EFFECTS OF CHARGED CHOLESTERYL ESTERS ON MYCOPLASMA GROWTH

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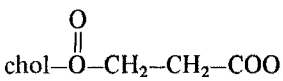
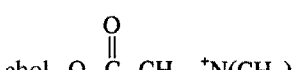
1. Introduction

The structural features of sterol molecules essential for their function in biological membranes include a planar (*trans*-fused) tetracyclic ring system, an aliphatic side chain and an unblocked equatorial hydroxyl group at position C-3 [1]. The same structural features are also required of sterols to be capable of promoting growth of mycoplasmas [2]. Doubts about the generality of these requirements were raised in [3–6] where several sterols and sterol esters which did not possess all of the above structural features were found to satisfy the sterol requirement of *Mycoplasma capricolum*.

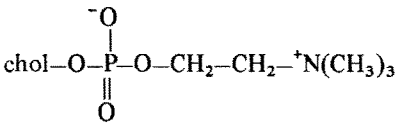
The claim that a free hydroxyl group is not always essential for the biological activity of sterols is somewhat surprising in light of its ubiquity in membrane sterols and the great importance attributed to it in sterol–phospholipid interactions and in keeping the sterol in the perpendicular orientation with regard to the lipid bilayer [1]. It appeared, therefore, worthwhile to re-examine the ability of *M. capricolum* to grow with sterol esters and compare it with other sterol-requiring mycoplasmas. The common serum cholesteryl esters which contain long-chain fatty acids, are extremely hydrophobic and are essentially insoluble in the aqueous growth media. We made use, therefore, of hydrophilic synthetic cholesteryl esters with charged head groups:



cholesteryl hemisuccinate



cholesteryl betainate



cholesteryl phosphorylcholine

Unlike the natural cholesteryl esters they are appreciably soluble in water and are readily incorporated into liposomes and red cell membranes [7,8]. Moreover, the esters resemble cholesterol in their effects on microviscosity and degree of order of lipid bilayers [7,8].

These results show that of the 3 cholesteryl esters tested, only cholesteryl betainate could support *M. capricolum* growth. However, none of the other sterol-requiring mycoplasmas tested could grow with any of these esters. It thus appears that *M. capricolum*, and possibly closely related strains, is outstanding among the mycoplasmas in its broad sterol specificity, so that conclusions drawn for this mycoplasma may not apply to other mycoplasmas.

2. Materials and methods

2.1. *Organisms and growth conditions*

Acholeoplasma laidlawii (oral strain), *Mycoplasma capricolum* (California kid, ATCC 27343) and *Mycoplasma gallisepticum* (A 5969) were grown in a modified Edward medium [9] supplemented with 0.5% (w/v) glucose, 0.5% (w/v) bovine serum albumin (fraction V, Sigma), 10 μg/ml palmitate and 10 μg/ml oleate. For growth of *M. capricolum* and *M. gallisepticum* used for inoculum the above medium was supplemented with 0.3% (v/v) and 4% (v/v) horse serum, respectively, and the pH of the medium was adjusted to 8.0. *Mycoplasma hominis* (ATCC 15056 and

ATCC 23114) and *Mycoplasma arginini* (G230) were grown in the same medium with 4% horse serum but with 20 mM L-arginine replacing the glucose supplement, and the initial pH in the medium was adjusted to 6.5.

2.2. *Sterol donors*

Cholesterol of high purity was purchased from Sigma. Cholesteryl hydrogen succinate (>99% pure) was purchased from Pfaltz and Bauer (New York). Cholesteryl phosphorylcholine was synthesized and purified according to [10]. The batch used in the uptake experiments was 92% pure, the impurities being cholesteryl phosphate (5%), cholesteryl bromoethyl phosphate (2%) and cholesterol (<1%). Cholesteryl betainate (chloride form) was prepared by bubbling trimethyl amine gas through a solution of cholesteryl chloroacetate (Sigma) in chloroform at 60°C. The reaction was followed by liberation of Cl^- and reached completion after 18 h. The product was crystallized from chloroform, and was found to be 93% pure with <5% residual cholesteryl chloroacetate and 1–2% cholesterol. The sterols were added to the medium either as ethanolic solutions or as part of lipid vesicles. When added as ethanolic solution, the concentration of ethanol in the medium was ≤0.3% (v/v). The lipid vesicles containing the sterol and phosphatidylcholine (egg lecithin, Makor Chemicals, Jerusalem) in a 0.9:1.0 molar ratio were prepared according to [11].

2.3. *Effect of sterols on growth*

Double dilutions of the sterols were prepared in 5 ml growth medium devoid of horse serum. Test tubes containing medium without sterol, and medium with 5% (v/v) of horse serum served as controls. A 3% inoculum of a young mycoplasma culture was added to each test tube. A_{640} of the cultures was measured after 48 h incubation at 37°C.

2.4. *Measurement of sterol uptake by isolated membranes*

M. capricolum and *A. laidlawii* were grown in Edward medium as above. *M. capricolum* was grown with 0.3% horse serum and *A. laidlawii* with no serum. The organisms were harvested after 20–24 h incubation at 37°C, when A_{640} of culture reached ~0.17 for *M. capricolum* and 0.30 for *A. laidlawii*. The sedimented organisms were washed twice and resuspended in 0.25 M NaCl. Cell membranes were isolated by osmotic lysis of the mycoplasmas, as in [9]. The sterol uptake experiment was done in a mixture consisting of 0.05 M phosphate buffer (pH 7.0) containing sterol–phosphatidylcholine vesicles (to yield 52 nmol sterol/ml) and membranes (0.1 mg protein/ml). The suspension was incubated at 37°C for 2–4 h and the membranes were collected by centrifugation at 38 000 × *g* for 25 min, washed twice in 0.25 M NaCl and resuspended in 0.05 M NaCl. The washed membranes were analyzed for their protein and lipid content.

2.5. *Analytical procedures*

Protein was determined according to [12]. Lipids were extracted from membranes with chloroform/methanol 2:1 [13]. The lipid extracts were analyzed for their sterol content by the colorimetric technique in [14]. This technique, devised originally for cholesterol, was found by us to be also adequate for quantitation of the cholesteryl esters. Lipid phosphorus was determined by the method in [15] after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. Sterols were separated on silica gel G(Merck) plates, using benzene/ethyl acetate (5:1, v/v) as the developing solvent. The sterol spots were detected by iodine vapor and identified by sulfuric acid–acetic anhydride spray reagent (5 ml sulfuric acid and 5 ml acetic anhydride in 80 ml ethanol).

3. Results

Growth of *M. capricolum* increased linearly on increasing the cholesterol concentration in the medium up to 13 nmol cholesterol/ml (fig.1). Cholesteryl betainate at low concentrations was even more effective than cholesterol in growth promotion. However, at higher concentrations of this cholesterol ester growth was inhibited. The two other cholesterol esters were essentially ineffective as growth promoters of *M. capricolum*. The same experiment carried out with *M. gallisepticum* (not shown) revealed the growth-dependency of this organism on cholesterol. However, none of the cholesterol esters could support growth of this mycoplasma. The sterol-nonrequiring *A. laidlawii* was used as a control for the toxicity of the sterol esters. Fig.1 shows that cholesteryl hemisuccinate did not inhibit *A. laidlawii* growth at the range of concentrations tested, whereas cholesteryl phosphorycholine and cholesteryl betainate inhibited growth at 13 and 26 nmol/ml, respectively.

To minimize the growth inhibitory effect of the

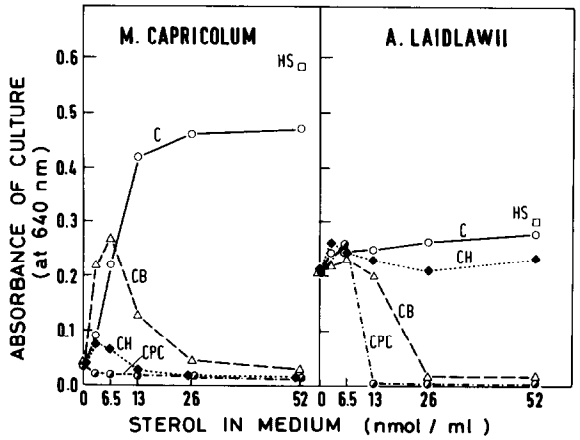


Fig.1. Growth of *M. capricolum* and *A. laidlawii* in the presence of: cholesterol, ○ (C); cholesteryl betainate (△, CB); cholesteryl hemisuccinate, (◆, CH); cholesteryl phosphorylcholine (◉, CPC); medium supplemented with 5% horse serum (□, HS). The sterols were added to the growth medium as ethanolic solutions.

cholesterol esters the albumin concentration of the growth medium was raised from 0.5–2% (w/v), but this did not decrease the toxicity of the esters and even diminished growth by itself. The incorporation of cholesteryl betainate in lipid vesicles made of phosphatidylcholine decreased toxicity of the sterol ester and enabled *M. capricolum* growth to a level almost as high as with cholesterol (fig.2). However, even when supplemented in lipid vesicles this cholesterol ester could not replace cholesterol in the growth of *M. arginini* (fig.2), neither could it support growth of the *M. hominis* strains (not shown).

To test whether the different cholesterol esters are incorporated into mycoplasma membranes, vesicles containing the derivatives with phosphatidylcholine were incubated with isolated membranes of *M. capricolum* and *A. laidlawii* for 4 h in phosphate buffer. Table 1 shows that significant quantities of all the cholesterol esters were taken up by the membranes of the two organisms. Cholesteryl betainate was incorporated best. The uptake of the cholesterol esters was accompanied by an increase in lipid P_i, apparently due to phosphatidylcholine uptake. On the other hand there was no phospholipid uptake from cholesterol–phosphatidylcholine vesicles.

4. Discussion

Our data show that cholesteryl betainate, a derivative of cholesterol in which the equatorial hydroxyl group is substituted by betainate, can replace cholesterol in growth of the sterol-requiring *M. capricolum*. This supports the findings in [3,4] that the sterol requirement of this mycoplasma can be satisfied by cholesteryl acetate or cholesteryl methyl ether, in which the 3-hydroxyl group is blocked. These sterol derivatives were recovered unchanged from the bacterial cells [4] indicating that *M. capricolum* resembles other mycoplasmas in its inability to hydrolyse cholesteryl esters [5]. However, our results show that cholesteryl betainate under the same conditions failed to support growth of other sterolrequiring mycoplasmas, including *M. gallisepticum*, *M. arginini* and *M. hominis*. *M. capricolum* and the closely related *M. mycoides* subsp. *capri* differ from

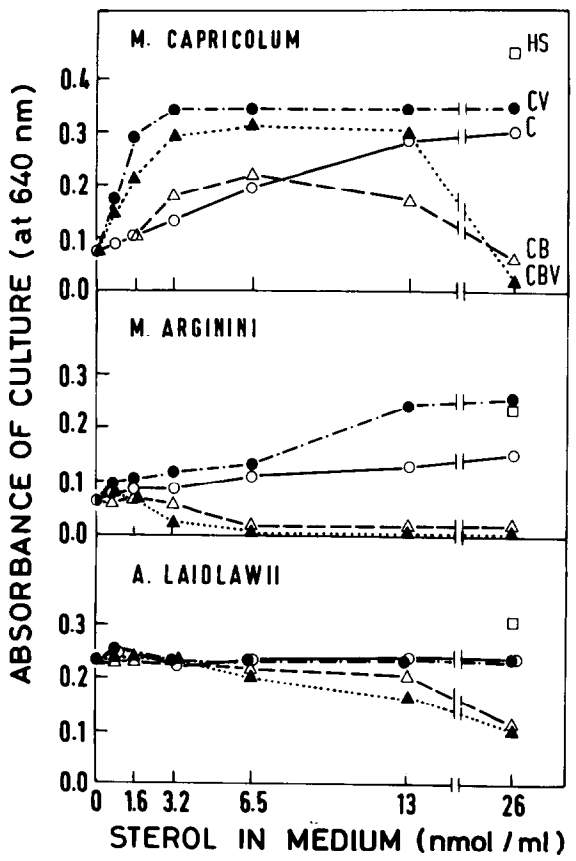


Fig.2. The effect of cholesterol and cholesteryl betainate on growth of *Mycoplasma* and *Acholeplasma* species: cholesterol added as ethanolic solution (○, C); cholesterol added in phosphatidylcholine vesicles (●, CV); cholesteryl betainate added as ethanolic solution (△, CB); cholesteryl betainate added in phosphatidylcholine vesicles (▲, CBV); medium supplemented with 5% horse serum (□, HS).

Table 1
Net uptake of sterols and phosphatidylcholine (μmol/mg membrane protein) by isolated membranes of *M. capricolum* and *A. laidlawii*[a]

Mycoplasma	Sterol in vesicles	Lipid taken up	
		Sterol[b]	Lipid P_i[b]
M. capricolum	cholesterol	0.04	0
	cholestryl betainate	0.66	0.44
	cholestryl phosphorycholine	0.19	0.16
	cholesteryl hemisuccinate	0.31	0.31
A. laidlawii	cholesterol	0.10	0
	cholestryl betainate	0.70	0.54
	cholestryl phosphorylcholine	0.55	0.55
	cholesteryl hemisuccinate	0.27	0.28

[a] The membranes were incubated for 4 h at 37°C with vesicles made of sterol and phosphatidylcholine (0.9:1.0 molar ratio)

[b] The net values for sterol and phosphatidylcholine uptake were obtained by subtracting the values of cholesterol and lipid P_i at zero time. The values were 0.36 μmol lipid P_i and 0.37 μmol cholesterol/mg membrane protein for *M. capricolum* and 0.43 μmol lipid P_i/mg membrane protein and zero cholesterol for *A. laidlawii*

other sterol-requiring *Mycoplasma* species in their ability to grow with low amounts of cholesterol in their membrane [5,6,16,17]. In addition, *M. capricolum* binds much more cholesterol esters from serum lipoproteins than other sterol-requiring mycoplasmas [11]. It appears, therefore, that conclusions drawn from studies with *M. capricolum* and *M. mycoides* subsp. *capri* as to sterol specificity may not be valid for other sterol-requiring mycoplasmas.

The toxicity shown by the synthetic cholesterol esters complicates the interpretation of results, and limits their use as cholesterol substitutes in biological systems. As expected from the amphipatic structure of the charged cholesterol ester, they may act as mild detergents. In fact, cholesteryl phosphorylcholine was found to lyse human red blood cells [7]. Hence, there is a need to distinguish between growth inhibition caused by the detergent action of the cholesterol esters, and the failure of the esters to replace cholesterol in growth promotion. The experiments with the sterol-nonrequiring *A. laidlawii* fulfilled this need. Cholesteryl phosphorylcholine had the highest toxicity, while cholesteryl hemisuccinate was essentially not toxic to this mycoplasma (fig.1). Yet, the hemisuccinate ester showed very little or no promotion of growth of the sterol-requiring mycoplasmas, while the more toxic cholesteryl betainate promoted *M. capricolum* growth at low concentrations; at higher concentrations the growth inhibitory activity of this derivative prevailed. The incorporation of cholesteryl betainate into phosphatidylcholine vesicles improved its growth-promoting activity (fig.2) apparently by lowering the concentration of the unbound ester, reducing in this way its toxic effects.

Significant quantities of the synthetic cholesterol esters were taken up by isolated mycoplasma membranes. Uptake of the derivatives was particularly notable with *A. laidlawii* membranes, where cholesterol uptake is usually restricted [11]. Of the 3 esters tested, the betainate derivative was taken up in highest quantities, possibly due to its net positive charge, expected to facilitate its binding to the negatively-charged phospholipids characteristic of mycoplasma membranes [2]. The simultaneous uptake of phosphatidylcholine by the membranes is of interest. Isolated mycoplasma membranes fail to incorporate this phospholipid from vesicles made with cholesterol (table 1 and [11]). The data in table 1 show that the molar ratio of the cholesterol esters to the phospholipid taken up is close to the ratio of these components in the vesicles (~1.0). It can be suggested that the detergent activity of the cholesterol esters enhanced the ability of the membranes to incorporate exogenous phospholipids. Our data do not rule out the possibility that at least some of the lipid vesicles adhered to or fused with the membranes.

Acknowledgement

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