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Cholesterol Incorporation into Bacterial Membranes

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The wall-covered bacteria *Micrococcus lysodeikticus*, *Bacillus megaterium*, and *Proteus mirabilis* incorporated exogenous cholesterol into their cytoplasmic membrane in quantities resembling those incorporated by sterol-nonrequiring mycoplasmas. Cholesterol incorporation into the outer membrane of *P. mirabilis* was much more restricted than into the cytoplasmic membrane.

The ability to synthesize sterols is rarely found in prokaryotes. Consequently, when bacteria or blue-green algae are grown in sterol-free media, sterols are not usually detected in their membranes (11). The lack of cholesterol in microbial membranes is thus frequently cited as a feature distinguishing them from eukaryotic cell membranes. The wall-less mycoplasmas resemble other prokaryotes in their inability to synthesize sterols. Yet, unlike the other prokaryotes, most of the mycoplasmas require exogenous cholesterol for growth and incorporate large quantities of it into their cell membrane (7). Some mycoplasmas, included in the genus *Acholeplasma*, do not require cholesterol and exhibit a much lower cholesterol-binding capacity (6). The finding of marked differences between the sterol-requiring and sterol-nonrequiring mycoplasmas in the cholesterol-binding capacity of their cell membranes prompted us to investigate cholesterol binding by wall-covered eubacteria. Our main aims were to compare the cholesterol binding ability of the plasma membrane of eubacteria to that of mycoplasmas, and to determine whether the bacterial cell wall interferes or participates in the cholesterol binding process.

The organisms listed in Table 1 were grown at 37 C in a modified Edward medium (8) devoid of antibacterial inhibitors and supplemented with 2% (vol/vol) of PPLO serum fraction (Difco) which served as a cholesterol source. The organisms were harvested at the late exponential phase of growth. The eubacteria were washed twice in deionized water and the mycoplasmas were washed twice in 0.25 M NaCl. The cytoplasmic membranes of the gram-positive bacteria were isolated by treatment of the washed cells and suspended in deionized water with lysozyme (100 μg/ml) and pancreatic deoxyribonuclease (10 μg/ml). The mycoplasma membranes were isolated by osmotic lysis of the washed organisms (8). The outer and cytoplasmic membranes of *Proteus vulgaris* were isolated by a modification of the method of Osborn et al. (5) as described by Hasin et al. (3). The washed cells and membranes were subjected to the following analyses: (i) total protein content, measured according to Lowry et al. (4); (ii) lipid phosphorus, determined by the method of Ames (1) on the membrane lipid fraction which was extracted from the isolated membranes with chloroform-methanol (2:1, vol/vol) for 2 h at 45 C; and (iii) cholesterol content, determined on the extracted lipid fraction by the colorimetric technique of Rudel and Morris (10).

Table 1 shows that the wall-covered bacteria incorporated appreciable quantities of cholesterol when grown in its presence. Most of the bound cholesterol was retained in the cytoplasmic membrane fraction isolated from the gram-positive bacteria by lysozyme treatment. The ratio of cholesterol to protein and to phospholipid in these membranes resembled that of the sterol-nonrequiring *Acholeplasma laidlawii*, but was remarkably lower than that of the sterol-requiring *Mycoplasma mycoides* var. *capri*.

Separation of the outer from the cytoplasmic membrane of *P. mirabilis* enabled the determination of the distribution of the exogenously incorporated cholesterol in the two membrane types. Table 2 shows that most of the cholesterol was incorporated into the cytoplasmic membrane although in the intact cell the outer membrane obviously has better access to the cholesterol in the medium. The fact that the cytoplasmic membrane contains about twice as much phospholipids as the outer membrane (3) is likely to contribute to the higher cholesterol binding capacity of the former since studies with mycoplasma membranes have shown that the amount of cholesterol incorporated is largely determined by their phospholipid or total lipid content (6). Yet, this explanation seems incom-

TABLE 1. *Cholesterol content of eubacteria and mycoplasmas grown in a cholesterol-supplemented medium*[a]

Organism	Cholesterol content		
	μg/mg of cell protein	μg/mg of membrane protein	μg/μg of lipid phosphorus
Micrococcus lysodeikticus	15.1 (13.2-25.0)	44.6 (30.1-74.5)	5.2 (1.2-6.0)
Bacillus megaterium	23.2 (7.0-44.3)	45.6 (38.6-75.0)	5.5 (3.8-6.2)
Proteus mirabilis strain 19	9.4 (7.4-10.6)	See Table 2	See Table 2
Escherichia coli B	7.3 (4.9-9.0)	ND	ND
Acholeplasma laidlawii (oral strain)	24.9 (18.2-35.0)	52.2 (35.1-64.8)	6.4 (4.8-6.8)
Mycoplasma mycoides var. *capri* (PG3)	53.0 (38.2-56.0)	132.6 (85.4-178.9)	12.4 (11.0-13.5)

[a] Data shown represents the mean and range (in parentheses) of determinations carried out on three to six different batches of cells and membranes. ND, Not done.

TABLE 2. *Distribution of cholesterol in the outer and cytoplasmic membranes of P. mirabilis*

Preparation	Protein content (mg)	Phospholipid content (μg of P_i/mg of protein)	Cholesterol content	
			μg/ml of protein	μg/μg of lipid phosphorus
Outer membrane	5.7	13.9	17.0	1.2
Cytoplasmic membrane	3.1	24.2	119.2	5.2

plete as Table 2 also shows that the cholesterol-to-phospholipid ratio in the outer membrane is remarkably lower than that found in the cytoplasmic membrane, despite the phospholipid composition being qualitatively very similar in the two membranes (9). Hence, it appears that the phospholipid component of the outer membrane does not exhibit its full cholesterol binding capacity. Possible explanations for this may be based on the higher viscosity of the lipid domain of the outer membrane, as measured by electron-spin resonance spectroscopy, and on the finding that a significant part of the phospholipids in the outer membrane resist extraction with aqueous acetone, suggesting their tighter binding to other membrane components (9), factors which may act to limit cholesterol incorporation.

The cholesterol binding capacity of bacterial membranes was also tested in an experimental system consisting of washed cells or isolated membranes, labeled cholesterol, Tween 80, and buffer (2). The kinetics of cholesterol uptake by isolated *M. lysodeikticus* membranes resembled that of *A. laidlawii* membranes, though the uptake values were generally lower for the bacterial membranes (Fig. 1). Washed *M. lysodeikticus* cells were also capable of cholesterol uptake in the experimental system (Fig. 1). The cholesterol taken up did not undergo any chemical change as all the label in the membranes or cells was found to be associated with free

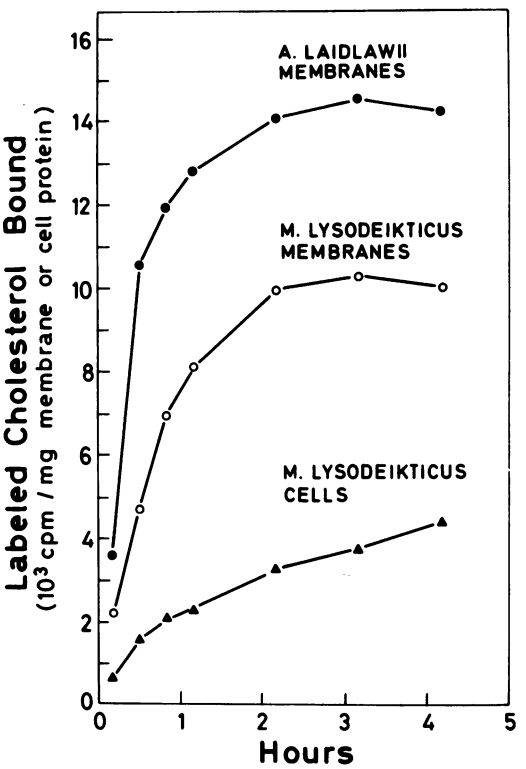

FIG. 1. *Uptake of cholesterol by washed cells and membranes incubated at 37 C with 10^{-6} M [4-^{14}C]-cholesterol in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.0, supplemented with 0.01% Tween 80 and 5 mM $MgCl_2$. Cholesterol uptake was measured as described by Gershfeld et al. (2).*

cholesterol. Digestion by lysozyme of the wall of the cholesterol-loaded cells suspended in 1 M NaCl released less than 15% of the labeled cholesterol into the medium, leaving the rest bound to the protoplasts, again indicating that in the gram-positive bacterium most of the cholesterol is bound to the cytoplasmic membrane rather than to the cell wall.

Although the number of organisms tested in this study is small, the results suffice to indicate that exogenous cholesterol can be incorporated into the cytoplasmic membrane of growing bacteria, despite its being covered by a cell wall. The quantities of cholesterol incorporated are comparable to those incorporated into membranes of the sterol-nonrequiring mycoplasmas, but fall far below those incorporated into membranes of the sterol-requiring mycoplasmas.

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