Effects of Sterol Structure and Exogenous Lipids on the Transbilayer Distribution of Sterols in the Membrane of *Mycoplasma capricolum*†

Sanda Clejan, Robert Bittman,* and Shlomo Rottem

ABSTRACT: Stopped-flow kinetic measurements of the association of filipin with sterols in intact cells and isolated membranes of *Mycoplasma capricolum* were used to study the effects of varying the phospholipid composition and the sterol structure on sterol distribution in the membrane. The phospholipid composition and content of the membrane were varied by growing cells in an albumin-containing medium with cholesterol, palmitic and oleic acids, and various concentrations of exogenous phospholipids. The exogenous phospholipids (phosphatidylcholine, sphingomyelin, and phosphatidic acid) were incorporated up to levels of approximately 50% of the total membrane phospholipids but had no effect on the distribution of cholesterol between the two halves of the membrane bilayer. The sterol structure was varied by growing the cells with 10 μg/mL of either cholesterol, β-cholestanol, 4,6-cholestadien-3β-ol, ergosterol, β-sitosterol, or stigmasterol. With cholesterol, β-cholestanol, and 4,6-cholestadien-3β-ol, approximately 65% of the sterol was found to be present in the outer half of the lipid bilayer. With ergosterol, β-sitosterol, and stigmasterol, about 89% of the sterol is localized in the outer half of the membrane bilayer. Thus, the behavior of the alkyl-substituted sterols differs from that of cholesterol. The extent to which a sterol is distributed asymmetrically between the two halves of the bilayer is not related to the extent to which maximum growth is produced. These results suggest that growth-supporting sterols need not be translocated extensively.

Recent studies have indicated that the distribution of cholesterol in the membrane of *Mycoplasma capricolum* can be examined on the basis of the rapid kinetics of filipin binding with cholesterol (Clejan et al., 1978). In another sterol-requiring mycoplasma, *M. gallisepticum*, the estimate of the distribution of cholesterol between the two halves of the bilayer obtained by filipin binding (Bittman & Rottem, 1976) was the same as that obtained by the kinetics of spontaneous cholesterol exchange with high-density lipoproteins (Rottem et al., 1978). The filipin-binding approach was also used to examine the rate of transbilayer movement of cholesterol under physiological conditions, using an adapted strain of *M. capricolum* which grew on media containing low cholesterol concentrations (Clejan et al., 1978). Since the sterol specificity of *M. capricolum* is relatively broad [Rodwell et al., 1970; Odriozola et al., 1978; for review, see Smith (1979)] and since filipin binds to various sterols (Bittman & Fischkoff, 1972), we have used this approach to measure the transbilayer localization of a variety of sterols in the membrane of *M. capricolum*.

The recent discovery that exogenous lipids are incorporated extensively into the membrane of *M. capricolum* (Z. Gross, S. Rottem, and R. Bittman, unpublished results) has been used in the present study to investigate the effects of uptake of phosphatidylcholine (PC)[1], SPM, phosphatidic acid, and cholesterol oleate on the distribution and transbilayer movement of cholesterol in the membrane of *M. capricolum*.

Materials and Methods

Lipids. Cholesterol and 5α-cholestan-3-ol (cholestanol) were from Sigma; stigmasterol, β-sitosterol, and ergosterol were from Steraloids; 4,6-cholestadien-3β-ol was from Research Plus Steroid Laboratories. The structures of these sterols are shown in Figure 1. All sterols were recrystallized at least 3 times from ethanol. The purity was checked by thin-layer chromatography on silica gel in ether–petroleum ether (9:3 v/v) and by gas–liquid chromatography (as trimethylsilyl ethers) on SE-54 glass columns (4 ft × 2 mm) at 230 °C with nitrogen as carrier gas, using 5α-cholestane as an internal standard. Gas–liquid chromatography was kindly performed by Dr. T. S. Parker of Rockefeller University. No cholesterol was detected in cholestanol, stigmasterol, sitosterol, ergosterol, and 4,6-cholestadien-3β-ol. Only the batches of bovine serum albumin (Sigma, fraction V) that were found to be cholesterol free by gas chromatography were used in this study. A small amount of polar impurity (possibly oxygenated sterol) was found in 4,6-cholestadien-3β-ol by thin-layer chromatography on charring with sulfuric acid. Cholesterol oleate, egg PC, bovine-brain SPM, and egg phosphatidic acid were purchased from Sigma, diluted in chloroform, and stored at −30 °C. The purity of each phospholipid was checked by thin-layer chromatography on silica gel G plates in chloroform–methanol–water (65:25:4 v/v) using 0.005% butylated hydroxytoluene as an antioxidant.

Growth of M. capricolum and Isolation of Membranes. For incorporation of exogenous lipids, *M. capricolum* (California kid) was grown in a modified Edward medium (Razin & Rottem, 1976) that contained 1% bovine serum albumin (fatty acid poor, Sigma, fraction V), 10 μg/mL cholesterol, and palmitic and oleic acids (10 μg/mL of each). For determination of the incorporation of phospholipids, [^{14}C]SPM or [^{14}C]PC (New England Nuclear) was diluted with the unlabeled SPM or PC (2.5–30 μg/mL). The lipids were added as ethanolic solutions, and the final concentration of ethanol did not exceed 1% (v/v). For growth in the presence of various sterols, the concentration of bovine serum albumin was increased from 1 to 2% in order to minimize cell lysis. Solutions of sterols and fatty acids in ethanol were mixed and added

† From the Department of Chemistry, Queens College of The City University of New York, Flushing, New York 11367 (S.C. and R.B.), and Department of Membranes and Ultrastructure, The Hebrew University—Hadassah Medical School, Jerusalem, Israel (S.R.). *Received August 7, 1980.* This work was supported in part by the United States–Israel Binational Science Foundation.

[1] Abbreviations used: PC, phosphatidylcholine; SPM, sphingomyelin.

0006-2960/81/0420-2200$01.25/0

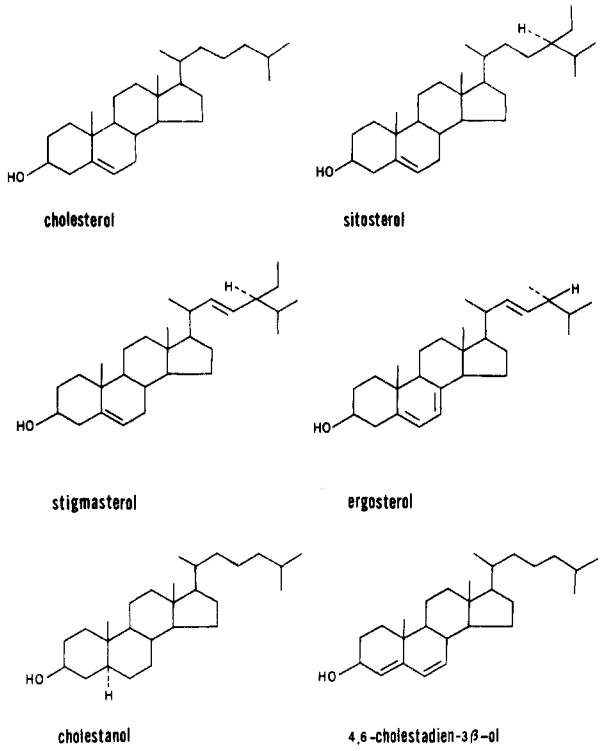

FIGURE 1: Structures of sterols used to support growth of *M. capricolum*.

together to the growth medium. The order of addition was, first, the sterol–fatty acid solution, then (when present) cholesterol oleate, and finally albumin. Crystals of lipids were not observed after incubation with the medium when the sterol concentration was 10 μg/mL or less. So that we could obtain *M. capricolum* cultures containing sterols other than cholesterol, the organisms were passed at least 3 times with the desired sterol (10 μg/mL) prior to inoculation of a large volume of growth medium. Growth was followed by measuring the absorbance of the culture at 640 nm. Most experiments were performed with mid-exponential phase cells (A_{640} = 0.15–0.22). To follow exogenous lipid incorporation, we grew cells in the medium containing bovine serum albumin, fatty acids, and 10 μg/mL of cholesterol to an absorbance at 640 nm of 0.1. The various phospholipids were then added, and growth was continued for up to 6 h. The cells were harvested by centrifugation at 12000*g* for 20 min, washed twice, and resuspended in cold 0.25 M NaCl solution. Membranes were prepared, pelleted, and washed as described previously (Clejan et al., 1978).

Lipid Analysis. Lipids were extracted from intact cells and isolated membranes by the method of Bligh & Dyer (1959). Thin-layer chromatography of total membrane lipids were performed with silica gel HR plates (Analtech). The plates were first developed at room temperature with petroleum ether (bp 40–60 °C)–acetone (3:1 v/v) and then at 4 °C with chloroform–methanol–water (65:25:4 v/v). Lipid spots were detected by iodine vapor. The iodine was removed by evaporation. For determination of radioactivity in the lipid spots, the compounds were scraped from the plate into scintillation vials containing 5 mL of Bray's solution. The radioactivity was measured in a Beckman scintillation spectrometer. For determination of the phosphorus concentration in phospholipid spots, the compounds were scraped from the plate into test tubes and digested with 0.5 mL of 10 N sulfuric acid in the presence of silica gel. Total phosphorus was determined by the method of Taussky & Shorr (1953). Neutral lipids were separated by thin-layer chromatography on silica gel G plates.

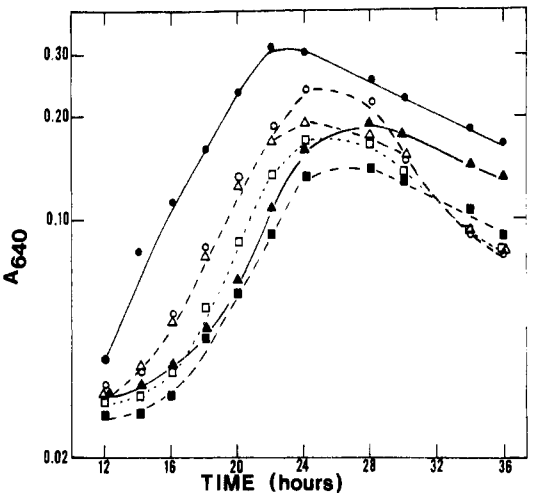

FIGURE 2: Growth curves of *M. capricolum* cultured at 37 °C in Edward medium supplemented with bovine serum albumin (2% w/v), palmitic and oleic acids (10 μg/mL of each), and sterol (10 μg/mL). The sterols were (●) cholesterol, (○) β-sitosterol, (△) stigmasterol, (□) ergosterol, (▲) β-cholestanol, and (■) 4,6-cholestadien-3β-ol.

The plates were developed at room temperature with benzene–ethyl acetate (5:1 v/v). The spots were scraped from the plates and extracted into chloroform–methanol (2:1 v/v) at 45 °C for 1 h. Stock solutions of 10, 20, and 30 μg/mL of free cholesterol and cholesterol oleate were made and used as standards. After centrifugation and evaporation of the chloroform layer, the free cholesterol and cholesterol ester separated in this way were assayed by the method of Zlatkis & Zak (1969).

Kinetic Measurements. The filipin complex was purified as described previously (Bittman & Rottem, 1976). The final concentration of filipin, after mixing with cells or membranes, was 10 μM, unless noted otherwise. Initial rates of filipin–cholesterol association were measured as described previously (Clejan et al., 1978). The initial rate of filipin association with cholesterol in cells grown in media containing PC, SPM, phosphatidic acid, or cholesterol oleate was first order in each reactant. The initial rate of filipin–sterol association in cells and membranes containing the various sterols remained first order in each reactant. Membranes obtained from mycoplasma cells by sonication or osmotic lysis are known to be largely unsealed, based on morphological (Rottem & Razin, 1966) and transport (Cirillo & Razin, 1973) studies. Therefore, the ratio of the rate constants for filipin–cholesterol association in cells relative to membranes is a measure of the distribution of this sterol between the two surfaces of the mycoplasma membrane (Bittman, 1978).

Miscellaneous Methods. For determination of whether cells that had incorporated PC, SPM, phosphatidic acid, cholesterol oleate, or various sterols remained intact, the percent efflux of [^{3}H]thymidine-labeled components and of NADH oxidase was determined as described previously (Clejan et al., 1978).

Protein and phospholipid analyses were made as described previously (Clejan et al., 1978). Total sterol was measured colorimetrically (Zlatkis & Zak, 1969). The sterols were analyzed at the following wavelengths: cholesterol, 550 nm; β-cholestanol, 530 nm; stigmasterol and β-sitosterol, 542 nm; ergosterol, 560 nm; 4,6-cholestadien-3β-ol, 560 nm.

Results

Growth of M. capricolum with Various Sterols. When sterols are added to a medium in which serum is replaced by bovine serum albumin and palmitic and oleic acids, growth of *M. capricolum* occurs with the following relative activities

Table I: Sterol Incorporation and Transbilayer Distribution in the Membrane of *M. capricolum* Cells[a]

sterol added to growth medium (10 μg/mL)	free sterol content (μg/mg of membrane protein)	$10^{-4}k_{cells}$[b] (M^{-1} s^{-1})	$10^{-4}k_{membranes}$[b] (M^{-1} s^{-1})	$k_{cells}/k_{membranes}$[c]	sterol distribution[d] (μg/mg of membrane protein)	
					outer half	inner half
cholesterol	153	4.2 ± 0.3	6.4 ± 0.5	0.66 ± 0.04 (4)	101	52
4,6-cholestadien-3β-ol	126	5.5 ± 0.5	8.7 ± 0.9	0.65 ± 0.03 (2)	82	44
β-cholestanol	98	4.1 ± 0.2	6.3 ± 0.8	0.65 ± 0.07 (2)	64	34
ergosterol	85	5.3 ± 0.6	6.3 ± 0.6	0.89 ± 0.07 (3)	76	9
β-sitosterol	84	2.4 ± 0.2	2.8 ± 0.2	0.87 ± 0.05 (2)	73	11
stigmasterol	142	17.8 ± 0.6	20.0 ± 1.1	0.89 ± 0.02 (2)	126	16

[a] Sterols were added to the medium as ethanolic solutions. The initial rates of filipin–sterol association were measured at 10 °C at various sterol concentrations in intact cells and isolated membranes. The extent of cell lysis did not exceed 15%. [b] A representative example of second-order rate constants analyzed from one of the cultures. The results are expressed as the average value of the rate constant ± standard error of the mean. [c] Average ratio of second-order rate constants for filipin–sterol association in cells and membranes obtained from different cell cultures. The number of cell cultures investigated is indicated in parentheses. [d] The sterol distribution (in μg/mg of membrane protein) was calculated for a representative example from $k_{cells}/k_{membranes}$ × free sterol content, which equals the sterol content in the outer monolayer.

(Figure 2): cholesterol > β-sitosterol ~ stigmasterol > ergosterol > β-cholestanol ~ 4,6-cholestadien-3β-ol. The maximum extent of growth is greatest with cholesterol and poorest with 4,6-cholestadien-3β-ol, and the lag phase is longer with β-cholestanol, ergosterol, and 4,6-cholestadien-3β-ol than with the other sterols (Figure 2). The specificity with which the various sterols support growth is rather small, however, since the maximum slopes of the logarithmic phases of growth are very similar with all of the sterols investigated. Thus, introduction of an alkyl substitutent at C-24 (ergosterol, β-sitosterol, and stigmasterol), unsaturation at C-22 (ergosterol and stigmasterol) or at C-4 and C-6 (4,6-cholestadien-3β-ol), or reduction of the Δ^5 bond (β-cholestanol) is not sufficient to abolish growth or to markedly affect the rate of the logarithmic phase of growth after their initial lag phase.

Distribution of Sterols between the Two Halves of the Membrane Bilayer. Table I summarizes the transbilayer distributions of various sterols, as determined by analysis of the initial rates of filipin–sterol association in intact cells and isolated membranes. Sixty-five percent of the cholesterol, 4,6-cholestadien-3β-ol, and β-cholestanol that reacted with filipin was calculated to be present in the outer monolayer. We reported previously that two-thirds of the cholesterol remained in the outer half of the bilayer of cholesterol-grown cells (Bittman & Rottem, 1976). The ratio of the rate constants k_{cells} and $k_{membranes}$ indicates that approximately 85–90% of the ergosterol, β-sitosterol, and stigmasterol is present in the outer monolayer. In other experiments with cells grown on the various sterols (data not shown), the $k_{cells}/k_{membranes}$ ratio was not changed when the ergosterol content was 110 and 123 μg/mg of membrane protein; similarly, membranes containing 109 μg of β-sitosterol and 91 μg of stigmasterol per mg of membrane protein gave the same rate ratio as the membrane preparations shown in Table I. Therefore, there does not appear to be a correlation between the amount of sterol incorporated and the $k_{cells}/k_{membranes}$ ratio.

Effect of PC and Cholesterol Oleate on the Translocation of Cholesterol from the Outer to the Inner Monolayer. Cells of *M. capricolum* and related species can be obtained with a very low cholesterol content in the membrane by making serial transfers of the organisms into media containing one-half of the sterol concentration of the previous medium (Rottem et al., 1973; Clejan et al., 1978). The growth rate of cells "adapted" to grow on 1.25 μg/mL cholesterol is enhanced dramatically when an early logarithmic culture is transferred to a medium containing a high cholesterol concentration (10 μg/mL). Table II shows the distribution of cholesterol in the cell membrane of cells grown for times up to 4 h in the cholesterol-rich medium. The value of $k_{cells}/k_{membranes}$ is lower in the adapted strain than in the native strain, and approximately 45% of the cholesterol available to filipin is present in the outer monolayer after a 4-h growth period in cholesterol-rich medium. The difference in cholesterol distribution between the adapted and native strains may result from differences in membrane composition and physiology of the organism upon adaptation (Clejan et al., 1978). The effects of cholesterol oleate and egg PC on the rate of cholesterol translocation from the outer to inner monolayer were tested by including these exogenous lipids in the cholesterol-rich medium. The distribution of cholesterol in the membranes enriched in cholesterol oleate and PC was the same as that in membranes lacking these exogenous lipids.

Table II: Effect of Incorporation of Exogenous Lipids on Cholesterol Transbilayer Movement across the Membrane of *M. capricolum* upon Transfer to Cholesterol-Rich Medium[a]

time of incubation in medium containing 10 μg/mL cholesterol (h)	$k_{cells}/k_{membranes}$ lipid added to growth medium			
	none	cholesterol oleate (5 μg/mL)	egg PC (5 μg/mL)	cholesterol oleate + egg PC (5 μg/mL of each)
0	0.58	0.70	0.72	0.70
1	0.55	0.54	0.55	0.55
2	0.45	0.45	0.45	0.52
4	0.43	0.43	0.46	0.48

[a] Cells were adapted to grow on 1.25 μg/mL of cholesterol by serial passaging of a culture through media containing one-half of the cholesterol concentration of the previous medium, as described before (Clejan et al., 1978). The adapted cells were grown to A_{640} of 0.1 and were then transferred to a medium containing 10 μg/mL of cholesterol and the various lipids. Growth was continued for up to 4 h, and cells were harvested at the times indicated. The final concentration of filipin was 16 μM. The content of free cholesterol (FC) and cholesterol ester (CE) in the membrane was as follows. No CE added to medium: 0 h, FC = 35 μg/mg of membrane protein; 1 h, FC = 48; 2 h, FC = 92; 4 h, FC = 133. The CE content was about 2 μg/mg of membrane protein and did not vary during the incubation with FC-containing medium. CE (5 μg/mL) added to medium: 0 h, FC = 30, CE = 27 μg/mg of membrane protein; 1 h, FC = 42, CE = 38; 2 h, FC = 86, CE = 80; 4 h, FC = 124, CE = 110. The values of $k_{cells}/k_{membranes}$ found by using cells grown in the presence of 2.5 and 10 μg/mL of PC were similar to those obtained with 5 μg/mL. Growth in media containing 2.5 and 10 μg/mL of CE gave $k_{cell}/k_{membranes}$ values similar to those with 5 μg/mL.

Table III: Effect of Incorporation of Exogenous Phospholipids on the Transbilayer Distribution of Cholesterol ($k_{cells}/k_{membranes}$) in the Membrane of *M. capricolum*[a]

exogenous phospholipid added to growth medium[b]	concn (μg/mL)	$10^{-4} \times k_{cells}$ ($M^{-1} s^{-1}$)	$10^{-4} \times k_{membranes}$ ($M^{-1} s^{-1}$)	$k_{cells}/k_{membranes}$
PC	0	4.2	6.4	0.66 ± 0.04 (4)
	5	4.5	7.2	0.62 ± 0.06 (3)
	10	5.3	8.8	0.60 ± 0.02 (5)
	15	7.6	11.7	0.65 ± 0.05 (4)
SPM	5	5.9	9.0	0.62 ± 0.06 (5)
	10	8.1	11.9	0.68 ± 0.05 (5)
	15	7.2	10.9	0.66 ± 0.03 (5)
PC + SPM	10 (of each)	7.3	10.8	0.68 (1)
phosphatidic acid	5	4.7	7.0	0.67 ± 0.05 (2)
	10	6.0	9.2	0.65 ± 0.05 (2)

[a] Cells were grown in a medium containing bovine serum albumin (1%), cholesterol (10 μg/mL), palmitic and oleic acids (10 μg/mL of each), and increasing concentrations of phospholipids. [b] The extent of incorporation of exogenous phospholipid into the membranes, in terms of the percentage of total phospholipid, was the following: PC, 20%, 30%, and 33% for 5, 10, and 15 μg/mL, respectively. The free cholesterol content of the membrane was unaffected by the incorporation of exogenous phospholipids (47–54 μg of cholesterol per mg of cell protein).

Effect of Exogenous Phospholipids on Cholesterol Distribution. When *M. capricolum* cells are cultured on a medium containing PC and SPM, a massive incorporation of these phospholipids results, up to levels of about 50% of the total phospholipids (Z. Gross, S. Rottem, and R. Bittman, unpublished results). Despite the striking increase in the polar lipid content of the membrane caused by growth in the presence of PC, SPM, and phosphatidic acid, the ratio of the rate constants k_{cells} and $k_{membranes}$ remained the same; i.e., approximately 65% of the cholesterol was present in the outer monolayer (Table III).

Discussion

Mycoplasmas have convenient characteristics that make a study of the structural features of sterols required for translocation very attractive. These sterol-requiring procaryotes are unable to synthesize sterols or to modify the fatty acyl chains of membrane phospholipids. Thus, *M. capricolum* is unable to isomerize, alkylate, esterify, or carry out other conversions of sterols (e.g., Odriozola et al., 1978; Dahl et al., 1980a). The sterol is taken up exclusively into the plasma membrane (the only membrane present) and affects the physical state of membrane lipids and some of the cell's biochemical and physiological properties (Rottem et al., 1973; Razin & Rottem, 1978; Rottem, 1980). Cholesterol is an especially efficient regulator of bulk membrane fluidity in *M. capricolum* and helps maintain its cell membrane in a homogeneous physical state despite variations in temperature and fatty acid composition of the growth medium (Rottem, 1980; Dahl et al., 1980b). In previous work, we found that the extent of cholesterol incorporation is decreased when growth is inhibited, suggesting that a mechanism may be present in growing mycoplasmas that catalyzes cholesterol incorporation into the membrane (Clejan et al., 1978). The present study demonstrates that sterols bearing alkyl substituents in the side chain are distributed between the two halves of the bilayer to a different extent than cholesterol. Although the factors responsible for this difference in distribution are not yet known, one hypothesis is that interactions occur in the membrane which are specific for the cholesterol molecule or derivatives having the cholestane-type side chain.

M. capricolum was also chosen for the study of how changes in the composition of the polar and nonpolar lipids affect cholesterol distribution and movement in the two halves of the bilayer because this strain is capable of incorporating high levels of cholesterol esters, PC, and SPM into its membrane (Z. Gross, S. Rottem, and R. Bittman, unpublished results). Furthermore, *M. capricolum* grows well when exogenous phospholipids are incorporated into the membrane. In most mycoplasma species, exogenous phospholipids that are incorporated from the growth medium are not altered (Rottem, 1980). Measurement of the initial rates of filipin association with cholesterol in intact cells and open, isolated membranes obtained from cells enriched in exogenous lipids shows that incorporation of egg PC, brain SPM, egg phosphatidic acid, and cholesterol oleate did not alter cholesterol distribution and movement between the two halves of the bilayer (Tables II and III). Incorporation of egg PC, brain SPM, and egg phosphatidic acid caused the rates of filipin–cholesterol binding to increase in both intact cells and isolated membranes (Table III), but the ratio of $k_{cells}/k_{membranes}$ was not changed. The increase in rate constants may reflect an increased accessibility of cholesterol for reaction with filipin; an increase in membrane fluidity was suggested in spin-label and permeability studies on incorporation of unsaturated exogenous phospholipids into the *M. capricolum* membrane (Z. Gross, S. Rottem, and R. Bittman, unpublished results).

Although *M. capricolum* will accept various sterols into its membrane without dramatic changes in the logarithmic-phase growth rates (Figure 2), our studies of transbilayer distribution of sterols indicate that the nature of the alkyl side chain of the sterol affects the extent of sterol translocation from the external to the cytoplasmic surface of the *M. capricolum* bilayer. Sterols with the cholesterol side chain (β-cholestanol and 4,6-cholestadien-3β-ol) are translocated to the same extent as cholesterol (Table I). However, sterols containing a 24α-ethyl group (β-sitosterol and stigmasterol), a 24β-methyl group, and a Δ^7 bond (ergosterol) or a Δ^{22} bond (ergosterol and stigmasterol) remain localized predominantly in the outer monolayer. Alkylation at C-24 and unsaturation at Δ^{22} increase the bulk of the side chain and consequently may cause steric interference in the movement of the sterol molecule across the lipid bilayer. Two modifications of the sterol nucleus, namely absence of the Δ^5 bond in cholestanol and introduction of $\Delta^{4,6}$ bonds in place of the Δ^5 bond, were found to cause no change in transbilayer distribution compared with cholesterol (Table I). Cholesterol (which produced the highest extent of growth among the sterols studied) and 4,6-cholestadien-3β-ol (the poorest growth supporter we studied) are translocated to the same extent, and β-sitosterol, stigmasterol, and ergosterol are not translocated extensively (Table I). Since growth of *M. capricolum* does not seem to require extensive transbilayer movement of sterol from the outer to inner half of the bilayer, the physiological consequences of how sterols are integrated into the membrane remain to be established. It also remains to be shown whether mycoplasmas contain an agent that recognizes cholesterol (including its side chain) and catalyzes its movement into the inner half of the bilayer or whether adjustments in phospholipid or protein asymmetry may compensate for the differences observed in sterol asymmetry.

References

Bittman, R. (1978) *Lipids 13*, 686.

Bittman, R., & Fischkoff, S. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3795.

Bittman, R., & Rottem, S. (1976) *Biochem. Biophys. Res. Commun. 71*, 318.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Cirillo, V. P., & Razin, S. (1973) *J. Bacteriol. 113*, 212.
Clejan, S., Bittman, R., & Rottem, S. (1978) *Biochemistry 17*, 4579.
Dahl, C. E., Dahl, J. S , & Bloch, K. (1980a) *Biochemistry 19*, 1462.
Dahl, J. S., Dahl, C. E., & Bloch, K. (1980b) *Biochemistry 19*, 1467.
Odriozola, J. M., Waitzkin, E., Smith, T. L., & Bloch, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4107.
Razin, S., & Rottem, S. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., Ed.) pp 3–26, Chapman and Hall, London.
Razin, S., & Rottem, S. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, 51.
Rodwell, A. W., Peterson, J. E., & Rodwell, E. S. (1970) *Pathog. Mycoplasmas, Ciba Found. Symp.*, 123.
Rottem, S. (1980) *Biochim. Biophys. Acta 604*, 65.
Rottem, S., & Razin, S. (1966) *J. Bacteriol. 92*, 714.
Rottem, S., Yashouv, J., Ne'eman, Z., & Razin, S. (1973) *Biochim. Biophys. Acta 323*, 495.
Rottem, S., Slutzky, G. M., & Bittman, R. (1978) *Biochemistry 17*, 2723.
Smith, P. F. (1979) in *The Mycoplasmas* (Barile, M. F., & Razin, S., Eds.) Vol. I, pp 231–258, Academic Press, New York.
Taussky, H. H., & Shorr, E. (1953) *J. Biol. Chem. 202*, 675.
Zlatkis, A., & Zak, B. (1969) *Anal. Biochem. 29*, 143.

Effect of Cholesterol in Membranes. Pulsed Nuclear Magnetic Resonance Measurements of Lipid Lateral Diffusion†

Göran Lindblom,* Lennart B.-Å. Johansson, and Gösta Arvidson

ABSTRACT: Lateral diffusion coefficients of lipids in a bilayer can be measured directly in a macroscopically aligned sample by use of a pulsed NMR method with pulsed magnetic field gradients [Lindblom, G., & Wennerström, H. (1977) *Biophys. Chem. 6*, 167]. This technique has been utilized to investigate the influence of cholesterol on the lipid diffusion of egg yolk lecithin, palmitoyloleoyllecithin, and dioleoyllecithin. It is found that cholesterol has a very small effect on the phospholipid diffusion. On the other hand, cholesterol has a great influence on the molecular ordering in the bilayer and on the lipid phase structure. It is therefore suggested that cholesterol exerts its dominant effect on the lipid membrane stability.

The effects of cholesterol on the physicochemical properties of lipid bilayers have been studied for many years by various physical methods, including, in particular, different forms of spectroscopy. Investigations of this kind should increase our understanding of the role that membrane-bound cholesterol might play in such important biological phenomena as endo- and exocytosis, membrane fusion, permeability regulation, etc. A possible function for cholesterol in these processes is indeed indicated by many studies. Exactly how cholesterol influences the properties of membranes at a molecular level is, however, still a matter under debate. The purpose of the present paper is to show the fundamental importance of making a distinction between static and dynamic parameters when discussing the interactions of cholesterol with phospholipid molecules in bilayers. We also want to draw attention to a hitherto somewhat neglected aspect on cholesterol function in membranes, viz., its effects on the stability of the lamellar phase structure itself and the relevance of such effects for the possible occurrence of phase transitions in biological membranes.

Material and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOL) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POL) were synthesized according to the methods described by Gupta et al. (1977). The egg yolk lecithin (EYL) was prepared from freshly extracted egg yolk lipids by column chromatography on alumina. The lipids were then purified by silic acid column chromatography until the purity was ≥99% as judged by thin-layer chromatography. The macroscopic alignment of the lamellar samples was performed as described in previous papers (deVries & Berendsen, 1969; Lindblom, 1972). The diffusion coefficients were measured at about 61 MHz on a Bruker 322s spin–echo NMR spectrometer by using the pulsed magnetic field gradient technique developed by Stejskal & Tanner (1965). The spectrometer was supplemented with a home-built pulsed magnetic field gradient unit, permitting digital settings for all parameters used, i.e., magnetic field gradient width, δ, the distance between gradient pulses, Δ, and the amplitude of the gradients, g. The diffusion experiment was done as follows: a spin–echo was produced by the common two-pulse sequence $\pi/2-\tau-\pi$ and the magnetic field gradient was also applied as two pulses, one before and one after the π-rf pulse. The advantage of this method is that it is possible to use much larger magnetic field gradients than can be used in the static gradient technique and thus smaller diffusion coefficients can be measured. The translational diffusion of the molecules will attenuate the spin–echo amplitude, E, at 2τ accordingly:

$$\ln (E_g/E_0) = -(\gamma\delta g)^2 D[\Delta - (\delta/3)]$$

where E_g/E_0 is the echo attenuation. In a typical experiment, Δ and δ were kept constant at about 30 and 5 ms, respectively, and g was varied between 0 and 3.5 T m^{-1}. The echo amplitude was measured repeatedly several times on an oscilloscope. The diffusion coefficient, D, was calculated from a least-squares determination of the slope in a plot of ln E_g vs.

† From the Division of Physical Chemistry 2, Chemical Centre, University of Lund, S-220 07 Lund, Sweden (G.L. and L.J.), and the Department of Physiological Chemistry, University of Uppsala, Biomedical Centre, Uppsala, Sweden (G.A.). *Received September 24, 1980.* This work was supported by the Swedish Natural Science Research Council.

0006-2960/81/0420-2204$01.25/0