<td>3.2</td>
<td>68</td>
</tr>
<tr>
<td>C32</td>
<td>0.047</td>
<td>ND$^{d}$</td>
<td>ND$^{d}$</td>
</tr>
</tbody>
</table>

$^{a}$ The absorbance of the culture at 640 nm was measured after 20 h of growth. The values are representative examples from one culture with each sterol.

$^{b}$ Cells were grown in 500 ml of medium.

$^{c}$ Values are the average of two different cell preparations.

$^{d}$ Not determined because of poor growth and extensive cell lysis.

![Fig. 3. First order kinetic plots of $^{14}$C-sitosterol or $^{14}$C-desmosterol exchange from *M. gallisepticum* cells to sitosterol/PC or desmosterol/PC vesicles at 37 °C. The molar ratio of sterol/phospholipid was 0.90. A, sitosterol exchange in STM buffer in the presence of albumin (2%, w/v). The values of percentage of $^{14}$C-sitosterol remaining in the cells are plotted ± S.E. versus time of incubation with vesicles; cells were separated at intervals as described under "Methods." The arrow represents the size of the rapidly exchanging kinetic pool (see text). B, desmosterol exchange in STM buffer in the presence (○) and absence (○) of albumin (2%, w/v).](http://www.jbc.org/)[Fig. 3. First order kinetic plots of $^{14}$C-sitosterol or $^{14}$C-desmosterol exchange from *M. gallisepticum* cells to sitosterol/PC or desmosterol/PC vesicles at 37 °C. The molar ratio of sterol/phospholipid was 0.90. A, sitosterol exchange in STM buffer in the presence of albumin (2%, w/v). The values of percentage of $^{14}$C-sitosterol remaining in the cells are plotted ± S.E. versus time of incubation with vesicles; cells were separated at intervals as described under "Methods." The arrow represents the size of the rapidly exchanging kinetic pool (see text). B, desmosterol exchange in STM buffer in the presence (○) and absence (○) of albumin (2%, w/v).](http://www.jbc.org/)
Kinetic parameters for exchange of \(^{14}\text{C}\text{-sterols between } M.\text{ gallisepticum cells and sterol/egg PC vesicles}

The kinetic parameters were determined as described under "Methods" using the derivation of Bloj and Zilversmit (19). For the exchange of \(^{14}\text{C}\text{-sitosterol (Fig. 3A) or of }^{[14]}\text{C}\text{-cholesterol (18) from } M.\text{ gallisepticum cells, } g_1 \text{ and } g_2 \text{ are the rapid and slow exponential rate constants. The size of the outer pool, } R_\text{o}\text{, is obtained from } R_\text{o} = \frac{k_\text{a} / (k_\text{a} + k_\text{o})}{t_\text{in}} \text{ the time required for the }^{14}\text{C}\text{-sterol in the more accessible pool to be reduced by 50%. For }^{[14]}\text{C}\text{-desmosterol exchange only one kinetic pool was found (Fig. 3B). Values are the average of two different cell preparations for sitosterol and desmosterol and four different preparations for cholesterol. The sterol/phospholipid molar ratio was 0.9. The exchange experiments were conducted at 37 °C in STM buffer containing 2% albumin. For the exchange of }^{[14]}\text{C}\text{-desmosterol in the absence of albumin (Fig. 3B), the values of } g_1 \text{ and } t_\text{in} \text{ were } -0.105 \text{ h}^{-1} \text{ and 6.5 h, respectively. The slopes of the rapid and slow phases (} g_1 \text{ and } g_2 \text{) in the semilogarithmic plots (Fig. 3) were calculated by a least squares regression analysis of the data points. The correlation coefficients for } g_1 \text{ and } g_2 \text{ of the }^{14}\text{C}\text{-sterols presented in this table ranged from 0.93 to 0.96.}

\begin{table}[b]
\centering
\caption{Kinetic parameters for exchange of \(^{14}\text{C}\text{-sterols between } M.\text{ gallisepticum cells and sterol/egg PC vesicles}}
\begin{tabular}{|l|c|c|c|c|}
\hline
\text{Sterol} & \text{\(g_1\)} & \text{\(g_2\)} & \text{\(R_\text{o}\)} & \text{\(t_\text{in}\)} \\
\hline
\text{Cholesterol} & 0.277 & 0.167 & 0.58 & 6.2 & 2.5 \\
\text{Sitosterol} & 0.204 & 0.165 & 0.76 & 4.9 & 3.4 \\
\text{Desmosterol} & 0.196 & 1.0 & & & 3.5 \\
\hline
\end{tabular}
\end{table}

Second order rate constants for association of filipin with sterols in \( M.\text{ gallisepticum cells and membranes}

The second order rate constants were calculated from stopped flow traces of the initial rates of filipin-sterol association at 10 °C as described previously (7). Initial rates were measured using at least four sterol concentrations in cells and membranes from each culture. The filipin concentration was maintained constant (7 to 9 \(\mu\text{M} \text{ final concentration). The second order rate constants presented are the averages from two cell cultures of } M.\text{ gallisepticum adapted to grow with each sterol with the exception of sitosterol and cholesterol, with which three cultures were grown, and desmosterol, with which four cultures were grown. The error limits are standard errors of the mean.}

\begin{table}[t]
\centering
\caption{Second order rate constants for association of filipin with sterols in \( M.\text{ gallisepticum cells and membranes}}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\text{Sterol} & \text{\(k_{\text{cells}}\)} & \text{\(k_{\text{membranes}}\)} & \text{\(k_{\text{cells}}/k_{\text{membranes}}\)} \\
\hline
\text{Sitosterol} & \(3.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}\) & \(4.2 \pm 0.4\) & \(0.86 \pm 0.06\) \\
\text{Desmosterol} & \(8.1 \pm 0.6\) & \(8.6 \pm 0.8\) & \(0.94 \pm 0.06\) \\
\text{Cholest-5-22E,24-} & \(6.6 \pm 0.4\) & \(7.0 \pm 0.3\) & \(0.94 \pm 0.05\) \\
\text{trans-3β-ol} & & & & \\
\text{cis-22-Dehydro-} & \(6.5 \pm 0.5\) & \(7.1 \pm 0.5\) & \(0.91 \pm 0.07\) \\
\text{cholesterol} & & & & \\
\text{trans-22-Dehydro-} & \(6.7 \pm 0.3\) & \(7.2 \pm 0.3\) & \(0.93 \pm 0.04\) \\
\text{cholesterol} & & & & \\
\text{Cholesterol} & \(6.1 \pm 0.2\) & \(11.7 \pm 0.3\) & \(0.52 \pm 0.05\) \\
\text{20-Isocholesterol} & \(14.4 \pm 0.7\) & \(24.4 \pm 0.8\) & \(0.59 \pm 0.04\) \\
\text{C22} & \(7.6 \pm 0.8\) & \(12.9 \pm 1.0\) & \(0.59 \pm 0.09\) \\
\text{C24} & \(6.7 \pm 0.9\) & \(12.2 \pm 1.0\) & \(0.55 \pm 0.10\) \\
\text{C26} & \(7.2 \pm 1.0\) & \(13.1 \pm 0.9\) & \(0.57 \pm 0.09\) \\
\text{C28} & \(7.0 \pm 1.1\) & \(13.2 \pm 0.5\) & \(0.60 \pm 0.05\) \\
\text{C30} & \(8.8 \pm 1.0\) & \(14.7 \pm 1.1\) & \(0.59 \pm 0.09\) \\
\hline
\end{tabular}
\end{table}

rate of cholesterol translocation into the internal membrane surface.

**DISCUSSION**

In this paper we have assessed how sterol transbilayer distribution in \( M.\text{ gallisepticum}\) is influenced by shortening and extending the length of the aliphatic side chain and by enlarging it in bulk by introducing double bonds or alkyl substituents. In a previous study we reported that sitosterol, stigmasterol, and ergosterol are localized predominantly in the outer leaflet of the \( M.\text{ capricolum}\) cell membrane (6). It has been pointed out by Razin that conclusions about sterol structural specificity drawn from studies with \( M.\text{ capricolum}\) may not be valid for other sterol-requiring mycoplasmas (21), since a very broad range of sterols can support growth of this organism (e.g. Refs. 6, 22–24). We have now analyzed sterol transbilayer distribution in \( M.\text{ gallisepticum}\) membranes. Sterols bearing alkyl substituents or unsaturation in the side chain are localized predominantly in the outer leaflet (Table III), indicating that \( M.\text{ gallisepticum}\) and \( M.\text{ capricolum}\) share the same characteristic of accumulating sterols with bulky or rigid side chains in the outer half of the bilayer. Alkylation at C-24 (as in sitosterol, stigmasterol, and ergosterol) increases the steric bulk of the side chain, and (because of branching) decreases the conformational freedom. Unsaturation (as in

![Fig. 4](http://www.jbc.org/)

Stopped flow kinetics of association of filipin with C22 and sitosterol in \( M.\text{ gallisepticum cells and membranes}

At 10 °C, a photograph of multiple traces of the initial transmittance changes at 360 nm as a function of time (50 ms/large division) obtained on mixing filipin solutions with intact cells containing C22. The vertical axis is 50 mV/division; initial signal, 1.6 V. The initial 100–150-ms disturbance period was ignored, and initial rates were calculated from the linear transmittance increases (see Ref. 7). The final C22 concentration in the cell suspension, after mixing with filipin, was 11.2 \(\mu\text{M}\). The final filipin concentration was 9 \(\mu\text{M}\). B, plot of the initial rate of absorbance change/s, \(dA/dt\) (which was calculated from the increase in the transmittance of filipin on binding to sitosterol) versus sitosterol concentration in intact cells (C) and open isolated membranes (D). At least six measurements of the initial rate were made at each concentration. The final filipin concentration was 7.5 \(\mu\text{M}\).
ergosterol, stigmasterol, desmosterol, cis- and trans-22-dehydrocholesterol, and cholesta-5,22E,24-trien-3β-ol also decreases the flexibility and increases the bulk of the side chain.

The differences in the distribution of cholesterol and derivatives containing alkyl groups or double bonds suggest that the latter experience steric interference during translocation across *M. gallisepticum* and *M. capricolum* membranes. The kinetics of sitosterol and stigmasterol translocation from the outer leaflet of adapted *M. capricolum* cells (which is in contact with the exogenous supply of sterol) into the inner leaflet confirm this conclusion (Table IV); about one-half of the cholesterol moved into the inner half of the bilayer rapidly in these growing cells, whereas about three-fourths of the newly acquired sitosterol and stigmasterol was present in the outer half. In *M. gallisepticum*, the high ratios of \( k_{\text{cell}}/k_{\text{membrane}} \) in the filipin-binding experiments (Table II) indicate that 86 ± 6% of the sitosterol and 94 ± 6% of the desmosterol are localized in the outer leaflet. The \( [^{14}\text{C}] \) sitosterol and \( [^{14}\text{C}] \) desmosterol exchange data are in good agreement with these results, since 76% and 100% of these sterols, respectively, were found in the more accessible pool (Fig. 3, Table II). In contrast to the effects of side chain bulk and rigidity on sterol localization in the membrane, side chain length does not appear to impose an important constraint on sterol distribution between the inner and outer halves of the *M. gallisepticum* membrane. Cholesterol and its analogs having shorter (C22, C24, C26) and longer (C28 and C30) side chains than isocoyl were found to have the same localization (Table III). Thus the absence of branching at C-25 in the \( 20(R) \)-n-alkylpregn-5-en-3β-ols does not influence sterol distribution. Furthermore, 20-isocholesterol and cholesterol have an identical distribution between the two leaflets of the membrane, suggesting that the orientation of the side chain at C22 with respect to the C/D ring system does not influence sterol localization in *M. gallisepticum*. It therefore appears that the translocation process is particularly sensitive to inhibition by the insertion of double bonds at C-22 or C-24 or alkyl groups at C-24 of the sterol side chain.

All of the sterols we used are taken up into membranes spontaneously because of their lipophilicity, and become intercalated between fatty acyl chains of phospholipid molecules. Sterols with alkyl substituents or double bonds in the side chain may fit into spaces between adjacent phospholipids less well than the unalkylated, saturated analogs (Refs. 4 and 25 and references cited therein). The looser packing of these sterols with phospholipids in the membrane is expected to decrease the ability of the rigid sterol ring to restrict the trans-gauche isomerizations of the fatty acyl chains. Since the sterol side chain extends deeply into the interior of the bilayer (26, 27), van der Waals interactions in this region would be weakened when sterols with bulky side chains are present. In fact, sterols with very long aliphatic side chains have been postulated to penetrate into the adjacent leaflet of the bilayer (28). This insertion would disrupt the membrane bilayer and increase the fluidity of the membrane in the liquid-crystalline state; it may explain why C30 and, in particular, C32 failed to function as effective growth supporters of *M. gallisepticum*. It is not known why cis-22-dehydrocholesterol was more effective than the trans isomer with respect to growth support and cell yield. The kinked conformation of the side chain has been postulated to reduce the ability of this sterol to interdigitate between phospholipid fatty acyl chains, producing a lethal effect on mouse fibroblast cells (29). It should be noted, however, that the cis isomer was not toxic to many species of *Drosophila*, whereas trans-22-dehydrocholesterol was toxic (13).

Several other factors, besides looser packing of the bulk lipids, may be responsible for the differences in growth and cell yield we found with the various sterols (Table I). The abilities of sterols with alkylated, unsaturated, or very long side chains to act as spacer and filler molecules may be diminished relative to cholesterol. In its role as a spacer molecule, cholesterol is considered to separate phospholipid head groups and disrupt head group interactions (30-33). Small amounts of cholesterol may also exert a regulatory role on phospholipid biosynthesis by interacting with specific membrane-bound enzymes (35). It remains to be determined if low concentrations of other sterols can also interact specifically with protein(s) in the membrane to enhance enzymatic activity, resulting in modification of phospholipid synthesis.

The physiological role of extensive sterol localization in each leaflet of the membrane bilayer remains to be established. The results we obtained in *M. gallisepticum* (Tables II and III) and in *M. capricolum* (6) indicate that growth-supporting sterols need not be translocated extensively into

**Table IV. Growth of *M. capricolum* cells, sterol content, and sterol transbilayer distribution in the membrane of adapted cells transferred to sterol-rich medium**

<table>
<thead>
<tr>
<th>Time after transfer to 10 μg of sterol/ml</th>
<th>Cholesterol*</th>
<th>Sitosterol</th>
<th>Stigmasterol</th>
<th>Cholesterol*</th>
<th>Sitosterol</th>
<th>Stigmasterol</th>
<th>Cholesterol*</th>
<th>Sitosterol</th>
<th>Stigmasterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>μg/mg membrane protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.08</td>
<td>0.10</td>
<td>0.10</td>
<td>33</td>
<td>25</td>
<td>20</td>
<td>0.73</td>
<td>0.83</td>
<td>0.89</td>
</tr>
<tr>
<td>1</td>
<td>0.16</td>
<td>0.11</td>
<td>0.11</td>
<td>44</td>
<td>33</td>
<td>32</td>
<td>0.53</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.24</td>
<td>0.13</td>
<td>0.13</td>
<td>92</td>
<td>42</td>
<td>41</td>
<td>0.45</td>
<td>0.74</td>
<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>0.26</td>
<td>0.20</td>
<td>0.20</td>
<td>127</td>
<td>62</td>
<td>59</td>
<td>0.45</td>
<td>0.75</td>
<td>0.79</td>
</tr>
<tr>
<td>6</td>
<td>0.23</td>
<td>0.22</td>
<td></td>
<td>77</td>
<td>70</td>
<td></td>
<td>0.70</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

*Data with cholesterol represent results after transfer to 10% fetal calf serum (5).
the inner half of the bilayer. For example, sitosterol, cholesta-5,22E,24-trien-3β-ol, and cis-22-dehydrocholesterol supported growth of *M. gallisepticum* and were incorporated into the membrane in high amounts, but with different distributions between the two leaflets compared with cholesterol. On the other hand, 20-isocholesterol and C30 gave the same trans-bilayer distribution as cholesterol, but were poor growth supporters and were taken up at lower levels. Similarly, in *M. capricolum* (6), sitosterol, ergosterol, and stigmasterol supported growth nearly as well as cholesterol but were localized differently than cholesterol in the two leaflets of the bilayer; cholesterol analogs with modifications in the steroid nucleus were distributed identically as cholesterol but were poorer growth supporters. No information is available concerning the possibility that other membrane components may be redistributed differently between the two halves of the bilayer of cholesterol-containing membranes compared with, for example, sitosterol-containing membranes. It is also not known how much sterol is needed in the inner leaflet of mycoplasmas for optimal membrane function or whether some specific membrane functions may be altered when the sterol distribution is changed.

Acknowledgments—We are grateful to Drs. Toshiyuki Akiyama and Henry Kircher for kindly donating the cholesterol analogs. We thank Sylvia Schaffel for typing the manuscript and Bruce Robinson for preparing the art work.

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Distribution and movement of sterols with different side chain structures between the two leaflets of the membrane bilayer of mycoplasma cells.

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