Volume 232, number 2, 354–358 FEB 05880 May 1988

Effect of 7β-hydroxycholesterol on growth and membrane composition of *Mycoplasma capricolum*

I. Lelong, B. Luu*, M. Mersel* and S. Rottem

Department of Membrane and Ultrastructure Research, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel and **Centre de Neurochimie du CNRS, 67804 Strasbourg, France*

Received 12 February 1988

7β-OH cholesterol in a cholesterol rich growth medium (5–10 μg/ml) extended the lag period and slowed down the growth rate of *Mycoplasma capricolum* cells. In a cholesterol poor medium (0.5 μg/ml) inadequate to support growth, 7β-OH cholesterol exerts a synergistic effect on growth. The 7β-OH cholesterol was incorporated unchanged from the growth medium and could be recovered exclusively in the membrane fraction. The incorporation of the 7β-OH cholesterol has no effect on the total phospholipid content but the DPG to PG ratio was markedly decreased. Exchange studies with lipid vesicles revealed that whereas most of the cholesterol underwent exchange, only about 20% of the 7β-OH cholesterol was exchanged.

7β-Hydroxycholesterol; Membrane composition; Sterol exchange; (*Mycoplasma capricolum*)

1. INTRODUCTION

7β-OH cholesterol exhibits a strong cytotoxicity towards various cells [1–4]. The cytotoxicity of this component as well as of other hydroxylated sterols was attributed mainly to the inhibition of the HMG-CoA reductase, a key enzyme in cholesterol biosynthesis [1–3]. Recently it has been suggested that hydroxylated sterols affect the physical state of the cell membrane as well [5]. To extend our understanding on the mechanism of the cytotoxic effect of hydroxylated sterol, we undertook this investigation to study the effect of 7β-OH cholesterol on mycoplasmas.

Mycoplasmas are among the simplest and most useful microorganisms known for membrane studies [6]. These prokaryotes lack a rigid cell wall and are bound by a single membrane [6]. Unlike other prokaryotes, cholesterol constitutes a major component in the membrane of these organisms [7]. Yet, mycoplasmas cannot synthesize cholesterol but incorporate it unchanged from the growth media. Mycoplasmas vary widely in their cholesterol content, and some species were adapted to grow with low levels of cholesterol [8–10]. Among these species is *Mycoplasma capricolum*. This organism showed a surprisingly broad sterol specificity and sterol requirements could be fulfilled even with lanosterol and cycloartenol that contain angular methyl groups on the face of the molecule. This organism may therefore provide a unique system for our investigation.

The data presented in this study demonstrate the insertion of 7β-OH cholesterol into the cytoplasmic membrane of *M. capricolum*, its effect upon the phospholipid composition of the membrane and suggests its tight association with a membrane component.

Correspondence address: S. Rottem, Department of Membrane and Ultrastructure Research, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

2. MATERIALS AND METHODS

2.1. *Organisms, growth conditions and the isolation of cell membranes*

M. capricolum was grown at 37°C in a modified Edward medium [13] containing 0.4% delipidated bovine serum albumin [14] and supplemented with oleic and palmitic acid (20 μg/ml of each), cholesterol (0.5–10 μg/ml) and 7β-OH

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

cholesterol (2.5–5 μg/ml). To label cell phospholipids, 0.01 μCi/ml of [^{3}H]palmitic acid or [^{3}H]oleic acid (50 μCi/mmol, The Radiochemical Center Amersham, England) was added to the growth medium. To label cells for the sterol incorporation experiments, 0.02 μCi/ml of [^{3}H]cholesterol ([1,2,6,7,-^{3}H(N)]-cholesterol, 60–90 Ci/mmol, NEN) or [^{3}H]7β-OH cholesterol (synthesized as previously described [15] 1.85 Ci/mmol) was added to the medium as an ethanolic solution. To label cells for the sterol exchange experiments, 0.01 μCi/ml of [^{14}C]cholesterol (55 mCi/mmol NEN) and 0.1 μCi/ml of [^{3}H]7β-OH cholesterol were added to the growth medium as an ethanolic solution. The final concentration of ethanol never exceeded 0.5%.

Growth was followed by measuring the absorbance of the culture at 640 nm. Cells were harvested at the mid-exponential phase of growth (A_{640} 0.2–0.35) by centrifugation at 12000 × *g* for 10 min, washed once and resuspended in 0.25% NaCl solution. Membranes were isolated after lysing cells preloaded with glycerol [16]. The membranes were collected by centrifugation at 34000 × *g* for 30 min and washed once in 0.25% NaCl solution.

Proteins in cells or membrane preparations were determined by the method of Lowry et al. [17].

2.2. *Lipid extraction and analysis*

Lipids were extracted according to the Bligh and Dyer procedure [18]. Lipid analysis was performed by thin-layer chromatography on silica gel HR 60 (Merck) 0.8 mm thick layer plates. The plates were developed first at room temperature with petroleum ether (b.p. 40–60°C)/acetone (3 : 1) then at 4°C with chloroform/methanol/water, 65:25:4. Lipid spots were detected by exposing the plates to iodine vapor. For determining radioactivity in the lipid spots, the spots were scraped off the plates into scintillation vials and counted by a liquid scintillator spectrometer utilizing 4 ml of 40% Lumax (Luma, Schaesberg, The Netherlands) in toluene. Phosphorus in the lipid spots was determined by the method of Ames [19] and sterol content was determined utilizing the cholesterol 20 kit (Sigma).

2.3. *Sterol exchanged with lipid vesicles*

Lipid vesicles were prepared according to Rottem et al. [20] using egg phosphatidylcholine, cholesterol and 7β-OH cholesterol. The ratio of cholesterol plus 7β-OH cholesterol to egg phosphatidylcholine was similar to that found in the mycoplasma cell grown with both sterols (5 μg/ml each).

Cells (approx. 1 mg of cell proteins/ml) and lipid vesicles (a 50–100-fold excess with respect to lipid concentration) were incubated in 0.4 M sucrose, 20 mM $MgCl_2$ and 50 mM Tris-Cl adjusted to pH 6.5, at 37°C with gentle shaking for periods up to 18 h. Penicillin (5000 units/ml), deoxyribonuclease (20 μg/ml) and albumin (1%) were added to the incubation medium. At defined time intervals duplicates of 0.75 ml were withdrawn and the cells were pelleted by centrifugation at room temperature in an Eppendorf centrifuge for 2 min. After removal of the supernatant by vacuum aspiration, the pellets were cut off from the Eppendorf tube using a razor blade and transferred to scintillation vials containing 4 ml of scintillation fluid. Radioactivity was determined in a liquid scintillation spectrometer.

The exchanges of [^{14}C]cholesterol and [^{3}H]7β-OH cholesterol were estimated from the percentage of residual radioactivity in the cell pellet.

3. RESULTS AND DISCUSSION

3.1. *The effect of 7β-OH cholesterol on growth of* M. capricolum

It has been reported that some mycoplasma species can be adapted to grow with low levels of cholesterol by multiple serial passages to media containing lower cholesterol concentration than that in the previous medium [9]. To examine the ability of 7β-OH cholesterol to replace cholesterol in the growth medium and to check the effect of 7β-OH cholesterol on cells grown in 7β-OH cholesterol containing medium, *M. capricolum* was adapted to grow in a medium containing as low as 1 μg/ml cholesterol. The cells grow neither with 0.5 μg/ml of cholesterol in the growth medium nor with 2.5 μg/ml of 7β-OH cholesterol. Yet in the presence of 0.5 μg/ml of cholesterol along with 7β-OH cholesterol (2–5 μg/ml) reasonable growth rates were obtained. As is apparent from fig.1, the growth response is synergistic rather than additive and resembles the response of *M. capricolum* to low levels of cholesterol that were combined with lanosterol [10]. Our results are in support of the hypothesis by Bloch and colleagues [10–12] that cholesterol may serve a dual role in membranes: one as a bulk component and another of a more specializing nature. The requirement for a bulk component is satisfied in our study by 7β-OH cholesterol that may affect membrane fluidity [5] or serve to space the excess negative charges present in *M. capricolum* membranes [12]. Fig.1 also shows that when the 7β-OH cholesterol (5 μg/ml) was added to a growth medium containing a sufficient concentration of cholesterol, growth was inhibited as evident by the prolonged lag period and the longer generation time. As 7β-OH cholesterol was found to be incorporated exclusively into the cell membrane without any further modification (see below) its toxicitiy may be due either to the decrease in membrane fluidity or to a specific effect on membrane component(s).

3.2. *The effect of 7β-OH cholesterol on the chemical composition of* M. capricolum *membranes*

The total phospholipid to protein ratio was

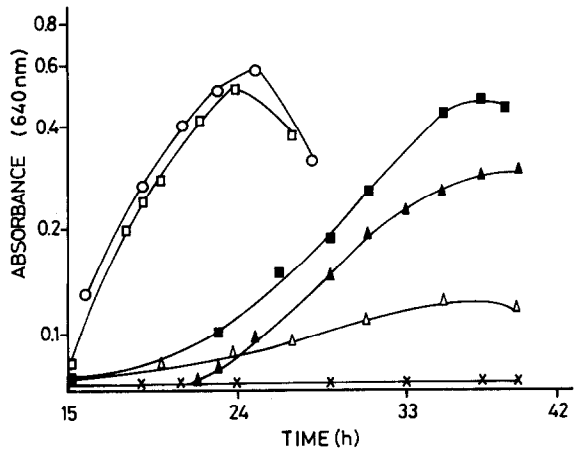

Fig.1. Growth of *M. capricolum* cells at 37°C with various concentrations of sterols. The medium was supplemented with 0.4% delipidated albumin, palmitic and oleic acid (20 μg/ml of each) and various concentrations of sterols. (Open symbols) Cholesterol alone; (closed symbols) cholesterol with 7β-OH cholesterol. (○) 10 μg/ml, (□) 5 μg/ml, (△) 0.5 μg/ml, (■) 5 μg/ml of each, (▲) 0.5 μg/ml of cholesterol plus 2.5 μg/ml 7β-OH cholesterol, (×) 2.5 μg/ml 7β-OH cholesterol alone.

essentially unchanged in membranes prepared from *M. capricolum* cells grown in a medium containing 5–10 μg cholesterol per ml with or without 7β-OH cholesterol (5 μg/ml) and harvested at the mid-exponential phase of growth. Table 1 also shows that the incorporation of radioactive oleate and palmitate was almost the same in membrane preparations from cells grown with cholesterol alone or with cholesterol alone or with cholesterol and 7β-OH cholesterol in combination.

Both cholesterol and 7β-OH cholesterol were incorporated exclusively into *M. capricolum* cell membrane and could be recovered unmodified. Table 1 shows that the sterol concentration in the isolated membrane preparations mimicked the sterol concentrations in the growth medium. Thus in cells grown with equal amounts of both sterols (5 μg/ml of each), cholesterol and 7β-OH cholesterol were incorporated to almost the same extent. The sterol to phospholipid molar ratio in membranes isolated from cells grown with 5 μg/ml cholesterol was 0.6, whereas in membranes isolated from cells grown with both sterols (5 μg/ml of each) the total sterol to phospholipid molar ratio was 1:2.

When grown in a lipid preextracted medium, the major de novo synthesized phospholipids of *M. capricolum* are phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) that account for about 80% of the total polar lipid fraction [14]. Whereas the DPG to PG ratio in *M. capricolum* membranes was only slightly affected by varying the cholesterol content of the growth medium, the 7β-OH cholesterol added to the growth media resulted in a two-fold decrease in the DPG to PG ratio. As in *M. capricolum* PG serves as the only precursor for the synthesis of DPG, the low DPG to PG ratio suggests that in the presence of 7β-OH cholesterol, the conversion of PG to DPG was markedly inhibited.

In *E. coli* it was suggested that the PG to DPG conversion plays a vital role in cell growth [21]. The molecular basis for such a role is yet

Table 1

Lipid composition of *M. capricolum* membranes from cells grown with or without 7β-OH cholesterol

Sterol added to medium (μg/ml)	Fatty acid incorporation (dpm/mg membrane proteins)		Sterol incorporation (μg/mg membrane proteins)			DPG to PG ratio determined by	
	[^{14}C]Oleate	[^{14}C]Palmitate	Total	Cholesterol	7β-OH cholesterol	Lipid phosphorus	Radioactivity
Cholesterol (10)	31180	55250	74.1	74.1	–	0.74	0.80
Cholesterol (5)	33600	66500	51.1	51.1	–	0.66	0.61
Cholesterol (5) + 7β-OH cholesterol (5)	32100	45350	103	57.7	47.3	0.32	0.42

Cells were grown in a medium containing 0.4% delipidated albumin, oleic and palmitic acid (20 μg/ml of each) and either [1-^{14}C]oleate or [1-^{14}C]palmitate. The medium was supplemented with cholesterol and 7β-OH cholesterol. The cells were harvested in mid-exponential phase of growth. Lipid analysis was performed as described in section 2

unknown. However, since DPG may be induced in the presence of cytosolic Ca^{2+} to form nonlamellar phases [22], it has been suggested that the DPG to PG ratio is part of a control mechanism to maintain an intermediate membrane lipid structure in *M. capricolum* [23]. These structures would contain a balanced mixture of bilayer and nonbilayer lipids that would have to satisfy the structural role as well as participate in various membrane-mediated processes [24].

3.3. *Sterol exchange between* M. capricolum *and lipid vesicles*

The spontaneous exchange of cholesterol between mycoplasmas and lipid vesicles was intensively studied with the relative stable cells of *M. gallisepticum* that remain intact and even viable throughout the prolonged incubation period [20,25,26]. In the present study, successful exchange experiments were performed with the osmotically sensitive cells of *M. capricolum*. The cells utilized were passed 20 times through the lipid preextracted growth medium supplemented with albumin (0.4%) oleic and palmitic acid (20 μg/ml of each) and 5 μg/ml of cholesterol. Such passages increased to a large extent the osmotic stability of the organisms that remained intact throughout the exchange experiment as judged from the unchanged absorbance of the cell suspension and the very little decrease (< 10%) in the NADH dehydrogenase activity in the cells [20].

The exchange studies were performed by incubating *M. capricolum* cells grown in a medium containing [^{14}C]cholesterol and [^{3}H]7β-OH cholesterol with excess of non-radioactive lipid vesicles. The lipid vesicles contain approximately the same ratio of cholesterol and 7β-OH cholesterol to phospholipid as do the mycoplasma cells. This ratio was chosen to minimize depletion of mycoplasma sterols during the incubation period. Indeed determinations of the cholesterol and 7β-OH cholesterol content in cells at the end of the incubation period showed no significant changes from that at the start. This observation indicates the lack of net sterol transfer and suggests that the decrease in the radioactivity of *M. capricolum* cells upon incubation with the lipid vesicles represent sterol exchange. Fig.2 shows the results of an exchange study utilizing cells and vesicles containing a cholesterol/phospholipid

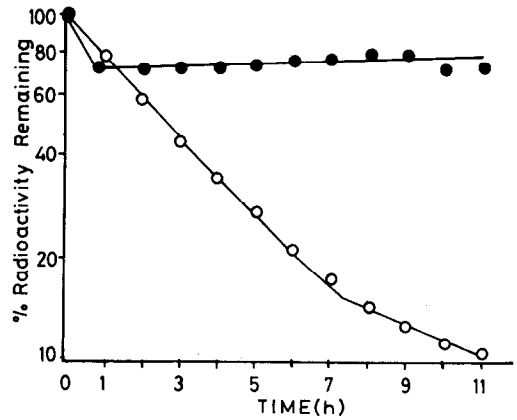

Fig.2. Kinetics of [^{14}C]cholesterol and [^{3}H]7β-OH cholesterol exchange between *M. capricolum* cells and PC/sterols vesicles. The exchange was performed as described in section 2. (○) [^{14}C]Cholesterol, (●) [^{3}H]7β-OH cholesterol.

ratio of 0.6 and a total sterol/phospholipid molar ratio of 1 : 1. Whereas over 95% of the cholesterol underwent exchange with a biphasic kinetics only about 25% of the 7β-OH cholesterol was exchanged. Desorption of sterols from the donor membrane is thought to be the rate limiting step in exchange or transfer to excess acceptor [26,27]. The rate of desorption is affected by the lipid-lipid and the lipid-protein contacts within the membrane as well as by the aqueous solubility of the monomeric lipid [26]. As the aqueous solubility of 7β-OH cholesterol is higher than that of cholesterol [28], the low or nonexistent exchange rate of most ($\geq$75%) of the 7β-OH cholesterol pool in *M. capricolum* cells strongly suggests tight interactions of the 7β-OH cholesterol with *M. capricolum* membrane components.

REFERENCES

[1] Breston, J.L., Lothor, D.A., Spauling, D.R. and Kandutsch, A.A. (1975) Biochim. Biophys. Acta 398, 10–17.
[2] Kandutsch, A.A. and Chen, H.W. (1977) J. Biol. Chem. 252, 409–415.
[3] Kandutsch, A.A., Chen, H.W. and Heiniger, H.J. (1978) Science 201, 498–501.
[4] Hietter, H., Trifilieff, E., Richert, L., Beck, J.P., Luu, B. and Ourisson, G. (1984) Biochem. Biophys. Res. Commun. 120, 657–664.
[5] Richert, L., Castagna, M., Beck, J.P., Rong, S., Luu, B. and Ourisson, G. (1984) Biochem. Biophys. Res. Commun. 120, 192–198.

[6] Razin, S. (1973) Adv. Microbiol. Physiol. 10, 1–80.
[7] Rottem, S. (1980) Biochim. Biophys. Acta 604, 65–90.
[8] Rottem, S., Yashow, J., Ne'eman, Z. and Razin, S. (1973) Biochim. Biophys. Acta 323, 495–508.
[9] Clejean, S., Bittman, R. and Rottem, S. (1978) Biochemistry 17, 4579–4583.
[10] Odriozola, J.M., Waitzkin, E., Smith, T.L. and Bloch, K. (1978) Proc. Natl. Acad. Sci. USA 75, 4107–4109.
[11] Dahl, J.S., Dahl, C.E. and Bloch, K. (1980) Biochemistry 19, 1467–1472.
[12] Dahl, J.S. and Dahl, C.E. (1983) Proc. Natl. Acad. Sci. USA 80, 692–696.
[13] Razin, S. and Rottem, S. (1976) in: Biochemical Analysis of Membranes (Maddy, A.H. ed.) pp.3–26, Chapman and Hall, London.
[14] Gross, Z., Rottem, S. and Bittman, R. (1982) Eur. J. Biochem. 122, 169–174.
[15] Heitter, H., Gouyette, A., Van Dorsselaer, A. and Luu, B. (1988) J. Steroid Biochem., in press.
[16] Rottem, S., Stein, O. and Razin, S. (1968) Arch. Biochem. Biophys. 125, 46–56.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.R. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[18] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[19] Ames, B.N. (1966) Methods Enzymol. 8, 115–116.
[20] Rottem, S., Shinar, D. and Bittman, R. (1981) Biochim. Biophys. Acta 649, 572–580.
[21] Michel, G., Di Sarino, D. and Starka, J. (1977) J. Bacteriol. 128, 145–150.
[22] De Kruijff, B., Cullis, P.R. and Verkleij, A. (1980) Trends Biochem. Sci. 5, 79–81.
[23] Gross, Z. and Rottem, S. (1986) J. Bacteriol. 167, 986–991.
[24] Goldfine, H. (1984) J. Lipid Res. 25, 1501–1507.
[25] Rottem, S., Slutzky, G.M. and Bittman, R. (1978) Biochemistry 17, 2723–2726.
[26] Clejan, S. and Bittman, R. (1984) J. Biol. Chem. 259, 441–448.
[27] McLean, L.R. and Phillips, M.C. (1981) Biochemistry 20, 2893–2900.
[28] Theunissen, J.J.H., Jackson, R.L., Kempken, H.J.M. and Demel, R.A. (1986) Biochim. Biophys. Acta 860, 66–74.