Effect of cholesterol and lanosterol on the structure and dynamics of the cell membrane of *Mycoplasma capricolum*

Deuterium nuclear magnetic resonance study

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ABSTRACT Deuterium nuclear magnetic resonance (NMR) techniques were employed to study the effect of sterols on the composition and dynamics of the membrane lipids of *Mycoplasma capricolum,* a natural fatty acid auxotroph that requires sterols for growth. The membrane lipids of cells grown in modified Edwards medium supplemented with cholesterol, oleic acid (OA), and palmitic acid (PA) were composed primarily of phosphatidylglycerol (PG) (60%) and cardiolipin (CL) (35%). The incorporation of cholesterol and the cellular OA/PA ratio increased nonlinearly with increases in exogenous cholesterol level, whereas the levels of phospholipid increased only slightly. At the growth temperature, 37°C, the residual deuterium quadrupole splittings were found to be 43–46 kHz for cells grown with (7,7,8,8-$^2H_4$) PA and 1.25 μg/ml (30 mol%) to 10 μg/ml (50 mol%) cholesterol, respectively, similar to that found in the cholesterol/lecithin binary dispersions of similar cholesterol contents. Deuterium T_{2e} of these samples were found to be 170 ± 10 μs and were independent of cellular cholesterol content. In comparison, T_{2e} of the corresponding lipid extracts were longer (320–420 μs) and dependent on cholesterol content. Thus, lipid-protein interactions in the cell membrane is the dominant mechanism responsible for the reduced T_{2e}. At lower temperatures, spectra indicative of the coexistence of gel and liquid-crystalline states were observed for cells having low cholesterol levels. For both cell membrane and membrane lipid extract containing 50 mol% cholesterol, T_{2e} was found to be constant at the temperature range from 15 to 40°C. On the other hand, T_{2e} of cell membrane containing 30 mol% cholesterol decreased linearly at 3.2 μs/°C. T_{2e} of the corresponding lipid extract showed much stronger temperature variation. Cells containing 39 mol% lanosterol were found to have a quadrupole splitting of 39 kHz, broader than that of the cholesterol-free lecithin dispersion (< 30 kHz) but less than that of cell membrane containing 30 mol% cholesterol (43 kHz). T_{2e} of the lanosterol sample was found to be 130 ± 10 μs which decreased linearly at a slope similar to that observed for the low cholesterol sample. Therefore, although lanosterol appeared to be capable of modulating cell membrane physical properties it is less effective than cholesterol. When growth rates were correlated with NMR parameters, we found that the membranes of faster growing cells were also more ordered. In contrast, the T_{2e} of the cells of *M. capricolum* seemed to be maintained at a relatively constant value around 170 μs.

INTRODUCTION

Cholesterol is an ubiquitous component in animal cells and is essential for some prokaryote such as *Mycoplasma* species. The biological importance of cholesterol is well documented (1–3). Early investigations on mixtures of phospholipids and cholesterol revealed that cholesterol has the ability to exert a "plasticizer" effect on membrane bilayers resulting in a bilayer having lipids in a physical state intermediate between gel and liquid-crystalline states (4, 5). On the cellular level, *de novo* cholesterol synthesis is required for cell proliferation and has been correlated with cell transformation (2). In *Mycoplasma capricolum* and in yeast, cholesterol has been reported to exert a synergistic effect in controlling protein and lipid synthesis, possibly through specific lipid-protein interactions (6–8). Thus, cholesterol is believed to serve dual biological roles, maintaining membrane structural integrity through its "plasticizer" effect and controlling protein function through specific cholesterol/lipid/protein interactions.

On the molecular level, the details of the lipid/cholesterol interaction have been examined by a variety of techniques including differential scanning calorimetry (DSC) (4, 5, 9, 10), electron spin resonance (ESR) (11, 12), fluorescence polarization (13, 14), x-ray diffraction (5, 15–17), electron diffraction (18), neutron scattering (19), freeze fracture electron microscopy (16, 20), and nuclear magnetic resonance (NMR) (21–27). These studies demonstrated the condensing effect of cholesterol in both model and biological membrane. The broadening of the transition temperature and the decrease in transition entropy were observed by DSC measurements. The addition of cholesterol induces rotational diffusion and reduces the cross-sectional area. In addition, a detailed phase diagram consisting of several domains was constructed (27).

Even though biological membranes are much more heterogeneous deuterium NMR studies on *Achole-*

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plasma laidlawii B have shown that there is a remarkable similarity between the ^{2}H-NMR results for model and biological membranes. In particular, the order parameter profiles observed for *A. laidlawii* membrane containing selectively deuterated palmitic and oleic acid closely resemble those observed in phospholipid dispersions (26, 28). Similar results were observed in the *E. coli* system (29–33). In the absence of cholesterol the membrane lipids of *A. laidlawii* undergo a relatively well defined gel to liquid-crystalline phase transition similar to those observed in model system. The addition of cholesterol removed the phase transition. Whereas the order parameter profile in the liquid-crystalline phase region maintains its general shape, the cholesterol-containing membrane have a higher average order. Although *A. laidlawii* can incorporate cholesterol into its membrane, it does not require cholesterol for growth. Thus, the biological significance of the effect of cholesterol on *A. laidlawii* is hard to assess.

Mycoplasma species are unusual in that they have only a cytoplasmic membrane with no cell wall or other lipid containing structures, the size of their genome is the smallest known for self-replicating free-living prokaryote, and they have an absolute growth requirement for cholesterol (31). *Mycoplasma* species are incapable of synthesizing fatty acids or cholesterol, making them dependent on the supply of these lipid molecules in the growth medium. As a result the cell membrane lipid composition is relatively simple and labeled fatty acids, lipids, and sterols can be incorporated into cell membrane for spectroscopic studies. The ability to introduce controlled alterations in the fatty acid composition and sterol content has been utilized extensively over the past decade to study relationships between molecular organization and the physical state of membrane lipid and various transport mechanisms (34–38). This paper reports biochemical and deuterium NMR studies of the effect of cholesterol and lanosterol on the structure and dynamics of the cytoplasma membrane of *M. capricolum* in whole cells and in total lipid extracts.

MATERIAL AND METHODS

Mycoplasma capricolum ATCC 27342 was grown at 37°C in a modified Edwards medium. The antibacterial agents penicillin G and thallium acetate were not used. Serum or serum fractions were not used as lipid sources, instead lipid components were added as ethanol solutions to Edwards medium containing 0.5% essentially fatty acid- and globulin-free bovine albumin (Sigma Chemical Co., St. Louis, MO) (39, 40). The amount of ethanol did not exceed 0.5% of the medium and had no apparent effect on the growth of cultures. Lipid-depleted, modified Edwards medium was prepared by extracting medium components with choloroform-methanol (41). The amounts of contaminating fatty acids and other lipids in all media and lipid supplements were determined by lipid extraction, silicic acid paper chromatography, and gas-liquid chromatography of methyl ester derivatives (described in the next section).

Lipid extraction and chromatography

Cells were harvested by centrifugation at 8,000 *g*, washed in 0.25 M NaCl and extracted by the method of Bligh and Dyer (42). Total lipid extracts were chromatographed in two dimensions on silica gel loaded paper (Model SG81; Whatman, Inc., Clifton, NJ) as described previously (43) to separate polar lipids. Fatty acid methyl ester derivatives of acylated fatty acids were prepared by base-catalyzed transesterification with sodium methoxide (44). Fatty acid methyl ester derivatives of all lipids including free fatty acids were prepared by dissolving lipid extracts in 0.2 ml chloroform and 0.2 ml boron trifluoride-methanol (Supelco, Inc., Bellefonte, PA), boiling for three minutes, addition of 1 ml water to stop the reaction, and addition of 0.2 ml chloroform. The chloroform layer containing the methyl esters was dried and dissolved in hexane. Fatty acid methyl esters were separated by gas-liquid chromatography using a fused silica 50 m carbowax 20 M capillary column (Hewlett-Packard Co., Palo Alto, CA) in a 5890 Hewlett-Packard gas chromatograph and 3390 integrator for quantitation.

Labeling of cellular-lipids

Cells were grown in base medium with 0.5% bovine albumin to which was added oleic acid, 10 μg/ml; palmitic acid, 30 μg/ml; and various amounts of cholesterol or lanosterol. Inocula for experiments were started from frozen cultures and transferred at least once in the above medium containing cholesterol at 10 μg/ml. Two percent inocula were normally employed. Cultures were checked frequently for typical cellular morphology by phase and bright field microscopy and colony morphology on solid media. Double labeling of cellular lipids was accomplished by adding [1-^{14}C]-palmitic acid (0.05 μCi/ml) (specific activity, 2–10 mCi/nmol) and ^{32}P-orthophosphate (0.5 μ Ci/ml) (initial specific activity, 233 Ci/mg) (New England Nuclear, Boston, MA) to the growth medium before inoculation. After chromatography of the lipid extracts, the individual lipids were cut from the chromatographs and counted as previously described (45). Deuterium labeling of lipids were accomplished by supplementing the growth medium with 30 μg/ml (7,7,8,8-d_4, or perdeuterated PA. Based on our fatty acid analyses and the results of Rigaud and Leblanc (46) we estimated that under our growth conditions *M. capricolum* cell membrane PG (and CL) would have the following fatty acyl chain compositions; OPPG (54%), POPG (13%), and DPPG (33%). Therefore, OA will be predominately on *sn*-1 chain, in contrast to those isolated from *E. coli* (47).

Synthesis of (7,7,8,8-d_4) palmitic acid

(7,7,8,8-d_4)PA was synthesized by the method of Ames with some modifications (48). An w-bromocarboxylic acid was alkylated with lithium acetylide. The deuterium was introduced by a deutreo-diimide reduction of the acetylenic moiety. The diimide was generated *in situ* by means of a $^2H^+$ catalysis of potassium azodicarboxylate. Typically, we found an average of 3.6 deuterons per palmitic acid by mass spectrometry.

Deuterium NMR spectroscopy

Deuterium quadrupole NMR spectra were obtained at 46 MHz on a home-built NMR spectrometer with model 300/89 magnet (Oxford Instruments Limited, Oxford, England). The spectrometer consists

of a model 1280 computer (Nicolet Instrument Corp., Madison, WI) with a 293B pulse programmer, two PTS-200 frequency synthesizers (Programmed Test Source, Littleton, MA), an ENI-550L radio frequency (RF) power amplifier (ENI Inc., Rochester, NY), Henry Radio 2002A power amplifier (Henry Radio, Los Angeles, CA), a home-built RF receiver with a Nicolet transient recorder (Nicolet Instrument Corp.) (Receiver overall bandwidth ± 1.0 MHz), and a home-built low-power superheterodyne transmitter. An analogue four-phase box was constructed according to Jeffrey (49). Deuterium NMR spectra were obtained with a quadrupole echo pulse sequence of an echo delay time of 35 μs and recycle time of 0.2 s (50). The 90° pulse is typically 2 μs for the small coil (5 mm × 12 mm) and 3 μs for the large coil (8 mm × 25 mm). The small coil was used for samples of total cellular lipid extracts or samples labeled with perdeuterated PA. Whole-cell samples were prepared by packing ~1.2 g of pelleted cells in an 8 mm thin-walled glass tube without aeration. Each sample contained ~20 mg (7,7,8,8-d_4) (or 2 mg of perdeuterated) PA. Each spectrum was obtained in ~30–90 min for whole-cell samples containing (7,7,8,8,-d_4) PA and 5–20 min for lipid extracts and samples containing perdeuterated PA. Quadrature phase detection was employed. Signals were digitized at 2 MHz beginning a few microseconds before echo top. The data were then left-shifted to the top of the echo before Fourier transformation. The temperature was controlled to within 0.5°C, and at least 15 min was allowed for the temperature to stabilize.

Using samples labeled with perdeuterated PA, spectra for determining transverse relaxation time, T_{2e}, were obtained by varying the delay time between two 90° pulses in the quadrupole echo experiment. Peak-heights of the outermost perpendicular edges were measured and were fitted to a single exponential decay curve. Some of these results were compared with samples labeled with (7,7,8,8-$^2H_4$)PA and found to be similar.

Chemical tests and materials

Cholesterol (51), lipid phosphorus (52), and protein (53) were determined by colorimetric tests. Radioisotope labeled compounds were obtained from New England Nuclear, fatty acid methyl ester standards and other lipids from Supelco, Inc., and Sigma Chemical Co. Lanosterol was obtained from Sigma Chemical Co. and used without further purification.

RESULTS

Effect of cholesterol on the growth of *M. capricolum*

The effect of exogenous levels of cholesterol and lanosterol on cell growth, as measured by generation times, is shown in Fig. 1. Generation times were determined spectrophotometrically from the increase in OD at 640 nm. Monotonic increases in cell growth rate (decreases in generation time) were observed when the exogenous cholesterol concentration was increased from 1.25 to 20 μg/ml. Replacing cholesterol with lanosterol markedly increased generation times (reduced the growth rate) at all concentrations. The generation time at the highest lanosterol level was longer than that at the lowest cholesterol level.

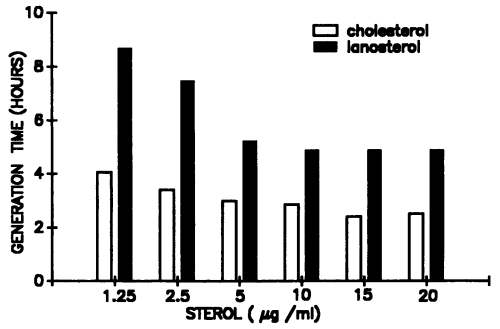


FIGURE 1 Effect of sterols on cell generation time (hour). Cells were grown at 37°C in modified Edwards medium supplemented with 30 μg/ml palmitic acid, 10 μg/ml oleic acid, and various amounts of cholesterol or lanosterol.

Cholesterol and phospholipid composition of cells

Fig. 2 *a* shows the variation of membrane cholesterol and phospholipid levels, in μmol/g cell protein, with the exogenous cholesterol concentration. Cholesterol level increased from 17 μmol/g protein at 1.25 μg/l cholesterol concentration to 78 μmol/g protein at 20 μg/l cholesterol concentration. In contrast the cellular phospholipid level increased only slightly from ~40 to 52 μmol/g. As a result the cellular CHOL/PL ratio increased from 30 to 60 mol% (Fig. 2 *b*).

Cell membrane lipid composition

Changes of polar and neutral lipids as a result of changes in exogenous cholesterol levels were followed with ^{14}C-labeled palmitic acid and ^{32}P-phosphorus incorporation (Fig. 3). The polar lipids represented 95% of cellular lipids and consisted primarily of phosphatidylglycerol (PG) and cardiolipin (CL), similar to the phospholipids synthesized *de novo* in most mycoplasmas (34). Other lipids detected included glycolipid (0.2–1.5%), diglyceride (1.8–2.2%) and free fatty acids (0.5–1.2%). With increasing amounts of cholesterol the relative amount of PG increased slightly with a proportional decrease in CL level.

Cell fatty acid composition

The fatty acid ratios of cells grown in different cholesterol concentrations is shown in Fig. 4. The initial levels of oleic acid and palmitic acid in the growth medium were kept constant at 10 and 30 μg/ml), respectively, but the relative amounts of these two fatty acids varied considerably in the cells. The fatty acid methyl ester

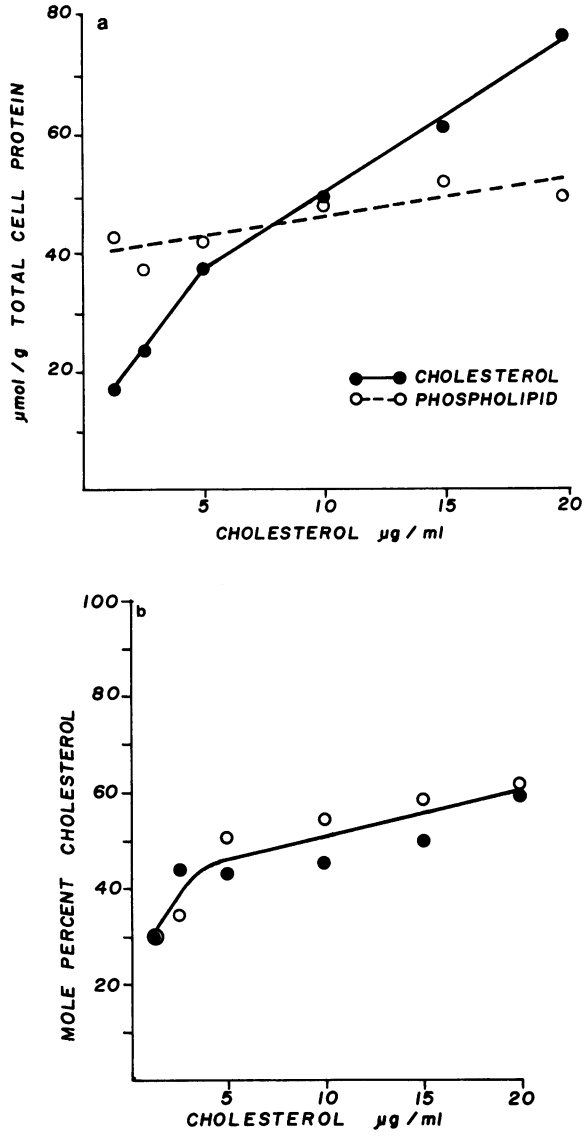


FIGURE 2 Effect of exogenous cholesterol on *M. capricolum* cell membrane cholesterol (*solid circle*) and phospholipid (*open circle*) content as measured in micromole/gram total cell protein. (*b*) Effect of exogenous cholesterol on cell membrane cholesterol/phospholipid molar percent ratio. Solid and open circles represent two independent experiments. Cultures of 150 ml were grown with different amounts of cholesterol. Total cell protein was determined on washed cell samples from each culture. The remaining portions were extracted for total lipid, and the amount of cholesterol and total lipid phosphorus in the lipid extracts determined.

derivatives were derived by transesterification, therefore, representing only the acyl groups esterified to polar and neutral lipids, and not due to the possible nonspecific adsorption of fatty acids to cells. After an initial increase, palmitic acid decreased with increasing cholesterol, whereas the relative amount of oleic acid continued to increase. The OA/PA ratio increased from 0.27 to 0.52 as the exogenous cholesterol concentration increased from 1.25 to 20 μg/ml in a nonlinear manner similar to the nonlinear changes in mole percent cholesterol. Similar behavior was also observed in cells grown in lanosterol supplements.

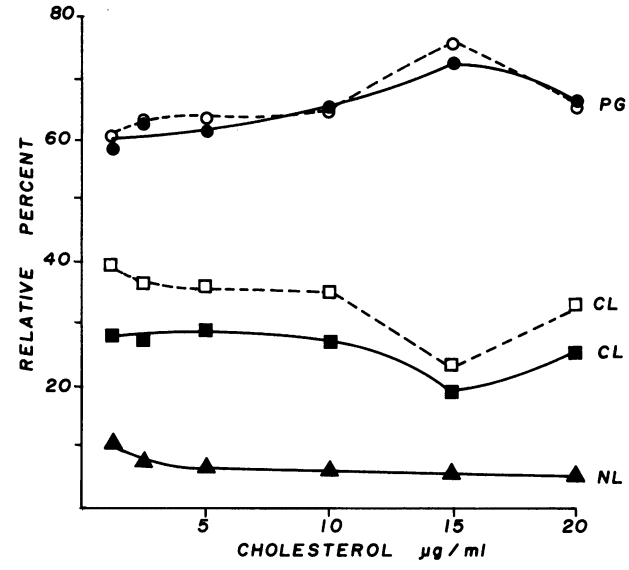


FIGURE 3 Relative percent phospholipid and neutral lipid. Culture were grown to late log phase (OD_{640nm} = 0.1–0.2) in media containing (1-^{14}C)palmitic acid (*solid symbols*) and ^{32}P-phosphoric acid (*open symbols*) and different concentrations of cholesterol. PG, phosphatidylglycerol (*circle*); CL, cardiolipin (*square*); NL, neutral lipid (*triangle*).

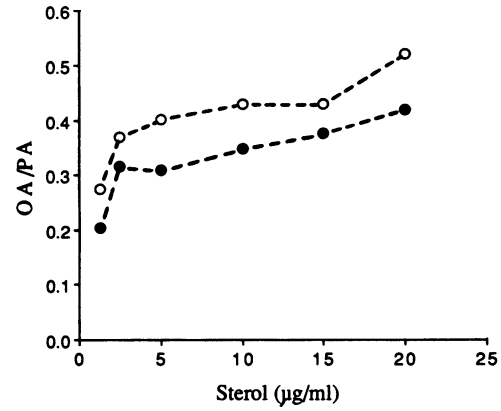


FIGURE 4 Variation of cellular molar oleic acid/palmitic acid ratios with exogenous cholesterol (*open circles*) or lanosterol (*solid circles*) concentrations. Cultures (50 ml) were grown to midlog phase and fatty acid ratios of total lipid extracts determined by gas-chromatography.

Deuterium NMR

The temperature variation of the ^2H NMR spectra of *M. capricolum* cells (Fig. 5, *a–c*), or cell lipid extracts (Fig. 5 *d*), grown in Edwards medium supplemented with 10 μg/ml OA, 30 μg/ml (7,7,8,8-d_4)PA, and various amounts of sterols are shown in Fig. 5. At the growth temperature (37°C) a double-horn shape spectrum, the Pake pattern, was observed in all samples. The two horns were normally referred to as the perpendicular edges because the absolute value of the frequencies of the two horns correspond to the perpendicular component of the symmetric deuterium electric field gradient tensor of the methylene deuterons. The residual splittings, $\Delta\nu_Q$, i.e., the separation between the two perpendicular edges, were found to be 43 ± 1 kHz and 46 ± 1 kHz for the low (1.25 μg/ml) and high (10 μg/ml) cholesterol samples and 39 ± 1 kHz for the lanosterol (10 μg/ml) sample, respectively. For rigid methylene deuterons, such as those in gel-state membrane, the expected splitting is 120 kHz (29). The observation of a single Pake pattern of narrow splitting suggested that the membrane lipids were in liquid-crystalline state at the growth temperature (10). At lower temperatures the residual splitting of the membranes of cells grown in 10 μg/ml cholesterol (membrane contains 50 mol% CHOL) (Fig. 5 *a*) broadened only slightly, suggested that the membrane remained in liquid-crystalline state even at 0°C. In contrast, the spectra at 20°C of cells grown in 1.25 μg/ml cholesterol (membrane contains 30 mol% CHOL) Fig. 5 *b*) or in 10 μg/ml lanosterol supplement (membrane contain 39 mol% LAN) (Fig. 5 *c*) were composites of gel (broad) and liquid-crystalline (narrow) components. The transition from totally narrow to totally broad component occurred over the temperature range 5–20°C. The temperature variation of the deuterium NMR spectra of the lanosterol sample was very similar to that of the low cholesterol sample.

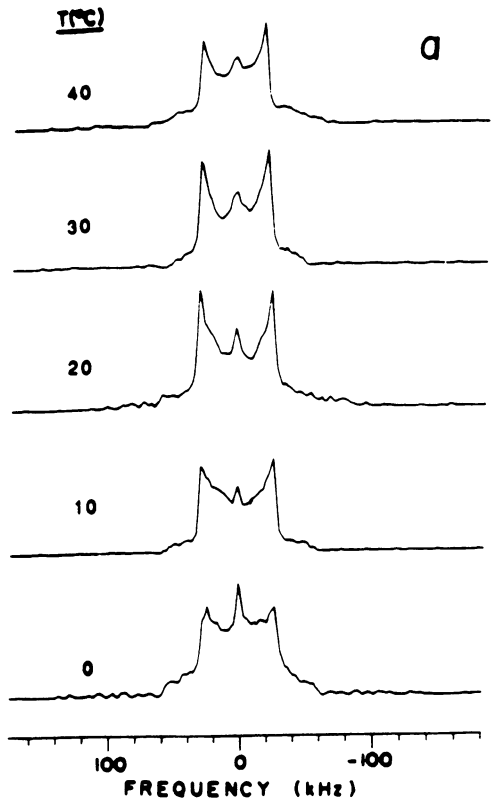
The ^2H NMR spectra of cell lipid extracts of cells grown in 10 μg/ml cholesterol are shown in Fig. 5 *d*. Again a single component was observed. In agreement with previous observations (29–33), the spectra of the lipid extracts were much sharper than that of the corresponding whole-cell spectra. Fig. 6 shows the temperature variations of the residual deuterium quadrupole splittings, $\Delta\nu_Q$, of the spectra shown in Fig. 5. All three samples showed a similar temperature variation of 0.4 kHz/°C.

The effects of echo delay time on deuterium NMR spectra are shown in Fig. 7 for samples containing cells grown in either perdeuterated PA (Fig. 7 *a*), or (7,7,8,8-$^2H_4$PA (Fig. 7 *b*). The intensities of the two perpendicular edges, i.e., the most prominent edges at ~ ±20 kHz, were employed to determine the transverse relaxation time, T_{2e}, at various temperatures and sterol compositions (Fig. 8). The prominent features of Fig. 8 are (*a*) at 40°C T_{2e} = 170 ± 10 μs was obtained for all cells grown with cholesterol supplement independent of the membrane cholesterol content. For cells grown in Lanosterol the T_{2e} = 130 ± 10 μs. (*b*) Upon lowering temperature, the T_{2e} for the high cholesterol cells increased to a maximum of 200 μs at 20°C and then decreased at lower temperatures. In contrast, the T_{2e}'s for the low cholesterol or lanosterol samples decreased linearly with a slope of 3.2 μs/°C. Therefore, at temperatures <40°C, the T_{2e} of sample of cells with highest cholesterol content was the longest whereas it was the shortest for cells grown with lanosterol. (*c*) The T_{2e}'s of lipid extracts were much longer and exhibited strong dependence on both sterol content and temperature variation. In contrast to results with whole cells, the T_{2e} of the lipid extract with the highest cholesterol content was the shortest and remained relatively constant at 320 ± 20 μs from 40 to 15°C. However, the T_{2e}'s of lipid extract extracts from cells with lower cholesterol level were highly temperature dependent. For example, the T_{2e} for the 30 mol% CHOL (1.25 μg/ml) sample increased from 280 ± 20 μs at 15°C to a maximum of 488 ± 20 μs at 30°C and decreased to 420 ± 20 μs at 40°C. Thus, a high level of cholesterol appeared to have the ability of maintaining a constant T_{2e} in both whole cells and the corresponding lipid extract. It is also interesting to note that the effect of cholesterol on the T_{2e} was much less pronounced in samples of whole cells than it was in the lipid extracts.

DISCUSSION

Effect of cholesterol on cell membrane composition

Mycoplasmas are unable to synthesize or acylate sterols or synthesize or elongate fatty acids. They are considered to be natural fatty acid auxotrophies (54–56). However, as indicated in this study, the cellular lipid composition of *M. capricolum* did not reflect a simple passive incorporation of exogenous lipid. The concentration of membrane cholesterol increased in a nonlinear manner from 17 μmol/g total cell protein at 1.25 μg/ml cholesterol to 78 μmol/g protein at 20 μg/ml cholesterol (Fig. 2). The amount of phospholipid, which is synthesized *de novo* from exogenously supplied fatty acids, increased from 40 to 52 μmol/g protein over the same ranges of cholesterol supplements. Consequently, cellular molar cholesterol concentration increased from 30 to 60%. Increases in the cholesterol level also enhanced the incorporation of oleate into the cell membrane.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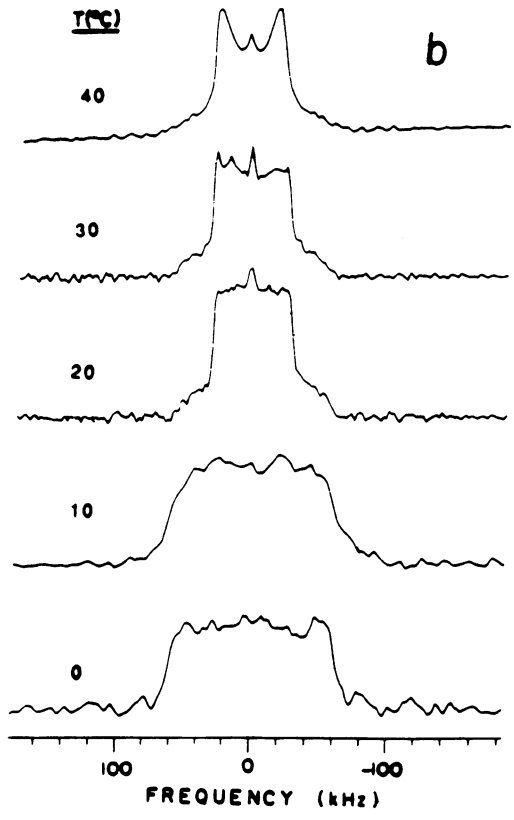
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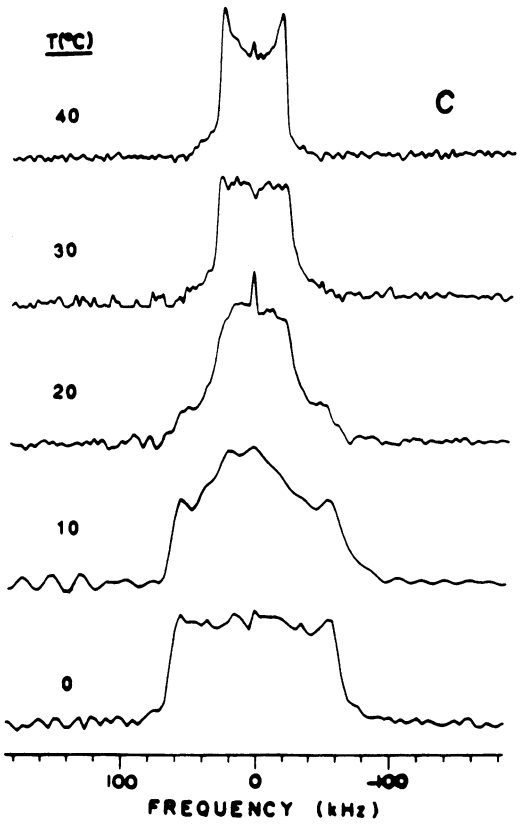
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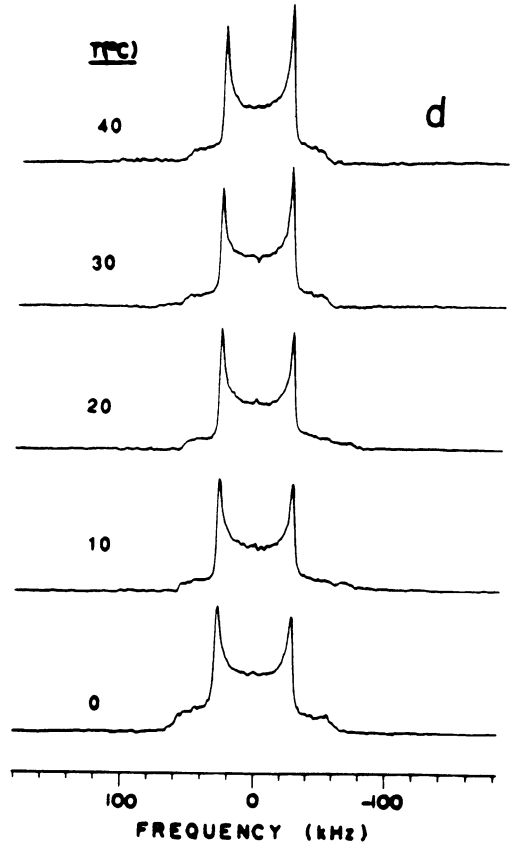
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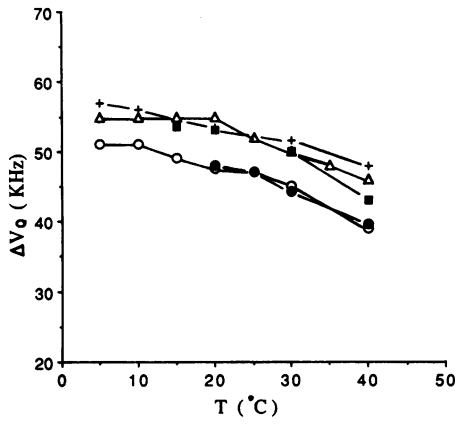

FIGURE 6 Variation with temperature of the deuterium quadrupole splittings of spectra shown in Fig. 5. Cell sterol conditions: (*solid square*) 1.25 μg/ml CHOL, whole cell. (*open triangle*) 10 μg/ml CHOL, whole cell. (+) 10 μg/ml CHOL, lipid extracts. (*solid circle*) 10 μg/ml LAN, whole cell. (*open circle*) 10 μg/ml LAN, lipid extracts.

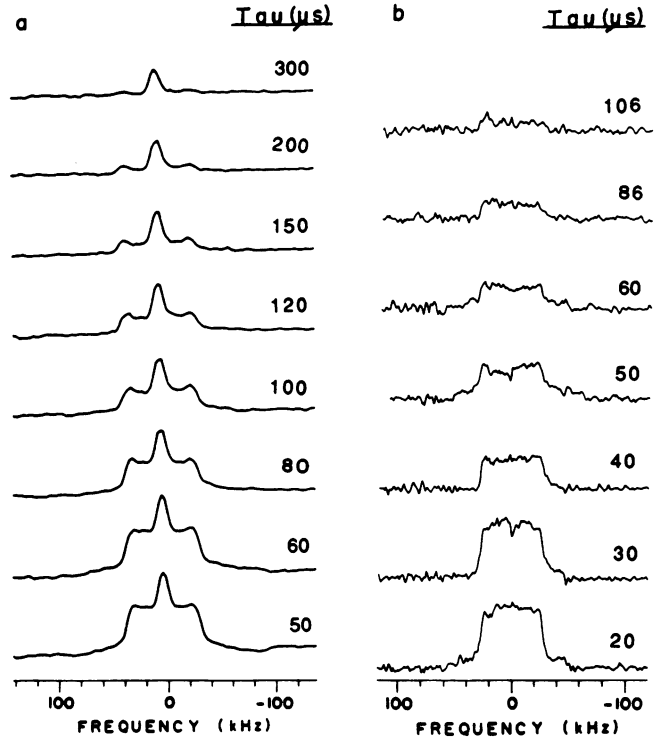

FIGURE 7 Variation of deuterium NMR spectra of *M. capricolum* cells with quadrupole echo delay time, tau. Spectra were obtained at 30°C with quadrupole echo technique as described in the text. Cell growth conditions: (*a*) 2.5 μg/ml CHOL supplemented with 30 μg/ml perdeuterated PA. (*b*) 10 μg/ml LAN supplemented with 30 μg/ml (7,7,8,8-$^2H_4$) PA. Other conditions are the same as described in the text.

Thus, in log phase the OA/PA ratios increased from 0.27 at 1.25 μg/ml cholesterol to 0.52 at 20 μg/ml cholesterol. These alterations in lipid composition were concomitant with increases in cell growth rate at higher cholesterol concentrations (Fig. 1).

The effect of cholesterol on membrane lipid order

Deuterium NMR has been shown to be a particularly informative, nonperturbing method for studying molecular structure and dynamics (29–33). The fact that quadrupolar interactions of deuterons are much greater than dipolar and chemical shift anisotropic interactions renders the analysis of deuterium NMR parameters particularly simple. The presence of molecular motions averages the deuterium quadrupole powder spectrum to a lineshape which is characteristic of the rate and the mechanism of molecular motion. Thus, the structure and dynamic of a given carbon segment can be deduced from deuterium NMR parameters of deuterons attached directly to the specific carbon atom as explained below (29–33). For lipid molecules exerting fast rotational diffusional motion along its molecular axis in the liquid-crystalline state in lipid dispersion, Pake patterns were observed. The residual quadrupole splitting of the pattern, $\Delta\nu_Q$, is related to the C-D bond orientational order parameter S_{CD} by the following relation,

$$\Delta\nu_Q = 3/4(e^2qQ/h)S_{CD},$$

with the segmental order parameter, S_{CD}, given by

$$S_{CD} = \langle[3\cos^2\theta(t) - 1]/2\rangle,$$

where e^2_{qQ}/h = 170 kHz is the deuterium quadrupole coupling constant of the methylene deuterons and $\theta(t)$ is the angle between the C-D bond and the bilayer normal at time t. The angular brackets represent an ensemble average. In the liquid-crystalline state the C-D bond is perpendicular to the bilayer normal and the expected S_{CD} is 0.5, corresponding to $\Delta\nu_Q$ = 60 kHz which is much broader than 30 kHz measured in pure phospholipid dispersions. The reduced S_{CD} is attributed to the pres-

FIGURE 5 Temperature variations of deuterium NMR spectra of *M. capricolum* cells (*a–c*) and cell membrane lipid extracts (*d*). Cells were grown in 10 μg/ml cholesterol (*a*), 1.25 μg/ml cholesterol (*b*), or 10 μg/ml lanosterol (*c*). Spectra of *d* were obtained with the total lipid extract from sample *a* (10 μg/ml cholesterol). Spectra were obtained as described in the text.

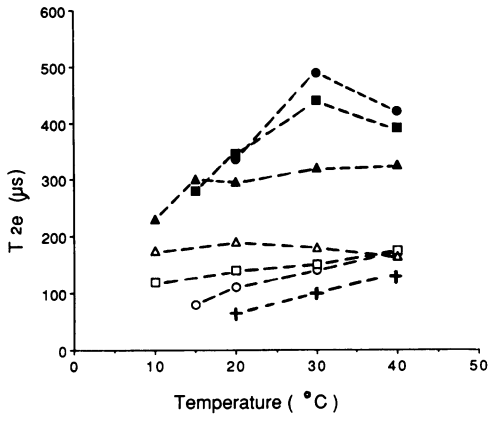

FIGURE 8 Temperature variations of deuterium transverse relaxation times, T_{2e}, of cells (*open symbols*) and cell lipid extracts (*solid symbols*). Cells were grown in modified Edwards medium supplemented with 10 μg/ml OA, 30 mg/ml (7,7,8,8-D_4) PA or perdeuterated PA, and various amounts of CHOL or LAN. Sterol conditions: (*triangle*) 10 μg/ml CHOL, (*square*) 2.5 μg/ml CHOL, (*circle*) 1.25 μg/ml CHOL, (+) 10 μg/ml LAN. T_{2e} were determined as described in the text.

ence of *trans-gauche* isomerization which gives rise to the fluid nature of the acyl chain characteristics of the liquid-crystalline phase (29, 57). Thus, S_{CD} can be taken as a measure of the lipid order and allows the determination of the average orientation of the C-D bond with respect to the lipid bilayer normal. The broader $\Delta\nu_Q$, hence, larger S_{CD}, observed in the presence of cholesterol suggests that cholesterol hinders the *trans-gauche* isomerization and results in a more ordered lipid environment.

The $\Delta\nu_Q$ of lipid acyl chain deuterons is sensitive to the environment and has been employed to determine bilayer thickness, lipid phases, and other thermodynamic properties (29, 58). In this study at 40°C we observed deuterium quadrupole splitting of 43 ± 1 kHz and 46 ± 1 kHz, corresponding to S_{CD} = 0.34 and 0.37, for cells grown in 1.25 and 10 μg/ml cholesterol supplement (membrane cholesterol molar ratios of 0.3 and 0.5, respectively). Upon lowering the temperature a gradual increase in $\Delta\nu_Q$ of 0.5 kHz/°C to 56 kHz at 20°C was observed, corresponding to S_{CD} of 0.44. The splitting remained constant upon further lowering the temperature. In comparison, in model membrane studies the quadrupole splitting of the C-6 deuteron in a DPPC/cholesterol dispersion varies substantially with both temperature and cholesterol near the transition temperature (27; Huang, T.-h., and R. G. Griffin, to be published). For a 50 mol% cholesterol/DPPC mixture, quadrupole splitting increased linearly from 42 to 52 kHz when the temperature was decreased from 60 to 40°C. Therefore, the splittings observed for whole-cell samples at 40°C are qualitatively similar to that of model membranes, suggesting that cell membrane lipids exist in a state similar to that of a high cholesterol/DPPC complex, the "β" phase (21). This similarity further suggests that membrane proteins have little effect on lipid chain order. In contrast, over the same temperature range a sigmoidal increase of splitting from 32 to 49 kHz was observed for 30 mol% cholesterol/DPPC dispersion. Sigmoidal behavior was not observed in our whole cell and total lipid extract samples. This was most likely due to the intrinsic behavior of our cell membrane system which was more heterogeneous, containing PG and CL of mixed OA and PA chains with a probable T_c range from −5 to 40°C.

Deuterated PA was incorporated into both PG and CL, primarily in the *sn*-2 chain. Phase separation could have occurred due to the size difference between PG and CL, the presence of cholesterol (59), and/or due to lipid-protein-cholesterol interactions which are stronger for PG and CL (31–33). Thus, one would expect to observe the presence of multiple components. However, multiple components were not observed at the growth temperature, presumably due to the fast exchange of lipid molecules among various environments, further illustrating the dynamic nature of biological membranes. The absence of broad components in all samples at the growth temperature is consistent with the general notion that protein-immobilized "boundary lipids" are invisible to deuterium NMR. This is somewhat surprising because PG and CL are thought to be involved in strong specific interactions with membrane proteins. At lower temperatures, spectra indicative of the coexistence of gel and liquid-crystalline states were obtained. The onset of this coexistence occurs at 20°C for the 1.25 μg/ml sample and at 0°C for the 10 μg/ml sample with the transformation taking place over a temperature range of ±10°C. The complexity of these spectra precludes a detailed characterization of the nature of these components at this stage in our investigation.

The effect of cholesterol on membrane lipid dynamics

In general, in the liquid-crystalline phase the presence of proteins does not drastically change the spin lattice relaxation time, T_1. However the value of T_{2e} is always decreased markedly by the presence of even small concentrations of protein. Because T_1 is affected by motional processes near or above the Larmor frequency whereas, T_{2e} is also affected by spectral density at zero frequency, the differential effect of proteins on T_1 and T_{2e} is taken to indicate that the protein-lipid interaction introduces a new slow motion of the phospholipid molecules which is capable of modulating the quadrupo-

lar interactions sufficiently to influence T_{2e}, but which is too slow to affect T_1(32, 60). The nature of the slow motions of acyl chains induced by the lipid-protein interaction has not been established. Some of the candidates are: exchange of lipids between boundary and bulk sites (60); rotation of proteins or motion of their secondary structural elements such as α-helix relative to each other (61); reorientational motion of lipids while bound to the protein surface; changes in the rate of lateral diffusion parallel to the curved surfaces (62, 63); and retardation in fatty acyl chain fluctuation, rotation and *trans-gauche* isomerization (64). If we adapt the two-site exchange model of Paddy et al. (46) using the order parameter determined from the residual splittings and assuming that the lipid molecules are equally likely to be associated with protein as well as in the lipid environment then the correlation time can be estimated from T_{2e} (Eq. 16 of reference 60),

$$1/T_{2e} = (4\pi^2 \Delta\upsilon_Q^{2/5}) x_p \tau_c,$$

where $x_p = 0.5$ is the fractional time that a lipid is in the protein environment. For $\Delta\upsilon_Q$ = 43 kHz and T_{2e} = 170 μs one finds the correlation time $\tau_c = 8 \times 10^{-7} S$. This value may reflect the time scale of motion on the protein surface and/or it may reflect the time scale of the exchange of lipid between the protein surface or the free lipid.

Meier et al. recently reported a detailed study on the interaction of myelin proteolipid apoprotein with DMPC (64). They found that T_1, T_{2e}, and spectral lineshape were strongly affected by the presence of this integral protein. By fitting these results to the numerical solution of the stochastic Liouville equation, using a model that includes the inter and intramolecular motions, they concluded that in the fluid phase protein had little influence on either the chain order of the population of gauche rotational isomers but strongly retarded chain dynamics. Thus, at both high protein/lipid molar ratios ($>4 \times 10^{-2}$), a T_{2e} of 170 μs was observed, compared to 1,200 μs in pure lipid. These results were interpreted in terms of the increase in the correlation times for chain fluctuations from 20 to 650 ns, for chain rotation from 10 to 180 ns, and for the *trans-gauche* isomerization from 0.15 to 1.75 ns for pure lipid and the protein complex, respectively. Because the protein/lipid molar ratio in *M. capricolum* cell membrane is likely to be higher than 4×10^{-2}, it was expected that protein alone would reduce T_{2e} to 170 μs. In fact, 170 ± 10 μs was the value obtained. Our data does not allows us to distinguish whether the reduced T_{2e} in cell membrane is due to the exchange process as proposed by Paddy et al. (60) or the retardation in local chain dynamics as described by Meier et al. (64). It is also likely that both processes contribute to the decreases in T_{2e}.

At 40°C the cellular cholesterol concentration appeared to have no effect on T_{2e}. However, when proteins were eliminated by examining total lipid extracts, the T_{2e} increased by more than twofold to 320 and 450 μs for the 30 and 50 mol% samples, respectively. Thus, the effect of different concentrations of cholesterol on T_{2e} is discernable without the presence of protein. This result suggests that membrane protein is the dominant factor causing the reduction in T_{2e}. The consequence of this finding is that the lipid/cholesterol domain size must be relatively small such that diffusion of lipid molecules to the protein surface is not limited by the presence of cholesterol. It should also be mentioned that T_{2e}'s of membrane lipid extracts are still much less than 1,200 μs which was observed by Meier et al. in pure DMPC. Therefore, cholesterol alone is capable of exerting significant effects on retarding lipid dynamics although it appears to be relatively ineffective in modulating the effect of proteins on the dynamics of cellular lipid.

The temperature variation of T_{2e}, as shown in Fig. 8, provides additional information concerning the effect of cholesterol and proteins on membrane dynamics. The presence of cholesterol suppressed the temperature variation of the T_{2e} in both whole cells and total lipid extracts, suggesting that cholesterol is capable of ordering lipid bilayer, as measured by $\Delta\upsilon_Q$, and restricting lipid dynamics, as measured by T_{2e}, to a state which is relatively insensitive to temperature variations. The lack of variation with temperature is in accord with the observation of a broad, even the disappearance of, phase transition in the presence of cholesterol. Comparing samples that contain the same amounts of cholesterol, the T_{2e} for whole cells are much less temperature dependent than lipid extracts, suggesting that the presence of proteins also stabilizes the membrane dynamics. The presence of T_{2e} maxima and the strong temperature variation of T_{2e} of low cholesterol total lipid extracts is quite distinct. Part of the variation was probably due to presence of phase transitions in the low cholesterol samples. Such phase transition most likely induces additional motion which is different from that which reduces T_{2e} at high temperature. More detailed calorimetric and relaxation studies are needed to understand this unusual behavior in T_{2e}.

The effect of lanosterol on cell membrane structure and dynamics

There are four lines of evidence that suggest that lanosterol is much less effective in modulating membrane order (as measured by $\Delta\upsilon_Q$) and dynamics (as measured by T_{2e}): (*a*) the residual quadrupole splitting

of lipids of cells containing 39 mol% lanosterol was 39 kHz (S_{CD} = 0.31). The corresponding value for cells containing 30 mol% cholesterol is 43 kHz (S_{CD} = 0.34). Therefore, cell membrane containing 39 mol% lanosterol is even less ordered than that containing 30 mol% cholesterol. (*b*) At the growth temperature, the T_{2e} of lipids of lanosterol-grown cells (130 μs) was shorter than that of the cholesterol samples (170 μs). In light of the finding by Meier et al. (64) that high concentration of protein alone reduces the T_{2e} to 170 μs the observation of T_{2e} of 130 μs for lanosterol cells is unexpected, indicating that lower than 170 μs can be obtained with different protein-lipid systems. (*c*) The temperature dependence of the T_{2e} of lanosterol cells resembled that of the low cholesterol sample and is in contrast with that of cell membrane containing 50 mol% cholesterol which is virtually temperature independent. (*d*) The temperature variation of the deuterium spectral lineshape of the lanosterol cells is very similar to that of the low cholesterol cells. Therefore, we can conclude that the state of the cell membrane containing 39 mol% lanosterol is similar to, but is less order than, that of the cell membrane containing 30 mol% cholesterol. It should be emphasized that $\Delta\nu_Q$ of 39 kHz for the lanosterol cells is still much broader than the <30 kHz splitting observed for cholesterol-free lipid dispersion. Thus, lanosterol is also able to order membrane lipid, albeit less effective than that of cholesterol. Differential effects of cholesterol and lanosterol on the residual splitting of the C-9 deuteron of oleic acid was reported by Yeagle (65) and the fluorescence decay lifetime of dye introduced into the membrane of *M. capricolum* was reported by Dahl et al., (12). The conclusions of these studies agree with our results.

Correlation between deuterium NMR parameters and cell growth rates

Regulation of cell growth is a complex process. The absolute growth requirement *M. capricolum* for sterols provides an opportunity to assess whether the physical properties which are modulated by the presence of sterols, and which can be monitored by physical techniques such as deuterium NMR, can be correlated with the growth rate. To make such correlation less complicated we varied only the sterol condition in the growth medium. As we have shown in this paper, even under such a constraint the membrane lipid composition, especially the oleic acid to palmitic acid ratio, varied with cholesterol concentration. The effect of the change in fatty acid composition is hard to assess and will be ignored for the following discussion. Furthermore, we believe that the specific effect of cholesterol on cell growth, i.e., the synergistic effect, is unimportant under our experimental conditions for the following reason. Dahl et al. suggested that the synergistic effect of cholesterol in promoting cell growth in medium supplemented with lanosterol was probably due to the ability of cholesterol in facilitating the transport of fatty acids across the membrane. Such synergistic effect became unimportant when large quantity of fatty acids (>10 μg/ml) were added in the growth medium (12). Thus, under our growth condition (30 μg/ml palmitic acid and 10 μg/ml oleic acid) the proposed biochemical roles of cholesterol can be ignored.

When cell growth rates were correlated with the deuterium NMR results (Fig. 1) we observed that: (*a*) the order parameter correlates with the growth rate in the sense that the fastest growing cells is also the most ordered species. Thus, the generation times of the three sets of samples are: 3 h (50% cholesterol), 4 h (30% cholesterol), and 5 h (39% lanosterol). The corresponding order parameters at the growth temperature are: 0.39, 0.34, and 0.31, respectively. (*b*) The correlation between T_{2e} at the growth temperature and cell growth rate is not obvious. On one hand, T_{2e} is the same for cells grown with cholesterol even though a 33% difference in growth rate was found between high and low cholesterol cells. On the other hand, the slow growth rate of cells with lanosterol as the supplement corresponded to a shorter T_{2e}. In fact, the slope of T_{2e} versus temperature is better correlated with growth rate with the steeper slope corresponding to a slower growth species. More studies are needed to establish these relationships.

CONCLUSIONS

In summary, we have characterized *M. capricolum* cell membrane lipid compositions and found that under our growth conditions, the lipids are composed primarily of phosphatidylglycerol (60%) and cardiolipin (35%). Cell membrane cholesterol and lanosterol concentrations can be controlled by growing cells with various amounts of exogenous sterol supplements. Cell generation times were determined and were correlated with deuterium NMR parameters. Results of our study showed: (*a*) at the growth temperature the deuterium NMR spectra showed only one component in all samples, suggesting that the lipids of this complex system were homogeneous to deuterium NMR. (*b*) The temperature variation of orientational order of acyl chains, as determined from the residual deuterium quadrupole splitting, was similar to that of the corresponding lecithin/cholesterol dispersions containing high cholesterol. Therefore, the presence of protein had little effect on acyl chain order even in the presence of a high concentration of cholesterol. (*c*) Protein had a dominating effect on reducing the T_{2e}

in the membranes of whole cells. (*d*) Cholesterol was capable of retarding and stabilizing chain dynamics in the membrane, with or without the presence of proteins. (*e*) There appeared to be some correlation between the acyl chain order parameter and cell growth rate. The T_{2e} of *M. capricolum* may have to be maintained in a relatively constant value for optimal growth. (*f*) Lanosterol was found to be able to order membrane lipid. However, it is less effective than cholesterol as a growth supplement and in modulating the cell membrane in both orientational order and dynamics. The poor growth rate of cultures grown with lanosterol may be due partially to the inability of lanosterol to modulate the physical properties of the cell membrane.

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