CHOLESTEROL IS REQUIRED TO PREVENT CRYSTALLIZATION OF *MYCOPLASMA ARGININI* PHOSPHOLIPIDS AT PHYSIOLOGICAL TEMPERATURE

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1. Introduction

Mycoplasmas are the only prokaryotes that require cholesterol for growth [1]. Cholesterol is incorporated unchanged from the growth medium, reaching levels comparable to those found in the plasma membrane of animal cells (25–30%, by wt of total membrane lipids). Studies were artificial lipid mixtures [2] have shown that the addition of cholesterol disordered the acyl chains of phospholipids at temperatures below the gel–liquid crystalline (order–disorder) phase transition and ordered the acyl chains at temperatures above this phase transition. It was therefore proposed that [2,3] cholesterol maintains an intermediate fluid state in biological membranes by eliminating the phase transition at low temperatures and decreasing membrane fluidity at high temperatures. This conclusion was supported from studies with mycoplasmas. *Mycoplasma mycoides* subsp. *capri* cells, adapted to grow with low cholesterol concentrations, increased the saturated fatty acid content of its membrane phospholipids [4]. This increase was interpreted to compensate for the loss of sterol and preserve the bilayer in its optimal ordered form [4,5]. Further support comes from an animal cell system, LM-cell mutant, defective in sterol biosynthesis [6]. Sterol depletion of these cells resulted in a marked increase in the unsaturation of membrane phospholipids [6,7].

This work shows that *M. arginini* cells, like *M. hominis* [8] and *Spiroplasma citri* [9], preferentially incorporate saturated fatty acids into their de novo synthesized membrane phospholipids. The incorporation of large amounts of cholesterol into the membrane of *M. arginini* was found to be necessary to prevent the highly saturated membrane phospholipid from being in an entirely ordered form at the physiological temperature. It is suggested that the strict cholesterol requirement of several parasitic mycoplasmas is associated with the inability of these cells to incorporate unsaturated fatty acids from the growth medium.

2. Materials and methods

2.1. *Organism and growth condition*

Mycoplasma arginini was kindly donated by Dr G. E. Kenny (University of Washington, Seattle WA). The organism was grown in a modified Edward medium [10] in which 0.5% bovine serum albumin (fraction V, Sigma, St Louis MO) cholesterol (15 μg/ml) and oleic and palmitic acids (12.5 μg/ml of each) replaced the horse serum. To label membrane lipids, 0.001 μCi [1-^{14}C]oleate or [1-^{14}C]palmitate were added/ml growth medium. The cultures were grown at 37°C for 18–22 h and the cells (at the mid-exponential phase of growth having an absorbance of 0.18–0.20 at 640 nm) were harvested by centrifugation at 12 000 × *g* for 20 min, washed once and resuspended in a cold 0.25 M NaCl solution.

2.2. *Lipid analyses*

Lipids were extracted from intact cells as in [11] and the solvent was evaporated under nitrogen. Traces of water were removed by overnight freeze-drying of the preparation and the lipids were redissolved in chloroform. Polar lipids were separated from neutral lipids by silicic acid chromatography [12]. The lipid

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fractions were dried under nitrogen, weighed and redissolved in chloroform. The neutral lipid fraction was chromatographed on silica gel G plates as in [12]. Phospholipids of the polar lipid fraction or total membrane lipids were separated on silica gel HR plates. The plates were first developed at room temperature with petroleum ether (b.p. 40–60°C)–acetone (3:1, by vol.) and then at 4°C with chloroform–methanol–water (65:25:4, by vol.). Lipid spots were detected by iodine vapor. The identification of the lipid spots and determination of their radioactivity were performed as in [12].

The fatty acid content of membrane phospholipids was determined by gas–liquid chromatography. Methyl esters of the fatty acids were prepared by heating the phospholipid samples for 15 min at 72°C in 14% of boron trifluoride in methanol (Sigma, St Louis MO). The methyl ester derivatives were extracted with *n*-hexane and separated in a Perkin-Elmer model 900 gas liquid chromatograph equipped with a 1.8 m column of 10% SP 2330 (Supelco, Belfonte PA) and programmed to increase the column temperature from 150–210°C at 6°C/min. Fatty acids were identified by their retention time relative to standard methyl ester derivatives.

2.3. *Physical measurements*

Microviscosity measurements of lipid vesicles were performed using 1,6-diphenyl-1,3,5-hexatriene (DPH) in an Elscint model MV-1A microviscometer. For labelling of vesicle suspension, an equal volume of 1 μM DPH solution was added to an equal volume of a multilamellar vesicle suspension (1 μM phospholipids in 0.25 M NaCl solution). The mixture was incubated at 37°C for 30 min. Fluorescent depolarization of the DPH incorporated into the membranes was then measured over 7–45°C and microviscosity (η) was calculated as in [13].

Differential scanning calorimetry (DSC) was performed as in [14]. Samples of the polar lipid fraction or of total membrane lipids were dried on microscope slides under nitrogen and then placed under vacuum for 1 h. The dried lipids were scraped off of the slides and loaded into Perkin-Elmer volatile sample pans. Lipid samples were hydrated by adding water to ≥5-times the sample mass. Calorimetry was performed using a modified Perkin-Elmer DSC II.

2.4. *Analytical methods*

Protein was determined according to [15]. Total phosphorous in the lipid fraction was determined as in [16] after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. Cholesterol content of the total lipids, the neutral lipid fraction and lipid spots resolved by chromatography was measured colorimetrically [17].

3. Results and discussion

Mycoplasma arginini cells, grown in a horse serum-free medium supplemented with cholesterol and fatty acids, contain phospholipids (63–68% of total lipids) and unesterified cholesterol (25–30%) as the major lipids. The cholesterol to phospholipid molar ratio was 0.80–0.85. The remaining lipids (~7%) were comprised mainly of glycerides and cholesterol esters, apparently incorporated from the growth medium. The lipids were found to be exclusively located in the cell membrane.

The phospholipid composition of *M. arginini* was found to be rather simple, resembling that of *M. hominis* [8] and *M. gallisepticum* [12]. The phospholipid fraction was comprised mainly of phosphatidylglycerol (PG), which constitutes 92–95% of total membrane phospholipids (table 1). When grown

Table 1
Properties of *Mycoplasma arginini* membrane phospholipids[a]

Phospholipid	% of total membrane P_i	Incorporation of ^{14}C-labeled fatty acid (cpm/mg phospholipid)		Fatty acid composition (% of total)				
		Palmitate	Oleate	14:0[b]	16:0	18:0	18:1	18:2
Phosphatidylglycerol	92–95	54 200	4800	0.5	76.5	11.0	10.2	<0.5
Phosphatidylcholine	3–16	<500	<500	1.7	11.4	38.0	12.0	34.8

[a] The organisms were grown in an Edward medium [10] supplemented with either [1-^{14}C]palmitate or [1-^{14}C]oleate to A_{640} = 0.20
[b] The first number indicates chain length and the second the number of double bonds

with radioactive palmitate or oleate, radioactivity was fully recovered in the PG fraction (table 1). Furthermore, practically all the radioactivity was found in the PG spot after thin-layer chromatography in chloroform–methanol–acetic acid–water (65:25:1:4, by vol.) and chloroform–methanol–ammonia–water (60:35:2.5:2.5, by vol.) solvent systems, and in a two-dimensional system [18]. This suggests that PG is the only phospholipid synthesized by this organism de novo. The low amounts of phosphatidylcholine (PC) found are apparently due to PC molecules incorporated unchanged from the growth medium. The incorporation of exogenous phospholipids by several species of the wall-less mycoplasmas is well documented.

Table 1 also shows that when the cells were grown with a radioactive fatty acid, the de novo synthesized PG was intensively labelled by palmitate but very little by oleate. Moreover, the fatty acid composition of the PG of *M. arginini* contained mainly saturated fatty acids with a saturated to unsaturated fatty acid ratio of 8.5–9.0. The fatty acid composition of the *M. arginini* PC, apparently incorporated from the growth medium, contained approximately equal amounts of saturated and unsaturated fatty acids (table 1). This further suggests that *M. arginini* cells, like *M. hominis* [8] do not possess the ability to incorporate a variety of fatty acids from the growth medium but make a preferential use of saturated fatty acids for PG biosynthesis.

The physical state of membrane lipids depends largely on the nature of the hydrocarbon chain [20]. The highly saturated nature of the hydrocarbon chains of the PG of *M. arginini* is expected to raise the temperature of the gel–liquid crystalline phase transition. DSC analysis of an aqueous dispersion of the polar lipid fraction of *M. arginini* from 35–54°C is shown in fig.1a. In both upscans and downscans the transition behavior was typical of phospholipids in excess water. It is apparent from the thermogram that at the physiological growth temperature (37°C) the phospholipid fraction of *M. arginini* is highly ordered. As the physical properties of membrane lipids are vitally important to mycoplasmas [21] it is expected that *M. arginini* cells with a membrane containing mainly the highly saturated PG will not grow at 37°C, where its lipids exist almost entirely in the gel state. *M. arginini* prevents this highly ordered state by incorporating large quantities of cholesterol during growth, offsetting the condensing effect of the saturated PG. When the total lipid fraction of *M. arginini* containing both the phospholipids and cholesterol were analyzed (fig.1b) no order–disorder transition was detectable by our calorimetry. The effect of temperature on the physical state of the lipids, as presented in an Arrhenius plot of the microviscosity (in poise) *vs* $1/T$, is shown in fig.2. The plot of lipid dispersions made from the polar lipid fraction exhibited prominent discontinuities at 35°C and 40°C. In con-

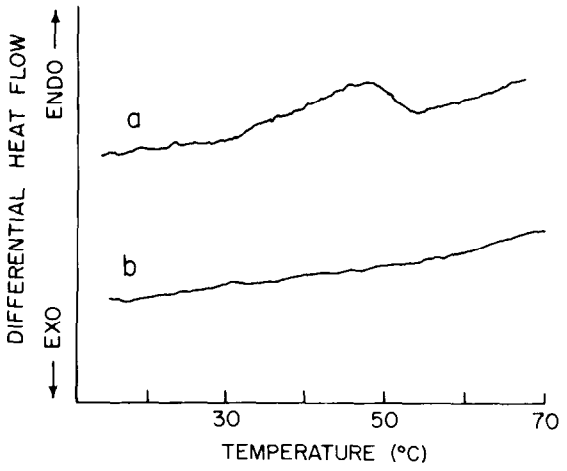

Fig.1. Thermograms of *M. arginini* lipid dispersions: (a) polar lipid fraction; (b) total membrane lipids. The lipids were hydrated by adding water and cycling them in the calorimeter repeatedly above and below the lipid transition temperature.

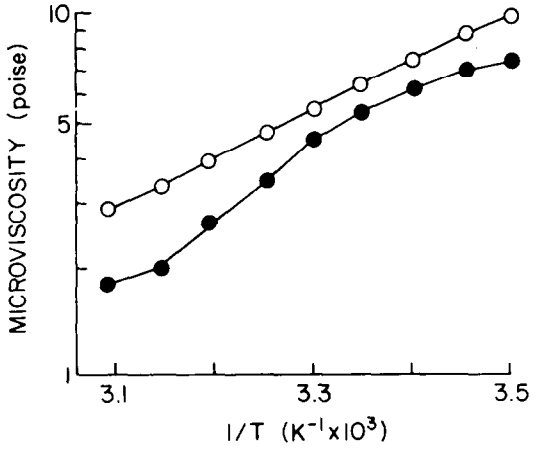

Fig.2. Effect of temperature on the microviscosity of lipid dispersions. Microviscosity was determined by fluorescent depolarization measurements using DPH: (○) dispersion of total membrane lipids; (●) dispersion of the polar lipid fraction.

trast, with dispersions of total membrane lipids the relationship was strictly linear. It is therefore suggested that in mycoplasmas that preferentially incorporated saturated fatty acids into their membrane phospholipids, the incorporation of cholesterol prevents crystallization of membrane lipids at their optimal growth temperature.

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